Supplementary information

List:

Supplemental data

Supplemental Experimental Procedures

Supplemental Data

Table S1. Protein containing diet compositions.

	10F - 20P		10F - 5P		60F - 20P		60F - 5P	
kcal/gm	3,8		3,8		5,2		5,2	
	gram (%)	**kcal (%)**	**%gm**	**%kcal**	**%gm**	**%kcal**	**%gm**	**%kcal**
Protein	19	20	5	5	26	20	7	5
Carbohydrate	67	70	82	85	26	20	46	35
Fat	4	10	4	10	35	60	35	60
Ingredient	**gram**	**kcal**	**gram**	**kcal**	**gram**	**kcal**	**gram**	**kcal**
Casein	200	800	50	200	200	800	50	200
L-Cystine	3	12	0,75	3	3	12	0,75	3
Corn Starch	315	1260	527,25	2109	0	0	125	500
Maltodextrin 10	35	140	75	300	125	500	152,3	609
Sucrose	350	1400	250	1000	68,8	275	68,8	275
Cellulose, BW200	50	0	50	0	50	0	50	0
Soybean Oil	25	225	25	225	25	225	25	225
Lard	20	180	20	180	245	2205	245	2205
Mineral Mix S10026	10	0	10	0	10	0	10	0
DiCalcium Phosphate	13	0	13	0	13	0	13	0
Calcium Carbonate	5,5	0	5,5	0	5,5	0	5,5	0
Potassium Citrate, 1 H2O	16,5	0	16,5	0	16,5	0	16,5	0
Vitamin Mix V10001	10	40	10	40	10	40	10	40
Choline Bitartrate	2	0	2	0	2	0	2	0
FD&C Yellow Dye #5	0,05	0	0	0	0	0	0	0
FD&C Red Dye #40	0	0	0,025	0	0	0	0,05	0
FD&C Blue Dye #1	0	0	0,025	0	0,05	0	0	0
Total	1055,05	4057	1055,05	4057	773,85	4057	773,9	4057

Table S2. Amino acid containing diet compositions.

	18% AA		4.5% AA	
kcal/gm	3,8		3,8	
	gram (%)	**kcal (%)**	**gram (%)**	**kcal (%)**
Protein	17	18	4	4,5
Carbohydrate	68	72	81	85
Fat	4	10	4	10
Ingredient	**gram**	**kcal**	**gram**	**kcal**
L-Arginine	6	24	1,5	6
L-Histidine-HCl H2O	4,6	18	1,15	5
L-Isoleucine	7,6	30	1,9	8
L-Leucine	15,8	63	3,95	16
L-Lysine	13,2	53	3,3	13
L-Methionine	5,1	20	1,275	5
L-Phenylalanine	8,4	34	2,1	8
L-Threonine	7,2	29	1,8	7
L-Tryptophan	2,1	8	0,525	2
L-Valine	9,3	37	2,325	9
L-Alanine	5,1	20	1,275	5
L-Aspartic Acid	12,1	48	3,025	12
L-Cystine	4,2	17	1,05	4
L-Glutamic Acid	38,2	153	9,55	38
Glycine	3	12	0,75	3
L-Proline	17,8	71	4,45	18
L-Serine	10	40	2,5	10
L-Tyrosine	9,2	37	2,3	9
Corn Starch	315	1260	429,3	1717
Maltodextrin 10	35	140	55	220
Sucrose	350	1400	350	1400
Cellulose	50	0	50	0
Soybean Oil	25	225	25	225
Lard	20	180	20	180
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5,5	0	5,5	0
Potassium Citrate, 1 H2O	16,5	0	16,5	0
Sodium Bicarbonate	7,5	0	7,5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Yellow Dye #5	0,05	0	0	0
FD&C Red Dye #40	0	0	0,05	0
Total	1038	3959	1039	3960

Table S3. Human study: diet compositions.

	Habitual diet	Protein restricted diet
Energy, MJ/d	9.4 ± 0.8	12.8 ± 0.6
Alcohol	0 ± 0	0 ± 0
Protein %E	20.2 ± 0.5	9.0 ± 0
CHO %E	43.6 ± 0.6	71.0 ± 0
Fat %E	36.1 ± 0.9	20.0 ± 0
Protein, g/d	111 ± 11	73 ± 3
Protein, g/kg BW/d	1.57 ± 0.22	0.97 ± 0.03

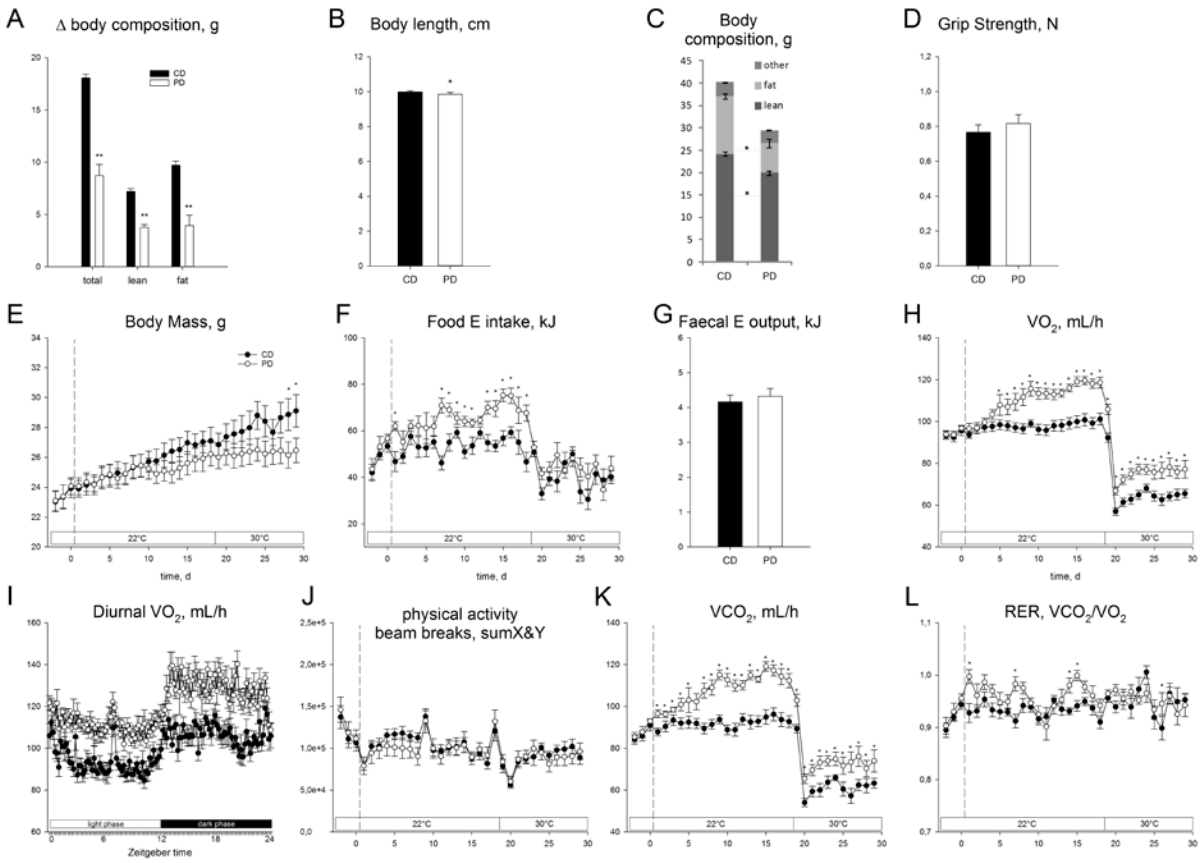

Figure S1.

