Supporting Information

Adipocyte-Specific Over-Expression of Retinol-Binding Protein 4 (RBP4) Causes Hepatic Steatosis in Mice

Seung-Ah Lee[1], Jason J. Yuen[1], Hongfeng Jiang[1], Barbara B. Kahn[2] and William S. Blaner[1]

[1]Department of Medicine, College of Physicians and Surgeons, Columbia University, 650 W. 168th Street, New York, NY 10032 and [2]Division of Endocrinology, Diabetes, and Metabolism, Department of Medicine, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA 02215

EXPERIMENTAL PROCEDURES

***Targeting Construct and Generation of Adipocyte-specific hRBP4 (adi-hRBP4) Transgenic Mice*.** We employed a ROSA26 targeting vector (STOP-eGFP-ROSA26TV) obtained from Addgene (Cambridge, MA) to generate adipocyte-specific hRBP4 transgenic mice. A full-length cDNA encoding hRBP4 was cloned into a modified version of pROSA26-1 as described by others (1). The hRBP4 cDNA was preceded by a *loxP*-flanked *neo*^{r}-stop cassette, followed by an internal ribosomal entry site-enhanced green fluorescent protein (EGFP) cassette flanked with a *Flp* recombinase-recognition target, and a polyadenylation signal. The resulting sequence was inserted into the ubiquitously expressed ROSA26 locus (**Figure 1A**). The targeting vector was electroporated into ES cells originally derived from 129X1/SvJ mice and G418-resistant colonies were analyzed by the Transgenic Mouse Core Facility of the Columbia University Herbert Irving Comprehensive Cancer Center. Southern blot analysis identified that one hRBP4-EGFP colony out of four carried the targeted allele. The targeted ES cells were injected into C57BL/6J blastocysts and three chimeric male progeny were bred with wild type C57BL/6J females to produce heterozygous and subsequently homozygous hRBP4 knock-in mice. Germline transmission was confirmed by the Agouti coat color of the offspring and by a combination of different PCR analyses of genomic DNA obtained from mouse tail biopsies. All three chimeric mice showed germline transmission. Heterozygous siblings were interbred and the resulting F_2 and F_3 generations were analyzed by PCR. To generate adipocyte-specific hRBP4 (adi-hRBP4) transgenic mice, the hRBP4 knock-in mice were bred with *adiponectin-Cre* mice (2). This deleted the *loxP*-flanked *neo*^{r}-stop cassette specifically

in adipocytes. Littermates lacking the *adiponectin-Cre* transgene were used as controls in our studies. All experiments were approved by the Columbia University Institutional Animal Care and Use Committee, in accordance with the National Research Council's Guide for the Care and Use of Laboratory Animals (3).

Diet Studies. After weaning, mice were maintained on a standard chow diet (Purina Animal Nutrition LLC, Gray Summit, MO) providing 15 IU vitamin A per g diet. For studies exploring the effects of a high-fat diet on adi-hRBP4 mice, the mice were fed a chow diet until they reached 7-8 weeks of age when they were switched to a high-fat diet providing 60% of calories as fat (Research Diets, #D12492, New Brunswick, NJ) and 4 IU vitamin A per g diet. Mice were maintained on a 12-h light/dark cycle and had *ad lib* access to both diet and water. Body weights were measured twice a week.

Metabolic Measurements. For glucose tolerance tests, mice were fasted for 5 to 6 h and subsequently given by intraperitoneal (IP) injection a glucose challenge (1-2 g glucose/kg of body weight). Blood glucose levels were measured by glucometer at intervals up to 2 h after injection. Body composition was analyzed by the Mouse Phenotyping Core at the Columbia University Medical Center using the Minispec mq10 NMR analyzer (Brucker Optics, Woodlands, TX).

Biochemical analyses. Commercial kits were used to assess plasma triglycerides (TG) (Thermo Fisher Scientific, Middleton, VA), plasma free fatty acids (FFA) (Wako Diagnostics, Richmond, VA), plasma total cholesterol (TC) (Genzyme Diagnostics, Cambridge, MA), plasma leptin (EMD Millipore Corporation, Darmstadt, Germany), plasma alanine transaminase (ALT) (Genzyme diagnostics, Cambridge, MA) and plasma and tissue tumor necrosis factor-α (TNFα) (BD Biosciences, San Jose, CA).

