Genetically Altered Expression of Spermidine/Spermine N\textsuperscript{1}-Acetyltransferase Affects Fat Metabolism in Mice via Acetyl-CoA*

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The acetylating enzyme, spermidine/spermine N\textsuperscript{1}-acetyltransferase, participates in polyamine homeostasis by regulating polyamine export and catabolism. Previously, we reported that overexpression of the enzyme in cultured tumor cells and mice activates metabolic flux through the polyamine pathway and depletes the N\textsuperscript{1}-acetyltransferase coenzyme and fatty acid precursor, acetyl-CoA. Here, we investigate this possibility in spermidine/spermine N\textsuperscript{1}-acetyltransferase transgenic mice in which the enzyme is systemically overexpressed and in spermidine/spermine N\textsuperscript{1}-acetyltransferase knock-out mice. Tissues of the former were characterized by increased N\textsuperscript{1}-acetyltransferase activity, a marked elevation in tissue and urinary acetylated polyamines, a compensatory increase in polyamine biosynthetic enzyme activity, and an increase in metabolic flux through the polyamine pathway. These polyamine effects were accompanied by a decrease in white adipose acetyl- and malonyl-CoA pools, a major (20-fold) increase in glucose and palmitate oxidation, and a distinctly lean phenotype. In SSAT-ko mice, the opposite relationship between polyamine and fat metabolism was observed. In the absence of N\textsuperscript{1}-acetylation of polyamines, there was a shift in urinary and tissue polyamines indicative of a decline in metabolic flux. This was accompanied by an increase in white adipose acetyl- and malonyl-CoA pools, a decrease in adipose palmitate and glucose oxidation, and an accumulation of body fat. The latter was further exaggerated under a high fat diet, where knock-out mice gained twice as much weight as wild-type mice. A model is proposed whereby the expression status of spermidine/spermine N\textsuperscript{1}-acetyltransferase alters body fat accumulation by metabolically modulating tissue acetyl- and malonyl-CoA levels, thereby influencing fatty acid biosynthesis and oxidation.

The polyamines putrescine (Put), spermidine (Spd), and spermine (Spm) are known for their critical role in supporting cell proliferation, albeit in ways that have not yet been clearly defined. For the most part, polyamines do not incorporate into macromolecules but rather bind electrostatically to negatively charged molecules, such as DNA, RNA, and phospholipids. Thus, as metabolically distinct entities, homeostatic control of intracellular polyamines is critical to their role in supporting cell proliferation. This is achieved by effector systems that regulate biosynthesis, catabolism, uptake, and export of these molecules. The enzyme, spermidine/spermine N\textsuperscript{1}-acetyltransferase (SSAT), catalyzes the transfer of acetyl groups from acetyl-CoA to the terminal amines of polyamines and, thus, readies the molecule for export or catabolism via polyamine oxidase. The enzyme is short lived, sensitively regulated by intracellular polyamine pools, and highly inducible by polyamine analogues and various cytotoxic agents (1, 2).

Although most antiproliferative strategies targeting the polyamine pathway seek to deplete intracellular pools by inhibiting biosynthesis, we have been investigating the alternative approach, of activating SSAT-mediated polyamine acetylation (3). Our initial expectation was that acetylation of polyamines would facilitate export and catabolism, leading to pool depletion and inhibition of cell growth. In the absence of a selective inducer of SSAT activity, we used genetic approaches to overexpress SSAT and test this hypothesis. Those studies detected an apparent linkage between polyamine homeostasis and fat metabolism (4, 5). More specifically, conditional overexpression of SSAT in prostate tumor cells inhibited cell growth in a manner that correlated with depletion of acetyl-CoA pools (5). This occurs via a metabolic scenario whereby SSAT overexpression gives rise to a compensatory increase in polyamine biosynthesis and sustained metabolic flux (Fig. 1) through the biosynthetic and catabolic arms of the polyamine pathway (4–6). More particularly, acetylation of polyamines promotes their degradation and export out of the cell as we predicted.

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3 The abbreviations used are: Put, putrescine; AcSpd, N\textsuperscript{1}-acetylsperrmidine; AcSpm, N\textsuperscript{1}-acetylspermine; MRI, magnetic resonance imaging; ODC, ornithine decarboxylase; SAMDC, 5-adenosylmethylene decarboxylase; Spd, spermidine; Spm, spermine; SSAT, spermidine/spermine N\textsuperscript{1}-acetyltransferase; SSAT-ko, SSAT knock-out mouse; SSAT-tg, SSAT-transgenic mouse; SSAT-wt, SSAT wild-type mouse; WAT, white adipose tissue; Syn, synthase.
However, this initial decline in Spd or Spm pools gives rise to derepression of the biosynthetic enzymes, ornithine and S-adenosylmethionine decarboxylase (ODC and SAMDC, respectively). By providing a continuous supply of polyamines for acetylation and export, this deregulated metabolic cycling depletes the SSAT co-factor acetyl-CoA and restricts its availability for fatty acid biosynthesis. The associated growth inhibition is presumed to be linked to impaired fat-dependent processes, such as membrane biosynthesis and energy regulation, although these possibilities have not yet been investigated.

Consistent with this interpretation, we note that transgenic overexpression of SSAT in mice is associated with a distinctly lean phenotype (7, 8). In agreement with our findings, a Finnish group has also associated altered SSAT expression with altered metabolic flux (9). In addition, Rodriguez-Caso et al. (10) recently used mathematical modeling to conduct an in silico analysis of polyamine metabolism and arrived at the prediction that altered SSAT activity will affect acetyl-CoA availability.

