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Supplemental Information

Decreased Blood Lipids, Increased Energy Expenditure and Improved Glucose Tolerance in Carboxylesterase 3/Triacylglycerol Hydrolase Deficient Mice

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SUPPLEMENTAL EXPERIMENTAL PROCEDURES:

Materials - Polyclonal goat anti-human apoB antibodies were purchased from Chemicon International (Temecula, CA). Monoclonal anti-mouse microsomal triglyceride transfer protein (MTP) antibodies were from BD biosciences. Mouse anti-goat, goat anti-mouse and goat anti-rabbit secondary antibodies conjugated to horseradish peroxidase (HRP) were obtained from Pierce Biotechnology Inc (Rockford, IL). Anti-protein disulfide isomerase (PDI) antibodies were from Stressgen Biotechnologies (Victoria, BC, Canada). Polyclonal antibodies against mouse and human TGH and rat albumin were raised in rabbits and affinity purified in our laboratory. Poloxamer 407 (P-407) was a gift from BASF Customer Care. ECL® immunoblotting reagents, [U-^{14}C]glycerol (149 mCi/mmol) and [9,10(n)-^{3}H]oleic acid (10 Ci/mmol) were purchased from Amersham-Pharmacia Canada (Oakville, ON, Canada). Oleic acid, essentially fatty acid-free bovine serum albumin (BSA) and fumed silica (Cab-O-Sil) were purchased from Sigma Chemical (St. Louis, MO). [L-^{35}S]Methionine (1175 Ci/mmol) was obtained from PerkinElmer (Boston, MA). Dulbecco's modified Eagle medium (DMEM), sodium pyruvate, penicillin and streptomycin, FBS were from GIBCO BRL (Life Technologies, Grand Island, NY). All

other chemicals and solvents were of reagent grade or better quality and were obtained from local suppliers.

RNA Isolation and PCR Analysis Mice that had a free access to chow diet (fed state) and mice that were fasted for 16 hours (fasted state) were terminated and livers were collected. Total RNA was isolated using Trizol® reagent (Life Technologies, Inc.) according to the manufacturer's instructions. First-strand cDNA synthesis from 2 μg of total RNA was performed using SuperScript™ II reverse transcriptase (Invitrogen) primed by oligo$(dT)_{12–18}$ primers. The PCR program included denaturation at 94 °C for 30 s, annealing at 59 °C for 30 s, and elongation at 72 °C for 2 minutes (30 cycles). The PCR product was separated by electrophoresis on 1% (w/v) agarose gels.

Real-time qPCR was performed using Platinum® Quantitation PCR supermix (Invitrogen), SYBR Green I (Molecular Probes), and intron-spanning, gene-specific oligonucleotides (250 nM of each primer) in a total volume of 25 μl. Transcripts were detected by real-time qPCR with a Rotor-Gene 3000 instrument (Montreal Biotech, Montreal, Quebec, Canada). Data were analyzed using the Rotor-Gene 6.0.19 program. A standard curve was used to calculate mRNA level relative to that of a control gene, cyclophilin. The specificity of products was confirmed by agarose gel electrophoresis and sequence analysis. All primers were synthesized at the DNA Core Facility of the University of Alberta. Primers for different genes are listed in Table S1.

Subcellular fractionation - Liver samples in 1 ml of ice cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 250 mM sucrose and 1 mM EDTA) were homogenized by a Potter

Elvhjem homogenizer. Unbroken tissues were pelleted by brief centrifugation and the resulting homogenates were centrifuged at 99,000 rpm (TL100.2 rotor) for 45 min to yield supernatants (cytosol containing cytosolic lipid droplets) and membrane pellets. Membranes were washed with Tris-buffered saline (TBS) and re-suspended in TBS by sonication (Lehner et al., 1999; Wei et al., 2005). Protein concentrations were determined with BioRad protein assay reagent using BSA as a protein standard.

