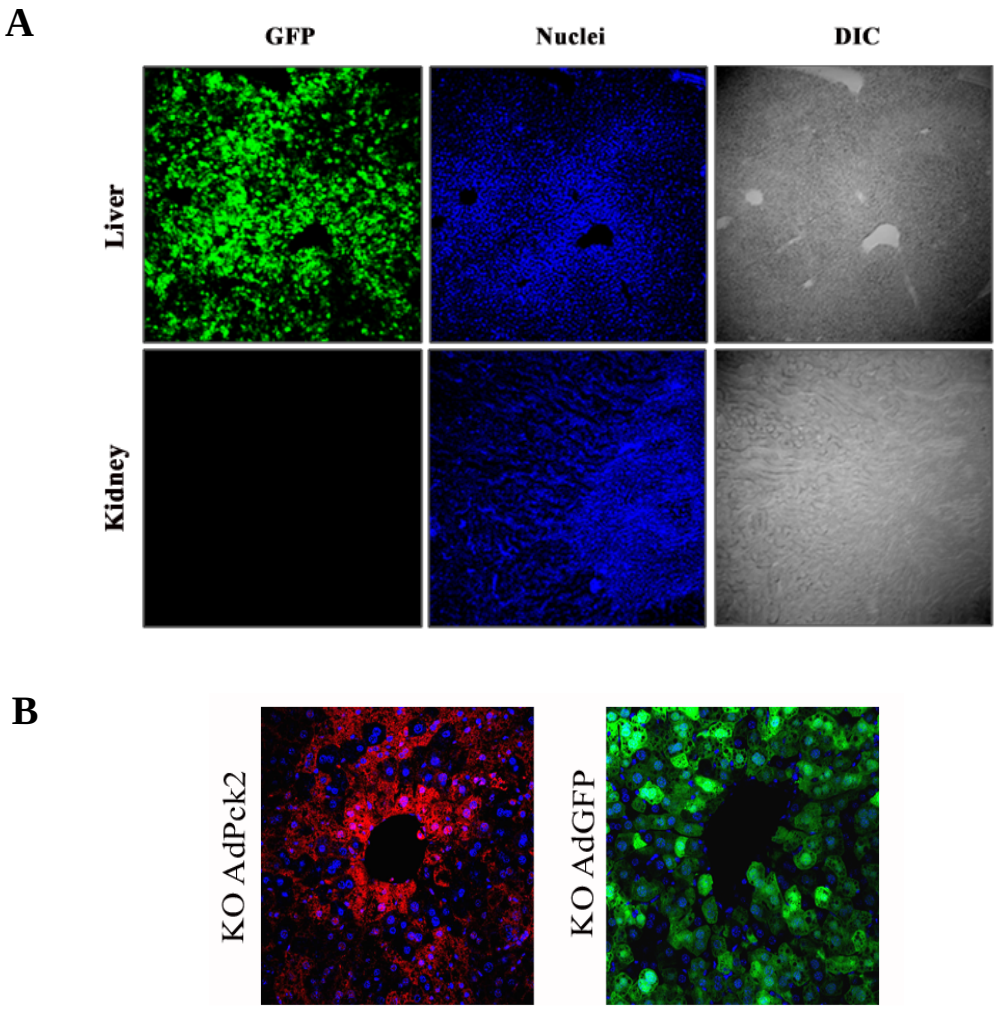
Supplemental Information

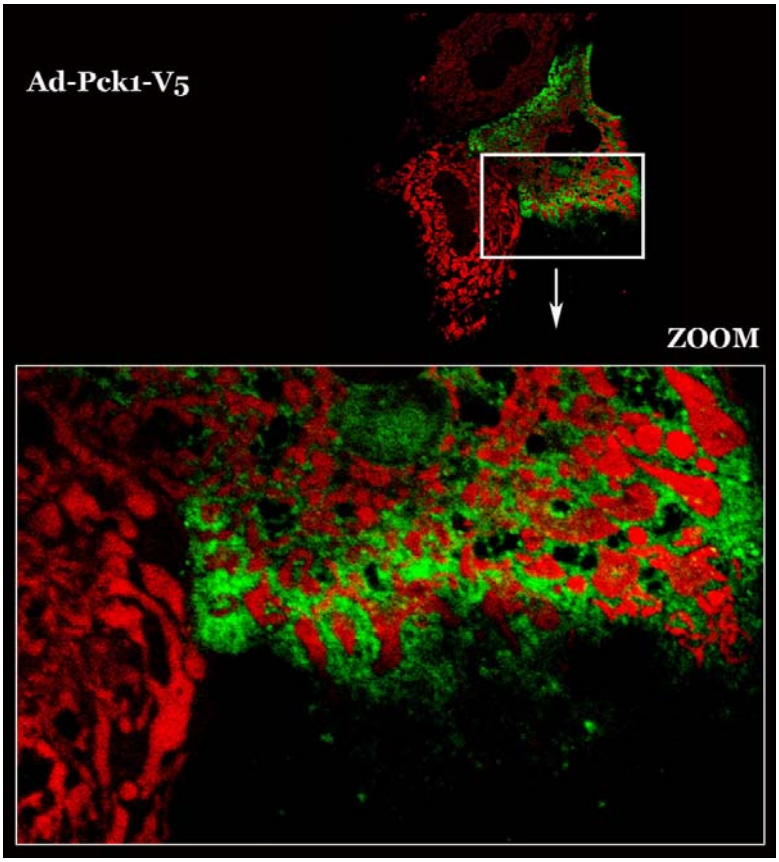
Supplementary Figure 1.



