Supplemental Materials and Methods

Intestine farnesoid X receptor agonist and the gut microbiota activate G-protein bile acid receptor-1 signaling to improve metabolism

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Oral glucose and insulin tolerance tests

For oral glucose tolerance test, wild-type and *db/db* mice were fasted 6 h and were orally gavaged with glucose (2 g/Kg). Blood samples were collected via tail vein and serum glucose was measured at intervals over 2 h using a OneTouch Ultra Mini glucometer (LifeScan; Milpitas, CA). For insulin tolerance tests, wild-type and *db/db* mice were fasted for 6 h and insulin (0.75 units/Kg) was administered by i.p. injection and serum glucose was measured at intervals over 2 h.

Immunoblot analysis

Total tissue and cell lysates were prepared in RIPA buffer (Cell Signaling Technology, Danvers, MA) and proteins were resolved on 10% SDS-PAGE gels. Monoclonal antibodies against AKT and $pAKT_{473}$ were purchased from Cell Signaling Technology (cat # 9272 and 9271, respectively). Anti-TGR5 antibodies were purchased from LS Bioscience (cat #LS-C47388, Seattle, WA). Loading control histone blot was performed by stripping and re-probing the blot with histone antibody (cat #9715, Cell Signaling Technology). For analysis of bile acid synthesis enzymes, microsomes were isolated from mouse liver after gavage with FEX (50 mg/Kg) or vehicle (0.2% DMSO) as previously described [(1)]. Immunoblot analysis was performed using polyclonal antibodies against regulatory enzymes CYP7A1 and CYP8B1 (cat# Sc25536, Sc23515, SantaCruz Biotechnology, Dallas, TX) and CYP7B1 (Ab136801, Abcam, Cambridge, MA). Liver

lysates were used for immunoblot of mitochondrial CYP27A1 (cat # Ab126785, Abcam). Calnexin (cat # 2679, Cell Signaling Technology) was used as loading control for microsomes and Gapdh (cat # 2118, Cell Signaling Technology) was used as a loading control for liver lysates.

Bile acid pool size

C57BL/6J mice were gavaged with FEX (n=8, 50 mg/Kg) for 9 days. Mice were fasted for 6 h before sacrifice and bile acids were isolated from 100 mg liver, whole intestine, and whole gallbladder by a series of ethanol and methanol extractions overnight at 65°C. Bile acid content was quantified by kit (Genzyme Diagnostic; Cambridge, MA) and bile acid pool size was determined by totaling bile acids in liver, gallbladder, and intestine.

Quantitative real-time PCR assay (qPCR)

Total RNA was isolated with Tri-Reagent (Sigma Aldrich). All primers/probe sets for qPCR were ordered from TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). SYBR-green primers were used for Sult2a1, Sult2a2 and Sulg2a8 amplification. Amplification of Gapdh was used as an internal control. Relative mRNA expression was quantified using the comparative CT (ΔCt) method and expressed as $2^{-\Delta\Delta Ct}$.

Serum AST or ALT measurement

Serum AST and ALT levels were calculated using Infinity AST or ALT liquid reagent (ThermoFisher Scientific) according to the manufacturer's protocol.

Staining and immunohistochemistry

Wild-type and db/db mice were gavaged with fexaramine (50 mg/Kg). Mice were fasted 6h and inguinal fat (iWAT) and epididymal (eWAT) fat pads were isolated. Tissue were fixed in 4% paraformaldehyde for 48 h. Paraffin embedding was performed after overnight tissue processing. Tissue sections were stained with hematoxylin and eosin (H&E) using a standard protocol. A portion of samples were de-paraffinized and antigen unmasking was performed using citrate

buffer (pH = 6). IHC was performed against UCP-1 (Abcam #ab10983) with a VECTASTAIN ABC kit (Vector Laboratories, Burlingame, CA) according to the manufacturer's protocol.

