

Hepatocyte β -Klotho regulates lipid homeostasis but not body weight in mice

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ABSTRACT β -Klotho (β -KL), a transmembrane protein expressed in the liver, pancreas, adipose tissues, and brain, is essential for feedback suppression of hepatic bile acid synthesis. Because bile acid is a key regulator of lipid and energy metabolism, we hypothesized potential and tissue-specific roles of β -KL in regulating plasma lipid levels and body weight. By crossing β -kl^{-/-} mice with newly developed hepatocyte-specific β -kl transgenic (Tg) mice, we generated mice expressing β -kl solely in hepatocytes (β -kl^{-/-}/Tg). Gene expression, metabolomic, and *in vivo* flux analyses consistently revealed that plasma level of cholesterol, which is over-excreted into feces as bile acids in β -kl^{-/-}, is maintained in β -kl^{-/-} mice by enhanced *de novo* cholesterol synthesis. No compensatory increase in lipogenesis was observed, despite markedly decreased plasma triglyceride. Along with enhanced bile acid synthesis, these lipid dysregulations in β -kl^{-/-} were completely reversed in β -kl^{-/-}/Tg mice. In contrast, reduced body weight and resistance to diet-induced obesity in β -kl^{-/-} mice were not reversed by hepatocyte-specific restoration of β -KL expression. We conclude that β -KL in hepatocytes is necessary and sufficient for lipid homeostasis, whereas nonhepatic β -KL regulates energy metabolism. We further demonstrate that in a condition with excessive cholesterol disposal, a robust compensatory mechanism maintains cholesterol levels but not triglyceride levels in mice.—Kobayashi, K., Tanaka, T., Okada, S. Morimoto, Y., Matsumura, S., Manio, M. C. C., Inoue, K., Kimura, K., Yagi, T., Saito, Y., Fushiki, T., Inoue, H., Matsumoto, M., Nabeshima, Y.-i. Hepatocyte β -Klotho regulates lipid

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Obesity and dyslipidemia are leading, closely related risk factors for cardiovascular morbidity and mortality. In humans, body mass index is positively correlated with plasma triglyceride and total cholesterol levels (1). Moreover, weight loss lowers plasma triglyceride levels and improves dyslipidemia (2). Although the molecular linkage between obesity and dyslipidemia has not been fully elucidated, recent research suggests that bile acids may be involved.

Bile acid is a class of molecules that is synthesized enzymatically from cholesterol in the liver (3, 4). Indeed, bile acid synthesis is the predominant catabolic route of cholesterol in mammals. Bile acid-binding resins, which remove bile acid from the intestinal lumen, have long been used as cholesterol-lowering agents (5). Bile acid also serves as endogenous detergents, being essential for intestinal absorption of dietary lipids and lipid-soluble vitamins (3, 4). In humans, it has been shown that bile acid synthesis is well correlated with triglyceride metabolism. For example, patients with hypertriglyceridemia show enhanced bile acid synthesis compared to control subjects, whereas patients with hypercholesterolemia do not (6). Based on these biochemical, physiologic, and epidemiologic studies, it seems likely that changes in bile acid

Abbreviations: β -KL, β -Klotho; ALT, alanine aminotransferase; ANGPTL, angiopoietin-like protein; AST, aspartate aminotransferase; BAT, brown adipose tissue; CM, chylomicron; CoA, coenzyme A; CYP7A1, cholesterol 7 α -hydroxylase; EGFP, enhanced green fluorescent protein; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FXR, farnesoid X

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metabolism are closely associated with changes in both cholesterol and triglyceride metabolism. Furthermore, administration of bile acid reduces body weight by increasing energy expenditure in mice (7). These findings support the idea that bile acid dysregulation plays a role in both obesity and dyslipidemia.

β -Klotho (β -KL), an essential regulator of bile acid homeostasis, is a transmembrane protein expressed in the liver, pancreas, white adipose tissue (WAT), brown adipose tissue (BAT), and some specific areas in the brain (8, 9). In the liver, β -KL suppresses mRNA expression of cholesterol 7 α -hydroxylase (CYP7A1) (10), the rate-limiting enzyme in hepatic bile acid synthesis (3, 4), by binding to and mediating the signals of fibroblast growth factor (FGF) 15/19 and its receptor (FGFR) 4 (10–13). FGF19 is the human ortholog of rodent FGF15 and thus referred to collectively here. In adipose tissues and brain, β -KL serves as a cofactor for FGF21 activity to mediate various physiologic and possibly some pharmacologic actions (8, 14–17). We previously reported that mice lacking β -KL (β -KL^{-/-} mice) show elevated mRNA expression of CYP7A1 (10). It is noteworthy that we also found that β -KL^{-/-} mice exhibit lower body weight than their wild-type (WT; β -KL^{+/+}) and heterozygous (β -KL^{+/-}) littermates (10). The marked induction of *Cyp7a1* expression and consequent increase in bile acid excretion in β -KL^{-/-} mice led us to hypothesize that β -KL expressed in the liver plays a vital role in the regulation of lipid metabolism and energy homeostasis specifically through its modulatory effect on bile acid synthesis.

In this study, we first generated a novel β -KL^{-/-} mouse model in which β -KL is expressed transgenically solely in hepatocytes. We then used this model to investigate the potential hepatocyte-specific function of β -KL on lipid and energy homeostasis.

MATERIALS AND METHODS

Animals

Full-length mouse β -KL cDNA was cloned from WT mouse liver. The mouse β -KL cDNA was inserted upstream of an enhanced green fluorescent protein (EGFP) gene in the pEGFP-N2 vector (Clontech Laboratories, Mountain View, CA, USA). The EGFP-fused mouse β -KL cDNA was inserted downstream of a 2.3-kb albumin promoter and rabbit β -globin gene in the pBluescript KS vector. The transgene was then excised with *NotI/XhoI* restriction enzymes and purified. The fragment was microinjected into the pronucleus of fertilized C57BL/6J mouse eggs, and transgenic (Tg) founder mice were generated. Genomic DNA from tails of founder mice was digested with *BglII* and analyzed by Southern blot hybridization with a mouse β -KL cDNA probe. Expression of the β -KL transgene in cDNA was analyzed with primers specific to the 5'-UTR sequence of β -KL transgene (sense primer 5'-GAA

CTA GTG GAT CCC CCT GG-3', antisense primer 5'-GTT GAC ACC TCT CAG GTG TGA GT-3'). β -KL^{-/-} mice were generated as described (10). Mice were maintained on a 12 h light/12 h dark cycle and given free access to food and water. Mice were given CE-2 (12% kcal fat and 59% kcal carbohydrate; CLEA, Tokyo, Japan) or MFG (14% kcal fat and 60% kcal carbohydrate; Oriental Yeast, Tokyo, Japan), unless otherwise mentioned, and changed to a control diet (no. D06041501: 10% kcal fat and 70% kcal carbohydrate; Research Diets, New Brunswick, NJ, USA) or a high-fat diet (HFD) (no. D12492: 60% kcal fat and 20% kcal carbohydrate; Research Diets) as described in the figure legends. Recombinant human FGF19 (BioVendor, Brno, Czech Republic) was injected into the tail vein of mice at the dose of 1 mg/kg body weight. At 6 h after injection, mice were killed under isoflurane anesthesia, and the livers were excised for total RNA preparation. Very low-density lipoprotein (VLDL) secretion assay (18), dual-energy X-ray absorptiometry analysis (19), and indirect calorimetry (20) were performed as previously described. Adipocyte size and number of epididymal fat pads from mice fed an HFD for 8 wk were determined using a Coulter counter (Beckman Coulter, Indianapolis, IN, USA) as described (21). All experiments were performed with male mice fed *ad libitum*, and were approved by the Animal Research Committees of Kyoto University Graduate School of Medicine, RIKEN Center for Developmental Biology, and Foundation for Biomedical Research and Innovation and were carried out in accordance with the laws and guidelines of each institution.

Isolation of hepatocytes and nonhepatocyte fraction

Mouse hepatocytes were isolated from male C57BL/6J mice (6 wk of age; SLC, Shizuoka, Japan) by collagenase perfusion of the inferior *vena cava* using a modification of a method described previously (22). Nonhepatocyte fraction was prepared using a gentleMACS Dissociator (Miltenyi Biotec, Bergisch Gladbach, Germany). In brief, whole liver was dissociated in DMEM containing 500 CDU/ml collagenase IV (Sigma-Aldrich, St. Louis, MO, USA) and protease inhibitor cocktail (cOmplete, Mini, EDTA-free; Roche, Mannheim, Germany) in gentleMACS C Tubes (Miltenyi Biotec). After incubation for 30 min at 37°C, the liver homogenate was filtered through a cell strainer (100 μ m mesh size). The flow-through fraction was centrifuged at 20 g for 4 min at 4°C, and the supernatant was used as nonhepatocyte fraction.

Quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA, USA) or an RNeasy Mini Kit (QIAGEN, Venlo, The Netherlands), treated with DNase I (Promega, Madison, WI, USA) and transcribed into first-strand cDNA using a SuperScript III First-Strand Synthesis SuperMix for quantitative RT-PCR (Life Technologies) according to the manufacturer's instructions. Quantitative real-time PCR (QPCR) analysis was performed with a 7300 Real-Time PCR System (Applied Biosystems, South San Francisco, CA, USA) or StepOnePlus Real-Time PCR System (Applied Biosystems) using TaqMan Expression Assay Primers (Applied Biosystems). Relative expression levels were calculated by the relative standard curve method by using 18S rRNA as the internal control.

Western blot analysis

Tissue homogenates were prepared in buffer [20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, 150 mM NaCl, and 0.5% Nonidet P-40 (pH 7.4)] containing protease inhibitor cocktail (cOmplete, Mini, EDTA-free), and supernatant protein (25–60

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receptor; HFD, high-fat diet; HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A; LC-MS, liquid chromatography mass spectrometry; LPL, lipoprotein lipase; PPAR, peroxisome proliferator-activated receptor; QPCR, quantitative real-time PCR; SHP, small heterodimer partner; SREBF, sterol regulatory element-binding factor; Tg, transgenic; TLC, thin-layer chromatography; VLDL, very low-density lipoprotein; WAT, white adipose tissue; WT, wild-type

μg) was used for SDS-PAGE. Western blot analyses were performed using a rat mAb against mouse β-KI (10) or a mouse mAb against β-actin (ab8226; Abcam, Cambridge, United Kingdom).

Biochemical and histologic analysis

Bile acid pool size and fecal bile acids were determined using the Total Bile Acids Test (Wako Pure Chemical Industries, Ltd., Osaka, Japan) after ethanol extraction, as previously described (23). Triglyceride and total cholesterol levels in plasma, liver, and feces were determined using the Triglyceride E-Test Wako and Cholesterol E-Test Wako (Wako Pure Chemical Industries, Ltd.), respectively. Lipid content in the liver and feces were extracted by hexane/isopropanol as previously described (24). Plasma lipoproteins were separated by HPLC and quantified (LipoSearch; Skylight Biotech Inc., Akita, Japan) as described previously (25).

