Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat

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Obesity resistance and multiple mechanisms of triglyceride synthesis in mice lacking Dgat

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Triglycerides (or triacylglycerols) represent the major form of stored energy in eukaryotes. Triglyceride synthesis has been assumed to occur primarily through acyl CoA:diacylglycerol transferase (Dgat), a microsomal enzyme that catalyses the final and only committed step in the glycerol phosphate pathway[1–3]. Therefore, Dgat has been considered necessary for adipose tissue formation and essential for survival. Here we show that Dgat-deficient (*Dgat*$^{-/-}$) mice are viable and can still synthesize triglycerides. Moreover, these mice are lean and resistant to diet-induced obesity. The obesity resistance involves increased energy expenditure and increased activity. Dgat deficiency also alters triglyceride metabolism in other tissues, including the mammary gland, where lactation is defective in *Dgat*$^{-/-}$ females. Our findings indicate that multiple mechanisms exist for triglyceride synthesis and suggest that the selective inhibition of Dgat-mediated triglyceride synthesis may be useful for treating obesity.

We previously identified and cloned a mammalian *Dgat* cDNA (ref. 4). To identify its biological functions, we inactivated *Dgat* in mice by replacing approximately 1 kb of the gene with a *neo* gene (Fig. 1*a*). *Dgat*$^{-/-}$ mice were viable (Fig. 1*b*) and were found in the expected 1:2:1 mendelian distribution from heterozygous intercrosses (wild type, 49; *Dgat*$^{+/-}$, 108; *Dgat*$^{-/-}$, 45; P=0.574 by χ^2 test). *Dgat*$^{-/-}$ mice were healthy and fertile. *Dgat* mRNA expression was undetectable in *Dgat*$^{-/-}$ mice and reduced in *Dgat*$^{+/-}$ mice (Fig. 1*c*). Tissue Dgat activity, measured by the incorporation of [^{14}C]oleoyl CoA into triglycerides in membrane preparations, was absent or reduced in *Dgat*$^{-/-}$ mice; we detected residual activity in *Dgat*$^{-/-}$ duodenum (~150 pmol/mg prot/min; Fig. 1*d*).

Although deficient in Dgat activity, *Dgat*$^{-/-}$ mice were capable of synthesizing triglycerides. Fasting serum triglyceride levels in *Dgat*$^{-/-}$ mice were similar to those in wild-type mice (Fig. 2*a*). *Dgat*$^{-/-}$ mice also had white adipose tissue, which appeared to be normal (Fig. 2*b*) and contained triglycerides as determined by thin-layer chromatography (Fig. 2*c*). The fatty acid composition of adipose tissue triglycerides was similar in chow-fed *Dgat*$^{-/-}$ and wild-type mice (data not shown).

We hypothesized that Dgat deficiency might result in less white adipose tissue and lower body weights in mutant mice. Wild-type and *Dgat*$^{-/-}$ mice fed a chow diet had similar body weights (Fig. 3*a*), but the weight of the total fat pads in *Dgat*$^{-/-}$ mice was lower than that in age-, weight- and sex-matched wild-type mice (Fig. 3*b*). Total carcass triglyceride content tended to be lower in 5–7-month-old *Dgat*$^{-/-}$ mice (7.7±2.9% versus 9.8±3.3% of body weight for wild-type mice; n=10 males each), although the differ-