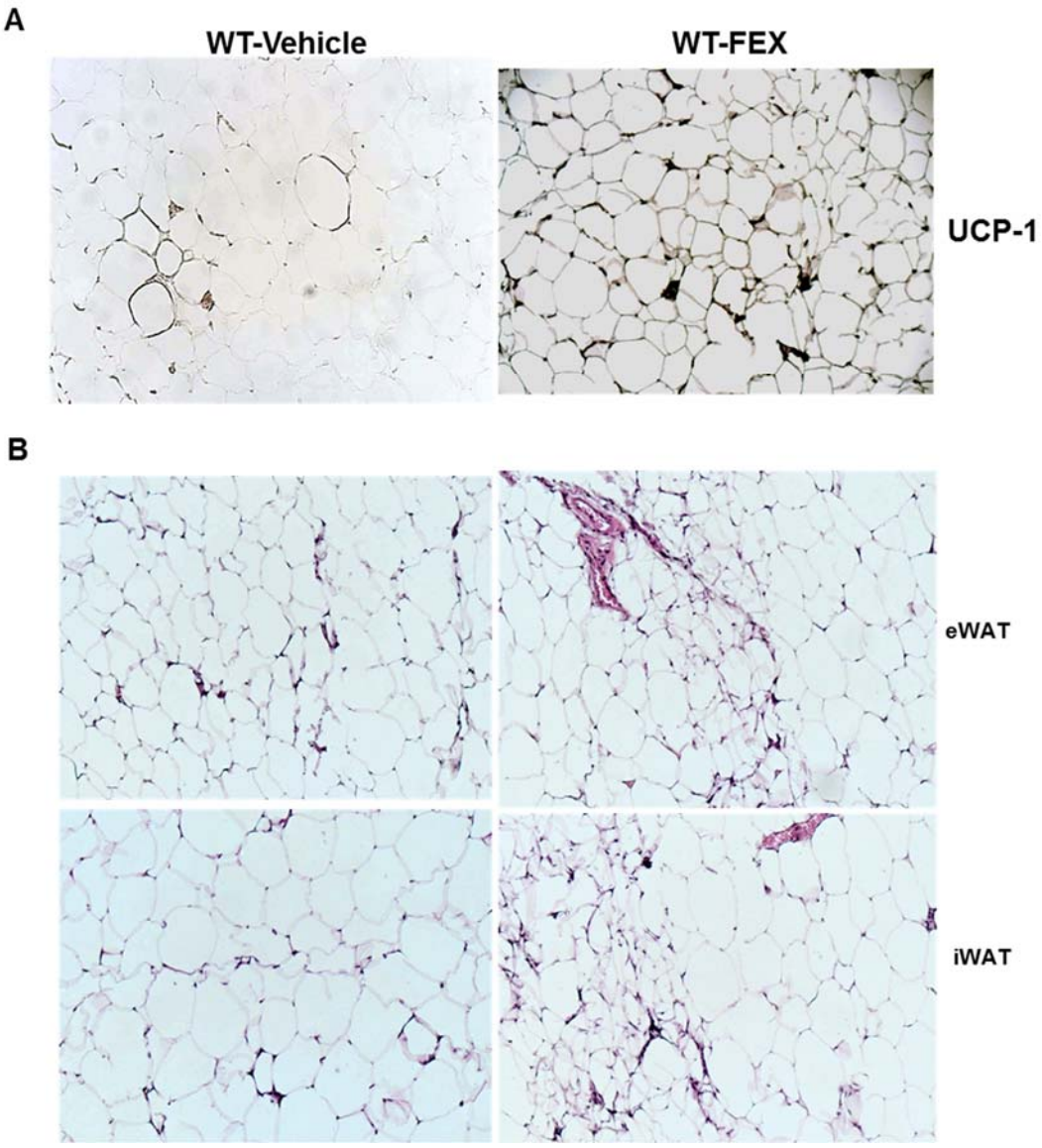


Fig. S1. FEX treatment increased white adipocyte tissue browning in wild-type mice.

Wild-type mice were treated with FEX or vehicle as described under Methods. (A) Immunohistochemical staining of inguinal fat (iWAT) using UCP-1 antibody followed by DAB staining. (B) H&E staining of epididymal (eWAT) and inguinal fat (iWAT).

