Adipocyte-specific Inactivation of Acyl-CoA Synthetase Fatty Acid Transport Protein 4 (Fatp4) in Mice Causes Adipose Hypertrophy and Alterations in Metabolism of Complex Lipids under High Fat Diet*

Lena-Solveig Lenz 1, Jana Marx 1, Walee Chamulitrat 3, Iris Kaiser 5, Hermann-Josef Gröne 5, Gerhard Liebisch 6, Gerd Schmitz 6, Christoph Elsing 6, Beate K. Straub 7, Joachim Fülekrug 8, Wolfgang Strommel 1 2, and Thomas Herrmann 1 4 2, 3

From the 3 Department of Internal Medicine IV, University of Heidelberg, 69120 Heidelberg, the 5 Department of Cellular and Molecular Pathology, German Cancer Research Center, 69120 Heidelberg, the 4 Institute of Clinical Chemistry, University of Regensburg, 93042 Regensburg, the 1 Department of Internal Medicine, St. Elisabeth Hospital, 46225 Dorsten, the 2 Department of General Pathology, Institute of Pathology, 69120 Heidelberg, and the 3 Institute of Clinical Medicine I, Klinikum Idar-Oberstein, 55743 Idar-Oberstein, Germany

Fatp4 exhibits acyl-CoA synthetase activity and is thereby able to catalyze the activation of fatty acids for further metabolism. However, its actual function in most tissues remains unresolved, and its role in cellular fatty acid uptake is still controversial. To characterize Fatp4 functions in adipocytes, we generated a mouse line with adipocyte-specific inactivation of the Fatp4 gene (Fatp4−/−). Under standard conditions mutant mice showed no phenotypical aberrance. Uptake of radiolabeled palmitic and lignoceric acid into adipose tissue of Fatp4−/− mice was unchanged. When exposed to a diet enriched in long chain fatty acids, Fatp4−/− mice gained more body weight compared with control mice, although they were not consuming more food. Pronounced obesity was accompanied by a thicker layer of subcutaneous fat and greater adipocyte circumference, in contrast to these findings, in the only study using cultured knockdown adipocytes no decline of fatty acid influx into the cell could be shown (10). In contrast to these findings, in the only study using cultured Fatp4 knockdown adipocytes no decline of fatty acid influx into the cell could be shown (10).

The mechanism of fatty acid uptake into the cell is still under debate. In recent years there has been growing evidence for fatty acid uptake across the plasma membrane by specific protein transport systems rather than by mere diffusion processes (1). In line with this perception, there has been increasing interest in an evolutionarily conserved group of genes encoding fatty acid transport proteins (Fats). The Fatp family consists of six members (Fatp1–6) of which Fatp1 was first described and is the best characterized (2). A 60% homologue to that founding member of the Fatp family is Fatp4. Like other members of this gene family, it shows a tissue-specific expression pattern. It can be detected in skin, liver, adipose tissue, brain, skeletal muscle, and heart and is the only Fatp found in small intestine (3). For most of these tissues, the physiological function of Fatp4 is unknown. In several experiments, overexpression of Fatp4 in different cultured cell lines resulted in an increased cellular influx of fatty acids (4–7). In line with these observations, Fatp4 was initially presumed to be a typical transmembrane transport protein (4). Meanwhile, there is emerging evidence that Fatp4 is not plasma membrane-associated but is localized to the endoplasmic reticulum (8) or other intracellular compartments (5, 9). Furthermore, like other members of the gene family, Fatp4 exhibits acyl-CoA synthetase activity and is thereby able to catalyze the activation of fatty acids for further metabolism (3). Referring to this enzymatic property, Fatp4 was attributed to play a role in the so-called vectorial acylation, i.e. promoting the uptake of lipids by activating and thereby “trapping” them within the cell rather than being a transporter per se (8). In contrast to these findings, in the only study using cultured Fatp4 knockout adipocytes no decline of fatty acid influx into the cell could be shown (10). According to its high expression in small intestine, Fatp4 was proposed to be the major transport protein responsible for intestinal lipid absorption shortly after its discovery (4). In more recent studies, this hypothesis could not be confirmed (11). Astoundingly, it turned out that Fatp4 plays a crucial role....
in the epidermis. In a murine knock-out model created in our laboratory, constitutive Fatp4 depletion leads to hyperproliferative hyperkeratosis with disturbed barrier function and restrictive dermopathy causing the perinatal death of the animals (12). These phenotypical abnormalities are associated with an altered fatty acid composition of epidermal ceramides (12). Recently, a corresponding mutation in humans has been identified, with affected individuals suffering from congenital ichthyosis, hence showing a predominant skin phenotype as well (13). In Caenorhabditis elegans, the loss of acs-20 and acs-22 genes that are homologous to mammalian Fatp4 induced severely disrupted barrier function, and this is associated with reduced incorporation of exogenous very long chain fatty acids into sphingomyelin (14).

Nonetheless, the actual role of Fatp4 in tissues other than skin still remains unresolved. The conflicting results yielded by experiments from cultured cells suggest that it might be questionable to draw conclusions regarding metabolically active tissue in vivo. Furthermore, mice featuring a constitutive Fatp4 knock-out die shortly after birth and therefore are not available for further in vivo studies (12). For this reason, we herein generated a mouse model with adipocyte-specific inactivation of the Fatp4 gene to elucidate its function in this complex tissue highly active in fatty acid metabolism.