Metabolite measurements, metabolomics, and *in vivo* flux analyses

Contents of acetyl-coenzyme A (CoA) derivatives were analyzed by liquid chromatography mass spectrometry (LC-MS) (Shimadzu Techno-Research Inc., Kyoto, Japan) as described previously (26), with some modifications. For metabolomics, the liver samples were snap-frozen in liquid nitrogen, and the extract was analyzed by capillary electrophoresis mass spectrometry (Human Metabolome Technologies Inc., Yamagata, Japan) as described previously (27). For flux analyses, mice were intraperitoneally injected with [1-¹⁴C]sodium acetate (15 μCi/mouse; PerkinElmer, Boston, MA, USA) and killed 30 min after the injection (28). Liver tissue (50 mg) was homogenized in 0.6 ml hexane/isopropanol (3:2, v/v), and lipids were extracted. [³H]Cholesterol (1 μCi) was added to the extraction tube as a recovery control. Extracted lipids were evaporated and resolubilized in 15 μl chloroform/methanol (2:1, v/v). [³H]Cholesterol radioactivity in 2 μl of the same sample was determined by a TopCount counter (PerkinElmer). Samples (10 μl) were loaded onto silica thin-layer chromatography (TLC) plates (Merck Millipore, Darmstadt, Germany) and separated using freshly prepared hexane/diethyl ether/acetate (80:30:1, v:v:v) as the mobile phase. TLC nonpolar Lipid Mixture B (Matreya, Pleasant Gap, PA, USA) was loaded as a loading control. The autoradiography of the TLC plate was captured with a storage phosphor imaging system (BAS-5000; GE Healthcare, Little Chalfont, United Kingdom). The signal intensity corresponding to cholesterol, cholesteryl ester, fatty acid, and triglyceride in each animal was quantified using Multi Gauge, version 3.1 (Fuji Film, Tokyo, Japan), and normalized by internal recovery standard [³H]cholesterol radioactivity. Signals were multiplied by each liver weight and expressed by the normalized relative intensity per liver in 30 min.

VLDL uptake analysis

VLDL uptake into tissue was determined as previously described (29). In brief, male ICR mice (SLC, Shizuoka, Japan) were anesthetized with Avertin (Wako Pure Chemical Industries, Ltd.) and injected with 0.1 μCi of [9,10-³H]palmitic acid (PerkinElmer) dissolved in 200 μl of 2 mg/ml bovine serum albumin-saline *via* the tail vein. After 25 min, blood was collected, and plasma was pooled. VLDL was isolated by ultracentrifugation at a density of 1.006. For the uptake experiment, mice anesthetized with Avertin were injected with 1 mg ³H-labeled VLDL (231,600 cpm/mouse) *via* the tail vein. After 15 min, mice were anesthetized with isoflurane, and tissues were collected. Lipids in the liver (200 mg) were extracted by hexane/isopropanol as described above and evaporated and resolubilized in 60 μl hexane.

The ³H radioactivity in the extracted lipids (50 μl) and the plasma (70 μl) was determined by a TopCount counter and multiplied by whole-liver weight and total plasma volume, respectively. Total plasma volumes of mice were calculated from the equation volume (milliliters) = 0.04706 × body weight (grams), as described previously (30). The VLDL uptake activity was expressed as the percentage of the injected ³H-labeled VLDL radioactivity (100%).

Statistics

Statistical analyses were performed using a 2-tailed *t* test (2 groups) or 1-way ANOVA with Tukey-Kramer test (>2 groups) (Ekuseru-Toukei 2012; Social Survey Research Information Company, Limited, Tokyo, Japan). Data are presented as the means ± SEM; *P* < 0.05 was considered significant. The number of replicates is given in each figure or table legend.

RESULTS

Generation of hepatocyte-specific β-kl Tg mice

β-KI is highly expressed in the liver of adult mice, but the cell type that expresses β-KI has not been identified. Therefore, we examined the expression of β-kl mRNA in hepatocyte and nonhepatocyte fractions freshly isolated from mouse liver. QPCR analysis showed that β-kl mRNA was predominantly expressed in hepatocytes (Fig. 1A).

Next, to examine hepatocyte-specific function of β-KI, we generated Tg mouse lines expressing murine β-kl full-length cDNA under the control of hepatocyte-specific albumin promoter (31) (Fig. 1B). Among the Tg lines confirmed by Southern blot analysis (data not shown), we selected a line with the hepatic β-kl mRNA level of 2.5-fold higher than that in WT littermates (Fig. 1C). There was no difference in β-kl mRNA levels in pancreas or adipose tissues between the WT and Tg mice (Fig. 1C). By means of PCR using transgene-specific primers, we confirmed that the transgene-derived transcript was detectable only in the liver but not in any other tissues tested (Fig. 1D). We observed no difference in mRNA expression of CYP7A1 or sterol 12α-hydroxylase, 2 key enzymes in bile acid synthesis, between WT and Tg mice (Fig. 1E). The pool size and fecal excretion of bile acid in Tg were also comparable to those in WT mice (Fig. 1F, G).

Hepatocyte-specific restoration of β-KI normalizes bile acid homeostasis

To generate a mouse model in which β-KI is expressed solely in hepatocytes, we crossed Tg with systemic β-kl^{-/-} mice (10) and obtained 4 genotypes: β-kl^{+/+}, β-kl^{-/-}, β-kl^{+/+}/Tg, and β-kl^{-/-}/Tg. To obtain sufficient numbers of mice for experiments, we undertook β-kl^{+/+} and β-kl^{-/-} mating and used β-kl^{+/+} mice as controls. Compared with control β-kl^{+/+} mice, β-kl mRNA levels in the liver from β-kl^{+/+}/Tg and β-kl^{-/-}/Tg mice were 4.3- and 3.0-fold higher, respectively (Fig. 2A). Western blot analysis confirmed the expression of transgene-derived EGFP-tagged β-KI in the liver of β-kl^{+/+}/Tg and β-kl^{-/-}/Tg mice; no endogenous β-KI protein was detected in β-kl^{-/-} or β-kl^{-/-}/Tg mice (Fig. 2B). We also confirmed that transgene-derived

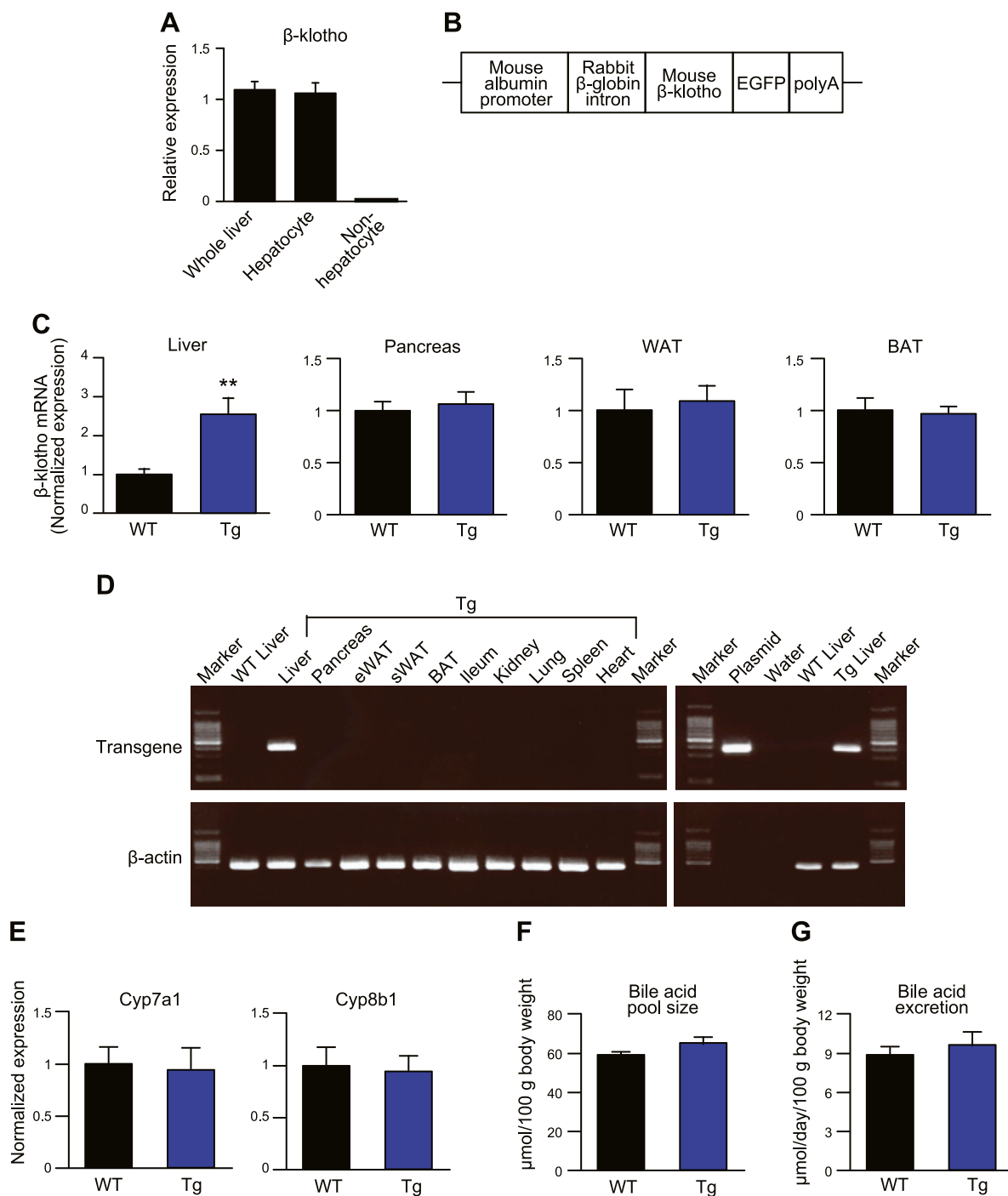


Figure 1. Generation of Tg mice over-expressing β -kl in hepatocytes. **A**) β -kl mRNA levels in whole liver, hepatocyte fraction, and nonhepatocyte fraction from C57BL/6J mice ($n = 3-4$). The same amount (50 ng) of RNA was used for each sample. mRNA levels were normalized by 18S rRNA expression. **B**) A schematic drawing of the transgene construct, consisting of albumin promoter and EGFP-fused mouse β -kl cDNA. **C**) β -kl mRNA expression in the liver, pancreas, epididymal WAT, and interscapular BAT from WT and β -kl Tg mice ($n = 8-10$). **D**) Representative PCR analysis using primers specific to the 5' UTR sequence of transgene-derived β -kl ($n = 3$). WT mouse liver (lane 1) and β -kl Tg mouse liver (lane 2), as well as Tg mouse pancreas (lane 3), epididymal WAT (eWAT) (lane 4), s.c. WAT (sWAT) (lane 5), BAT (lane 6), ileum (lane 7), kidney (lane 8), lung (lane 9), spleen (lane 10), and heart (lane 11) are shown. β -actin was included as an internal control. The same amount (50 ng) of cDNA was used for each sample. The plasmid carrying EGFP-tagged mouse β -kl (shown in **B**) was included as a positive control. **E**) *Cyp7a1* and *Cyp8b1* (sterol 12 α -hydroxylase) expression in the liver ($n = 10$). For **(C)** and **(E)**, mRNA levels were normalized by 18S rRNA expression, and the data are shown as fold increase over WT mice. **F**) Bile acid pool size ($n = 10$) and **(G)** fecal excretion of bile acids ($n = 9-10$) in WT and Tg mice. Pool size is expressed as micromoles of bile acid per 100 g body weight. Fecal excretion of total bile acids is expressed as micromoles per day per 100 g body weight. Stool samples were collected daily for 3 consecutive days. Data are presented as the means $\pm$ SEM. ** $P < 0.01$.