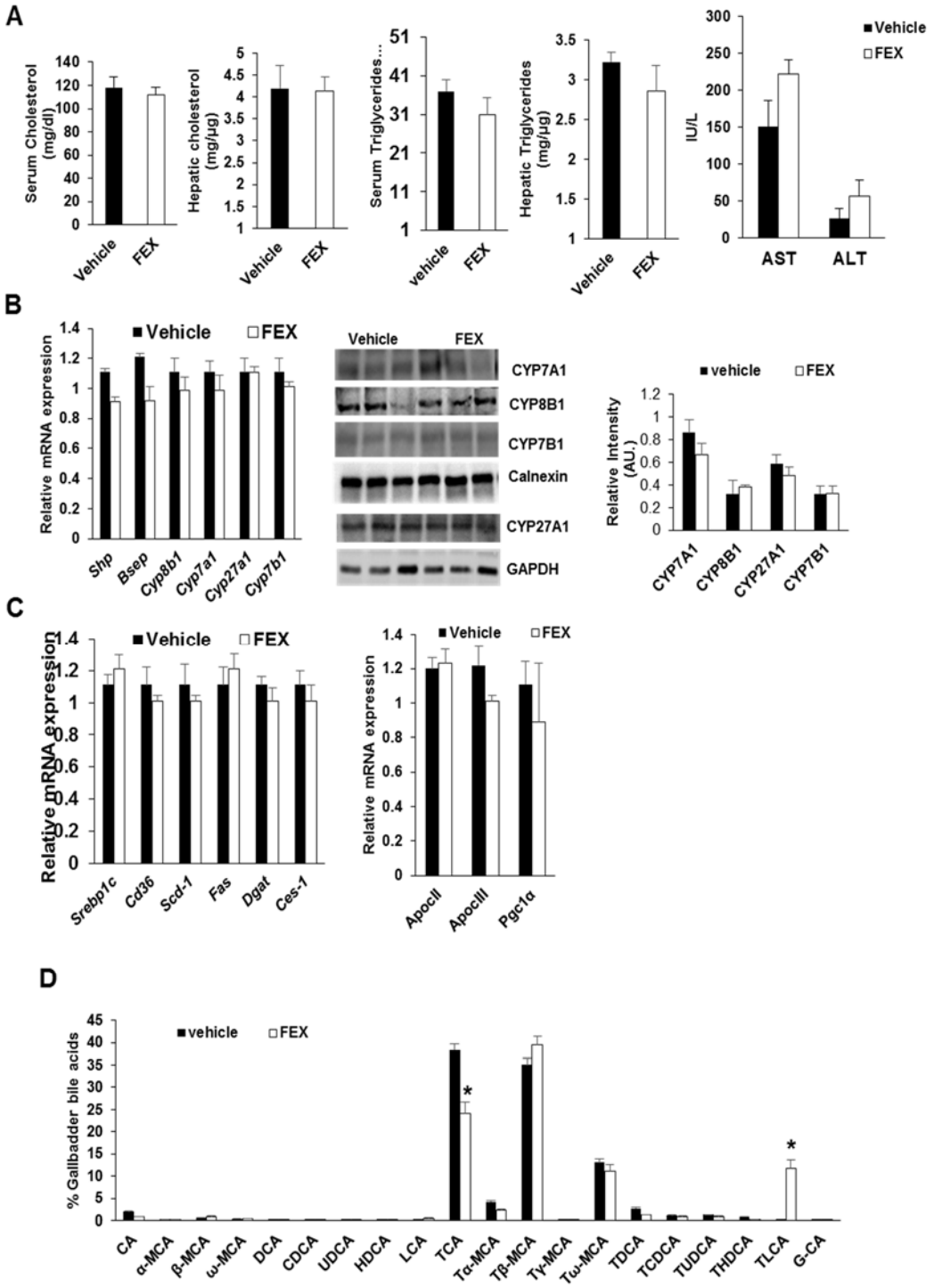


Fig. S2. FEX treatment did not affect serum lipid profiles or mRNA expression of liver bile acid synthesis and lipid metabolism genes. Wild-type mice were treated with FEX or vehicle as described under Methods. (A) Serum and liver cholesterol and triglycerides. Serum AST and ALT

level in vehicle and FEX-treated mice. (B) qPCR and immunoblot analyses of bile acid synthesis enzyme mRNA and protein in liver extracts. (C) qPCR analysis of liver lipogenesis and lipoprotein mRNA expression. (D) Gallbladder bile acid composition (%) in vehicle and fexaramine treated mice. An "*" indicates statistically significant difference by Student's *t*-test ($p \leq 0.05$), FEX vs. vehicle treated wild-type mice.

