Disruption of the Acyl-CoA-binding Protein Gene Delays Hepatic Adaptation to Metabolic Changes at Weaning*

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Ditte Neess‡1, Maria Bloksgaard‡1, Signe Bek‡1, Ann-Britt Marcher‡1, Ida C. Elle‡1, Torben Helledie‡2, Marianne Due‡1, Vasileios Pagmantidis‡3, Bente Finsen‡1, Johannes Wilbertz‡1, Mogens Kruhøffer‡1, Nils Færgevand‡1, and Susanne Mandrup‡4

From the 1Department of Biochemistry and Molecular Biology and the 9Institute of Molecular Medicine, University of Southern Denmark, 5230 Odense M, Denmark, the 2Karolinska Institute, Karolinska Center for Transgene Technologies, 171 77 Stockholm, Sweden, and 3AROS Applied Biotechnology A/S, 8200 Aarhus N, Denmark

The acyl-CoA-binding protein (ACBP)/diazepam binding inhibitor is an intracellular protein that binds C14–C22 acyl-CoA esters and is thought to act as an acyl-CoA transporter. In vitro analyses have indicated that ACBP can transport acyl-CoA esters between different enzymatic systems; however, little is known about the in vivo function in mammalian cells. We have generated mice with targeted disruption of ACBP (ACBP−/−). These mice are viable and fertile and develop normally. However, around weaning, the ACBP−/− mice go through a crisis with overall weakness and a slightly decreased growth rate. Using microarray analysis, we show that the liver through a crisis with overall weakness and a slightly decreased growth rate. Using microarray analysis, we show that the liver

In conclusion, lack of ACBP interferes with the normal metabolic adaptation to weaning and leads to delayed induction of the lipogenic gene program in the liver.

The mouse acyl-CoA-binding protein (ACBP)5/diazepam binding inhibitor is a 10-kDa intracellular protein consisting of 86 amino acids. It is highly conserved throughout evolution and expressed in all cell types in the eukaryotes investigated (1, 2). This, together with the characteristics of the ACBP promoter (3, 4), implies a housekeeping function of the gene. However, expression levels vary markedly between tissues (5) and in response to different metabolic stimuli (6–9), thereby indicating that ACBP might perform more specialized functions in some cell types. The ACBP protein binds C14–C22 acyl-CoA esters with high affinity and specificity (10, 11) and has very little or no affinity toward other ligands (11–13). From in vitro studies, ACBP is known to protect acyl-CoA esters from hydrolysis (14–16) and to relieve acyl-CoA inhibition of a number of enzymes, including long chain acyl-CoA synthetase, acetyl-CoA carboxylase (ACC), adenine nucleotide translocase, fatty acid synthetase (FAS), carnitine palmitoyltransferase, and acyl-CoA:cholesterol acyltransferase (9, 16–18). In addition, ACBP is known to donate acyl-CoA esters to phospholipid, glycerolipid, and cholesteryl ester (CE) synthesis (14, 18–21). Finally, proteolytic products of secreted ACBP have been shown to have signaling functions in Dictostelium as well as mammalian cells (22). Targeted disruption of the yeast ACBP gene (ACBI) revealed that ACBP deficiency results in increased levels of C18:0 acyl-CoA esters and a decrease in the amount of total C26:0 fatty acids, indicating that transport of FA toward elongation is impaired by lack of ACBP. Furthermore, sphingolipid and ceramide amounts were reduced, membrane structure was altered, and vesicular transport was compromised (23–25).

The functions of ACBP in lipid metabolism have been further studied in different mammalian cell culture systems and animal models by both knockdown strategies and overexpression of the protein. It has been reported that knockdown of ACBP by small interfering RNA causes growth arrest and lethality in three different mammalian cell lines (26); however, data from our laboratory show that ACBP can be knocked down in many different cell systems without affecting growth and survival (27). Recently, knockdown of ACBP in HepG2 cells was shown to suppress the expression of a number of genes involved in lipid biosynthesis and lead to decreased levels of saturated and monounsaturated fatty acids (28). In 3T3-L1 preadipocytes, knockdown of ACBP caused a mild impairment of adipocyte differentiation and accumulation of triacylglycerol (TAG) (27), whereas overexpression of ACBP in McA-RH7777 rat hepatoma cells resulted in increased intracellular TAG accumulation (29). Overexpression of ACBP in transgenic mice resulted in accumulation of different lipid classes, including TAG in the liver (30). These results suggest

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that ACBP may play a role in TAG synthesis. Furthermore, transgenic rats overexpressing ACBP show decreased hepatic mRNA levels of peroxisome proliferator-activated receptors γ and δ and SREBP-1 (sterol regulatory element-binding protein 1) in the fed but not the fasted state (31). Recently, mice carrying the nm1054 mutation (~400-kb deletion on chromosome 1, including the Acbp sequence and five other genes) were characterized. These mice have sparse hair with a greasy appearance and sebocyte hyperplasia (32). Furthermore, lipid analyses showed that they have a decreased amount of TAG on the fur compared with control mice, whereas TAG levels in the skin and liver were similar. While this paper was in preparation, a new report was published showing that disruption of ACBP in mice causes preimplantation embryonic lethality (33). The molecular basis for these findings, which are at odds with results from our laboratory and those from the nm1054 mice, is not clear.

During the suckling-weaning transition, where pups change diet from the high fat breast milk to the standard carbohydrate-rich chow, the liver undergoes significant metabolic changes to adapt to the alterations in energy substrate (reviewed for rats in Ref. 34). Immediately after birth, mice feed exclusively on breast milk provided by the mother. Subsequently, the mice begin natural weaning, gradually increasing the intake of chow while still suckling. This natural weaning continues until the age of ~3–4 weeks, from which point the mice feed exclusively on chow. During the suckling period, the liver produces glucose and ketone bodies; however, at the suckling-weaning transition, the need for hepatic glucose production by gluconeogenesis ceases due to the increase in consumption of carbohydrate-rich chow. Coordinately, the hepatic fatty acid oxidation and ketone body production is reduced. At the suckling-weaning transition, where the high fat breast milk diet is substituted with the carbohydrate-rich chow, the hepatic synthesis of fatty acids from carbohydrates increases as a consequence of the increased expression and activity of lipogenic enzymes (e.g. ACC, FAS, and ATP citrate lyase (ACLY) (reviewed for rats in Ref. 34). These inductions of lipogenic genes are thought to be mediated by an increase in the expression of the mature nuclear form of SREBP-1 (35).