**EXPERIMENTAL PROCEDURES**

**Generation of Adipose Tissue-specific Fatp4 Mutant Mice**—The method for generation of mutant mice with tissue-specific Fatp4 inactivation has been established in our laboratory as shown previously for knock-out in the epidermis (15). To generate mutant animals with adipocyte-specific Fatp4 inactivation, we used mice heterozygous for the Fatp4floxed allele and the floxed Fatp4 allele (Fatp4flx). Interbreeding with aP2-Cre transgenic mice that express Cre recombinase under the control of the adipose-specific aP2 promoter (strain 005069 B6.Cg-Tg(Fabp4-Cre)1Rev/J, The Jackson Laboratory, Bar Harbor, ME) resulted in the excision of exon 3 (Fatp43lox3) specifically in adipose tissue (Fatp43lox3/−/−). Genomic DNA was isolated from tissues by standard techniques. Fatp4 genotyping was performed by PCR with primer pairs specific for the Fatp4flx and the Fatp43lox3, respectively (15).

**Immunoblot Analysis**—Tissues were homogenized in PBS containing protease inhibitor. Samples were separated by SDS-PAGE and transferred to PVDF membranes for detection. The characterization of affinity-purified antibodies against Fatp4 has been described (8). Protein abundance was quantified by densitometry using the ImageJ software.

**Animal Experiments**—Animals were maintained on regular laboratory chow (V1554, sniff, Soest, Germany) or on a high fat diet (content of long chain fatty acids (LCFA), 20%; metabolizable energy from lipids, 41%; E15144-34 sniff, Soest, Germany) for feeding experiments starting at the age of 6 weeks. Body weight and food consumption were assessed weekly for an observation period of 18 weeks. Four hours before sacrifice, mice were deprived of food. For a distinct set of experiments, mice fed a standard chow diet were fasted for 48 h. Body weight was assessed before and after fasting, and serum was analyzed for NEFA and glycerol. All experiments and procedures were in compliance with the guidelines of the institutional animal care and use committees and in accordance with governmental guidelines.

**Blood Parameters**—Serum adipokine and insulin levels were determined using commercial ELISA kits (R&D Systems, Wiesbaden, Germany; Millipore, Schwalbach, Germany). Serum levels of glucose, glyceral, triacylglycerol, and NEFA were quantified using commercial enzymatic kits (BioVision, Heidelberg, Germany). Serum phospholipids, sphingolipids, cholesterol, and cholesteryl esters were quantified by electrospray ionization-tandem mass spectrometry as described before (12).

**Histological Analysis and Immunohistochemistry**—Organs were fixed in 4% neutral formaldehyde solution and embedded in paraffin. Sections were cut at 3 μm and stained with hematoxylin/eosin. Measurements of adipocyte circumference and subcutaneous adipose tissue thickness were conducted using CellA software (Olympus, Hamburg, Germany).

Hepatic fat content was determined microscopically on H&E-stained slides with the point-counting technique in a high power field (magnification ×400) using a special ocular grid. For immunohistochemical examination of lipid droplet morphology, paraffin sections were stained with mouse monoclonal antibodies against perilipin (clone Peri112.17, catalog no. 651156; Progen Biotechnik, Heidelberg, Germany) and guinea pig polyclonal antibodies against adipophilin (catalog no. GP40; Progen Biotechnik).

**Real Time PCR**—Total RNA was isolated using a commercially available kit (RNAqueous, Ambion, Darmstadt, Germany), followed by DNase digestion (DNA-free, Ambion) and 1 μg of total RNA was reverse-transcribed using the Transcriptor First Strand cDNA synthesis kit (Roche Applied Science), following the manufacturer’s instructions. Quantitative real time PCR was carried out in a 20-μl reaction volume using SYBR Green I dye on the LightCycler carousel-based system (Roche Applied Science). Primers were specifically designed to amplify Fatp1, Fatp2, Fatp5, Fas, Scd-1, Acc-1, Fat/CD36, Fabp4, caveolin-1, Atg1, Hsl, and perilipin. Primer sequences are indicated in Table 1. Transcript abundance was quantified relative to the expression of the housekeeping gene β-actin, using ReQuant software (Roche Applied Science). Adipose mRNA expression of TNFα, MCP-1, diacylglycerol O-acyltransferase 1 (DGAT1), diacylglycerol O-acyltransferase 2 (DGAT2), 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2), and glycerol-3-phosphate acyltransferase was analyzed by real time PCR using the Assays-on-Demand TaqMan gene expression assays with TaqMan® universal PCR master mix (Applied Biosystems) and run on an Applied Biosystem 7500 real time PCR machine using Assays-on-Demand TNFα (Mm0043258), MCP-1 (Mm004412), DGAT1 (Mm00515643), DGAT2 (Mm0127395), AGPAT2 (Mm00458880), and glycerol-3-phosphate acyltransferase (Mm00833328) TaqMan® primers. mRNA of the target was normalized to housekeeping gene GAPDH.

**Postprandial Lipid Distribution**—After a 4-h fast, mice were given an intragastric 10 ml/kg olive oil bolus containing 0.75 μCi/100 μl [9,10-3H]palmitic acid and 0.75 μCi/100 μl [1-14C]lignoceric acid (Hartmann Analytic, Braunschweig, Germany). After additional 4 h of food deprivation, mice were sacrificed. Tissues were homogenized and 3H and 14C content
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### TABLE 1