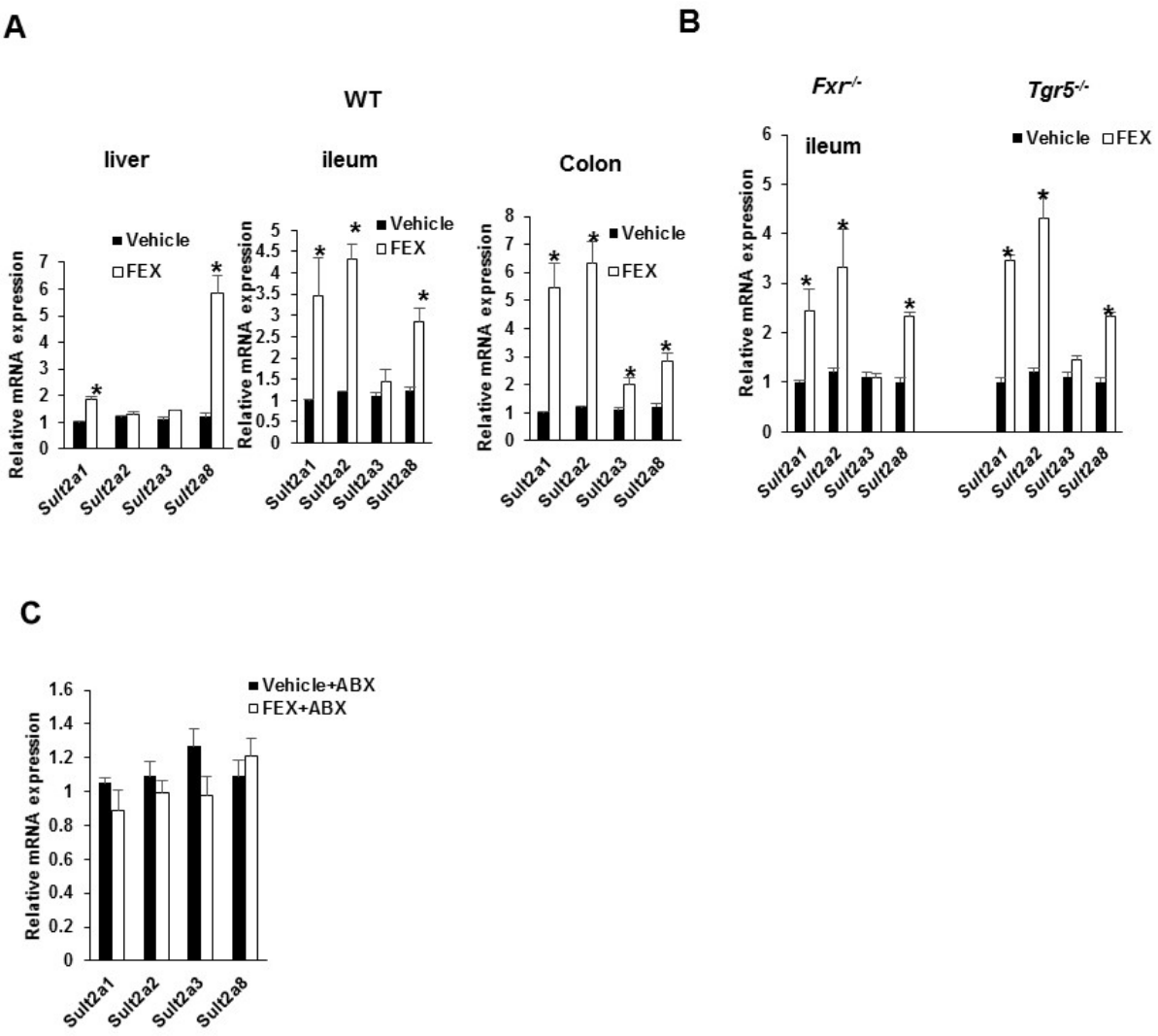


Fig. S3. FEX treatment induced sulfotransferase (Sult) mRNA expression levels and antibiotics prevented the effect of FEX on Sults. Wild-type C57BL/6J mice were treated with antibiotics (ABX) and FEX. (A) Relative mRNA expression of Sult mRNAs in the liver, ileum and colon of wild-type mice. (B) Relative mRNA expression levels in the ileum of *Tgr5*$^{-/-}$ and *Fxr*$^{-/-}$ mice. (C) Effect of FEX on Sult mRNA expression levels in ileum of ABX-treated mice. Results were expressed as mean ± standard error. An "*" indicates statistically significant difference by Student's *t*-test ($p \leq 0.05$), FEX treated vs. vehicle treated wild type mice.