The members of the SREBP family are important regulators of hepatic lipogenesis (36–38). SREBP-1c expression is activated transcriptionally by insulin and by oxysterols through activation of liver X-activated receptors, whereas SREBP-2 activity is primarily regulated by posttranslational processing (39, 40). However, the Srebp-1c and -2 genes are also autoactivated in a feed-forward regulatory loop involving sterol regulatory element sites in their promoters (41, 42). The SREBPs are synthesized as inactive precursors (pSREBP), which are bound to the SREBP cleavage-activating protein (SCAP) in the ER membrane. Retention of the pSREBP-SCAP complex in the ER membrane is determined by insulin-induced gene (Insig) proteins that reside in the ER membrane and interact with the pSREBP-SCAP complex in a steroid-dependent manner (43–46). When steroid levels are low, the pSREBP-SCAP complex translocates to the Golgi, where pSREBP is cleaved to generate the mature nuclear form (nSREBP) (47, 48). The nSREBPs then enter the nucleus, where they bind as dimers to target sites and promote transcriptional activation of a number of lipogenic and cholesterologenic genes (49–51).

We have generated mice with targeted disruption of the ACBP gene (ACBPΔ/Δ) and show here that depletion of ACBP results in a delayed adaptation of the liver to weaning due to a delayed induction of lipogenic pathways. This is caused by a compromised expression and maturation of SREBP precursors and a reduced binding of SREBP to their target gene promoters.

MATERIALS AND METHODS

Generation and Maintenance of ACBP-deficient Mice—The mouse ACBP genomic sequence was derived from a 129SV mouse genomic library (lambda FIX® II (Stratagene)). The targeting vector was generated by replacing exon 2 and parts of introns 1 and 2 with a 2-kb loxP-flanked neomycin resistance cassette under control of the PGK1 (phosphoglycerate kinase 1) promoter. The vector also contained a cassette for negative selection (PGK1 promoter-driven thymidine kinase cassette 5′ of the Acbp gene). R1 embryonic stem (ES) cells (kindly provided by Dr. Andreas Nagy, Mount Sinai Hospital, Toronto, Canada) were electroporated with linearized vector and submitted to G418 and ganciclovir selection. Clones were characterized by Southern blotting and PCR analysis. Targeted embryonic stem cells with no additional random insertions were injected into C57Bl/6N blastocysts, which were transferred to pseudopregnant foster mothers. The chimera offspring were mated with C57BL/6BomTac mice to generate ACBPΔ/Δ founders, which were backcrossed to C57BL/6BomTac wild type mice for more than 10 generations.

Mice were housed at the Biomedical Laboratory, University of Southern Denmark, under standard laboratory conditions, including a 12-h light/dark cycle and free access to water and food (altromin 1324 for maintenance, altromin 1314 for breeding and lactation). The room was controlled at ~55% relative humidity at 22 ± 3°C. Breeding of transgenic mice and animal experiments were approved by the Danish Animal Experiment Inspectorate.

Animals—Mice used for the growth curve experiment were weighed every other day in the period 3–35 days of age and every fourth day thereafter. Besides the normal chow diet supplied in the cage lid throughout the period, mice were provided a soaked chow diet and water in the bottom of the cage from day 14 to 35. The mice were housed along with their mother and littermates until 21 days of age. At 21 days of age, pups were removed from their mother and housed with littersmates until day 35. Mice were divided by gender, and pups from different litters were mixed in order to have groups of mice consisting of 4–7 individuals/cage. The pups sacrificed at day 21 or earlier were removed from their mother immediately before euthanasia. Mice at all ages and for all experiments were killed between 8 and 11 a.m. unless otherwise stated.

For determination of gene expression by DNA microarray and single gene real-time PCR, protein analyses by Western blotting and ChIP, the mice were euthanized by cervical dislo-

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7 S. Mandrup, unpublished data.
cation, and tissues were dissected. All tissue used for RNA and protein extracts were immediately frozen in liquid nitrogen.

**Plasma Insulin**—Fed state 21-day-old mice were anesthetized in the morning (8–11 a.m.) by 25% hypnorm, 25% dromicium in sterile water (0.01 ml/g mouse), and blood was sampled by heart puncture into EDTA-coated tubes. Plasma was separated by centrifugation and analyzed by a mouse insulin ELISA kit (10-1149, Mercodia) according to the manufacturer’s instructions by use of Victor2 multilabel counter (Wallac) ELISA plate reader.

**Immunohistochemistry**—Paraffin sections were mounted on SuperFrostPlus® microscopic slides and dried (60 °C, 60 min), paraffin was removed with xylene, and sections were rehydrated with ethanol and H$_2$O. Endogenous peroxidase activity was quenched by immersion in 1.5% H$_2$O$_2$ in 50 mM Tris-HCl, pH 7.4, containing 0.5 mM EGTA (1000 W until boiling and 440 W for 15 min). Slides were rinsed in tap water for 5 min and washed twice for 2 min in TBS containing 0.05% Tween 20 (TNT). Prior to incubation with antibody, sections were incubated with 10% FCS in TBS (30 min at room temperature). For detection of ACBP, sections were incubated with polyclonal rabbit anti-Rat ACBP antibody (442, SN11/7-95 no. 27; kindly provided by Dr. Karsten Kristiansen), diluted 1:500 in 10% FCS in TBS. Subsequently, sections were washed in TNT buffer and incubated for 30 min with secondary antibody Rabbit EnVision + System-HRP-labeled polymer from DakoCytomation (Dako K4003). After rinsing with TNT buffer, sections were incubated in diaminobenzidine solution (0.5 mg/ml diaminobenzidine; 0.015% hydrogen peroxide in TBS) for 10 min and H$_2$O for 5 min. Stained sections were dehydrated and mounted with AquaTex (Merck Eurolab) coverslips. In some sections, nuclei were stained with hematoxylin.