The following studies examine the relationship between polyamine and fat metabolism more closely by comparative studies with SSAT wild-type (SSAT-wt), SSAT overexpressing transgenic (SSAT-tg), and SSAT knock-out (SSAT-ko) mice (11), all in the C57BL/6 background. The hypothesis being tested is that by controlling flux through the polyamine metabolic pathway, SSAT modulates levels of acetyl-CoA and malonyl-CoA, thereby influencing fat homeostasis and accumulation of body fat. The possibility that SSAT expression status may affect fat metabolism could have implications for controlling obesity. The importance of the latter is clearly indicated by the fact that within the past 5 years, the health risks associated with obesity have risen to levels exceeding those of tobacco or alcohol consumption (12, 13).

MATERIALS AND METHODS

Breeding of Mice—Mice were maintained in the C57BL/6 background for more than 10 generations. SSAT-tg mice carry ~10–20 copies of the full-length mouse Sat1 gene and express elevated levels of SSAT enzyme activity in most tissues (7). The transgene is under the endogenous, murine SSAT promoter. Carriers of the transgene are readily identified by hair loss, which begins at ~3–4 weeks of age. Because female SSAT-tg mice are infertile except at a very young age, the line is maintained by breeding male SSAT-tg mice with SSAT-wt C57BL/6 female mice. Mouse embryonic stem cells, which carry a targeted inactivating mutation within the X-linked Sat1 gene, were used to generate the SSAT-ko mice (11). The presence or absence of the SSAT-ko allele (located on the X chromosome) was determined by standard PCR techniques. Through interbreeding, we developed female SSAT-ko mice with the SSAT gene homozygously deleted, which, when bred with SSAT-ko male mice, yield 100% SSAT-ko pups (6). Phenotypic characterization was performed at or around 30 weeks, since pilot studies revealed phenotypic differences in fat accumulation between SSAT-tg, SSAT-wt, and SSAT-ko mice at this age.

Polyamine Metabolism—Tissues (i.e. liver and white adipose tissue (WAT)) were excised, snap-frozen, and stored at ~70 °C for less than 2 weeks before analysis. Frozen tissues were crushed into a fine powder using a Bio-Pulverizer (BioSpec Products, Inc., Bartlesville, OK). Tris/EDTA (pH 7.0) breaking buffer was added to ~50–100 mg of sample for sonication and centrifugation to obtain the soluble supernatant extracts used in enzyme and polyamine pool analyses. SSAT activity was assayed radiochemically as described previously (3, 5) and expressed as nmol of N⁵-[¹⁴C]acetylperoxime (AcSpd) generated/min/mg of protein. Both ODC and SAMDC activities were determined by a CO₂ trap assay as described previously (3, 5) and reported as μmol of radiolabeled CO₂/h/mg of protein. For polyamine pool analysis, buffered tissue extracts were further extracted with an equal volume of 1.2 n perchloric acid. Solubilized extracts were then dansylated and analyzed by reverse phase high performance liquid chromatography using methods refined by Kramer et al. (14). This methodology detects and quantifies Put, Spd, and Spm and the SSAT products, AcSpd, N⁵-acetylperoxime (AcSpm), and N⁴,N¹¹-diacyetylperoxime, an important SSAT product first reported in mammalian systems by the Porter laboratory (3).

Urinary Polyamines—Excreted mouse urine was collected from hand-held mice by pipette. The volume was brought to 205 μl and extracted with 0.6 M perchloric acid for 10 min at 4 °C and centrifuged at 15,000 rpm at 4 °C. Supernatant was transferred to a microcentrifuge tube and neutralized with 220 μl of 2 M Na₂CO₃. Polymamines were dansylated and separated as described above. The levels of polymamines in urine were normalized to urinary creatinine measured using a kit (Phenomenex, Torrance, CA) following the manufacturer’s instructions. Data were expressed as μg of polyamines/mg of creatinine.

Measurement of Acetyl-CoA and Malonyl-CoA—The separation and quantitation of acetyl-CoA in biological samples follows the high performance capillary electrophoresis assay of Liu et al. (15) as described previously (3) with some modifications to separate malonyl-CoA and separate it from interfering peaks. Cells were lysed and processed by Sep-Pak plus solid phase extraction. Purified extracts were then analyzed on a Beckman P/ACE MDQ capillary electrophoresis system (Fullerton, CA) equipped with a photodiode array detector and an uncoated fused silica CE column of 50-μm inner diameter and 60 cm in length with 50 cm from inlet to the detection window (Polymerico Technologies, Phoenix, AZ). Electrophoretic conditions were as described by Liu et al. (15). Briefly, the capillary was preconditioned with 0.1 M NaOH and Milli-Q water for 5 min each at 20 p.s.i. and then equilibrated with 100 mM NaH₂PO₄ separating buffer containing 0.1% β-cyclodextrin (pH 6.0) for 10 min. After each run, the capillary was rinsed with 1 M NaOH, Milli-Q water, and running buffer for 3 min each. The injection was done hydrodynamically at a pressure of 5 p.s.i. for 10 s. Injection volume was calculated using CE Expert Lite software from Beckman. Separation voltage was ~30 kV at a constant capillary temperature of 15 °C. In order to establish the standard calibration curves, solutions containing acetyl-CoA, malonyl-CoA, and the internal standard (isobutyryl-CoA; 41 nmol) were prepared at concentrations ranging from 1 to 200 nmol. Standards were processed as described above for cell lysates and resuspended in 15 μl of water. The detector response (r > 0.99) for all acetyl-CoA species was within the above concentration range. Coenzymes A were monitored with a photodiode array detector at the maximum absorbance wave-
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length (253.5 nm). Data were collected and processed by Beckman P/ACE 32 Karat software version 7.0. Cellular acetyl-CoA and malonyl-CoA levels were expressed as nmol/10⁶ cells.