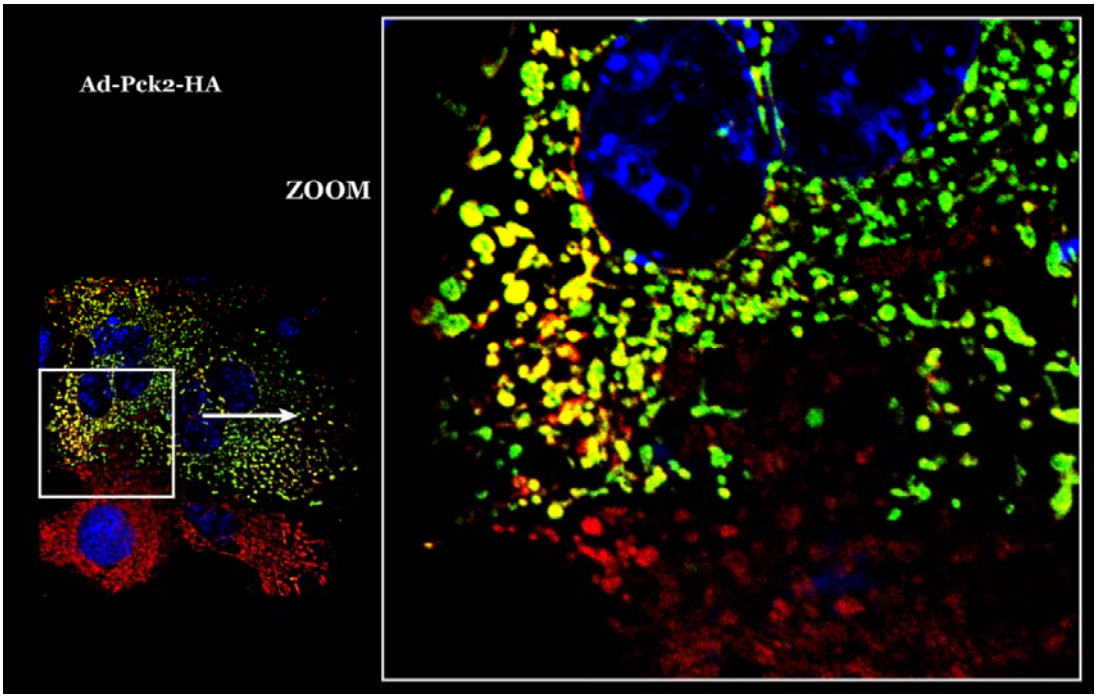
Supplementary Figure 1 (Legend). Experimental Model. (A) Specificity of adenovirus mediated hepatic targeting *in vivo.* Absence of renal infection. Laser scanning confocal microscopy images of liver (upper panel) and kidney (lower panel) cryosections from mice infected with adenovirus expressing Green Fluorescent protein (AdGFP). Images show GFP expression in green (left), nuclei staining with TO-PRO®-3 in blue (middle) and laser scanning confocal 'light transmitted' images (right), demonstrating homogeneous hepatic transduction and absence of renal infection. **(B)** Indirect immunodetection using confocal microscopy of PEPCK-M (left) and GFP fluorescence (right) performed on fixed liver cryosections of *pck*$^{lox/lox}$+Alb*Cre* treated with AdPck2 or AdGFP. PEPCK-M (red) is visualized with α-PEPCK-M antibody, GFP fluorescence is shown in green and nuclei are stained in blue using TO-PRO®-3.

Supplementary Figure 2:

