Supplementary Information

The transcription factor CREB-H Regulates Triglyceride Metabolism

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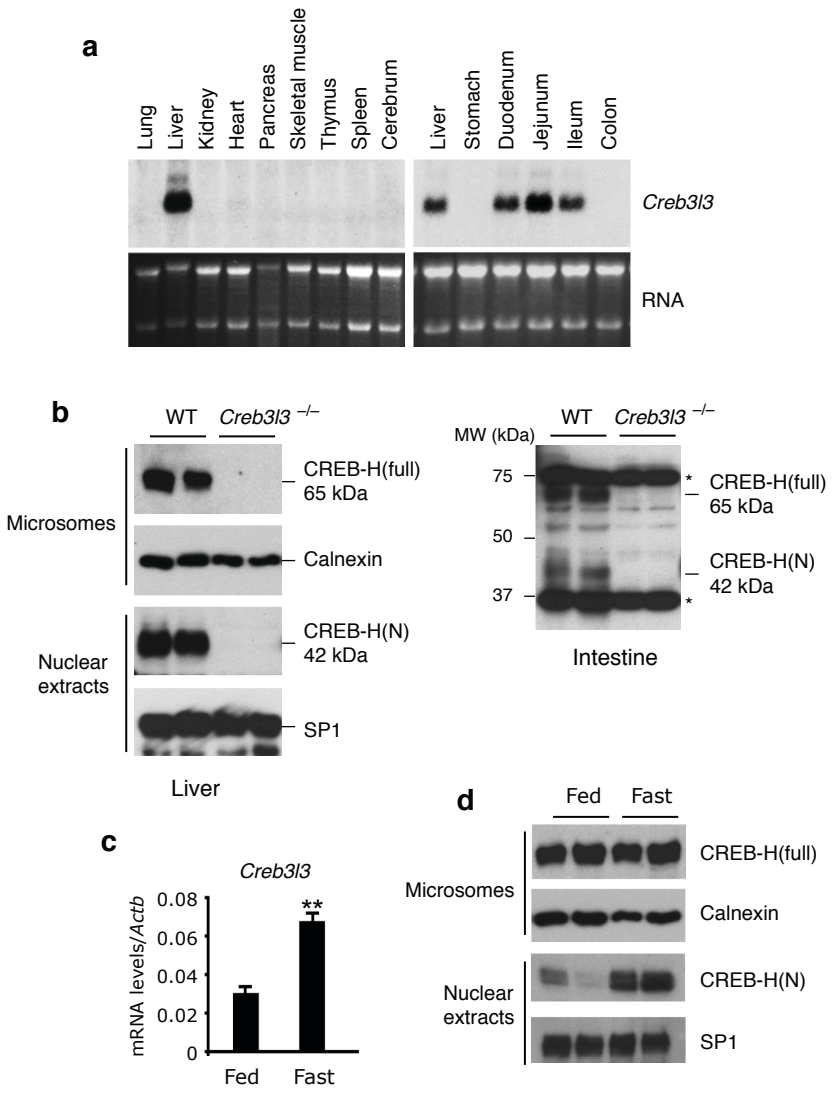
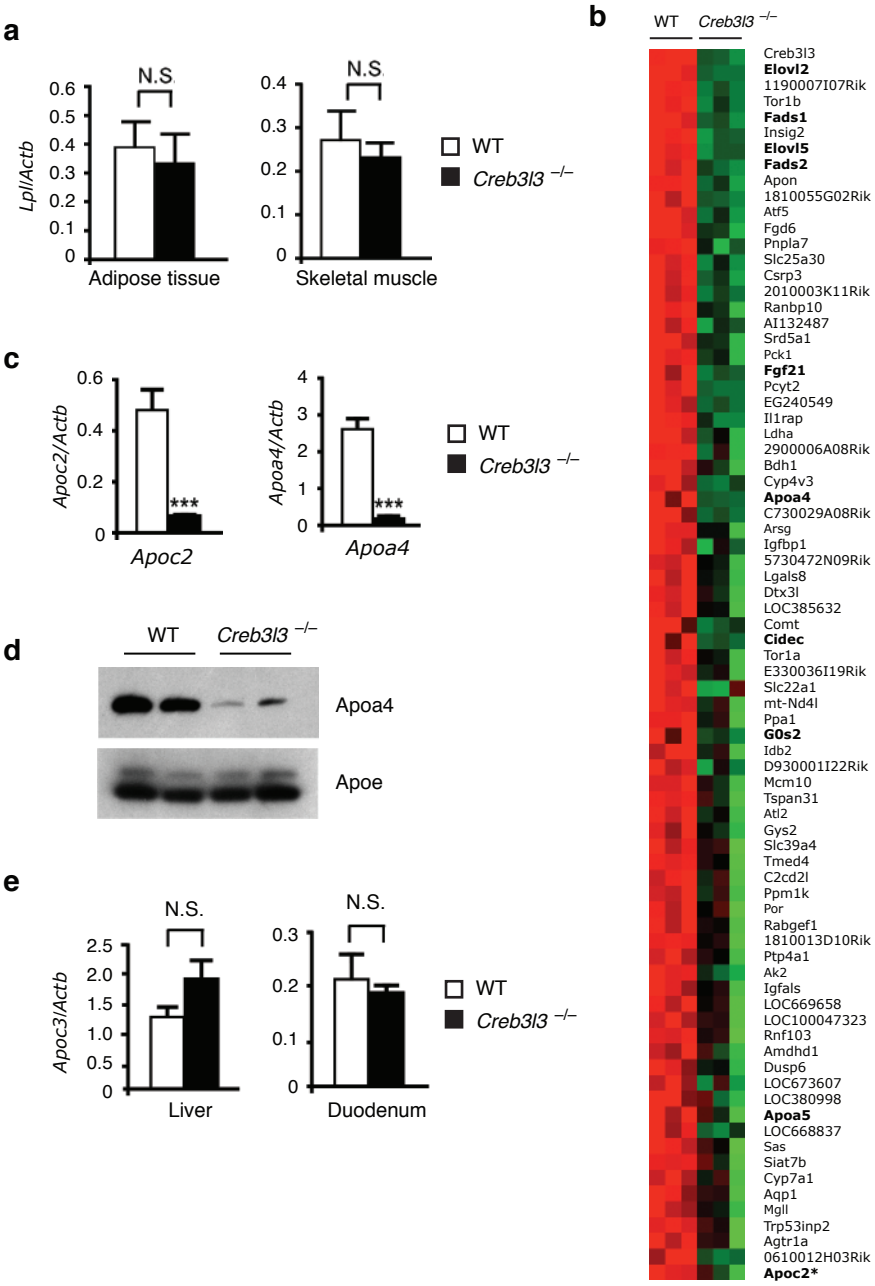
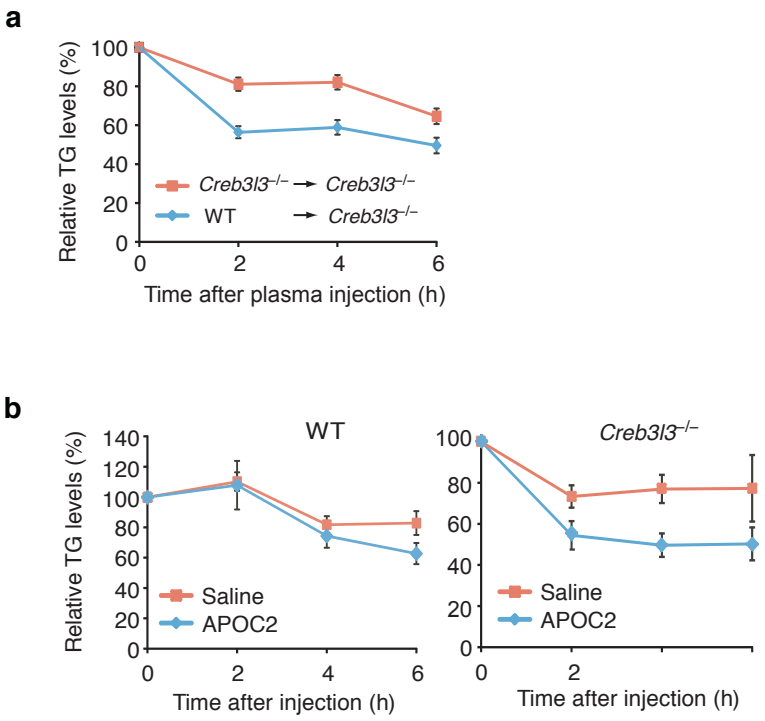