| Primer sequences for RT-PCR | F is forward and R is reverse |
|----------------------------|-----------------------------|
| Fatp1 F                    | 5′-CGG TTT CTT CGG GCT ATC GTC TGC AAG-3′ |
| Fatp1 R                    | 5′-AAG ATG CAC GGG ATC GTG TCT-3′ |
| Fatp2 F                    | 5′-CTG ATG ATC GAC GGT GAG AA-3′ |
| Fatp2 R                    | 5′-TAC CAG TCC CAC GAT GTC AG-3′ |
| Fatp5 F                    | 5′-GCT TTT TGG ATC ATT CCT GTG GA-3′ |
| Fatp5 R                    | 5′-GAA GGG TGG GTT CTT TGG AA-3′ |
| CD36 F                     | 5′-TGG AGC GTG TAT TGG TGC AG-3′ |
| CD36 R                     | 5′-TGG GTG TTT CAC ATC AAA GA-3′ |
| Caveolin-1 F               | 5′-GGG AGA ACA GAC ATG TCT TGG-3′ |
| Caveolin-1 R               | 5′-TTT AAA ACA GAC ACT GGC ATG G-3′ |
| Fabp4 F                    | 5′-ACC GCA GAC GAC AGG AAG-3′ |
| Fabp4 R                    | 5′-GCC CTG TCA TAA ACT CTT GTG G-3′ |
| Aco-1 F                    | 5′-GGG GGA GGA GCT CCT AAT TC-3′ |
| Aco-1 R                    | 5′-GGT GAT TTT CTT CTT GAC CAG G-3′ |
| Scd-1 F                    | 5′-GGG AACT ATG GAT GGT GAG GGG-3′ |
| Scd-1 R                    | 5′-TCC GCA AAA AGG AAA GAA G-3′ |
| Fab F                      | 5′-CAG CCT CCT AAG CCA GGA G-3′ |
| Fab R                      | 5′-CCT CCA CAG ACA AGA TAG G-3′ |
| Atgl F                     | 5′-AGC AGG TGG AAC TGC GAC-3′ |
| Atgl R                     | 5′-TGG TGC AGA AGA GAC CCA G-3′ |
| Perilipin F                | 5′-GGT AGA AGA GGG TCC ATG AGG-3′ |
| Perilipin R                | 5′-TGG AAG GTG ATG GGT GAG GGG-3′ |

**Lipid Analysis**—Lipid composition in adipose and liver tissues was assessed by electrospray ionization-tandem mass spectrometry and by TLC as described before (12, 16). For the determination of NEFA, adipose tissue was homogenized in a buffer containing 50 mM Hepes, 137 mM NaCl, 1 mM MgCl2, 1 mM CaCl2, 10 mM sodium pyrophosphate, 10 mM sodium fluoride, 2 mM EDTA, 1% Nonidet P-40, 0.25 mM PMSF, and proteinase inhibitor followed by extraction with 2:1 chloroform/methanol. The organic phase was dried under a stream of nitrogen, and lipids were taken up in 3:2 hexane/isopropanol. Free fatty acid quantification kit (BioVision) was used to determine NEFA content in subcutaneous adipose tissue.

**Fatty Acyl-CoA Synthetase Assay**—Samples were assayed for total acyl-CoA synthetase activity by the conversion of [3H]oleate to its CoA derivative as described elsewhere (8).

**Statistical Analysis**—Data are shown as mean values ± S.E. Statistical analysis was performed using Student’s t test (two-tailed, unequal variance). p values of <0.05 and <0.01 were considered significant and highly significant, respectively.

**RESULTS**

**Generation of Fatp4 Mutant Mice**—Mutant mice with adipose tissue-specific Fatp4 inactivation were generated by interbreeding Fatp4<sup>floxed/excised</sup> mice with p2-Cre transgenic mice (Fig. 1A). PCR analysis was used to determine the different genotypes and to detect Cre-mediated adipocyte-specific recombination of the allele Fatp4<sup>floxed</sup> to the allele Fatp4<sup>excised</sup>. PCR (Fig. 1B) yielded specific conversion of the floxed Fatp4 allele (Fatp4<sup>flox</sup>) into the null allele Fatp4<sup>excised</sup> in p2-Cre transgenic mice. Using Western blot analysis, a residual Fatp4 protein expression of ~11% in adipose tissue of Fatp4<sup>−/−</sup> mice could be detected (Fig. 1C).

**Evidence for Undisturbed Uptake of Fatty Acids into Fatp4-deficient Adipocytes**—To investigate postprandial distribution of different fatty acids, an intragastric bolus of a [9,10-3H]palmitic acid and [1-14C]lignoceric acid mixture was administered to both normal and Fatp4 mutant mice. Four hours after oil gavage, the amount of radioactivity was measured in different organs.

In both types of adipose tissue, no significant differences in radioactivity could be detected among normal and mutant mice (Fig. 2). All values are expressed as percentage of total radioactivity administered, related to 100 mg of tissue wet weight. There was an uptake of 0.042 ± 0.009% of total [3H]palmitic acid in visceral and subcutaneous white adipose tissue (WAT) in 10-week-old mice. After a 4-h fast, mice were given an intragastric fat load containing [3H]palmitic acid and [1-14C]lignoceric acid. After additional 4 h of food deprivation, mice were sacrificed and adipocytes were homogenized, and radioactivity was quantified by liquid scintillation counting. No significant differences could be detected regarding content of long chain and very long chain fatty acids in visceral and subcutaneous adipose tissue. Data represent mean ± S.E., n = 5 (n = 3 for subcutaneous white adipose tissue control). epid. WAT, epididymal white adipose tissue; dorsol.WAT, dorsolumbar white adipose tissue.

**3H]palmitic acid (C16:0) and [1-14C]lignoceric acid (C24:0) mixture** was administered to both normal and Fatp4 mutant mice. Four hours after oil gavage, the amount of radioactivity was measured in different organs.