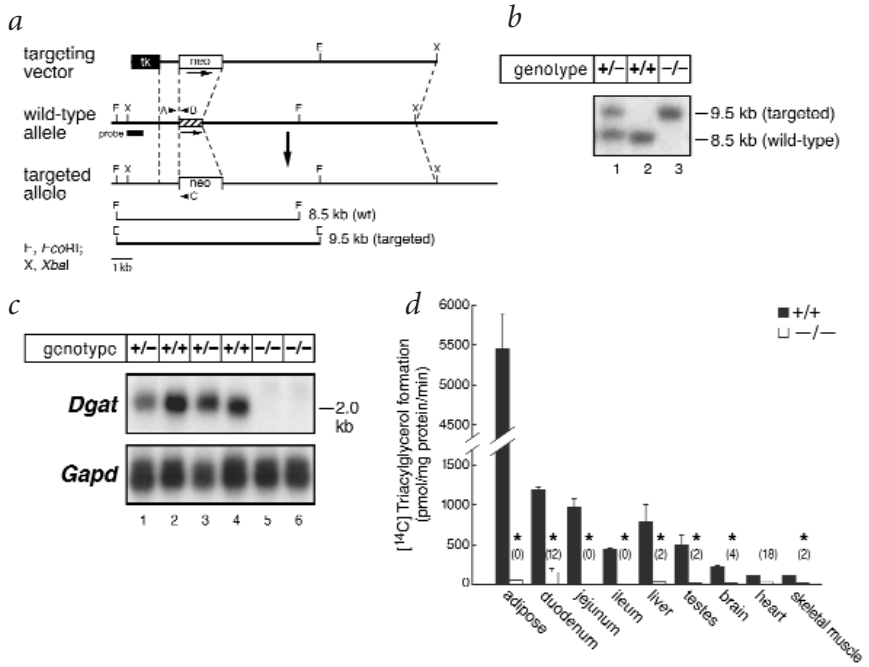

Fig. 1 Generation of *Dgat*-mutant mice. ***a***, Targeting strategy. Homologous recombination of the disruptive vector with the wild-type allele results in the replacement of ~1 kb of *Dgat* sequences (hatched bar) with *neo* and deletes the carboxy-terminal 27% of the Dgat protein. The targeted allele is identified by a 1-kb increase in an *Eco*RI restriction fragment when probed with the indicated 5′ external fragment. Targeting frequency was ~1 in 500. A, B and C represent primers used for genotyping mice. ***b***, Southern blot demonstrating *Dgat*$^{-/-}$ mice. Tail DNA was isolated from three-week-old F_1 offspring, and the genotype was determined by Southern-blot analysis. ***c***, Northern blot of *Dgat* mRNA expression in testes. Blots were reprobed with a *Gapd* fragment as a control to assess loading equivalency. ***d***, Dgat activity assays. Membrane preparations from various tissues were assayed for incorporation of [^{14}C]oleoyl CoA into triglycerides. Numbers in parentheses indicate the per cent of residual enzyme activity in the mutant membranes compared with wild-type values. Testes values are from four male mice of each genotype. Duodenum, ileum, jejunum, heart, skeletal muscle and brain values are from 2–4 female mice of each genotype. Adipose tissue (reproductive fat pads) and liver values are from four wild-type and four *Dgat*$^{-/-}$ mice of mixed sexes; values were similar for male and female mice. *P<0.01.

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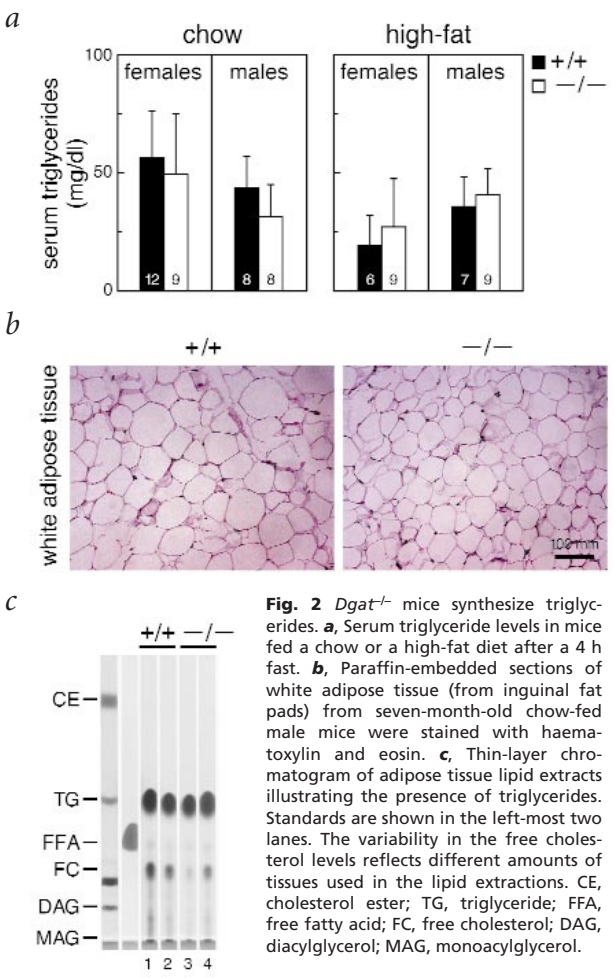