Fat Content by Magnetic Resonance Imaging (MRI)—Whole body fat quantitation in mice was carried out using a 4.7-Tesla, 33-cm horizontal bore MR magnet (GE NMR Instruments, Fremont, CA) incorporating AVANCE digital electronics and a 72-mm radio frequency quadrature coil (Bruker BioSpec platform with Paravision® version 3.02 operating system; Bruker Medical, Billerica, MA). Prior to imaging, mice were anesthetized with 4% isoflurane and positioned in the MR scanner. Animals were screened for fat/water ratios using MR spectroscopic methods. Spectroscopic data were converted from time domain to frequency domain using a Fourier transformation and base line-corrected with polynomial fitting (MestReC 4.4.6; Mestrelab Research, Santiago de Compostela, Spain). The spectra were referenced by assigning the chemical shift of the water peak to 4.74 ppm, and the relative concentrations of water and fat protons were calculated by integrating the area under the peaks with ranges of 2.0–9.0 ppm for water and ~1.0–2.0 ppm for fat.

Following determination of fat/water ratios, representative animals from each group were selected for whole body imaging. Whole body, high resolution three-dimensional images were acquired using a T1-weighted, fast spin-echo imaging protocol that resulted in high contrast, hyperintense signal from adipose tissue versus nonadipose tissue due to the short T1 relaxation time and the intrinsic hyperintensity of fat with fast spin-echo imaging techniques (16).

Image segmentation of fat from whole body data sets was performed through the creation of object maps using Analyze 5.0 (Analyze Direct, Lenexa, KS). Three-dimensional, whole body surface renderings were created from segmented image data using commercially available software (Amira 3.1; Mercury Computer Systems, Chelmsford, MA). Adipose tissue was rendered opaque as a yellow surface, whereas the whole mouse body was rendered partially transparent with a transparency value of 0.7.

Serum Leptin Analysis—Analysis of serum leptin levels (17) was performed at the Mouse Metabolic Phenotypic Center (Vanderbilt University, Nashville, TN) following protocol instructions to a commercially obtained ELISA-based kit (DuoSet; R&D, Minneapolis, MN).

Whole Body Calorimetry—Experiments were performed with the assistance of the Mouse Metabolic Phenotyping Center (Dr. Owen McGuiness, Nashville, TN) following standard methodologies (18). Briefly, whole body oxygen volume (VO₂) and carbon dioxide volume (VCO₂), heat, and respiratory exchange ratio were measured continuously for 21 h in single animal cages using conscious mice in the Oxymax Deluxe System (Columbus Instruments, Columbus, OH).

Fatty Acid and Glucose Oxidation—Palmitate and glucose oxidation were measured on liver slices and WAT spreads as described by Roduit et al. (19). Liver and abdominal fat pad WAT were excised and placed in cold KRB buffer (pH 7.4), and liver samples were sliced manually to ~1-mm thickness. Liver slices and white fat spreads were weighed and placed in KRB buffer at 37 °C for 2 h with one of the following substrates: 10 mm glucose containing 1 μCi of D-[U-14C]glucose for glucose oxidation studies or 0.5 mm sodium palmitate containing 1 μCi of [1-14C]palmitate for fatty acid oxidation studies. A sealed test tube was filled with an insert containing 0.3 ml of NaOH to trap radiolabeled CO₂ released from the reaction mixture over a 2-h period. The reaction was terminated by adding 0.5 ml of 1 N HCl added to the mixture, and samples were then allowed to incubate for 45 min at 37 °C. The CO₂ traps were removed and added to 7.5 ml of scintillation fluid. Activities were expressed as nmol/g of tissue/h.

High Fat Diet—The synthetic high fat diet used for the diet-induced obesity model was composed of 60% fat calories, 20% carbohydrate calories, and 20% protein calories (5.2 kcal/g) as obtained from Research Diets, Inc. (New Brunswick, NJ) (stock number D12492I). Control animals were fed a diet of standard rodent chow composed of 13% of calories from fat, 58% from carbohydrate, and 29% from protein (4.1 kcal/g) (Prolab RMH 2500, PMI Nutrition International, Inc., Brentwood, MO). Mice began consumption of experimental diets immediately postweaning at 5–6 weeks of age and were monitored weekly for food consumption and weight gain for up to 30 weeks of age. Previous studies have shown significant differences in accumulation of abdominal fat in C57BL/6 mice within 2 weeks of high fat diet feeding (20).

Statistics—Analysis of data obtained from the three mouse groups was performed using a Dunnett’s test within the one-way analysis of variance framework, where α level was reduced from 0.05 to 0.025. Unless indicated otherwise, p values typically derive from comparisons of SSAT-tg or SSAT-ko mice with SSAT-wt mice.

RESULTS

Tissue Polyamine Metabolism—Mice were first compared for the effect of altered SSAT expression on various aspects of polyamine metabolism. As reported earlier (7), global overexpression of SSAT (SSAT-tg) was found to produce a significant increase in polyamine biosynthesis at the levels of ODC and SAMDC activities. In that previous study, ODC activities were increased 4–10-fold relative to SSAT-wt in various tissues, with ODC being more affected than SAMDC. Here, we analyzed enzyme activities in liver and WAT, which was not included in our earlier study (Table 1). SSAT activity was increased 4–10-fold in both tissues, leading to at least a 2–3-fold increase in ODC and SAMDC activities. Deletion of SSAT (SSAT-ko) lowered acetylation activity in both liver and WAT but did not completely eliminate it due to the presence of nonspecific acetylating enzymes (21). In general, ODC and SAMDC activities were not remarkably affected in SSAT-ko mice. Polyamine pools, including those of acetylated polyamines, were also measured in the same tissues noted above and found to provide a clearer indication of metabolic activity (Table 1).