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**FIGURE 1. Fatp4 knock-out strategy and recombination verification. A, tissue-specific Fatp4 inactivation strategy by means of Cre-mediated recombination. Cre recombines into the allele Fatp4<sup>excised</sup>, lacking exon 3. loxP sites are depicted as open triangles. B, PCR genotyping of different tissues isolated from 10-week-old mice heterozygous for the floxed Fatp4 allele Fatp4<sup>flox</sup> and the null allele Fatp4<sup>−/−</sup> carrying the p2-Cre transgene. Recombination of Fatp4<sup>flox</sup> into Fatp4<sup>−/−</sup> occurs specifically in adipose tissue in these mice. WAT, white adipose tissue. C, Western blot analysis of protein isolated from epididymal adipose tissue of mice heterozygous for the floxed Fatp4 allele Fatp4<sup>flox</sup> carrying the p2-Cre transgene (Fatp4<sup>−/−</sup>; Blot of two representative animals) and mice homozygous for the floxed Fatp4 allele Fatp4<sup>flox</sup> (control).**

**FIGURE 2. No alterations in fatty acid uptake in Fatp4 mutant mice. Content of 9,10-3H-labeled fatty acids (A) and 1-14C-labeled fatty acids (B) in subcutaneous and visceral white adipose tissue (WAT) in 10-week-old mice. After a 4-h fast, mice were given an intragastric fat load containing [3H]palmitic acid and [1-14C]lignoceric acid. After additional 4 h of food deprivation, mice were sacrificed and adipocytes were homogenized, and radioactivity was quantified by liquid scintillation counting. No significant differences could be detected regarding content of long chain and very long chain fatty acids in visceral and subcutaneous adipose tissue. Data represent mean ± S.E., n = 5 (n = 3 for subcutaneous white adipose tissue control). epid. WAT, epididymal white adipose tissue; dorsol.WAT, dorsolumbar white adipose tissue.**
assessed in skeletal muscle, heart, and liver. Again, no significant differences in fatty acid uptake could be detected (data not shown). Thus, the lack of Fatp4 in visceral and subcutaneous adipocytes did not impair the uptake of long chain (palmitic acid, C16:0) or very long chain (lignoceric acid, C24:0) fatty acids. Likewise, adipocyte deficiency of Fatp4 did not cause alterations in postprandial fatty acid uptake into other organs involved in lipid metabolism.

**Phenotype of Fatp4 Mutant Mice**—Mice with adipocyte-specific Fatp4 deficiency were born at a normal Mendelian ratio. Mutant mice of both sexes were viable and fertile and showed no gross anatomic or behavioral abnormalities. For further analyses, only male mice were used.

Under standard breeding conditions while being fed a normal laboratory chow, Fatp4<sup>−/−</sup> mice displayed unremarkable development. Compared with normal animals, there were no statistical differences regarding body weight at different ages (6 and 24 weeks and 10 months; data not shown). During an observation period of 18 weeks, Fatp4 mutant and normal mice exhibited similar weight gain when fed a standard laboratory chow diet (69.8 ± 8.2% versus 65.6 ± 5.8% of initial weight, respectively; Fig. 3A). When sacrificed at an age of 24 weeks, no statistically significant differences between the groups regarding organ weight of epididymal adipose tissue, liver, heart, spleen, or kidney could be observed (data not shown). Gross histological examination of these organs of adipocyte Fatp4<sup>−/−</sup> mice yielded no abnormalities, and in particular epididymal as well as subcutaneous adipose tissue exhibited normal morphology.

Thus, under standard conditions no obvious phenotypical changes in mutant mice could be detected. Furthermore, total acyl-CoA synthetase activity for oleate remained unchanged in adipose tissue of Fatp4<sup>−/−</sup> mice under standard conditions (12.3 ± 2.1 versus 11.7 ± 2.4 pmol of oleoyl-CoA/min/μg of protein in normal and mutant mice, p > 0.05).

To reveal more subtle effects of adipocyte Fatp4 deficiency, normal and mutant animals were administered a special diet containing 20% of long chain fatty acids and holding 40% metabolizable energy from lipids. Body weight and food intake were assessed regularly for 18 weeks. During this observation period, mice lacking Fatp4 in adipocytes gained significantly more weight compared with controls (72.1 ± 3.26 kcal/g in knock-out versus 117.2 ± 5.01 kcal/g in normal mice; *, p < 0.01, n = 6).

The more pronounced weight gain in Fatp4<sup>−/−</sup> mice could not be attributed to an altered food consumption, as food intake in relation to body weight was virtually identical in the two groups (Fig. 3C). More strikingly, Fatp4 mutant animals even needed significantly less calories from food to gain 1 g of body mass compared with normal mice (92.1 ± 3.26 kcal/g in knock-out versus 117.2 ± 5.01 kcal/g in normal mice; *, p < 0.01; Fig. 3D). The difference in weight gain reached statistical significance after week 10 on the diet.

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**Fatp4 Mutant Mice Develop Adipocyte Hypertrophy and Fatty Liver**—Being fed a diet rich in fat and long chain fatty acids, Fatp4<sup>−/−</sup> knock-out mice developed a higher grade of obesity, although they were not consuming more food. Histological examination revealed that their pronounced weight gain was reflected in a significantly thicker layer of subcutaneous fat (465.3 ± 39.17 μm in knock-out versus 315.3 ± 31.85 μm in normal mice; p < 0.05; Fig. 4A). Furthermore, adipocytes in subcutaneous fat lacking Fatp4 possessed a significantly greater circumference compared with normal adipocytes (233.35 ± 8.95 μm in knock-out versus 196.51 ± 8.82 μm in normal mice;
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FIGURE 5. Hepatic steatosis in Fatp4 mutant mice. A, degree of fatty hepatocyte degeneration with 33.0 ± 8.56% versus 13.1 ± 3.67% fat in hepatocytes in knock-out and normal mice, respectively (p = 0.06, n = 6). B, representative H&E-stained slides of liver tissue of control and Fatp4A/-/ mice (×10 magnification).