Related to Figure 1.

(A): The change in total body mass and composition, measured by ECHO-MRI, between wk 0 and wk 16 in mice fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. N = 5-6/group.

(B): Body length at wk 16 of mice as in (A).

(C): Body composition measured by ECHO-MRI of mice at wk 16 as in (A).

(D): Grip strength of mice treated similarly as in (A).

(E): Body mass accrual in mice fed CD or PD before (pre) and during 3 wk at an ambient temperature of 22°C and a subsequent 2 weeks at 30°C. n= 10/group.

(F): Daily food energy (E) intake from mice as in (E).

(G): Faecal E output from mice as in (E).

(H): 24h-averaged oxygen consumption rate (VO2) from mice as in (E).

(I): Diurnal VO2 response during d16 from mice as in (E).

(J): Physical activity as measured by beam breaks in both horizontal axes by mice as in (E).

(K): 24h-averaged carbon dioxide production rate (VCO2) from mice as in (E).

(L): Respiratory exchange ratio (RER) from mice as in (E).

Data are mean ± SEM. Significant difference between CD and PD; *P<0.05, **P<0.01, ***P<0.001.

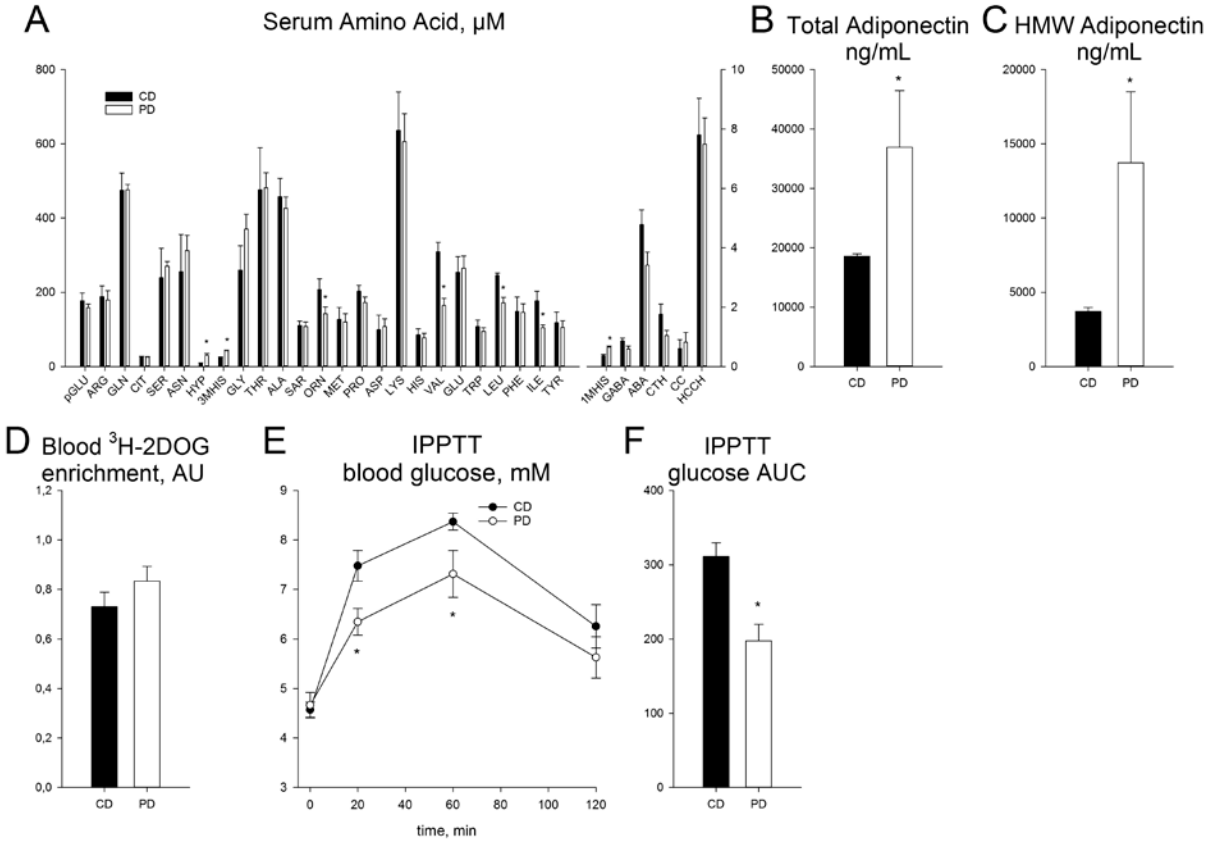

Figure S2.

Related to Figure 2.

(A): Blood serum amino acid profile in mice fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. N = 5-6/group.

(B): Total blood serum adiponectin levels in mice as in (A).

(C): Blood serum high molecular weight (HMW) adiponectin levels in mice as in (A).

(D): Average blood ^{3}H-2-deoxy-D-glucose enrichment in mice during an intraperitoneal glucose tolerance test after 2wk administration of CD or PD. N=6/group.

(E): Blood glucose excursion during an intraperitoneal pyruvate tolerance test (IPPTT) from mice treated similarly as in (D). N=6/group.

(F): The glucose area under the curve from (E).

Data are mean ± SEM. Significant difference between CD and PD; *P<0.05, **P<0.01, ***P<0.001.

