Supplemental data

Supplemental material and methods

Animal care and use

All animal protocols were approved by the Animal Experimentation Ethics Committee of IDIBELL (AAALAC accredited facility, B9900010) and IRB-Barcelona, and by the corresponding Department of Generalitat de Catalunya according to EU directive 2010/63/EU. Experiments were carried out with the highest scientific, humane, and ethical principles. Pure genetic background C57BL/6J mice were maintained in a 12h light-dark cycle, in temperature and humidity-controlled room. Until beginning of experiments, animals were housed in sterile cages with free access to food (Teklad Global 14% Protein Diet, Harlan Laboratories, IN, USA) and water.

dKO LAT2-TAT1 mice generation

Single loss-of-function mouse models for LAT2 (null knockout)[1-3] and TAT1 (premature STOP codon at position Y88)[4] were crossed to obtain double heterozygous mice, which were backcrossed to get the F2 generation with the 9 expected genotypes, including dKO LAT2-TAT1 (dKO) mice.

For genotyping analyses, genomic DNA was isolated from tail tissue. LAT2 genotype was confirmed by PCR (30 cycles at 58ºC annealing temperature), based on a 3 primer strategy (F: 5'-GGAGCGATCTGCGGAGTGA-3'; R Wt-specific: 5'-ACAGAGTGCGCTCCTACCCT-3'; R KO-specific: 5'-CGGTGGGCTCTATGGGTCTA-3') allowing to distinguish genotypes by generating 458 bp and 180 bp fragments from the WT and KO alleles, respectively. For TAT1 genotype, an amplification PCR was set (30 cycles at 60.8ºC annealing temperature; F: 5'-GGGACCCTCGGATGTCTC-3'; R: 5'-GGCCATGTTGTCATCGTCCTTGG-3', product size 226 pb), followed by sequence analysis (StabVida, Portugal). The presence of the point mutation C>A was analysed at position 482 using Sequencher DNA sequence analysis software (Gene Codes Corporation, MI, USA).

Experimental protein diets

For exacerbation of renal phenotype, a high protein diet was used (40% casein, Harlan Laboratories, IN, USA). As control, mice of each genotype were fed with 20% casein diet (Ssniff Spezialdiäten GmbH, Germany). Animals were fed with the corresponding experimental diet for 8 days, with free access to water.

Urine, plasma and tissue collection

Male mice from each genotype were individually housed in metabolic cages for 4 days during which experimental diet was maintained. Water and food intake, and faeces and urine excreted were monitored. 24h urine samples from two consecutive days were collected and kept at -20ºC until further analysis. The last experimental day, intracardiac puncture was performed under deep anaesthesia (IsoFlo, Esteve Veterinaria, Spain) to obtain up to 1 mL of blood. Blood was collected in EDTA-tubes and plasma was obtained by centrifugation at 3000 rpm, 10 min, 4ºC, and stored at -80ºC until analysis. Organs of interest (i.e. epididymal white adipose tissue, pancreas, liver, kidney, spleen, gastrocnemius, soleus, quadriceps and brain) were harvested, weighed, frozen in liquid nitrogen and stored at -80ºC until further processing for RNA and/or protein extraction.

Amino acid and creatinine analysis

Amino acid and creatinine concentrations in plasma and urine samples were determined as reported elsewhere.[5] Briefly, amino acid were analysed by ion exchange chromatography with nynhydrin derivatization and spectrometric detection (Biochrom 30, Chromsystems, UK). Creatinine concentration was determined by an automated spectrophotometric assay in the Architect c8000 analyzer (Abbott, IL, USA).

RNA extraction, quantification and quality assessment

Total RNA from kidney was isolated using TriPure Isolation Reagent (Roche LifeScience, Switzerland) following manufacturer's instructions. RNA was accurately quantified by a fluorimetric method (Qubit RNA BR Assay kit, Molecular Probes, OR, USA) and RNA quality was analysed with the RNA 6000 Nano kit and the Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA). Only samples with RNA Integrity Number (RIN) > 8 were used.

Gene expression

Gene expression analysis was done by reverse transcription quantitative PCR (RT-qPCR), in combination with the universal probe library (UPL) detection probes (Roche LifeScience, Switzerland) and the microfluidic chip technology from Fluidigm (CA, USA). Genes analyzed, primer pairs and matching UPLs used are listed in Supplemental Table 6 (information provided based on MIQE's recommendations[6]). For the RT reaction, 25-50 ng of RNA were reverse-transcribed into cDNA using the Reverse Transcription Master Mix (Fluidigm) following manufacturer's conditions. Then a pre-amplification step of 16 cycles using PreAmp Master Mix kit (Fluidigm) following manufacturer's instructions. Real-time qPCR was performed using the FastStart Universal Probe Master with ROX (Roche) and qPCR was set in a 96.96 dynamic array (Fluidigm) carefully following company instructions. The chip was primed and placed into Biomark HD

instrument (Fluidigm) for thermal cycling and real-time fluorescence detection (reaction conditions in Supplemental Table 7). Quantification cycle (C_q) values were determined and $2^{-\Delta Cq}$ values were calculated.

Immunoblotting

Total membrane proteins were extracted from frozen kidneys in ice-cold homogenization buffer (25 mM HEPES, 250 mM sacarose, 4 mM EDTA and protease inhibitors). Samples were centrifuged at 3500x g, 20 minutes, 4ºC, and the supernatant was subsequently centrifuged at 20000x g, 1 hour, 4ºC. The pellet was resuspended in HEPES buffer with proteases using a 25G syringe. Samples were quantified using the Pierce BCA Protein Assay kit (Thermo Scientific, MA, USA). 50-100 µg of protein were separated on 8% non-denaturing SDS-polyacrylamide gels in Tris/glycine but, and transferred to PVDF (for y^+LAT1 detection) or nitrocellulose (for LAT2 and TAT1 proteins) membranes. Primary antibodies were probed overnight at 4ºC at 1:500 (TAT1[7]) and 1:1000 dilution (LAT2[8] and y^+LAT1). Protein detection was done by ECL reaction (GE Healthcare, UK) and film exposure (y^+LAT1); or with fluorescent secondary antibodies using Odyssey Infrared Imager (Li-Cor Biosciences, NE, USA) (LAT2 and TAT1). Densitometric analysis of y^+LAT1 was done using ImageJ software. LAT2 and TAT1 were quantified using Odyssey v3.0 software (Li-Cor Biosciences).

Immunofluorescence

Organ collection of kidney was followed by fixation in 4% paraformaldehyde (PFA) over-night, 15% sucrose solution over-day, then 30% sucrose solution over-night, and embedding in OCT matrix for cryosections (CellPath, UK) or by directly embedding in OCT and freezing in liquid propane. Sections 5 µm thick were cut with cryotome (Leica) and mounted on Superfrost Plus slides (Menzel glasses, Thermo scientific, Germany). Tissues that were directly frozen were post fixed in cold methanol at -20 °C for 90 sec and immediately washed in PBS. Antigen retrieval was performed in 10 mM Na-Citrate solution (pH 6.0) at 98 °C for 10 min in a pressure cooker (Histos 3, Milestone, USA) and after that blocking with 1% BSA solution for 30 min. Previously described antibodies were used for TAT1[7] and LAT2[8]. Secondary antibody (Alexa Fluor 488) and 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) were applied at a dilution of 1:500, cover slips mounted with DAKO-Glycergel, and the sections were analysed with a fluorescent light microscope (Nikon, TE300 Eclipse or Zeiss, 200M Axiovert).

Statistical tools

GraphPad Prism 6.03 (GraphPad Software, CA, USA) was used for statistical data analysis and plotting. Student's t-test was used for the statistical analysis of amino acid (urine excretion, plasma concentration, renal clearance and tubular reabsorption) and protein expression analysis. BootstRatio[9] was used to assess statistical differences on results obtained by RT-qPCR, using the option "With control" (http://regstattools.net/br). Significance was set in all cases at $p < 0.05$.

