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Fat/Vessel-derived Secretory Protein (Favine)/CCDC3 Is Involved in Lipid Accumulation*

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Background: Coiled-coil domain-containing 3 (*Ccdc3*) mRNA is highly expressed in adipose tissue and vessels, but its function remains unknown.
Results: CCDC3 overexpression promoted adipogenesis of 3T3-L1 adipocytes, whereas CCDC3 deficiency reduced adiposity and inhibited age-related hepatic steatosis in mice.
Conclusion: CCDC3 positively regulates lipid accumulation.
Significance: CCDC3 is a possible therapeutic target to age-related obesity and diseases.

We previously identified a novel gene encoding Favine/CCDC3 (NCBI protein entry NP_083080), a possible secretory factor, the mRNA of which is highly expressed in adipose tissue and the aorta. The *Favine* mRNA levels are increased in the course of differentiation of rat primary adipocytes and are more elevated in the adipose tissue of genetically obese and diet-induced obese mice than in lean mice. However, its biological function has not yet been elucidated until now. Here, we tested the hypothesis that Favine is involved in lipid metabolism in adipocytes. We found that overexpression of Favine promoted 3T3-L1 adipocyte differentiation. To further investigate the function of Favine *in vivo*, we generated Favine knock-out (KO) mice. Favine KO mice exhibited a lean phenotype as they aged. The weights of white adipose tissue and liver were less, and adipocyte size was smaller in Favine KO mice compared with wild-type littermates (WT). Expression levels of lipogenic genes, such as fatty-acid synthase (*FAS*), acetyl-CoA carboxylase α (*ACC1*), and diacylglycerol *O*-acyltransferase-2 (*Dgat2*), were decreased in adipose tissue of Favine KO mice. In 1-year-old mice, Favine deficiency decreased the number of inflammatory cells in white adipose tissue and diminished hepatic steatosis. *In vitro*, deficiency of Favine attenuated differentiation of primary adipocytes. Taken together, these data demonstrate that Favine has adipogenic and lipogenic effects on adipocytes.

Adipose tissue serves as a reservoir for energy and is also considered as an endocrine organ (1). Adipocyte differentiation, also called adipogenesis, is a complex and highly orchestrated program, which is accompanied by morphological changes, expression of adipocyte-specific genes and many lipogenic genes, extensive lipid accumulation, and the establishment of sensitivity to many hormones (2–4). In differentiated adipocytes, excess carbohydrate is stored as fat via lipogenesis, which is the metabolic process in synthesis of fatty acids and triglyceride (5, 6). An increase in both adipogenesis and lipogenesis is an important contributor to increased fat mass (7). In diet-induced obesity, increased lipid burden in adipose tissue and ectopic lipid accumulation in non-adipose tissues causes multiple dysfunction of the organs (8). Recently, aging has also been recognized to be linked with obesity (9, 10).

Previously, we searched for novel secretory factors associated with obesity and identified coiled-coil domain-containing 3/fat/vessel-derived secretory protein (CCDC3/Favine). Favine is a secretory protein predominantly expressed in adipose tissue and the aorta. The expression levels of *Favine* mRNA are increased in murine adipose tissue of genetic and diet-induced obesity models and induced in the course of differentiation of rat primary adipocytes. In differentiated adipocytes, mRNA expression levels of *Favine* are augmented by insulin and peroxisome proliferator-activated receptor γ (PPARγ)[2] agonist and suppressed by TNF-α, norepinephrine, and isoproterenol (11). Eberlein *et al.* (12) showed that mRNA expression levels of *Favine* in bovine muscle are correlated with the amount of intramuscular fat deposition. Ugi *et al.* (13) observed that human omental *Favine* mRNA is correlated to body mass index and waist circumference. Although these observations suggest that Favine is involved in lipid accumulation, the function of Favine has not been elucidated.

Here, we show for the first time that Favine has adipogenic and lipogenic effects by gain-of-function study in murine 3T3-L1 preadipocyte cell line and loss-of-function study in mice.

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[2] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; CAR, Coxsackie-adenovirus receptor; FAS, fatty-acid synthase; ACC1, acetyl-CoA carboxylase α; Dgat, diacylglycerol *O*-acyltransferase; ChREBP, carbohydrate-responsive element-binding protein; ATGL, adipose triglyceride lipase; HSL, hormone-sensitive lipase; C/ebpα, CCAAT/enhancer-binding protein α; Pref-1, preadipocyte factor-1; Ctgf, connective tissue growth factor.; Favine, fat/vessel-derived secretory protein.

EXPERIMENTAL PROCEDURES

Animals—Mice had *ad libitum* access to water and chow (MF, Oriental Yeast, Suita, Japan). All animals were housed in a temperature-controlled room under a 12-h light/12-h dark cycle. Animals were weighed at 10 a.m. in the fed state. Food intake was determined in individually housed mice by weighing remaining diet. Mice were sacrificed at 1 p.m. Tissues of each mouse were dissected out, washed with phosphate-buffered saline (PBS), and immediately weighed and deposited into liquid nitrogen or into 4% buffered formaldehyde. Male Favine KO mice and wild-type littermates were used for experiments. All experimental protocols were approved by the Ethics Review Committee for Animal Experimentation of Osaka University.

Cell Cultures—3T3-L1 cells were purchased from National institute of Biomedical Innovation, JCRB Cell Bank (Japan), and were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Two days post-confluence, the cells were treated with adipogenic mixture containing 0.5 mM 3-isobutyl-1-methylxanthine (Nacalai Tesque, Kyoto, Japan), 1 μM dexamethasone (Sigma), and 0.1 or 1 μM insulin (Nacalai Tesque) for 48 h. After 48 h, medium was replaced with DMEM supplemented with 10% FBS. The stable Coxsackie-adenovirus receptor (CAR)-expressing 3T3-L1 adipocytes (CAR-3T3-L1 adipocytes) were maintained in DMEM supplemented with 10% FBS and 2 μg/ml puromycin (Nacalai Tesque) and differentiated into mature adipocytes with adipogenic mixture containing 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, and 1 μM insulin for 48 h (14). HEK293A cells and HEK293T cells were maintained in DMEM supplemented with 10% FBS.

Generation of Stable Favine-overexpressing 3T3-L1 Cells—The retroviral vector, pMXs-IG, and Plat-E cells were kindly provided by Dr. T. Kitamura (Institute of Medical Science, the University of Tokyo) (15). C-terminal His-tagged Favine was inserted into pMXs-IG (named as pMXs-IG-Favine). The retrovirus vectors, empty pMXs-IG and pMXs-IG-Favine, were transfected into Plat-E cells using Lipofectamine 2000 reagent (Invitrogen). 48 h after transfection, the supernatants were harvested as viral stock solutions. 3T3-L1 cells stably expressing Favine were obtained by infection with viral stocks, followed by a collection of GFP-positive cells with FACS Aria (BD Biosciences).