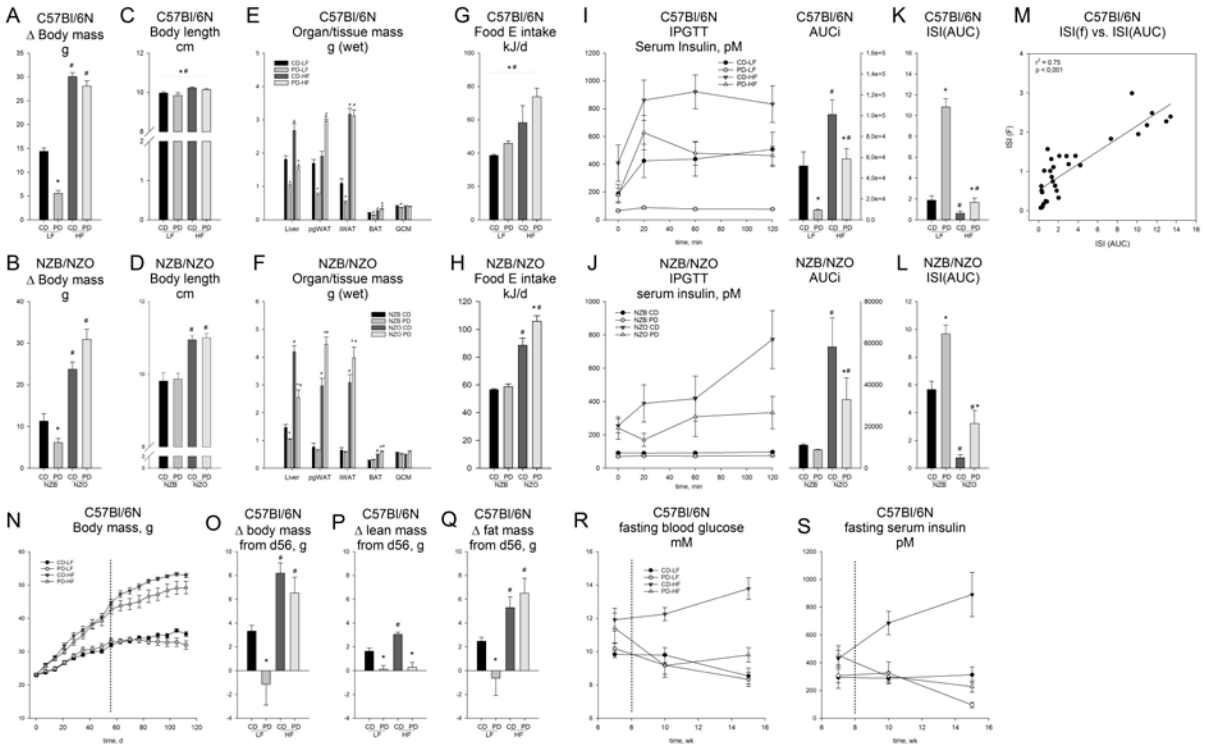

Figure S3.

Related to Figure 3.

(A): C57Bl/6N mice fed either control diets (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate; with either 10% (LF) or 60% (HF) calories from fat. N = 7-8/group.

(B): The change in body mass between the start and end of the dietary intervention in New Zealand Black (NZB) or New Zealand Obese (NZO) mice fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. N = 6-8/group.

(C): Body length at wk 16 from mice as in (A).

(D): Body length at wk 10 from mice as in (B).

(E): Tissue/organ mass upon necropsy at wk 16 from mice as in (A). pgWAT: perigonadal white adipose tissue; iWAT: inguinal white adipose tissue; GCM: gastrocnemius complex muscle; BAT: brown adipose tissue.

(F): Tissue/organ mass upon necropsy at wk 10 from mice as in (B). pgWAT: perigonadal white adipose tissue; iWAT: inguinal white adipose tissue; GCM: gastrocnemius complex muscle; BAT: brown adipose tissue.

(G): Food energy (E) intake rate from mice as in (A).

(H): Food energy (E) intake rate from mice as in (B).

(I): Blood serum insulin excursion during an intraperitoneal glucose tolerance test (IPGTT) in mice as in (A). AUCi: insulin area under the curve.

(J): Blood serum insulin excursion during an intraperitoneal glucose tolerance test (IPGTT) in mice as in (B).

(K): An insulin sensitivity index derived from the inverse of the product of the glucose and insulin AUC (ISI(AUC))from the IPGTT

(L): ISI(AUC) from mice as in (B).

(M): A scatter plot demonstrating a positive linear relationship between the ISI(AUC) and the ISI derived from the fasting glucose and insulin levels (ISI(f)).

(N): Body mass accrual from mice before and after a maintenance on the same diet or a diet switch (d56) to a respective protein-diluted diet. N = 7-8/group.

(O): The change in body mass between d56 and end of the dietary intervention from mice as in (N).

(P): The change in lean mass between d56 and end of the dietary intervention from mice as in (N).

(Q): The change in fat mass between d56 and end of the dietary intervention from mice as in (N).

(R): The change in blood glucose from at selected time points before and after the dietary intervention from mice as in (N).

(S): The change in blood serum insulin from at selected time points before and after the dietary intervention from mice as in (N).

Data are mean ± SEM. Significant effect of dietary protein: *P<0.05. Significant effect of dietary fat/strain: $^{\#}P < 0.05$.

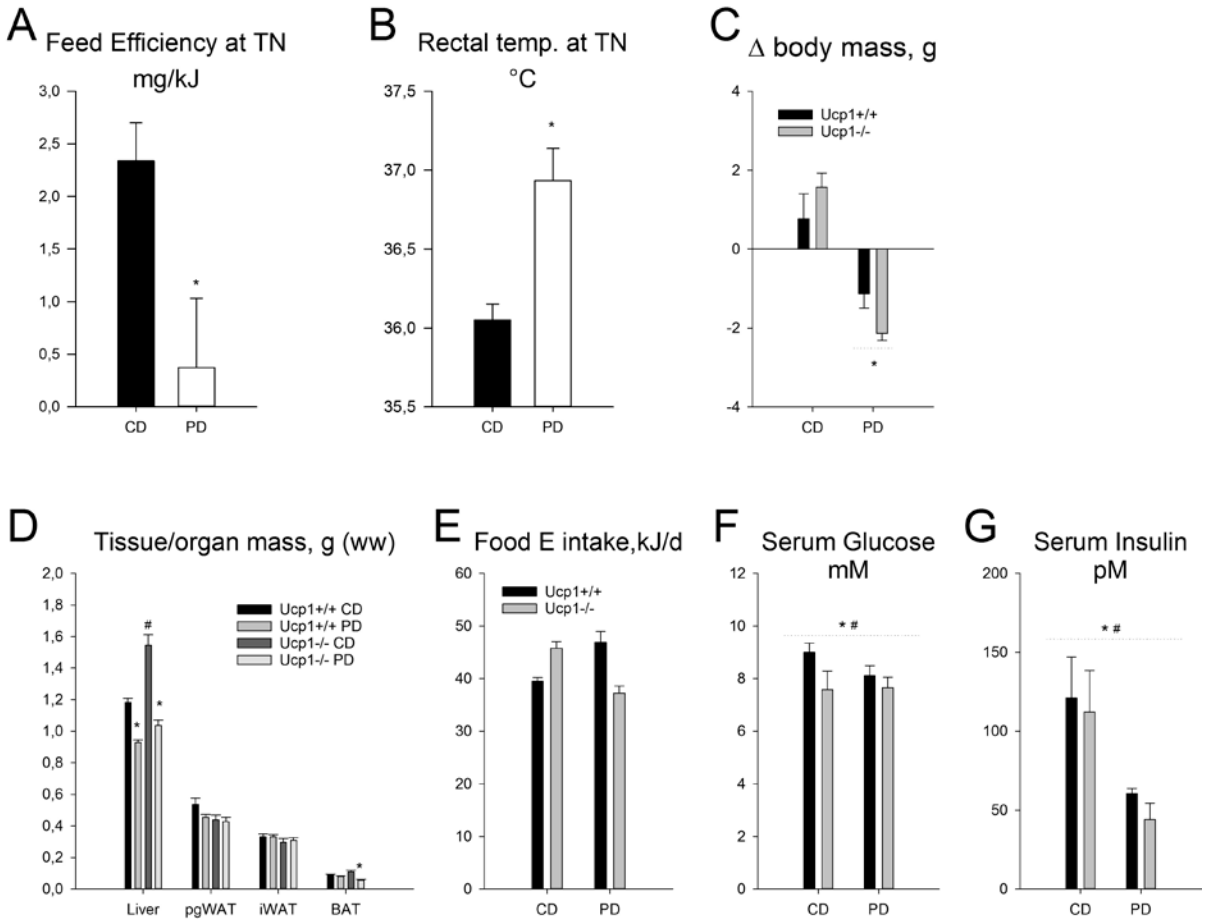

Figure S4.