Supplemental equations

Supplemental Equation 1. Renal clearance (RC). *K* is the clearance (ml/min), C_u is the urine concentration of the amino acid (mmol/l), *Q* is the urine flow in volume/time (ml/min, usually ml/24 hours), and C_B is the plasma concentration of the amino acid (mmol/l).

$$\mathrm{K} = \frac{\mathrm{C_u} \cdot \mathrm{Q}}{\mathrm{C_B}}$$

Supplemental Equation 2. Glomerular filtration rate (GFR). The glomerular filtration rate was calculated using the creatinine concentrations in urine and plasma. *Q* is the urine flow (in ml/24 h). $[\mathrm{Creatinine}]_{\mathrm{urine}}$ and $[\mathrm{Creatinine}]_{\mathrm{plasma}}$ are in mM units.

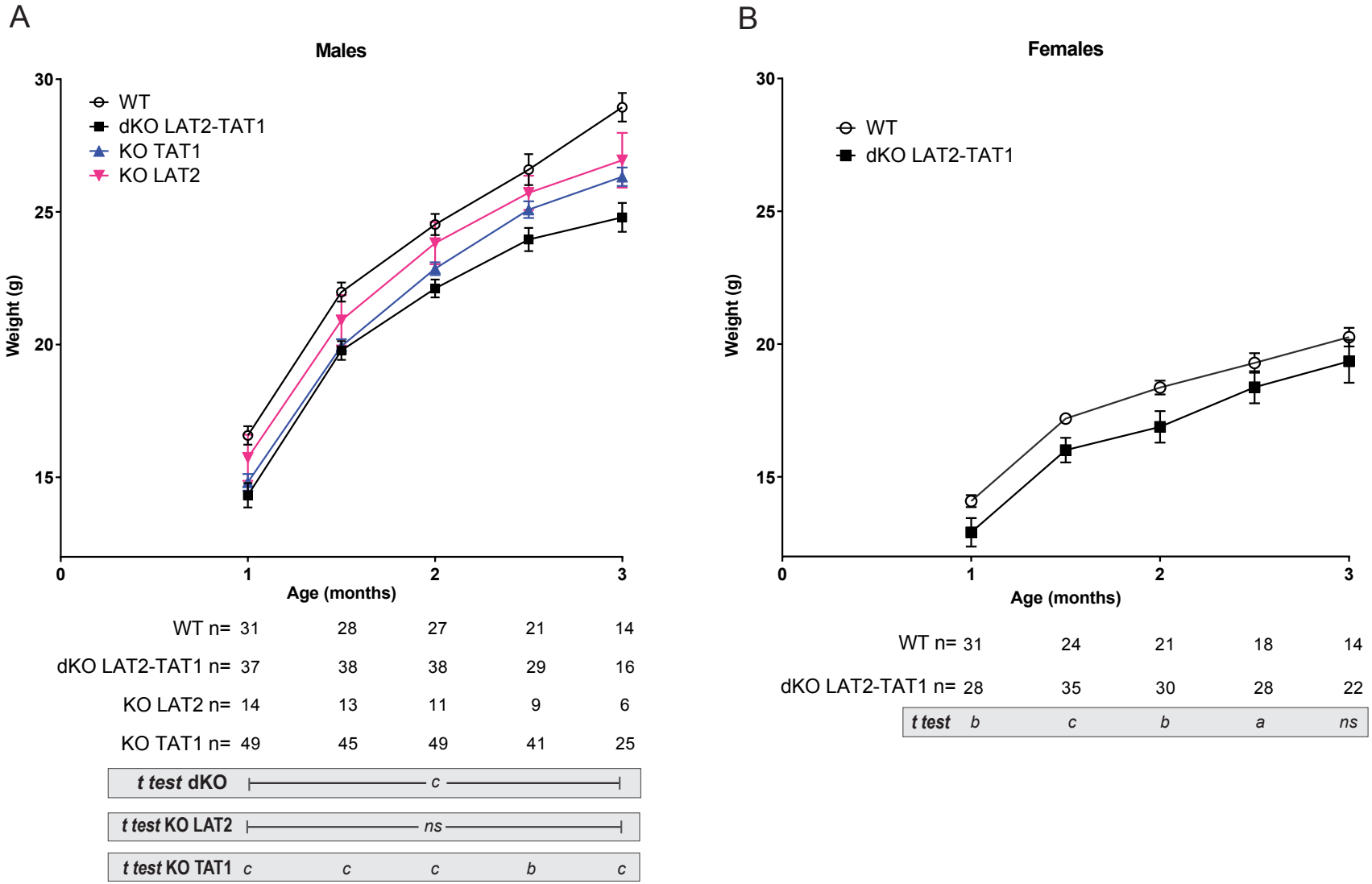
$$\mathrm{GFR} = \frac{[\mathrm{Creatinine}]_{urine} \cdot \mathrm{Q}}{[\mathrm{Creatinine}]_{plasma}}$$

Supplemental Equation 3. Tubular reabsorption (TR). TR is given as a %. $[AA]_{plasma}$ is the concentration of the amino acid in plasma, *GFR* is the glomerular filtration rate (Equation 2) and *Tubular excretion* the absolute amount of amino acids excreted in 24 h.

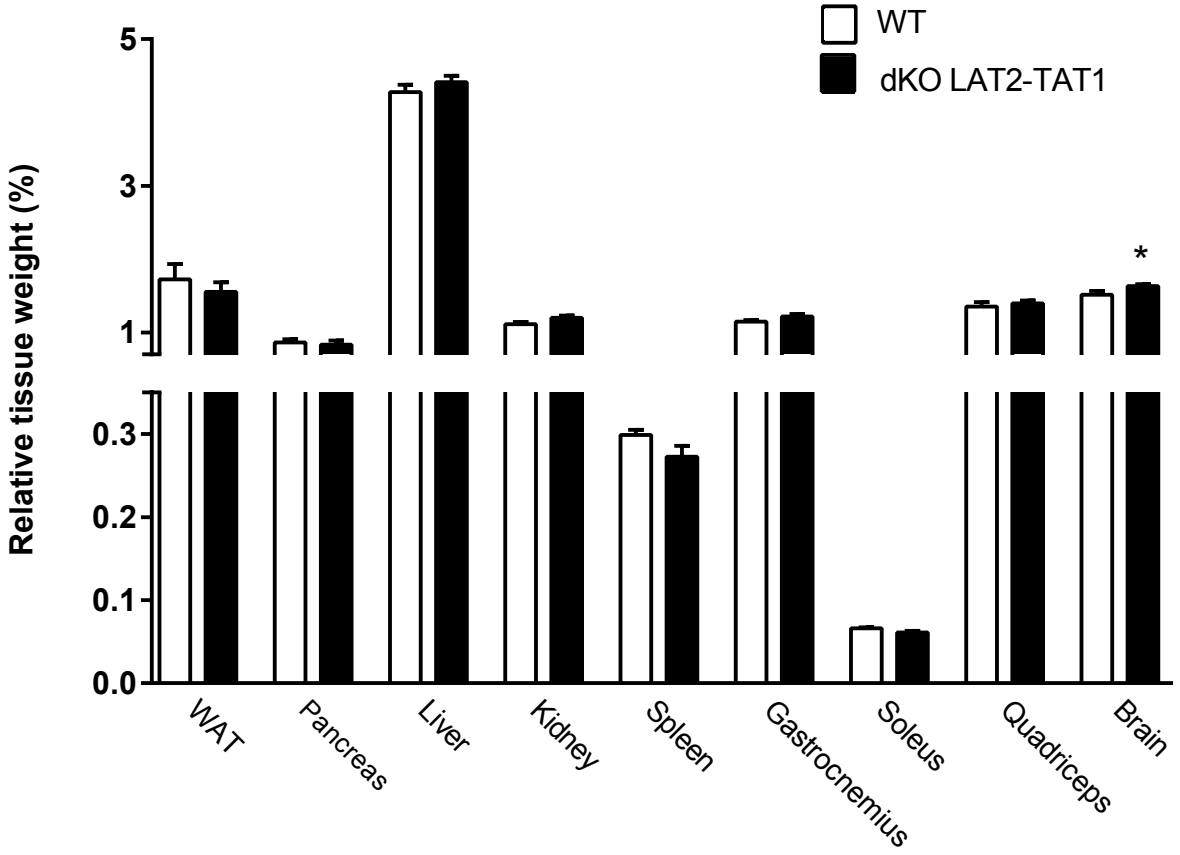
$$\%\mathrm{TR} = \frac{\left([\mathrm{AA}]_{\mathrm{plasma}} \cdot \mathrm{GFR}\right) - \text{ Tubular excretion}}{[\mathrm{AA}]_{\mathrm{plasma}} \cdot \mathrm{GFR}} \cdot 100$$