A

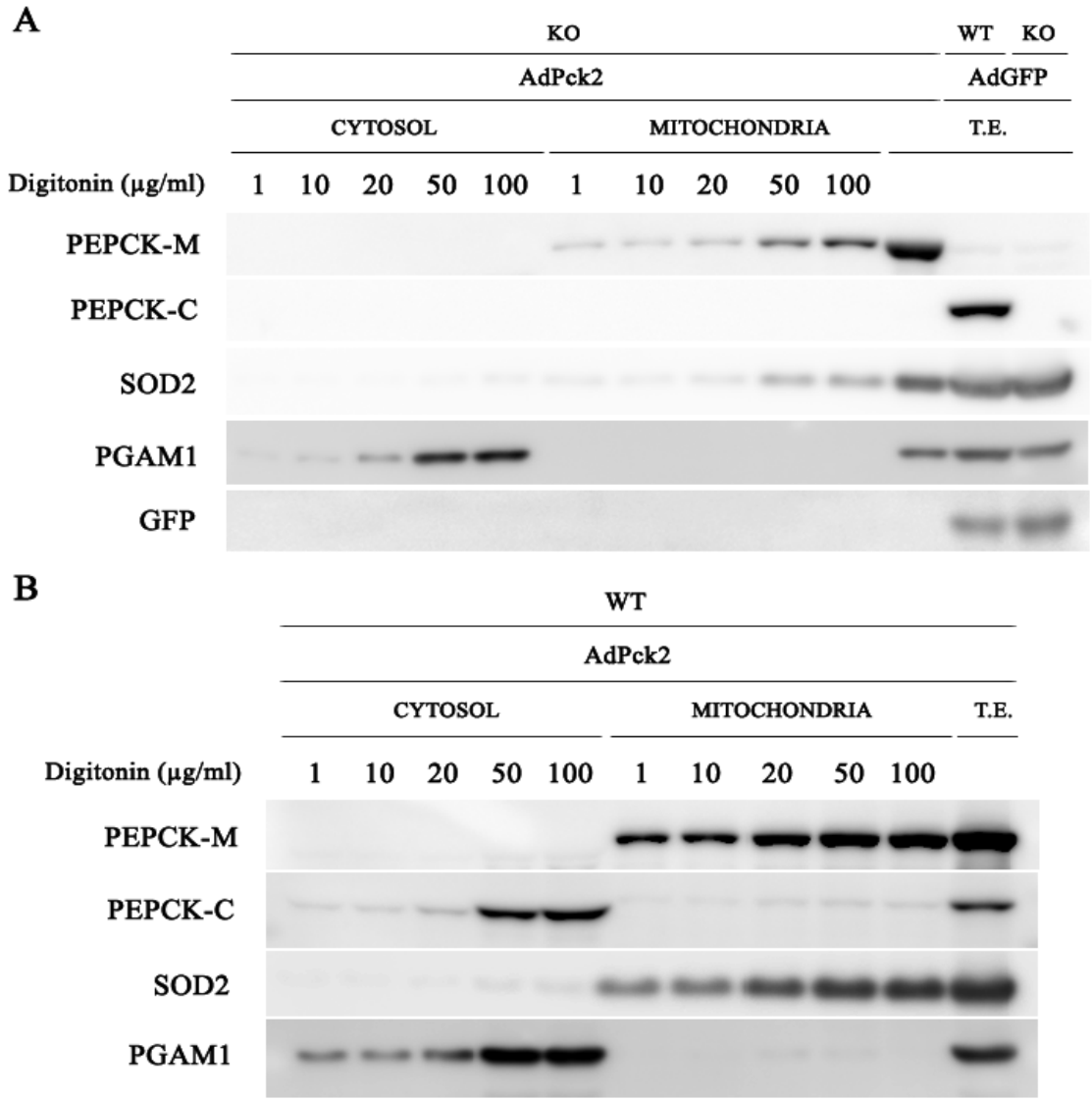


B



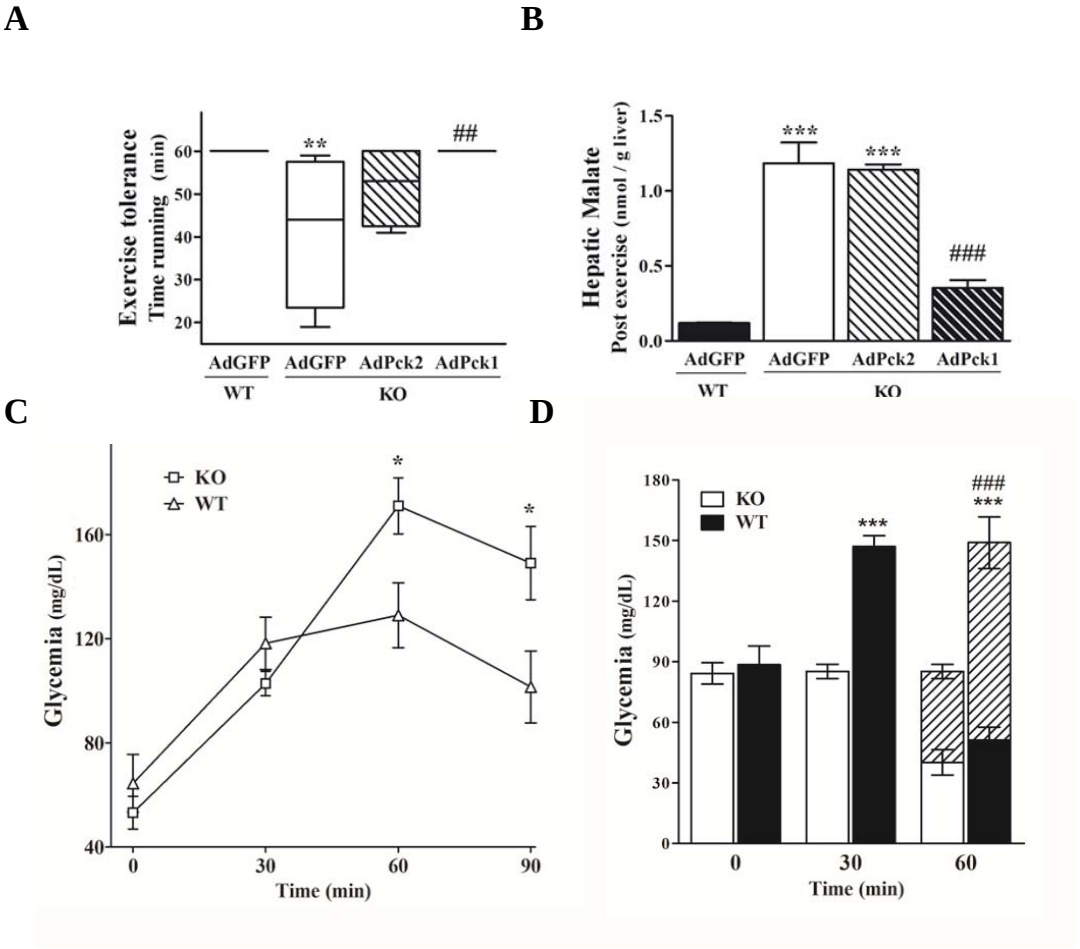
Supplementary Figure 2 (Legend). Adenovirus mediated overexpression in the isolated hepatocytes. Immunofluorescent staining of cultured hepatocytes isolated from mice. Cells were treated with 40 MOI of AdPck1-V5 **(A)** or AdPck2-HA **(B)** during 48 hours. V5 and HA tags were detected using mouse monoclonal antibodies. V5 and HA staining are shown in green. Mitochondria and nuclei are visualized using MitoTracker Red CMXRos (red) and YOYO®-1 (blue), respectively. Merged pictures and enlargement of representative areas are shown. Note the presence of infected and uninfected hepatocytes.

Supplementary Figure 3:



Supplementary Figure 3: Adenovirus-overexpressed PEPCK-M is exclusively located in the mitochondrial compartment. Liver mitocondria were isolated from freshly isolated hepatocytes using digitonin extraction method. Hepatocytes were isolated from fasted KO **(A)** and WT **(B)** mice 7 days after AdPck2 treatment, as detailed in experimental methods. Digitonin titration of release of cellular proteins was used to determine the correct subcellular localization of overexpressed PEPCK-M. Equal fractions related to fresh tissue were loaded in each lane corresponding to each dose of digitonin for comparing the extraction efficiency and protein expression in both compartments. Mitochondrial (M) and cytosolic (C) PEPCK isozymes were analyzed by Western blot. Mitochondrial superoxide dismutase (SOD2) and phosphoglycerate mutase 1 (PGAM1) were used as mitochondrial and cytosolic markers respectively. Total extracts (T.E.) from hepatocytes with 0 µM digitonin are shown as control. WT and KO mice treated with AdGFP were use as PEPCK-C positive and negative controls, respectively.

Supplementary Figure 4:



Supplementary Figure 4. Glucose metabolism *in vivo* in resting and exercising mice. (In reference to Figure 4). (A)(B) Endurance test. Overnight fasted mice (n=5-6) performed exercise on a motordriven treadmill (see also methods). Exercise was divided in three 20 min running periods with increasing intensities (12, 15 and 18 m/min). **(A)** Total running time is presented as box and whiskers. **(B)** Hepatic malate content after the exercise test. Data are presented as mean ± SEM. $^{**}P<0.01$; $^{***}P<0.001$ relative to WT AdGFP. $^{\#\#}P<0.01$; $^{\#\#\#}P<0.001$ relative to KO AdGFP group. One-way ANOVA, with a Newman-Keuls post-hoc test. **(C)** Glycerol challenge was performed using 2g/kg dose i.p. of glycerol, in 10 µl/g of physiological saline, in 16-h-fasted $pck^{lox/lox}$ (Δ) or $pck^{lox/lox}$+Alb*Cre* (□) mice (n=4). Glucose levels were measured at indicated time points. Data are presented as mean ± SEM., n=5; $^{*}P<0.05$, Two-way ANOVA with a Bonferroni post-hoc test. **(D)** Labeled Pyruvate challenge experiment was performed using a 2g/kg dose i.p. of total Pyruvate containing 10% of uniformly labeled Pyruvate [U-^{13}C3]Na-pyruvate in 16 hours fasted $pck^{lox/lox}$+Alb*Cre* (open bars) or $pck^{lox/lox}$ mice (solid bars) (n=4). Glycemia was measured at the indicated times. At the time point=60 min blood was collected from the tail vein, and plasma glucose was isolated to measure glucose enrichments by GC-MS. M+1,2,3 was monitored and indicated conversion of pyruvate to glucose [1]. Glucose enrichment from 10% of labeled pyruvate was used to calculate the portion of total blood glucose containing exogenous carbons (hatched bars). Results are mean ± SE, n=4; $^{***}P<0.001$ for glycemia. $^{\#\#\#}P<0.001$ for contribution of exogenous carbons to glucose with respect to KO group. One-way ANOVA

Supplementary Table 1:

			Liver Metabolites		
	Body weigth (g)	Relative liver weight (%)	Glycogen (mg/g)	TAG (mg/g)	Malate (nmol/g)
FASTED					
WT AdGFP	23.1 ± 1.0	4.2 ± 0.1	2.24 ± 0.4	94.7 ± 3.9	0.15 ± 0.01
WT AdPck2	22.9 ± 0.7	3.95 ± 0.2	2.18 ± 0.43	85.5 ± 5.0	0.19 ± 0.01
KO AdGFP	24.1 ± 0.8	5.34 ± 0.2 ***	0.13 ± 0.01 ***	118.4 ± 3.3 *	1.23 ± 0.06 ***
KO AdPck2	23.1 ± 1.7	5.56 ± 0.1 ***	0.17 ± 0.03 ***	108.7 ± 4.6	1.37 ± 0.08 ***
KO AdPck1	25.0 ± 1.1	4.80 ± 0.2 ##	1.59 ± 0.6 #	98.2 ± 9.5 #	0.83 ± 0.07 ### / ***
FED					
WT AdGFP	26.1 ± 1.1	4.8 ± 0.1	31.94 ± 4.4	35.7 ± 3.3	0.14 ± 0.01
WT AdPck2	26.2 ± 0.65	4.7 ± 0.2	32.96 ± 4.5	30.1 ± 2.1	0.11 ± 0.08
KO AdGFP	27.7 ± 1.1	5.3 ± 0.2 *	34.85 ± 4.0	35.1 ± 4.9	0.35 ± 0.03 ***
KO AdPck2	25.9 ± 1.0	5.3 ± 0.1 *	32.40 ± 2.5	31.7 ± 2.8	0.25 ± 0.01 ## / **