Related to Figure 4.

(A): Feed efficiency of mice housed under thermoneutral (TN; 30°C) conditions and fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. Studies were conducted for 3wk after a one week period of adaptation to 30°C. N = 5-6/group.

(B): Rectal temperature from mice as in (A).

(C): The change in body mass of wild-type (Ucp1+/+) and Ucp1 knockout (Ucp1-/-) mice fed a control diet (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate. Studies were conducted for 6 wk. N = 6-9/group.

(D): Tissue/organ mass at the endpoint necropsy of mice as in (C). pgWAT: perigonadal white adipose tissue; iWAT: inguinal white adipose tissue.

(E): Food energy (E) intake rates from mice as in (C).

(F): Fasting blood glucose from mice as in (C).

(G): Fasting serum insulin from mice as in (C).

Data are mean ± SEM. Significant effect of dietary protein, *$P<0.05$. Significant effect of dietary fat or genotype, $^{\#}P<0.05$.

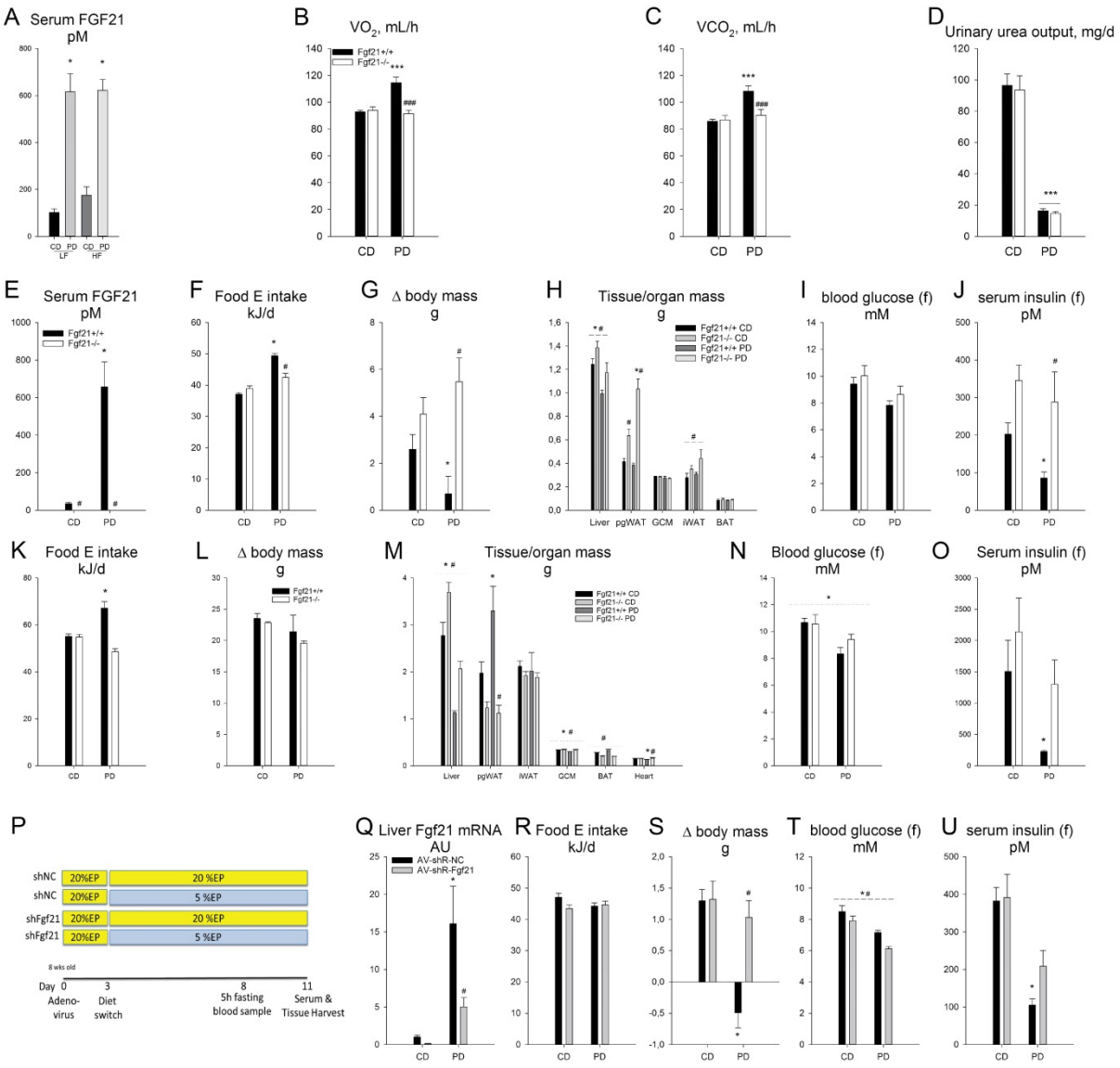

Figure S5

Related to Figure 5.

(A): Serum FGF21 levels from C57Bl/6N mice fed either control diets (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate; with either 10% (LF) or 60% (HF) calories from fat. N = 7-8/group.

(B) Oxygen consumption rate (VO_2) of whole-body Fgf21-/- and Fgf21+/+ mice studied on low-fat CD or PD in a balanced, cross-over manner. N =8-10/group.

(C) Carbon dioxide production rate (VCO2) of mice as in (B).

(D) 24h urinary urea output of mice as in (B).

(E) Serum FGF21 levels in whole-body Fgf21-/- and Fgf21+/+ mice following CD or PD feeding for 7 wk. N=5-6/group.

(F) Average daily energy (E) intake over the first four weeks of CD or PD feeding for mice in (B).

(G) Delta body mass over the food intake measurement period in (C).

(H) Endpoint tissue/organ masses of mice in (B).

(I) Blood glucose levels from 5 hour fasted mice (from (B)) following 5 weeks of diet feeding.

(J) Serum insulin levels measured in parallel with (F).

(K) Food E intake in whole-body Fgf21-/- and Fgf21+/+ mice over an 8wk period during 16 wk of high-fat CD or PD feeding. N=5-6/group.

(L) Body mass accrual over the 8wk period as in (H).

(M) Tissue/organ masses of mice treated and sacrificed at wk 16 as in (H).

(N) 5-6h fasting blood glucose levels at wk 15 from mice as in (H).

(O) Serum insulin levels measured in parallel with (H).

(P) Schematic of adenovirus study designed to test the role of hepatic knockdown of Fgf21 on the metabolic adaptation to PD feeding.

(Q) Liver Fgf21 transcript at the study endpoint in (H).

(R) Food intake between days 3 and 11 for mice in (H).

(S) Changes in body mass over the period in (J).

(T) Blood glucose on day 8 following a 5 hour fast for mice in (H).

(U) Serum insulin levels measured in parallel with (L).

Data are presented as means ± SEM. *, effect of PD. #, effect of genotype/virus. $P<0.05$.