Supplemental Figures

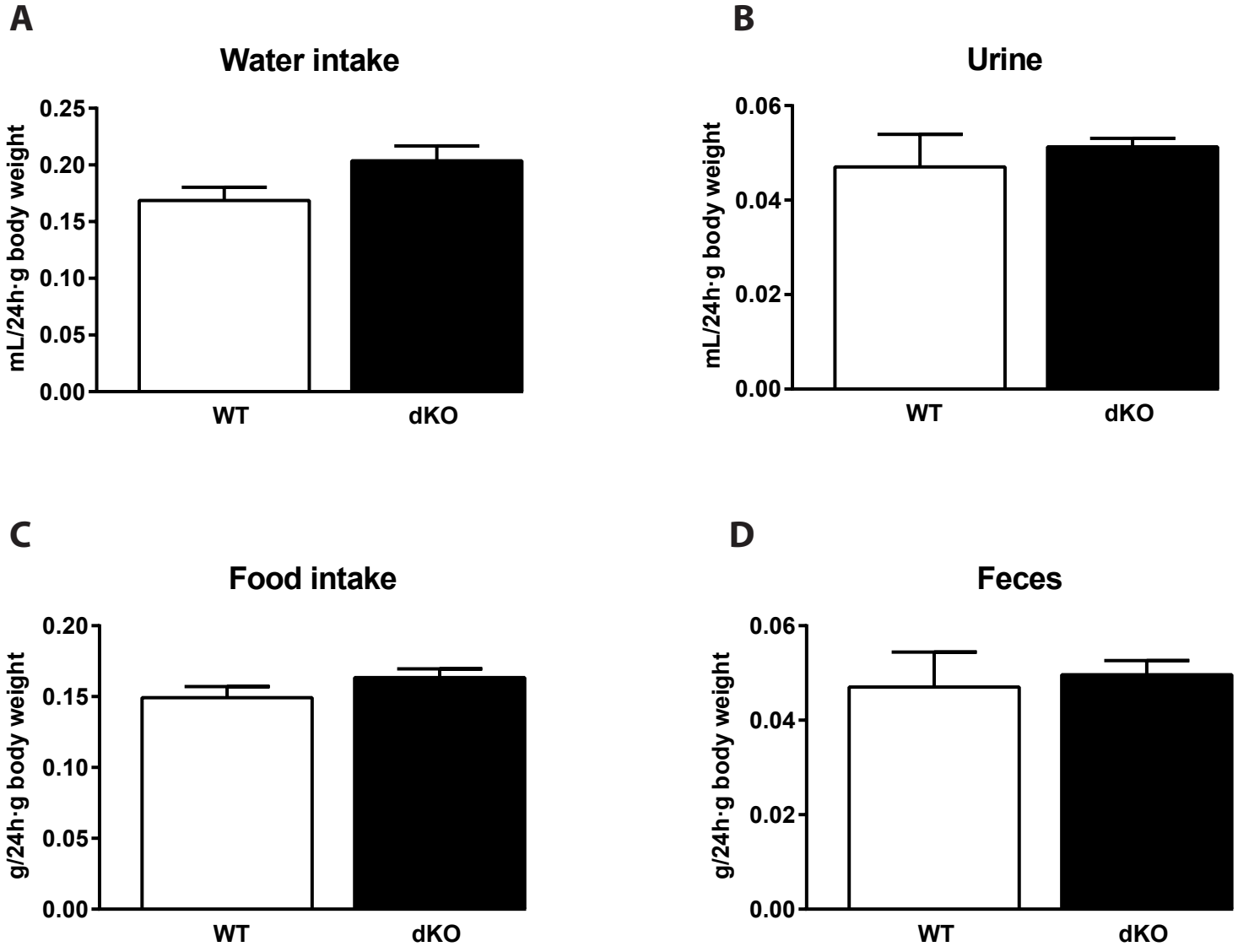
Supplemental Figure 1. Body weight of single and double LAT2 and TAT1 knockout mice. Body weight of male (A) and female (B) mice with the indicated genotypes between months 1 and 3 of age. The number of animals at each age and genotype is shown below. Genotypes: WT, wild type, open circles; KO LAT2, LAT2 homozygotes, pink inverted triangles; KO TAT1 homozygotes, blue triangles; dKO, dKO LAT2-TAT1, black squares. Unpaired t-test results shown are for comparisons of KO LAT2 or KO TAT1 vs WT. A letter-code is used to indicate statistical differences: a for $p \leq 0.05$; b for $p \leq 0.01$; c for $p \leq 0.001$; ns for not significant differences between values.



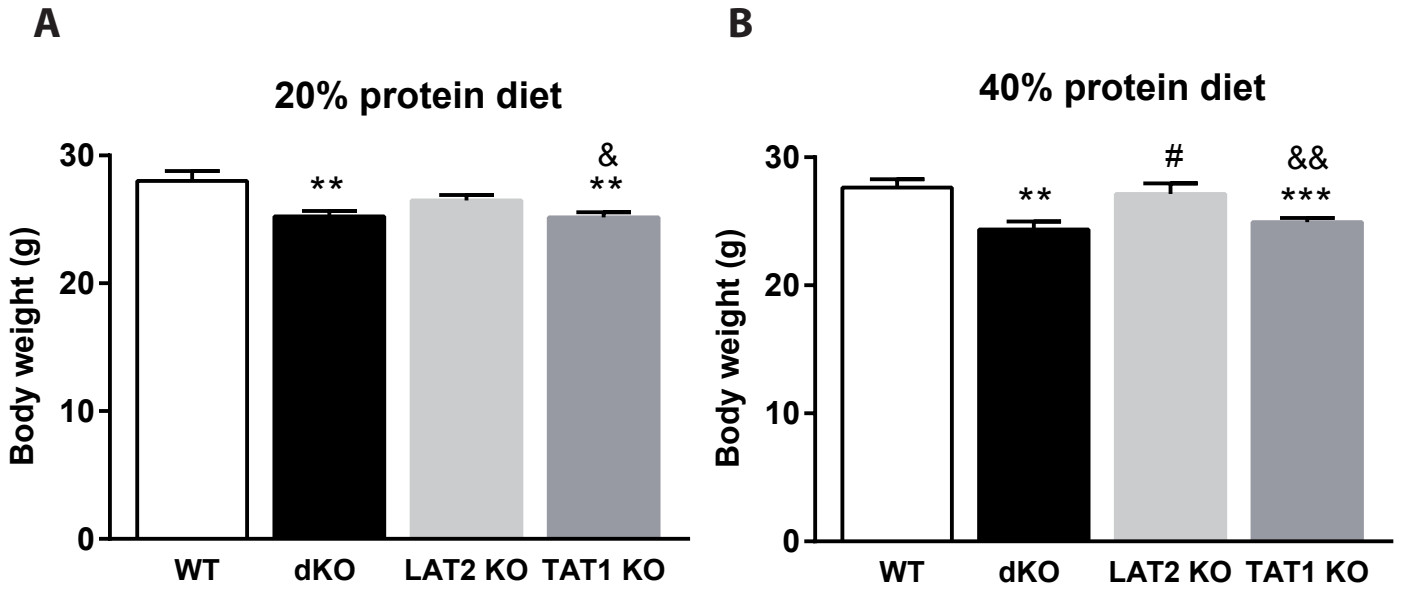
Supplemental Figure 2. Weight of tissues and organs in double LAT2-TAT1 knockout mice under standard 14% protein-content diet. Relative tissue and organ weights are shown as percentage of total body weight. Data (mean ± SEM) from 5 wild type male mice (open bars) and 8 dKO LAT2 TAT1 (black bars) male mice, at 3-4 months of age and fed with standard 14% protein-content diet. t-test was performed to compare values from WT and dKO LAT2-TAT1 mice with the following statistical significance: * $p \leq 0.05$. Kidneys and skeletal muscle weights correspond to the sum of both right and left organs. WAT: epididymal white adipose tissue.