	Serum Metabolites						
	Glucose (mg/dL)	Insulin (pg/ml)	BHBA (mmol/L)	NEFA (mmol/l)	TAG (mg/dL)	Urea (mg/dL)	CHOL (mg/dL)
FASTED							
WT AdGFP	66.6 ± 3.5	268.2 ± 11	2.16 ± 0.13	1.12 ± 0.07	108.1 ± 9.4	61.3 ± 2.5	125.5 ± 6.8
WT AdPck2	58.0 ± 5.4	276.5 ± 28	2.17 ± 0.13	1.11 ± 0.06	93.37 ± 11.3	53.6 ± 2.46	110 ± 10.5
KO AdGFP	60.8 ± 5.1	261.2 ± 11	0.90 ± 0.09 # / ***	1.50 ± 0.12 *	174.7 ± 18.8 *	59.7 ± 2.4	138.0 ± 5.3
KO AdPck2	60.2 ± 4.1	258.6 ± 11	0.84 ± 0.06 ***	1.23 ± 0.07	142.7 ± 19.5	66.9 ± 4.5	122.0 ± 8.3
KO AdPck1	78.0 ± 6.1 p=0.06	273.3 ± 15	1.37 ± 0.13 ***	1.20 ± 0.10	94.3 ± 11.3 #	61.6 ± 3.8	114.6 ± 9.3
FED							
WT AdGFP	112.1 ± 5.3	379.5 ± 70	0.11 ± 0.01	0.93 ± 0.1	152.7 ± 25	45.7 ± 1.7	127.5 ± 5.3
WTAdPck2	100.2 ± 4.1	585 ± 122	0.14 ± 0.01	0.8 ± 0.06	119.3±20.1	49.53±1.61	148.5 ± 4.5
KO AdGFP	105.4 ± 4.9	481.9 ± 94	0.11 ± 0.01	0.82 ± 0.1	104.4 ± 12	45.0 ± 1.5	128.0 ± 5.7
KO AdPck2	107.5 ± 3.2	339.2 ± 34	0.12 ± 0.00	0.84 ± 0.1	96.9 ± 10	44.6 ± 1.7	128.6 ± 6.0

Supplementary Table 1 (Legend): Effect of treatment with PEPCK isoenzymes on hepatic and serum metabolic profile in fed and overnight-fasted *pck*$^{lox/lox}$+Alb*Cre* mice. Body and liver weight, plasma and hepatic metabolite concentrations in fed and overnight fasted treated *pck*$^{lox/lox}$ and *pck*$^{lox/lox}$+Alb*Cre* mice. Data are presented as mean ± SEM. **P*<0.05; ***P*<0.01; ****P*<0.001 relative to WT AdGFP. $^{\#}$*P*<0.05; $^{\#\#}$*P*<0.01; $^{\#\#\#}$*P*<0.001 relative to KO AdGFP group, One-way ANOVA, with a Newman-Keuls post-hoc test.

Supplementary Table 2:

Non-essential amino acids (µM)				
Amino acid	**WT AdGFP**	**KO AdGFP**	**KO AdPck2**	**KO AdPck1**
Glutamine	1531,0 ± 112,4	2068,6 ± 141,9 *	2055,7 ± 90,1 **	2081,0 ± 213,9 *
Glutamate	56,8 ± 9,2	153,1 ± 32,5 *	152,0 ± 15,9 *	86,3 ± 14,4
Glycine	65,7 ± 3,3	49,5 ± 3,4 *	56,3 ± 3,6	57,2 ± 3,4
Alanine	173,4 ± 3,0	202,8 ± 19,3 *	229,3 ± 16,6 *	172,6 ± 20,9
Serine	69,8 ± 2,9	52,1 ± 8,2	64,2 ± 5,1	73,6 ± 7,0
Proline	189,3 ± 8,9	210,8 ± 11,0	225,4 ± 12,3	225,2 ± 14,5
Aspartate	36,7 ± 9,6	176,9 ± 53,3 *	123,5 ± 24,3 *	61,2 ± 7,1
Tyrosine	63,6 ± 9,3	50,5 ± 5,2	54,1 ± 3,6	56,9 ± 7,3

Essential amino acids (µM)				
Amino acid	**WT AdGFP**	**KO AdGFP**	**KO AdPck2**	**KO AdPck1**
Valine	153,2 ± 13,4	177,9 ± 13,0	182,9 ± 12,6	190,9 ± 19,2
Leucine	141,4 ± 15,4	140,2 ± 11,4	167,0 ± 15,3	169,8 ± 27,2
Isoleucine	39,5 ± 3,9	30,6 ± 2,8	35,5 ± 2,7	39,4 ± 7,1
Threonine	146,3 ± 15,3	138,5 ± 7,5	156,2 ± 11,0	177,5 ± 18,0
Phenylalanine	81,8 ± 2,7	81,0 ± 5,5	95,6 ± 6,5	86,9 ± 10,8
Tryptophan	35,0 ± 1,4	26,1 ± 1,9 ***	23,7 ± 1,6 ***	31,7 ± 1,1 ##
Methionine	50,5 ± 2,8	45,3 ± 2,0	49,0 ± 4,4	57,7 ± 7,8
Lysine	466,7 ± 33,7	595,1 ± 41,7	711,3 ± 38,6 **	520,6 ± 65,0

Effect of treatment with PEPCK isoenzymes on plasma amino acids concentrations in overnight-fasted *pck*$^{lox/lox}$+Alb*Cre* mice. Amino acids were detected by mass spectrometry (ESI-MS/MS) using the MRM mode by monitoring specific transitions under positive electro spray ionization as previously reported [2]. Quantification was done by comparison of individual ion peak areas to that of an internal ^{13}C standard (Cambridge Isotope Laboratories, Inc.). Data are presented as mean ± SEM, n=5-6. *P<0.05; **P<0.01; ***P<0.001 relative to WT AdGFP. $^{\#}P$<0.05; $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001 relative to KO AdGFP group. One-way ANOVA, with a Newman-Keuls post-hoc test.

Supplementary Table 3: Effect of treatment with PEPCK isoenzymes on hepatic mRNA expression in overnight-fasted *pck*$^{lox/lox}$+Alb*Cre* mice.