Fig. 2 *Dgat*$^{-/-}$ mice synthesize triglycerides. ***a***, Serum triglyceride levels in mice fed a chow or a high-fat diet after a 4 h fast. ***b***, Paraffin-embedded sections of white adipose tissue (from inguinal fat pads) from seven-month-old chow-fed male mice were stained with haematoxylin and eosin. ***c***, Thin-layer chromatogram of adipose tissue lipid extracts illustrating the presence of triglycerides. Standards are shown in the left-most two lanes. The variability in the free cholesterol levels reflects different amounts of tissues used in the lipid extractions. CE, cholesterol ester; TG, triglyceride; FFA, free fatty acid; FC, free cholesterol; DAG, diacylglycerol; MAG, monoacylglycerol.

ence was not significant. Carcass protein content was similar in wild-type and *Dgat*$^{-/-}$ mice (12.2±1.1% and 13.5±0.5% of body weight for five wild-type and five *Dgat*$^{-/-}$ mice, $P>0.06$). Intrascapular brown adipose tissue was similar in quantity (6.4±2.6 and 5.1±1.2 mg/g body weight for 1-week-old wild-type and *Dgat*$^{-/-}$ mice, $P=0.44$) and histologic appearance in wild-type and *Dgat*$^{-/-}$ mice.

Wild-type male mice fed a high-fat diet reached an average weight of 45–50 g, but *Dgat*$^{-/-}$ male mice stabilized at 30–35 g ($P<0.01$; Fig. 3*c*), a weight comparable to that of chow-fed mice. Similar results were observed in females (Fig. 3*c*). As in chow-fed mice, fat stores were reduced in *Dgat*$^{-/-}$ mice versus controls (Fig. 3*d*). Total carcass triglycerides were also lower in *Dgat*$^{-/-}$ mice (14.7±1.4 versus 21.8±1.7% of body weight for seven *Dgat*$^{-/-}$ and nine wild-type mice, $P=0.007$), whereas carcass protein was similar (6.5±0.3 versus 7.2±0.4% of body weight for *Dgat*$^{-/-}$ and wild-type mice, $P=0.19$). Thus, when fed a high-fat diet, *Dgat*$^{-/-}$ mice were resistant to obesity.

We investigated two possible mechanisms for the obesity resistance caused by Dgat deficiency: decreased caloric absorption and increased energy expenditure. We found no evidence for fat malabsorption. Fecal output and fecal caloric content (3.633±0.015 kcal/g in wild-type mice and 3.573±0.304 kcal/g in *Dgat*$^{-/-}$ mice) were similar in wild-type and *Dgat*$^{-/-}$ mice. There was also no evidence of increased fecal fat content in *Dgat*$^{-/-}$ mice on a chow or a high-fat diet (data not shown). To examine energy expenditure, we analysed weight-matched wild-type and *Dgat*$^{-/-}$ male mice in metabolic cages on a chow diet for two days and then for seven days on a high-fat diet. During chow feeding, *Dgat*$^{-/-}$ mice had higher metabolic rates than wild-type mice (0.526±0.068 versus 0.431±0.075 kcal/d/g, $P=0.021$); energy intakes were not significantly different in wild-type and *Dgat*$^{-/-}$ mice, (0.403±0.132 versus 0.333±0.130 kcal/d/g, $P=0.31$). During high-fat feeding, wild-type mice gained more weight than *Dgat*$^{-/-}$ mice (Fig. 3*e*). Energy intakes were not significantly different for wild-type and *Dgat*$^{-/-}$ mice ($P=0.42$), but *Dgat*$^{-/-}$ mice had higher metabolic rates ($P=0.003$; Fig. 3*f*).