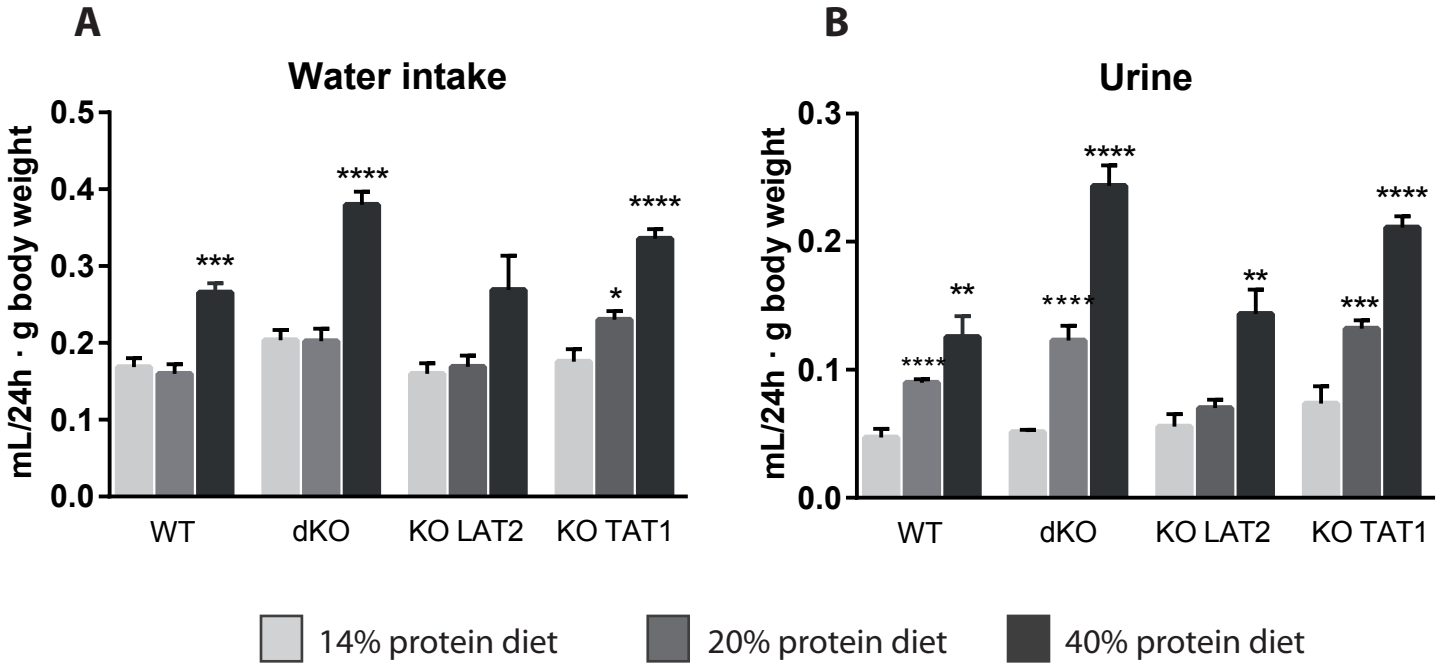
Supplemental Figure 3. Metabolic data in double LAT2-TAT1 knockout mice under standard 14% protein diet. (A) Water intake, in mL of water drunk in 24 h per gram of body weight. (B) Volume of urine excreted in 24 h per gram of body weight. (C) Food intake, in grams of food in 24h per gram of body weight. (D) Amount of feces excreted in 24h per gram of body weight. Data (mean ± SEM) from 5 control male mice (open bars) and 8 dKO LAT2-TAT1 male mice (black bars) at three months of age. No statistical differences between wild-type and dKO mice were observed in any of the parameters analyzed. WT and dKO denote wild-type mice and dKO LAT2-TAT1 homozygotes.



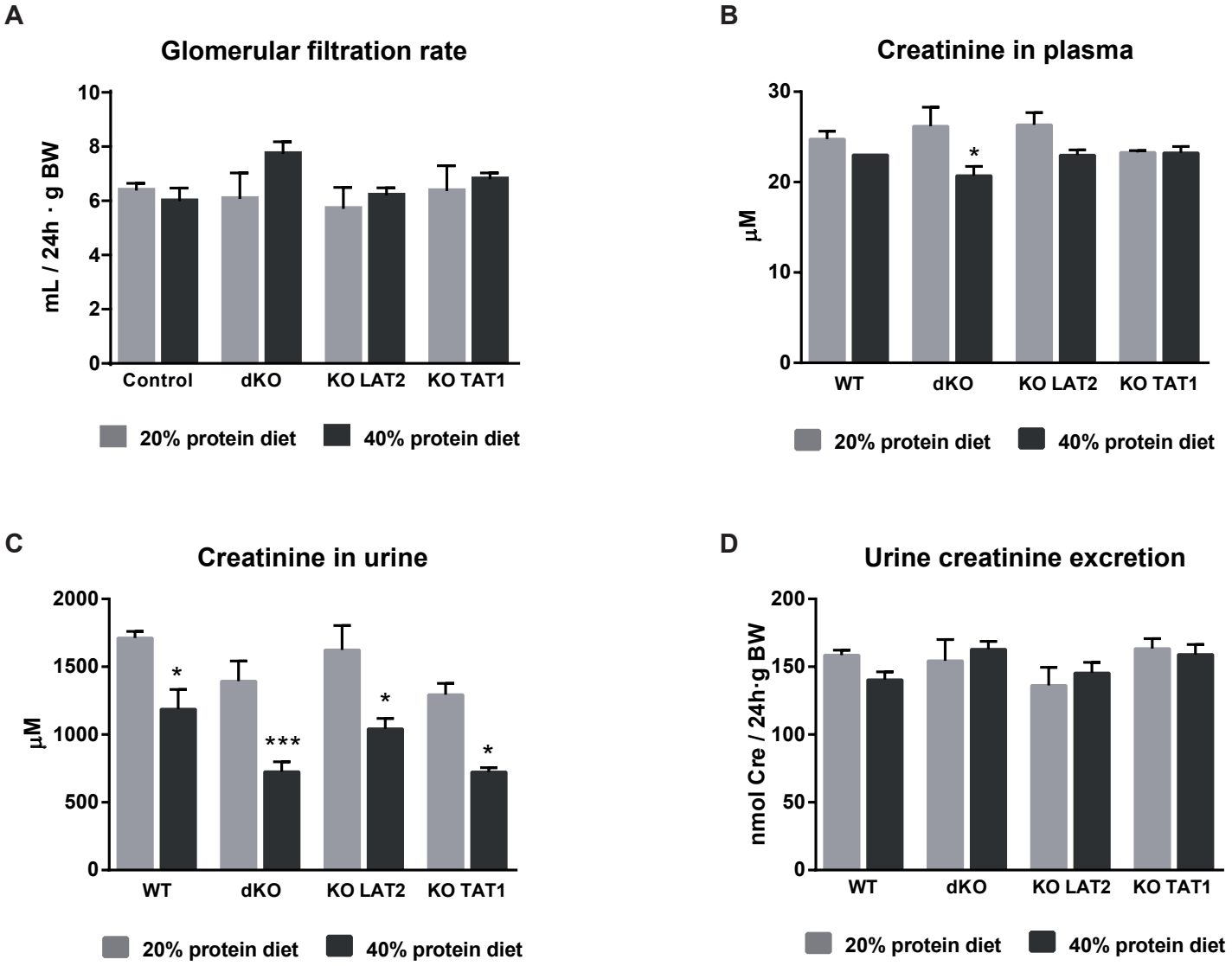
Supplemental Figure 4. Body weight of single and double LAT2 and TAT1 knockout mice under 20% and 40% protein diets. Mice with the indicated phenotype and under diets with 20% (A) or 40% (B) protein content were studied. Body weight comparing both diets was also plotted (C). Data (mean ± SEM) are from male mice at 3-4 months of age. The number of mice studied per group was: 8, 11, 10 and 11 (WT, dKO, KO LAT2, KO TAT1, respectively) in A, and 5, 11, 8 and 18 (WT, dKO, KO LAT2, KO TAT1, respectively) in B. Statistical differences (t-test analysis): * is used to compare with WT, # to compare with dKO; and & to compare LAT2 KO. Number of symbols indicates p-values: 1 symbol for $p \leq 0.05$; 2 for $p \leq 0.01$; and 3 for $p \leq 0.001$. WT and dKO denote wild-type mice and dKO LAT2-TAT1 homozygotes.