Hepatic TG, FFA, and TC levels were measured using the same kits listed above. For these analyses, total hepatic lipids were first extracted from 10% (w/v) tissue homogenates as described by others (4). The N_2-dried extracts were resuspended in 2% Triton X-100 prepared in H_2O and aliquots were taken for lipid measures.

HPLC Analysis of Retinol and Retinyl Esters. Plasma and tissue total retinol (retinol + retinyl esters) levels were analyzed by HPLC according to our standard protocol (5). Briefly, after protein denaturation in 100% ethanol containing a known amount of internal standard, total retinol was extracted from plasma or tissue homogenates into hexane. After evaporation of the hexane, the lipid residue including the retinol and retinyl esters was dissolved in benzene and separated on a Symmetry C18 column (Waters, Milford, MA, USA). For quantification of extracted retinoids, the areas under the absorbance peaks at 325 nm were measured and adjusted for recovery of known amounts of internal standard (retinyl acetate, Sigma-Aldrich, St. Louis, MO).

Liquid Chromatography Tandem Mass Spectrometry (LC/MS/MS) Analyses of All-trans-Retinoic Acid And Neutral Lipids. Tissue levels of all-*trans*-retinoic acid and the acyl-composition of tissue and plasma FFAs were determined by LC/MS/MS using a Waters Xevo TQ MS ACQUITY UPLC system (Waters, Milford, MA). For analysis of all-*trans*-retinoic acid levels, tissue homogenates were extracted using the two-step acid-base extraction described by Kane et al. (6). Initially, 0.4 mL of 0.025 M KOH in methanol was added to a 5% (w/v) tissue homogenate, along with 10 ng of penta-deuterated all-*trans*-retinoic acid (ATRA-d5) (Toronto Research Chemicals, Ontario, Canada) dissolved in absolute ethanol to serve as an internal standard. The aqueous phase containing non-polar lipids was then extracted with hexane. The

remaining aqueous phase was acidified with 30 µL of 4M HCl and polar lipids including all-*trans*-retinoic acid were extracted with hexane. After phase separation, the organic phase was removed and dried under N_2. The all-*trans*-retinoic acid-containing extract was resuspended in acetonitrile for LC/MS/MS analysis. All-*trans*-retinoic acid concentrations were determined as our laboratory has previously reported (7). Acyl-compositions of free fatty acids (FFAs) present in the extract were determined by LC/MS/MS according to protocols we have previously described in detail (8).

Histochemistry and Measurement of Adipocyte Size. Liver sections were fixed overnight in 10% buffered formalin: one for paraffin embedding and subsequent hematoxylin and eosin (H&E) staining, and the other was transferred to a 30% sucrose solution and processed for cryosectioning and Oil Red O staining. Epididymal (visceral) adipose tissue was fixed in 10% buffered formalin, embedded in paraffin, sectioned, and stained with H&E. All histology was performed by the Pathology Core Facility at the Columbia University Medical Center and images were captured using an Olympus FSX100 microscope (Olympus, Center Valley, PA). Adipocyte surface area was quantified in H&E stained adipose tissue sections using and Olympus FSX100 microscope and software. The mean adipocyte surface area was calculated from a minimum of 100 adipocytes present in 5-8 randomly chosen fields for two mice per group.

Measurement of Hepatic de novo Lipogenesis. To determine the rate of hepatic *de novo* lipogenesis, mice were given an intraperitoneal injection of 1 mCi of $[^3H]H_2O$ (ART 0194, 50 mCi/ml, American Radiolabeled Chemicals Inc., St Louis, MO). One h after injection, blood and liver were collected for analysis. ^3H-labeled FAs were

isolated after saponification of liver homogenates in 25 µL of 2.5 M KOH at 75 ^{o}C for 4 h. After extraction of the nonsaponifiable lipids and acidification with H_2SO_4, ^{3}H-labeled FFAs were extracted into hexane, dried under N_2, redissolved in $CHCl_3$, spotted onto silica thin layer chromatography (TLC) plates (Analtech, Newark DE) developed in hexane/diethyl ether/glacial acetic acid (70:20:1), and visualized through exposure to iodine vapor. The FFA fraction was scraped from the TLC plates into a counting vial containing scintillation fluid and ^{3}H-cpm assayed in a Beckmann LS 6500 liquid scintillation counter. The specific activity of plasma water (cpm/nmol) was used to calculate the rate of FFA synthesis in terms of micromoles of [^{3}H]H_2O incorporated into the hepatic fatty acid pool per hour per gram liver tissue (µmol [^{3}H]H_2O incorporated/h/g) (9).