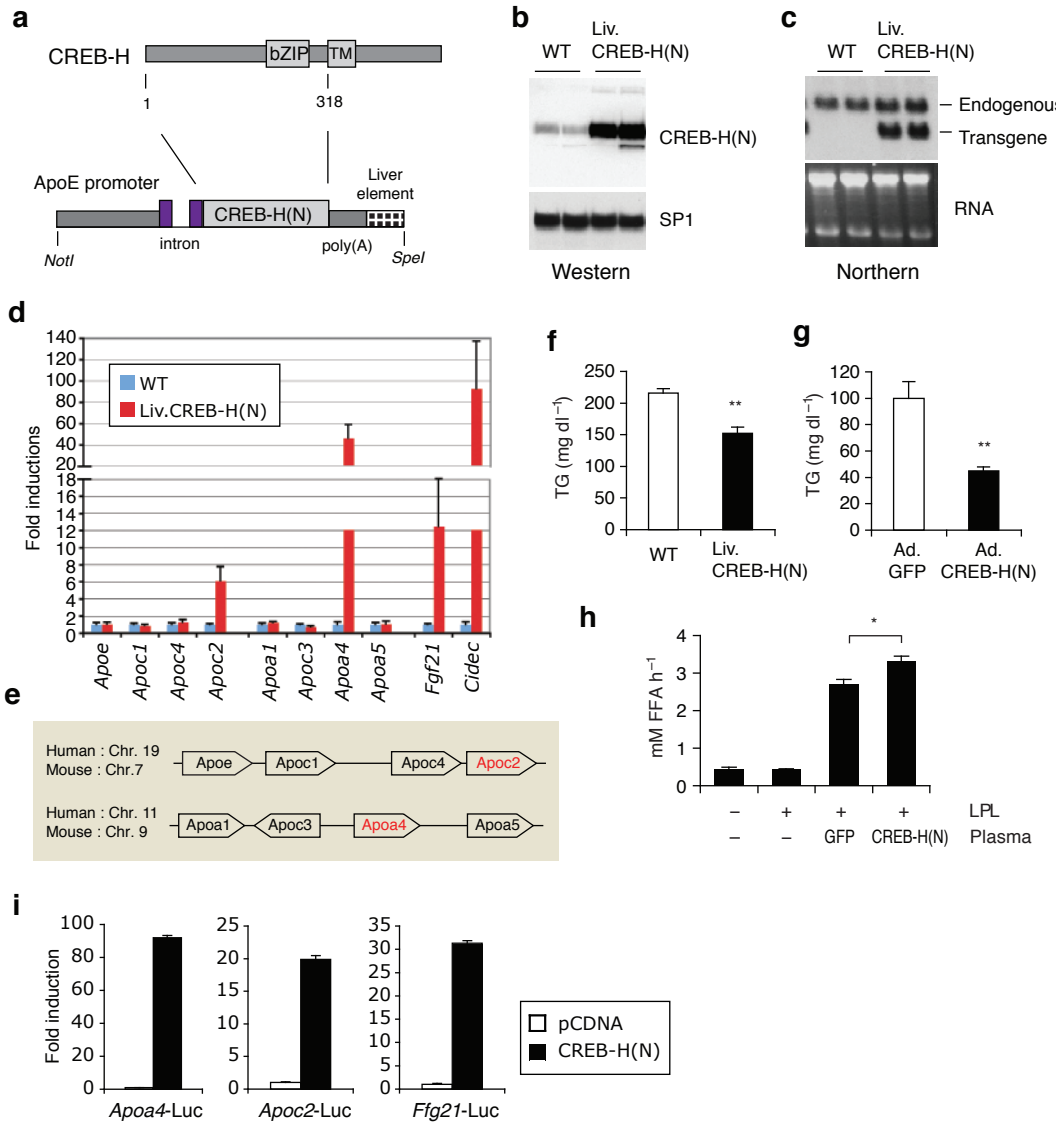
Supplementary Figure 1. *Creb3l3* is expressed in liver and small intestine and induced by fasting in mouse liver. **(a)** Total RNAs were isolated from various organs of C57BL/6 male mice and subjected to northern blot analysis to detect *Creb3l3* mRNA. **(b)** Western blot of CREB-H in microsomal fractions and nuclear extracts of the liver or whole lysates of intestinal epithelial cells of WT and *Creb3l3*$^{-/-}$ mice. *Nonspecific bands. **(c)** Hepatic *Creb3l3* mRNA level at fed state or after a 24 h fast determined by qRT-PCR. $n = 4$ mice per group. **(d)** CREB-H precursor and the processed CREB-H(N) levels in hepatic microsomal fractions and nuclear extracts, respectively. Calnexin and SP1 proteins served as loading controls. **$P < 0.01$compared to fed mice.