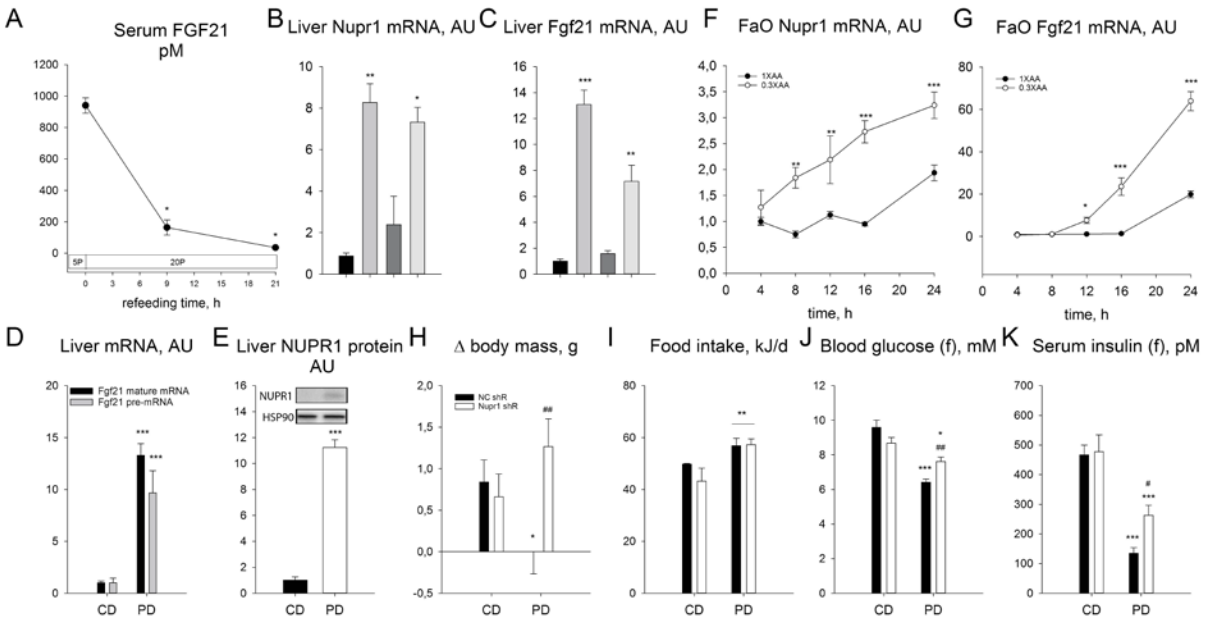

Figure S6

Related to Figure 6.

(A) Serum FGF21 following adaptation to PD (2wk) is rapidly reversed following switch to CD. N=5/group.

(B) Liver Nupr1 mRNA expression from C57Bl/6N mice fed either control diets (CD) containing 20% caloric energy from protein (CD) or a protein-diluted (PD) diet containing 5% caloric energy from protein, diluted by added carbohydrate; with either 10% (LF) or 60% (HF) calories from fat. N = 6-8/group.

(C) Liver Fgf21 mRNA expression from mice as in (B).

(D) Liver Fgf21 mature and immature (i.e. pre-mRNA) mRNA levels from ice as in Fig. 1A.

(E) Liver NUPR1 and housekeeping protein HSP90 expression in overnight fasted and then 6h refed mice following a 2wk adaptation to the respective diets. N=6/group.

(F) Nupr1 mRNA levels from cultured Fao hepatic cells following exposure to normal (1X) or restricted (0.3X) media total AA concentrations for selected times. N=3/group.

(G) Fgf21 mRNA levels from Fao cells as in (E).

(H) Body mass accrual of CD or PD-fed mice given adenoviruses overexpressing negative control (NC) or Nupr1-selective shRNAs in the liver using a similar experimental design as in Fig. SF5P. N=5-6/group.

(I) Food energy intake of mice as in (G).

(J) Fasting blood glucose levels of mice as in (G).

(K) Fasting serum insulin levels of mice as in (G).

Data are presented as means ± SEM. *, difference between diets. #, difference between genotype/virus. E-F: * difference vs 4h. *P<0.05, **P<0.01, ***P<0.001.

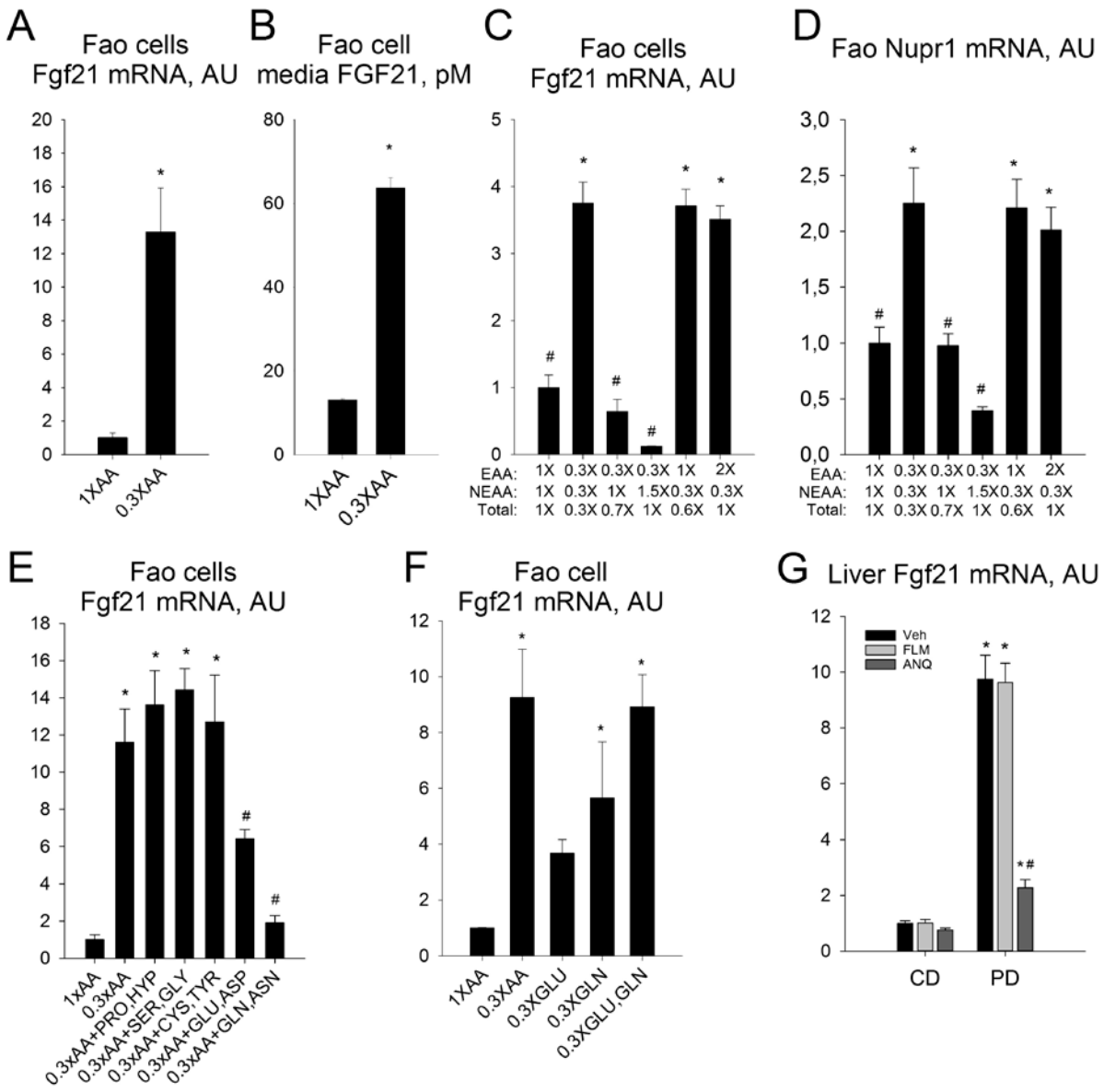

Figure S7.