	WT AdGFP	KO AdGFP	KO AdPck2	KO AdPck1
acaca	1.10 ± 0.19	0.58 ± 0.06 *	0.61 ± 0.04 **	0.52 ± 0.05 **
acacb	1.05 ± 0.22	1.17 ± 0.23	1.64 ± 0.18	0.79 ± 0.11
acly	1.02 ± 0.13	0.62 ± 0.07	0.59 ± 0.04	0.66 ± 0.18
akt2	1.04 ± 0.11	0.90 ± 0.10	0.84 ± 0.12	0.96 ± 0.10
cpt1a	1.02 ± 0.07	0.92 ± 0.09	0.98 ± 0.09	0.99 ± 0.10
cpt2	1.01 ± 0.06	1.29 ± 0.05 *	0.95 ± 0.09 #	0.89 ± 0.09 #
dusp4	1.10 ± 0.22	0.78 ± 0.12	0.86 ± 0.12	0.62 ± 0.05
fasn	1.09 ± 0.19	0.46 ± 0.04 **	0.56 ± 0.07 *	0.61 ± 0.10 **
foxo1	1.05 ± 0.17	0.68 ± 0.10	0.76 ± 0.07	1.02 ± 0.13
g6pc	1.04 ± 0.11	0.65 ± 0.07 *	0.68 ± 0.08 *	0.71 ± 0.07*
gck	1.08 ± 0.17	0.42 ± 0.07 **	0.44 ± 0.10 **	0.79 ± 0.11#
gpd1	1.03 ± 0.10	0.64 ± 0.12 *	0.64 ± 0.08 *	0.75 ± 0.10
gsk3b	1.02 ± 0.09	1.08 ± 0.07	1.14 ± 0.13	1.07 ± 0.06
hmgcs2	1.01 ± 0.05	1.12 ± 0.09	1.14 ± 0.13	1.11 ± 0.10
hnf1a	1.02 ± 0.07	0.65 ± 0.05	0.71 ± 0.08	0.77 ± 0.08
hnf4a	1.04 ± 0.11	0.78 ± 0.06	0.79 ± 0.07	0.80 ± 0.07
ikbkb	1.02 ± 0.08	0.56 ± 0.05***	0.59 ± 0.03***	0.75 ± 0.06**
il6	1.04 ± 0.12	0.55 ± 0.13 **	0.50 ± 0.04 **	0.43 ± 0.10 **
inppl1	1.01 ± 0.07	1.30 ± 0.13	1.38 ± 0.11	1.20 ± 0.12
mapk8	1.00 ± 0.03	0.83 ± 0.08	1.01 ± 0.12	0.84 ± 0.06
me1	1.02 ± 0.08	1.24 ± 0.13	1.05 ± 0.10	1.02 ± 0.07
nfkb1	1.01 ± 0.06	0.85 ± 0.10	0.97 ± 0.06	0.84 ± 0.08
nos2	1.14 ± 0.24	0.82 ± 0.31	0.73 ± 0.15	0.45 ± 0.11
nr1h3	1.03 ± 0.09	0.74 ± 0.13	0.84 ± 0.07	0.89 ± 0.06
nrf1	1.02 ± 0.08	0.90 ± 0.17	0.82 ± 0.06	0.69 ± 0.04
parp1	1.01 ± 0.06	0.71 ± 0.07 **	0.64 ± 0.07 **	0.76 ± 0.06 *
*pck1 **	1.07 ± 0.15	0.10 ± 0.01 ***	0.14 ± 0.02 ***	0.75 ± 0.05 */###
pck2	1.04 ± 0.12	2.00 ± 0.41	134.7 ± 30.9 ***/###	0.81 ± 0.09
pfkfb3	1.05 ± 0.12	1.00 ± 0.09	1.08 ± 0.08	1.00 ± 0.19
ppara	1.00 ± 0.06	0.62 ± 0.12 **	0.69 ± 0.08 **	0.93 ± 0.12
ppargc1a	1.03 ± 0.09	2.18 ± 0.25 **	2.05 ± 0.27 **	1.12 ± 0.15 ##
ppargc1b	1.06 ± 0.14	1.01 ± 0.16	1.26 ± 0.26	1.01 ± 0.13
pparg	1.04 ± 0.12	0.74 ± 0.11	1.00 ± 0.14	0.84 ± 0.09
pygl	1.01 ± 0.04	0.39 ± 0.04 ***	0.44 ± 0.04 ***	0.61 ± 0.05 ***/##
scd1	1.02 ± 0.07	0.71 ± 0.08 *	0.48 ± 0.12 **	0.54 ± 0.09 **
sirt1	1.01 ± 0.05	0.77 ± 0.07 *	0.89 ± 0.05	0.84 ± 0.06
sirt3	1.01 ± 0.06	0.65 ± 0.13 *	0.91 ± 0.06	1.01 ± 0.09 #
slc2a2	1.03 ± 0.11	0.33 ± 0.04 ***	0.43 ± 0.05 ***	0.59 ± 0.08 ***/#
slc2a4	1.16 ± 0.24	0.34 ± 0.11 *	0.37 ± 0.14 *	0.48 ± 0.21 *
sod1	1.01 ± 0.04	0.72 ± 0.08 *	0.68 ± 0.05 *	0.91 ± 0.08 #
sod2	1.04 ± 0.12	0.88 ± 0.07	0.90 ± 0.06	0.94 ± 0.11
srebf1	1.02 ± 0.07	0.71 ± 0.09	0.88 ± 0.07	0.86 ± 0.07
tkt	1.05 ± 0.13	0.90 ± 0.13	0.91 ± 0.11	0.76 ± 0.08
ucp2	1.11 ± 0.18	3.05 ± 0.57 **	2.36 ± 0.27*	1.82 ± 0.36

Supplementary Table 3 (Legend): Effect of treatment with PEPCK isoenzymes on hepatic mRNA expression in overnight-fasted *pck*$^{lox/lox}$+Alb*Cre* mice. Total RNA was extracted using RNAeasy mini kit (Qiagen). cDNA synthesis from 2 µg RNA was performed using Ready-To-Go You-Prime First Strand Beads (Amersham Biosciences) with random hexamers. mRNA levels of selected genes were quantified using a Low Density Array (Applied Biosystems) in a HT7900 Real-Time RT-PCR system (Applied Biosystems). Gene expression was normalized with Dataassist^{TM} global normalization method. Data analysis is based on the ΔΔCt method. The relative mRNA expression of selected genes was simultaneously analyzed using TaqMan Low-Density Arrays (as described for Supplemental table 3). Normalized values are shown as fold difference relative to WT AdGFP group. Data are presented as mean ± SEM, n=8. **P*<0.05; ***P*<0.01; ****P*<0.001 relative to WT AdGFP. $^{\#}P$<0.05; $^{\#\#}P$<0.01; $^{\#\#\#}P$<0.001 relative to KO AdGFP. One-way ANOVA, with a Newman-Keuls post-hoc test. (*) *pck1* probe detects the truncated *pck1* mRNA (untranslated) resulting from the *Cre*-recombination.

Supplementary Table 4: Effect of treatment with PEPCK isoenzymes on hepatic mRNA expression in fed *pck*$^{lox/lox}$+Alb*Cre* mice.