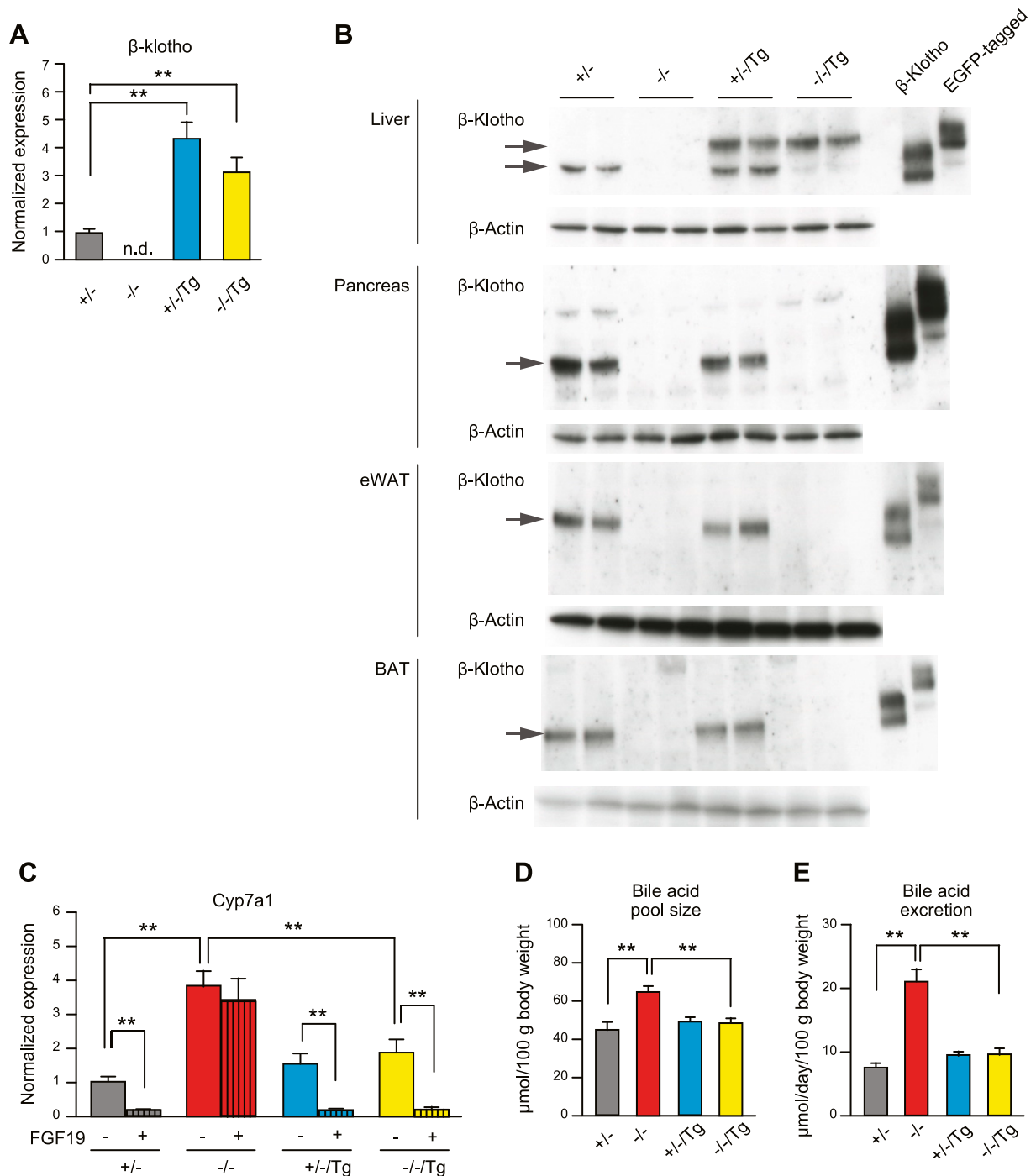


Figure 2. Restoration of hepatocyte β -Kl normalizes bile acid homeostasis. *A*) β -kl mRNA levels in the liver of β -kl heterozygous (β -kl^{+/+}; $n = 8$), β -kl knockout (β -kl^{-/-}; $n = 3$), β -kl^{+/+}/Tg ($n = 6$), and β -kl^{-/-}/Tg ($n = 7$) mice. n.d., not detected (Ct >40). Data were normalized by 18S rRNA expression and are shown as fold increase over β -kl^{+/+} mice. *B*) Western blot of β -Kl protein in the liver, pancreas, epididymal WAT (eWAT), and BAT from β -kl^{+/+}, β -kl^{-/-}, β -kl^{+/+}/Tg, and β -kl^{-/-}/Tg mice with β -Actin as a loading control. Cell lysates from HeLa cells transfected with mouse β -Kl or EGFP-tagged mouse β -Kl were loaded as positive controls. *C*) *Cyp7a1* expression in the liver. Mice were treated with intravenous injection of recombinant human FGF19 (1 mg/kg) or vehicle, and the liver was excised 6 h later ($n = 8$ mice per group for β -kl^{+/+}; $n = 3$ mice per group for β -kl^{-/-}; $n = 6-7$ mice per group for β -kl^{+/+}/Tg; $n = 6-7$ mice per group for β -kl^{-/-}/Tg). Data were normalized by 18S rRNA and are shown as fold increase over vehicle-treated β -kl^{+/+} mice. *D*) Bile acid pool size ($n = 5-6$) and *E*) fecal excretion of bile acids ($n = 6-8$) in β -kl^{+/+}, β -kl^{-/-}, β -kl^{+/+}/Tg, and β -kl^{-/-}/Tg. Pool size is expressed as micromoles of bile acid per 100 g body weight. Fecal excretion of total bile acids is expressed as micromoles per day per 100 g body weight. Stool samples were collected daily for 3 consecutive days. Data are presented as the means $\pm$ SEM. $**P < 0.01$.

β -Kl protein was undetectable in pancreas and adipose tissues of β -kl^{-/-} and β -kl^{-/-}/Tg mice (Fig. 2B).

In order to examine whether the transgene-derived β -Kl protein is functional as a receptor component for FGF15/19 *in vivo*, we measured *Cyp7a1* mRNA levels in the liver of β -kl^{-/-}/Tg mice under basal (vehicle-treated) and FGF19-stimulated conditions. Consistent with our previous reports (10, 12), *Cyp7a1* mRNA level was 3.8 times higher in β -kl^{-/-} mice compared with β -kl^{+/+} mice in the basal condition (Fig. 2C). There was no difference in *Cyp7a1* expression between β -kl^{+/+} and β -kl^{+/+}/Tg mice (Fig. 2C). In β -kl^{-/-}/Tg mice compared with β -kl^{-/-} mice, *Cyp7a1* expression level was decreased by Tg rescue of β -Kl expression in hepatocytes and was comparable to the level in β -kl^{+/+} mice (Fig. 2C). Treatment with FGF19 suppressed *Cyp7a1* mRNA level in β -kl^{+/+} and β -kl^{+/+}/Tg mice, whereas it failed to suppress *Cyp7a1* expression in β -kl^{-/-} mice (Fig. 2C). In contrast to β -kl^{-/-} mice, administration of FGF19 resulted in a significant reduction of *Cyp7a1* expression level in β -kl^{-/-}/Tg mice (Fig. 2C).

Furthermore, increased pool size (1.4-fold) and augmented fecal excretion (2.8-fold) of bile acid in β -kl^{-/-} mice were both ameliorated in β -kl^{-/-}/Tg mice (Fig. 2D, E).

These data provide direct *in vivo* evidence that the transgene-derived β -Kl protein is functional in β -kl^{-/-}/Tg mice and that β -Kl in hepatocytes is necessary and sufficient for bile acid homeostasis.

Hepatocyte β -Kl is necessary and sufficient for plasma lipid regulation

To explore a putative role of hepatocyte β -Kl in lipid homeostasis, we first measured plasma lipid levels. We observed only a tendency of decrease in plasma cholesterol level in β -kl^{-/-} mice compared with β -kl^{+/+} mice (-15%; Fig. 3A). In contrast, plasma triglyceride level in β -kl^{-/-} mice was significantly decreased (-44%; Fig. 3C). By hepatocyte-specific expression of β -Kl, both plasma cholesterol and triglyceride levels in β -kl^{-/-}/Tg were reversed and comparable to the levels of β -kl^{+/+} mice (Fig. 3A, C).

We further analyzed plasma lipoprotein profile. β -kl^{-/-} mice showed decreased cholesterol in VLDL (-45%) with a tendency of decrease in LDL (-29%) compared with β -kl^{+/+} mice (Fig. 3B). No difference was observed between β -kl^{+/+} and β -kl^{-/-} mice in the levels of cholesterol

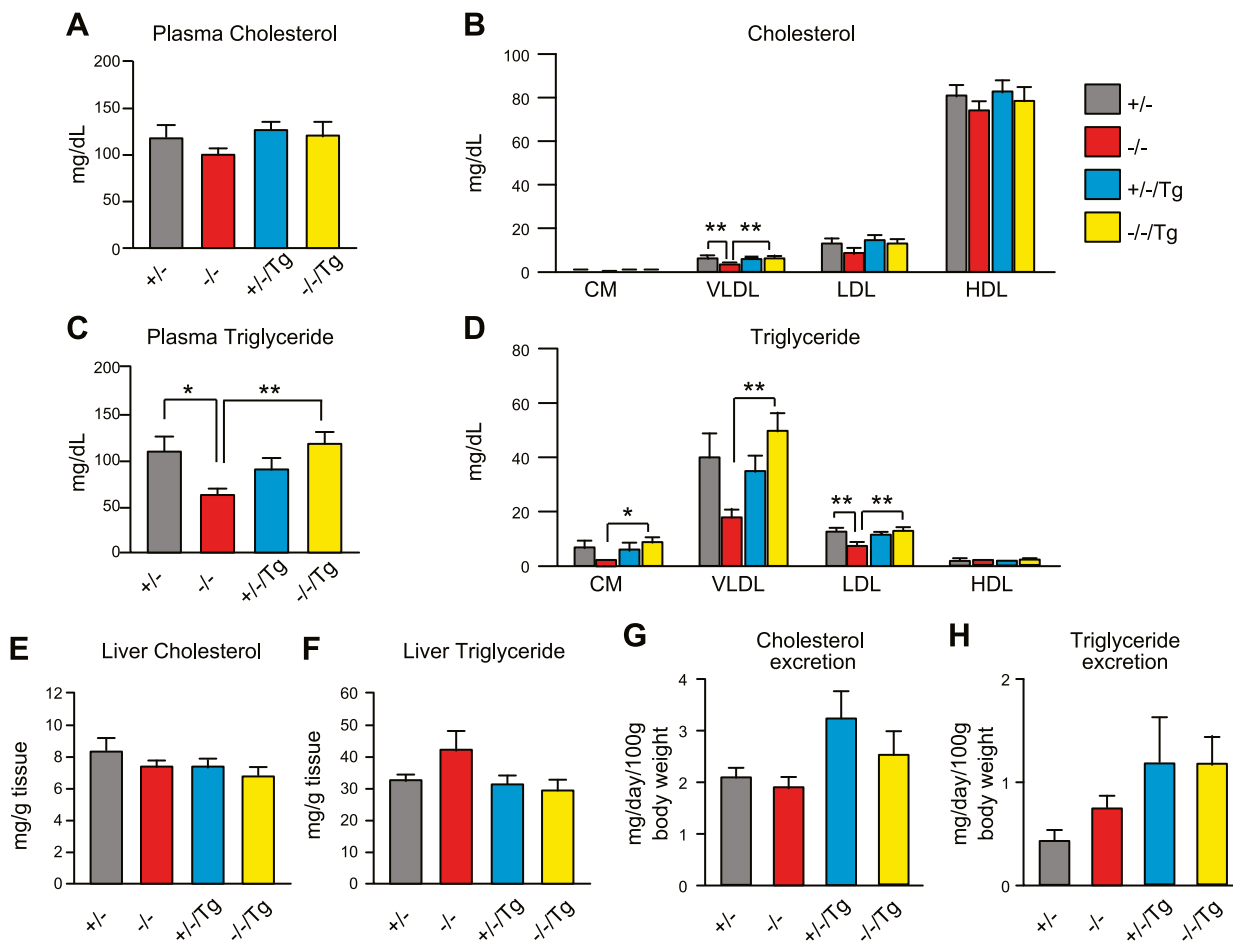


Figure 3. Hepatocyte β -Kl is necessary and sufficient for plasma lipid regulation. A) Plasma total cholesterol and (C) triglyceride levels ($n = 7-8$). B) Cholesterol and (D) triglyceride levels in plasma associated with each lipoprotein fraction ($n = 7-8$). E) Liver total cholesterol and (F) triglyceride contents ($n = 7-8$). Fecal excretion of total cholesterol (G) and triglycerides (H) ($n = 7-8$). Fecal excretion of lipids is expressed as milligrams per day per 100 g body weight. Samples were obtained from β -kl^{+/+}, β -kl^{-/-}, β -kl^{+/+}/Tg, and β -kl^{-/-}/Tg mice fed a control diet for 8 wk starting from 9 to 11 wk and fed *ad libitum* on the day of sampling. Data are presented as the means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