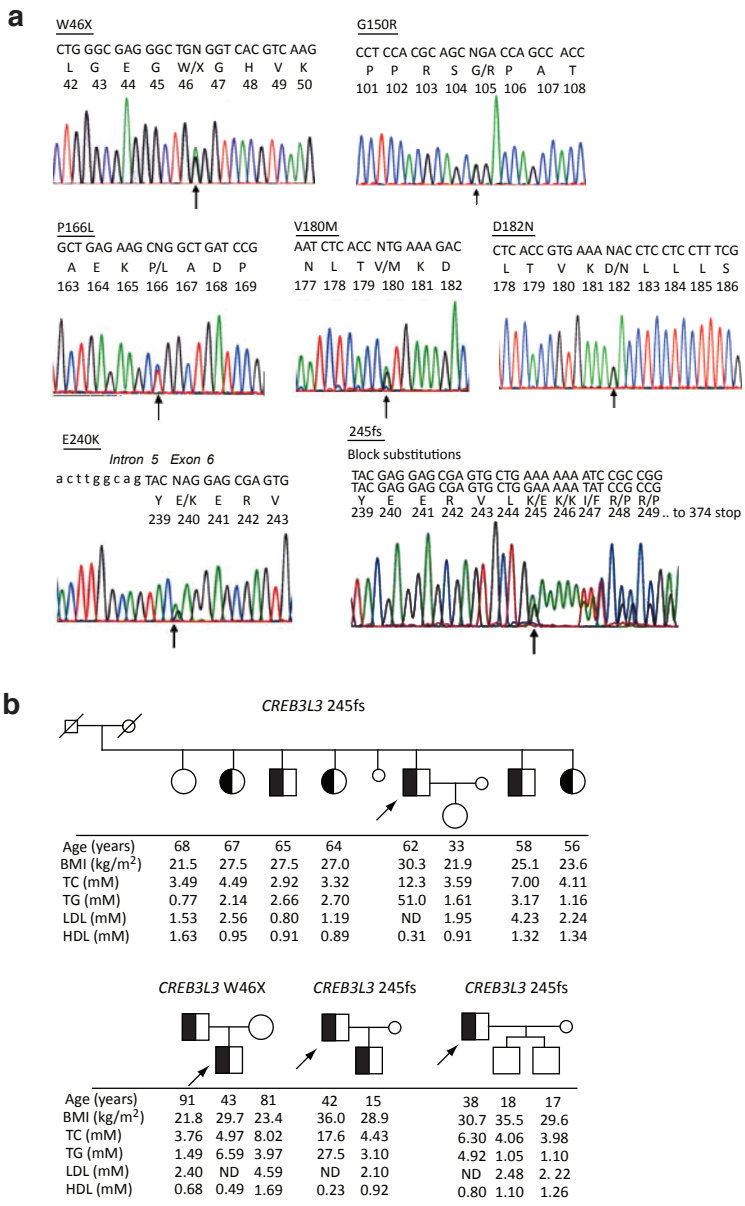
Supplementary Figure 2. Gene expression analysis of *Creb3l3*$^{-/-}$ mice. **(a)** *Lpl* mRNA levels in adipose tissue and skeletal muscle determined by qRT-PCR . $n = 4$ per group. **(b)** Microarray analysis of total RNAs isolated from liver after fasting the animals for 24 h. Listed are genes which are downregulated in *Creb3l3*$^{-/-}$ mice by < 67% with P values of < 0.05 for comparison between WT vs. *Creb3l3*$^{-/-}$. *$P = 0.086$. Genes that are known to be associated with TG metabolism in human or mice are highlighted in bold face. **(c)** *Apoa4* and *Apoc2* mRNAs levels in the duodenum of WT and *Creb3l3*$^{-/-}$ mice determined by qRT-PCR . $n = 8$ mice per group. **(d)** Western blot of plasma Apoa4. Apoe served as loading control. **(e)** *Apoc3* mRNA expression in liver and small intestine. $n = 8$ mice per group. *** $P < 0.0001$, N.S., not significant ($P > 0.05$).