This increased energy expenditure in Dgat deficiency was unexpected, and its mechanism is unclear. On the high-fat diet, *Dgat*$^{-/-}$ mice were twice as active as wild-type mice (Fig. 3*g*), indicating that increased activity contributed to the higher energy expenditure. Increased activity levels, however, did not account for the increased energy expenditure in chow-fed *Dgat*$^{-/-}$ mice. This increased energy expenditure did not result from greater lean body mass, which was similar in wild-type and *Dgat*$^{-/-}$ mice. Thermogenesis also appeared to be normal in *Dgat*$^{-/-}$ mice, as core body temperatures measured at 25 °C and after exposure to 4 °C for 24 hours were similar during both chow and high-fat feeding (data not shown). In addition, serum thyroxine levels were not elevated in *Dgat*$^{-/-}$ mice (data not shown).

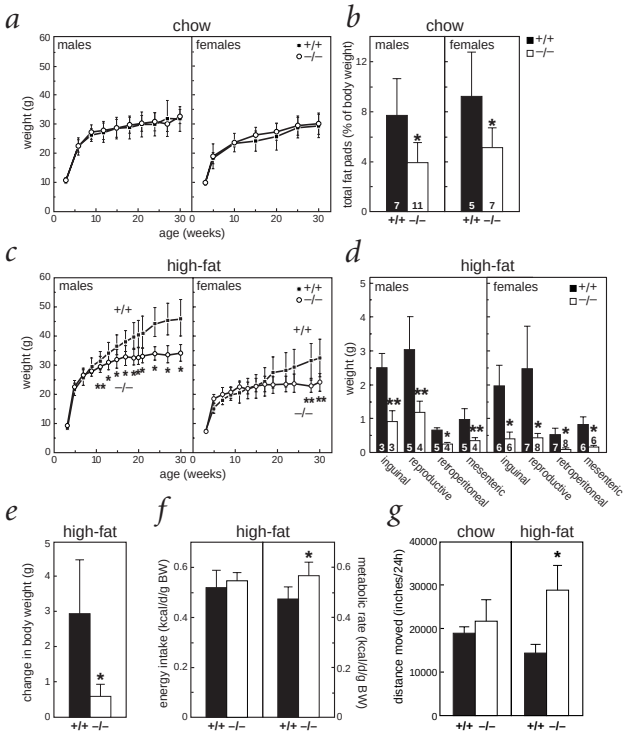

Fig. 3 *Dgat*$^{-/-}$ mice are resistant to diet-induced obesity and have increased energy expenditure. ***a***, Body weights of male (eight wild type and 14 *Dgat*$^{-/-}$) and female (14 wild type and 13 *Dgat*$^{-/-}$) mice fed a chow diet. ***b***, Total fat-pad weights of mice fed a chow diet for 32 weeks. Body weights for wild-type and mutant mice were similar. **P*<0.05. ***c***, Body weights of male (12 wild type and 13 *Dgat*$^{-/-}$) and female (15 wild type and 11 *Dgat*$^{-/-}$) mice fed a diet containing 21% fat by weight. **P*<0.001. ***P*<0.05. ***d***, Fat-pad weights from mice fed a diet containing 21% fat for 32 weeks. The data are expressed in absolute weight of fat pads because body weights differed between wild-type and *Dgat*$^{-/-}$ mice. **P*<0.001. ***P*<0.05. ***e***, Changes in body weights during the 7-d high-fat feeding period for wild-type (n=7) and *Dgat*$^{-/-}$ (n=5) male mice housed in metabolic cages. **P*=0.007. ***f***, Energy intakes and metabolic rates in wild-type (n=9) and *Dgat*$^{-/-}$ (n=7) mice during high-fat feeding. **P*=0.003. ***g***, Home cage activity in wild-type (n=8) and *Dgat*$^{-/-}$ mice (n=7) fed a chow diet and after 7 d of high-fat feeding. **P*=0.05. BW, body weight.