Related to Figure 7.

(A) Fgf21 mRNA levels from cultured Fao hepatic cells following exposure to normal (1X) or restricted (0.3X) media total AA concentrations. N=3/group.

(B) Media FGF21 levels from Fao cells as in (A).

(C) Effect of media total AA restriction, with or without differential essential (EAA) or non-essential (NEAA) amino acid supplementations on Fgf21 mRNA in cultured rat Fao hepatic cells. N=3/group.

(D) Nupr1 mRNA levels from Fao cells as in (C).

(E) Effect of media total AA restriction, with selected groups of NEAA supplementations on Fgf21 mRNA in cultured rat Fao hepatic cells. N=3/group. All indicated amino acids were added to match their concentration in the 1x AA group.

(F) Fao cell Fgf21 mRNA in response to total amino acid restriction or restriction of select amino acids. N=3/group.

(G) Liver Fgf21 mRNA levels following overnight fasting and refeeding in mice on adapted for 2wk to CD or PD combined with intraperitoneal administration of vehicle (0.9% NaCl) select EAA (i.e. Phe, Leu, Met; FLM), or NEAA (i.e. Ala, Asn, Gln; ANQ) (8mg each, 24mg; ~1mg/g BM). N=4/group.

Data are presented as means ± SEM. *, different from t=0 (A) or CD/1x AA (D-F). #, different from AA-restricted group (0.1x AA) or Veh. $P<0.05$.

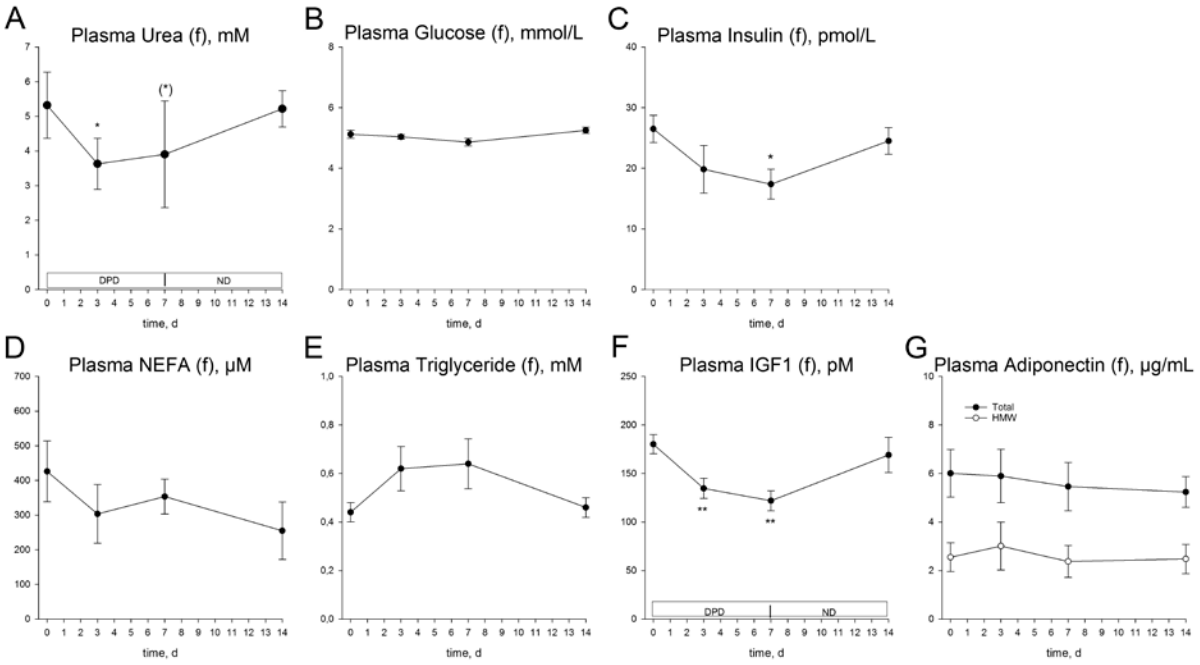

Figure S8.

Related to Figure 8.

(A): Fasting blood plasma urea levels in young men subjected to a 7d period of consumption of a protein diluted diet (DPD) followed by consumption of their regular mixed diet (ND) for a further 7d. N=5.

(B): Fasting plasma glucose levels in men as in (A).

(C): Fasting plasma insulin levels in men as in (A).

(D): Fasting plasma non-esterified fatty acid (NEFA) levels in men as in (A).

(E): Fasting plasma triglyceride levels in men as in (A).

(F): Fasting plasma insulin like growth factor 1 (IGF1) levels in men as in (A).

(G): Fasting plasma adiponectin levels in men as in (A). HMW: high molecular weight.

Data are mean ± SEM. Significant effect of dietary protein/AA level; *P<0.05. Significant effect of time, $^{\#}$P<0.05.

Supplemental Experimental Procedures

Materials. All materials were from Sigma-Aldrich unless stated otherwise.

Animal experiments. Mice used were male C57Bl/6NCrl (027; Charles River Laboratories, Sultzfeld, DEU), as well as News Zealand Black (000993; NZB/BlNJ) and New Zealand Obese (002105; NZO/HlLtJ, Jackson Laboratories, USA) strains. In addition, male *Ucp1*-/- (1), *Fgf21*-/- (2, 3) and *Nupr1*-/- (4) and corresponding +/+ littermate mice from +/- crossings on the C57Bl/6J background were used. For diet studies, 7 wk old mice were initially fed a standard high-carbohydrate diet (Research diets D12450Bi, New Brunswick, USA) for one week after which they were either maintained on this diet or switched to one of the following diets: high-carbohydrate with low protein, or a high-fat diet containing either normal or low protein. The same strategy was used for amino acid containing diets.

To test the effects of nutrition *per se* on metabolic/hormonal responses to dietary treatments, mice were adapted to diets for at least 7d, after which time mice were fasted overnight followed by readministration of the respective diets. Blood samples were taken in the fasted state as well as at 6h and 24h of refeeding. To test the effects of specific amino acids on the responses to refeeding, the same protocol was followed but with food withdrawl after 2h of refeeding with a single intraperitoneal administration of a mixture of selected essential amino acids (i.e. Leu, Met, Phe), non-essential amino acids (i.e. Ala, Asn, Gln), or vehicle control (i.e. 0.9% NaCl). The amino acid mixtures were delivered in a volume of 200µL with 8mg of each amino acid which constituted a total of 24mg and thus ~1 mg/g body mass. The mice rested for a further 4h after IP injection. Tail vein blood was collected and the mice were subsequently sacrificed and tissues harvested.