Immunoblot analyses - Protein samples were electrophoresed on an SDS-10% polyacrylamide gel and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4 °C with 5% (w/v) skimmed milk in 20 mM Tris/HCl, pH 7.4, 150 mM NaCl and 0.1% Tween 20 then incubated for 1 h with antibody raised against the specified protein followed by a 1h wash and 1 h incubation with secondary goat anti-rabbit (detection of TGH, albumin and PDI), goat anti-mouse (detection of MTP) or mouse anti-goat (detection of apoB) conjugated to HRP. Immunoreactivity was detected by the ECL™ system according to the manufacturer's instructions. Where indicated, primary and secondary antibodies were stripped from membranes by incubation with 100 mM 2-mercaptoethanol/2% SDS/62.5 mM Tris/HCl (pH 6.7) for 30 min at 50 °C, and the blot was re-probed with another antibody. For analysis of plasma apoB48 and apoB100 levels blood was collected from each mouse via cardiac puncture in the presence of trace amounts of 250 mM EDTA, and plasma was isolated by centrifugation. Plasma (2 µl) was resolved on a SDS-4-15% polyacrylamide gradient gel. Proteins were transferred to nitrocellulose membranes and probed with anti-apoB and anti-albumin antibodies.

Lipid analysis by thin-layer chromatography — Lipids were extracted from samples with chloroform/methanol (2:1, v/v) in the presence of non-radioactive lipid carriers (phosphatidylcholine [PC], OA, trioleoylglycerol, and cholesteryl oleate) (Gilham et al., 2003). The chloroform phase containing lipids was dried under nitrogen, redissolved in a small volume of chloroform, and applied to silica gel H thin-layer chromatography plates. The plates were developed to one-third the height with chloroform/methanol/acetic acid/water (25:15:4:2, by vol) to allow separation of PC from other polar glycerolipids, followed by development in heptane/isopropyl ether/acetic acid (60:40:4, by vol) to separate neutral lipids [fatty acids, TG, and cholesteryl esters (CE)] (Gilham et al., 2003). The lipid classes were visualized by exposure to iodine vapor, the bands were scraped, and the associated radioactivity was determined by scintillation counting (Gilham et al., 2003).

Pancreatic Islet Isolation - Islets were isolated from each donor group using previously established techniques (Ohzato et al., 1991). Briefly, the donor pancreata was distended via the descending bile duct with a collagenase (type V, Sigma) solution. After storage on ice the distended pancreata were digested with the same collagenase solution for 14 minutes at 37 °C. Following the digest the islets were purified using a Dextran/Euro-Collins continuous density gradient and cultured in supplemented Hams F10 (Sigma). The cultured islets were incubated overnight at 37 °C in humidified air (5% CO_2, 95% air).

Islet Insulin Secretion Response to Glucose **-** Each islet donor group was examined for in vitro viability using a static incubation assay. Islets from WT and TGH knockout mice were isolated and cultured as a suspension overnight at 37°C in Ham's F10 (Invitrogen) culture media containing 0.5% bovine serum albumin (Korbutt et al., 1996). Cultured islets were washed (without centrifugation), sampled for total cellular insulin and DNA content, and aliquots of 50-100 islets were incubated for 120 min in 1.5ml of Ham's F10 supplemented with 2 mmol/L L-glutamine, 0.5% BSA and either 2.8 mM or 20.0 mM glucose. After incubation, media were collected and frozen for later analysis of insulin content using the rodent insulin radioimmuno assay by Linco. After analysis for insulin for both content and secretion the stimulation index was determined by dividing the percent insulin release at 20 mM glucose by that at 2.8mM glucose.

SUPPLEMENTAL REFERENCES

Gilham, D., Ho, S., Rasouli, M., Martres, P., Vance, D.E., and Lehner, R. (2003). Inhibitors of hepatic microsomal triacylglycerol hydrolase decrease very low density lipoprotein secretion. FASEB J *17*, 1685-1687.

Korbutt, G.S., Elliott, J.F., Ao, Z., Smith, D.K., Warnock, G.L., and Rajotte, R.V. (1996). Large scale isolation, growth, and function of porcine neonatal islet cells. J Clin Invest *97*, 2119-2129.

Lehner, R., Cui, Z., and Vance, D.E. (1999). Subcellullar localization, developmental expression and characterization of a liver triacylglycerol hydrolase. Biochem J *338 (Pt 3)*, 761-768.

Ohzato, H., Gotoh, M., Monden, M., Dono, K., Kanai, T., and Mori, T. (1991). Improvement in islet yield from a cold-preserved pancreas by pancreatic ductal collagenase distention at the time of harvesting. Transplantation *51*, 566-570.

Wei, E., Lehner, R., and Vance, D.E. (2005). C/EBPalpha activates the transcription of triacylglycerol hydrolase in 3T3-L1 adipocytes. Biochem J *388*, 959-966.