Supplementary Figure 3. Reduction of plasma TG levels in *Creb3l3*$^{-/-}$ mice by transfusion of WT serum or recombinant APOC2. **(a)** TG levels in mice fasted for 4 h and then *i.v.* injected with 100 µl of freshly prepared plasma from WT or *Creb3l3*$^{-/-}$ mice (n = 10 per group), or **(b)** 25 µg of recombinant APOC2 proteins or saline (n = 4 per group). Data are shown as mean ± SEM. *$P < 0.05$, **$P < 0.01$, ***$P < 0.0001$, compared to WT mice.



Supplementary Figure 4. Identification of CREB-H target genes by transgenic or adenoviral overexpression. **(a)** Transgenic vector contains amino acids 1-318 of CREB-H (CREB-H(N)), encompassing the N-terminal portion of the protein extending to the predicted S2P protease cleavage site. **(b-c)** Western and Northern blot analysis of the endogenous and transgenic CREB-H. **(d)** qRT-PCR analysis of liver RNA. (n = 4 mice per group). **(e)** Schematic representation of *Apoe/c1/c4/c2* and *Apoa1/c3/a4/a5* loci. **(f)** Fed Plasma TG levels determined at 9 wks of age. n = 5–6 male mice per group. **(g)** Fed plasma TG levels determined 3 days after adenoviral infection. n = 4–8 female mice per group. $^{**}P$ <0.001 **(h)** Recombinant LPL was incubated with triolein substrate in the presence of plasma collected from GFP or CREB-H(N) adenovirus infected mice. Values represent FFA concentration released from triolein. n = 4–8 per group. $^{**}P$ <0.05 **(i)** Luciferase reporter constructs containing 0.43 kb *Apoa4*, 0.5 kb *Apoc2* or 1.5 kb *Fgf21* promoters were transfected into Hepa1.6 cells together with control or CREB-H(N) expression plasmid for luciferase assays.



Supplementary Figure 5. Identification of nucleotide sequence variations in *CREB3L3*. **(a)** *CREB3L3* sequencing electropherograms**.** The panel shows the heterozygous *CREB3L3* mutations found in HTG patients. The trivial name for each mutation is indicated at the top of each panel. The electropherogram sections show the mutant sequence in each patient; the lines of text show normal codons, and the amino acid change predicted by the mutant codon. Mutation sites are indicated by arrows. **(b)** Pedigrees in which *CREB3L3* nonsense mutations segregate. The probands are indicated by arrows. The lipid profiles are shown below each individual. Abbreviations: BMI, body mass index; TC, total cholesterol; TG, triglycerides; LDL, low-density lipoprotein cholesterol; HDL, high-density lipoprotein cholesterol; ND, not determined.