To test whether the metabolic effects of 5P feeding could be influenced by quality of supplementary carbohydrates, we compared in a head-to-head manner the metabolic effects of 5P:85C feeding for 4 weeks to our conventional 20P:70C diet as well as an additional 20P:70C diet differing from the first only in carbohydrate quality (lower sucrose, higher complex carbohydrates). Interestingly, the salutary effects of protein restriction on key metabolic parameters (metabolic efficiency, glucose homeostasis, serum TGs) remained significantly changed regardless of the carbohydrate quality of the 20P comparator group and importantly, 20P diets differing only in carbohydrate composition produced identical metabolic profiles in mice (data not shown).

Details of the diet compositions are outlined in Supplementary Tables 1 & 2 (Research diets, New Brunswick, USA). Animal experiments were conducted according to local, national, and EU ethical guidelines, and adhered to ARRIVE guidelines.

Adenovirus studies. Adenoviruses expressing FGF21-specific (target sequence GCATGGTAGAGCCTTTACAGG), NUPR1-specific (target sequence GATACAGGACCTTGGAGAAAT) or nonspecific negative control (target sequence GATCTGATCGACACTGTAATG) shRNAs under the control of the U6 promoter were generated as previously described (5). Male C57Bl/6N mice, aged 8 weeks, received respective adenoviruses i.v. ($2x10^9$ ifu/mouse) (day 0). At day 3, half of the mice from each virus group were switched to protein restriction (5P:85C:10F). A 5h fasting blood sample was taken from the tail vein (day 8) for calculation of ISI, and mice were killed and tissues were harvested on day 11. A schematic picture of the experiment design can be found in Fig. S5H.

ISRIB experiment. Mice were adapted to diets for at least 12d, after which time mice were fasted overnight followed by readministration of the respective diets for 2h. Food was then withdrawn and mice were intraperitoneally administered *trans*-ISRIB (5095840001, Merck-Millipore, DEU; 2.5mg/kg; ~60µg in 100µL ca. 12.5% DMSO, 12.5% PEG400, 75% 0.9% NaCl) or corresponding vehicle (Sidrauski et al. 2013) in a total volume of 100µL. The mice rested for a further 4h after IP injection. Tail vein blood was collected and the mice were subsequently sacrificed and tissues harvested.

Behavioural and metabolic phenotyping. For all metabolic experiments, general considerations outlined by the EMPRESS protocols (http://empress.har.mrc.ac.uk) were followed. Mice were individually housed for about one week prior to entering the PhenoMaster Cage System (TSE Systems, Bad Homburg, Germany) which enables simultaneous determination of indirect calorimetry, 3D activity, as well as food and water consumption. Mice were then housed individually in the system for a total of 35d. Calculations of oxygen consumption, carbon dioxide production rates and respiratory exchange ratio were performed according to established guidelines (6, 7), beginning after a 3d period of adaptation to the system. All mice were maintained on a 12h light-dark cycle at 22°C with unrestricted access to food and water. Feed efficiency was calculated based upon the quotient of body mass accrual (mg) and food energy intake (kJ) during a selected growth period. Upon exiting the system, body composition was determined by an Echo magnetic resonance imaging (ECHO-MRI) body composition analyser (Echo Medical Systems, Houston, USA). Faecal energy output was measured with the IKA C7000 calorimeter (IKA, Staufen, Germany) from an aliquot of lyophilised faecal material collected over a 24h period. 24h urine collection was performed in custom-made individual housing units as conducted previously (8). Nutrient oxidation rates were calculated from 24h rates of O_2 consumption,

CO_2 production and urinary urea production as described (9). Rectal temperature was measured using a sensor (N856-1) and digital measurement device (Almemo 2390-1; Ahlborn Mess- und Regelungstechnik GmbH, Germany), and were made between ZT3-5 on two separate days in a randomised order at standard laboratory temperature (i.e. 22-24°C). Neuromuscular function was assessed using a grip strength tester (BIO-GS3, BIOSEB, Vitrolles Cedex, France) with the average of two measurements taken.

Assessment of glucose homeostasis. Blood glucose measurements for all tests were determined with an Accu-Chek Performa glucometer (Roche, Mannheim, Germany). For the intraperitoneal glucose tolerance test, following a 5-6 h fast, mice were injected i.p. with glucose (1g/kg body weight). Blood samples were taken from the tail vein before and 20, 60 and 120 min after the injection. For the intraperitoneal insulin tolerance test, following a 5-6 h fast, mice were injected intraperitoneally with insulin (Huminsulin Normal, 0.6 U/kg, Eli Lilly, Bad Homburg Germany) in 0.9% NaCl. Tail vein blood glucose was monitored before and 20, 60 and 120 min after the injection. For the intraperitoneal pyruvate tolerance test, following a 16-18h fast, pyruvate was injected intraperitoneally (2g/kg body weight) and tail vein blood glucose measured before and 20, 60 and 120 min after the injection. Glucose (AUCg) and insulin (AUCi) integrated areas under the curve from 0 mM were calculated and an insulin sensitivity index (ISI(AUC)) was calculated from the following equation: $1x10^9$/(AUCg x AUCi). An ISI was also calculated based upon 5-6h fasting blood glucose and serum insulin values (ISI(f)): 1000/(glucose (mM) x insulin (pM)). For human analyses, the quantitative insulin sensitivity check index (QUICKI) was calculated from the inverse of the sum of the logarithms of the fasting insulin and fasting glucose: 1 / (log(fasting insulin µU/mL) + log(fasting glucose mg/dL)) (10).

For the assessment of tissue glucose uptake *in vivo*, mice were fasted for 5-6 hours before intraperitoneal injection of Humulin insulin (0.6U/kg) together with 2-[1,2-3H(N)]-Deoxy-D-glucose (NET328A, Perkin Elmer). Blood samples were taken from the tail vein before and 15, 30 and 45 min after the injection of tracer/insulin for blood glucose monitoring as well as for determination of specific activity of glucose tracer in the blood. Subsequently, mice were sacrificed and tissues were excised, weighed and rinsed in ice-cold PBS and subsequently snap frozen in LN_2. Tissue glucose disposal rates were calculated from the average blood glucose specific activity as well as the tissue accumulation of phosphorylated-2DOG, essentially as described previously (11, 12).

Human experiments. Five healthy, lean male volunteers, age 25.6 ± 0.4 years, body weight 75.9 ± 5.3 kg (mean ± SEM), participated in the human diet study. Subjects consumed a controlled diet low in protein for 7 days followed by a wash-out period for 7 days on their habitual, mixed diet. The low protein diet comprised 9 E% protein, 71 E% carbohydrates and 20 E% fat. The protein content was significantly lower in DPR compared to the habitual, mixed diet which amounted to 20 E% protein. The diet compositions are outlines in Table S3. The protein restricted diet comprised of (by weight) 51% fruits and vegetables, 25% bread and pasta, 17% juice, conserves and condiments, 4% sweets, 1% meat, 1% dairy products, and 1% nuts and oils. Expressed per kg bodyweight protein intake averaged 0.94±0.03 and 1.57±0.22 g/kg/day in the DPR and habitual, mixed diet, respectively. Subjects followed a fixed meal plan and food items, weighed to 1 gram of accuracy, were delivered to the subjects from the Metabolic kitchen.