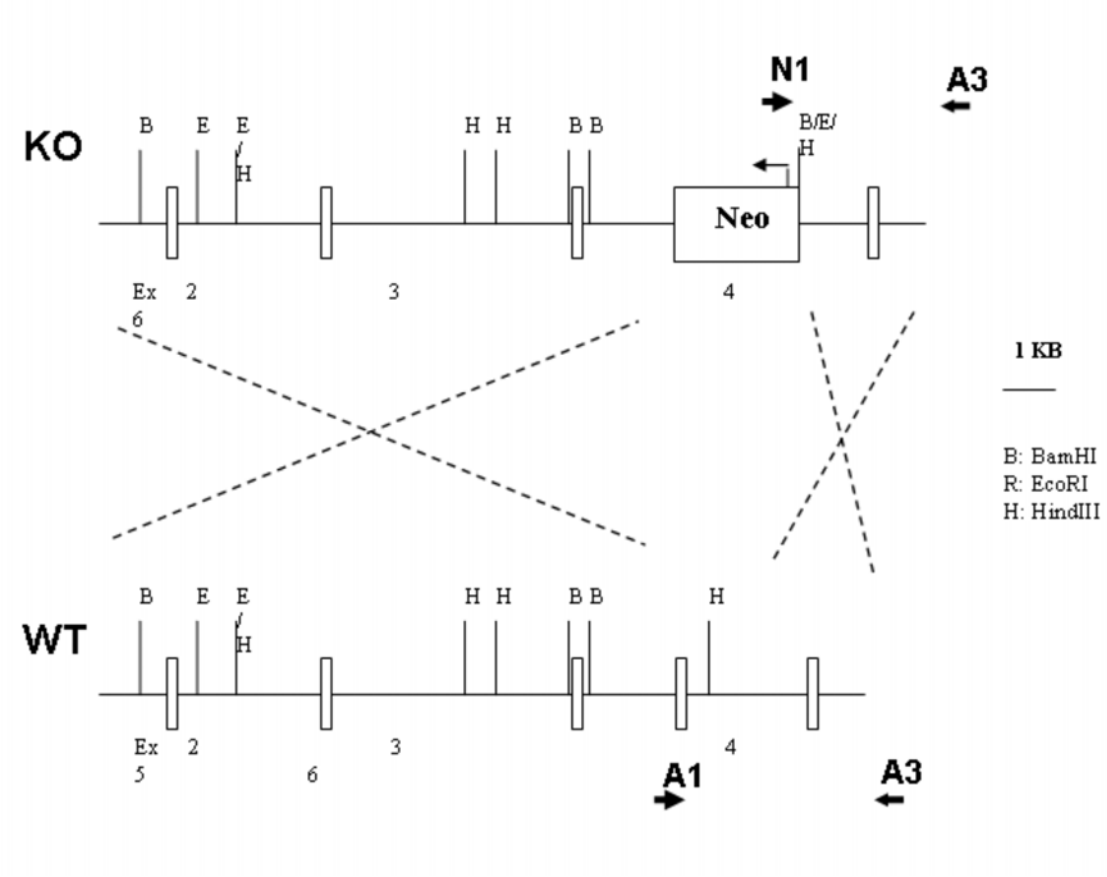

Figure S1. Gene-targeting strategy for the generation of TGH-deficient mice. Homologous recombination of the targeting vector with TGH allele replaces 850 bp including exon 5 with a neo cassette. Primers N1 and A3 amplify a 2.3 kb product in the targeted allele, primers A1 and A3 amplify a 2.9 kb product in the WT allele.

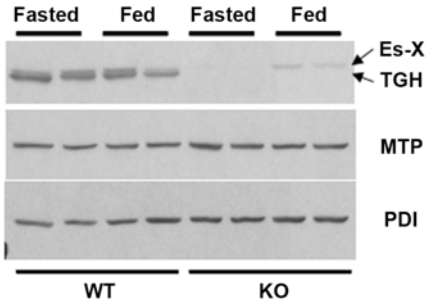

Figure S2. Analysis of hepatic MTP level in TGH-deficient mice. Adult (12-14 week old) male mice were fasted overnight for 16 h (fasted group) or allowed free access to a chow-diet (fed group). Liver homogenates were prepared and used for protein analysis. Immunoblots of liver homogenates with anti-mTGH, anti-MTP and anti-PDI (loading control) antibodies.