Supplementary Table 1. Primers for qRT-PCR and genomic sequencing of *CREB3L3*

Gene	Forward	Reverse	Source
Creb3l3	GGCCATTGACCTGGACATGT	TTCACAGTGAGGTTGAAGCGG	This study
Fgf21	GGAGCTCTCTATGGATCGCCT	TGTAACCGTCCTCCAGCAGC	PrimerBank*
Fads1	AGCACATGCCATACAACCATC	TTTCCGCTGAACCACAAAATAGA	PrimerBank
Fads2	AAGGGAGGTAACCAGGGAGAG	CCGCTGGGACCATTTGGTAA	PrimerBank
Elovl2	CCTGCTCTCGATATGGCTGG	AAGAAGTGTGATTGCGAGGTTAT	PrimerBank
Elovl5	ATGGAACATTTCGATGCGTCA	GTCCCAGCCATACAATGAGTAAG	PrimerBank
Cidec	ATGGACTACGCCATGAAGTCT	CGGTGCTAACACGACAGGG	PrimerBank
G0s2	TAGTGAAGCTATACGTTCTGGGC	GTCTCAACTAGGCCGAGCA	PrimerBank
apoa1	TCCTGACAGGGAGCCAGG	TGTCCCATTGGGACTGGG	PrimerBank
apoa4	CCAGCTAAGCAACAATGCCA	TGGAAGAGGGTACTGAGCTGC	PrimerBank
Apoa5	AGGCAGCAGTTGAAACCCTA	TGAGCCTTGGTGTCTTCTCC	This study
Apoc1	TCCTGTCCTGATTGTGGTCGT	CCAAAGTGTTCCCAAACTCCTT	PrimerBank
Apoc2	GCATGGATGAGAAACTCAGGG	AAAATGCCTGCGTAAGTGCTC	PrimerBank
Apoc3	TACAGGGCTACATGGAACAAGC	CAGGGATCTGAAGTGATTGTCC	PrimerBank
Apoc4	GAGCTGTCCAGGGCTTTATG	GGCTGTGGGTCTTGTTTAGG	PrimerBank
Apoe	CTGACAGGATGCCTAGCCG	CGCAGGTAATCCCAGAAGC	PrimerBank
Lpl	GGGAGTTTGGCTCCAGAGTTT	TGTGTCTTCAGGGGTCCTTAG	PrimerBank
CREB3L3 exon1	ACAGAGGGCTGTGAGCTTG	TTCTCTGGGCCTCAGTCTTC	
CREB3L3 exon2	AGCGGCAACTGAACTCTAGC	GCTAAAATCAAGCACCGTGA	
CREB3L3 exon3	CATCTTGGAGAAGGGAAGGACACC	ACACCTAGCCAAGGGAGACACGTG	
CREB3L3 exon4	CTTGGGGACTCCAAACTCTG	AAATTCCACGCCTTTCTGTG	
CREB3L3 exon5	CCTGGGGTGATAGTGTTTTCG	AAGCTGAGATCGTGCCACTG	
CREB3L3 exon6	CCTGACCTCAGGTGATACGC	ATCTTTCCATCCCTGCAATC	
CREB3L3 exon7	TGGGTTCTCTTGGCTTGTAACGTGAGG	TGAGATTACAGGCGTGAGCCACTGCAC	
CREB3L3 exon8&9	CCTTGAAGAATGGATGGAATTTGG	ACAAGGTGGAGGTGGGGTCCTATG	
CREB3L3 exon10	TTGCGCCTGTACGAGGTAG	ATCTCTGCTGGGGTCTTGG	

*http://pga.mgh.harvard.edu/primerbank/

Supplementary Table 2. Clinical features of mutation carriers in *CREB3L3* gene

HTG or CONTROL	GL	Mutation Type	Mutation	Polyphen prediction	Age	Sex	BMI	TC (mM)	TG (mM)	HDL (mM)	DM
HTG	1329	nonsense	W46X	truncation	43	male	29.7	4.97	6.59	0.49	no
HTG	818	missense	G105R	possibly damaging	58	male	27.8	9	5.23	ND	no
HTG	1042	missense	G105R	possibly damaging	54	male	22.7	8	5.99	1.98	no
HTG	1591	missense	P166L	benign	60	male	32.2	6.1	11.8	0.95	yes
HTG	1040	missense	P166L	benign	43	female	26.8	7.1	4.61	1.26	no
HTG	4901	missense	V180M	benign	35	female	29	15.1	56.3	0.66	no
HTG	1020	missense	D182N	possibly damaging	53	female	25.8	6.22	6.03	0.76	no
HTG	4887	missense	E240K	possibly damaging	58	male	28.3	11.7	32.5	1.72	no
HTG	2430	missense	E240K	possibly damaging	36	male	32	8.3	5.2	1.2	no
HTG	2657	nonsense	245fs	truncation	57	male	30.3	12.3	51	0.31	yes
HTG	1189	nonsense	245fs	truncation	42	male	36	17.6	27.5	0.23	yes
HTG	4953	nonsense	245fs	truncation	33	male	40.7	6.3	4.92	0.8	no

Abbreviations: HTG, hypertriglyceridemia; GL, identification number; BMI, body mass index; TC, total cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol;DM, Diabetes Mellitus; ND, not determined.