associated with chylomicrons (CMs) or HDL (Fig. 3B). Triglyceride in the LDL fraction was significantly decreased in β -kl^{-/-} mice compared with β -kl^{+/+} mice (-39%; Fig. 3D). CM (-71%) and VLDL (-56%) triglyceride levels in β -kl^{-/-} mice showed a tendency of decrease, but not significant, and there was no difference in HDL triglyceride (Fig. 3D). The changes in plasma lipoprotein profiles in β -kl^{-/-} mice were all completely normalized in β -kl^{-/-}/Tg mice (Fig. 3B, D). We observed no significant difference in liver cholesterol or triglyceride contents (Fig. 3E, F). No significant difference was observed in fecal cholesterol or triglyceride contents (Fig. 3G, H). These data clearly demonstrate that β -Kl in hepatocytes is necessary and sufficient for plasma lipid and lipoprotein homeostasis in mice.

Plasma cholesterol level is maintained by a robust homeostatic regulation of cholesterologenesis

To obtain insight into the molecular basis for the dysregulation of lipid metabolism observed in β -kl^{-/-} mice, we first analyzed gene expression in the liver. Expression levels of sterol regulatory element-binding factor (*Srebf*)2,

3-hydroxy-3-methylglutaryl-Coenzyme A (HMG-CoA) synthase 1, and HMG-CoA reductase, which are all critical genes in cholesterologenesis, were concertedly elevated in β -kl^{-/-} mice (Fig. 4A). In β -kl^{-/-}/Tg mice, the elevated expression levels of all the aforementioned cholesterologenic genes were restored and comparable to the level in β -kl^{+/+} mice (Fig. 4A). In contrast to the cholesterologenic genes, no difference was observed in the mRNA levels for the critical genes in lipogenesis: *Srebf*1, fatty acid synthase, and stearoyl-CoA desaturase 1 (Fig. 4A). It has been reported that farnesoid X receptor (FXR), a nuclear receptor-type bile acid receptor, transcriptionally induces small heterodimer partner (SHP) to suppress SREBP-1c expression (32). However, we did not detect any significant difference in the levels of *Fxr* or *Shp* among the 4 genotypes (Fig. 4A).

Second, we measured the content of intermediate metabolites in cholesterologenic and lipogenic pathways in the liver by LC-MS. The analysis revealed that acetyl-CoA and HMG-CoA contents tended to be higher in β -kl^{-/-} than β -kl^{+/+} mice, and both were significantly lower in β -kl^{-/-}/Tg than β -kl^{-/-} mice (Fig. 4B). In contrast, we observed no difference in malonyl-CoA contents among β -kl^{+/+}, β -kl^{-/-}, and β -kl^{-/-}/Tg mice (Fig. 4B), further

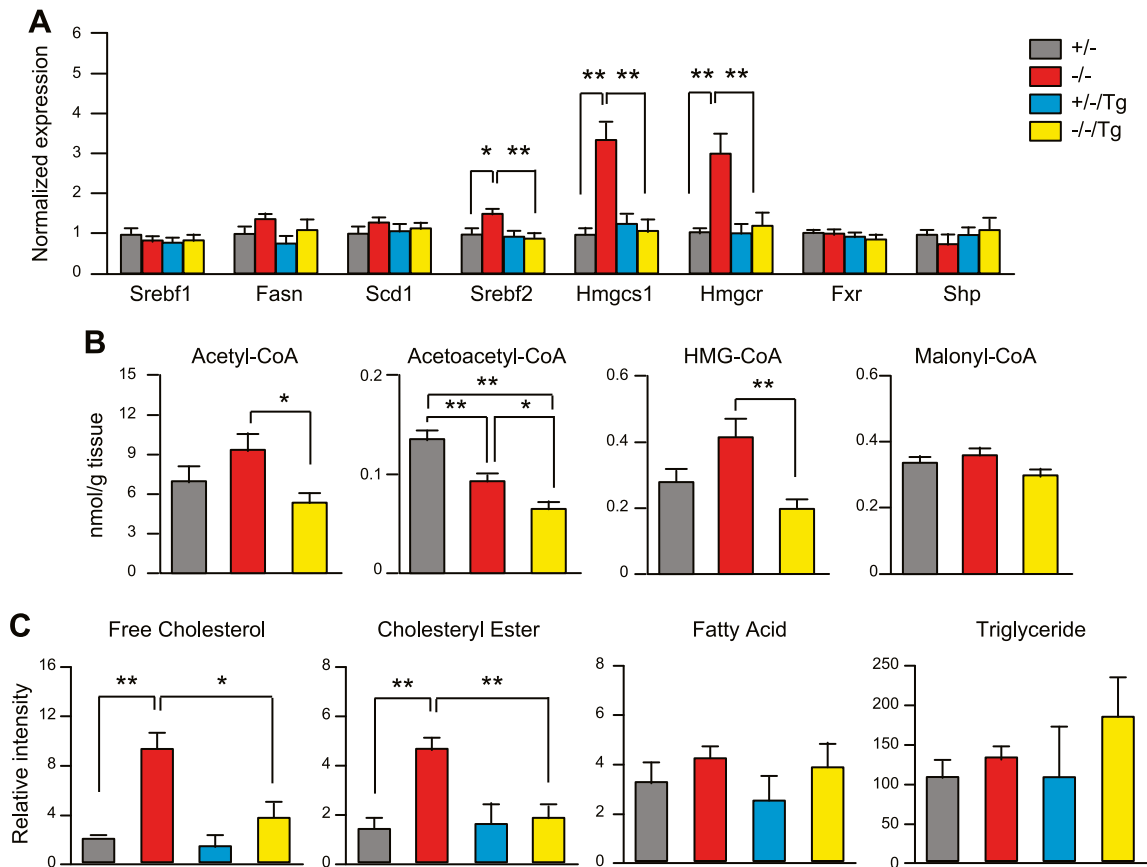


Figure 4. Plasma cholesterol level is maintained by a robust homeostatic regulation of cholesterologenesis. A) mRNA expression of genes involved in the regulation of lipid metabolism in the liver ($n = 7-8$). *Fasn*, fatty acid synthase; *Hmgcr*, HMG-CoA reductase; *Hmgcs1*, HMG-CoA synthase 1; *Scd1*, stearoyl-CoA desaturase 1. Data were normalized by expression of 18S rRNA and are shown as fold increase over β -kl^{-/-} mice. B) The levels of metabolites involved in cholesterologenesis and lipogenesis in the liver, measured by LC-MS ($n = 5$). For (A) and (B), samples obtained from mice fed a control diet for 8 wk starting from 9 to 11 wk were used. C) Incorporation of ¹⁴C into free cholesterol, cholesteryl ester, fatty acid, and triglyceride in the liver ($n = 5$). Mice were given an intraperitoneal injection of [¹⁴C]acetate, and the liver was sampled 30 min later. Mice fed a control diet for 12 wk starting from 9 to 10 wk were used. Data are presented as the means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

supporting the view that cholesterologenesis, but not lipogenesis, was enhanced in β -*kl*^{-/-} mice. Acetoacetyl-CoA level was decreased both in β -*kl*^{-/-} and β -*kl*^{-/-}/Tg mice compared with β -*kl*^{+/-} mice (Fig. 4B). In order to describe the global metabolic landscape apart from our hypothesis this time, we further measured the liver content of 99 core molecules in nutrient metabolism by capillary electrophoresis mass spectrometry. NADPH, an essential cofactor in cholesterol and fatty acid synthesis, showed a tendency of decrease in β -*kl*^{-/-} (0.9-fold) compared with β -*kl*^{+/-} and increase in β -*kl*^{-/-}/Tg (1.1-fold) compared with β -*kl*^{+/-} mice (Table S1). Compared with β -*kl*^{+/-} mice, ribose 5-phosphate tended to be increased both in β -*kl*^{-/-} (1.5-fold) and β -*kl*^{-/-}/Tg (2.1-fold) mice (Table S1). Ribulose 5-phosphate also tended to be increased in β -*kl*^{-/-} (1.3-fold) and β -*kl*^{-/-}/Tg (1.3-fold) mice, whereas glucose 6-phosphate levels were not changed (Table S1). Although indirect, these changes provide supporting evidence for a presumed alteration in β -*kl*^{-/-} mice of the flux of pentose pathway, a major provider of NADPH and thereby a vital element in lipid biosynthesis.

Third, to determine *de novo* cholesterologenesis and lipogenesis rates *in vivo*, we performed a tracer experiment by administering mice with ¹⁴C-labeled sodium acetate. The amounts of incorporated ¹⁴C into free cholesterol and cholesteryl ester in β -*kl*^{-/-} mice were 4.5- and 3.2-fold higher, respectively, than in β -*kl*^{+/-} mice (Fig. 4C). Again, incorporations into both were significantly lower in β -*kl*^{-/-}/Tg mice (Fig. 4C). Consistent with the gene expression and metabolite contents in the liver, no difference was observed in the incorporation of ¹⁴C into fatty acid and triglyceride among the 4 genotypes (Fig. 4C). These data show that a loss of β -KI in hepatocytes provokes *de novo* cholesterologenesis, but not lipogenesis.

Hepatocyte β -KI is required for the maintenance of plasma triglyceride levels

VLDL secretion from the liver and lipoprotein clearance in peripheral tissues are 2 major factors that control plasma triglyceride levels. To uncover the pathophysiology of hypotriglyceridemia in β -*kl*^{-/-} mice, we first measured VLDL secretion from the liver. Plasma triglyceride level in the basal condition was lower in β -*kl*^{-/-} mice, but we observed no difference in the rates of VLDL secretion among the genotypes (Fig. 5A). Consistent with unchanged secretion, we found no difference in the expression levels of microsomal triglyceride transfer protein and apolipoprotein B, 2 key regulators of VLDL secretion (Fig. 5B).

