Supporting Information

Charalambous et al. 10.1073/pnas.1406119111

SI Experimental Procedures

Serum and Tissue Biochemistry. Enzymatic assay kits were used for determination of plasma FFAs (Roche), TAGs and glycerol (Sigma-Aldrich), and cholesterol and ALT (Dade-Behring). ELISA kits were used for measurements of leptin and insulin (Crystalchem), IGF1 (Rat/Mouse IGF1 ELISA; IDS), total thyroxine and total triiodothyronine (TT4 and TT3, DELFIA; Perkin-Elmer), adiponectin (mouse adiponectin/Acrp30, Quantikine ELISA kit; R&D Systems), resistin (mouse resistin Quantikine ELISA kit; R&D Systems), growth hormone (Rat/Mouse GH ELISA kit; Millipore), adrenaline and noradrenaline (IBL), and corticosterone (IDS) (all according to the manufacturers' instructions). Blood glucose levels were measured using a glucose meter (One Touch Ultra; LifeSpan). The methods for DLK1 ELISA and tissue determination of TAG have been described previously (1).

Glucose and Insulin Tolerance Tests. GTTs and ITTs were carried out on 6-mo-old mice according to published protocols (2).

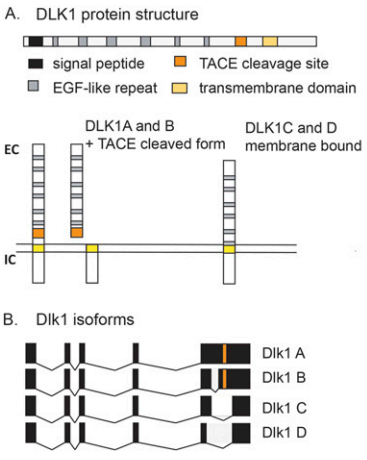
Morphometry of the Abdominal Adipose Tissue. Images of each WAT section were acquired using a digital camera and microscope (Olympus BX41). Adipocyte cross-sectional area was measured using AnalySIS software (Soft Imaging System). Two fields from each section from the epididymal adipose tissue depot ($n = 4$ mice per genotype) were analyzed to obtain the mean cell area per animal.

Real-Time Quantitative PCR. mRNAs were analyzed by RT-PCR as described (1). Quantification was performed using the relative standard curve method, and target gene expression was normalized to the expression of *Hprt* or *β-2 Microglobulin* (*β2M*), the expression of which did not differ between the groups. All primers (sequences in Table S7) were amplified with efficiency greater than or equal to 85%.

Determination of Isoform Use of Dlk1. Levels of mRNA encoding cleavable vs. membrane-tethered Dlk1 were determined using RT-qPCR with isoform-specific primers (Table S7). The *Dlk1-AB* primers contain sequences within the exon 5 spliced region whereas *Dlk1-all* primers detect sequences common to all transcripts. Both sets of primers amplify with high efficiency (>0.9). The relative proportion on *Dlk1AB* to all was calculated as a percentage of *β2M* normalized values from each primer set.

Western Blotting. Sample preparation, gel electrophoresis, and Western blotting have been described previously (3), with Abcam anti-DLK1 (ab21682, which recognizes the intracellular domain of the protein) at 1:500. Anti-alpha tubulin (clone B-5-1-2; Sigma-Aldrich) was used as a loading control at 1:10,000. Anti-FAS 1:1,000 (3180; Cell Signaling Technology) and anti-CD36 1:1,000 (ab133625; Abcam) were used to assess lipogenic protein in the liver, and Gapdh was used as a loading control at 1:5,000 (sc166574; Santa Cruz). Signaling studies were performed using the Pathscan Akt Signaling Antibody Array Kit (5301; Cell Signaling Technology).

1. Charalambous M, et al. (2012) Imprinted gene dosage is critical for the transition to independent life. *Cell Metab* 15(2):209–221.
2. Medina-Gomez G, et al. (2005) The link between nutritional status and insulin sensitivity is dependent on the adipocyte-specific peroxisome proliferator-activated receptor-gamma2 isoform. *Diabetes* 54(6):1706–1716.
3. da Rocha ST, et al. (2009) Gene dosage effects of the imprinted delta-like homologue 1 (dlk1/pref1) in development: Implications for the evolution of imprinting. *PLoS Genet* 5(2):e1000392.