Gene	Group	Avg	SE	Gene	Group	Avg	SE	Gene	Group	Avg	SE
	WT GFP	1.00	0.05		WT GFP	1.02	0.11		WT GFP	1.04	0.17
acaca	**KO GFP**	**0.69**	**0.16**	***hnf4a***	KO GFP	1.25	0.16	***ppargc1a***	KO GFP	1.24	0.17
	KO Pck2	**0.49**	**0.03**		KO Pck2	0.99	0.04		KO Pck2	1.01	0.12
	WT GFP	1.03	0.17		WT GFP	1.00	0.03		WT GFP	1.01	0.09
acacb	**KO GFP**	**0.53**	**0.10**	***ikbkb***	KO GFP	1.02	0.04	***ppargc1b***	KO GFP	1.20	0.09
	KO Pck2	**0.53**	**0.07**		KO Pck2	1.00	0.03		**KO Pck2**	**0.89**	**0.11**
	WT GFP	1.16	0.33		WT GFP	1.04	0.17		WT GFP	1.02	0.11
acly	KO GFP	0.86	0.32	***il6***	KO GFP	0.82	0.18	***pparg***	KO GFP	1.60	0.53
	KO Pck2	0.61	0.06		KO Pck2	0.90	0.20		KO Pck2	1.51	0.40
	WT GFP	1.02	0.11		WT GFP	1.00	0.03		WT GFP	1.02	0.10
akt2	KO GFP	0.97	0.08	***inppl1***	KO GFP	0.98	0.04	***pygl***	KO GFP	1.12	0.17
	KO Pck2	1.32	0.24		KO Pck2	1.00	0.03		KO Pck2	0.87	0.07
	WT GFP	1.02	0.12		WT GFP	1.01	0.07		WT GFP	1.02	0.10
cpt1a	KO GFP	1.47	0.24	***mapk8***	KO GFP	1.08	0.09	***scd1***	KO GFP	0.65	0.25
	KO Pck2	1.19	0.09		KO Pck2	0.92	0.13		KO Pck2	0.69	0.12
	WT GFP	1.01	0.07		WT GFP	1.04	0.16		WT GFP	1.01	0.06
cpt2	**KO GFP**	**1.26**	**0.08**	***me1***	KO GFP	0.98	0.31	***sirt1***	KO GFP	1.02	0.10
	KO Pck2	**0.97**	**0.04**		KO Pck2	0.91	0.15		KO Pck2	0.92	0.02
	WT GFP	1.01	0.09		WT GFP	1.01	0.08		WT GFP	1.00	0.05
dusp4	KO GFP	0.97	0.13	***nfkb1***	KO GFP	1.04	0.03	***sirt3***	KO GFP	1.10	0.09
	KO Pck2	0.81	0.06		KO Pck2	1.00	0.12		KO Pck2	1.10	0.22
	WT GFP	1.01	0.09		WT GFP	1.15	0.29		WT GFP	1.03	0.14
fasn	**KO GFP**	**0.55**	**0.25**	***nos2***	KO GFP	1.54	0.23	***slc2a2***	KO GFP	0.95	0.20
	KO Pck2	**0.42**	**0.08**		KO Pck2	1.10	0.29		KO Pck2	0.80	0.07
	WT GFP	1.00	0.04		WT GFP	1.01	0.08		WT GFP	1.14	0.30
foxo1	KO GFP	1.08	0.19	***nr1h3***	KO GFP	1.25	0.05	***slc2a4***	KO GFP	1.09	0.10
	KO Pck2	0.86	0.06		KO Pck2	1.16	0.17		KO Pck2	0.99	0.03
	WT GFP	1.02	0.13		WT GFP	1.00	0.04		WT GFP	1.01	0.07
g6pc	KO GFP	0.87	0.25	***nrf1***	KO GFP	1.19	0.05	***sod1***	KO GFP	1.01	0.05
	KO Pck2	0.59	0.07		**KO Pck2**	**0.87**	**0.08**		KO Pck2	1.01	0.13
	WT GFP	1.05	0.19		WT GFP	1.01	0.09		WT GFP	1.01	0.06
gck	KO GFP	1.19	0.24	***parp1***	KO GFP	1.08	0.07	***sod2***	KO GFP	1.09	0.07
	KO Pck2	1.23	0.28		**KO Pck2**	**0.88**	**0.04**		KO Pck2	0.99	0.05
	WT GFP	1.04	0.17		WT GFP	1.06	0.18		WT GFP	1.04	0.16
gpd1	KO GFP	1.38	0.14	***pck1****	**KO GFP**	**0.25**	**0.06**	***srebf1***	KO GFP	1.01	0.18
	KO Pck2	0.99	0.15		**KO Pck2**	**0.19**	**0.04**		KO Pck2	0.84	0.06
	WT GFP	1.01	0.07		WT GFP	1.01	0.09		WT GFP	1.03	0.14
gsk3b	KO GFP	1.05	0.10	***pck2***	KO GFP	1.07	0.06	***tkt***	KO GFP	0.96	0.12
	KO Pck2	0.84	0.10		**KO Pck2**	**123**	**27.8**		KO Pck2	0.93	0.07
	WT GFP	1.01	0.07		WT GFP	1.00	0.05		WT GFP	1.01	0.09
hmgcs2	**KO GFP**	**1.40**	**0.11**	***pfkfb3***	KO GFP	1.34	0.18	***ucp2***	**KO GFP**	**1.43**	**0.10**
	KO Pck2	1.05	0.09		KO Pck2	1.16	0.14		**KO Pck2**	**1.52**	**0.18**
	WT GFP	1.06	0.22		WT GFP	1.00	0.04				
hnf1a	KO GFP	1.50	0.14	***ppara***	KO GFP	1.12	0.17				
	KO Pck2	0.97	0.10		KO Pck2	1.08	0.13				

Supplementary Table 4 (Legend): Effect of treatment with PEPCK isoenzymes on hepatic mRNA expression in fed *pck*$^{lox/lox}$+Alb*Cre* mice. The relative mRNA expression of selected genes was simultaneously analyzed using TaqMan Low-Density Arrays (described under Supplemental Procedures). Normalized values are shown as fold difference relative to WT AdGFP. Data are presented as mean ± SEM, n=4-6. $P<0.05$ is considered significative. **IN RED:** statistically significant differences with respect to WT AdGFP. **IN GREEN**: statistically significant differences with respect to WT GFP and KO GFP. **IN BLUE**: statistically significant differences with respect to KO GFP. One-Way ANOVA, Newman-Keuls post-test. (*) *pck1* probe detects the truncated *pck1* mRNA (untranslated) resulting from the *Cre*-recombination.

Supplemental Materials and Methods

Experimental animals and adenovirus.

Liver-specific PEPCK-deficient (*pck*$^{lox/lox}$+Alb*Cre*) and control (*pck*$^{lox/lox}$) mice were generated as previously described [3]. Mice were maintained in a constant 12-h light/dark cycle, and fed a standard rodent chow and water ad libitum. At the beginning of the experiment, animals were 12 – 16 weeks old. All animal protocols were approved by the ethics committee at the University of Barcelona or the UTSWMC Institutional Animal Care and Use Committee. Recombinant E1-E3 deficient adenovirus (serotype 5) expressing full-length cDNA of mouse *pck1* (AdPck1) and *pck2* (AdPck2) genes were generated in our laboratory using Gateway and Virapower technologies (Invitrogen) according to the manufacturer's protocol. A clonal stock was amplified and purified for its use *in vivo* in UPV-CBATEG (Bellaterra, Spain). Adenovirus encoding *Aequorea victoria green fluorescent* protein (AdGFP) (UPV-CBATEG) was used as control treatment. Adenovirus were administered by tail vein injection of 3×10^{10} plaque-forming units (pfu)/kg in 200 µl physiological saline. Surgery was performed under isofluorane anesthesia (Abbot). Tissues were immediately snap-frozen in liquid nitrogen and stored at −80°C until analysis. Blood was collected by inferior cava puncture.