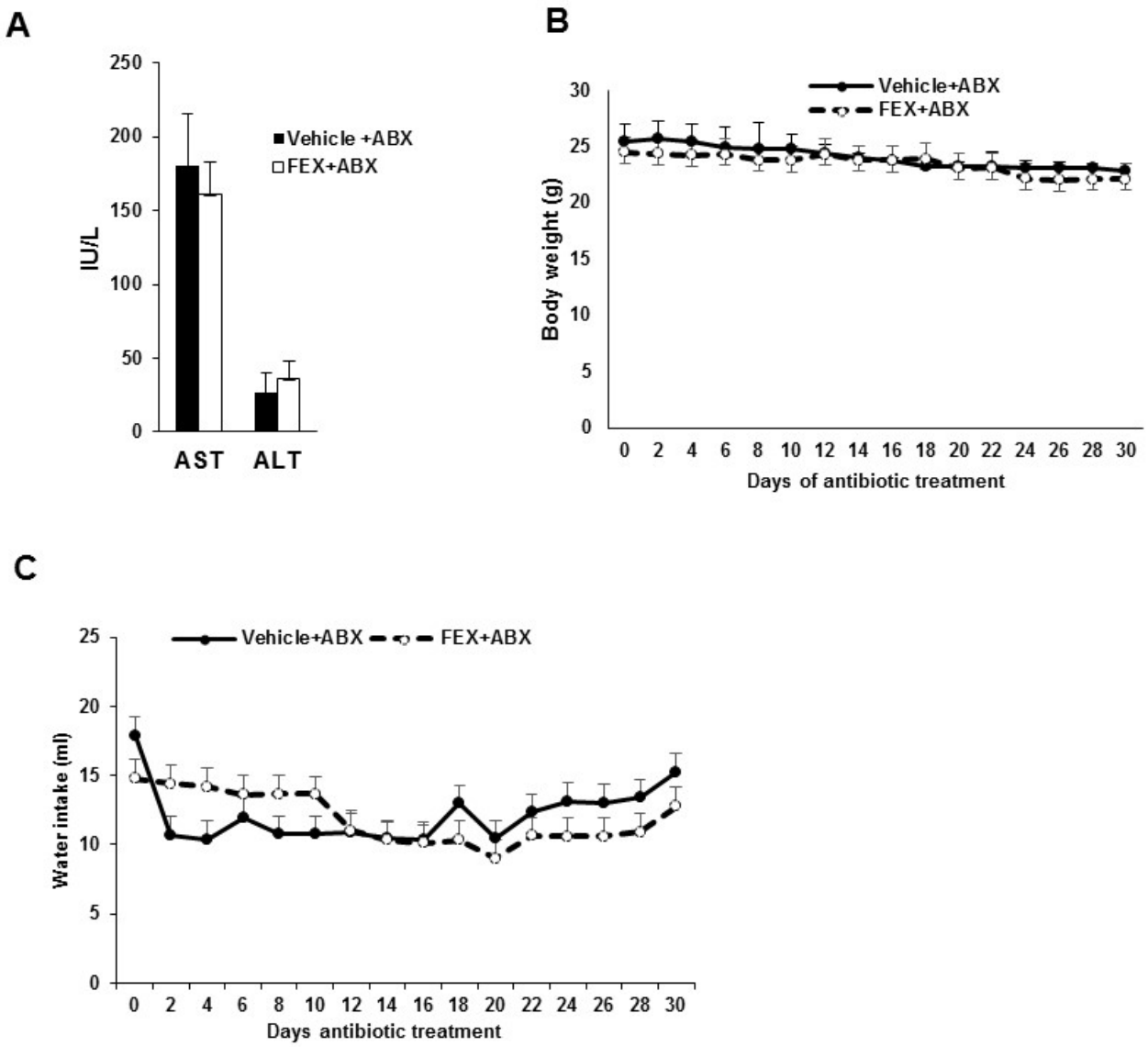
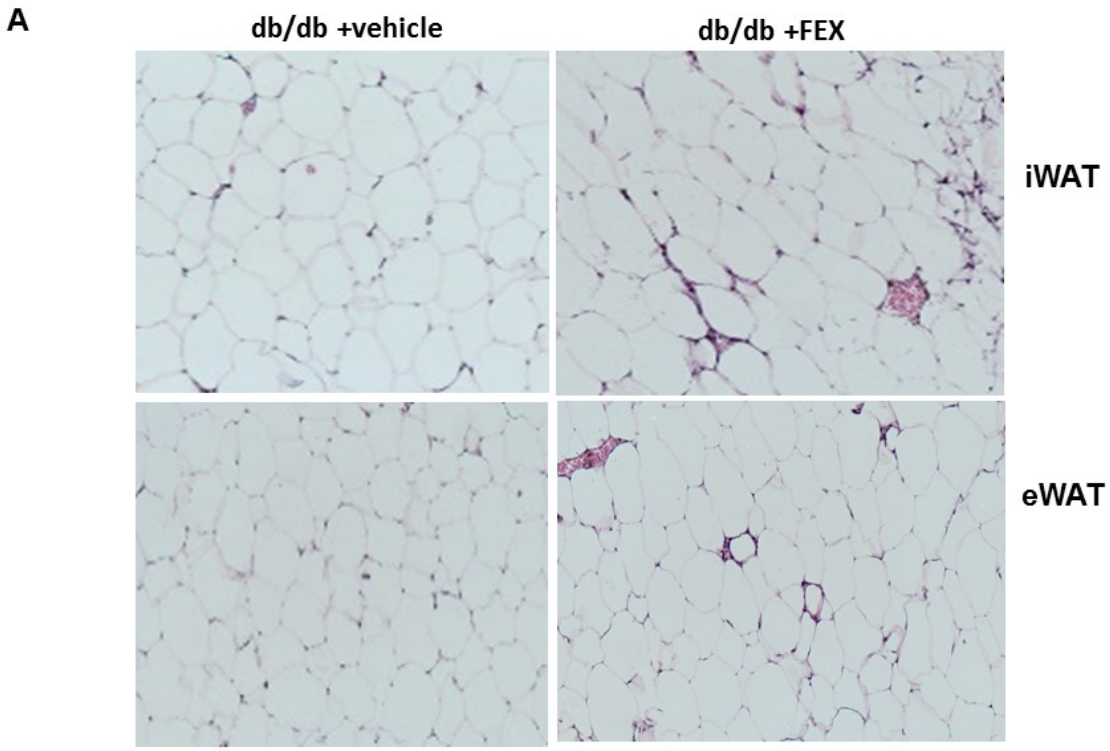