FIGURE 6. No signs of increased insulin resistance in Fatp4 mutant mice. A, serum glucose levels under high fat/high LCFA diet and chow diet. B, serum insulin levels in Fatp4 knock-out and control mice being fed a high fat/high LCFA diet (11.7 ± 1.9 milliunits/liter versus 14.3 ± 0.5 milliunits/liter; p > 0.05). C, quantitative insulin sensitivity check index (QUICKI) (6.0 ± 0.87 versus 5.8 ± 1.29) and HOMA index (0.3 ± 0.01 versus 0.3 ± 0.01) in Fatp4 mutant and control mice being fed a high fat/high LCFA diet. Data represent mean ± S.E., n = 5–6.

p < 0.05; Fig. 4B). Epididymal adipocytes of knock-out animals displayed a tendency toward a greater circumference (322.7 ± 10.1 μm in knock-out versus 282.8 ± 17.45 μm in normal mice; p = 0.07).

Although there were no differences regarding organ weight between animal groups under a standard diet, epididymal fat depots of Fatp4A/-/ mice fed a high fat/high LCFA diet were significantly heavier (1.82 ± 0.11 g in knock-out versus 1.37 ± 0.16 g in normal mice; p < 0.05; Fig. 4C). Adipocyte hypertrophy in visceral adipose tissue of Fatp4A/-/ animals under a high fat/high LCFA diet, however, was not accompanied by significant changes in expression levels of genes involved in triacylglycerol synthesis (AGPAT2, glycerol-3-phosphate acyltransferase, DGAT1, DGAT2; data not shown).

No further histological abnormalities could be detected in adipose tissue with Fatp4 deficiency exposed to a high fat diet. There were no hints of any increased inflammation within adipose tissue; mRNA levels of TNFα and MCP-1 remained unchanged (data not shown).

Immunohistochemical staining for perilipin also reflected the larger circumference of fat droplets in Fatp4 knock-out compared with normal adipose tissues (data not shown). No increased staining for adipophilin (synonyms: perilipin-2; adipose differentiation-related protein), which is known to be expressed in maturing or brown adipose tissue, was detected in Fatp4A/-/ mice. Thus, increased body weight in Fatp4 mutant animals under a high fat diet reflected adipocyte hypertrophy of subcutaneous and visceral fat depots without accompanying alterations in morphology or inflammation.

Skeletal muscle, myocardium, and small intestine of Fatp4A/-/ mice showed no histological signs of increased lipid accumulation (data not shown). However, Fatp4A/-/ animals exhibited a greater incidence of fatty liver, which was reflected in significantly higher liver weights of mutant mice (2.7 ± 0.24 g in knock-out versus 2.0 ± 0.03 g in normal mice; p < 0.05). Almost all livers of adipocyte-specific Fatp4 knock-out mice displayed significant degrees of fatty degeneration (33.0 ± 8.56% versus 13.1 ± 3.67% fat in hepatocytes of knock-out and normal mice, respectively; p = 0.06; Fig. 5, A and B).

Obesity in Fatp4 Mutant Mice Does Not Induce Insulin Resistance—As obesity frequently is accompanied by abnormalities in glucose homeostasis, we investigated serum levels of glucose and insulin in Fatp4 mutant and normal mice.

The type of diet administered had no influence on serum glucose levels; animals administered a diet enriched in fat and LCFA had similar serum glucose levels compared with animals under a chow diet (207 ± 3.3 mg/dl versus 204 ± 4.8 mg/dl; p > 0.05; Fig. 6A). Also, no inter-group differences regarding serum glucose levels could be identified. Furthermore, insulin levels between normal and Fatp4A/-/ mice were comparable under chow as well as under high fat/high LCFA diet (Fig. 6B).

To evaluate insulin sensitivity, homeostasis model assessment (HOMA) index and quantitative insulin sensitivity check index were calculated. Again, no hints at an increased insulin resistance in Fatp4 mutant animals could be detected (HOMA, 6.0 ± 0.87 versus 5.8 ± 1.29; quantitative insulin sensitivity check index, 0.3 ± 0.01 versus 0.3 ± 0.01 in normal and mutant animals respectively, p > 0.05; Fig. 6C). Hence, adipose hypertrophy and fatty liver of mice with adipocyte-specific Fatp4 deficiency were not associated with decreased insulin sensitivity.

Adipokine Levels in Fatp4 Mutant Mice—To further elucidate the increase in body weight of Fatp4A/-/ mice, serum levels of major adipokines in samples of animals under high fat diet were determined.
In both Fatp4 mutant and normal mice being fed a high fat diet, serum levels of resistin and adiponectin were identical (resistin, 23.1 ± 2.77 ng/ml; adiponectin, 6.98 ± 0.42 ng/ml; p > 0.05). The increase in adipose fat mass was contrasted by a decrease in total phosphatidylethanolamine as well as fatty acid fractions with regard to number and location of double bonds and chain length. Under standard diet for both types of adipose tissue, total amounts of phospholipids, cholesteryl esters (CE), free cholesterol, sphingomyelins (SM), ceramides (CER), and plasmalogens (PE-pl) in subcutaneous adipose tissue of Fatp4 knockout animals contained significantly higher serum levels of leptin (30.99 ± 3.94 ng/ml versus 17.76 ± 2.77 ng/ml; p < 0.05; Fig. 7). In contrast, mice lacking Fatp4 in adipocytes exhibited significantly higher serum levels of resistin (6.98 ± 0.42 ng/ml versus 23.1 ± 2.77 ng/ml; p < 0.05; Fig. 8). In contrast, mice lacking Fatp4 in adipocytes, triacylglycerol content was significantly less phospholipids, cholesteryl esters (CE), free cholesterol as well as fatty acid fractions with regard to number and location of double bonds and chain length. Table 2 shows the lipid composition of subcutaneous adipose tissue of Fatp4A−/− knockout and control mice, respectively. Data represent mean ± S.E., n = 6.

In summary, although under standard conditions no significant changes in fatty acid composition of adipose tissue due to Fatp4 deficiency could be demonstrated, exposure to a high fat diet had profound effects on different lipid species in subcutaneous adipose tissue of Fatp4A−/− mice (2.26 ± 0.3 versus 2.74 ± 0.21 nmol/mg, p > 0.05).