Liver Perfusion Experiments and NMR Analysis

Briefly, livers from were isolated after a 18 hr fast and perfused without recirculation for 60 min as previously detailed [4-6]. The perfusion media consisted of Krebs-Henseleit bicarbonate buffer containing 1.5 mM lactate, 0.15 mM pyruvate, 0.25 mM glycerol, 0,221 g/l free fatty acids mixture (22.13% palmitic acid, 5.20% palmitoleic acid, 2.70% stearic acid, 27.02% oleic acid, 37.71% linoleic acid, 2.44% γ-Linolenic acid, 2.80 % decosahexanoic acid) bound to BSA (30 g/l), 0.1 mM [U-$^{13}C_3$]-propionate, and 3% v/v D_2O. Effluent perfusate was collected for assays of glucose production as well as isolation of glucose for NMR analysis as previously described [4-6]. Livers were freeze clamped after the perfusion period. Glucose purified from the effluent perfusion media was converted to the 1,2-diisopropylidene glucofuranose derivative (monoacetone glucose, MAG) and analyzed by NMR at 14.1 T to determine the positional enrichment of ^2H and ^{13}C [4, 6]. The relative deuterium enrichments in glucose H_2, H_5, and H_{6s} were determined by ^2H NMR, and these values were used to determine the rates of gluconeogenesis and glycogenolysis [7]. Pathways intersecting the TCA cycle were evaluated by ^{13}C isotopomer analysis of glucose C2. The ^{13}C NMR multiplets in glucose generated by the tracer [U-$^{13}C_3$]propionate were evaluated to determine flux through PEPCK and pyruvate cycling [8]. Fractional glycogenolysis and gluconeogenesis measured by ^2H NMR were combined with PEPCK, pyruvate cycling and gluconeogenesis relative to TCA cycle flux measured by ^{13}C NMR, and the absolute rate of glucose production (µmol/min/g wet liver tissue) to yield the absolute fluxes through each of these pathways [4]. [U-$^{13}C_3$] propionate (99%) and ^2H_2O (99%) were purchased from Cambridge Isotopes.

Substrate challenges

Pyruvate and glycerol challenges were performed by i.p. injection of 2g/kg of total substrate. A 2 g/kg Na-Pyruvate, or glycerol (Sigma-Aldrich), in 10 µl/g of physiological saline, bolus was injected intraperitoneally in 16-h-fasted mice on day 7 after adenoviral infection. Glucose levels were measured at indicated time points. Labeled pyruvate challenge experiment was performed after

administering a 10% [$^{13}C_3$]Na-pyruvate (Cambridge Isotope Laboratories, Inc. MA, USA) solution as intra-peritoneal injection at a dose of 2g/kg body weight in 16 hours fasted mice. Blood glucose concentrations were measured at 0, 30 and 60 minutes after pyruvate administration using a glucose meter. At time point 60 min, ~50µL of blood was collected from the tail vein, and plasma glucose was isolated to measure glucose enrichment from pyruvate using GC-MS. Briefly, the di-*O*-isopropylidene acetate derivative of glucose was injected on to a capillary column (HP-5; 30 m × 0.25 mm × 0.25 µm; Agilent Technologies, Palo Alto, CA) before selective ion monitoring on a 5973N Mass Selective Detector (Agilent Technologies, Palo Alto, CA), under electron ionization mode [1]. Mass isotopomer enrichments of the glucose derivative fragments with mass-to-charge (*m*/*z*) 287 (unlabeled), 288 (M+1), 289 (M+2) and 290 (M+3) were determined and corrected for background enrichments. Total glucose enrichment arising from [$^{13}C_3$]pyruvate was used to calculate the portion of total blood glucose containing exogenous carbons.

RNA extraction and quantitative RT-PCR.

Total RNA was extracted using RNAeasy mini kit (Qiagen). cDNA synthesis from 2 µg RNA was performed using Ready-To-Go You-Prime First Strand Beads (Amersham Biosciences) with random hexamers. mRNA levels of selected genes were quantified using a Low Density Array (Applied Biosystems) in a HT7900 Real-Time RT-PCR system (Applied Biosystems). Gene expression was normalized with Dataassist^{TM} global normalization method. Data analysis is based on the ΔΔCt method.

Western blot.

Tissue was homogenized in radioimmunoprecipitation assay buffer supplemented with protease and phosphatase inhibitors and centrifuged at 15,000g for 15 min at 4°C. Western blots were performed with 20–50 µg tissue extract. Proteins were separated in 8–12% SDS-PAGE and transferred to an Immobilon membrane (Millipore). All membranes were normalized using monoclonal anti-α-tubulin (Sigma-Aldrich) at 1:10,000. Horseradish peroxidase activity linked to secondary antibody was detected with ECL substrate (Pierce) in a Fujifilm LAS 3000 Intelligent Dark Box IV imaging system). Densitometry was performed using Multi Gauge software. Hepatic PEPCK-M expression in different species was analyzed in total liver extracts of four different species. Two specimens were analyzed for each species. Rat, mouse and rabbit livers were homogenized as described above. Human whole normal liver lysates of two adults were purchased to Novus Biologicals (NB820-59233 and NB820-59232). Western blot was performed with 40 µg tissue extract. Two different primary antibodies were used to detect PEPCK-M; Ab1: rabbit anti-PEPCK-M (Abcam - ab70359) and Ab2:goat anti-PEPCK-M (Everest). Due to the unavailability to normalize expression with a marker equally expressed and equally detected in the four species the membranes were normalized using Ponceau staining. Peptide sequence used to generate the PEPCK-M Ab2 is 100% homologous in mouse, rat, rabbit, and human (KPWKPGDKEPCAH, from the internal region of the PEPCK-M protein sequence - Everest Biotech). Synthetic peptide used to generate the PEPCK-M Ab2 was derived from within residues 400 - 500 of Human PEPCK-M. Note that the detection pattern is similar with both antibodies**.**

Histology and immunofluorescence.

A portion of the third hepatic lobule was fixed for at least 24 h in 4% paraformaldehyde, equilibrated in 30% sucrose, embedded in Tissue-Tek OCT compound (Sakura), and stored at −80°C until 7 and 30-μm-thick cryosections were obtained. PEPCK was immunostained as previously described [9], except that a rabbit anti-PEPCK-M primary antibody (Abcam) was used. Hepatocytes were fixed by incubating in 4% paraformaldehyde for 10 min temperature and permeabilized by incubating with 0.2% Triton X-100 in PBS for 10 min. Cells were then blocked by incubating with 3% BSA in PBS for 30 min and treated with the indicated primary antibody for 16 hr. After four washes with 0.1% Triton in PBS, cells were incubated with anti-rabbit Alexa Fluor® 488, 555 or 647 secondary antibodies (Invitrogen) for 1 hr, during this incubation nucleis markers were added where indicated. After being washed with PBS 0.1% triton three times, For mitochondrial labeling cultured hepatocytes were incubated in culture media containing a 0.1 μM final concentration of MitoTracker Red CMXRos dissolved in dimethyl sulfoxide for 20 min in a 37°C, 5% CO_2 gas incubator. The cells were washed with PBS and fixed with 4% paraformaldehyde in PBS for 10 min. For the detection of intracellular lipid droplets, sections were incubated with a 2.5 μg/mL Nile Red (Sigma-Aldrich) solution, in 75% aqueous glycerol, during 10 minutes. Samples were examined using a confocal microscope (TCS SL Leica) and LCS Lite software (Leica) was used to collect digital images.