Table 1 • Plasma glucose, insulin and free fatty acid levels

Genotype	Sex	Diet	Measurement conditions	n	Glucose (mg/dl)	FFA (mM)	Insulin (ng/ml)
wild type	m	chow	fed	8	168±29	0.23±0.06	2.6±3.2[a]
Dgat$^{-/-}$	m	chow	fed	8	151±20	0.23±0.06	0.6±0.2[a]
wild type	m	high fat	fed	6	198±25	0.42±0.10	—
Dgat$^{-/-}$	m	high fat	fed	6	191±22	0.42±0.14	—
wild type	m	chow	30 min after glucose load	7	338±118	—	1.90±1.48
Dgat$^{-/-}$	m	chow	30 min after glucose load	4	215±76	—	1.80±2.21
wild type	f	chow	30 min after glucose load	4	268±56	—	1.13±0.83[b]
Dgat$^{-/-}$	f	chow	30 min after glucose load	4	166±24[c]	—	0.44±0.14

[a]Insulin levels were measured on a separate set of animals (n=5 of each genotype). [b]n=2. [c]*P*=0.015 versus wild-type females.

Finally, increased serum leptin levels did not account for the increased energy expenditure. Leptin levels were lower in *Dgat*$^{-/-}$ mice fed either a chow or a high-fat diet (chow diet, 3.4±1.0 versus 8.0±4.6 ng/ml for wild-type mice; high-fat diet, 6.0±1.7 versus 15.2±4.5 ng/ml), probably reflecting the difference in white adipose tissue content between the groups. Further analyses (for example, measuring the expression of uncoupling proteins and introducing Dgat deficiency into genetic models of obesity) are underway and should help to clarify the mechanisms involved in the increased energy expenditure.

Because several mouse models with reduced white adipose tissue and serum leptin levels exhibit insulin resistance[5–7], we examined insulin and glucose metabolism. Wild-type and *Dgat*$^{-/-}$ mice had similar basal plasma levels of glucose, insulin and free fatty acids (Table 1). *Dgat*$^{-/-}$ mice, however, tended to have lower glucose and insulin levels after a glucose tolerance test, suggesting increased insulin sensitivity. Therefore, reduced serum leptin levels in *Dgat*$^{-/-}$ mice do not appear to cause insulin resistance. This is noteworthy because several *in vivo* studies in animals suggest that leptin improves glucose metabolism[8–12].

Dgat deficiency also altered triglyceride metabolism in several other tissues. For example, *Dgat*$^{-/-}$ females had a complete absence of milk production. As a result, pups born to *Dgat*$^{-/-}$ mothers required transfer to foster mothers for survival. In sections of mammary tissue, neutral lipid-staining droplets were abundant in the apical regions of the epithelial cells and in the ductal lumens of lactating wild-type females (Fig. 4*a*), but were rare in postpartum *Dgat*$^{-/-}$ females (Fig. 4*b*). Dgat therefore appears to be crucial for lactation. *Dgat*$^{-/-}$ mice fed a high-fat diet also accumulated less triglyceride in the liver (Fig. 4*c*,*d*). Dgat deficiency also had effects on skin and fur (data not shown). These mutant mice will provide an opportunity to explore the contribution of Dgat to triglyceride metabolism in these and other tissues.

Our results indicate that Dgat activity is not the only mechanism for triglyceride synthesis. What other mechanisms might exist? One possibility is that multiple Dgats exist, a situation similar to that for acyl CoA:cholesterol acyltransferase enzymes[13], and that our assay conditions were not optimized to detect a second Dgat. Indeed, preliminary data suggest that Dgat activity can be increased modestly in several tissues by altering enzyme assay conditions (unpublished data). Alternatively, fatty acyl groups may be transferred directly from one diacylglycerol (or a phospholipid) to a second diacylglycerol acceptor through transacylase activity[14] that does not involve a fatty-acyl-CoA intermediate.

The findings that *Dgat*$^{-/-}$ mice are viable, lean and resistant to diet-induced obesity suggest that disrupting triglyceride synthesis may offer a new target for obesity treatment. The reduced adiposity associated with Dgat deficiency did not cause insulin resistance and, in fact, appeared to improve glucose metabolism. Our findings also indicate that other enzymes contribute to triglyceride synthesis. The *Dgat*$^{-/-}$ mouse provides a tool to examine other mechanisms of triglyceride synthesis and the relationship between triglyceride synthesis and obesity.