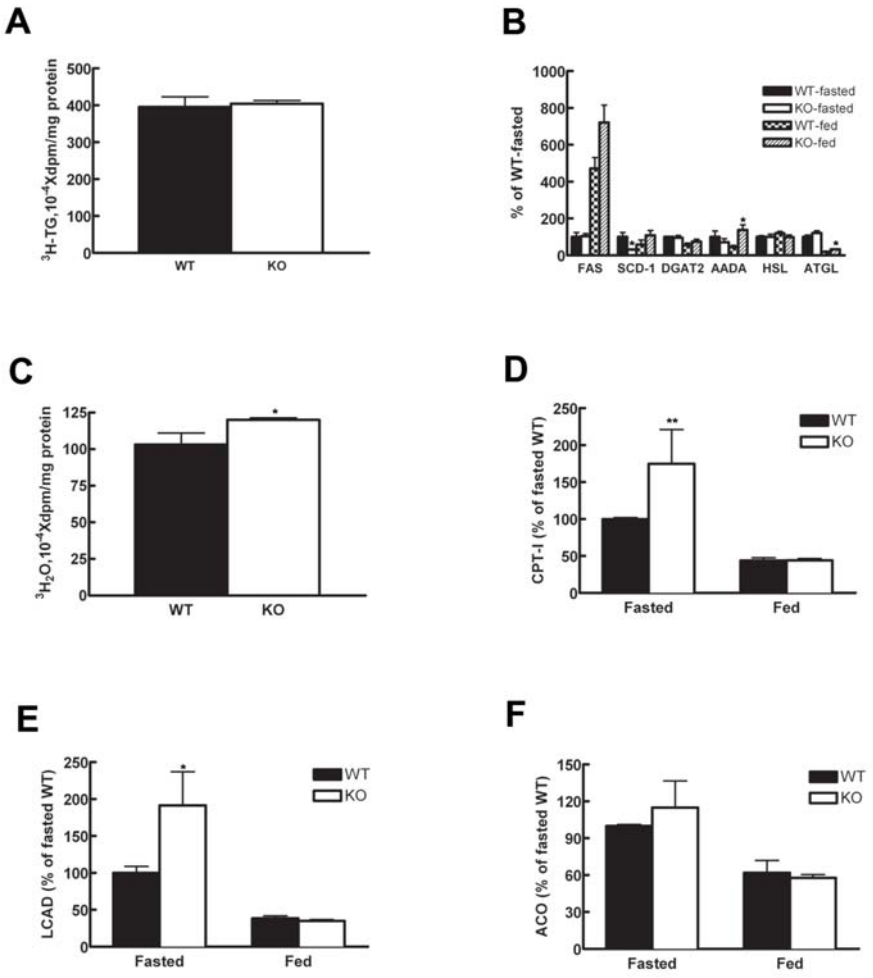

Figure S3. Hepatic TG synthesis is not altered by TGH deficiency but fatty acid oxidation is increased. (A) Primary mouse hepatocytes were incubated for 4 h in DMEM containing 0.4 mM [^{3}H]OA complexed to 0.5% fatty acid-free BSA. Cells were harvested and lipids were extracted and radioactivity in TG was determined. (B) Messenger RNA levels of hepatic FAS, SCD-1, DGAT2, AADA, HSL and ATGL were analyzed by quantitative real time PCR. (C) Primary hepatocytes were incubated for 4 h with 0.4 mM [^{3}H]OA complexed to BSA. Media were harvested, and ^3H_2O was extracted and analyzed. (D-F) RNA was extracted from the livers of fasted and fed mice and reverse transcribed into

cDNA. The expression of CPT-I, LCAD and ACO in the liver were evaluated by real-time PCR. Data are presented as the ratio of expression of a given gene to cyclophilin. Values are normalized to WT fasted mice where the gene/cyc ratio was set as 1. *$P < 0.05$, **$P < 0.01$ vs WT .