Blood and liver biochemical analysis.

Blood glucose was measured using a Glucocard Memory 2 apparatus (Menarini) by tail clipping. Serum metabolites were measured by the Veterinarian Clinical Biochemistry Service, Veterinary Hospital, Universitat Autònoma de Barcelona (Barcelona, Spain). Hepatic glycogen was measured essentially as previously described [10]. Hepatic triglycerides and fatty acid content were quantified using a TAG kit (Sigma-Aldrich) and NEFA kit (Wako), respectively, in 3 mol/l KOH, 65% ethanol extracts, based on the method of Salmon and Flatt for liver saponification. Hepatic malate and phosphoenolpyruvate concentrations were determined by enzymatic assays as previously described . PEPCK activity was assayed in liver extracts homogenized with a *Polytron i*n appropriate lysis buffer. Activity was measured spectrophotometrically by coupling the conversion of phosphoenolpyruvate to oxaloacetate by PEPCK to the subsequent conversion to malate by malate dehydrogenase [11]. Serum insulin was determined using Ultrasensitive Mouse Insulin ELISA (Mercodia). For quantitation of plasma amino acids, thawed plasma samples were immediately spiked with labeled amino acid internal standard (Cambridge Isotope Laboratories, Inc.) and four volumes of acetonitrile. The samples were then centrifuged at 14,000 rpm for 10 min and 2 uL of the supernatant was injected for separation of amino acids on to a reverse phase C18 column (Atlantis T3, Waters, Milford, MA; 150×2.1 mm, 3.0 μm) with a gradient elution. Amino acids were detected using the MRM mode by monitoring specific transitions under positive electro spray as previously reported [2] on an API 3200 triple quadrupole LC/MS/MS mass spectrometer (Applied Biosystems/Sciex Instruments). Quantification was done by comparison of individual ion peak areas to that of an internal standard.

Endurance exercise study

24-week-old male *pck*$^{lox/lox}$+Alb*Cre* mice and their wild type littermates were infected with adenovirus expressing the indicated proteins. Overnight fasted mice performed an exercise test on a motordriven treadmill (Columbus Instruments). *M*ice were acclimated to the treadmill inactivated belt 3 minutes and then 3 minutes at 6 m/min. Exercise was composed by three 20-min running periods, and a 4-min

rest between running periods. The speed in these periods was 12, 15 and 18 m/minute. Inclination was 0°. Blood glucose levels were determined *before and after each running period* by tail clipping. If any of the animals arrives to exhaustion before the completion of the test (60 minutes, 900 meters), *exercise time = End* is considered.

Hepatocyte studies

Hepatocytes were isolated from 4 hours fasted *pck*$^{lox/lox}$+Alb*Cre* or overnight fasted wild type mice as previously described [12] with some modifications. Short-term fasting in KO mice avoided the low cell culture viability and impaired efficiency of disaggregation typical of steatotic livers. Briefly, portal vein cannulation was performed under Ketamine/Xylazine (100/10 mg/kg i.p; Merial/Calier) anesthesia, and heparin (100 IU mouse i.v.) was administered. Liver was pre-perfused with calcium-free Hanks' balanced salt solution (HBSS) (Sigma-Aldrich) containing EGTA at 37°C before perfusion (7 ml/min) with Ca^{2+}-containing HBSS and a final concentration of 33 µg/ml of Liberase TM (Roche) during 10 minutes. Hepatocytes were filtered through a 70 µM nylon cell strainer (Becton Dickinson) and cleared by repeated centrifugation at 50g for 2 min in washing media (MEM-EAGLE for suspension culture plus 2mM glutamine; Biological industries). Viability (>80%) was assessed by trypan blue exclusion. Isolated hepatocytes were seeded in 12 -24 wells collagen-coated plates (5 µg/cm^2) with seeding media (Williams E supplemented with 2 mM glutamine, 100 units/ml penicillin, 0.1 mg/ml streptomycin, and 10% fetal bovine serum). Infection was performed at this step with a multiplicity of infection of 40 (unless otherwise indicated) for 4 hours. Following initial infection, the supernatant was replaced with fresh HepatoZYME-SFM media (Gibco) supplemented with 2 mM glutamine and antibiotics, and incubated for 20 hours. Gluconeogenesis was induced by conditioned medium (Williams E supplemented with 200 U/ml penicillin/streptomycin, 2 mM glutamine, 2 mM Pyruvate, 20 mM Lactate and 100 nM dexamethasone) for 24 hours before the experiment. Experiments were performed 48 hours post-infection. Cells were washed 4 times with PBS buffer and medium was replaced by assay media (DMEM medium w/o glucose plus 100nM dexamethasone, 2mM glutamine) containing indicated substrates and inhibitors. After 6 hours, the supernatant was collected for determination of glucose content (Sigma-Aldrich Kit 510- A). Cells were harvested and frozen for subsequent analysis of protein content and transgene expression.

Subcellular fractionation

Hepatocytes were isolated as described above, except for an additional wash step with Isolation buffer (IB) (Tris-HCl 10 mM, 250 mM sucrose, 0.5 mM Na_2EDTA, 1 g/l BSA, pH 7.4). Hepatocytes were resuspended in 7 ml of IB, supplemented with protease inhibitors. Equal aliquots of hepatocytes (1 ml) were incubated with various concentrations of digitonin (0-100 µg/ml) for 4 min on ice. Cytosol fraction was obtained by centrifuging the preparations at 12000 g for 10 min. This step prevents excessive damage to mitochondrial membranes due to digitonin action. Supernatant 1 was centrifuged again at 20000 g for 20 min and 300 µl were mixed with 100 µl of standard 4X SDS sample buffer (0.250 M Tris pH 6.8, 8% SDS, 40% glycerol, 4% β-mercaptoethanol, 0.04% bromophenol blue). Pellet 1 was gently resuspended with 1 ml of IB and centrifuged at 1200 g for 10 minutes to remove large cell and tissue fragments and cell nuclei. Pellet 2 was discarded and mitochondria were pelleted by centrifuging the supernatant 2 at 12000 g for 10 minutes. Pellet containing mitochondria was resuspended in 1 ml of SDS buffer (Tris-HCl 25 mM, 1 mM EDTA, 1 mM EGTA, 1% SDS, pH 7.4) and 300 µl were mixed with 100 µl 4X SDS sample buffer. 0 µg/ml (vehicle) digitonin hepatocytes

were directly resuspended in equal volume of SDS buffer to obtain a total extract. Western blots were performed with equal volume of of cytosolic and mitocondrial fractions of each digitonin concentration, to maintain a correct corrrelation relative to fresh tissue. Proteins were separated in 12% SDS-PAGE. Primary antibodies used to detect PEPCK-M and PEPCK-C, were rabbit anti-PEPCK-M (Abcam - ab70359) and sheep anti-PEPCK-C (generous gifts from Dr. Daryl Granner). Note a slight cross reactivity, under determinate astringency conditions, of PEPCK-C antibody vs PEPCK-M protein.

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