Fatty Acid Uptake Studies and Measurement of Ketone Bodies. Tissue uptake of FFAs was assessed as previously described (10). [^{3}H]Oleic acid (54.6 mCi/mmol, NEC317, Perkin Elmer, Waltham, MA) was dried under a stream of N_2 and resuspended in 6% fatty acid free bovine serum albumin (BSA) (1 µCi/100 µl) in phosphate buffered saline (PBS). Each mouse received a 100 µl injection of [^{3}H]oleic acid:BSA via the femoral vein. Small amounts of blood were collected before injection and 0.5, 1, 2, and 5 min after injection. Immediately after the 5 min blood draw, mice were sacrificed and liver and visceral (epididymal) adipose tissue excised for assessment of ^{3}H-cpm by liquid scintillation counting. Tissue FFA uptake values, expressed as total tissue ^{3}H-cpm, were corrected to total plasma FFA concentrations at baseline. To measure hepatic ketone body formation, liver homogenates were precipitated with 100% trichloroacetic acid (TCA) such that the final mixture was 20%

with regards to TCA. This solution was vortexed, incubated on ice, and spun at 13,000 g in an Eppendorf desktop centrifuge at 4 ^{o}C. The soluble fraction was collected and taken for ^{3}H-cpm measurement.

Hepatic VLDL Production. Mice were fasted for 4-5 h and then injected intraperitoneally with the total lipase inhibitor P-407, at 1 mg/g body weight (5, 7). Retro-orbital blood samples were taken at immediately prior to injection (0 h) and after injection (2 and 4 h) for plasma TG measurement as described above.

Quantitative PCR and Western blotting. Total RNA was extracted from liver and visceral (epididymal) adipose tissue for cDNA synthesis and quantitative PCR (qPCR) as previously described (11). Primer sequences are available upon request. Changes in mRNA expression levels for selected genes were confirmed at the protein level by Western blotting using standard protocols. In brief, 30-50 µg of whole liver and visceral adipose tissue proteins were analyzed from each animal. Primary antibodies raised against homogeneously purified rat plasma RBP4 (1:3000), EGFP (1:1000; Cell Signaling Biotechnology, Danvers, MA), FAS (1:1,000; BD Biosciences, San Jose, CA), SREBP-1 (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), GAPDH (1:1000; Cell Signaling Biotechnology, Danvers, MA) and β-actin (1:5,000; Sigma-Aldrich, St. Louis, MO) were used. Densitometric analysis of protein expression levels was performed using ImageJ (National Institutes of Health), with β-actin serving as the loading control.

Statistical Analysis. Data are given as means ± SEM if not specified. Statistical significance ($P < 0.05$) was determined either by unpaired Student *t*-test or by a one-way ANOVA followed by Tukey's post-hoc analysis.