Generation of Transient Favine-overexpressing CAR-3T3-L1 Adipocytes—The adenoviral vector, pAD/CMV/V5-DEST Gateway vector, was purchased from Invitrogen. β-Galactosidase (LacZ) and Favine were inserted into the pAd/CMV/V5-DEST Gateway vector, respectively (named as pAD-LacZ and pAD-Favine). The adenoviral vectors, pAD-LacZ and pAD-Favine, were transfected into HEK293A cell lines to produce adenoviral stock (named as Ad-LacZ and Ad-Favine, respectively). Amplified adenoviral stock was purified by Adeno-X Maxi purification kit (Clontech) as per the manufacturer's instructions. The CAR-3T3-L1 adipocytes on the 4th day after induction were transfected with Ad-LacZ and Ad-Favine, incubated for 3 days, and used for lipogenesis assay and oxygen consumption assay.

Lipogenesis Assay—The stable Favine-overexpressing 3T3-L1 adipocytes and adenovirus-infected CAR-3T3-L1 adipocytes were serum- and glucose-deprived and assayed as described (16) with some modifications. Cells were incubated for 3 h prior to assay in Krebs-Ringer bicarbonate buffer supplemented with 30 mM HEPES, 0.5% bovine serum albumin (KRBH/BSA), and 2.5 mM glucose. The cells were washed once with PBS and incubated for another 15 min in KRBH/BSA without glucose. 100 nM insulin were added, and the cells were incubated for 15 min. The reaction was initiated by the addition of [U-^{14}C]glucose (1 μCi/well) and glucose (5 mM final concentration). The assay was terminated after 1 h by washing three times with ice-cold PBS. Radiolabeled glucose incorporation into lipid was assessed by placing cells into 0.5 ml of PBS and shaking vigorously with 2.5 ml of Betafluor Liquid Scintillation Fluid (National Diagnostics). After samples settled overnight, radioactivity in 1.5 ml of the organic phase was determined by scintillation counting.

Western Blot Analysis—Cell lysates and culture media of 3T3-L1 cells were harvested. Culture media were concentrated by absorption onto a nickel resin (GE Healthcare). Nickel-Sepharose-bound proteins and 10 μg of cell lysates were subjected to SDS-PAGE under reducing conditions, transferred, and blotted with HRP-conjugated anti-His antibody (Qiagen, Santa Clarita, CA) or anti-rabbit GFP antibody 2956 (Cell Signaling Technologies, Danvers, MA) followed by HRP-conjugated rabbit IgG antibody (Amersham Biosciences). The membrane was stripped and reproved with anti-mouse β-actin Clone AC-15 (Sigma), followed by HRP-conjugated mouse IgG antibody (Amersham Biosciences).

Oil Red O Stains in Adipocytes—Lipid accumulation in adipocytes was examined by Oil Red O staining (17). Cells were fixed with 4% formaldehyde, washed with water, dried, and stained with 0.18% Oil Red O (Nacalai Tesque) in 60% isopropyl alcohol (Nacalai Tesque). Stained cells were washed with water and dried. The tissue culture plates were scanned on a flatbed scanner. Dye was extracted using dimethyl sulfoxide (Nacalai Tesque), and its absorbance was determined at $A_{540\ nm}$.

RNA Isolations—Total RNA was extracted from each tissue using RNA STAT-60 (Tel-Test, Friendswood, TX) or Sepasol®-RNA I Super G (Nacalai Tesque) according to the instructions supplied by the manufacturers.

Quantitative Real Time RT-PCR—Quantitative real time RT-PCR was performed as described previously (11). The sequences of primers used in real time RT-PCR are listed in Table 1.

Targeted Disruption of Ccdc3/Favine Gene—Favine KO mice were generated at Unitech, Co. Ltd. (Chiba, Japan). The *Ccdc3/Favine* chromosomal gene was isolated from bacterial artificial chromosome clones of C57BL/6J mice, and its exon1 and most of exon2 were replaced by a coding neomycin resistance (*Neo*) gene (shown in Fig. 2*A*). Embryonic stem cells were electroporated, and 7 of 41 screened colonies were found to be correctly targeted, followed by injection into C57BL/6J blastocysts to generate chimeric mice. The chimeric mice were crossed with C57BL/6J mice to obtain heterozygous Favine KO mice. Mice were genotyped by PCR using two *Favine*-specific primers (primer A, 5′-AGG TCT GGT GGT CAA GGC GTT G-3′;

TABLE 1
Sequences of PCR primers used for quantitative real time RT-PCR

36B4	Forward	5′-GCTCCAAGCAGATGCAGCA-3′
	Reverse	5′-CCGGATGTGAGGCAGCAG-3′
CypA	Forward	5′-CAGACGCCACTGTCGCTTT-3′
	Reverse	5′-TGTCTTTGGAACTTTGTCTGCAA-3′
β-actin	Forward	5′-GTGACGTTGACATCCGTAAAGA-3′
	Reverse	5′-GCCGGACTCATCGTACTCC-3′
Favine	Forward	5′-TGGTCCAGGACTACTCTTATTTCTT-3′
	Reverse	5′-GAAAACATCCTTCTGTTCTCCTG-3′
FAS	Forward	5′-CATCCACTCAGGTTCAGGTG-3′
	Reverse	5′-AGGTATGCTCGCTTCTCTGC-3′
Dgat1	Forward	5′-TGGTGTGTGGTGATGCTGATC-3′
	Reverse	5′-GCCAGGCGCTTCTCAA-3′
Dgat2	Forward	5′-GGCTACGTTGGCTGGTAACT-3′
	Reverse	5′-CACTCCCATTCTTGGAGAGC-3′
ChREBP	Forward	5′-CACTCAGGGAATACACGCCTAC-3′
	Reverse	5′-ATCTTGGTCTTAGGGTCTTCAGG-3′
ChREBP-β	Forward	5′-CGAGGTCCCAGGATCCGA-3′
	Reverse	5′-TCCCGGCATAGCAACTTGAG-3′
SREBP-1c	Forward	5′-TGGACTACTAGTGTTGGCCTGCTT-3′
	Reverse	5′-ATCCAGGTCAGCTTGTTTGCGATG-3′
MTP	Forward	5′-ACTCTGGCTCTGGCATTCTG-3′
	Reverse	5′-GGCTTCAATCACCACCTGAC-3′
C/ebpα	Forward	5′-TTTGCACCTCCACCTACATCCC-3′
	Reverse	5′-CCCGTGTCCTCCTATCCC-3′
ACC1	Forward	5′-GAGGTACCGAAGTGGCATCC-3′
	Reverse	5′-GTGACCTGAGCGTGGGAGAA-3′
PPARγ	Forward	5′-CCAGAGTCTGCTGATCTGCG-3′
	Reverse	5′-GCCACCTCTTTGCTCTGCTC-3′
adiponectin	Forward	5′-GATGGCAGAGATGGCACTCC-3′
	Reverse	5′-CTTGCCAGTGCTGCCGTCAT-3′
Ap2	Forward	5′- CCGCAGACGACAGGA-3′
	Reverse	5′- CTCATGCCCTTTCATAAACT-3′
Pref-1	Forward	5′- TCTGCAAGGATGGCTGGGAC-3′
	Reverse	5′- CACAGGCAGGAAGCATGCGA-3′
TGF-β1	Forward	5′-ACCATGCCAACTTCTGTCTG-3′
	Reverse	5′-CGGGTTGTGTTGGTTGTAGA-3′
Ctgf	Forward	5′-GCATCTCCACCCGAGTTA-3′
	Reverse	5′-TTGACAGGCTTGGCGATT-3′