**Protein Extraction, Western Blotting, and ECL Detection**—For whole cell mouse liver extracts, frozen tissue was homogenized in SDS lysis buffer (2.3% SDS, 50 mM Tris-HCl, pH 6.8, 10% glycerol, 10 mM DTT, 10 mM β-glycerophosphate, 10 mM sodium fluoride, 0.1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), and 1× complete protease inhibitor (Roche Applied Science), boiled, and treated with benzonase (Merck). Livers used for nuclear extracts were harvested at 6 a.m. Nuclear extracts were prepared from three livers from 21-day-old mice. The livers were washed and homogenized in PBS containing 1 μg/ml leupeptin, 1.4 μg/ml pepstatin, 0.2 mg/ml PMSF, 1 mM EDTA, and 1 mM EGTA. Cells were centrifuged, and nuclei were released by Dounce homogenization in cell lysis buffer (5 mM PIPES, 85 mM KCl, 0.5% Nonidet P-40, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 0.2 mg/ml PMSF, 50 μg/ml ALLN). Nuclei were resuspended in 6 ml of homogenization buffer (10 mM HEPES, pH 7.6, 25 mM KCl, 1 mM EDTA, 1 mM EGTA, 2 mM sucrose, 10% glycerol, 0.15 mM spermine, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 0.2 mg/ml PMSF, 50 μg/ml ALLN), centrifuged through 10 ml of homogenization buffer (120,000 × g, 4 °C, 1 h), and resuspended in nuclear lysis buffer (50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 1% SDS, 10 μg/ml leupeptin, 5 μg/ml pepstatin, 0.2 mg/ml PMSF, 50 μg/ml ALLN). Protein concentration was determined using the RCDC protein assay kit (Bio-Rad). Equal amounts of proteins from individual mice were pooled according to the different groups. For Western blotting of nuclear extracts, liver from fasted/refed and lovastatin/Zetia-fed mice were used for SREBP-1- and SREBP-2-positive controls, respectively.

Proteins were separated by SDS-PAGE (20 mA/gel) and blotted onto a polyvinyl difluoride membrane (GE Healthcare) by wet blotting (400 mA, 1 h). Subsequently, the membranes were blocked in 5% nonfat milk in PBS, pH 8.9, 0.1% Tween 20 or in TBS, pH 7.6, 0.1% Tween 20 (Sigma). The membranes were incubated with primary antibody in 2–5% nonfat milk in PBS or TBS, 0.1% Tween 20 (room temperature, 1 h to overnight) and incubated with secondary antibody in 2–5% nonfat milk in PBS or TBS (room temperature, 1 h). The membranes were washed in PBS or TBS before ECL detection (Amersham Biosciences). The light emitted by the oxidized luminal was detected by Kodak X-OMAT UV film. Equal loading was confirmed by Amido Black staining of the membrane and ECL detection of transcription factor IIB (TFIIB).

Primary antibodies used were rabbit antiserum against mouse ACBP (1:1500), kindly provided by Dr. Jens Knudsen, rabbit anti-human TFIIB C-18 (1:1000) (sc-225, Santa Cruz Biotechnology, Inc. (Santa Cruz, CA)), FAS (1:1000) (H-300, Santa Cruz Biotechnology, Inc.), HMGCR (1:1000) (C-18 sc-27578, Santa Cruz Biotechnology, Inc.), and SREBP-1 and -2 (1:1000), kindly provided by Dr. Timothy F. Osborne. Secondary antibody used was horseradish peroxidase-conjugated swine anti-rabbit IgG (1:1000) (Dako).

**RNA Extraction, cDNA Synthesis, and Real-time PCR**—Frozen liver tissue was homogenized in 1 ml of TRIzol reagent (Invitrogen) and RNA was isolated according to the manufacturer’s instructions. RNA concentration and quality was evaluated by use of the NanoDrop™ 1000 spectrophotometer and by gel electrophoresis. RNA was treated with DNase I (Invitrogen), and cDNA was synthesized as described previously (52). The resulting cDNA was diluted in 300 μl of H$_2$O. Real-time PCR was carried out using SYBR Green Jumpstart Taq Ready Mix (Sigma). All real-time PCRs were run on MX3000P (Stratagene) at the following conditions: 2 min at 96 °C (hot start), 40 cycles of 10 s at 96 °C, 15 s at 60 °C, and 15 s at 72 °C.

Primers used were as follows: *Acbp* forward, 5′-TTTCGG-CATCCGTATCACC; *Acbp* reverse, 5′-TTTGTCAAAATC-AGCCGTGAGACA; *Accs2* forward, 5′-GCTTTCTTCCATCTTCCGTT; *Accs2* reverse, 5′-CCCCGACTTACATCGA-TG; *Fasn* forward, 5′-ATTTGGTGTGTTGGAATGTC; *Fasn* reverse, 5′-CCGAGCCCTCCATCCTCCTCCT; *Elovl6* forward, 5′-TCAGAAAGACCCCGAACTAG; *Elovl6* reverse, 5′-CTGTTTCTCTCAAGAATGTAATCCTG; *Hmgcr* forward, 5′-ACGCTTTGGAATGTCCT; *Hmgcr* reverse, 5′-GGACGCGCTGACATGGTG; *Sqle* forward, 5′-TGAC-AAACGAGGCGTCCT; *Sqle* reverse, 5′-GGTGCCCTACGG- TTATGCAT; *Lss* forward, 5′-TTATGGTGTTGCAGG- CTCC; *Lss* reverse, 5′-ATGTACAGGTAATCAGAGA-
CCTG; Srebp-1c forward, 5’-GGAGCCATGGATTGACATTTG; Srebp-1c reverse, 5’-CAAATAGGCCAGGAACTC; Srebp-2 forward, 5’-TGAAGCTGCGCAATCAGAA; Srebp-2 reverse, 5’-AGTCCTGGAGCACTGATGTTG; TfIIb forward, 5’-GGTTCTGCTCACCCTTGCTCT; TfIIb reverse, 5’-TGTGTAGCTGCCATCTGACTT.

DNA Microarray—The DNA expression microarray analysis was performed on the Mouse Genome 430_2.0 Array GeneChip® (Affymetrix). Complementary cDNA was synthesized from RNA and made double-stranded. An oligo(dT) primer, which contains a T7 RNA polymerase site, was used for cDNA synthesis. The cRNA was labeled with biotin and fragmented to 100–120 base fragments, which were hybridized to the array chip. Steptavidin-phycocerythrin and antistreptavidin-phycocerythrin were added, and signals were detected by a laser scanner. Data were processed using standard Affymetrix software MASS. The full array data set is available in the Gene Expression Omnibus database with accession number GSE24451.