C. Regulation of Dlk1 isoforms in WT and WT/TG tissues.

Tissue	WT fold expression	Total Dlk1		%Dlk1A&B/Total		n
		mean	s.e.	mean	s.e.	
Embryo		67.85	11.20	19.0	2.3	3
Placenta		4.76	0.67	38.8	0.4	3
Adrenal WT	1	3.11	0.59	53.2	5.0	4
Adrenal WT/TG	4	12.09	5.40	38.5	10.6	4
Muscle WT	1	0.03	0.01	32.2	1.4	4
Muscle WT/TG	100	3.01	0.95	27.9	3.3	3
WAT WT	1	0.02	0.00	47.9	9.3	3
WAT WT/TG	8	0.16	0.06	41.5	6.7	4
BAT WT	1	0.06	0.01	39.7	3.0	4
BAT WT/TG	8	0.48	0.04	24.7	1.0	4
Pituitary WT	1	32.29	6.47	20.7	0.7	4
Pituitary WT/TG	4	116.60	20.42	24.6	4.9	3
Brain WT	1	2.04	0.16	22.7	0.9	3
Brain WT/TG	1	1.73	0.30	31.7	0.8	3
Intestine WT	not detectable					
Intestine WT/TG						
Liver WT	not detectable					
Liver WT/TG						

Fig. S1. Expression of Dlk1 from the 70-kb BAC transgenic recapitulates endogenous tissue and splice-form specific expression. (*A*) Protein structure of full-length DLK1. The extracellular portion (EC) of the protein contains six EGF-like repeat domains (gray boxes) and a signal peptide for membrane targeting (black box). The protein may be cleaved from an extracellular cleavage site (orange box) to release a 50-kDa ectodomain by the Tnfα-converting enzyme TACE/ADAM17. The uncleaved protein contains a single pass transmembrane domain (yellow box) and a small intracellular domain (IC). (*B*) Presence or absence of the sequences encoding the TACE cleavage site (orange box) in the mRNA sequence is regulated by alternative splicing. The cleavage site is included in proteins translated from *Dlk1*A and -B, but not in proteins encoded by *Dlk1*C and -D. (*C*) Relative expression of total Dlk1 and %Dlk1A/B in prenatal and adult male tissues from hemizygotes (WT/TG) of the 70C line compared with WT littermates. Relative expression of WT/TG to WT is shown as WT-fold expression. WT embryo and placenta are shown for reference; WT/TG are not shown for these tissues but have been reported previously (3).