In epididymal adipose tissue, the consequences of Fatp4 knock-out on lipid composition were much less distinctive. In comparison to normal tissue, a decrease in total phosphatidylethanolamine-based plasmalogens, and phosphatidylglycerol was comparable (Tables 2 and 3). The amount of nonesterified fatty acids was not changed in dorsolumbar adipose tissue of Fatp4A−/− mice (2.26 ± 0.3 versus 2.74 ± 0.21 nmol/mg, p > 0.05).

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**TABLE 2**

| Lipid Class       | Fatp4A−/− | Control | p value |
|-------------------|-----------|---------|---------|
| Phosphatidylcholine (PC) | 0.020** 0.058*  | 0.013 0.3  | **0.05** |
| Phosphatidylethanolamine (PE) | 0.005 0.052  | 0.017 0.022 | **0.02** |
| Phosphatidylserine (PS) | 0.001 0.019  | 0.001 0.016 | **0.01** |
| Phosphatidylglycerol (PG) | 0.000 0.008  | 0.001 0.008 | **0.01** |
| Ceramide (CER) | 0.001 0.020  | 0.001 0.014 | **0.01** |
| Plasmalogen (PE-pl) | 0.000 0.008  | 0.001 0.016 | **0.01** |

* Values are presented as nmol/mg wet weight.

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neous adipose depots. Only minor alterations were found in visceral adipose tissue of Fatp4 mutant mice. 

Alterations in Serum and Hepatic Lipid Composition in Fatp4 Mutant Mice—Fatp4 knock-out did not result in detectable alterations of lipid and fatty acid composition in adipose tissue of mice fed standard chow diet. In accordance with results from adipose tissue, no significant abnormalities in lipid content and composition neither in blood samples nor in hepatic tissue of those mice could be observed (data not shown).

Having been fed a diet enriched in fat and LCFA, Fatp4 mutant mice developed adipocyte hypertrophy and marked reduction in complex lipids and cholesteryl esters. Furthermore, Fatp4 deficiency in adipocytes of those animals affected serum lipid composition. Total content of phosphatidylcholine (3278.09 ± 175.8 versus 3879.42 ± 89.09 μM, p < 0.05), lysophosphatidylcholine (983.46 ± 51.67 versus 1172.13 ± 39.64, p < 0.05), CER (9.05 ± 0.71 versus 11.22 ± 0.54, p < 0.05), and CE (4826.96 ± 298.79 versus 5592.28 ± 86.66, p < 0.05) as well as triacylglycerol (0.36 ± 0.04 versus 0.53 ± 0.06 mmol, p < 0.05) was augmented in knock-out mice. However, serum level of nonesterified fatty acids was elevated only by trend (0.21 ± 0.02 versus 0.26 ± 0.03 nmol/μl, p > 0.05).

Livers of Fatp4-deficient animals under high fat diet weighed more and showed a higher degree of fatty degeneration. Therefore hepatic content of phospholipids, other complex lipids, cholesteryl esters, and triacylglycerol was analyzed. There were no significant differences in phosphatidylcholine, phosphatidylethanolamine, phosphatidylglycerol, phosphatidylglycerol, lysophosphatidylcholine, sphingomyelin, CER, phosphatidylethanolamine-based plasmalogens, and CE between normal and mutant animals (data not shown), whereas there was a strong trend toward increased liver triacylglycerol content in knock-out animals (798 ± 284 versus 1788 ± 498 μg/mg protein; p > 0.05).

Adipocyte-specific Fatp4 Deficiency Is Associated with Changes in Gene Expression—To investigate molecular changes associated with Fatp4 deletion in adipose tissue, expression of different lipid metabolic genes was investigated by quantitative real time PCR. Enzymes known to be involved in transmembrane and intracellular transport of fatty acids in adipocytes are fatty acid transport protein 1 (Fatp1), fatty acid translocase (Fat/CD36), caveolin-1, and fatty acid-binding protein 4 (Fabp4). Fatty-acid synthetase, acetyl-CoA carboxylase 1 (Acc1), and stearoyl-CoA desaturase 1 (Scd1) participate in de novo lipogenesis, whereas hormone-sensitive lipase (Hsl), adipose triglyceride lipase (Atgl), and perilipin are major enzymes of lipolysis or involved in the regulation of lipolysis.

Expression levels of Fat/CD36, Fabp4, and Fatp1 in Fatp4 mutant mice were decreased by 9.9% (2.6 ± 0.06 versus 2.34 ± 0.04 relative mRNA abundance; p < 0.01), 16.5% (0.194 ± 0.008 versus 0.162 ± 0.006; p < 0.05), and 26.4% (0.31 ± 0.06 versus 0.23 ± 0.02; p < 0.05), respectively. In addition, a tendency toward reduced mRNA levels of caveolin-1 (1.73 ± 0.09 versus 1.5 ± 0.05; p = 0.06) could be noted (Fig. 8A). No differences were found in Fas-, Acc1-, and Scd1-expression, suggesting regulation of de novo lipogenesis in adipocytes lacking Fatp4 was not disturbed.

In epididymal adipose tissue, a significant down-regulation of lipolytic genes by 24.8% for Hsl (0.78 ± 0.04 versus 0.58 ± 0.04; p < 0.05) and by 19.8% for Atgl (0.93 ± 0.03 versus 0.75 ± 0.04; p < 0.01) was observed, whereas the expression of perilipin showed a tendency to be increased (5.33 ± 0.68 versus 6.91 ± 0.48, p > 0.05; Fig. 8B).