Chromatin Immunoprecipitation—50 mg of frozen liver tissue was homogenized in 1.5 ml of PBS and cross-linked for 10 min in 1% formaldehyde (with agitation at room temperature). Glycine was added to a final concentration of 0.125 M, 10 min. Cells were precipitated by centrifugation, washed twice in cold PBS, resuspended in 300 μl of ChIP lysis buffer (0.1% SDS, 1% Triton X-100, 0.15 M NaCl, 1 mM EDTA, 20 mM Tris, pH 8) and incubated for 10 min at room temperature. Samples were sonicated 12 times for 30 s at maximum setting (BioruptorTM, Diagenode) and centrifuged (10,000×g, 4 °C, 1 min). 200 μl of ChIP lysis buffer was added to the supernatant, and 100 μl of 50% for this was used for one ChIP reaction. ChIP lysis buffer (300 μl) and 2 μl of SREBP-2 antisera (kindly provided by Dr. Timothy F. Osborne) were added to the 100 μl of chromatin, and samples were incubated for 3 h at 4 °C with rotation. Protein A beads (Amersham Biosciences) (20 μl/reaction) were washed three times in ChIP lysis buffer and incubated with 1 μg of BSA in a total volume of 100 μl for 2 h (4 °C, with rotation). Prepared beads (100 μl) were added to the chromatin and incubated overnight at 4 °C on a rotor. Beads were washed, and DNA-protein complexes were diluted and decross-linked as described previously (53). DNA was purified by phenol/chloroform extraction and redissolved in 200 μl of H2O. Real-time PCR was run as described above. Primers used were as follows: BG1 forward, 5’-TGGTCGCTCAGGAGGCTTG; BG1 reverse, 5’-ATCCAGAAGTGGGACACAGCT; BG2 forward, 5’-GGGTCTCTTCTCTCTTACCCGAT; BG2 reverse, 5’-ACCTTGGCTGGGCATGGAAC; Ascs2 forward, 5’-GGAGTGTCAACCCAGAGAA; Ascs2 reverse, 5’-TGGATGTCGACTGAGGAGGAC; Acly forward, 5’-GGTTCCCTCTCATCCTTGCAG; Acly reverse, 5’-GGTCGCTTCAGGAGCAGAGGAC; Hmgcr forward, 5’-GGCTCGGAGACCAATAGGA; Hmgcr reverse, 5’-CGAAGGCTTCTCCTAAACAC.

De Novo Lipid Synthesis and Total Lipid Quantification—For determining de novo lipid synthesis, 21-day-old ACBP+/+ (n = 7) and ACBP−/− (n = 8) pups were injected i.p. with 5 μCi of [14C]acetate (1-[14C]acetic acid, sodium salt (Amersham Biosciences)) in 0.9% saline and sacrificed 1 h later by cervical dislocation. Tissue was frozen in liquid nitrogen and stored at −80 °C. Total lipids were extracted from 100 mg of liver tissue by the standard Bligh and Dyer method (54) and dissolved in butylated hydroxytoluene (50 mg/liter) in chloroform/methanol (1:1).

Equal amounts of lipid extracts, including standards, were sprayed onto silica plates (Merck High Phase Thin-Layer Chromatography) and left to air-dry for 15 min. Plates were developed in a horizontal development chamber with a solvent mixture of hexane/diethyl ether/acetic acid (v/v/v) (80:30:1) for separation of natural lipids and dried. For visualization of 14C-labeled lipids, plates were placed on an X-sensitive PhosphorImager screen for 2 weeks, visualized by a Typhoon Trio scanner (Amersham Biosciences), and quantified by Quantity One 1-D Analysis Software 4.6.7 (Bio-Rad). For visualization of total lipids, plates were sprayed with 10% CuSO4 (w/v) in 8% H3PO4, dissolved in H2O, dried, and burned at 180 °C. Total CE were determined by development with FeCl3·6H2O (0.5 mg/ml) in 5% H2SO4 and 5% CH3COOH in H2O, burned at 110 °C, and quantified as described above.

Quantitation of Triacylglycerol and Cholesterol—Total lipids extracted by the Bligh and Dyer method (54) from −2.5 mg of liver tissue of ACBP−/− and ACBP+/+ (n = 8 in each group) mice were dried and resuspended in 15 μl of LPL buffer (28.8 mM Pipes, 57.4 mM MgCl2·6H2O, 0.57 mg/ml fatty acid-free BSA; dissolved in 0.1% SDS). Samples were sonicated for 30 s, and tubes were left overnight in an orbital shaker at room temperature, protected from light. The next day, the samples were sonicated three times for 30 s each. TAG content from liver tissue was determined using the Tri- glyceride Assay kit (Zen Bio) according to the manufacturer’s instructions. The amount of TAG in plasma from 21-day-old ACBP−/− (n = 9) and ACBP+/+ (n = 10) mice was determined using the Serum Triglyceride Assay kit (Zen Bio) according to the manufacturer’s instructions. Absorbance was measured at A540 (FLUOstar Omega, BGM LABTECH).

Total lipids extracted by the Bligh and Dyer method (54) from −2.5 mg of liver tissue of ACBP−/− (n = 20) and ACBP+/+ (n = 22) mice were dried, resuspended in 57 μl of 1× reaction buffer, and sonicated for 30 s in a water bath sonicator. Free hepatic cholesterol was determined by use of the Amplex Red Cholesterol Assay kit (Invitrogen) according to the manufacturer’s instructions except that no cholesterol esterase was used. Total plasma cholesterol was determined by 21-day-old ACBP−/− (n = 10) and ACBP+/+ (n = 11) mice using the Amplex Red Cholesterol Assay kit (Invitrogen). Quantification was based on a number of standards, which were treated as samples.