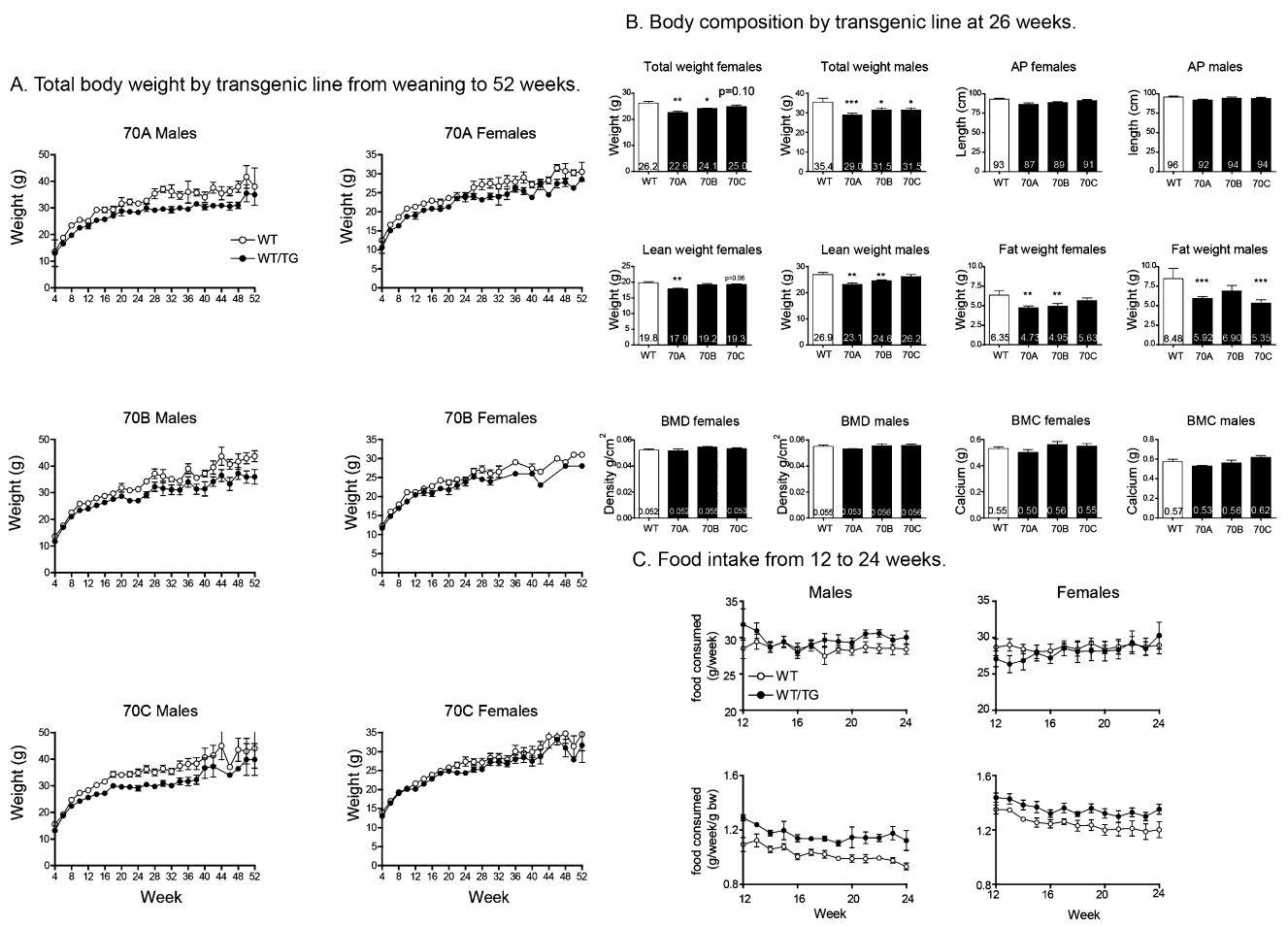


Fig. S2. Adult size, body composition, and food intake. (*A*) Dlk1-TG animals are consistently smaller than WT littermates. n = 3–24 animals per line per sex per genotype; all lines are compared with WT littermates. (*B*) Body composition of three Dlk1-BAC transgenic lines (70A, 70B, and 70C) compared with WT littermates from all three crosses combined. Female (n = 3–12) and male (n = 4–12) mice were terminally anesthetized and then scanned in a DXA scanner to determine lean and fat composition, bone mineral density (BMD), and bone mineral calcium (BMC). All three lines consistently show a reduction in total body mass and lean and fat mass, but no change in anterior–posterior length (AP) or bone density. Numbers at the bottom of the bar show the mean of the data. TG lines were compared with WT using one-way ANOVA with Bonferroni's post hoc test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. (*C*) Food intake of 70B-TG mice and WT littermates over a 12-wk period, displayed as grams of food consumed per week (*Upper*) and normalized for body weight (*Lower*). Females, WT, n =7; TG, n = 5; males, WT, n = 6; TG, n = 6. Mean food consumption does not differ between WT (males 28.5 ± 0.6 g/wk, females 28.5 ± 0.7 g/wk) and TG (males 29.9 ± 0.6 g/wk, females 27.5 ± 1.1 g/wk) but is significantly different when normalized to body weight in males (WT 1.03 ± 0.01 g/wk per g body weight; TG 1.16 ± 0.02 g/wk per g body weight, $P < 0.001$) and females (WT 1.25 ± 0.02 g/wk per g body weight; TG 1.36 ± 0.02 g/wk per g body weight, $P < 0.05$). Data compared by Mann–Whitney U test. All values show mean ± SEM.