As previously reported (6), transgenic overexpression of SSAT led to variable tissue-specific shifts in Spd and/or Spm pools and a consistent increase in Put pools indicative of increased ODC activity and SSAT-facilitated use of the back-conversion pathway (see Fig. 1). Spm pools were decreased in liver and WAT and Spd pools were increased in both tissues. The fact that the polyamine pools were not depleted is due to
Activity and polyamine metabolic flux within the mouse. As relevant to the latter, we measured urinary polyamine levels oxidized by polyamine oxidase or transported out of the cell. With the understanding that these molecules are either rapidly polyamines in liver and WAT of SSAT-tg mice are consistent through its cofactor, acetyl-CoA. Acetylated polyamines can also be back-synthesized in the tissues of SSAT-tg mice relative to SSAT-wt mice. As a result of this, leading to extracellular release of polyamines and their eventual excretion into the urine. Under this interpretation, there is reason to believe that in SSAT-ko mice, this flux level is significantly reduced (or unable to be induced) relative to SSAT-wt mice.

**Table 1**

| Tissue polyamine enzymes and pools as affected by mouse SSAT expression status |
|-----------------------------------------------|
| Tissue | SSAT status | Polyamine enzymes | Polyamine pools |
| | | SSAT | ODC | SAMDC | Put | Spd | Spm | AcSpd |
| | | nmol/min/mg | μmol/h | μmol/h | pmol/μg protein |
| Liver (n = 6) | ko | 20 ± 7 | 14 ± 7 | 93 ± 15 | 33 ± 7 | 7,632 ± 592a | 8,563 ± 998 | <10 |
| | wt | 30 ± 10 | 11 ± 5 | 91 ± 4 | 48 ± 8 | 5,866 ± 732 | 7,705 ± 440 | <10 |
| | tg | 160 ± 32b | 29 ± 8 | 194 ± 27b | 2,210 ± 114b | 9,696 ± 575c | 3,891 ± 466c | 270 ± 45c |
| WAT (n = 7) | ko | 147 ± 76c | <5 | <5 | 314 ± 168 | 13,725 ± 1420 | 5,976 ± 739 | <10c |
| | wt | 344 ± 97c | <5 | <5 | 623 ± 244 | 12,849 ± 2032 | 4,856 ± 1291 | <10 |
| | tg | 1,276 ± 408c | 41 ± 17c | 73 ± 32c | 8,122 ± 1,702b | 9,635 ± 2,163 | 2,832 ± 794 | 96 ± 6c |

* N1-acetylspermidine; neither N1-acetylspermine nor N1,N1-diacytalspermine was detectable in any sample.  
  + Dunnett's test, p < 0.05 relative to SSAT-wt.  
  + Dunnett's test, p < 0.005 relative to SSAT-wt.

**Table 2**

| Urinary polyamines as affected by mouse SSAT expression status |
|-----------------------------------------------|
| Urinary polyamine pools | Mouse (n = 4) | Put | Spd | Spm | AcSpd |
| | | μg/mg creatinine |
| SSAT-ko | 46.51 ± 6.53 | 4.10 ± 0.72a | 0.29 ± 0.04 | NQb |
| SSAT-wt | 109.64 ± 14.80 | 2.61 ± 0.20 | 0.27 ± 0.02 | NQ |
| SSAT-tg | 132.90 ± 10.82 | 2.78 ± 0.50 | 0.88 ± 0.34 | 0.04 NQ |

* NQ, N1-acetylspermine or N1,N1-diacytalspermine not quantifiable due to low levels and a competing unidentified chromatographic peak, presumed to be N0-acetylspermidine (21).

**Figure 1.** Polyamine metabolism and SSAT-induced metabolic flux. ODC and SAMDC regulate biosynthesis of polyamines (left), a process culminating in the production of Spd and Spm. SSAT regulates catalysis by acetylation through its cofactor, acetyl-CoA. Acetylated polyamines can also be back-converted from Spd and Put via the enzyme polyamine oxidase (right). Overexpression of SSAT leads to increased polyamine export and a compensatory increase in polyamine biosynthetic activity at the level of ODC and SAMDC. The large shaded arrow indicates the presumed metabolic cycling or flux that takes place through the biosynthetic and catabolic/export arms of the pathway. PAO, polyamine oxidase; SMO, spermine oxidase; MTA, S’-deoxy-S’-(methylthio)adenosine.

The compensatory increase in polyamine biosynthesis, as indicated by elevated ODC and SAMDC activities noted above. Further indication of metabolic cycling was evidenced by the tissue accumulation of the SSAT product AcSpd in liver and WAT. There was no comparable accumulation of DA Spm or AcSpm, perhaps, because they were rapidly oxidized by polyamine oxidase. These same trends were previously noted in the initial characterization of the SSAT-tg mice (6). In SSAT-ko mice, polyamine pools were only marginally affected. As expected, acetylated polyamines were absent, and nonacetylated polyamine pools remained roughly similar to those of SSAT-wt mice with the exception of Put, which was modestly decreased (p < 0.0145).

**Urinary Polyamines**—The relatively low levels of acetylated polyamines in liver and WAT of SSAT-tg mice are consistent with the understanding that these molecules are either rapidly oxidized by polyamine oxidase or transported out of the cell. Relevant to the latter, we measured urinary polyamine levels among the SSAT variant mice as a systemic sum total of SSAT activity and polyamine metabolic flux within the mouse. As shown in Table 2, Put was by far the predominant polyamine found in SSAT-wt urine, followed by Spd and Spm. Put is known to be especially high in male mouse urine due to an androgen-inducible kidney ODC (22). In SSAT-tg mice, the main perturbation was a large increase in AcSpd, reflecting the synthesis and export of massive amounts of the acetylated polyamines by various tissues. In SSAT-ko and SSAT-wt urine, AcSpd was difficult to quantify due to low levels and a competing unidentified chromatographic peak, presumed to be N0-acetylspermidine (23).