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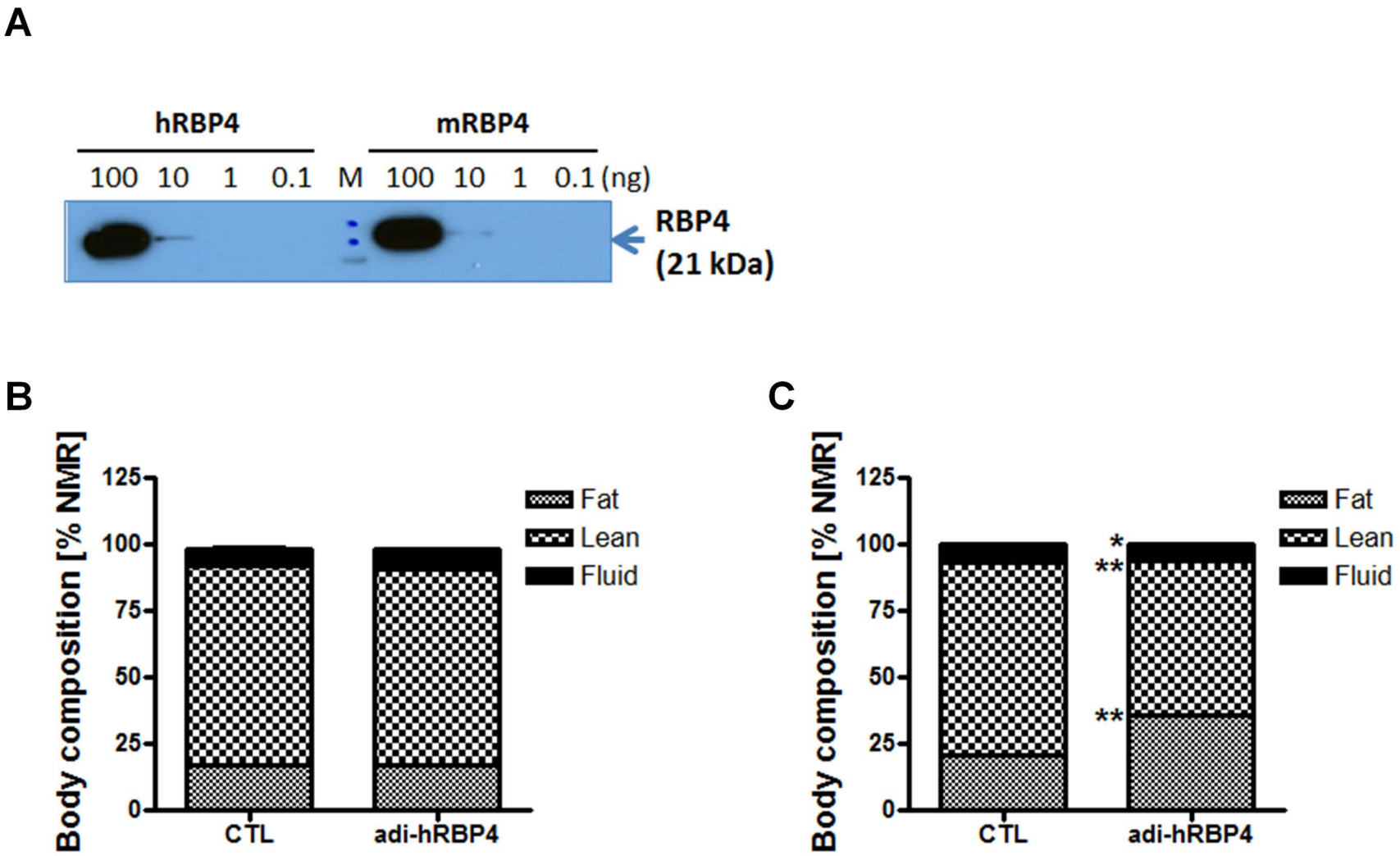
LEGEND TO SUPPLEMENTARY FIGURE

Supplementary Figure 1. Characterization of adi-hRBP4 mice fed a chow diet. (A) Equal specificity of anti-RBP4 antibody to known amounts of purified recombinant human (hRBP4) or mouse (mRBP4) RBP4. Body compositions of matched male adi-hRBP4 and littermate control (CTL) mice fed either a chow diet (B) or a high-fat diet (C) as determined by NMR analyses. Fat, lean, and fluid mass are normalized to body weight; n = 8 for each genotype.

Supplementary Figure 2. Characterization of adi-hRBP4 mice fed a high-fat diet for 24 days. (A) Glucose clearance curves for fasting adi-hRBP4 and matched control (CTL) mice maintained on the high-fat diet. Mice were given an intraperitoneal dose of glucose (2 g/kg body weight). (B) Area under the curve (AUC) values for the glucose clearance curves reported in Panel A. (C) Immunoblot showing SREBP-1 protein expression levels in liver homogenates prepared from adi-hRBP4 and control (CLT) mice maintained on the high-fat diet. (D) Serum triglyceride levels of fasted adi-hRBP4 and matched control (CTL) mice that had been given an intraperitoneal dose of P-407 at the time of the first blood draw (t = 0 hours) to block lipolysis of circulating hepatically-derived triglyceride. (E) Ketone body levels in livers from adi-hRBP4 and matched control (CTL) mice.

Supplementary Figure 1.



Supplementary Figure 2.