Methods

Generation of *Dgat*-mutant mice. We obtained a P1 clone containing mouse *Dgat* (Genome Systems) and subcloned an ~15-kb *Xba*I fragment into pBSSKII. A sequence replacement vector was constructed in pKSloxPNT (a gift from A. Joyner; ref. 15) by amplifying and subcloning a 0.85-kb upstream short-arm fragment containing 5′ coding sequences (primers: 5′–GTTCATCGATCTTTATTCCTACCGGGATG–3′ and 5′–AGAAGGTC GACCACAGCATT GAGACAGGAGTG–3′) and a 12-kb downstream long-arm fragment containing the *Dgat* stop codon and polyadenylation signal (primers: 5′–tgctttagggcgcgccTGAGGTACTGCCAAAGGCCAG–3′, lowercase indicates nucleotides added to engineer an *Asc*I site for cloning purposes; and a plasmid T3 primer). This vector was used to generate targeted embryonic stem cells and mice[13]. The disrupted allele was confirmed by hybridizing *Eco*RI-digested genomic DNA with a ^{32}P-labelled 0.6-kb fragment located 5′ of the short arm of homology. Subsequent genotyping in mice was performed by PCR with primers A (5′–CTCCATGAAGCC CTTCAAGG–3′), B (5′-TGTGCACG GGGATATTCCAG–3′) and C (5′–TACCGGTGGATGTGGAATGTGTGCG–3′) in a 35-cycle reaction (96 °C for 30 s, 50 °C for 1 min, and 72 °C for 2 min). A and B amplify an ~450-bp fragment from the wild-type allele, and A and C amplify an ~550-bp frag-

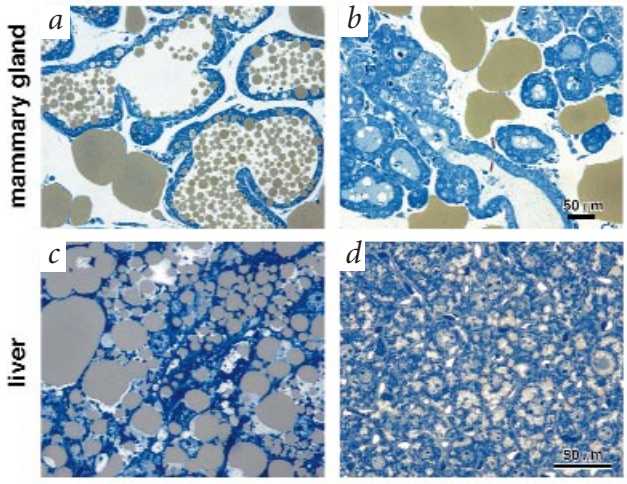

Fig. 4 Histologic findings in mammary gland and liver of *Dgat*$^{-/-}$ mice. *a*,*b*, Osmium-tetroxide–stained sections of mammary glands from six-month-old chow-fed mice (2 d postpartum). The wild-type mouse (***a***) has numerous lipid-staining droplets in the apical regions of mammary epithelial cells and in the ductal lumens not seen in the *Dgat*$^{-/-}$ mouse (***b***). Lipid-rich adipocytes surround the mammary epithelial tissue in both wild-type and mutant mice. *c*,*d*, Osmium-tetroxide–stained sections of liver from male mice fed a diet containing 21% fat for 32 weeks. Numerous irregularly sized lipid-staining droplets are visible in the wild-type mice (***c***). The lipid accumulation is reduced in *Dgat*$^{-/-}$ mice (***d***). Average hepatic triglyceride content was 157±8 and 28±16 mg/g for male wild-type and *Dgat*$^{-/-}$ mice (n=2 each), respectively.

ment from the mutant allele. For northern blots, we isolated total RNA from tissues with Trizol (Life Technologies), and hybridized samples (10 µg) with a ^{32}P-labelled 0.6-kb *Dgat* cDNA fragment from the 5′ region of the gene.

We studied mice of a mixed (50% C57BL/6J and 50% 129/SvJae) genetic background. Mice were housed in a pathogen-free barrier facility (12 h/12 h light/dark cycle) and fed rodent chow (Ralston Purina) or a high-fat diet containing 21% fat by weight (TD88137, Harlan Teklad). We measured body temperature with a model 4600 thermometer and model 402 colonic probe (YSI).