There was also a significant decrease in Put and an increase in Spd in SSAT-ko urine. The latter may represent a compensatory response to the inability of these mice to synthesize and excrete AcSpd or to convert it to Put. We note that these urinary polyamine profiles were found to be reliably predictive of mouse SSAT expression status (i.e. SSAT-tg mice typically had high urinary AcSpd and SSAT-ko mice had low urinary Put and high Spd relative to SSAT-wt mice).

**Metabolic Flux**—The urinary and tissue polyamine findings reveal that large amounts of acetylated polyamines were being synthesized in the tissues of SSAT-tg mice relative to SSAT-wt mice. The biochemical data indicate that this synthesis was sustained by the compensatory activation of polyamine biosynthesis at the levels of ODC and SAMDC. As a result of this response, metabolic cycling through the biosynthetic and acetylating arms of the polyamine pathway was activated (see Fig. 1), leading to extracellular release of polyamines and their eventual excretion into the urine. Under this interpretation, there is reason to believe that in SSAT-ko mice, this flux level is significantly reduced (or unable to be induced) relative to SSAT-wt mice.

**Tissue Acetyl- and Malonyl-CoA**—As previously reported (5), our model predicts that the increased metabolic flux seen in SSAT-tg mice will produce a decrease in acetyl-CoA pools, while the decrease in flux presumed to occur in SSAT-ko mice will lead to an increase in acetyl-CoA pools. To test this hypoth-
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We next considered that the depletion of acetyl-CoA and malonyl-CoA pools seen in SSAT-tg mice may be due to thermoregulatory responses related to the fact that the mice are hairless (25). To investigate the contribution of hairlessness, we obtained hairless (cub/cub) C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) for comparison with SSAT-wt mice. These mice differ from wild-type C57BL/6 animals by a single mutation involving the cub gene (26). The biological function of the cub gene has not yet been determined, but our own studies indicate that it is unrelated to polyamine metabolism (data not shown). Unlike the typical nu/nu mice, which are athymic and immunocompromised, these mice have an intact immune system and, thus, provide a meaningful control for determining the contribution of hairlessness to the physiological phenotype of SSAT-tg mice. Mice homozygous for the mutation (cub/cub) lack hair, whereas heterozygous mice (cub/+ ) have hair and were therefore used as a control. As shown in Table 3, the cub/cub hairless mice had liver and WAT acetyl- and malonyl-CoA pools that were nearly identical to those seen in SSAT-wt mice.

Controlling for Lack of Hair—We next considered that the depletion of acetyl-CoA and malonyl-CoA and malonyl-CoA pools seen in SSAT-tg mice may be due to thermoregulatory responses related to the fact that the mice are hairless (25). To investigate the contribution of hairlessness, we obtained hairless (cub/cub) C57BL/6 mice from Jackson Laboratories (Bar Harbor, ME) for comparison with SSAT-wt mice. These mice differ from wild-type C57BL/6 animals by a single mutation involving the cub gene (26). The biological function of the cub gene has not yet been determined, but our own studies indicate that it is unrelated to polyamine metabolism (data not shown). Unlike the typical nu/nu mice, which are athymic and immunocompromised, these mice have an intact immune system and, thus, provide a meaningful control for determining the contribution of hairlessness to the physiological phenotype of SSAT-tg mice. Mice homozygous for the mutation (cub/cub) lack hair, whereas heterozygous mice (cub/+ ) have hair and were therefore used as a control. As shown in Table 3, the cub/cub hairless mice had liver and WAT acetyl- and malonyl-CoA pools that were nearly identical to those of the cub/+ furred mice. Thus, the cub/cub data indicate that the perturbations in acetyl-CoA and malonyl-CoA pools seen in SSAT-tg mice are not due to a lack of hair but rather to transgenic overexpression of the enzyme. Additional comparative studies between SSAT-tg and cub/cub mice are described below.

Body Fat Analysis—The above data indicate that changes in acetyl-CoA and malonyl-CoA related to SSAT-mediated shifts in metabolic flux through the polyamine pathway could have an effect on fat homeostasis in the intact animal. Thus, the possible relationship between fat-related parameters and SSAT expression status was examined in 30-week-old male mice. We began with a comparison of body weight (Table 4), where as a trend, SSAT-tg consistently weighed less (24.00 ± 3.04 g) than SSAT-wt mice (30.55 ± 2.42 g), and SSAT-ko mice weighed about the same (30.62 ± 1.35 g). The former difference was statistically significant (p = 0.001), but the difference between SSAT-ko and SSAT-wt mice was clearly not (p = 0.95). To evaluate the fat content of the mice, we compared the reproductive fat pad weight among the SSAT variant mice (Table 4). The average reproductive fat pad weights of 30-week-old mice revealed distinct phenotypic differences between the three

| Genotype (30 weeks) | Liver | SSAT-ko | SSAT-wt | SSAT-tg | cub/cub | Acetyl-CoA | Malonyl-CoA | Acetyl-CoA | Malonyl-CoA |
|---------------------|-------|---------|---------|---------|---------|-----------|------------|-----------|------------|
|                     | nmol/g tissue |       |         |         |         |           |            |           |            |
| SSAT-ko             | 23.3 ± 2.6  | 11.8 ± 1.7  | 45.8 ± 6.6  | 60.6 ± 13.0  |
| SSAT-wt             | 30.3 ± 4.5  | 8.4 ± 0.8  | 31.6 ± 2.9  | 34.7 ± 7.3  |
| SSAT-tg             | 38.6 ± 5.3  | 4.3 ± 1.1  | 9.7 ± 2.1  | 16.6 ± 2.3  |
| cub/cub             | 36.2 ± 2.6  | 3.6 ± 0.8  | 35.7 ± 2.7  | 20.1 ± 2.5  |
| cub/+               | 35.6 ± 2.3  | 3.4 ± 1.0  | 33.9 ± 5.1  | 18.7 ± 3.5  |

*a Dunnett’s test, p < 0.05 relative to SSAT-wt, n = 5.