Fig. S4. Antibiotics and FEX treatment did not alter antibiotic water intake, serum AST or ALT levels, or body weight. Wild-type mice were treated with antibiotics for 30 days. Mice were gavaged with vehicle or FEX (50mg/Kg, n=10) on day 23, for 7 days. (A) Serum AST and ALT levels. (B) Body weight during antibiotic water treatment. (C) Antibiotic water intake.



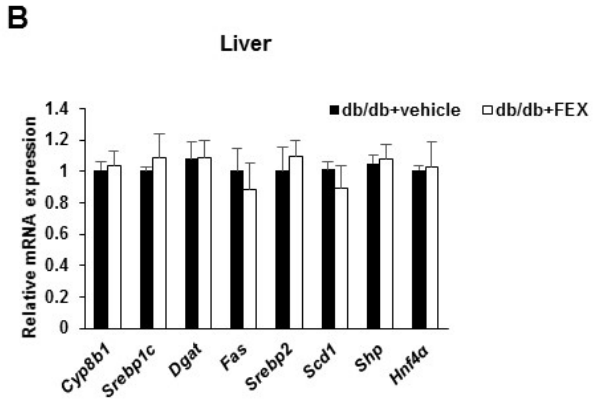


Fig. S5. Fexaramine induce white adipose tissue browning in db/db mice. Obese *db/db* mice were treated with FEX (50 mg/Kg, n=8) or vehicle (n=5) as described under Methods. (A) H&E staining of epididymal (eWAT) and inguinal fat (iWAT). (B) Liver lipogenesis gene mRNA expression.