primer B, 5′-TGG GTG CAC GGC TAG CTC TTC G-3′) and a *Neo*-specific primer (primer C, 5′-ACC GAC CAA GCG ACG TCT GA-3′) shown in Fig. 2*A*. For Southern blot, genomic DNA was isolated, digested with SacI or AseI, and detected using two probes in Fig. 2*A*.

Measurements of Parameters in Murine Plasma—Blood samples were collected from tail veins. Plasma glucose, triglyceride, insulin, free fatty acid, and leptin concentrations were measured using the glucose CII-test kit, triglyceride E-test (Wako Pure Chemical Industries, Tokyo, Japan), insulin enzyme-linked immunoassay kit (Morinaga, Yokohama, Japan), NEFA C-test (Wako), and mouse leptin ELISA kit (Morinaga), respectively, according to the manufacturers' instructions. Other plasma parameters were measured using Vetscan VS2 (Abaxis, Union City, CA).

Insulin tolerance Test—After 4 h of fasting, mice were injected with 1.0 milliunits/g body weight of human regular insulin intraperitoneally. Blood samples were collected from tail veins every 15–30 min for 120 min, and blood glucose was measured.

Measurements of Triglyceride in Murine Livers—Hepatic triglyceride was extracted as described (18) with some modifications, and measurement of the concentration of total lipids was carried with triglyceride E-test (Wako).

Hematoxylin and Eosin Staining—Tissues were fixed with 4% buffered formaldehyde for histological examination, embedded in paraffin, and sectioned at a thickness of 4 μm. The sections were stained with hematoxylin and eosin using a standard protocol.

Oil Red O Stains in Murine Livers—Tissues were frozen in Tissue-Tek OCT (Sakura Finetechnical, Tokyo, Japan) and were stained with Oil Red O using a standard protocol.

Determination of Adipocyte Cell Size—Adipocyte cell size was measured using BZ-X700 (KEYENCE, Osaka, Japan). The adipocyte area was evaluated in 5 or 7 mice from two genotypes.

Isolation of Murine Primary Adipocytes—Subcutaneous fat pads of two mice were extracted, and cells from the stromal vascular fraction were isolated as described (19) with some modifications. Adipose tissue was minced and digested at 37 °C in DMEM containing 20 mg of collagenase (Sigma). After digestion, the tissue remnants were removed by filtration through a 250-μm filter. Cells were suspended with DMEM containing 10% FBS, 1% antibiotic/antimycotic mixed solution (Nacalai Tesque), and 200 μM L-ascorbic acid and then filtered through a 25-μm filter. Isolated cells were plated on a collagen type 1 plate. Medium was replaced every 2 days. Two days after 100% confluency, the cells were treated with adipogenic mixture containing 0.5 mM 3-isobutyl-1-methylxanthine, 0.25 μM dexamethasone, 1 μM insulin, and 5 μM pioglitasone (Takeda Pharma). After 48 h, medium was replaced with DMEM containing 0.1 μM insulin and 10% FBS.

Statistical Analysis—Values were expressed as mean ± S.D. or ± S.E. Differences between the two groups were examined for statistical significance using the Student's *t* test. A p value less than 0.05 was considered statistically significant.

RESULTS

Retrovirus-mediated Overexpression of Favine Enhanced Adipogenesis and Lipogenesis in 3T3-L1 Cells—To elucidate the functions of Favine in adipocytes, we generated 3T3-L1 cells stably expressing Favine. 3T3-L1 cells were transduced with pMXs-Favine-IRES-green fluorescent protein (GFP) retrovirus (Favine-L1 cells) or pMXs-IRES-GFP retrovirus (Mock-L1 cells). Approximately equal amounts of intracellular GFP expression were confirmed between Favine-L1 cells and Mock-L1 cells by Western blot analysis (Fig. 1*A, upper panel*). Favine was detected in intracellular protein, and Favine-L1 cells secreted Favine into culture media as a 37-kDa protein (Fig. 1*B, upper panel*). β-Actin was blotted as loading controls for intracellular proteins (Fig. 1, *A, lower panel*, and *B, lower panel*). Morphologic differences were not observed between undifferentiated Favine-L1 cells and Mock-L1 cells, and the proliferation rate was similar between two types of cells (data not shown). Interestingly, after adipogenic induction, Oil Red O staining showed that the number of lipid-laden cells was increased in Favine-L1 cells compared with Mock-L1 cells (Fig. 1*C*). The size of the lipid drop was similar between Mock-L1 cells and Favine-L1 cells (Fig. 1*C, upper panel*). Then we measured the expression of genes involved in adipocyte differentiation and lipid metabolism. The mRNA expression levels of *PPARγ*, which is the master regulator of adipogenesis, adi-