The above data indicate that changes in acetyl-CoA and malonyl-CoA related to SSAT-mediated shifts in metabolic flux through the polyamine pathway could have an effect on fat homeostasis in the intact animal. Thus, the possible relationship between fat-related parameters and SSAT expression status was examined in 30-week-old male mice. We began with a comparison of body weight (Table 4), where as a trend, SSAT-tg consistently weighed less (24.00 ± 3.04 g) than SSAT-wt mice (30.55 ± 2.42 g), and SSAT-ko mice weighed about the same (30.62 ± 1.35 g). The former difference was statistically significant (p = 0.001), but the difference between SSAT-ko and SSAT-wt mice was clearly not (p = 0.95). To evaluate the fat content of the mice, we compared the reproductive fat pad weight among the SSAT variant mice (Table 4). The average reproductive fat pad weights of 30-week-old mice revealed distinct phenotypic differences between the three
TABLE 4
Effect of SSAT status on body weight, body fat, and related parameters

| Genotype (30 weeks) | Body weight (n = 25) | Reproductive fat pad (n = 24) | MRI total body fat (n = 5) | Serum leptin (n = 5) |
|---------------------|----------------------|-----------------------------|-------------------------|---------------------|
| SSAT-ko             | 30.62 ± 1.35         | 670 ± 107b                 | 3.90 ± 0.74             | 7.77 ± 3.71b       |
| SSAT-wt             | 30.55 ± 2.43         | 450 ± 84                    | 2.74 ± 0.02             | 4.13 ± 2.48        |
| SSAT-tg             | 24.01 ± 3.04b        | 10 ± 1b                     | 1.51 ± 0.42             | 1.25 ± 0.70b       |
| cub/cub (hairless)  | 22.9 ± 1.7           | 140 ± 50                    | NDb                     | ND                 |
| cub/+ (furred)      | 27.1 ± 1.5           | 340 ± 130                   | ND                      | ND                 |

a Dunnett’s test, p < 0.005 relative to SSAT-wt.
b Dunnett’s test, p < 0.05 relative to SSAT-wt.
c n = 6.
d ND, not determined.

FIGURE 3. MRI quantitation of body fat composition. Whole animal MRI ventral views of a SSAT-tg mouse (A), SSAT-wt mouse (B), and SSAT-ko mouse (C). The last mouse is representative of the ~20% of SSAT-ko mice that were overweight and obese. On average, the body fat composition was 6.3% for SSAT-tg, 8.9% for SSAT-wt, and 12.7% for SSAT-ko. The body fat composition for these particular mice was 4.8% for SSAT-tg, 10.1% for SSAT-wt, and 28.6% for SSAT-ko.

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FIGURE 4. Effect of SSAT status on food consumption and reproductive fat pad weight at 30 weeks. There was no significant difference in food consumption between SSAT-ko and SSAT-wt mice, although SSAT-ko displayed significantly larger reproductive fat pad weights (670 mg for SSAT-ko versus 450 mg for SSAT-wt). Note that despite being leaner, the SSAT-tg mice consumed significantly more food over a 7-day study (6.11 g/day) than SSAT-wt mice (4.51 g/day), yet SSAT-tg reproductive fat pad weights (10 mg) remained ~45 times lower than SSAT-wt. The implication is that the reproductive fat pad weight differences are metabolism-based as opposed to appetite-based. Top and bottom horizontal lines indicate the range of data, boxes show the upper and lower quartiles, and the middle horizontal line indicates the mean value. *, Dunnett’s test, p < 0.005 relative to SSAT-wt.

groups. The SSAT-tg fat pad weights (10 ± 1 mg) were statistically different (p < 0.001) from the SSAT-wt fat pads (450 ± 84 mg), which were statistically different (p < 0.005) from the SSAT-ko mice (670 ± 107 mg) with n > 25 animals per group. Similar to the SSAT-tg mice, the body weights and reproductive fat pad weights of the cub/cub hairless mice were less than those of the cub/+ mice or SSAT-wt mice, but they retained significantly heavier reproductive fat pads than those of SSAT-tg mice. Thus, lack of hair in the SSAT-tg mice fails to fully account for the effect of SSAT overexpression on accumulation of body fat.

MRI was used to obtain a more comprehensive determination of the whole body fat content of the SSAT variant mice (Table 4). By this analysis, fat loss and gain in SSAT-tg and SSAT-ko mice, respectively, was observed to occur uniformly throughout the body of these animals (Fig. 3). The average total fat content of SSAT-wt mice was about 8.9% of the total body mass. By comparison, the average fat content of SSAT-tg mice (6.3%) was less than that seen in SSAT-wt mice, whereas the average fat mass of SSAT-ko mice (12.7%) was considerably more than that of SSAT-wt mice. A certain proportion (~20%) of the SSAT-ko mice showed distinctly higher fat content than SSAT-wt mice (Fig. 3). The above measurements coincide closely with serum leptin measurements (Table 4), which were significantly decreased in the SSAT-tg mice (p < 0.05), in agreement with the lean phenotype, and significantly increased in the SSAT-ko mice (p < 0.05) in keeping with their tendency to accumulate fat. Taken together, these data strongly suggest an apparent relationship between SSAT expression status and fat accumulation in mice. Despite differences in body weight and body fat, SSAT-tg animals were found to consume more food than SSAT-wt mice, whereas SSAT-ko mice consumed a similar amount, suggesting that the underlying effect was metabolism-based as opposed to appetite-based. Fig. 4 shows a
comparison of food consumption versus fat pad weight for at least six representative mice from each group.