In subcutaneous adipose tissue, no significant changes in expression of lipolysis genes could be detected (data not shown). In liver tissue, expression levels of all investigated Fatps (Fatp1, Fatp2, Fatp4, and Fatp5) as well as of

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### TABLE 5

| Fatp4 Status | Status | Monounsaturated | Polyunsaturated | Total | FC |
|-------------|--------|-----------------|-----------------|-------|----|
| CE Control  | Mean† | 0.052 ± 0.008   | 0.010 ± 0.010   | 0.062 ± 0.002 | 1.163 ± 0.037 |
| Fatp4 A/−   | Mean† | 0.047 ± 0.008   | 0.010 ± 0.007   | 0.057 ± 0.001 | 1.247 ± 0.027 |
| SM Control  | Mean† | 0.040 ± 0.007   | 0.001 ± 0.001   | 0.041 ± 0.001 | 0.006 ± 0.001 |
| Fatp4 A/−   | Mean† | 0.039 ± 0.007   | 0.001 ± 0.001   | 0.040 ± 0.001 | 0.006 ± 0.001 |
| CER Control | Mean† | 0.008 ± 0.001   | 0.000 ± 0.000   | 0.008 ± 0.000 | 0.000 ± 0.000 |
| Fatp4 A/−   | Mean† | 0.007 ± 0.001   | 0.000 ± 0.000   | 0.007 ± 0.000 | 0.000 ± 0.000 |

### TABLE 4

| Phase | Lipid Class | Fatp4 Status | Control | Mean ± S.E. | p Value |
|-------|-------------|--------------|---------|-------------|---------|
| PC | Monounsaturated | Fatp4 A/− | 0.019 ± 0.01 | 0.014 ± 0.00 | 0.001 ± 0.01 |
| PE | Monounsaturated | Fatp4 A/− | 0.000 ± 0.01 | 0.000 ± 0.01 | 0.000 ± 0.01 |
| PE | Polyunsaturated | Fatp4 A/− | 0.000 ± 0.01 | 0.000 ± 0.01 | 0.000 ± 0.01 |
| PE | Monounsaturated | Fatp4 A/− | 0.000 ± 0.01 | 0.000 ± 0.01 | 0.000 ± 0.01 |
| PE | Polyunsaturated | Fatp4 A/− | 0.000 ± 0.01 | 0.000 ± 0.01 | 0.000 ± 0.01 |

* Further lipid classification was analogous to Table 2.
* Values are presented as nmol/mg wet weight.

Adipocyte-specific Fatp4 Deficiency Is Associated with Changes in Gene Expression—To investigate molecular changes associated with Fatp4 deletion in adipose tissue, expression of different lipid metabolic genes was investigated by quantitative real time PCR. Enzymes known to be involved in transmembrane and intracellular transport of fatty acids in adipocytes are fatty acid transport protein 1 (Fatp1), fatty acid translocase (Fat/CD36), caveolin-1, and fatty acid-binding protein 4 (Fabp4). Fatty-acid synthetase, acetyl-CoA carboxylase 1 (Acc1), and stearoyl-CoA desaturase 1 (Scd1) participate in de novo lipogenesis, whereas hormone-sensitive lipase (Hsl), adipose triglyceride lipase (Atgl), and perilipin are major enzymes of lipolysis or involved in the regulation of lipolysis.

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Adipocyte-specific Fatp4 Deficiency in Mice

Although Fatp4 was previously thought to play a role in transport of fatty acids across the plasma membrane, more recent data have shown that Fatp4 is dispensable for fatty acid uptake in different cell types (5, 10, 11). Supporting this notion, uptake of radiolabeled fatty acids into adipose tissue in our study was undisturbed in Fatp4−/− mice. Milger et al. (8) showed in various cell lines that Fatp4 is localized to the endoplasmic reticulum rather than near the plasma membrane. The membrane of the ER is known to embed proteins that act in synthesis and allocation of phospholipids. Furthermore, Fatp4 has been shown to exhibit acyl-CoA synthetase activity with some predilection for LCFA (3). Nonetheless, the actual physiological function of Fatp4 in most tissues still is unresolved.

In this study, adipocyte-specific Fatp4 knock-out in mice exposed to normal dietary fat content yielded no phenotypical abnormalities. Only when challenged with a diet rich in LCFA, Fatp4−/− mice developed pronounced obesity and held an altered adipose tissue lipid composition with a decreased content of different phospholipids, sphingolipids, and cholesteryl esters. Based on these results, we conclude that the physiological function of Fatp4 in adipose tissue is strongly associated with synthesis of phospholipids and other complex lipids, presumably by providing activated fatty acids via its acyl-CoA synthetase function. Evidence for a potential role for Fatp4 in phospholipid synthetic pathways has been presented in a number of reports. We have previously shown that constitutive Fatp4-null mice exhibit a considerable alteration in skin phospholipid composition (12). Furthermore, Jia et al. (5) found reduced incorporation of certain very long chain fatty acids into major phospholipid species using dermal fibroblast cell lines from Fatp4−/− mice. The Fatp4 knock-out in our experimental system was apparently not complete, as attested by the residual ~11% of Fatp4 protein detected by Western blot. When dietary fat uptake was within normal range, the lack of Fatp4 may have been compensated by other acyl-CoA synthetases with LCFA specificity, for example, so the remaining Fatp4 was sufficient to preserve phospholipid synthesis. In accordance with this hypothesis, total oleoyl-CoA synthetase activity in our study was unimpaired in adipocytes lacking Fatp4. Feeding a special diet containing 20% of LCFA severely enhanced metabolic work load of adipocytes, thereby leading to the observed phenotypical changes in knock-out mice.