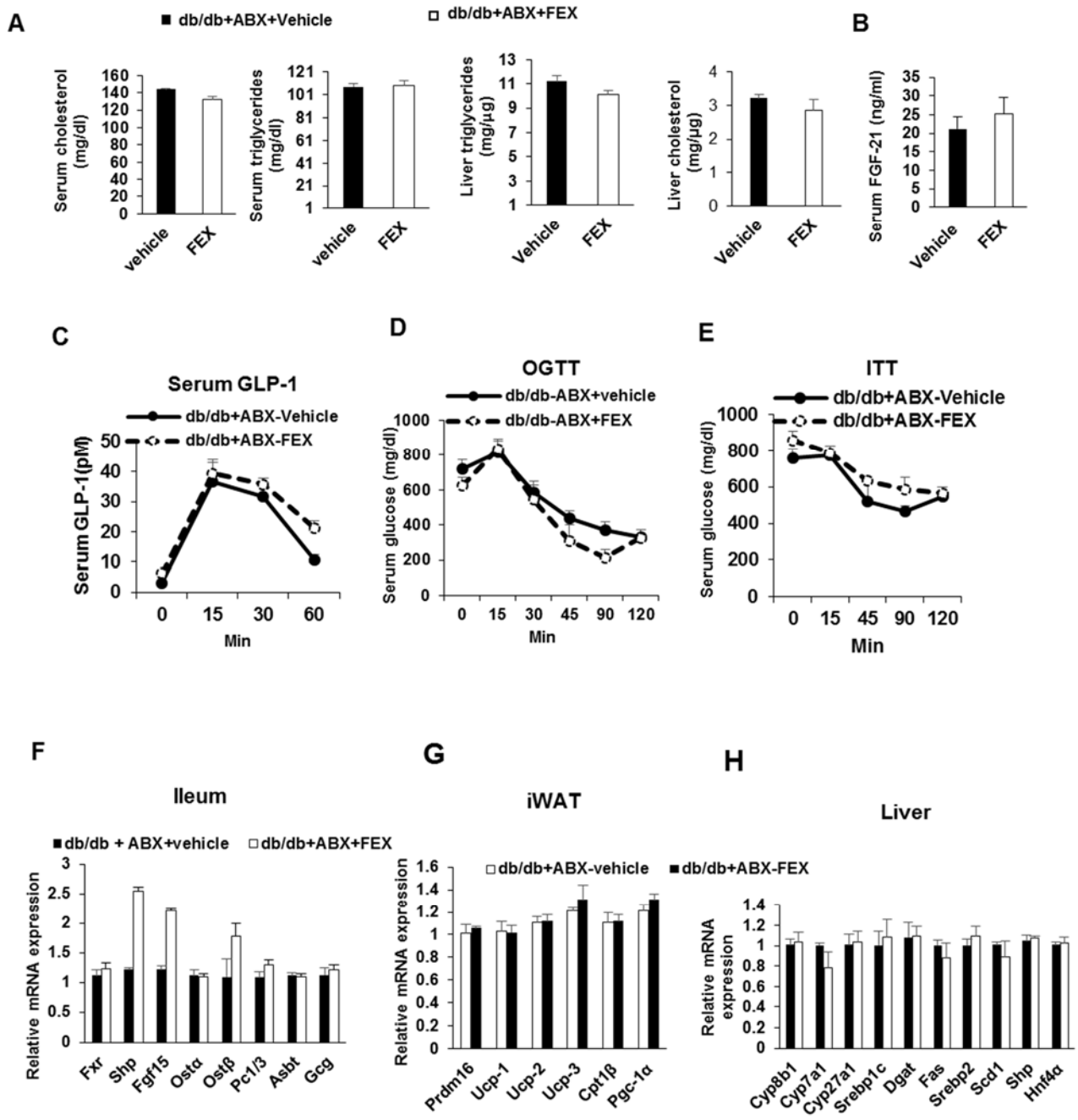


Fig. S6. Fexaramine did not induce white adipose tissue browning in antibiotic-treated *db/db* mice. Obese *db/db* mice were treated with antibiotics for 30 days. On day 20 mice were treated with FEX (50 mg/Kg, n=5) or vehicle (n=5) as described under Methods. (A) liver and serum lipid profiles; (B) serum FGF-21 levels; (C) GLP-1 secretion assay; (D) oral glucose tolerance test; (E) insulin tolerance test; (F) ileum FXR target gene mRNA expression; (G) inguinal WAT (iWAT)

browning factor mRNA expression; (H) liver mRNA expression. Student's *t*-test analysis showed no significant difference between FEX treated vs. vehicle control ($p>0.05$).