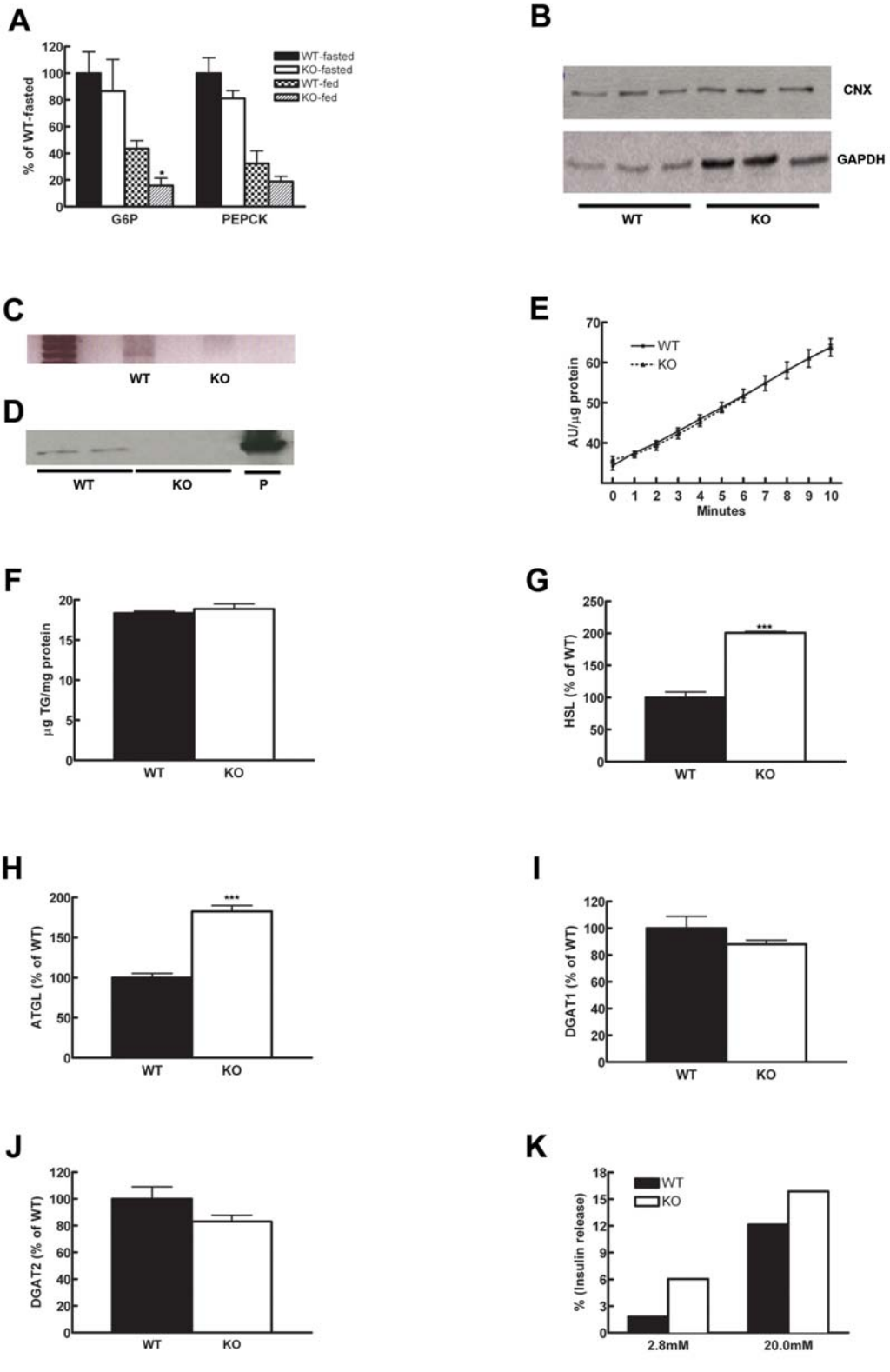
A
% of WT-fasted
WT-fasted
KO-fasted
WT-fed
KO-fed
G6P
PEPCK
B
CNX
GAPDH
WT
KO
C
WT
KO
D
WT
KO
P
E
AU/μg protein
WT
KO
Minutes
F
μg TG/mg protein
WT
KO
G
HSL (% of WT)
***
WT
KO
H
ATGL (% of WT)
***
WT
KO
I
DGAT1 (% of WT)
WT
KO
J
DGAT2 (% of WT)
WT
KO
K
% (Insulin release)
WT
KO
2.8mM
20.0mM

Figure S4. TGH deficiency decreases hepatic glucose production and increases glucose-stimulated insulin release by islets. (A) Hepatic gluconeogenesis related genes G6P and PEPCK mRNA levels were evaluated by quantitative real time PCR. (B) Immunoblot analysis of glycolysis protein GAPDH and calnexin (CNX) from primary mouse hepatocytes. (C) Islet TGH mRNA analyzed by RT-PCR. (D) Immunoblot with anti-TGH antibodies. Fifty μg of islet and white adipose tissue (WAT) proteins were analyzed. (E) Carboxylester hydrolase activity in islet homogenates. (F) TG levels in islets. (G-J) HSL, ATGL, DGAT1 and DGAT2 mRNA analyzed by quantitative real time PCR. (K) Glucose-stimulated insulin secretion. *P<0.05, ***P<0.001 vs WT.