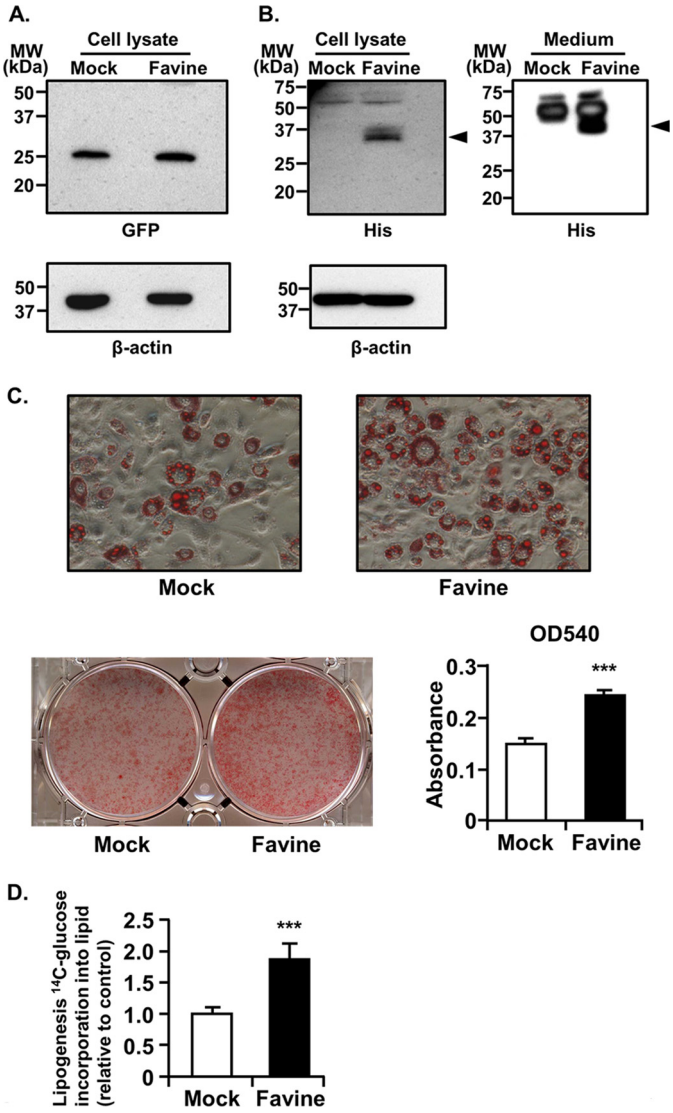

FIGURE 1. **Favine overexpression in 3T3-L1 cells.** *A,* Western blot of GFP (*upper panel*) and β-actin (*lower panel*) in cell lysates from undifferentiated Favine-L1 cells and Mock-L1 cells. *B,* Western blot of His-tagged Favine (*upper left panel*) and β-actin (*lower left panel*) in cell lysates and His-tagged Favine in culture media (*right panel*) from undifferentiated Favine-L1 cells and Mock-L1 cells. *C,* Oil Red-O stains of Favine-overexpressing 3T3-L1 cells. Mock-L1 cells and Favine-L1 cells were treated with adipogenic mixtures and stained with Oil Red-O on the 6th day after induction (*upper panel,* original magnification, ×200; *lower left panel,* scanning of stained culture plate). Extraction of the absorbed dye using DMSO and absorbance measurements at 540 nm (*lower right panel*). Data are mean ± S.D. ***, $p < 0.001$ *versus* Mock-L1 cells. *D,* lipogenic activity in Favine-L1 cells. D-[^{14}C]Glucose incorporation into lipid on the 7th day after induction was determined ($n = 6$). Data are mean ± S.D. ***, $p < 0.001$ *versus* Mock-L1 cells.

ponectin, and fatty acid-binding protein4 (*Ap2*), were increased more in Favine-L1 cells than in Mock-L1 cells (Table 2, left). The mRNA levels of major lipogenic enzymes, fatty-acid synthase (*FAS*), acetyl-CoA carboxylase α (*ACC1*), and diacylglycerol *O*-acyltranserase-1 and -2 (*Dgat1* and *Dgat2*), were also up-regulated in Favine-L1 cells (Table 2, left) compared with Mock-L1 cells. Actually, the rate of lipogenesis in Favine-L1 cells was increased (Fig. 1*D*). The expression levels of carbohydrate-responsive element-binding protein (*ChREBP*) and sterol regulatory element-binding protein 1c (*SREBP-1c*), major lipogenic transcription factors that regulate *de novo* lipogenic enzymes, were not changed (Table 2 left). These data suggest that overexpression of Favine enhanced adipogenesis and lipogenesis in 3T3-L1 cells.

Adenovirus-mediated Overexpression of Favine Promoted Lipogenesis in 3T3-L1 Cells—To analyze the functions of Favine in differentiated adipocytes, we constructed 3T3-L1 cells transiently expressing Favine. Favine overexpression increased the rate of lipogenesis in differentiated 3T3-L1 adipocytes more than the control LacZ-overexpressing adipocytes. Expression levels of *PPARγ* and adiponectin were not changed between Favine and LacZ-overexpressing cells (data not shown). Taken together, these data demonstrate that Favine is deeply involved in lipid accumulation in adipocytes.

Generation and Validation of Favine Null Knock-out Mice—To further investigate the physiological function of Favine *in vivo*, we generated Favine knock-out (KO) mice. The coding region of the mouse *Favine* gene includes three exons spanning about 93 kb on mouse chromosome 2. To disrupt the *Favine* gene, the 11-kb coding region of Favine polypeptide (exons 1 and 2) was removed using homologous recombination in embryonic stem cells. The targeting vector was designed to replace this region with SV40 polyadenylation signals and the *Neo* gene (Fig. 2*A*). Independent ES cell clones with homologous recombination were verified by Southern blot and injected into blastocysts to generate chimeras. Homologous recombination in offspring was confirmed by both Southern blot and PCR analysis (Fig. 2*B*). The absence of *Favine* mRNA in the knock-out mouse was validated by quantitative real time RT-PCR with cDNA generated from epididymal adipose tissue, aorta, liver and muscle RNA from F5 animals (Fig. 2*C*).

Favine KO Mice Showed Lean Phenotype—Favine KO mice were viable, fertile, and showed normal development. The body weight of Favine KO mice was the same as that of controls at 6 weeks of age, suggesting no congenital growth retardation. There was a trend toward a decrease of body weight as they got aged under a normal chow diet, and in the later stage Favine KO mice showed a statistically significant lean phenotype (Fig. 2*D*). Analysis of food intake did not reveal any differences between WT and Favine KO mice (Fig. 2*E*). Plasma glucose levels in the fed state were slightly higher in Favine KO mice compared with WT mice (Fig. 2*F*). Plasma insulin (data not shown), triglyceride, and free fatty acid levels of KO mice did not differ (Table 3). Results of the glucose tolerance test and the insulin tolerance test showed no significant difference at the 16 weeks of age between the two genotypes (data not shown), whereas 9-month-old KO mice showed higher insulin sensitivity (Fig. 2*G*).