Prior to the commencement of the low protein diet period, subjects arrived in the morning at the laboratory after an overnight fast (10h). After 30 min in the resting, supine position blood

samples were drawn from an antecubital vein. Thereafter the subjects commenced the low protein diet, which was consumed for 7 days. To study the acute effect of the dietary switch to low protein intake, a blood sample was obtained after three days on the diet and then again after 7 days. Thereafter subjects returned to their habitual, mixed diet for 7 days and another blood sample was obtained. All blood samples were obtained in the morning in the postabsorptive state as described for the first blood sample. Furthermore, a meal test was conducted before as well as at d7 of the experiment, whereby subjects ate an isocaloric meal (i.e. 60 kJ/kg BM) containing 15% or 9% protein with blood samples taken before as well as at selected times after the meal consumption. All subjects gave informed consent after the nature and possible consequences of the study were explained. The study was approved by the Copenhagen Ethics Committee (journal number H-3-2012-129) and was conducted in accordance with the code of ethics of the World Medical Association (Helsinki II declaration).

Cell culture. Fao rat hepatoma cells were subcultured in standard RPMI (Life technologies #21875091) containing 10% FBS and 1% penicillin/streptomycin (P/S). Cells were seeded with this medium in 12-well plates at a density of $4.5x10^5$ cells/well one day prior to experiments. A custom-made treatment media, containing salts and buffers based upon HBSS, was used for experiments, and additives (glutathione, glucose, and RPMI vitamins) added to match the composition of standard RPMI. Amino acids, at RPMI concentrations, as well as dialysed FBS and P/S were added separately.

Mouse primary hepatocytes were isolated from C57Bl/6 mice and plated on collagen-coated 12-well plates as described (13). Attachment media mimicked William's Media E (Genaxxon GmbH, Ulm, Germany), but contained amino acid concentrations based upon those in the rat portal vein (14) and contained 100 nM dexamethasone and 1.74 µM human insulin. Four

hours after plating, cells were washed and incubated overnight in the same media, but without insulin and dexamethasone. Cells were then treated for 18 hours with WME containing the indicated amino acid concentrations, 20 nM human insulin, 10% dialysed FBS and P/S. Media was centrifuged at 500x g for 10 mins to remove cell debris and supernatants frozen at -80°C until FGF21 measurement. Cells were harvested in Trizol and frozen until RNA extraction. Human cryopreserved primary hepatocytes (HMCPIS, Lot HU1359, Life technologies, DEU) were thawed and plated according to the manufacturer's specifications, and experiments conducted as described above for mouse primary hepatocytes.

Serum, urine & media metabolite & hormone analyses. For mouse samples, commercially available kits were used to measure serum urea (Z5030016, Biochain), cholesterol (CH200, Randox), non-esterified fatty acids (NEFA; NEFA-HR, Wako), glycerol/triglyceride (TR-0100; Sigma-Aldrich), β-hydoxybutyrate (Autokit 3-HB, Wako), insulin (10-1247-01, Mercodia), insulin-like growth factor 1 (EMI1001-1, AssayPro), thyroxine (T4; T4044T-100, Calbiotech), leptin (MOB00, R&D systems), adiponectin (47-ADPMS-E01, Alpco) and fibroblast growth factor 21 (FGF21; MF2100, R&D Systems). For serum insulin measurements during the IPITT, an ELISA was used with an antibody equally reactive to mouse and human insulin (EZRMI-13K, Merck Millipore, DEU). For mouse urine, urea and creatinine concentrations were determined using an Automated analyser (Cobas C311, Roche, DEU). For human samples, heparinised plasma glucose (ANC 767 kit, Roche), and non-heparinised plasma NEFA (NEFA-HR (2) 434-91795 and 436-91995, Wako, Germany) and triacylglycerol (TG GPO-PAP, Roche, Germany) were measured using an automated analyser (Hitachi 912 analyzer (Boehringer, Mannheim, Germany). Human plasma urea (Z5030016, Biochain), insulin (K621911, Dako), IGF1 (DG100, R&D Systems), FGF21

(DF2100, R&D Systems) and adiponectin (47-ADPHU-E01, Alpco) were measured using commercially available kits.

Serum amino acid profiling was conducted, including sample preparation and derivitization, by LC-MS/MS using the EZ:faast kit (Phenomenex) as described previously in detail (15). In particular, an electrospray ionization-triple quadrupol mass spectrometer (Quantum Ultra, Thermo) coupled to a liquid chromatography system (UltiMate3000, Dionex) controlled by Xcalibur 2.0.7 software with Dionex Chrom MS link 6.80 was used. Chromatographic separation was achieved on a EZ:faast AAA-MS column 250x2 mm (Phenomenex) at 35°C with a flow rate of 250µL/min; autosampler temperature was set to 10°C. A sample volume of 1µl was injected onto the column. Eluents consisted of 10 mM ammonium formate in water (A) and 10 mM ammonium formate in methanol (B). Initial conditions (0 min) were 68% B, then linear gradient was applied within 13 min to 83%B. The system returned to initial conditions within 4 min and equilibrated for 7 min, resulting in a total run time of 23 min per sample. The column flow was directly converted into the H-electrospray ionization (HESI) source of the mass spectrometer, which was operated in the positive ion mode. Capillary and vaporizer temperatures were maintained at 350°C and 50°C, respectively. Sheath gas and auxiliary gas were operated at 40 and 25 (pressure, arbitrary units), no ion sweep gas was applied. Collision energy and tube lens offset were adjusted accordingly to obtain the highest response for the specific amino acid. Quantification was performed via peak area ratios applied to internal standards provided in the kit.

RNA/protein extraction and analysis. RNA was extracted from tissues using Qiazol and cDNA synthesized using the Quantitect RT kit (Qiagen GmbH, Hilden, Germany). Quantitative PCR was conducted using Taqman master mix and Taqman primer-probe assays

(Life Technologies, Darmstadt, Germany). Tissue protein extraction and immunoblotting was performed using standard methods using UCP1 (#662045, 1:2000, Calbiochem, Darmstadt, Germany) and the housekeeping protein HSP90 (610418, BD Biosciences, Heidelberg, Germany).

***In Situ* Hybridization.** Following overnight fixation in 4% neutral-buffered formaldehyde at 4^{o}C, embedded in Tissue-Tek OCT compound, cut and stained with digoxigenin-labelled probes as previously described (16). The *Glul* probe (1 kb cloned from rat liver cDNA) was transcribed from a pBS vector kindly provided by W.H. Lamers (University of Amsterdam). The Fgf21 probe was transcribed from a pBS vector containing the full-length coding sequence of mouse liver Fgf21 (0.6 kb).

Statistical Analyses. For one-factorial designs, 2-tailed unpaired t-tests were performed. For multiple comparisons, statistical analyses were performed using a one-way or two-way analysis of variance (ANOVA), with or without repeated measures, with Holm-Sidak post-hoc tests when significant differences were detected, where appropriate (MS Excel and SigmaPlot 13, Systat Software GmbH, Erkrath, Germany). The significance level was set at $p < 0.05$.

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