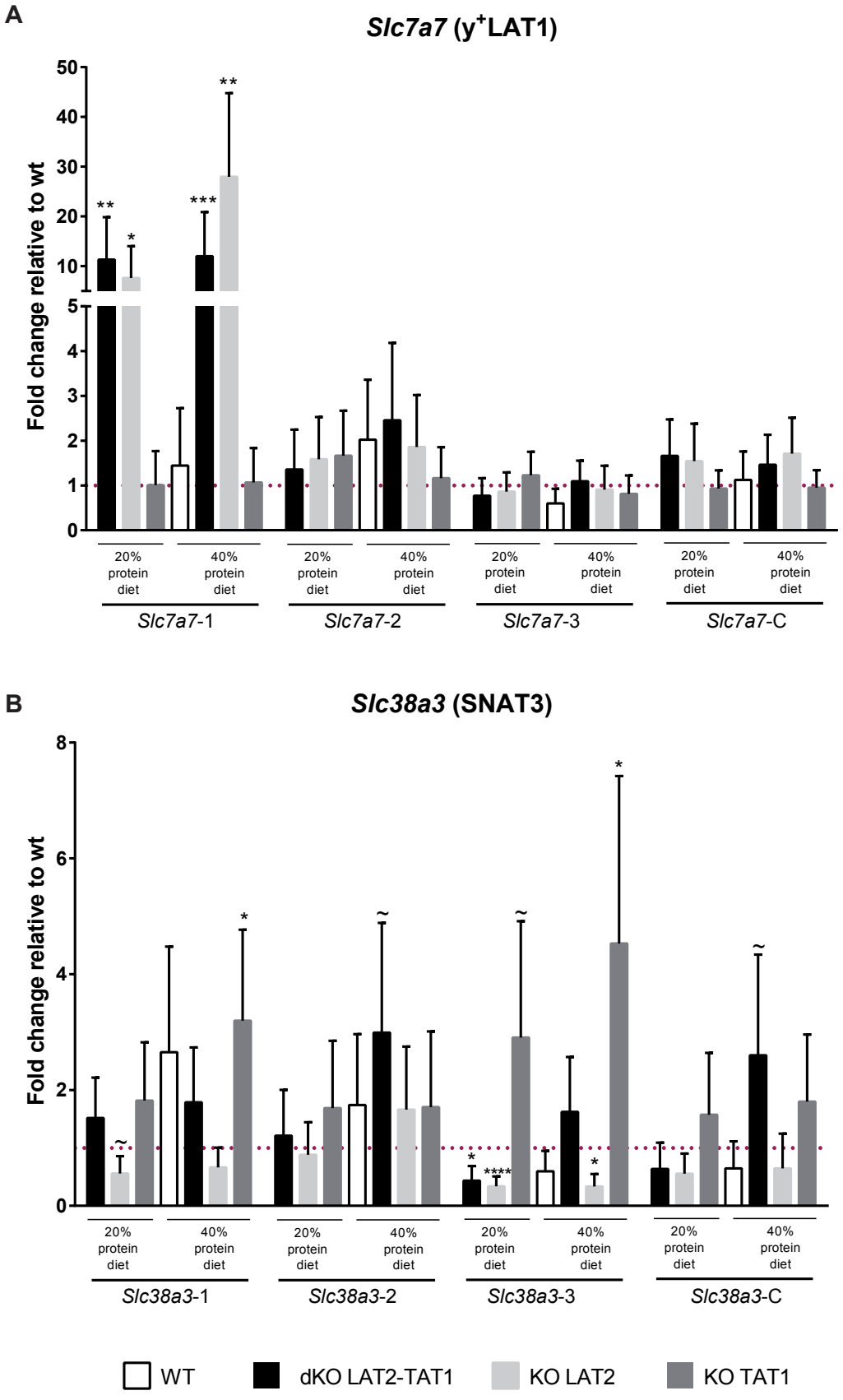
Supplemental Figure 5. Metabolic data from single and double LAT2 and TAT1 knockout mice under the three diets used. Water intake (A) and urine flow (B) is shown for the indicated genotypes and diets. Data (mean ± SEM) are from male mice at 3-4 months of age under the standard diet with 14% protein content or after 11 days under 20% or 40% protein diets as indicated. Unpaired t-test was performed to identify significant differences versus mice under the standard 14% protein diet: * $p < 0.05$; ** $p < 0,01$; *** for $p < 0,001$; **** $p < 0,0001$. WT and dKO denote wild-type mice and dKO LAT2-TAT1 homozygotes.



Supplemental Figure 6. Glomerular filtration rate in single and double LAT2 and TAT1 knockout mice. Glomerular filtration rate (A) was estimated using Equation 2 (see Supplemental equations) in mice with the indicated genotypes. Creatinine concentration (µM) in plasma (B) and urine (C) and excretion of creatinine in urine (nmol/ g of body weight in 24h (D) are also shown. In all cases, mice were analyzed after 11 days under 20% protein- or 40% protein-content diets. Data (mean ± SEM) from 5 male mice at 3 months of age. ANOVA with Tukey post-hoc analysis was performed to assess differences between groups. No significant differences were found in the glomerular filtration rate between genotypes and diets. Significant differences between mice kept under different diets are shown as follows: * for $p \leq 0.05$ and *** for $p \leq 0.001$.



Supplemental Figure 7. Gene expression of y$^+$LAT1 and SNAT3 transporters in the kidney of single and double LAT2 and TAT1 knockout mice. Fold changes of the mRNA variants of *Slc7a7* (y+LAT1) (A) and *Slc38a3* (SNAT3) (B) as indicated in the bottom of the graphs. C letter indicates that the primer pair was designed to detect all variants. Dotted line at fold-change 1 denotes the expression value of the control (wild type under 20% protein diet) to which all ratios are referred. Male mice were analyzed at 3-4 months of age and after 11 days under 20% protein- or 40% protein-content diets. Data (mean ± SD) are from 5-8 mice per group. Statistical significance of expression ratios is indicated according to BoostRatio results, with ~ for $p \leq 0.1$, * $p \leq 0.05$, ** $p \leq 0.01$ and *** $p \leq 0.001$.



Supplemental Tables

Supplemental Table 1. Genotype frequencies in the F2 generation obtained by crossing double heterozygous of LAT2 and TAT1 knockout mice. Data from 355 pups obtained from 10 different couples of F2 double heterozygous progenitors. The inlet shows $\chi 2$ test results (www.cnb.csic.es/~montoliu) showing that the observed genotype frequencies do not correspond to the expected frequencies of a Mendelian inheritance.

Genotype		n Observed	Frequence Observed (%)	Frequence Expected (%)
LAT2 *Slc7a8*	TAT1 *Slc16a10*			
+/+	+/+	21	5.92	6.25
+/+	+/−	31	8.73	12.5
+/+	−/−	15	4.23	6.25
+/−	+/+	59	16.62	12.5
+/−	+/−	89	25.07	25
+/−	−/−	54	15.21	12.5
−/−	+/+	23	6.48	6.25
−/−	+/−	53	14.93	12.5
−/−	−/−	10	2.83	6.25
	Total	355	100	100

χ^2 calculated value	21.73
χ^2 table	15.507
Confidence level	0.05
Calculated p-value	0.005
Degrees of freedom	8

Non-Mendelian inheritance

Supplemental Table 2. Excretion of amino acids in urine. Excretion, expressed as nmols of the indicated amino acid per gram of body weight in 24-hour sample, was assessed in mice with the indicated genotypes at 3-4 months of age and after 11 days under 20% protein- or 40% protein-content diets. Data (mean ± SEM) are from 5 male mice per 20% protein diet groups and for 40% protein diet groups: 4 for WT, 6 for dKO, 5 for KO LAT2 and 5 for KO TAT1. Unpaired T test was performed to assess differences between genotypes. * is used to indicate significant differences vs wild-type animals (WT), # differences vs dKO mice, and & vs KO LAT2 mice. Number of symbols denote p values, with 1 symbol for $p \leq 0.05$; 2 symbols for $p \leq 0.01$, and 3 symbols for $p \leq 0.001$. WT and dKO denote wild-type and dKO LAT2-TAT1 homozygotes. Amino acids are designated with the three-letter code.