In Adipose Tissue of Favine KO Mice, de Novo Lipogenesis Was Down-regulated and Age-related Adiposity Was Attenuated with Less Inflammatory Cells—For the study on leanness of Favine KO mice, mice were prepared for histological analysis at 14 weeks of age (young stage) and at 1 year of age (aged stage), respectively. At 14 weeks of age, the weight of epididymal and subcutaneous adipose tissue was significantly less in Favine KO mice compared with WT mice (Fig. 3*A*). Adipocyte size in both epididymal and subcutaneous adipose tissue was smaller in KO mice (Fig. 3, *B, upper panel*, and *C*), which could account for the decrease of the fat mass. At 1year of age, in epididymal adipose tissue of Favine KO mice the number of inflammatory cells was

TABLE 2

Summary of quantitative real time RT-PCR

Quantitative real time RT-PCR of adipogenic and lipogenic genes of differentiated Mock-L1 cells and Favine-L1 cells on the 6th day after induction (left column), 14-week-old murine epididymal adipose tissue (middle column), and primary adipocytes (right column). mRNA expression levels of listed genes were measured by quantitative real time RT-PCR. mRNA expression levels were normalized to *CypA* level in differentiated 3T3-L1 cells (Mock $n = 3$, Favine $n = 3$), to *36B4* level in 14-week-old murine epididymal adipose tissue (WT $n = 7$, KO $n = 5$), and to *β-actin* level in primary adipocytes (WT $n = 3$, KO $n = 3$). Data are mean ± S.D. or ± S.E. n.d. indicates not detected. +, $p = 0.07$; #, $p = 0.06$; [a], $p < 0.05$; [b], $p < 0.01$; [c], $p < 0.001$ *versus* Mock or WT.

	Overexpression in 3T3-L1 adipocytes		Adipose tissue in mice		Primary adipocytes from mice	
	Mock-L1	Favine-L1	WT	KO	WT	KO
Adipogenic						
PPARγ	1.0±0.02	1.61±0.17[b]	1.0±0.26	1.08±0.39	1.0±0.06	0.73±0.02[b]
adiponectin	1.0±0.05	1.61±0.11[a]	1.0±0.15	1.07±0.20	1.0±0.12	0.26±0.01[b]
C/ebpα	1.0±0.02	1.46±0.27	1.0±0.11	1.05±0.23	1.0±0.08	0.62±0.01[a]
Ap2	1.0±0.10	2.54±0.60[a]	1.0±0.18	1.13±0.25	1.0±0.13	0.4±0.05[b]
Lipogenic						
FAS	1.0±0.08	1.15±0.04[+]	1.0±0.97	0.19±0.13[#]	1.0±0.04	0.48±0.08[b]
ACC1	1.0±0.14	1.28±0.13[+]	1.0±0.77	0.24±0.08[a]	1.0±0.05	1.08±0.07
Dgat1	1.0±0.10	1.75±0.02[b]	1.0±0.38	1.02±0.20	1.0±0.06	0.73±0.02[b]
Dgat2	1.0±0.07	1.98±0.04[c]	1.0±0.45	0.35±0.14[b]	1.0±0.06	0.78±0.02[a]
Lipogenic Transcription Factors						
***ChREBP* total**	1.0±0.12	0.95±0.05	1.0±0.55	0.60±0.23[+]	1.0±0.08	0.63±0.06[b]
ChREBP-β	n.d.	n.d.	1.0±0.57	0.17±0.15[b]	1.0±0.05	0.64±0.12[a]
SREBP-1c	1.0±0.30	1.4±0.06	1.0±0.41	0.79±0.28	1.0±0.04	0.81±0.07[a]

significantly reduced compared with WT mice, besides a reduced fat mass with smaller adipocytes (Fig. 3*B, lower panel*).

We next measured the expression of genes involved in adipocyte differentiation and lipid metabolism in epididymal adipose tissue. The expression of genes involved in adipocyte differentiation, such as *PPARγ* and adiponectin, was not affected (Table 2, middle), suggesting that the decreased adipocyte size was not due to reduced adipocyte differentiation. The levels of plasma adiponectin in Favine KO mice were not changed, but the levels of plasma leptin in Favine KO mice were decreased (Table 3). Interestingly, expression levels of lipogenic genes (*FAS*, *ACC1,* and *Dgat2*) were significantly attenuated in epididymal adipose tissue of KO mice compared with WT mice (Table 2, middle), although mRNA levels of lipolytic genes (adipose triglyceride lipase (*Atgl*) and hormone-sensitive lipase (*HSL*)) were not altered (data not shown). Actually, basal lipolysis, fasting-induced lipolysis, and isoproterenol-stimulated lipolysis were unchanged in Favine KO mice (data not shown). *ChREBP-β*, a recently identified isoform of *ChREBP* in adipose tissue (20), was extremely down-regulated in KO mice. Total *ChREBP* expression, the sum of canonical isoform α and newly identified isoform β, in KO mice tended to be declined. *SREBP-1c* mRNA remained unchanged (Table 2, middle).

Lipid Accumulation in Livers of Aged Favine KO Mice Was Diminished—Liver is recognized to be a major lipogenic organ. Favine KO mice showed a lean phenotype (Fig. 2*D*) with reduced hepatic weight (Fig. 3*A*). Then, hepatic phenotypes were evaluated.

At 14 weeks of age, hepatic histological appearance was not different between genotypes (data not shown), and triglyceride contents in livers were not changed (Fig. 4*A*). Expression levels of three lipogenic genes in the liver; *FAS*, *Dgat2,* and microsomal triglyceride transfer protein (*MTP*), were not altered in Favine KO mice compared with WT mice (Fig. 4*B*).