Fatty Acid Profile—Liver fatty acid composition was analyzed by gas chromatography for five ACBP−/− and seven ACBP+/+ mice at day 21. Tissue (−25 mg) was homogenized, and C17:0 and C25:0 fatty acids were added to samples as internal standards. Butylated hydroxytoluene (15 μg, in chloroform/methanol (1:2)) was added to prevent oxidation. Lipids were extracted according to the Bligh and Dyer method (54) and dried, and another 15 μg of butylated hydroxytoluene was added. Samples were briefly ven-
tilated with N₂ and stored at −20 °C. Total fatty acids were methylated by incubation in oil bath (5 h, 75 °C) in 1:2 3 N HCl:methanol (anhydrous). After incubation, 2 ml of hexane was added, and the hexane phase containing the methylated fatty acids was transferred to fresh tubes and dried using N₂. Prior to analysis, methylated fatty acids were resuspended in 50 µl of hexane, and 10 µl was injected on a Chrompack CP 9002 equipped with a DB-WAX column (Agilent Technologies). The methylated fatty acids (C₁₄–C₂₄) were identified by comparison with standards (Larodan Fine Chemicals).

**RESULTS**

**Targeting of the Acbp Gene in Mice**—In order to investigate the function of the ACBP protein in a physiological context, mice with targeted disruption of the gene were generated. Using a sequence replacement vector, exon 2 and part of introns 1 and 2 were replaced by a *loxP*-flanked neomycin resistance cassette under the control of the PGK-1 promoter) replacing the *Acbp* exon 2 and parts of introns 1 and 2. Homologous recombination results in the targeted *Acbp* allele (c). *Acbp* mRNA (d) and protein (e) expressions in liver tissue from ACBP*+/−*, ACBP*−/−*, and ACBP*+/−* male mice were determined. RNA data are presented as the mean of six individuals with indication of S.E. (error bars). Protein was analyzed as pools of three individuals in each group. Immunohistochemistry on liver sections is representative of three ACBP*+/−* (f–i) and ACBP*−/−* (j and k) individuals in each group. Hematoxylin was used for background/nuclear staining of sections h–k.

**Acyl-CoA Profile**—Long chain acyl-CoA esters were extracted and analyzed from 30 mg of liver tissue (wet weight) essentially as described previously (55). Heptadecanoyl-CoA (2 pmol) was used as an internal standard and added just prior to extraction. Peak detection was accomplished by utilizing a Dionex RF2000 fluorescence detector (excitation 230 nm, emission 420 nm). Peak analysis was performed using Chromeleon Client version 6.80 (Dionex). The acyl-CoA peaks were identified by comparing the retention time with known standards. Each acyl-CoA was quantified by comparing the area under the peak with that of the internal standard.
This indicates that ACBP is expressed in an allele dosage-dependent fashion from both alleles. The immunohistochemical detections revealed that ACBP is distributed evenly in the hepatocytes from ACBP+/+ liver and that ACBP is present both in the cytosol and in the nucleus as has been reported for other cell types (57–60). Importantly, ACBP staining is completely absent from the liver of ACBP+/− mice, thereby demonstrating the specificity of the antibody.

**Growth and Feeding Status of the ACBP+/− Mice—**

ACBP+/− pups were born in a normal Mendelian ratio from heterozygous intercrosses and were viable and fertile. Phenotypical differences between ACBP+/+ and ACBP+/− pups become evident around 16 days of age, when a significant fur and skin phenotype develops.8 Around weaning, the ACBP+/− mice appear physically weak and display an increased mortality rate unless provided easy access to water and soaked food in the bottom of the cage. The growth rate of the ACBP+/− pups is similar to that of ACBP+/+ pups until 19 days of age (i.e. immediately before weaning at day 21) (Fig. 2a). When the ACBP+/− pups are 21 days old, they have a slightly decreased growth rate; however, their body weight has normalized to that of ACBP+/+ littersmate by day 31 and remains similar to that of ACBP+/+ mice throughout the life span. The weights of ACBP+/− pups are not significantly different from those of ACBP+/+ littersmate at any time (data not shown). These data indicate that targeted disruption of the Acbp gene leads to a decreased ability to adapt to weaning.

During the weaning period, it is not possible to measure the food intake of the individual pups because they are feeding from breast milk as well as an increasing amount of chow diet. Furthermore, the pups are housed with littersmate, thereby making it impossible to determine the food intake of the individual mice. In order to get an indirect measure of the feeding status, we measured plasma insulin at day 21 in ACBP+/+ and ACBP+/− pups (Fig. 2b). This shows that the fed plasma insulin level was similar in ACBP+/− and ACBP+/+ littersmate at day 21, suggesting that food intake is not largely different in ACBP+/− and ACBP+/+ mice.

**Gene Expression Profile of ACBP+/− Liver—**

To compare the metabolic status of the liver in ACBP−/− and ACBP+/+ mice at weaning, hepatic gene expression was assessed by expression microarray analysis of RNA from 21-day-old mice of both genotypes. The microarray data showed that the lipogenic pathways were most significantly deregulated in the ACBP−/− mouse livers. In fact, most genes involved in cholesterologenesis and many genes encoding proteins involved in fatty acid synthesis were expressed at significantly lower levels in ACBP−/− liver compared with that of ACBP+/+ mice at day 21 (Fig. 3). These results were validated by real-time PCR using cDNA from 21-day-old ACBP−/− and ACBP+/+ livers (Fig. 4a) and data not shown for acetyl-CoA carboxylase α (Acaca), Acly, 3-hydroxy-3-methylglutaryl-coenzyme A synthase (Hmgcs), mevalonate kinase (Mvk), mevalonate (diphospho)decarboxylase (Mvd), phosphomalvalate kinase (Pmvk), NAD(P)-dependent steroid dehydrogenase-like (Nsdhl), and farnesyl diphosphate farnesyltransferase (Fdf1t1)). Interestingly, all of the lipogenic genes deregulated in ACBP−/− liver are transcriptionally regulated by members of the SREBP family of transcription factors. SREBP-1c is known to be the main regulator of genes involved in hepatic fatty acid synthesis, whereas SREBP-2 is the main regulator of the cholesterologenic genes (50, 61). Thus, these results indicate that SREBP activity may be decreased in the liver of ACBP−/− mice at the time of weaning. The deregulation of target genes is most pronounced for the SREBP-2 target genes because nearly all known SREBP-2 target genes are down-regulated, whereas only a limited number of the known SREBP-1 target genes show reduced expression.