We then focused on lipoprotein clearance. We injected ³H-labeled VLDL into mice and then measured ³H radioactivity in the liver and plasma. Compared with β -*kl*^{+/-} mice, the ³H radioactivity in the liver was significantly increased in β -*kl*^{-/-} mice, whereas it was comparable in β -*kl*^{-/-}/Tg mice (Fig. 5C). In contrast, the ³H radioactivity in the plasma from β -*kl*^{-/-} mice was markedly decreased compared with β -*kl*^{+/-} mice (Fig. 5D). These data demonstrate that increased hepatic uptake and plasma clearance of VLDL are associated with decreased plasma triglyceride levels in β -*kl*^{-/-} mice. Lipoprotein lipase (LPL) is the primary enzyme for the clearance of triglyceride-rich lipoproteins in peripheral tissues (33). In

skeletal muscle, WAT, and BAT, no change was observed in *Lpl* expression levels among the genotypes (Fig. 5E). As for the liver lipoprotein clearance, expression of hepatic lipase, which facilitates clearance of triglyceride from VLDL particles (34), and LDL receptor showed no significant change in the liver from β -*kl*^{-/-} mice (Fig. 5B). Meanwhile, there was a significant increase of *Lpl* expression in the liver from β -*kl*^{-/-} mice (Fig. 5E). Although there are reports arguing that *Lpl* expression in the liver is negligible (35), *Lpl* mRNA expression was detected both in hepatocytes and nonhepatocyte fractions in our experiment (Fig. 5F). Taken together, elevated *Lpl* expression in the liver in β -*kl*^{-/-} mice may contribute at least in part to the decrease in plasma triglyceride-rich lipoprotein.

Liver phenotypes in the absence or presence of hepatocyte β -KI

We observed no significant difference in liver cholesterol or triglyceride contents (Fig. 3E, F), though hematoxylin and eosin and Oil Red O staining showed some indications of lipid accumulation in the liver of β -*kl*^{-/-} mice (Fig. 6A). In addition, β -*kl*^{-/-} mice showed slightly increased plasma levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST), which are markers of hepatocellular necrosis (Fig. 6B).

Recent reports have shown that in some animal models, hepatic steatosis is accompanied by an ectopic expression of peroxisome proliferator-activated receptor γ (*Pparg*) (36, 37). In accordance with these reports, QPCR analysis of liver from β -*kl*^{-/-} mice showed that the expression levels of *Pparg* and its transcriptional target gene CD36 antigen (*Cd36*) were significantly increased (Fig. 6C). We observed no difference in the expression levels of PPAR α (*Ppara*) and its target gene acyl-CoA oxidase 1 (Fig. 6C). Interestingly, however, expression of *Fgf21*, a reported target gene of PPAR α (38), was increased in β -*kl*^{-/-} mice but was restored to the level of β -*kl*^{+/-} mice in β -*kl*^{-/-}/Tg mice (Fig. 6C). These data suggest that a loss of β -KI in hepatocytes may promote lipid accumulation in the liver to a certain extent, possibly through enhanced expression of *Pparg*.

β -KI regulates body weight in a hepatocyte-independent manner

To explore a potential contribution of hepatocyte β -KI and consequent bile acid modulation to the regulation of energy metabolism, we measured the body weight of β -*kl*^{-/-} and β -*kl*^{-/-}/Tg mice. As we have reported previously (10), β -*kl*^{-/-} mice exhibited reduced body weight (Fig. 7A). In contrast to the recovery of hypotriglyceridemia, decreased body weight in β -*kl*^{-/-} mice was not recovered to the level of control β -*kl*^{+/-} mice in β -*kl*^{-/-}/Tg mice (Fig. 7A). Body weight in β -*kl*^{+/-}/Tg mice was comparable to β -*kl*^{+/-} mice (Fig. 7A). HFD led to a pronounced weight gain in all genotypes (Fig. 7B). However, even under HFD-fed conditions, not only β -*kl*^{-/-} but β -*kl*^{-/-}/Tg mice remained to weigh less than β -*kl*^{+/-} mice (Fig. 7B). No difference was observed in food intake among the genotypes either on the control diet or HFD (Fig. 7C). On an HFD, *Cyp7a1* expression in liver (Supplemental Fig. S1A) and *Fgf15*

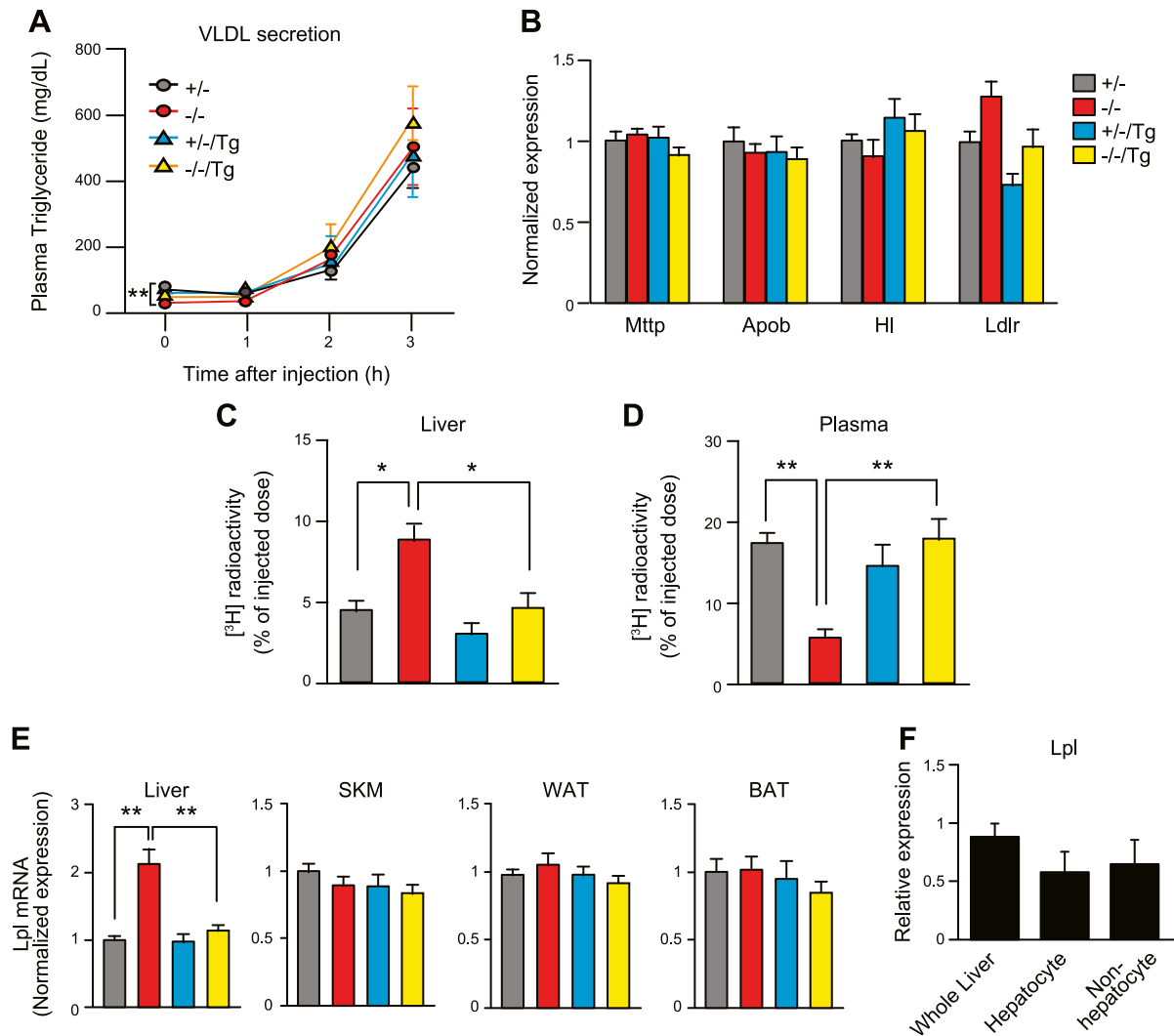


Figure 5. Hepatocyte β -KI is required for the maintenance of plasma triglyceride levels. **A**) VLDL secretion rate expressed as increment of plasma triglyceride levels over time after intraperitoneal injection of tyloxapol ($n = 4-5$). Mice fed a control diet for 13 wk starting from 9 to 10 wk were used. **B**) Expression levels of genes involved in lipoprotein metabolism in the liver ($n = 7-8$). *Apob*, apolipoprotein B; *Hl*, hepatic lipase; *Ldlr*, LDL receptor; *Mttp*, microsomal triglyceride transfer protein. **C**) VLDL uptake activity in the liver and **(D)** plasma. Mice were injected with ^3H -labeled VLDL. After 15 min, the liver and blood were collected, and ^3H radioactivity in the liver and the plasma was determined ($n = 4-5$). Mice fed a control diet for 28 wk starting from 10 wk were used. **E**) *Lpl* mRNA levels in the liver, skeletal muscle (SKM), WAT, and BAT ($n = 7-8$). For **(B)** and **(E)**, samples were obtained from mice fed a control diet for 8 wk starting from 9 to 10 wk. Data were normalized by 18S rRNA and are shown as fold increase over $\beta\text{-kl}^{+/+}$ mice. **F**) mRNA expression of *Lpl* in whole liver, hepatocyte fraction, and nonhepatocyte fraction from C57BL/6J mice ($n = 3-4$). The same amount (50 ng) of RNA was used for each sample. mRNA levels were normalized by 18S rRNA expression. Data are presented as the means $\pm$ SEM. * $P < 0.05$; ** $P < 0.01$.

expression in ileum (Supplemental Fig. S1B) in $\beta\text{-kl}^{+/+}$ mice were increased but were restored to the level of $\beta\text{-kl}^{+/+}$ in $\beta\text{-kl}^{+/+}/\text{Tg}$ mice, suggesting the recovery of β -KI-dependent bile acid regulation also on an HFD. No difference was observed in *Fgf4* mRNA expression in the liver (Supplemental Fig. S1C). The nose-to-hip length of both $\beta\text{-kl}^{+/+}$ and $\beta\text{-kl}^{+/+}/\text{Tg}$ mice was slightly but significantly shorter than that of $\beta\text{-kl}^{+/+}$ mice (Fig. 7D). These data demonstrate that β -KI has a role in determining body weight and length but at the same time provide evidence that β -KI expressed in hepatocytes is not involved in this action.

To obtain further mechanistic insights into the contribution of β -KI to body weight control, we subjected mice to indirect calorimetry. The volume of O_2 consumed by $\beta\text{-kl}^{+/+}$

mice was increased in both dark and light periods (Fig. 7E). The respiratory quotient, which reflects the carbohydrate:fat oxidation ratio, was not altered (Fig. 7F). We did not observe any difference in the expression of the genes involved in energy expenditure in BAT (Supplemental Fig. S2A).