Supplementary Methods

Animal experiments *Creb3l3*$^{-/-}$ mice have been described previously [1]. WT control and experimental groups (*Creb3l3*$^{+/-}$ and *Creb3l3*$^{-/-}$) were generated by intercrossing the heterozygous *Creb3l3*$^{+/-}$ mice. Age and sex matched littermates were used as controls throughout the study. Mice were housed in a specific pathogen free facility at the Harvard School of Public Health on a 12h light/dark cycles and had free access to standard chow diet (PicoLab Rodent diet 20, #5058, Lab diet). Animal studies and experiments were approved and carried out according to Harvard University's Standing Committee on Animals and National Institutes of Health guidelines for animal use and care.

Blood chemistry and lipid analysis Plasma TG, cholesterol and FFA concentrations were determined using assay kits from Sigma, Invitrogen and Wako Chemicals, respectively. VLDL secretion rate *in vivo* was measured as previously described [2]. Briefly, 4 h-fasted mice were injected with triton WR1339 (500 mg kg^{-1} in saline) via the tail vein. Blood samples were drawn at indicated time points for TG assays. For plasma and APOC2 transfusion, mice were fasted for 4 h and injected with 100 μl of plasma or 25 μg of APOC2 protein (Athens Biotechnology Company) through the tail vein. Plasma samples were collected at indicated time points after injection, and measured for TG levels. Liver tissues were homogenized and lipids were extracted with chloroform/methanol mixture (2:1 v/v), as described previously [3]. TG contents were determined using an assay kit from Sigma.

Lpl assay Lpl activity in post-heparin plasma was determined as described previously[4], with some modifications. Briefly, post-heparin plasma was prepared thirty minutes after i.p injection of 200U sodium heparin into mice. Substrate for Lpl was prepared by mixing 1.12 mCi of ^{3}H-triolein (99 μg), 300 mg of unlabeled triolein, and 18 mg of egg phosphatidylcholine. Radiolabeled substrate was mixed with 10 μl of post-heparin plasma and 15 μl of heat inactivated serum isolated from WT or *Creb3l3*$^{-/-}$ mice. Liberated FA was measured by scintillation counting.

Lpl assay using recombinant Lpl protein was performed as described elsewhere [5]. Briefly, the substrate was prepared by sonicating 83 mg of triolein (Sigma) in 1.785 ml of 0.2 M Tris buffer (pH 8.2) containing 150 mg ml^{-1} gum arabic. 150 μl of the sonicated substrate was mixed with 140 μl of 2x assay buffer containing 0.33 M NaCl, 165 mg ml^{-1} fatty acid-free albumin and 10 μl of plasma prepared from WT or *Creb3l3*$^{-/-}$ mice, and incubated for 80 min at 37°C to allow the transfer of serum Apoc to the substrate. 0.93 μg of purified Lpl (Sigma) was added to the substrate, and incubated for 30 min. The reaction was stopped by adding cold NaCl to a final concentration of 1 M. FFA content in the reaction mixture released from triolein by Lpl was measured as described above.

Fractionation of lipoproteins Five hundred μl of pooled plasma (3 per group) were fractionated by discontinuous gradient density ultracentrifugation as described previously [6]. Briefly, 1 g of potassium bromide (KBr) and 50 mg of sucrose were added to the plasma, which was then brought to a final volume of 3.5 ml with density solution

(ρ = 1.006 g ml^{-1} KBr). The discontinuous gradient consisted of plasma (3 ml) and density solutions with ρ = 1.21 g ml^{-1} KBr (2 ml), ρ = 1.08 g ml^{-1} KBr (3 ml), and ρ = 1.00 g ml^{-1} (3 ml) layered from bottom to top in a 12 ml ultracentrifuge tube. The samples were then centrifuged at 35,000 rpm for 18h at 8°C. Fractions (1 ml) were sequentially collected from top to bottom, determined for their densities, and assayed for TG and cholesterol contents. Fractions were desalted and concentrated into 50 μl of PBS using Centricon columns. Fifteen μl of fractions were separated on a 4–20% gradient SDS-polyacrylamide gel. The gel was stained by Coomassie Brilliant Blue G-250.

CREB-H(N) transgenic mice To generate CREB-H(N) transgenic mice, a cDNA fragment encompassing amino acids 1-318 of mouse CREB-H that extended to the predicted S2P protease cleavage site was cloned into the pLiv.7 transgenic vector that contained the apolipoprotein E (*Apoe*) promoter for liver specific transgene expression [7]. Microinjection of the transgenic DNA into the pronuclei of fertilized ICR embryos was performed as described previously [8].