The metabolism in the liver changes markedly during the weaning period due to the shift in diet from the high fat breast milk provided during suckling to the carbohydrate-rich standard chow diet. We therefore wanted to investigate whether this lower expression of SREBP target genes was a
ACBP Is Required for Normal Adaptation to Weaning

FIGURE 3. Disruption of ACBP leads to deregulation of SREBP target genes in the liver at day 21. RNA was isolated from ACBP$^{-/-}$ (n = 15) and ACBP$^{+/+}$ (n = 15) livers at day 21, and equal amounts of RNA from five individuals in each group were pooled. The six pools of liver RNA (three from ACBP$^{+/+}$ and three from ACBP$^{-/-}$) were processed as described in Materials and Methods. The primary goal of the study was to identify genes that are expressed in the liver according to the microarray. Genes shown in black type are all SREBP target genes, which are un-

transient state coupled to the weaning situation or whether it was a general pattern independent of age. To address this, we dissected ACBP$^{-/-}$ and ACBP$^{+/+}$ mice at different ages before, during, and after the weaning period and analyzed gene expression in the liver by single gene real-time PCR.

In keeping with previous reports (35, 62, 63), we found that mRNA expression of genes involved in fatty acid synthesis and elongation (i.e. acetyl-coenzyme A synthetase (Acas2), fatty acid synthase (Fasnl), and elongation of very long chain fatty acids protein (Elov6)) was strongly induced (14–39-fold) in ACBP$^{+/+}$ liver during weaning (Fig. 4a). Interestingly, we also observed an equally dramatic induction (8–50-fold) of cholesterogenic genes, such as 3-hydroxy-3-methylglutaryl-coenzyme A reductase (Hmgcr), squalene epoxidase (Sqle), and lanosterol synthase (Lss) (Fig. 4a). The suppression of these genes in the ACBP$^{-/-}$ liver was most prominent at day 21; however, for some genes, the lower expression level in the ACBP$^{-/-}$ compared with ACBP$^{+/+}$ livers persisted at days 23 and 28. The difference in expression levels between the different genotypes was most highly significant for the SREBP-2 target genes (Hmgcr, Sqle, and Lss). By day 35, the expression levels of all six SREBP target genes shown were similar in ACBP$^{-/-}$ and ACBP$^{+/+}$ livers. The decreased mRNA levels of SREBP target genes resulted in significantly decreased expression of the encoded proteins (e.g. expression of FAS and HMGCR proteins, which are products of typical SREBP-1 and SREBP-2 target genes, respectively, was reduced at days 19, 21, and 23) (Fig. 4b). Thus, targeted disruption of Acbp leads to significantly delayed induction of SREBP target genes in the liver at weaning, but 2 weeks following weaning, expression levels are similar between ACBP$^{-/-}$ and ACBP$^{+/+}$ mice.

To investigate whether the genotype of the mother affected the expression of SREBP target genes in the pups around weaning, we exchanged mothers of homozygote ACBP$^{+/+}$ intercrosses with mothers of homozygote ACBP$^{-/-}$ intercrosses at days 3–4. The results showed that the suppression of SREBP target genes in the ACBP$^{-/-}$ pups was independent of the foster mother (data not shown) and thus cannot be ascribed to, for example, a different composition of the milk in the ACBP$^{-/-}$ mothers.

Maturation of nSREBP Is Significantly Decreased in ACBP$^{-/-}$ Liver at Weaning—In order to investigate whether a decrease in SREBP activity is involved in the deregulation of lipogenic genes in the liver of ACBP$^{-/-}$ mice, we investigated the mRNA and protein levels of SREBP-1c and SREBP-2 in livers from ACBP$^{-/-}$ and ACBP$^{+/+}$ mice during the weaning period. The activity of the SREBP transcription factors in the liver is known to be regulated at several levels. SREBP-1c is mainly regulated at the transcriptional level by liver X-activated receptors and by autoactivation (42). These factors mediate the induction by oxysterols and insulin as well as the repression by glucagon. SREBP-1a and -2 are mainly regulated at the level of posttranslational processing via sterol or Insig-induced retention in the ER (43, 44, 64, 65). Comparison of Srebp mRNA levels during weaning showed that the level of Srebp-1c mRNA was similar between genotypes during weaning (Fig. 5a), whereas the mRNA expression of Srebp-2 was
modestly reduced in the liver from ACBP^-/- mice compared with ACBP^+/+ mice at weaning (Fig. 5b). Correspondingly, precursor SREBP-1 levels were similar between ACBP^+/+ and ACBP^-/- liver (Fig. 5c), whereas pSREBP-2 level was decreased in ACBP^-/- liver at days 19, 21, and 23 compared with ACBP^+/+ (Fig. 5d). Interestingly, for both SREBP-1 and SREBP-2, there was a very significant decrease in the amount of the mature nuclear form at 21 days of age with ACBP^-/- mice (Fig. 5a). In keeping with that, ChIP-PCR analyses showed that binding of SREBP-2 to target sites was significantly decreased in ACBP^-/- liver (Fig. 5e). The decrease in SREBP-2 activity may contribute to the decrease in Srebp-2 mRNA because the Srebp-2 gene has been shown to be autoactivated (41). Thus, these data show that there is a severely compromised activity of SREBP-1 and -2 in ACBP^-/- livers, which is likely to be the cause of the observed delayed up-regulation of SREBP target gene expression during weaning of ACBP^-/- mice.

Cholesterogenesis Is Suppressed in ACBP^-/- Liver at Weaning—The marked suppression of genes involved in fatty acid synthesis and cholesterogenesis in ACBP^-/- mice at weaning indicated that these metabolic programs may be suppressed in ACBP^-/- mice. We therefore investigated the rate of hepatic de novo TAG and cholesterol synthesis by i.p. injection of [14C]acetic acid into ACBP^-/- and ACBP^+/+ mice at day 21. In keeping with the significantly lower expression of cholesterogenic genes in the liver of ACBP^-/- mice, hepatic cholesterol synthesis was reduced by 50% in ACBP^-/- livers compared with ACBP^+/+ livers (Fig. 6a). However, hepatic de novo TAG synthesis was similar between ACBP^-/- and ACBP^+/+ mice (Fig. 6b). These results show that the delayed activation of SREBPs in the liver of ACBP^-/- mice leads to a significantly reduced de novo cholesterol synthesis compared with ACBP^+/+ livers at day 21.