Dgat enzyme assays. We performed Dgat assays as described[4]. The incorporation of [^{14}C]oleoyl CoA into triglyceride was measured under apparent V_{MAX} conditions by adding exogenous diacylglycerol substrate in acetone (4 mM solution). Partially purified membranes (25–100 µg) were used as the enzyme source. Tissues (except small intestine) were homogenized by 10 strokes of a motor-driven pestle in buffer B (100 mM sucrose, 50 mM KCl, 40 mM KH_2PO_4, 30 mM EDTA, pH 7.2). For small intestine, segments of tissue (<300 mg) were cut linearly and then rinsed in ice-cold saline. Segments were homogenized by five strokes of a motor-driven pestle in buffer A (75 mM sucrose, 10 mM DTT and 0.5 mg/ml trypsin inhibitor) followed by 10 strokes after addition of buffer B (5 ml) containing trypsin inhibitor (0.67 mg/ml). The homogenates were centrifuged twice (600*g* for 5 min at 4 °C, and 100,000*g* for 1 h at 4 °C), and the partially purified membrane pellets were resuspended in buffer B, dispensed into aliquots and stored at –80°C. We performed assays in 200 µl buffer (250 mM sucrose, 1 mM EDTA, 150 mM $MgCl_2$, 100 mM Tris-HCl, pH 7.5) containing bovine serum albumin (25 µg), DAG in acetone (40 nmol) and [^{14}C]oleoyl CoA (5 nmol; 40,000 dpm/nmol). Reactions were carried out for 5 min at 37 °C and the products were analysed as described[16].

Blood chemistries. We determined serum triglycerides and plasma free-fatty-acid concentrations by colorimetric kit assays (Triglycerides GPO, Cholesterol/HP and Free Fatty Acid Half Micro Test; Boehringer). Plasma glucose levels were determined with a kit assay (Sigma). Plasma leptin concentrations were determined by Ani Lytics, Inc. We measured plasma insulin levels by radioimmunoassay with a rat insulin RIA kit (Linco Research).

Tissue lipids. We measured tissue and carcass triglycerides with a Triglycerides 320A kit (Sigma) as described[5]. Fecal fat was determined by weighing the extracted lipids from 1-g aliquots of dried and ground feces. For qualitative analyses of tissue lipids, lipids were extracted from tissues[17] and separated on LK5D Silica Gel 150A TLC plates (Whatman) using hexane:ethyl ether:acetic acid (80:20:1). We determined the fatty acid species in the adipose tissue triglycerides by gas chromatography as described[18].

Histology. Tissues were fixed by immersion or perfusion in neutral buffered formalin, dehydrated in ethanols, transitioned into xylene and embedded in paraffin. We stained sections with haematoxylin and eosin. To enhance lipid staining, some formalin-fixed tissues were stained in 2% osmium tetroxide in sodium phosphate buffer (0.1 M, pH 7.4). These tissues were then dehydrated in an ethanol series, transitioned into propylene oxide and embedded in Epon resin. Sections (1 µm) were cut with a glass knife and stained in warm toluidine blue.

Energy metabolism studies. Energy balance studies were performed on wild-type (n=9) and mutant (n=7) male mice in metabolic chambers (Metabowl, Jencons Scientific). We measured energy intake by monitoring food consumption and multiplying this by the caloric content of the diet for chow and the high-fat diet (2.98 and 4.53 kcal/g, respectively). The metabolic rate was measured by indirect calorimetry[19] with an Oxymat/Ultramat 6 analyser (Siemens) that continuously monitors O_2 and CO_2 concentrations using data acquisition hardware (Analogic) and software (Labtech). We measured the caloric content of feces by bomb calorimetry (Pan Instrument). For activity measurements, male mice were housed individually with free access to food and water in a facility with constant temperature (18 °C) and a 12-h light cycle (6:00 to 18:00). Cages were placed into automated frames equipped with rows of infrared photocells interfaced with a computer (San Diego Instruments). Home cage activity was recorded for 24 h, and recorded beam breaks were used to calculate path lengths.

Statistical methods. Unless otherwise indicated, data are shown as mean±s.d. Measurements in wild-type and *Dgat*$^{-/-}$ mice were compared by *t*-tests or Mann-Whitney rank-sum tests. Differences in growth curves between groups were compared by ANOVA followed by the Tukey-Kramer test when appropriate.

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