	20% protein diet				40% protein diet			
Amino acid	**WT**	**KO LAT2**	**KO TAT1**	**dKO**	**WT**	**KO LAT2**	**KO TAT1**	**dKO**
TYR	4.7 ± 0.4	4.7 ± 0.9 ##	147.6 ± 26.1 ** # &&	328.6 ± 53.5 **	9.6 ± 1.3	9.4 ± 1.4 ####	538.5 ± 95.8 ** &&&	799.9 ± 90.7 ****
PHE	6.2 ± 0.8	4.2 ± 0.4 ##	8.6 ± 0.3 * # &&&&	18.1 ± 3.0 **	6.3 ± 0.5	6.2 ± 0.9 ###	26.7 ± 4.2 ** ## &&	74.2 ± 9.5 ***
ILE	2.7 ± 0.5	2.5 ± 0.5	1.5 ± 0.1 #	5.8 ± 1.4	5.2 ± 1.0	5.0 ± 0.4 ###	15.4 ± 2.9 * ### &	62.0 ± 8.9 ***
ALA	14.2 ± 2.7	12.1 ± 1.4	11.1 ± 2.3	18.3 ± 2.7	14.5 ± 2.4	14.1 ± 1.5 ####	29.5 ± 5.1 * ## &	61.2 ± 6.1 ***
VAL	5.7 ± 0.6	7.6 ± 0.8 #	7.9 ± 1.6 #	17.2 ± 3.4 *	15.5 ± 2.0	19.8 ± 1.8 ###	40.7 ± 6.5 * ### &&	132.6 ± 17.0 ***
LEU	6.1 ± 0.9	11.9 ± 4.2	6.1 ± 0.5 #	24.4 ± 6.1 *	17.2 ± 2.6	20.0 ± 1.2 ###	59.6 ± 10.5 ** ### &	202.8 ± 24.2 ***
SER	7.6 ± 1.6	9.7 ± 2.8 #	7.0 ± 1.1 #	31.1 ± 8.1 *	9.2 ± 1.2	15.9 ± 2.4 * ###	15.3 ± 2.5 ###	129.5 ± 17.6 ***
GLN	15.6 ± 1.5	30.5 ± 7.0	19.5 ± 3.1 #	72.0 ± 19.0 *	18.5 ± 1.2	46.1 ± 10.8 ###	50.0 ± 9.1 * ###	233.8 ± 28.4 ***
ASN	5.5 ± 0.3	5.0 ± 0.9	6.3 ± 1.0	11.1 ± 1.4	6.4 ± 0.6	8.0 ± 0.8 ##	8.0 ± 3.1 ##	41.0 ± 6.6 **
THR	21.5 ± 4.7	64.2 ± 11.2 *	36.9 ± 9.7 #	127.4 ± 27.0 *	23.6 ± 1.5	55.9 ± 15.3 ###	84.3 ± 15.7 * ###	379.4 ± 44.8 ***
MET	21.8 ± 4.3	23.5 ± 4.0 #	36.0 ± 4.0	99.0 ± 24.2 *	11.2 ± 0.5	12.6 ± 1.9 ###	21.9 ± 3.3 ## &	50.7 ± 6.2 **
GLY	25.9 ± 1.0	23.3 ± 1.1 #	25.3 ± 2.7 #	44.6 ± 7.5 *	27.1 ± 2.0	31.6 ± 2.3 ###	52.0 ± 5.7 ** ### &	198.7 ± 23.3 ***
HIS	3.7 ± 0.4	5.7 ± 1.1	7.1 ± 1.7 #	26.3 ± 7.8 *	4.8 ± 0.4	9.1 ± 2.3 ####	37.6 ± 8.3 * ### &	135.7 ± 15.9 ***
LYS	14.4 ± 1.4	14.2 ± 1.9	14.7 ± 1.5	16.1 ± 1.4	15.5 ± 2.1	13.8 ± 1.8 ##	18.7 ± 1.6	23.9 ± 2.1 *
ORN	2.3 ± 0.4	3.1 ± 0.8	2.0 ± 0.1 #	2.9 ± 0.3	4.0 ± 0.5	3.4 ± 0.5 ##	5.4 ± 0.9	7.6 ± 0.8 *
ARG	4.9 ± 1.1	4.9 ± 0.7 #	3.1 ± 0.4	2.7 ± 0.2	10.8 ± 1.8	6.5 ± 1.5	10.7 ± 1.9	10.2 ± 1.0
GLU	3.8 ± 0.9	4.0 ± 1.3	5.7 ± 1.9	6.8 ± 2.4	7.2 ± 2.0	4.0 ± 0.7 #	10.7 ± 2.6	11.8 ± 1.9
PRO	6.8 ± 1.4	26.9 ± 12.8	7.6 ± 0.4	14.8 ± 4.5	11.8 ± 3.0	26.0 ± 8.4 ##	39.1 ± 10.0 ##	153.0 ± 25.0 **

Supplemental Table 3. Amino acid concentrations in plasma. Plasma concentrations (μM) were determined in mice with the indicated genotypes at 3-4 months of age and after 11 days under 20% protein- or 40% protein-content diets. Data (mean ± SEM) are from the number of male mice per group indicated in the legend to Supplemental Table 2. Unpaired T test was performed to assess differences between genotypes. * is used to indicate significant differences vs wild-type animals (WT), # differences vs dKO mice, and & vs KO LAT2 mice. Number of symbols denote p values, with 1 symbol for $p \leq 0.05$; 2 symbols for $p \leq 0.01$,3 symbols for $p \leq 0.001$ and four symbols for $p \leq 0.0001$. (WT and dKO denote wild-type and dKO LAT2-TAT1 homozygotes). Amino acids are designated with the three-letter code.

	20% protein diet				40% protein diet			
Amino acid	**WT**	**KO LAT2**	**KO TAT1**	**dKO**	**WT**	**KO LAT2**	**KO TAT1**	**dKO**
TYR	84.2 ± 17.4	101.8 ± 14.6 ####	760.8 ± 79.8 **** &&&&	744.1 ± 62.2 ****	173.3 ± 26.8	113.5 ± 9.4 * ####	907.6 ± 63.2 **** ## &&&&	634.7 ± 56.6 ***
PHE	80.7 ± 10.2	78.0 ± 4.2 ##	172.4 ± 13.8 *** &&&	163.2 ± 18.3 **	94.6 ± 9.1	78.3 ± 1.6 #	180.8 ± 16.6 ** ## &&&	120.5 ± 12.3
ILE	133.5 ± 15.4	153.7 ± 16.8	141.2 ± 6.5	156.1 ± 17.4	287.7 ± 47.1	224.6 ± 19.2	234.1 ± 22.4	185.1 ± 17.3 *
ALA	575.8 ± 87.4	576.3 ± 99.0	428.2 ± 22.9	372.5 ± 40.2	719.1 ± 148.2	420.7 ± 45.9 ###	374.9 ± 35.4 * ##	229.1 ± 22.3 ***
VAL	310.2 ± 37.1	443.9 ± 69.4	379.1 ± 27.3	435.5 ± 43.6	722.5 ± 92.3	702.4 ± 65.1	594.9 ± 62.5	491.8 ± 68.5
LEU	201.2 ± 24.1	227.1 ± 25.9	214.9 ± 9.5	216.6 ± 28.7	460.7 ± 73.2	327.5 ± 30.6	391.9 ± 44.3 #	254.1 ± 25.9 **
SER	150.4 ± 23.3	170.6 ± 20.4	112.7 ± 11.3 &	119.7 ± 11.9	201.0 ± 37.6	138.1 ± 17.7 ##	85.5 ± 5.2 ** &	85.3 ± 5.5 ***
GLN	530.9 ± 61.0	586.7 ± 44.4 #	381.5 ± 12.1 * &&	407.6 ± 30.0	512.8 ± 42.7	481.1 ± 13.7	371.2 ± 16.1 ** &&&	403.4 ± 33.0
ASN	59.4 ± 1.7	64.4 ± 11.8	37.9 ± 4.3 **	35.0 ± 5.9 **	92.3 ± 19.6	56.0 ± 11.7 ##	28.9 ± 1.4 ** &	26.8 ± 3.1 ***
THR	238.5 ± 29.1	294.9 ± 47.3	207.7 ± 24.4	185.8 ± 20.4	376.7 ± 53.3	254.6 ± 34.4 ###	179.5 ± 15.7 ** ##	120.7 ± 12.8 ****
MET	91.9 ± 19.3	106.4 ± 20.1	110.0 ± 14.6	93.1 ± 15.0	121.0 ± 16.9	87.8 ± 11.6 ###	73.5 ± 7.4 * ##	43.1 ± 5.6 ****
GLY	229.8 ± 22.6	236.5 ± 37.0	134.0 ± 10.2 ** &	152.4 ± 7.0 *	204.4 ± 26.7	176.2 ± 15.0 ###	103.5 ± 6.4 ** &&&	107.1 ± 8.7 ***
HIS	62.0 ± 3.2	74.9 ± 5.1	72.6 ± 4.6	61.8 ± 4.2	88.5 ± 12.7	67.2 ± 7.1 #	67.2 ± 3.4 ##	47.8 ± 3.6 ***
LYS	385.7 ± 69.4	458.7 ± 67.7 #	295.8 ± 17.6 &	252.3 ± 20.5	459.5 ± 72.3	358.3 ± 29.4 ####	281.1 ± 38.1 * ##	163.0 ± 18.5 ****
ORN	100.3 ± 19.5	134.9 ± 16.1 ##	72.8 ± 7.6 &&	65.5 ± 5.1	107.5 ± 4.3	80.3 ± 5.9 * #	88.1 ± 12.7 #	52.2 ± 6.4 ***
ARG	51.6 ± 6.4	57.1 ± 21.3	55.1 ± 2.9	38.1 ± 4.6	73.5 ± 8.7	68.5 ± 4.5 ###	30.9 ± 7.0 ** &&	33.6 ± 5.0 ***
GLU	24.8 ± 4.8	24.0 ± 3.2	22.7 ± 2.2	26.9 ± 4.4	11.3 ± 1.2	19.0 ± 1.9 *	18.7 ± 2.9	25.6 ± 4.8
PRO	148.2 ± 37.9	156.8 ± 27.1	175.1 ± 24.9	127.9 ± 21.1	369.1 ± 72.1	180.0 ± 32.5 * ##	198.4 ± 22.7 * ###	86.0 ± 15.2 ****