Whole Body Calorimetry—With the assistance of the Mouse Metabolic Phenotypic Center at Vanderbilt (Dr. Owen McGuiness, Nashville, TN), representative mice were subjected to whole body calorimetry in order to examine the relationship between morphological phenotype and parameters such as energy expenditure. The calorimetric analysis revealed a significant increase in both oxygen consumption rate and caloric expenditure (heat production) in SSAT-tg mice, whereas, by contrast, the data for the SSAT-ko mice were similar to those of SSAT-wt (data not shown). Comparing average values for the 21-h measurement period, SSAT-tg mice consumed 37% more oxygen and expended 33% more calories (heat) than SSAT-wt mice with a high degree of significance (p < 0.001).

Glucose and Palmitate Oxidation—The above findings prompted us to examine glucose metabolism (i.e., oxidation to CO₂) and fatty acid (palmitate) oxidation in liver slices and WAT spreads from the different mice. On average, both parameters were ~20-fold higher in SSAT-tg WAT compared with SSAT-wt WAT (Table 5), whereas, by contrast, they were relatively unaffected in SSAT-tg liver slices. The increased palmitate oxidation finding is consistent with the above observation that malonyl-CoA, which is capable of negatively regulating fatty acid oxidation (24), is lowered in SSAT-tg WAT. These findings are further supported by the observation that circulating serum-free fatty acids were significantly increased (p = 0.004) in SSAT-tg mice (i.e., 0.90 ± 0.09 mmol/liter for SSAT-tg versus 0.50 ± 0.08 mmol/liter for SSAT-wt). Similarly, increased glucose utilization is consistent with the finding that acetyl-CoA, which can negatively affect glycolysis and tricarboxylic acid cycle progression (27, 28), is also lowered. It is relevant to again consider that thermoregulatory responses (25) associated with lack of hair might influence fatty acid biosynthesis and oxidation in SSAT-tg mice. As shown in Table 5, neither of these parameters were significantly increased in the hairless cub/cub mice relative to the furred cub/+ mice, suggesting again that the 20-fold effects seen in SSAT-tg mice are apparently related to SSAT-specific perturbations in acetyl- and malonyl-CoA pools that were not seen in cub/cub mice.

Oxidative activity in WAT spreads or liver slices from SSAT-ko mice were marginally but not significantly different from that of SSAT-wt mice, perhaps because the phenotypic difference is too subtle to detect in this manner. Glucose oxidation, however, was decreased by ~75%, possibly due to feedback inhibition on glycolysis and the tricarboxylic acid cycle (27, 28) by excess acetyl-CoA.

Effects of High Fat Diet—Since global SSAT overexpression gives rise to a lean phenotype and SSAT deletion gives rise to a fatty phenotype, we next examined whether SSAT expression status might modulate weight gain in mice placed on a high fat diet (Fig. 5). As tracked over a 6–28-week period, male SSAT-ko mice consistently gained more weight than SSAT-wt mice, whereas SSAT-tg failed to gain weight. At the end of 28 weeks, SSAT-wt mice on chow weighed 30.3 ± 4.0 g, whereas those on the high fat diet weighed 40.3 ± 4.3 g, a gain of 10 g or 33%. At the same age, SSAT-ko mice on the high fat diet weighed 52.7 ± 2.7 g, a gain of 22.8 g or 76% over SSAT-ko mice on chow. By contrast, the SSAT-tg mice on the high fat diet gained only 2.3 g or 8% over SSAT-tg mice on chow. Under the high fat diet, 100% of the SSAT-ko mice were clearly larger than SSAT-wt as compared with only 20% under chow diet. Thus, in addition to confirming the influence of SSAT expression status on fat accumulation, monitoring weight gain on the high fat diet exaggerated the SSAT-ko phenotype so that it was clearly distinguishable from SSAT-wt.

DISCUSSION

The present studies were initiated on the basis of in vitro and in vivo observations showing that overexpression of SSAT unexpectedly lowered the SSAT coenzyme and fatty acid precursor, acetyl-CoA, in cultured cells (5) and in prostates of TRAMP mice (4). They were further catalyzed by the observation that transgenic overexpression of SSAT gives rise to a distinctly lean phenotype (6–8). Because the association of SSAT overexpression with fat loss represents an experimental exaggeration of normalcy in mice, the implications were somewhat limited. However, our subsequent observation that SSAT-ko mice tend to accumulate fat lends more credible significance to

**TABLE 5**

| Genotype | Glucose oxidation | Palmitate oxidation |
|----------|-------------------|---------------------|
|          | Liver WAT         | Liver WAT           |
|          | mmol/g tissue/h   | mmol/g tissue/h     |
| SSAT-ko  | 962 ± 112         | 537 ± 65*           | 167 ± 21           | 99 ± 14 |
| SSAT-wt  | 870 ± 171         | 2,373 ± 606         | 182 ± 51           | 124 ± 26 |
| SSAT-tg  | 1,208 ± 288       | 48,085 ± 8,382*     | 177 ± 35           | 2,376 ± 574* |
| cub/cub (hairless) | 1,782 ± 318 | 1,675 ± 358         | 213 ± 46           | 165 ± 33 |
| cub/+ (furred)   | 1,331 ± 307       | 3,811 ± 976         | 222 ± 26           | 84 ± 10 |

a Dunnett’s test, p = 0.005 relative to SSAT-wt (n = 4).