We then examined body composition using dual-energy X-ray absorptiometry scanning. Both lean and fat masses were reduced in $\beta\text{-kl}^{+/+}$ compared with $\beta\text{-kl}^{+/+}$ or $\beta\text{-kl}^{+/+}/\text{Tg}$ mice (Fig. 7G). Adipocytes in epididymal WAT from $\beta\text{-kl}^{+/+}$ mice tended to be smaller than those of $\beta\text{-kl}^{+/+}$ mice (Supplemental Fig. S2B). We therefore examined the expression levels of lipases in WAT but found that the levels in $\beta\text{-kl}^{+/+}$ mice were comparable to $\beta\text{-kl}^{+/+}$ mice (Supplemental Fig. S2C). These data suggest that the

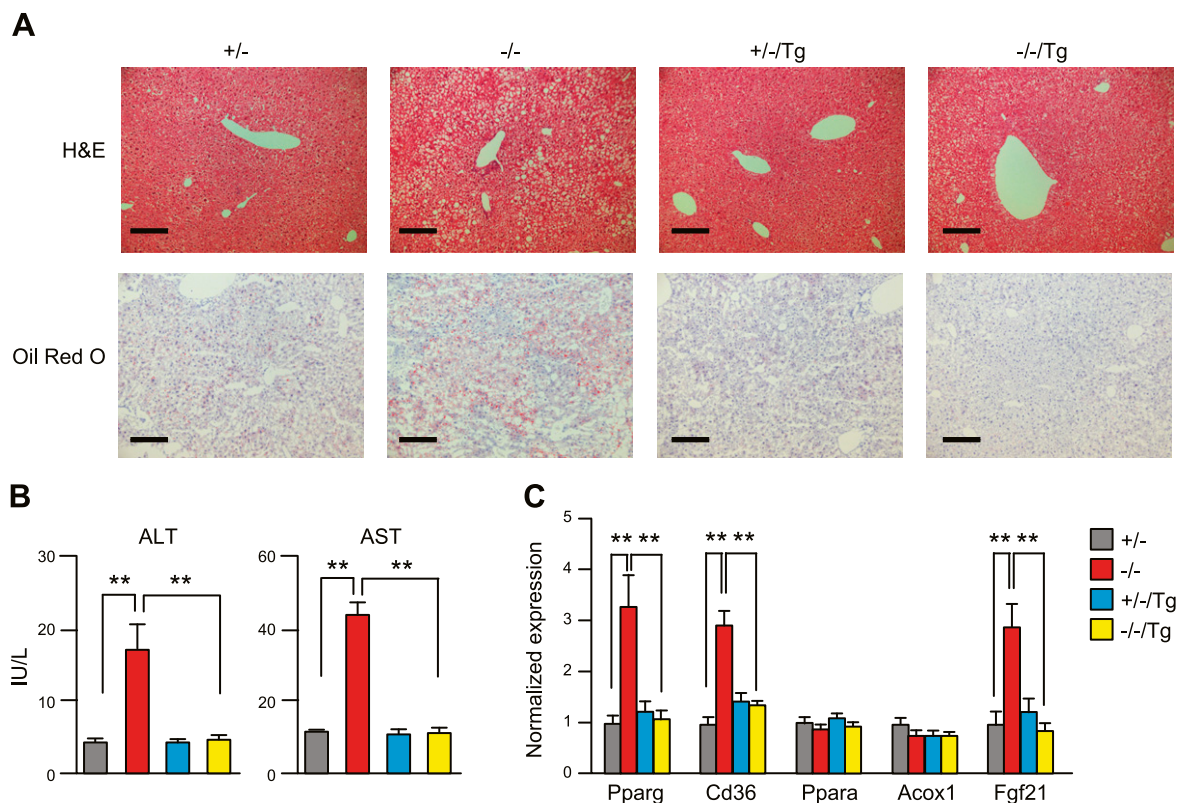


Figure 6. Liver phenotypes in the absence or presence of hepatocyte β -Kl. **A**) Representative hematoxylin and eosin (H&E) and Oil Red O-stained sections of liver ($n = 5$ mice per group). Scale bars, 200 μ m. **B**) Plasma ALT and AST levels ($n = 7-8$). **C**) Expression levels of *Pparg*, *Ppara*, and their target genes in the liver ($n = 7-8$). *Acox1*, acyl-CoA oxidase 1. Data were normalized by 18S rRNA and are shown as fold increase over β -kl^{-/-} mice. All samples were obtained from mice fed a control diet for 8 wk starting from 9 to 11 wk. Data are presented as the means $\pm$ SEM. ** $P < 0.01$.

lower body weight of β -kl^{-/-} mice may be partly explained by increased energy expenditure and shorter stature but is independent of bile acid excess.

DISCUSSION

β -Kl is an established regulator of bile acid metabolism, but its contributions to whole-body lipid and energy homeostasis have remained to be clarified. In this study, we demonstrate that β -Kl expressed specifically in hepatocytes is necessary and sufficient for lipid and bile acid homeostasis, but not body weight in mice. Our results further clarify the robustness of cholesterol metabolism in a condition with excessive cholesterol disposal.

The former conclusion is based upon 3 lines of evidence. First, restoration of hepatic β -Kl expression in β -kl^{-/-} mice leads to a complete recovery of FGF19-induced *Cyp7a1* suppression and normal bile acid metabolism. These findings show that hepatocyte-specific expression of β -Kl is necessary and sufficient for bile acid homeostasis and provide direct *in vivo* evidence that β -Kl/FGF15/19-mediated *Cyp7a1* suppression is cell autonomous in nature. Second, β -kl^{-/-} mice show decreased plasma triglyceride level, although plasma cholesterol level is maintained owing to enhanced *de novo* cholesterologenesis. The changes in plasma lipids and lipoprotein levels as well as enhanced cholesterologenesis in β -kl^{-/-} mice are completely

restored in β -kl^{-/-}/Tg mice to the levels of control β -kl^{-/-} mice, supporting our conclusion that β -Kl in hepatocytes is essential for whole-body lipid homeostasis. Third, reduced body weight and resistance to diet-induced weight gain in β -kl^{-/-} mice are not reversed in β -kl^{-/-}/Tg mice, suggesting that nonhepatocyte β -Kl, but not hepatocyte β -Kl, is involved in the regulation of body weight and energy homeostasis.

In our study, despite the elevation of hepatic bile acid synthesis from cholesterol, plasma cholesterol levels in β -kl^{-/-} mice do not show a significant decrease. Expression of *Srebf2*, a master regulator of cholesterologenesis, is increased in the liver of β -kl^{-/-} mice. Concomitantly, the expression levels of downstream cholesterologenic genes are up-regulated. Metabolomic and *in vivo* flux analyses show that *de novo* cholesterologenesis is markedly enhanced in β -kl^{-/-} mice, suggesting that augmented cholesterologenesis in β -kl^{-/-} mice compensates for the cholesterol depletion caused by unsuppressed cholesterol-to-bile acid conversion. In contrast to cholesterologenesis, no such compensatory response in lipogenesis is observed in β -kl^{-/-} mice, even in a condition where plasma triglyceride levels are markedly decreased. These data highlight the robustness with which plasma cholesterol level is maintained in this mouse model of enhanced cholesterol excretion. Furthermore, acetyl-CoA level shows a tendency of increase in β -kl^{-/-} mice and reversal in β -kl^{-/-}/Tg mice. We cannot see any obvious changes in acetyl-CoA-providing

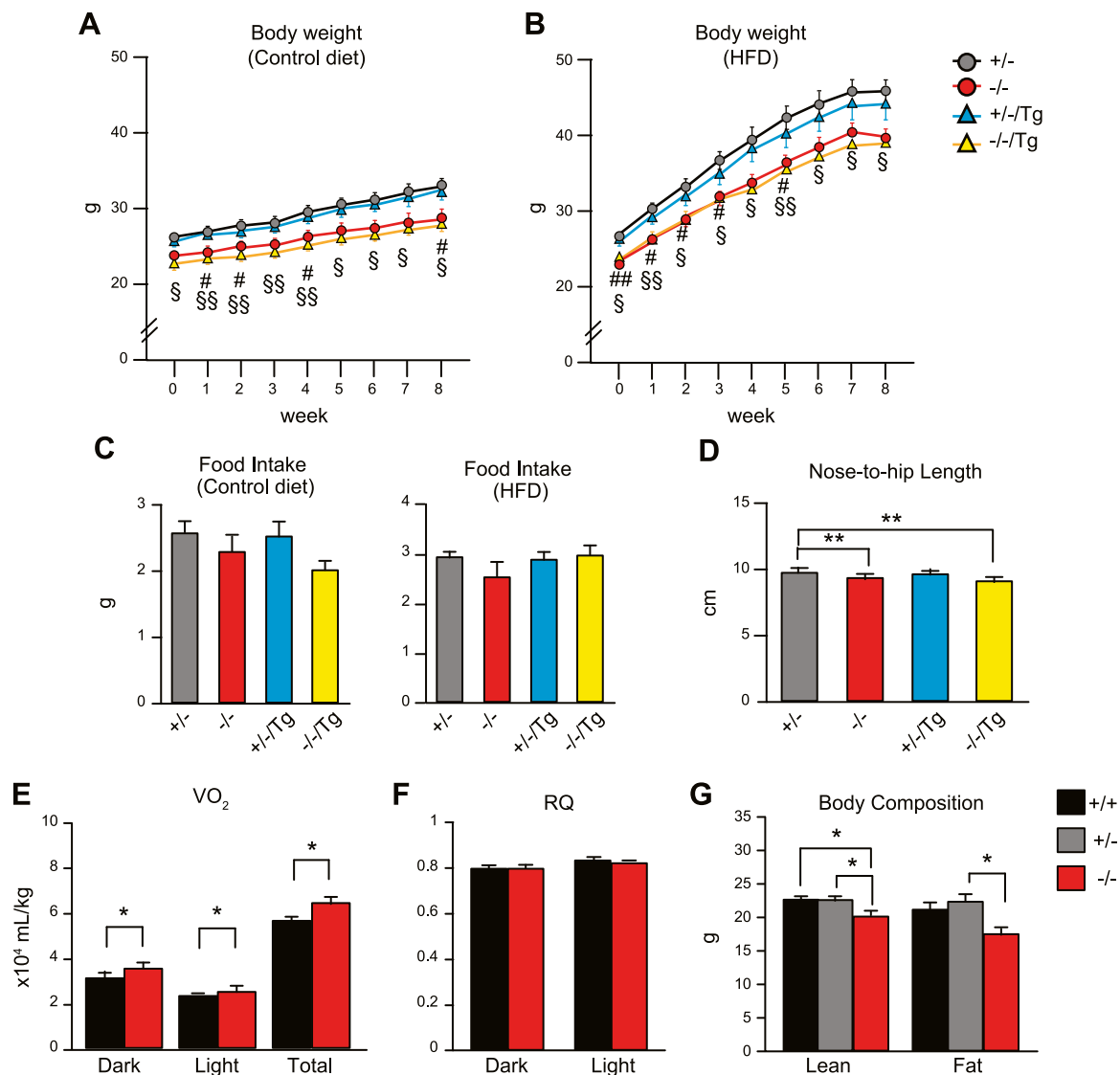


Figure 7. β -Kl regulates body weight in a hepatocyte-independent manner. Body weight changes in mice fed a (A) control diet ($n = 7-8$) and (B) HFD ($n = 9-12$) for 8 wk starting from 9 to 11 wk. Data are shown for $\beta\text{-kl}^{+/+}$ (gray circles), $\beta\text{-kl}^{-/-}$ (red circles), $\beta\text{-kl}^{-/-}/\text{Tg}$ (blue triangles), and $\beta\text{-kl}^{-/-}/\text{Tg}$ mice (yellow triangles). $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ between $\beta\text{-kl}^{-/-}/\text{Tg}$ and $\beta\text{-kl}^{-/-}$ mice; $^{\S}P < 0.05$ and $^{\S\S}P < 0.01$ between $\beta\text{-kl}^{-/-}/\text{Tg}$ and $\beta\text{-kl}^{+/+}$ mice. C) Average food intake in the control diet ($n = 7-8$) and HFD ($n = 5-8$) groups at 16–18 wk. Food weights were measured daily for 3 consecutive days. D) Nose-to-hip length in mice fed a control diet for 8 wk ($n = 7-8$). E) Volume of O_2 consumed (VO_2) and (F) respiratory quotients (RQs) were determined by indirect calorimetry over a 24 h period in WT ($\beta\text{-kl}^{+/+}$) and $\beta\text{-kl}^{-/-}$ mice fed an HFD for 3 wk from 16 to 18 wk ($n = 8$). VO_2 is expressed as milliliters of O_2 corrected for body weight. G) Body composition in mice fed an HFD for 6 wk from 12 to 15 wk ($n = 11-21$). Data are presented as the means $\pm$ SEM. See also Supplemental Figs. S1 and S2. $^*P < 0.05$; $^{**}P < 0.01$.

systems, including glycolysis, tricarboxylic acid cycle, or citrate shuttle. However, it seems reasonable to speculate that an augmented supply of acetyl-CoA in $\beta\text{-kl}^{-/-}$ mice may constitute part of a compensatory mechanism that enables enhanced *de novo* cholesterol synthesis, which can be a substantial demand for cytosolic acetyl-CoA as the substrate. Interestingly, acetoacetyl-CoA level in the liver is decreased both in $\beta\text{-kl}^{-/-}$ and $\beta\text{-kl}^{-/-}/\text{Tg}$ mice, whereas the rates of cholesterol and cholesteryl ester synthesis in $\beta\text{-kl}^{-/-}/\text{Tg}$ mice are comparable to the level of control. Considering that acetoacetyl-CoA also serves as the precursor in the pathway for ketone body synthesis, β -Kl expressed in nonhepatic tissues may potentially be involved in the ketogenic pathway. Similarly, increased

levels of ribose 5-phosphate both in $\beta\text{-kl}^{-/-}$ and $\beta\text{-kl}^{-/-}/\text{Tg}$ may also be the result of the lack of β -Kl in nonhepatic tissues.