Supplemental Table 4. Amino acid Renal clearance. Renal clearance (mL/24h·g body weight) was calculated in mice with the indicated genotypes at 3-4 months of age and after 11 days under 20% protein- or 40% protein-content diets. Data (mean ± SEM) are from the number of male mice per group indicated in the legend to Supplemental Table 2. Unpaired T test was performed to assess differences between genotypes. * is used to indicate significant differences vs wild-type animals (WT), # differences vs dKO mice, and & vs KO LAT2 mice. Number of symbols denote p values, with 1 symbol for $p \leq 0.05$; 2 symbols for $p \leq 0.01$, and 3 symbols for $p \leq 0.001$ and four symbols for $p \leq 0.0001$. WT and dKO denote wild-type and dKO LAT2-TAT1 homozygotes. Amino acids are designated with the three-letter code.

	20% protein diet				40% protein diet			
Amino acid	**WT**	**KO LAT2**	**KO TAT1**	**dKO**	**WT**	**KO LAT2**	**KO TAT1**	**dKO**
TYR	0.07 ± 0.01	0.05 ± 0.01 ###	0.19 ± 0.02 *** ## &&&	0.43 ± 0.05 ****	0.07 ± 0.02	0.08 ± 0.01 ##	0.58 ± 0.08 *** ## &&&	1.49 ± 0.30 **
PHE	0.08 ± 0.01	0.05 ± 0.00 ##	0.06 ± 0.01 ##	0.11 ± 0.01	0.07 ± 0.01	0.07 ± 0.01 ##	0.19 ± 0.04 * ## &	0.76 ± 0.16 *
ILE	0.02 ± 0.00	0.02 ± 0.00 #	0.01 ± 0.00 ** ##	0.03 ± 0.01 *	0.02 ± 0.01	0.02 ± 0.00 ##	0.08 ± 0.02 * ## &	0.40 ± 0.09 *
ALA	0.03 ± 0.00	0.02 ± 0.00 #	0.02 ± 0.00 #	0.05 ± 0.01 **	0.02 ± 0.01	0.03 ± 0.00 ###	0.13 ± 0.04 #	0.27 ± 0.04 **
VAL	0.02 ± 0.00	0.02 ± 0.00 ##	0.02 ± 0.00 #	0.04 ± 0.00 *	0.02 ± 0.00	0.03 ± 0.00 ##	0.08 ± 0.01 ** ## &&	0.31 ± 0.07 *
LEU	0.04 ± 0.01	0.03 ± 0.00 ##	0.03 ± 0.00 ##	0.10 ± 0.02 **	0.04 ± 0.01	0.07 ± 0.01 ##	0.20 ± 0.04 * ## &&	0.97 ± 0.20 **
SER	0.05 ± 0.00	0.06 ± 0.01 ##	0.06 ± 0.01 ##	0.24 ± 0.05 **	0.06 ± 0.01	0.12 ± 0.02 * ##	0.16 ± 0.01 *** ##	1.70 ± 0.34 **
GLN	0.03 ± 0.00	0.05 ± 0.01	0.05 ± 0.01 #	0.18 ± 0.05 *	0.05 ± 0.01	0.09 ± 0.03 ##	0.14 ± 0.02 * ##	0.66 ± 0.13 **
ASN	0.11 ± 0.01	0.09 ± 0.02 ####	0.17 ± 0.03 #	0.28 ± 0.01 ****	0.09 ± 0.03	0.16 ± 0.03 ##	0.22 ± 0.09 ##	1.76 ± 0.32 **
THR	0.15 ± 0.06	0.26 ± 0.09 #	0.19 ± 0.05 ##	0.66 ± 0.09 **	0.09 ± 0.02	0.21 ± 0.04 * ###	0.48 ± 0.07 *** ### &&	3.15 ± 0.46 ***
MET	0.41 ± 0.11	0.21 ± 0.04 ###	0.33 ± 0.04 ## &	1.0 ± 0.12 **	0.12 ± 0.02	0.14 ± 0.02 ##	0.30 ± 0.04 ** ## &&	1.47 ± 0.29 **
GLY	0.12 ± 0.01	0.12 ± 0.02 #	0.19 ± 0.02 * &	0.30 ± 0.06 *	0.15 ± 0.03	0.17 ± 0.01 #	0.46 ± 0.05 *** # &&&	2.26 ± 0.56 *
HIS	0.06 ± 0.01	0.08 ± 0.01 #	0.09 ± 0.02 #	0.41 ± 0.12 *	0.07 ± 0.01	0.14 ± 0.04 ##	0.55 ± 0.11 ** ## &&	3.37 ± 0.75 *
LYS	0.04 ± 0.01	0.02 ± 0.00 ###	0.05 ± 0.00 &&	0.06 ± 0.00 *	0.03 ± 0.00	0.04 ± 0.00 ##	0.08 ± 0.01 * # &&	0.18 ± 0.03 *
ORN	0.02 ± 0.00	0.02 ± 0.00	0.03 ± 0.00	0.04 ± 0.00 *	0.03 ± 0.00	0.04 ± 0.00 #	0.08 ± 0.02	0.19 ± 0.04
ARG	0.07 ± 0.01	0.05 ± 0.01	0.06 ± 0.01	0.07 ± 0.01 *	0.16 ± 0.03	0.09 ± 0.02 #	0.23 ± 0.04 &	0.39 ± 0.07
GLU	0.17 ± 0.07	0.16 ± 0.02	0.17 ± 0.02	0.13 ± 0.03	0.63 ± 0.24	0.17 ± 0.03 ###	0.69 ± 0.20 &	0.59 ± 0.07
PRO	0.40 ± 0.17	0.08 ± 0.02	0.05 ± 0.01 *	0.12 ± 0.03	0.04 ± 0.01	0.16 ± 0.05 #	0.23 ± 0.04 ** ##	2.28 ± 0.56 *

Supplemental Table 5. Amino acid Tubular reabsorption. The percentage of tubular reabsorption was estimated in mice with the indicated genotypes at 3-4 months of age and after 11 days under 20% protein- or 40% protein-content diets. Data (mean ± SEM) are from the number of male mice per group indicated in the legend to Supplemental Table 2. Unpaired T test was performed to assess differences between genotypes. * is used to indicate significant differences vs wild-type animals (WT), # differences vs dKO mice, and & vs KO LAT2 mice. Number of symbols denote p values, with 1 symbol for $p \leq 0.05$; 2 symbols for $p \leq 0.01$, 3 symbols for $p \leq 0.001$ and and four symbols for $p \leq 0.0001$. WT and dKO denote wild-type and dKO LAT2-TAT1 homozygotes. Amino acids are designated with the three-letter code.