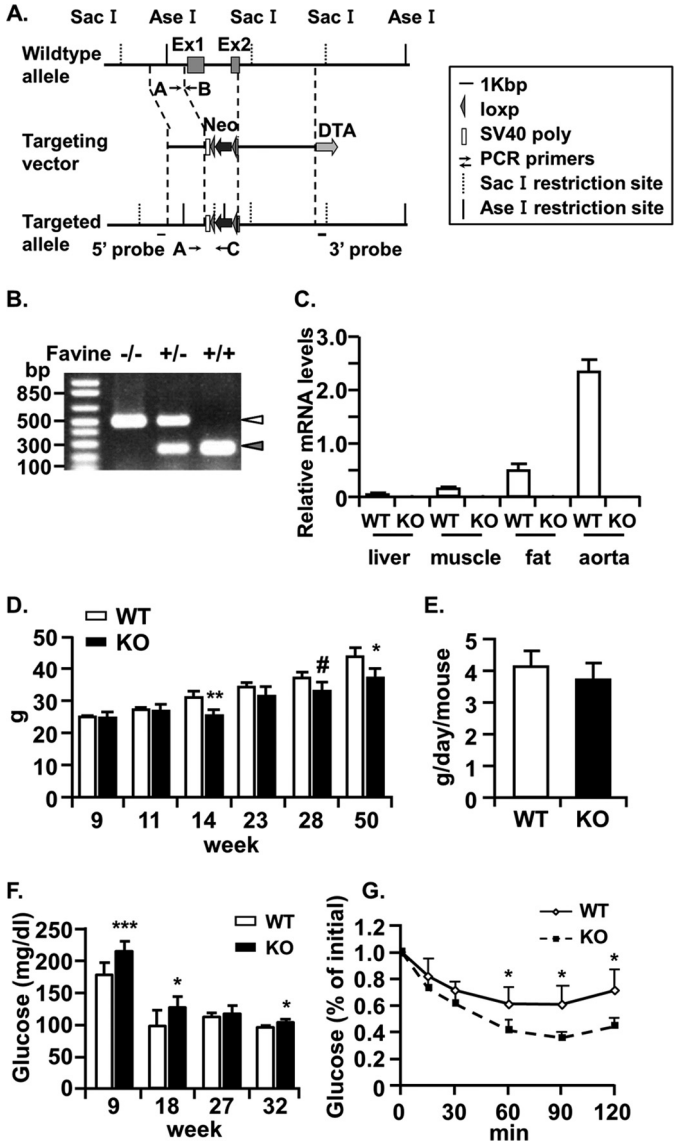

FIGURE 2. **Generation and characteristics of Favine KO mice.** *A,* schematic representation of the gene targeting strategy. Partial restriction map of the mouse *Favine* locus for WT allele (*top panel*). Targeting construct of mouse *Favine* was generated by replacing the regions of exons 1 and 2 containing the translation initiation site ATG and parts of intron 1 with the *Neo* cassette (*middle panel*). Transcriptional direction of the *Neo* gene is indicated by the *arrow*. Expected disrupted allele was obtained by homologous recombination (*bottom panel*). *B,* genotyping PCR with primers for wild-type and recombinant alleles. A 0.5-kb fragment was generated from the targeted allele (*white arrowhead*), and a 0.25-kb fragment was generated from the wild-type allele (*black arrowhead*). *C, Favine* mRNA expression in WT mice and Favine KO mice (WT $n = 4$, KO $n = 5$). mRNA expression level was measured by quantitative real time RT-PCR and normalized to the *36B4* level. Data are mean ± S.D. *Fat*, epididymal adipose tissue. *D,* average body weight of WT mice and Favine KO mice (WT $n = 4$ or 7, KO $n = 5$). Data are mean ± S.D. #, $p = 0.06$; *, $p < 0.05$; **, $p < 0.01$ *versus* WT. *E,* food intake in 19-week-old mice (WT = 5, KO = 5). Data are mean ± S.D. *F,* fed plasma glucose levels of WT mice and Favine KO mice (WT $n = 3$–6, KO = 5). Data are mean ± S.D. *, $p < 0.05$; ***, $p < 0.001$ *versus* WT. *G,* insulin tolerance tests. 9-Month-old mice were injected with 1.0 milliunits/g body weight of human regular insulin intraperitoneally. Blood glucose levels were measured from tail blood (WT $n = 5$, KO $n = 4$). Ratio relative to time 0 are expressed as mean ± S.D. *, $p < 0.05$ *versus* WT.

TABLE 3

Plasma parameters of Favine KO mice

Plasma triglyceride after 6 h of fasting and free fatty acid and adiponectin and leptin after 4 h of fasting levels were measured (WT $n = 4$ or 7, KO $n = 5$). Data are mean ± S.D. [a], $p < 0.01$ *versus* WT.

parameter	WT	KO
Triglyceride (mg/dl)	61.1±11.4	66.0±15.3
Free fatty acid (mEq/L)	0.36±0.07	0.34±0.12
Adiponectin (μg/ml)	36.0±9.3	29.5±2.3
Leptin (ng/ml)	6.27±2.59	2.01±0.91[a]

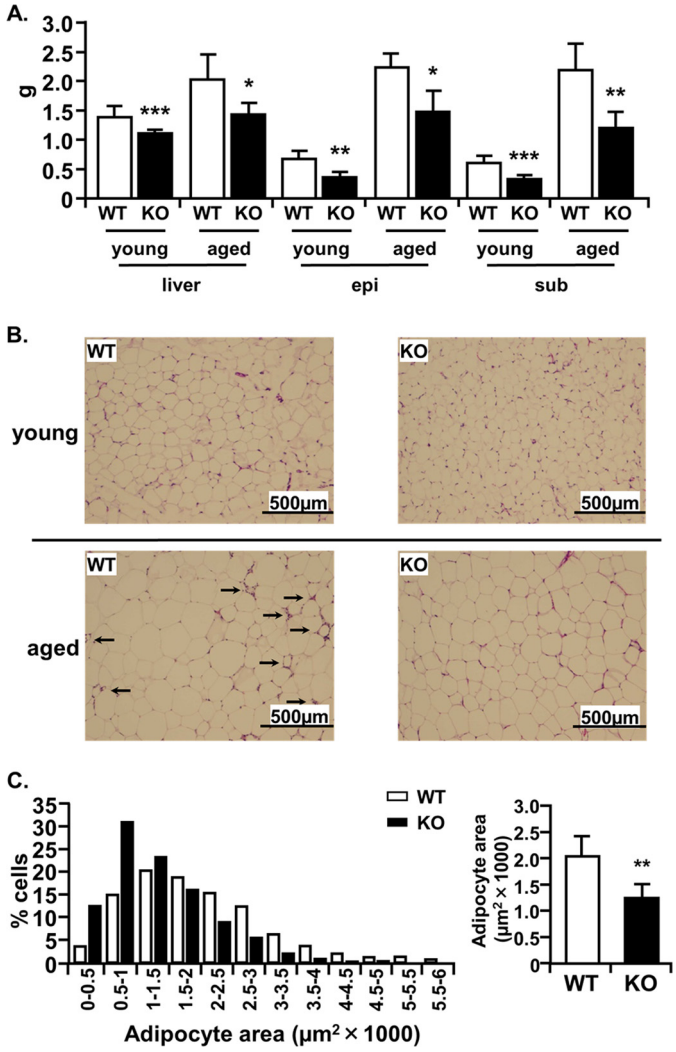

FIGURE 3. **Tissue weight and histological analysis of adipose tissue in Favine KO mice.** *A,* tissue weight of 14-week-old mice (*young*: WT $n = 7$, KO $n = 5$) and 1-year-old mice (*aged*: WT $n = 5$, KO $n = 4$) and data are mean ± S.D. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ *versus* WT. *epi*, epididymal adipose tissue; *sub*, subcutaneous adipose tissue. *B,* hematoxylin and eosin staining of 14-week-old murine epididymal adipose tissue (*upper panel*) and 1-year-old murine epididymal adipose tissue (*lower panel*) (original magnification, ×100). *Arrows* indicated inflammatory cells in epididymal adipose tissue. *C,* histological sections in 14-week-old murine epididymal adipose tissue were used for determining adipocyte size distribution (*left panel*) and mean adipocyte size (*right panel*) (WT $n = 7$, KO $n = 5$). Data are mean ± S.D. **, $p < 0.01$ *versus* WT.