$\beta\text{-kl}^{-/-}$ mice show elevated bile acid synthesis and concomitant decrease of plasma triglyceride. Plasma lipoprotein analyses show that $\beta\text{-kl}^{-/-}$ mice exhibit decreased levels of triglyceride-rich lipoprotein. To address the cause of hypolipoproteinemia mechanistically, we examined the synthesis, excretion, and clearance of plasma triglycerides. We observed no difference in hepatic *de novo* lipogenesis or triglyceride excretion into feces among the 4 genotypes. The mRNA expression of PPAR α , a master regulator of fatty acid oxidation, is not changed, either. The VLDL uptake analysis revealed increased hepatic uptake and

plasma clearance of VLDL in β -kl^{-/-} mice. Although *Lpl* mRNA expression in skeletal muscle and adipose tissues is not altered, *Lpl* expression in the liver is increased in β -kl^{-/-} mice. Although the physiologic role of LPL expressed in the liver remains controversial, liver-specific *Lpl*Tg mice have been reported and show reduced plasma triglyceride levels in a gene dose-dependent manner (39). Recent studies have observed that angiopoietin-like proteins (ANGPTLs) 3, 4, and 8, members of the ANGPTL family, are involved in the maintenance of plasma triglyceride levels (29, 40). We observe no significant difference in the expression of *Angptl3*, 4, or 8 in the liver from β -kl^{-/-} mice (data not shown). Taken together, it is highly suggested that decreased plasma triglyceride-rich lipoprotein levels in β -kl^{-/-} mice are attributable to increased lipoprotein clearance in the liver.

We found that β -kl^{-/-} mice show slightly elevated plasma ALT/AST levels and a tendency for triglyceride accumulation in the liver in histologic analyses. Ectopic expression of *Pparg* in the liver reportedly results in hepatic steatosis (36, 37). In accordance with this, our β -kl^{-/-} mice also show increased expression of *Pparg* and its transcriptional target gene in the liver. Furthermore, we observe increased *Fgf21* expression in the liver of β -kl^{-/-} mice, whereas the level in β -kl^{-/-}/Tg mice is comparable to control. Hepatic expression of *Fgf21* is classically known to be augmented through PPAR α induction during starvation (38). In our study, we observe no difference in hepatic *Ppara* expression among the 4 genotypes. Recent studies have reported the role of FGF21 in hepatic steatosis (41, 42). Interestingly, *Pparg* and *Fgf21* mRNA expression was increased in a rodent model of nonalcoholic steatohepatitis (43). Moreover, mice treated with a methionine- and choline-deficient diet, which causes a diet-induced nonalcoholic steatohepatitis model, showed robustly elevated *Fgf21* mRNA expression in the liver independent of PPAR α (44). These results together suggest that enhanced hepatic *Pparg* expression in β -kl^{-/-} mice may contribute to the increased *Fgf21* expression. Although β -Kl has been reported to serve as a cofactor not only for FGF15/19 but also FGF21, it remains unclear how FGF15/19 and 21 are related to each other as ligands in this pathway. Further study is required to clarify the relationship between FGF15/19 and 21 in terms of the liver function of β -Kl. However, care is needed in interpreting the data on FGF21 because we previously failed to detect any significant difference of hepatic *Fgf21* expression between β -k^{+/+} and β -kl^{-/-} mice (12). This apparent discrepancy might be due to differences in the diet used or the animal facility where our experiments have been undertaken.

We have previously shown that β -kl^{-/-} mice are viable, fertile, and grossly healthy but exhibit lower body weight from the time of weaning (10). Interestingly, oral administration of bile acid also reduces body weight by increasing energy expenditure in mice (7). We hypothesized that elevated bile acid synthesis in β -kl^{-/-} mice may also have a role in reducing the body weight. Contrary to our expectation, however, lower body weight and the resistance to diet-induced obesity in β -kl^{-/-} mice are not restored by Tg hepatic expression of β -Kl and consequent recovery of bile acid metabolism. β -kl^{-/-} and β -kl^{-/-}/Tg mice exhibit lower body weight without any significant change in the amount of food consumed. Instead, we found that β -kl^{-/-}

mice show a higher level of O₂ consumption than β -kl^{+/+} mice. These data suggest that the lower body weight in β -kl^{-/-} mice may be explained by increased energy expenditure, but through a bile acid-independent mechanism. Further studies are required to determine how β -Kl in nonhepatic tissues contributes to body weight control.

FGF21 has broad metabolic actions, including an anti-obesity effect in rodents and primates (45). Indeed, Tg mice with elevated circulating FGF21 levels exhibit lower body weight than WT mice (46). However, we have observed a decrease in body weight in β -kl^{-/-} mice (10). These data argue against the possibility that body weight change in β -kl^{-/-} mice is caused by a loss of FGF21 actions. Ding *et al.* (15) reported that adipose tissue-selective β -kl^{-/-} mice showed similar body weight and composition to their control mice. Furthermore, Bookout *et al.* (8) reported that mice lacking β -kl in hypothalamus or hind-brain showed body weight similar to control littermates. These findings argue against the possibility that β -Kl loss in adipose tissues or brain accounts for the reduction of body weight in whole-body β -kl^{-/-} mice. We previously reported that β -kl is expressed not only in adult tissues but also in the yolk sac in the embryo (9). Before placental maturation, the embryo absorbs maternal nutrients through the yolk sac. Thus, β -Kl in the adult pancreas and/or yolk sac during development may have a role in the determination of body weight. Further studies are required to determine how β -Kl in nonhepatic tissues contributes to fetal growth and body weight control.

FGFR4 is a selective receptor for FGF15/19 in the liver, and the phenotype of *Fgfr4*^{-/-} mice is similar to that of our β -kl^{-/-} mice (23). Huang *et al.* (47) reported that *Fgfr4*^{-/-} mice exhibit increased plasma triglyceride and cholesterol levels. Although there is a clear difference in the study design, an interesting possibility still remains that the discrepancy is due to a difference in the spectra of molecular functions between β -Kl and FGFR4.

We observed virtually no difference in bile acid or lipid metabolism between WT and Tg mice. A previous study has also reported that no difference is observed in bile acid metabolism in Tg mouse lines over-expressing human *Fgfr4* in hepatocytes (13). These data commonly suggest a possibility that expression of β -kl or *Fgfr4* at a supra-normal level does not necessarily result in a gene dose-dependent augmentation of FGF15/19 signaling or greater suppression of bile acid synthesis.

Serum FGF19 levels in human subjects are increased when orally administrated with bile acid and reduced by the treatment with bile acid-binding resin (48). Conversely, human *in vivo* study has shown that CYP7A1 activity, which is suppressed by FGF15/19 in experimental animals, is inhibited by bile acid and reciprocally activated by the resin (48). More importantly, the rate of triglyceride synthesis in humans is reduced by the oral administration of bile acid and increased by the resin (49). Although there are substantial differences in lipid regulation between rodents and humans, it seems reasonable to conclude that the β -Kl/FGF15/19-dependent regulatory mechanisms of bile acid and lipid metabolism essentially resemble each other in humans and rodents.

In conclusion, we have shown for the first time that β -Kl expressed in hepatocytes plays a critical role in whole-body lipid and lipoprotein homeostasis and that a

hepatocyte-independent role of β -KL is involved in the control of energy homeostasis. We further demonstrate that plasma cholesterol but not triglyceride levels are maintained by a robust compensatory mechanism in a mouse model of enhanced cholesterol disposal. **FJ**

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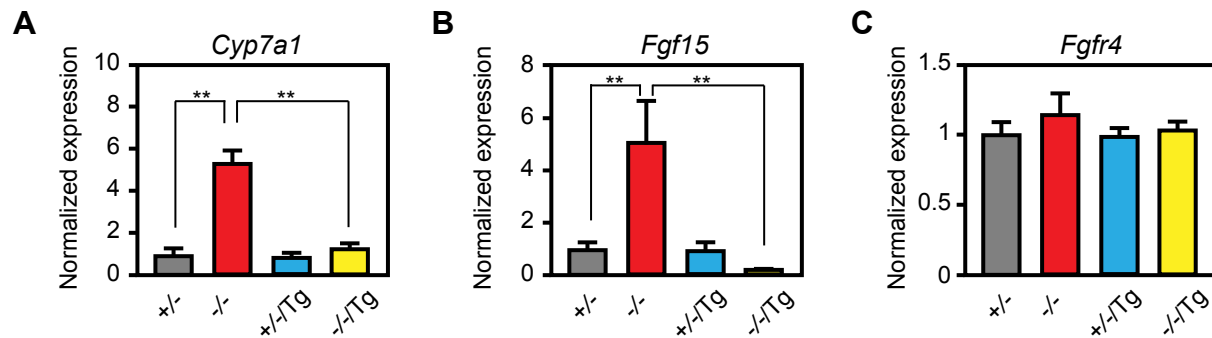


Figure S1, related to Figure 7. β -Klotho regulates body weight in a hepatocyte-independent manner.

(A) *Cyp7a1* mRNA expression in liver (n = 6-8). (B) *Fgf15* mRNA expression in ileum (n = 6-8). The small intestine was divided into four parts (0, 25, 50, 75 and 100%; relative distance from the stomach to distal ileum) and the 75-100% part was collected as the ileum. (C) *Fgfr4* mRNA expression in liver (n = 6-8). All samples were obtained from mice fed HFD for 8 weeks starting from 9-11 weeks old. Data were normalized by 18S rRNA and are shown as fold increase over β -kl^{+/+} mice. Data are presented as the mean $\pm$ SEM. **p < 0.01.