Table S1. Primers used for RT and Real-Time PCR Analysis

Gene	Sequence
TGH	F: 5′-GGAGGGCAGGTGCTCTCA-3′
	R: 5′-GCCTTCAGCGAGTGGATAGC-3′
Cyclophilin	F: 5′-TCCAAAGACAGCAGAAAACTTTCG-3′
	R: 5′-TCTTCTTGCTGGTCTTGCCATTCC-3′
Es-x	F: 5′-CTATTCTTCCATGATGTGGCTCTGTG-3′
	R: 5′-CAAACATGACTGGGCCTCCTG-3′
Es-22	F: 5′-TGTAGCCTCCTACCATGTGCC-3′
	R: 5′-GGCCACAATCATCTTCCATG-3′
ATGL	F: 5'-CTTCCTCGGGGTCTACCACA-3'
	R: 5'-GCCTCCTTGGACACCTCAATAA-3'
HSL	F: 5'-TCAACCGACCAGGAGTGCT-3'
	R: 5'-CTCGTTGCGTTTGTAGTGCTC-3'
DGAT1	F: 5'-GGATCTGAGGTGCCATCGT-3'
	R: 5'- CCACCAGGATGCCATACTTG-3'
DGAT2	F: 5'-GGCTACGTTGGCTGGTAACTT-3'
	R: 5'-TTCAGGGTGACTGCGTTCTT-3'
CPT1	F: 5′-TGAGTGGCGTCCTCTTTGG-3′
	R: 5′-CAGCGAGTAGCGCATAGTCATG-3′
LCAD	F: 5′-GCGAAATACTGGGCATCTGAA-3′
	R: 5′-TCCGTGGAGTTGCACACATT-3′
ACO	F: 5′-CAGCAGGAGAAATGGATGCA-3′

	R: 5′-GGGCGTAGGTGCCAATTATCT-3′
MCAD	F: 5′-TTACCGAAGAGTTGGCGTATG-3′
	R: 5′-ATCTTCTGGCCGTTGATAACA-3′
PGC-1α	F: 5′-ATACCGCAAAGAGCACGAGAAG-3′
	R: 5′-CTCAAGAGCAGCGAAAGCGTCACAG-3′
PPARα	F: 5′-GACCTGAAAGATTCGGAAACT-3′
	R: 5′-CGTCTTCTCGGCCATACAC-3′
FAS	F: 5′-TTCCGTCACTTCCAGTTAGAG-3′
	R: 5′-TTCAGTGAGGCGTAGTAGACA-3′
SCD-1	F: 5′-GTTGCCAGTTTCTTTCGTG-3′
	R: 5′-GGGAAGCCAAGTTTCTACACA-3′
PEPCK	F: 5′-GAACTGACAGACTCGCCCTAT-3′
	R: 5′-TTCCCACCATATCCGCTTC-3′
G6P	F: 5′-GGATTCCGGTGTTTGAACGTC-3′
	R: 5′-CGGAGGCTGGCATTGTAGATG-3′

F, forward; R, reverse.

TGH, triacylglycerol hydrolase; Es-x, carboxylesterase-x, Es-22, carboxylesterase-22; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; DGAT, acylCoA:diacylglycerol acyltransferase; CPT1, carnitine palmitoyltransferase I; LCAD, long-chain acyl-CoA dehydrogenase; ACO, acyl-CoA oxidase; PGC1α, peroxisome proliferator-activated receptor-gamma coactivator 1 alpha; PPARα, peroxisome proliferator-activated receptor alpha; FAS, fatty acid synthase; SCD-1, stearoyl-Coenzyme A desaturase 1; PEPCK, phosphoenolpyruvate carboxykinase; G6P, glucose 6 phosphatase.