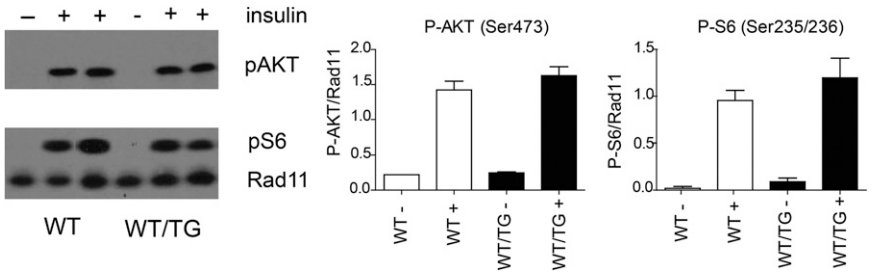


Fig. S3. Sensitivity to insulin is not compromised in the liver of Dlk1-TG mice. (*Left*) AKT and protein S6 phosphorylation after insulin stimulation of WT and WT/TG mice. Level of phosphorylated protein was normalized to total protein levels using the reporter Rad11 (*Lower*), and the mean ± SEM of four mice per genotype is charted *Center* and *Right*.

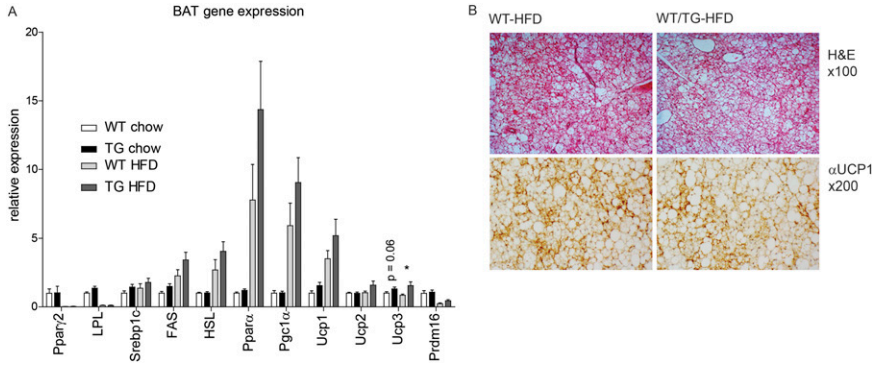


Fig. S4. BAT gene expression in Dlk1-overexpressing mice. (*A*) Relative expression of BAT genes from interscapular BAT at 6 mo (from 70C fasted male mice on a chow or HFD), measured by RT-qPCR. Data are normalized to reference gene expression and then presented relative to WT = 1.0. HSL, hormone-sensitive lipase; LPL, lipoprotein lipase. WT and WT/TG are compared by Mann–Whitney *U* test, $n > 6$ per genotype, $^*P < 0.05$. All values show mean ± SEM. (*B, Upper*) Hematoxylin/eosin staining of BAT from 6-mo-old fasted male WT and WT/TG (70C) mice fed a HFD. (*Lower*) Immunohistochemistry for UCP1 on the same samples as above. UCP1 protein expression is visible as a brown staining.