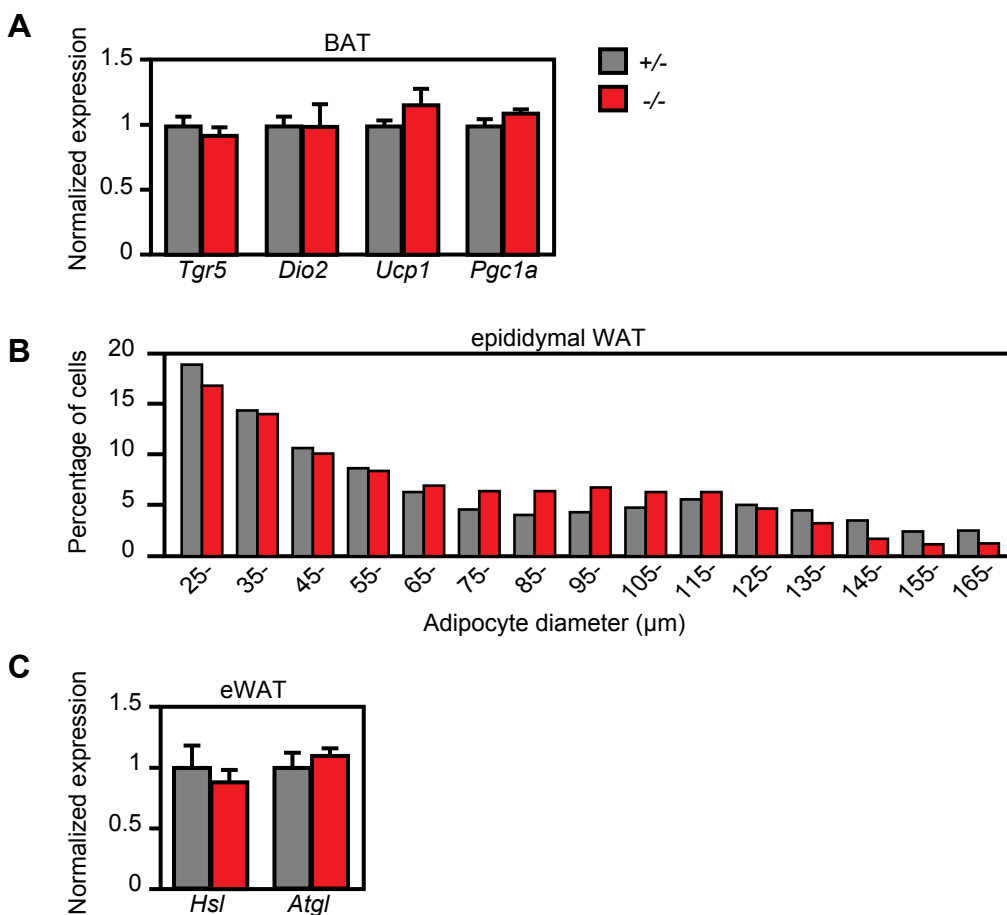


Figure S2, related to Figure 7. β -Klotho regulates body weight in a hepatocyte-independent manner.

(A) mRNA expression of genes involved in energy expenditure in brown adipose tissue (BAT) (n = 6-8). (B) Adipocyte size distribution in epididymal WAT (eWAT) (n = 5). (C) mRNA expression of lipases in eWAT (n = 6-8). All samples were obtained from mice fed HFD for 8 weeks starting from 9-11 weeks old. For (A) and (C), data were normalized by 18S rRNA and are shown as fold increase over β -kl^{+/+} mice. Data are presented as the mean $\pm$ SEM.

Table S1. Contents of metabolites in the liver determined by CE-MS.

		(nmol/g tissue)						
Pathway	Compound name	+/-		-/-		-/-/Tg		
Amino acid	Alanine	2669	± 183	2842	± 195	2374	± 230	
	Arginine	0.70	± 0.10	0.40	± 0.09	0.31	± 0.04	§
	Asparagine	107	± 7	96	± 6	100	± 5	
	Aspartic acid	376	± 28	254	± 18	330	± 4	†
	Cystathionine	39	± 8	26	± 6	20	± 4	
	Cysteine	3.8	± 0.5	3.5	± 0.4	5.3	± 0.4	†
	Glutamic acid	3157	± 129	3373	± 201	2748	± 200	
	Glutamine	4126	± 425	3352	± 313	4656	± 287	
	Glycine	1916	± 68	2098	± 80	1819	± 85	
	Histidine	662	± 43	591	± 33	615	± 50	
	Homoserine	4.2	± 0.3	3.1	± 0.3	3.0	± 0.2	§
	Hydroxyproline	23	± 4	27	± 1	16	± 1	††
	Isoleucine	197	± 20	177	± 14	185	± 13	
	Leucine	392	± 40	352	± 22	356	± 25	
	Lysine	490	± 45	453	± 34	448	± 34	
	Methionine	39	± 4	33	± 4	30	± 4	
	N-Acetylglutamic acid	53	± 8	78	± 12	36	± 4	†
	Phenylalanine	144	± 14	117	± 8	112	± 5	
	Proline	296	± 70	336	± 55	175	± 37	
	S-Adenosylhomocysteine	30	± 6	29	± 6	25	± 4	
	S-Adenosylmethionine	79	± 1	78	± 1	79	± 3	
	Serine	358	± 22	240	± 27	302	± 22	
	Threonine	358	± 32	305	± 17	246	± 14	§
	Tryptophan	39	± 3	37	± 2	38	± 3	
	Tyrosine	147	± 17	130	± 13	142	± 12	
	Valine	425	± 37	447	± 31	388	± 16	
	γ-Aminobutyric acid	10	± 1	9	± 1	10	± 1	
Choline	Betaine	333	± 41	193	± 34	227	± 33	
	Betaine aldehyde	0.73	± 0.16	0.5	± 0.12	1.03	± 0.18	
	Choline	56	± 7	36	± 6	62	± 4	†
	N,N-Dimethylglycine	12	± 1	16	± 2	11	± 1	
	Sarcosine	56	± 15	58	± 19	33	± 9	

Coenzyme	CoA	97 ± 9	139 ± 16	147 ± 18
	NAD ⁺	216 ± 21	254 ± 31	194 ± 11
	NADH	1.90 ± 0.03	1.90 ± 0.08	1.90 ± 0.11
	NADP ⁺	155 ± 5	139 ± 16	140 ± 11
	NADPH	1.7 ± 0.1	1.5 ± 0.1	1.9 ± 0.3
	β-Alanine	54 ± 4	54 ± 5	54 ± 4
Energy	ADP	726 ± 19	741 ± 59	689 ± 28
	AMP	1860 ± 54	1891 ± 62	1991 ± 89
	ATP	312 ± 30	391 ± 94	262 ± 29
	GDP	33 ± 2	34 ± 3	32 ± 1
	GMP	296 ± 6	300 ± 4	310 ± 12
	GTP	17 ± 2	20 ± 5	14 ± 2
Fatty acid	Carnitine	125 ± 9	129 ± 8	113 ± 6
Folate	Folic acid	0.064 ± 0.001	0.065 ± 0.004	0.062 ± 0.003
Glutathione	Glutathione (GSH)	5583 ± 348	5619 ± 73	5884 ± 97
	Glutathione (GSSG)	1451 ± 188	1326 ± 62	1149 ± 112
Glycogen	Fructose 1-phosphate	39 ± 3	36 ± 3	43 ± 3
	Galactose 1-phosphate	24 ± 2	21 ± 2	28 ± 3
	UDP-glucose	226 ± 34	246 ± 33	212 ± 45
Glycolysis	2,3-Diphosphoglyceric acid	111 ± 5	106 ± 6	102 ± 5
	2-Phosphoglyceric acid	0.61 ± 0.07	0.68 ± 0.08	0.54 ± 0.06
	3-Phosphoglyceric acid	6.3 ± 0.7	7.2 ± 1.0	5.7 ± 0.7
	Dihydroxyacetone phosphate	39 ± 3	27 ± 10	28 ± 5
	Fructose 1,6-diphosphate	13 ± 1	13 ± 1	9 ± 1 §,††
	Fructose 6-phosphate	295 ± 18	267 ± 15	256 ± 33
	Glucose 1-phosphate	67 ± 3	73 ± 4	68 ± 9
	Glucose 6-phosphate	652 ± 35	647 ± 27	634 ± 62
	Glyceraldehyde 3-phosphate	1.6 ± 0.1	1.3 ± 0.3	1.3 ± 0.2
	Glycerol 3-phosphate	1510 ± 80	1309 ± 84	1323 ± 111
	Lactic acid	7801 ± 790	9656 ± 767	7145 ± 502
	Phosphoenolpyruvic acid	1.6 ± 0.3	1.7 ± 0.3	1.3 ± 0.2
	Pyruvic acid	231 ± 15	256 ± 25	271 ± 17
Pentose	6-Phosphogluconic acid	68 ± 3	58 ± 4	70 ± 2
phosphate	Phosphoribosyl pyrophosphate	3.4 ± 0.2	3.6 ± 0.4	3.5 ± 0.2
	Ribose 1-phosphate	141 ± 4	119 ± 8	155 ± 11 †

	Ribose 5-phosphate	19 ± 1	29 ± 3	39 ± 6 §
	Ribulose 5-phosphate	13 ± 1	17 ± 2	17 ± 3
	Sedoheptulose 7-phosphate	119 ± 9	148 ± 16	146 ± 14
	Xylulose 5-phosphate	19 ± 2	20 ± 2	25 ± 2
Polyamine	Putrescine	2.9 ± 0.3	2.9 ± 0.4	2.3 ± 0.3
	Spermidine	31 ± 3	31 ± 1	30 ± 1
	Spermine	18 ± 2	16 ± 1	18 ± 2
Purine	Adenine	8.7 ± 1.1	8.0 ± 0.8	8.0 ± 0.6
	Adenosine	157 ± 12	142 ± 13	145 ± 16
	Adenylosuccinic acid	33 ± 3	23 ± 3	41 ± 5 †
	ADP-ribose	80 ± 3	70 ± 6	75 ± 4
	Hypoxanthine	41 ± 3	32 ± 5	47 ± 3 †
	Inosine	264 ± 19	177 ± 20 #	248 ± 21
	IMP	145 ± 21	125 ± 21	143 ± 12
	Uric acid	1.6 ± 0.2	1.5 ± 0.2	1.4 ± 0.1
	Xanthine	20 ± 1	22 ± 3	27 ± 2
	XMP	0.23 ± 0.02	0.24 ± 0.03	0.21 ± 0.01
Pyrimidine	N-Carbamoylaspartic acid	0.52 ± 0.13	1.44 ± 0.32 #	0.35 ± 0.09 ††
	Guanine	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.1
	Guanosine	43 ± 3	34 ± 3	34 ± 3
Signal	cAMP	0.36 ± 0.01	0.41 ± 0.04	0.33 ± 0.03
TCA cycle	2-Hydroxyglutaric acid	18 ± 2	30 ± 8	18 ± 2
	Citric acid	89 ± 11	79 ± 11	93 ± 10
	Fumaric acid	84 ± 11	53 ± 5	64 ± 7
	Succinic acid	836 ± 101	858 ± 110	775 ± 39
Urea	Argininosuccinic acid	3.0 ± 0.1	2.5 ± 0.1 #	2.5 ± 0.1 §
	Citrulline	29 ± 3	31 ± 4	19 ± 2 †
	Creatine	142 ± 12	125 ± 5	141 ± 10
	Creatinine	3.4 ± 0.6	3.0 ± 0.2	3.0 ± 0.1
	Ornithine	238 ± 26	230 ± 33	170 ± 14
	Phosphocreatine	1.7 ± 0.3	1.4 ± 0.3	1.6 ± 0.3
	Urea	6652 ± 285	7106 ± 379	6335 ± 451

Samples were obtained from mice fed control diet for 8 weeks starting from 9-11 weeks old (n = 5). Data are presented as the mean ± SEM. #p < 0.05; ##p < 0.01 between β -kl^{-/-} and β -kl^{+/-} mice, §p < 0.05 between β -kl^{-/-}/Tg and β -kl^{+/-} mice, †p < 0.05; ††p < 0.01 between β -kl^{-/-} and β -kl^{-/-}/Tg mice.