Adenovirus infections Recombinant adenoviruses that expressed mouse CREB-H(N) were produced using pAdTRACK-cmv shuttle vector as described previously [2]. We amplified recombinant adenoviruses in HEK293 cells and purified using a commercial kit (Virapur). Mice were infected with recombinant adenoviruses by tail vein injection. Each mouse received $3x10^9$ particles g^{-1} body weight in 0.15 ml of saline. Plasma was collected three days after virus infection for TG measurement and LPL assays.

RNA isolation, microarray, northern blot and real time PCR Total RNAs were isolated using TRIZOL (Invitrogen) according to the manufacturer's recommendation. Northern blot analysis of *Creb3l3* mRNA was performed as described previously [9]. Complementary DNAs were generated using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), and subjected to SYBR-based real-time PCR using the Mx3005P™ system (Stratagene). Primer sequences are listed in Table S2. Microarray and data analysis were carried out at the Harvard-Partners Center for Genetics and Genomics (Harvard Medical School). Three liver RNA samples per group were further purified using MiniElute Column (Qiagen), and hybridized on mouse WG-6 2.0 chips (Illumina).

Western blot analysis Liver nuclear extracts and microsomal fractions were prepared as described previously [2,10]. Primary mouse intestinal epithelial cells were isolated using dispase as described previously [11]. CREB-H western blot was performed using a rabbit polyclonal antibody that was raised against bacterially produced mouse CREB-H amino acids 1-232. Plasma Apoa4 (Santa Cruz, sc-19036) and Apoe (Biodesign, K23100R) levels were determined by western blot using specific antibodies. For Apoc3 western blotting using a rabbit polyclonal antibody (gift from Dr. B Monia), 5 μL of VLDL fractions were separated on a 12 % NuPAGE Novex Bis-Tris gel (Invitrogen) and transferred into nitrocellose membrane.

Plasmid constructs, cell culture and reporter gene assay Mouse *Creb3l3* cDNA was isolated by PCR amplification using a cDNA clone (IMAGE:4211480, BC010786) as template, and inserted into HA-Cruz-C mammalian expression vector (Santa Cruz). Cruz-CREB-H(N) which expressed amino aids 1-318 of CREB-H was also similarly generated by PCR with the following primers : forward, 5'-GATATCCTGGAAAGATGGCGTCCC-3'; reverse, 5'-AGATCTCAGGTGCCTGCATGGGCTG-3'. An EST clone containing human CREB-H cDNA (IMAGE: 8069010, BC101504) was obtained from OpenBiosystems and validated by DNA sequencing. Full length CREB-H and the N-terminal amino acids 1-322 were amplified by PCR and cloned into pCDNA3.1 vector (Invitrogen). W46X, G105R, P166L, V180M, D182N, E240K and 245fs mutations were introduced to the full length CREB-H and CREB-H(N) using QuikChange Site-Directed Mutagenesis Kit (Stratagene). Proximal *ApoA4* promoter (–430/+40) was isolated by PCR using RP24-302M3 BAC plasmid (BACPAC Resources Center, CHORI) containing *Apoa4* gene as template, and cloned into pGL3-basic vector (Promega). Sequential deletion and site directed mutagenesis were performed to generate additional shorter or mutated reporter constructs. *Apoc2* and *Fgf21* promoter-luciferase reporter plasmids were kindly provided by Dr. Peter A. Edwards (UCLA, Dept of Biological Chemistry and Medicine) and Dr. Steven A. Kliewer (UT Southwest Medical Center), respectively.

MODE-K, Hepa1.6 and 293T cells were maintained in DMEM medium supplemented with 10% fetal bovine serum. Cells were transfected using Lipofectamine 2000 (Invitrogen) and harvested for luciferase assays using Dual-luciferase reporter assay kit (Promega). Transfection efficiency was normalized to Renilla activity after cotransfection with pRL/CMV (Promega).

Sequencing of human genomic DNA. Sequencing, using primers and conditions shown in Table S2 (or available upon request), was performed on genomic DNA of patients who were part of a tertiary referral clinic screening cohort with hypertriglyceridemia (plasma TG > 1000 mg dL^{-1}) and matched controls, as described [12].

Supplementary References

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