Table S1. Organ weights at 6 mo from the 70B line

	Males				Females			
	WT		WT/TG		WT		WT/TG	
Tissue	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Muscle, g	0.184	0.003	0.173**	0.002	0.144	0.004	0.122*	0.005
Muscle, %	0.57	0.01	0.57	0.01	0.58	0.02	0.52*	0.01
Gonadal WAT, g	0.922	0.159	0.338*	0.028	0.659	0.297	0.202*	0.046
Gonadal WAT, %	2.74	0.40	1.23**	0.08	2.81	1.37	0.91*	0.21
Renal WAT, g	0.230	0.040	0.079*	0.011	0.209	0.036	0.118	0.031
Renal WAT, %	0.71	0.11	0.29**	0.04	0.85	0.14	0.53	0.14
Liver, g	1.512	0.07761	1.244*	0.0553	1.143	0.046	1.106	0.096
Liver, %	4.62	0.22	4.57	0.17	4.69	0.12	4.93	0.25
Brain, g	0.467	0.004	0.459	0.008	0.472	0.008	0.475	0.009
Brain, %	1.44	0.02	1.56*	0.03	1.91	0.05	2.05*	0.04
Interscapular BAT, g	0.135	0.022	0.075*	0.009	0.067	0.007	0.056*	0.009
Interscapular BAT, %	0.41	0.06	0.27	0.03	0.28	0.3	0.25	0.03

Organ weights at 6 mo from the 70B line. $n > 8$ males per genotype; $n > 5$ females. ANOVA with Bonferroni's posttest, $^*P < 0.05$, $^{**}P < 0.01$.

Table S2. Serum and tissue biochemistry in WT and WT/TG mice at 6 mo

	Males		Females	
	WT	WT/TG	WT	WT/TG
70B line				
Fasted glucose, mg/dL	126.9 ± 10.1	104.5 ± 7.1	97.5 ± 9.3	101.1 ± 6.3
Free-fed glucose, mg/dL	214.7 ± 22.5	188.0 ± 20.5	198.0 ± 36.1	137.3 ± 32.2
Fasted insulin, pmol/L	83.3 ± 8.3	70.9 ± 5.6	78.7 ± 8.2	101.0 ± 12.2
Free-fed insulin, pmol/L	100.7 ± 14.7	80.5 ± 18.9	67.8 ± 11.2	83.7 ± 1.3
Serum DLK1, ng/mL	20 ± 5	194 ± 33***	28 ± 4	198 ± 32**
70C line				
Fasted glucose, mg/dL	107.6 ± 7.6	100.8 ± 8.3	106.0 ± 6.8	99.0 ± 6.2
Free-fed glucose, mg/dL	210.1 ± 38.9	191.7 ± 22.6	153.9 ± 10.4	188.6 ± 25.8
Fasted insulin, pmol/L	61.0 ± 5.4	61.5 ± 7.0	106.0 ± 26.4	111.0 ± 14.4
Free-fed insulin, pmol/L	99.0 ± 17.7	100.3 ± 42.6	nd	nd
Leptin, ng/mL	2.5 ± 0.4	0.8 ± 0.2 **	2.4 ± 0.3	1.8 ± 0.3
Adiponectin, μg/mL	7.7 ± 0.7	7.3 ± 0.9	nd	nd
Resistin, ng/mL	14.8 ± 1.2	14.2 ± 1.2	26.8 ± 2.0	25.3 ± 2.6
Fasted triglyceride, CTE mg/dL	207 ± 22	199 ± 16	175 ± 14	161 ± 19
Free-fed triglyceride, CTE mg/dL	338 ± 36	236 ± 32	192 ± 15	205 ± 31
Fasted glycerol, CTE mg/dL	221 ± 2	295 ± 6	190 ± 20	248 ± 13
Free-fed glycerol, CTE mg/dL	326 ± 11	376 ± 16	297 ± 3	274 ± 3
Liver triglyceride, CTE mg/g tissue	76.7 ± 9.6	96.3 ± 7.7	nd	nd
Muscle triglyceride, CTE mg/g tissue	35.4 ± 9.0	16.7 ± 1.9	nd	nd
Fasted FFA, μmol/L	568 ± 57	792 ± 67*	nd	nd
Free-fed FFA, μmol/L	272 ± 31	322 ± 29	nd	nd
Serum DLK1, ng/mL	28 ± 3	339 ± 28***	54 ± 10	387 ± 46***

WT/TG from the 70B and 70C lines compared with WT by Mann–Whitney *U* test, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$. Data are shown as mean values ± SEM, $n = 4$–10 per genotype per sex.