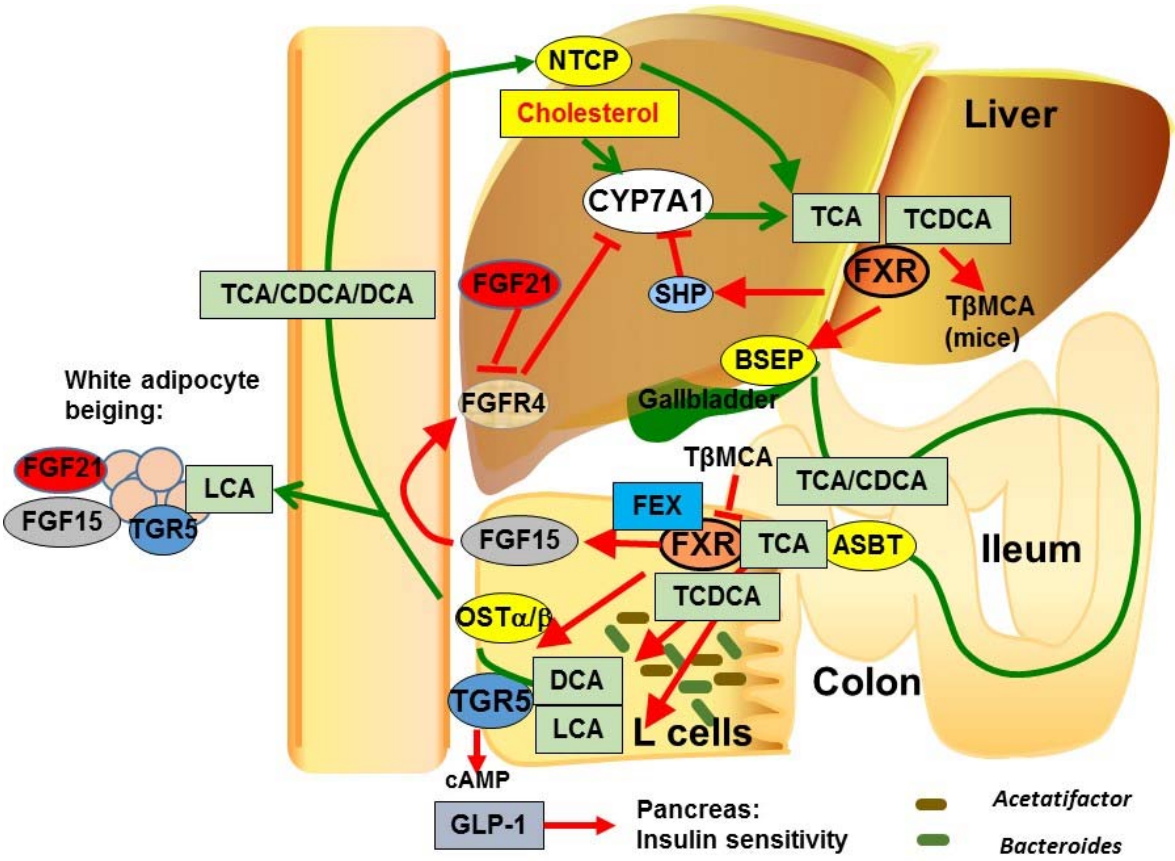


Fig. S7. Mechanism of FEX activation of FXR and TGR5 signaling in hepatic metabolism. In the intestinal L cells, FXR and TGR5 are co-expressed. FEX activates FXR to induce FGF15 and TGR5 expression, and shapes the gut microbiota to induce *Acetatifactor* and *Bacteroides*, which have high bile salt hydrolase and 7α/β-dehydrogenase activities to convert CDCA and UDCA to LCA. LCA activates TGR5 to stimulate GLP-1 secretion from L cells. GLP-1 stimulates insulin secretion from pancreatic β cells to improve insulin sensitivity. LCA also activates TGR5-cAMP signaling to induce browning factors, leading to beiging of white adipose tissues. FGF15 and FGF21 also promote WAT beiging. Free fatty acids released from adipose tissues activate PPARγ in adipose tissues and PPARα in hepatocytes to induce FGF21, which induces PGC-1α to stimulate energy metabolism and enhance insulin sensitivity in diabetes. FEX does not induced FXR/SHP pathway. FGF21 antagonizes FGF15/FGFR4 signaling and results in no effect on CYP7A1 expression and in bile acid synthesis.

References:

1. Li T, Chiang JY. A novel role of transforming growth factor beta1 in transcriptional repression of human cholesterol 7alpha-hydroxylase gene. Gastroenterology 2007;133:1660-1669.