Increased Amount of Hepatic CE and TAG in ACBP^-/- Mice during Weaning—The finding that de novo cholesterogenesis in the liver of ACBP^-/- mice at day 21 was decreased (Fig. 6a) prompted us to determine the steady state level of free hepatic cholesterol. Interestingly, although the hepatic de novo cholesterol synthesis was reduced, the total amount of cholesterol in the liver remained similar between ACBP^-/- and ACBP^+/+ mice during the weaning period (Fig. 7a), indicating that either there is an increased exogenous supply of cholesterol to the liver or an increased endogenous cholesterol production by, for example, turnover of CE within the liver. We investigated CE levels and found that there was an increase in hepatic CE in 19- and 21-day-old ACBP^-/- mice (Fig. 7b), whereas after weaning at days 23, 28, and 35, there was no difference between ACBP^-/- and ACBP^+/+ hepatic CE levels (Fig. 7b). Thus, the increase in CE in ACBP^-/- liver is a transient state that occurs only prior to weaning.

We also examined the levels of TAG in the liver of ACBP^-/- and ACBP^+/+ mice through the weaning period.
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![Graphs and figures related to ACBP and its effect on lipids and SREBP levels.](image)

**FIGURE 5.** SREBP-2 mRNA and protein levels are decreased in ACBP<sup>−/−</sup> liver at weaning. The mRNA expression levels of Srebp-1c (a) and -2 (b) in the liver of ACBP<sup>+/+</sup> and ACBP<sup>−/−</sup> mice were investigated by real-time PCR run on individual samples and shown as the mean with indications of S.E. (error bars). Two-way analysis of variance was used to compare ACBP<sup>−/−</sup> with ACBP<sup>+/+</sup> at the same age, and significance level was $p = 0.05$ (seven pairwise comparisons; $p = 0.0071$). * Differences between the two genotypes at the same age. The levels of pSREBP-1 (c) and -2 (d) were determined in livers of ACBP<sup>−/−</sup> and ACBP<sup>+/+</sup> mice with 10–20 individuals at each of the ages, 16, 19, 21, 23, 28, and 35 days. e and f, nuclear extracts were made from the livers of three ACBP<sup>−/−</sup> and ACBP<sup>+/+</sup> individuals at 21 days of age and analyzed for nSREBP-1 and -2 at 12 and 25 arbitrary units. g, SREBP-2 binding to well established target sites in the Acss2 and Hmgcr loci was quantified by ChIP-PCR on liver tissue from six ACBP<sup>−/−</sup> and six ACBP<sup>+/+</sup> male mice at 21 days of age. The background regions (BG1 and BG2) are regions that do not bind nSREBP-2. Unpaired t test (parametric) was used, and significance level was $p = 0.05$.

**FIGURE 6.** De novo synthesis of hepatic cholesterol is inhibited in ACBP<sup>−/−</sup> mice at day 21. Hepatic de novo synthesis of cholesterol (a) and TAG (b) was determined after i.p. injection of [1<sup>4</sup>C]acetate into 21-day-old ACBP<sup>+/+</sup> (n = 8) and ACBP<sup>−/−</sup> (n = 7) mice. One hour following injection, mice were euthanized. Lipids were extracted and analyzed as described under “Materials and Methods.” Data are shown as the mean with indication of S.E. (error bars). Unpaired t test (parametric) was used, and significance level was $p = 0.05$.

and showed that there was a 2-fold increase in the hepatic TAG level at the age of 21 days in ACBP<sup>−/−</sup> mice compared with that of ACBP<sup>+/+</sup> mice (Fig. 7c). Furthermore, there was a tendency toward a higher level of TAG in the ACBP<sup>−/−</sup> liver at days 16 and 19 (Fig. 7c); however, this difference is not statistically significant. Immediately after weaning at 23 days of age, the ACBP<sup>−/−</sup> mice had a reduction in the amount of TAG in the liver compared with ACBP<sup>+/+</sup> mice (Fig. 7c), which might reflect the decreased ability of these mice to cope with the dietary changes at weaning. From the age of 28 days, the ACBP<sup>−/−</sup> mouse livers had TAG levels comparable to those of ACBP<sup>+/+</sup> mice. Thus, the decreased hepatic TAG level is a transient state occurring immediately subsequent to weaning. In conclusion, the amount of TAG was increased in ACBP<sup>−/−</sup> liver at weaning, temporarily decreased immediately after weaning, and then normalized to ACBP<sup>+/+</sup> levels from the age of 28 days.

To investigate whether the lack of ACBP affected total fatty acid composition in the liver, we analyzed the fatty acid profile of livers from ACBP<sup>−/−</sup> (n = 5) and ACBP<sup>+/+</sup> (n = 7) mice at day 21 using gas chromatography. However, no major differences were detected in the amount of total fatty acids or in the fatty acid composition (data not shown). Ectopic expression of ACBP has been shown to increase the acyl-CoA pool size in yeast (66, 67) and in mouse and rat liver (30, 31). We therefore determined the level of acyl-CoA esters in livers from ACBP<sup>−/−</sup> and ACBP<sup>+/+</sup> mice. The total amount of acyl-CoA esters was reduced in ACBP-depleted livers (Fig. 8a), most significantly in the C16:0 and C18:2 species (Fig. 8b). These data indicate that
ACBP expression is critical for determining the intracellular acyl-CoA level. Thus, our results show that prior to weaning, ACBP/H11002 mice accumulate both TAG and CE in the liver, whereas the acyl-CoA level is reduced. However, the total amount of fatty acids and the fatty acid profile are similar in ACBP-depleted compared with wild type liver.

Plasma Cholesterol Is Increased at Day 21—The elevated amounts of hepatic CE and TAG led us to investigate cholesterol and TAG levels in plasma at day 21. We showed that total plasma cholesterol was elevated (Fig. 9a) whereas plasma TAG was similar (Fig. 9b) in ACBP/H11002 mice compared with ACBP/H11001 mice at day 21. The increase in plasma cholesterol in ACBP/H11002 mice at weaning may be caused by a decreased reverse cholesterol transport to the liver because expression of the low density lipoprotein (LDL) receptor gene, which is a well established SREBP-2 target gene (68), was decreased in ACBP/H11002 mice compared with ACBP/H11001 mice (data in microarray).