	20% protein diet				40% protein diet			
Amino acid	**WT**	**KO LAT2**	**KO TAT1**	**dKO**	**WT**	**KO LAT2**	**KO TAT1**	**dKO**
TYR	98.9 ± 0.1	98.4 ± 0.5 ##	96.6 ± 0.8 *#	92.2 ± 1.6 **	99.2 ± 0.1	98.4 ± 0.2 *#	89.0 ± 0.4 **** &&&&	75.2 ± 5.7 **
PHE	98.8 ± 0.1	98.6 ± 0.2	99.1 ± 0.1 #	98.0 ± 0.4	99.0 ± 0.2	98.9 ± 0.1 #	97.4 ± 0.7 #	88.3 ± 3.4 *
ILE	99.7 ± 0.0	99.5 ± 0.2	99.7 ± 0.1	99.3 ± 0.2	99.8 ± 0.1	99.6 ± 0.0 #	98.6 ± 0.3 *# &	93.3 ± 1.7 *
ALA	99.6 ± 0.1	99.3 ± 0.2	99.6 ± 0.1	99.1 ± 0.3	99.7 ± 0.0	99.4 ± 0.1 *#	99.1 ± 0.2 *#	96.7 ± 0.7 **
VAL	99.6 ± 0.1	99.5 ± 0.2	99.6 ± 0.1	99.3 ± 0.2	99.7 ± 0.0	99.5 ± 0.0 ** #	98.8 ± 0.2 ** # &	95.2 ± 1.2 *
LEU	99.4 ± 0.1	99.0 ± 0.3	99.4 ± 0.2 #	98.1 ± 0.5 *	99.5 ± 0.1	98.7 ± 0.1 ** #	97.1 ± 0.8 *#	84.1 ± 4.2 *
SER	99.2 ± 0.0	99.0 ± 0.1 #	98.9 ± 0.2 #	95.5 ± 1.3 *	99.3 ± 0.1	97.7 ± 0.2 *** #	97.2 ± 0.1 **** #	72.1 ± 7.3 *
GLN	99.2 ± 0.3	98.2 ± 0.7	99.1 ± 0.3	96.5 ± 1.4	99.2 ± 0.2	97.3 ± 0.9 #	96.6 ± 1.3	89.7 ± 2.7 *
ASN	98.3 ± 0.2	98.2 ± 0.6	97.1 ± 0.6	93.4 ± 2.3	99.0 ± 0.1	95.1 ± 1.7 #	94.1 ± 0.9 ** #	74.6 ± 6.6 *
THR	97.6 ± 0.8	95.0 ± 1.3	96.9 ± 0.8 #	87.5 ± 3.4 *	98.6 ± 0.3	95.6 ± 0.2 ***	89.9 ± 3.0 *	71.8 ± 11.3
MET	93.6 ± 1.7	92.0 ± 3.4	92.6 ± 1.9	81.3 ± 4.8 *	98.3 ± 0.3	97.2 ± 0.3 *	96.0 ± 0.6 *#	78.8 ± 6.6 *
GLY	98.2 ± 0.2	97.5 ± 0.6	96.8 ± 0.5 *	94.1 ± 2.0	97.5 ± 0.7	97.2 ± 0.3 #	93.9 ± 0.4 ** &&&	61.9 ± 12.0 *
HIS	98.7 ± 0.4	97.6 ± 0.8	98.3 ± 0.5	91.7 ± 3.4	98.9 ± 0.2	96.2 ± 1.1 ##	91.6 ± 2.3 *#	66.7 ± 9.2 *
LYS	99.4 ± 0.1	99.3 ± 0.2	99.2 ± 0.1	98.9 ± 0.2 *	99.5 ± 0.1	99.3 ± 0.0 *#	98.8 ± 0.3 *	97.5 ± 0.6 *
ORN	99.7 ± 0.0	99.6 ± 0.1	99.5 ± 0.2	99.3 ± 0.1 *	99.5 ± 0.0	99.3 ± 0.0 *	99.3 ± 0.1	96.8 ± 0.9 *
ARG	98.3 ± 0.5	97.8 ± 1.2	97.9 ± 1.1	98.6 ± 0.4	97.7 ± 0.5	98.2 ± 0.3 #	96.4 ± 1.0	94.3 ± 1.0 *
GLU	97.5 ± 1.0	93.0 ± 3.5	95.5 ± 1.6	95.3 ± 2.7	92.4 ± 3.7	96.7 ± 0.7 #	92.8 ± 3.7	91.6 ± 1.2
PRO	98.1 ± 0.9	96.9 ± 1.4	99.1 ± 0.2	97.7 ± 0.9	99.6 ± 0.1	96.3 ± 0.5 ***	96.6 ± 1.0 *	73.5 ± 11.9

Supplemental Table 6. Amino acid transporter genes and primers used for RNA screening. All gene variants included whenever possible. Information provided based on MIQE's recommendations[6].

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SLC family	Gene name	Protein name	Variant	RefSeq	Forward/ Reverse primers (5' - 3')	amplicon size (nt)
SLC1	*Slc1a4*	ASCT1		NM_018861.3	gcccacacatgacctctctc / cccttccacattcaccaca	79
	Slc1a5	ASCT2		NM_009201.2	attttcccctccaatctggt / tgggttcatatgaggtagcaaa	61
SLC3	*Slc3a1*	rBAT		NM_009205.2	ccatgtcaacggtgtaacca / gccagctggagtttccatac	65
	Slc3a2	4F2hc	C	NM_008577.4	caaagtgccaagaaaaagagc / ctgagcagggaggaaccac	87
			1	NM_001161413.1	no assay could be designed	
			2	NM_008577.4	no assay could be designed	
SLC6	*Slc6a15*	B^0AT2	C	NM_175328.3	taggtctggggaatgtgtgg / cggcaaaagatatgcacca	71
			1	NM_175328.3	no assay could be designed	
			2	NM_001252330.1	no assay could be designed	
	Slc6a19	B^0AT1		NM_028878.3	cttcccctacctatgccaga / aaggatgaggaatgggatca	61
	Slc6a20a	XTRP3A		NM_139142.2	gcccctacctcagtggtgt / ccaggcattgatgacattgta	83
SLC7	*Slc7a5*	LAT1		NM_011404.3	atgtggctccgattcaaga / ggagggccagattcacct	61
	Slc7a6	y^+LAT2		NM_178798.3	tatgtggcctgccgtctc / cgtaggcacagttcacaaatg	67
	Slc7a7	y^+LAT1	C	NM_011405.3	agctgtggcgctccctat / gcacagttaatgaaggttaagagaca	77
			1	NM_011405.3	ccagggtcctgtgtttgc / atgggggtgtgacttcagc	78
			2	NM_001253679.1	attgaggctggccttgaac / gggctagacatggtcattaaaact	na
			3	NM_001253680.1	ggagagactttgggggtca / aggcatgaatgaggcagttt	64
	Slc7a8	LAT2		NM_016972.2	ttactcttatgtgaaggacatcttcg / ccagcacagcaatccaca	68
	Slc7a9	$b^{0,+}AT$	C	NM_021291.3	aacggagctcttgcagtcc / cccaagatgctggatagagaa	71
			1	NM_021291.3	tggcttgacttgctgggta / gagaggagagtctcttgagtcagc	103
			2	NM_001199015.1	tgagagagtatcaccgactcaga / ccgaggtagctgaggtagga	114
			3	NM_001199016.1	no assay could be designed	
	Slc7a1	Asc-1		NM_017394.4	tggctggaacttcctcaact / gatggcacgaggtaggttct	70

Supplemental Table 7. Thermal protocol for 96.96 chip RT-qPCR.

Analysis mode	Segment	Temperature (ºC)	Time	Cycles
Thermal mix				
	T1	50	2 min	1
	T2	70	30 min	
	T3	25	10 min	
UNG and Hot Start				
	UNG	50	2 min	1
	Hot Start	95	10 min	
PCR Cycle				
	Denaturation	95	15 sec	40
	Annealing	70	5 sec	
	Extension	60	1 min	

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