At 1 year of age, we found that hepatic steatosis was markedly diminished in Favine KO mice, although livers of WT mice had increased the number of lipid droplets in hepatocytes and developed age-related steatosis (Fig. 4, *C* and *D*). Actually, amounts of hepatic triglycerides of aged KO mice were significantly less compared with aged WT mice (Fig. 4*A*). It is of inter-

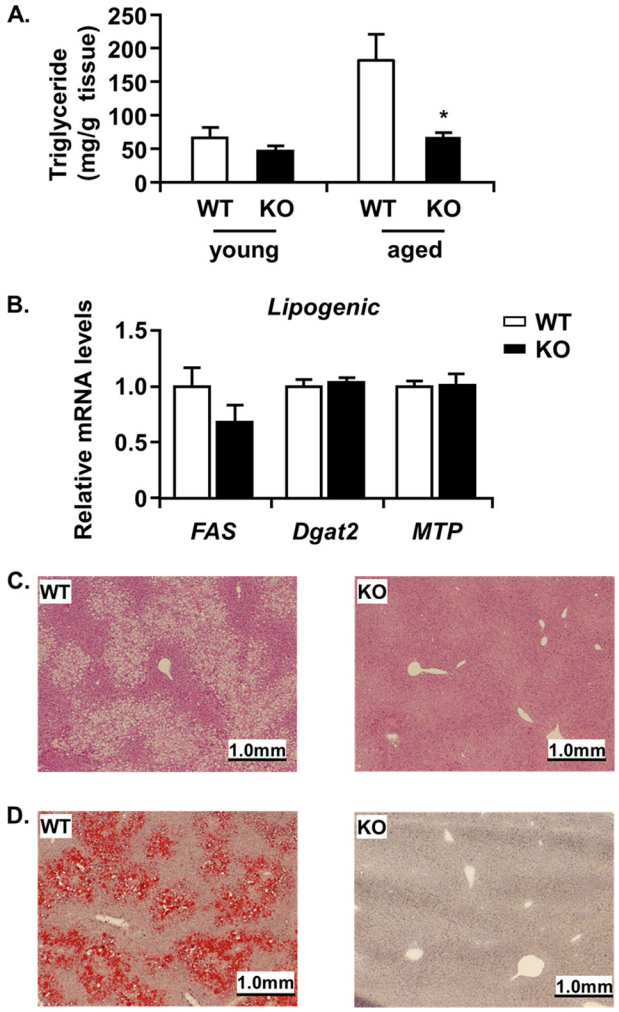

FIGURE 4. **Histological analysis of livers in Favine KO mice.** *A,* amount of hepatic triglyceride of young (14-week-old, WT $n = 4$, KO $n = 5$) and aged (1-year-old, WT $n = 9$, KO $n = 7$) mice. Mice were sacrificed, and tissues were frozen, followed by extraction of hepatic triglyceride. Data are mean ± S.E. *, $p < 0.05$ *versus* WT. *B,* quantitative real time RT-PCR of lipogenic genes of 14-week-old murine liver. Mice were sacrificed at 1 p.m. after 4 h of fasting. mRNA expression level of *FAS* (*left*), *Dgat2* (*middle panel*) and *MTP* (*right panel*) were measured by quantitative real time RT-PCR and normalized to the *36B4* level (WT $n = 4$, KO $n = 5$). Data are mean ± S.E. *C,* hematoxylin and eosin staining of 1-year-old murine livers (original magnification, ×40). *D,* Oil Red O stains of 1-year-old murine livers (original magnification, ×40).

est that expression levels of several genes involved in hepatic lipid metabolism showed no significant changes between the two aged groups (data not shown). No hepatic dysfunctions were observed by blood analysis in aged WT and KO mice (data not shown). Ectopic lipid accumulation in muscle, heart, aorta, and kidney was not observed in both genotypes.

Differentiation of Primary Adipocytes from Favine KO Mice Was Impaired—To further elucidate the function of Favine on adipocyte differentiation and lipogenesis, primary preadipocytes were isolated from subcutaneous fat pads of Favine KO mice and WT mice and subjected to adipocyte differentiation *in vitro*. There were no significant differences in cell growth rates between the two genotypes. After adipogenic induction, lipid accumulation in Favine-deficient adipocytes tended to be attenuated compared with WT adipocytes without statistical significance (data not shown). The expression levels of *PPARγ*

TABLE 4
Quantitative real time RT-PCR in primary undifferentiated adipocytes

Quantitative real time RT-PCR of anti-adipogenic genes of primary undifferentiated adipocytes is shown. mRNA expression levels of *Pref-1, TGF-β1, and Ctgf* in undifferentiated adipocytes were measured by quantitative real time RT-PCR and normalized to *β-actin* levels (WT $n = 3$, KO $n = 3$). Data are mean ± S.D. [a], $p < 0.01$; [b], $p < 0.001$ *versus* WT.

	WT	KO
Pref-1	1.0±0.10	0.93±0.13
TGF-β1	1.0±0.054	1.45±0.071[a]
Ctgf	1.0±0.072	2.6±0.065[b]

and CCAAT/enhancer-binding protein α (*C/ebpα*), which are master regulators of adipocyte differentiation, were less in Favine-deficient adipocytes than in WT adipocytes. Those of adiponectin and *Ap2*, which are adipocyte differentiation markers, were decreased (Table 2, right), and those of lipogenic genes (*FAS, Dgat1,* and *Dgat2*) and lipogenic transcription factors (*ChREBP-β* and *SREBP-1c*) were down-regulated in Favine-deficient adipocytes compared with WT adipocytes (Table 2, right). To reveal the possible mechanisms underlying the impairment of adipogenesis in Favine-deficient adipocytes, we measured expression levels of preadipocyte factor-1 (*Pref-1*), transforming growth factor β1 (*TGF-β1*), and connective tissue growth factor (*Ctgf*), all of which were shown to have anti-adipogenic effects in primary or 3T3-L1 adipocytes (21–23). Expression levels of *Pref-1* mRNA were not altered, but those of *TGF-β1* and *Ctgf* mRNA were higher in Favine-deficient preadipocytes than in WT preadipocytes (Table 4).