Table S3. Serum parameters in WT ob/ob and WT/TG ob/ob mice at 6 mo

	Males				Females			
	WT ob/ob ($n = 7$)		WT/TG ob/ob ($n = 6$)		WT ob/ob ($n = 8$)		WT/TG ob/ob ($n = 5$)	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Fasted glucose, mg/dL	190.8	32.4	147.6	19.8	180.0	30.6	183.6	32.4
Free-fed glucose, mg/dL	361.8	46.8	374.4	41.4	381.6	72.0	421.2	30.6
Fasted insulin, ng/mL	4.4	0.8	4.3	0.8	5.3	1.4	2.8	0.2
Adiponectin, μg/mL	6.4	0.5	8.1	1.0	nd	nd	nd	nd
Resistin, ng/mL	17.0	2.0	21.1	3.1	nd	nd	nd	nd
Fasted triglyceride, CTE mg/dL	199	16	179	10	173	23	163	17
Serum DLK1, ng/mL	51	8	365***	24	51	13	243**	85

WT/TG are from the 70C line. Serum DLK1 is elevated in WT/TG animals compared with WT littermates in both sexes, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, Mann–Whitney *U* test.

Table S4. Body composition of 70C line on a HFD

Body composition measure	WT		WT/TG	
	Mean	SEM	Mean	SEM
Total mass, g	40.8	1.7	35.6*	1.4
Lean mass, g	24.7	0.6	22.9*	0.3
Fat mass, g	14.0	1.2	11.4*	1.5
Fat mass, %	36.3	1.9	32.1	2.8
Length, cm	96	1	97	1
BMD, g/cm^2	0.059	0.001	0.059	0.001
BMC, g	0.55	0.02	0.58	0.01

Body composition of WT and WT/TG male mice at 24 wk from the 70C line on a HFD. $n > 8$ mice per genotype. Mann–Whitney *U* test, $*P < 0.05$.

Table S5. Serum parameters in WT and WT/TG male mice on a HFD at 6 mo

Hormone/metabolite	WT	WT/TG
Fasted glucose, mg/dL	243.0 ± 25.2	253.8 ± 14.4
Fasted insulin, pmol/L	366.6 ± 66.8	355.6 ± 79.4
Fasted triglyceride, CTE mg/dL	119 ± 11	129 ± 9
Fasted FFA, μmol/L	498 ± 18	482 ± 18
Cholesterol, mmol/L	4.5 ± 0.2	3.8 ± 0.2*
ALT, U/L	43.0 ± 8.0	22.3 ± 6.8*
Leptin, ng/mL	41.7 ± 6.8	28.3 ± 6.7
Adiponectin, μg/mL	9.6 ± 0.9	8.3 ± 1.1
Resistin, ng/mL	23.4 ± 3.1	23.5 ± 1.5
Serum DLK1, ng/mL	44 ± 5	380 ± 12**

WT/TG (70C) animals compared with WT littermates by Mann–Whitney *U* test, *$P < 0.05$, **$P < 0.01$. Values shown as mean ± SEM, WT $n = 8$, WT/TG $n = 9$.

Table S6. Serum endocrine parameters in WT and WT/TG adult animals

Hormone	WT	WT/TG
Males		
Adrenaline, ng/mL	3.4 ± 1.1	2.7 ± 0.8
Noradrenaline, ng/mL	2.6 ± 0.3	4.6 ± 1.5
Females		
Corticosterone, ng/mL	481 ± 65	434 ± 52
Total T3, nmol/L	0.7 ± 0.1	0.7 ± 0.1
Total T4, nmol/L	46.0 ± 3.4	40.8 ± 3.7

Catecholamine, corticosterone, and T3/T4 levels were measured in free-fed serum; WT and WT/TG animals were compared by Mann–Whitney *U* test, with $n = 6$–9 WT and 6–10 WT/TG. Values are shown as mean ± SEM.