The second striking result of this study was the pronounced weight gain Fatp4−/− mice exhibited under a special diet enriched in LCFA, reflected in adipocyte hypertrophy, heavier epididymal fat pads, and thicker subcutaneous fat layers. In contrast, mice deficient of Fatp1, another member of the FATP family expressed in adipose tissue, were protected against diet-induced obesity (17). However, the physiological role of Fatp1 in insulin-induced cellular uptake of fatty acids is much better characterized (18), although its homologue Fatp4 is involved neither in basal nor in insulin-stimulated fatty acid transport across the plasma membrane (10). Parallel to our findings, Jia et al. (5) observed an increased incorporation of labeled palmitate into triacylglycerol in Fatp4 mutant fibroblasts along with abnormal lipid droplet structure and size and concluded that increased triacylglycerol uptake and storage may be the underlying cause. Furthermore, in enterocytes of Fatp4-null mice with a rescued skin phenotype fed a Western diet, Shim et al. (11) detected a significant increase in triacylglycerol content. Accordingly, Lobo et al. (10) revealed a decreased incorporation of [3H]palmitate into acyl-CoA pools but not triglyceride pools in Fatp4 knockdown adipocytes. We hypothesize that the lack of Fatp4 acyl-CoA synthetase activity in our experimental system leads to fatty acid channeling to other functional compartments within the ER, and the subsequent incorporation of fatty acids likely activated by other acyl-CoA synthetases into triacylglycerols rather than into complex lipids. As the synthesis of triacylglycerols is energetically more favorable compared with the synthesis of phospholipids, this may explain the more efficient feed conversion observed in Fatp4−/− mice. However, alternative explanations as to an alteration in energy expenditure of Fatp4−/− animals also exist. It has long been accepted that phospholipids and phospholipid-derived molecules may function as signaling transmitters (19). Some of those lipid-derived transmitters are able to affect metabolic processes and energy homeostasis. For instance, endocannabinoids are anabolic lipid mediators that increase the intake, promote the storage, and decrease the expenditure of energy (20).

Phospholipids can also influence adipocyte metabolism in the context of them being integral components of the plasma membrane and organelle membranes. Phosphatidylinositol forms the precursor molecule for glycosylphosphatidylinositol as part of glycosylphosphatidylinositol-anchored proteins. A number of those glycosylphosphatidylinositol-anchored proteins on the other hand are known to regulate processes like lipolysis and lipogenesis (21, 22). However, it is unlikely that adipose hypertrophy in our study arose from increased de novo lipogenesis, because the expression of major enzymes involved in the latter remained unchanged.
Adipocyte-specific Fatp4 Deficiency in Mice

To further elucidate the pronounced weight gain and enhanced feed conversion in Fatp4A−/− mice, we studied plasma levels of major cytokines. Leptin, adiponectin and resistin are known to regulate energy expenditure and food consumption (23, 24). Plasma levels of the latter two adipokines were not changed in adipocyte Fatp4A knock-out animals. Only leptin was significantly increased in Fatp4A−/− mice. However, as obese subjects are known to develop leptin resistance, we interpret this as a secondary effect of increased body fat mass (25, 26).

The above specified changes in lipid composition could be observed only in subcutaneous but not in epididymal adipose tissue. In particular, the content of complex lipids in visceral adipose tissue was not altered. Notwithstanding, the circumstance of epididymal adipocytes showed a tendency to increase, and epididymal fat pads of Fatp4A mutant animals were significantly heavier compared with control mice. There is ample data highlighting multiple differences between visceral and subcutaneous adipose tissue concerning their biological function (27).

Thus, we conclude that Fatp4 has different physiological functions in visceral and subcutaneous adipose tissue, and its deficiency leads to different metabolic disturbances somehow or other culminating in adipocyte hypertrophy. Whereas in subcutaneous samples lipolysis seemed uninfluenced, in epididymal tissue there was a significant decrease of lipolysis enzyme expression levels accompanied by an (yet not significant) increase in perilipin expression. Lobo et al. (10) proposed a role for Fatp4 in re-esterification of fatty acids originating from basal lipolysis. Our findings with respect to epididymal adipose tissue support this theory in terms of a feedback mechanism, as the lack of Fatp4 leads to down-regulation of lipolysis and thereby preserves homeostasis by preventing an accumulation of potentially harmful nonesterified fatty acids within the cell. In another set of experiments, 15-week-old mice were fasted for 48 h after being fed a normal chow diet, and serum content of NEFA and glycerol was assessed. Here, no differences between mutant and normal mice were detected. Thus, the lack of Fatp4 alone seems to have no impact on fatty acid mobilization from adipose tissue.

Obesity is commonly linked with insulin resistance and disturbed glucose metabolism. In this study, Fatp4A−/− animals exhibited no signs of impaired glucose sensitivity compared with control mice. There are various other mouse models in which dissociation between obesity and insulin resistance was observed. Thus, the notion emerged that not the absolute fat mass but the capacity of adipose tissue to store lipids and thereby protecting other organs from fatty acid overload determines insulin sensitivity (28–30). In line with this, we did not detect any disruption of fatty acid uptake into adipose tissue or an increase of nonesterified fatty acids in the serum of Fatp4A mutant mice, although they showed a trend toward an increased hepatic steatosis, accompanied by an elevated triacylglycerol serum level. Furthermore, although obesity is frequently associated with chronic inflammation as a first step toward insulin resistance (31), mRNA levels of TNFα and MCP-1 in adipose tissue of Fatp4-deficient mice were unremarkable.

In summary, our findings suggest that Fatp4 plays a crucial and, depending on the kind of adipose tissue, distinct role in adipocyte metabolism. Adipocyte-specific Fatp4 deficiency results in profound disturbances in phospholipid, sphingolipid, and choleseryl ester synthesis in subcutaneous adipose tissue, whereas in visceral adipose tissue lipolysis is affected. As a consequence, obesity and hepatic steatosis without impairment of insulin sensitivity occur at the whole body level.

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