DISCUSSION

In this study, overexpression of Favine in 3T3-L1 cells enhanced adipocyte differentiation with increased expression of the master regulator of adipogenesis, *PPARγ*, and the adipocyte differentiation markers, adiponectin and *Ap2*. On the contrary, deficiency of Favine in primary adipocyte culture caused impairment of adipocyte differentiation with low expressions of *PPARγ*, *C/ebpα*, adiponectin, and *Ap2*. These data suggest that Favine is an adipogenic factor *in vitro*. We also found that mRNA levels of two anti-adipogenic factors, *TGF-β1* and *Ctgf*, were increased in Favine-deficient primary preadipocytes (21, 22, 24). TGF-β inhibits differentiation of 3T3-L1 cells into mature adipocytes at the early stage (25) and inhibits lipid accumulation in porcine primary adipocytes (26). CTGF is a downstream mediator of TGF-β signaling and has anti-adipogenic effects in murine primary adipocytes (22).

There is tight inter-regulation of adipogenic transcription and lipogenesis (4). The expression levels of a series of lipogenic enzymes were enhanced in differentiated stable Favine-overexpressing 3T3-L1 cells and attenuated in differentiated Favine-deficient primary adipocytes, indicating that Favine is involved in lipogenesis *in vitro*. Although it is difficult to distinguish the effects of adipogenesis and lipogenesis during adipocyte differentiation, the increased expression of *PPARγ*, the master regulator of adipocyte differentiation, in stable Favine-overexpressing adipocytes suggests that Favine primarily enhanced adipogenesis, and sequentially increased lipogenesis *in vitro*. Although we observed that Favine overexpression in preadi-

pocytes stimulated adipogenesis and lipogenesis, transient Favine overexpression in differentiated 3T3-L1 adipocytes increased the lipogenic capacity without significant increase of adipogenic markers, including PPARγ. All these data demonstrate that Favine is deeply involved in lipid accumulation in adipocytes. We further tested whether Favine contributed to *PPARγ* and *ChREBP* expression in non-adipose cells. Transient overexpression of FAVINE in HEK293T cells enhanced none of the mRNA expression levels of *PPARγ*, *ChREBP,* and the downstream target genes (data not shown), suggesting that the effect of Favine is exhibited specifically in adipocytes. Revealing the underlying mechanisms of how Favine promoted adipogenesis and/or lipogenesis awaits further study.

Favine KO mice exhibited a lean phenotype with reduced fat mass and smaller adipocyte size compared with WT mice. In adipose tissue of Favine KO mice, expression levels of lipogenic genes (*FAS*, *ACC1,* and *Dgat2*) were profoundly down-regulated, although expression level of *PPARγ*, which is essential not only for adipogenesis but also post-differentiation and survival of mature adipocytes *in vivo* (27, 28), was intact, and adiponectin mRNA and plasma adiponectin levels were not affected. We infer that the decrease of leptin was due to reduced fat mass. Reduced lipogenesis without impairment of adipocyte differentiation implies that Favine may be preferentially involved in lipogenesis *in vivo*. We speculate that some factors might compensate for loss of Favine-mediated adipogenic effects *in vivo*. In our preliminary experiments, hepatic overexpression of Favine induced the expressions of lipogenic genes such as *FAS* and *Dgat2* without significant increase of *PPARγ* mRNA, indicating that Favine could also have lipogenic effects in non-adipose tissues *in vivo* (data not shown).

ChREBP has been recognized as a central regulator of glycolysis and *de novo* lipogenesis in liver (29). Newly identified ChREBP-β is a highly active isoform of ChREBP in adipose tissue (20). ChREBP-β, induced by glucose or glucose metabolite, regulates the expression levels of lipogenic enzymes such as *FAS* and *ACC1* in adipose tissue. ChREBP null KO mice show modest glucose intolerance with hyperglycemia, accompanied by reduced adipose tissue weight (30). Our data showed that *ChREBP-β* expression was markedly reduced in adipose tissue of Favine KO mice, suggesting that Favine deficiency reduces transcription of ChREBP-β followed by coordinated down-regulation of *de novo* lipogenic genes. Favine KO mice developed a mild hyperglycemia in the fed state for reasons as yet unexplained. Down-regulation of adipose *ChREBP* may partially explain metabolic changes of both *de novo* lipogenesis in adipose tissue and glucose homeostasis in Favine KO mice.

Besides reduced lipogenesis *per se* in adipose tissue, changes in energy output and catabolic property of adipose tissue in Favine KO mice were possible mechanisms for reduced body weight and adipose tissue mass. In this study, basal oxygen consumption rate in Favine-overexpressing CAR-3T3-L1 differentiated adipocytes and lipolysis in Favine KO mice were unchanged (data not shown). Further analysis on metabolic states in other tissues will clarify the leanness of Favine KO mice.

Advancing age is accompanied by excessive lipid accumulation in not only adipose tissue but also non-adipose tissue such as the liver. Aging is also recognized to be linked with chronic inflammatory conditions in adipose tissue, resulting in insulin resistance (9, 10). However, the underlying mechanisms of age-related development of ectopic lipid accumulation and adipose tissue inflammation have been poorly understood. In this study, Favine deficiency in mice strongly inhibited age-related hepatic steatosis and decreased the number of inflammatory cells in epididymal adipose tissue, which could partly explain higher insulin sensitivity in aged Favine KO mice compared with WT mice, whereas the young KO mice showed normal insulin sensitivity.

The expression of hepatic mRNA involved in lipid metabolism did not change in either 14-week-old or 1-year-old Favine KO mice. However, the expressions of lipogenic genes in adipose tissue were decreased, and adipose tissue weight was reduced in Favine KO mice compared with WT mice. These data indicate that adipose tissue should be involved in protection of hepatic steatosis in Favine KO mice. One possible mechanism is that lack of Favine secretion from adipose tissue might inhibit hepatic steatosis in an endocrine manner. The other is that reduced fatty acid entry into liver repressed hepatic lipid accumulation in Favine KO mice.

We previously reported that Favine is expressed highly in murine aorta (11). In this study, Favine KO mice showed no morphological changes in the aorta, and blood pressure in Favine KO mice was similar to that of WT mice. The number of vessels stained with CD31 (31), one of endothelial cell markers, in adipose tissue in Favine KO mice was not changed (data not shown). Recently Azad *et al.* (32) showed that Favine represses tumor necrosis factor-α-stimulated vascular cell adhesion molecule-1 expression in endothelial cells *in vitro*. Further investigation on the function of Favine in endothelial cells *in vivo* and *in vitro* will help with understanding the mechanism of physiological action of Favine. To establish the system to measure plasma Favine levels will provide valuable information for significance of this factor in obesity and age-related diseases, which is now underway.

In summary, Favine is involved in adipogenesis and lipogenesis in adipose cells, and it also relates to age-related hepatic steatosis.

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Note Added in Proof—Tables 1–4 were misnumbered in the version of this article that was published as a Paper in Press on January 20, 2015. This has now been corrected.

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