Table S7. qPCR primers used in this study

Target gene	Forward	Reverse	Refs.
Dlk1-AB	CATCCTGAAGGTGTCCATGA	CAGGATGGTGAAGCAGATGG	This work
Dlk1-all	GCTTCGCAAGAAGAAGAACC	CTCATCACCAGCCTCCTTGT	This work
Ucp1	CTGGGCTTAACGGGTCCTG	CTGGGCTAGGTAGTGCCAGTG	(1)
Ucp2	GACCTCAAAGCAGCCTCCAG	GAGAAACGGGGGACCTTCAATC	(1)
Ppara	AGCAGTGCTGGCTACCTTCAA	AATATGTAGCCACCCCCTTGG	(1)
Pparg1	TTTAAAAACAAGACTACCCTTTTACTGAATT	AGAGGTCCACAGAGCTGATTCC	(1)
Pparg2	GATGCACTGCCTATGAGCACTT	AGAGGTCCACAGAGCTGATTCC	(1)
Pgc1a	GTAGGCCCAGGTACGACAGC	GCTCTTTGCGGTATTCATCCC	(1)
Pgc1b	CTCCAGGCAGGTTCAACCC	GGGCCAGAAGTTCCCTTAGG	(1)
Fas	CCTGGACTCGCTCATGGGT	ATTTCCTGAAGTTTCCGCAGC	(1)
Scd1	CCTGCGGATCTTCCTTATCATT	GATCTCGGGCCCATTCG	(1)
Srebp1c	CACGGAGCCATGGATTGC	CCCGGGAAGTCACTGTCTTG	(1)
Lpl	GTGGCCGAGAGCGAGAAC	AAGAAGGAGTAGGTTTTATTTGTGGAA	(1)
Glut4	GCTTTGTGGCCTTCTTTGAGAT	GACGGCAAATAGAAGGAAGACG	(1)
aP2	GCGTGGAATTCGATGAAATCA	CCCGCCATCTAGGGTTATGA	(1)
HPRT	CAGGCCAGACTTTGTTGGAT	TTGCGCTCATCTTAGGCTTT	(2)
Cd36	TGTGTGGAGCAACTGGTGGAT	CGTGGCCCGGTTCTACTAATT	(3)
PEPCK	CCACAGCTGCTGCAGAACAC	GAAGGGTCGCATGGCAAA	(1)
Igfbp1	CTGCCAAACTGCAACAAGAA	GACCCAGGGATTTTCTTTCC	This work
Cpt1a	TGAGTGGGCGTCCTCTTTGG	CAGCGAGTAGCGCATAGTCATG	(1)
Cpt1b	CCAAACGTCACTGCCTAAGCT	GGCCGCACAGAATCCAAGTA	(1)
Prl	AGGCCTATCCTGAAGCCAAAGGAA	TTTGGCACCTCAGGACCTTGAGAA	(4)
THRb	TGCCCGCACCATGTTACTCCTTAT	TGCAGTAGTTGGTTCTGACAGCCT	(3)
POMC	AGCAACCCGCCCAAGG	GCGTCTGGCTCTTCTCGG	This work
GHRHR	TTGTGAAGAGGGACTGCACCATCA	AATAGCCACGCAGAGGGCTACAAT	(3)
GH	TGGGCAGATCCTCAAGCAAACCTA	GAAGGCACAGCTGCTTTCCACAAA	(3)
GHRHR	TTGTGAAGAGGGACTGCACCATCA	AATAGCCACGCAGAGGGCTACAAT	(3)
Pdk4	TTTCTCGTCTCTACGCCAAGT	CAGCTTCGGAGCTCATCTG	This work

1. Lelliott CJ, et al. (2006) Ablation of PGC-1beta results in defective mitochondrial activity, thermogenesis, hepatic function, and cardiac performance. *PLoS Biol* 4(11):e369.
2. Charalambous M, et al. (2012) Imprinted gene dosage is critical for the transition to independent life. *Cell Metab* 15(2):209–221.
3. Barclay JL, et al. (2011) GH-dependent STAT5 signaling plays an important role in hepatic lipid metabolism. *Endocrinology* 152(1):181–192.
4. Zhu X, et al. (2006) Sustained Notch signaling in progenitors is required for sequential emergence of distinct cell lineages during organogenesis. *Genes Dev* 20(19):2739–2753.