DISCUSSION
ACBP is an intracellular lipid-binding protein that binds and is thought to transport acyl-CoA esters between different enzymatic compartments. However, the precise role of this protein in mammalian physiology remains elusive. Knock-out of the Acbp gene in mice has recently been reported to be embryonically lethal (33). This contradicts our findings and the report by Lee et al. (32), which show that mice depleted of ACBP are viable. The reason for the different outcomes of the studies remains unknown; however, as suggested by Landrock et al. (33), the deletion of large parts of the Acbp proximal promoter region in their study might have interfered with the regulation of other genes in the region, thereby causing lethality. Here we report that targeted disruption of the Acbp gene in mice interferes with the ability of the liver to adapt to the metabolic changes at weaning and to induce the lipogenic and cholesterogenic gene programs. This is due to a suppression of the expression and proteolytic processing of members of the SREBP family of transcription factors.

During weaning, mice gradually change diet from the high fat breast milk provided by the mother to the high carbohy-
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drivate standard chow diet. During the first 2 weeks of their life, the mice feed exclusively on breast milk, and after weaning (in this study at day 21) they feed fully on the standard chow diet provided. Between days 14 and 21, the pups feed from both diets, gradually increasing their consumption of chow as the mother naturally weans the pups (reviewed for rats in Ref. 34). This change in nutrient supply during weaning is accompanied by a metabolic change where hepatic de novo lipogenesis is induced. We show here that mRNA expression of the lipogenic and cholesterogenic gene programs in wild type liver is induced from a low level at day 16 to an 8–50-fold higher expression at day 28, at which time the mRNA levels of these genes reach their maximum. This is consistent with previous data reporting a significant induction of lipogenic genes at weaning in rats (35) and mice (62) and for the first time further extends these findings to also include cholesterogenic genes. It is unclear why such induction of cholesterogenic genes could not be detected in rats (35); however, species variation and differences in feed composition may play a role.

We show that the mRNA expression of the lipogenic and cholesterogenic genes is induced to the same level in ACBP+/− mouse liver but that the expression reaches its maximum at day 35 (i.e. one week later than in the liver of ACBP+/+ mice). The maximal difference in gene expression between ACBP+/+ and ACBP−/− mice is observed at day 21 (i.e. prior to removal of the pups from their mother). At that day, there is a dramatic (between 1.5- and 45-fold) suppression of the entire set of genes involved in de novo cholesterol synthesis and a less dramatic (2–3.5-fold) suppression of Fasn and a few other genes in the fatty acid synthesis pathway. The common regulators of these gene programs are members of the SREBP family of transcription factors (49–51), and we show that the expression and/or proteolytic processing of SREBP-1 and -2 to their mature nuclear form is blunted in ACBP−/− mouse liver at weaning. Furthermore, there is a significant decrease in the binding of the nSREBP-2 transcription factor to its target sites in ACBP−/− liver at weaning. This supports the notion that SREBP-1 plays a central role in switching on the lipogenic pathways at weaning and indicates that SREBP-2 plays an equally important if not more important role activating the cholesterogenic pathway at weaning. Furthermore, our data indicate that the failure to induce the lipogenic and cholesterogenic pathways in ACBP−/− liver at weaning can be explained by delayed activation of SREBP expression and processing.

In keeping with the significantly lower expression of genes involved in cholesterogenesis, we observe a 50% reduction in hepatic de novo cholesterol synthesis in the liver of ACBP−/− mice compared with ACBP+/+ mice. Similar reduction in hepatic de novo TAG synthesis could not be detected, indicating that the difference between the genotypes observed at the level of gene expression is not significant enough to be picked up at the level of TAG synthesis in this analysis. Interestingly, however, we find that the total level of hepatic TAG and CE is decreased levels of acyl-CoA esters are unclear, but such changes may affect specific enzymatic processes.

Based on the data shown here and the fact that SREBP posttranslational processing is known to be highly dependent on cellular lipid content, we hypothesize that the increased levels of TAG and/or CE are the primary causes of the deregulation of SREBP processing in ACBP−/− liver. This is supported by the fact that in our study, increases in hepatic TAG and CE appear prior to (days 19 and 21) the repression of lipogenic and cholesterogenic gene expression (days 21 and 23). It has previously been suggested that intracellular TAG can suppress SREBP processing, possibly by maintaining an intracellular acyl-CoA pool former. Functional implications of the decreased levels of acyl-CoA esters are unclear, but such changes may affect specific enzymatic processes.

Ectopic expression of ACBP in yeast (66, 67) and mouse and rat liver (30, 31) has been reported to increase the intracellular acyl-CoA level, indicating that ACBP is an acyl-CoA pool former. Curiously, however, the total acyl-CoA pool was unaffected in yeast by depletion of ACBP due to a general decrease in most acyl-CoA ester species accompanied by a significant increase in C18:0 acyl-CoA (24). Our current finding that acyl-CoA levels are significantly decreased in livers of 21-day-old ACBP−/− mice compared with livers of ACBP+/+ mice is consistent with the notion that ACBP is an intracellular acyl-CoA pool former. The functional implications of the decreased levels of acyl-CoA esters are unclear, but such changes may affect specific enzymatic processes.
ACBP−/− mice, it remains unclear whether these are primarily the result of hepatic ACBP deficiency or whether systemic effects due to the depletion of ACBP in other tissues play a role. In this regard, preliminary data show a 2-fold increase in plasma glucocorticoid levels in 21-day-old ACBP−/− mice, which may contribute to the induction of hepatic TAG accumulation because glucocorticoids are known to induce TAG accumulation in the liver ([71–73] and reviewed in Ref. 74). The recent report from Vock et al. (28) showing that knockdown of ACBP in HepG2 leads to a decreased expression of SREBP target genes suggests that the suppression of SREBP activity in the liver of ACBP−/− mice at weaning may be caused by ACBP deficiency in the liver per se. Notably, however, knockdown of ACBP in AML-12 cells did not lead to decreased expression of SREBP target genes. Another open question is whether the delayed adaptation of the liver to weaning contributes to the physical weakness of the mice at weaning.

In conclusion, we have shown that the adaptation to weaning and the concomitant up-regulation of lipogenic and cholesterogenic gene programs in the liver are significantly delayed in mice lacking ACBP. This is caused by a delayed induction of the proteolytic processing and expression of SREBP isoforms. We hypothesize that the increased accumulation of TAG and CE in the liver prior to weaning may interfere with the induction of SREBP processing. However, future investigations using tissue-specific knock-out models are needed to establish the role of ACBP in TAG and CE accumulation in the liver and in SREBP processing.

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