b Dunnett’s test, p = 0.05 relative to SSAT-wt (n = 4).
the association, since it infers that in wild-type mice, SSAT modulates fat metabolism to prevent overaccumulation. As further confirmation of the two phenotypes, serum leptin was found to be markedly elevated in SSAT-ko mice and reduced in SSAT-tg mice. Leptin is a fat-derived hormone (adipokine) involved in regulating food intake and energy expenditure (29), and serum leptin levels are generally proportional to total body fat mass (30). Taken together, the balanced association between SSAT expression status and body fat levels constitutes compelling evidence for a possible linkage between SSAT and fat metabolism. These findings, however, are not without their limitations; first, SSAT-tg mice lack hair, and this could affect evidence for a possible linkage between SSAT and fat metabolism. These findings, however, are not without their limitations; first, SSAT-tg mice lack hair, and this could affect

Although we initially expected that overexpression of SSAT would deplete polyamine pools, we now recognize that this manipulation is typically accompanied by a significant increase in polyamine biosynthetic activity, which tends to conserve polyamine pools (Fig. 1). The enzyme responses seen in liver and WAT are consistent with our earlier characterization of other tissues of the SSAT-tg mouse (7): SSAT, ODC, and SAMDC are all increased in activity. In addition, the enzyme product AcSpd was detected in all tissues analyzed (7), including both liver and WAT of SSAT-tg mice. The large amounts of urinary AcSpd (Table 2) in SSAT-tg samples is also indicative of massive cycling through both the biosynthetic and acetylation arms of the polyamine pathway (Fig. 1). Although deduced here, the metabolic fluxing due to SSAT activation has been previously proven to occur in cultured prostate carcinoma cells during conditional overexpression of SSAT (5).

Although characterization of polyamine metabolism in SSAT-tg tissues led to clear indications of heightened metabolic flux, a similar analysis of SSAT-ko mice was less defining. Our expectation based on the SSAT-tg mouse findings was that the biosynthetic enzymes and acetylated polyamines would be decreased in the SSAT-ko mice, but this was difficult to measure in WAT due to inherently low basal enzyme activities. Such a decrease was, however, seen in certain other tissues (i.e. intestine) that maintain higher basal levels of ODC and SAMDC (data not shown). Although we could not provide unequivocal evidence for a decrease in metabolic flux in SSAT-ko mice, it is reasonable to assume that in the absence of SSAT, basal and conditional flux are reduced in SSAT-ko mice relative to SSAT-wt mice, and certain of the polyamine findings support this conclusion. The SSAT-ko urinary polyamines showed consistent changes that suggest decreased flux at the systemic level. More particularly, Put was markedly decreased as would be expected with a fall in tissue ODC, and Spd was increased possibly to compensate for the inability of the SSAT-ko mice to generate and excrete AcSpd.

Our previous findings in cells and tumors (4, 5) predict that altered polyamine acetylation and polyamine metabolic flux in the three mouse types will differentially affect acetyl-CoA tissue levels. Consistent with this prediction, acetyl-CoA was found to be reduced in the SSAT-tg WAT and increased in SSAT-ko WAT relative to SSAT-wt. Such changes in acetyl-CoA can result in regulatory shifts in core metabolic pathways, such as glycolysis and the tricarboxylic acid cycle (27, 28), and may account for the changes in glucose oxidation seen in SSAT-tg mice. The additional and possibly more relevant observation that levels of malonyl-CoA, the downstream product of acetyl-CoA carboxylase, were similarly changed in the three mouse types has profound implications in fat metabolism.

In addition to serving as the source of two carbon units used in fatty acid biosynthesis, malonyl-CoA is well recognized as an allosteric inhibitor of carnitine palmitoyltransferase 1, which transports fatty acids into mitochondria for oxidation (24). When malonyl-CoA pools are low, as in SSAT-tg WAT, β-oxidation of fatty acids is activated in the mitochondria via activation of the fatty acid pathway (27, 28), and may account for the changes in glucose oxidation seen in SSAT-tg mice. The additional and possibly more relevant observation that levels of malonyl-CoA, the downstream product of acetyl-CoA carboxylase, were similarly changed in the three mouse types has profound implications in fat metabolism.

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Polyamine Acetylation and Fat Metabolism

FIGURE 6. Proposed metabolic shifts in SSAT-tg and -ko WAT. In the SSAT-wt mouse (B), acetyl-CoA is homeostatically partitioned between SSAT acetylation of polyamines and fatty acid (FA) metabolism. Transgenic overexpression of SSAT (A) gives rise to heightened metabolic flux and the diversion of acetyl-CoA from fatty acid metabolism to acetylation of polyamines, which are then excreted in the urine. Specifically, fatty acid oxidation increases due to a decline in malonyl-CoA, the regulator of carnitine palmitoyltransferase 1 (24). Deletion of SSAT (C) eliminates polyamine acetylation, rendering acetyl-CoA available for fatty acid biosynthesis.

Driven. Calorimetric measurements (i.e., energy expenditure and oxygen consumption) in SSAT-tg mice show an increased rate of oxidation. In support of this finding, oxidation of exogenous palmitate was much higher in SSAT-tg WAT than in SSAT-wt WAT, and glucose oxidation was increased to a similar extent. These observations are similar to those reported by Oh et al. (32) showing that primary adipocytes from mice lacking acetyl-CoA carboxylase 2, the enzyme responsible for malonyl-CoA synthesis, also show increased palmitate and glucose oxidation.

The possible contribution of hairlessness to the SSAT-tg phenotype was addressed by comparison studies with hairless C57BL/6 mice homozygous for the cub gene mutation (26). Although lacking hair, the mice are fully immunocompetent, and there was no indication that the cub mutation affects polyamine metabolism. Although the mice were also found to be lean, they were not nearly so lean as SSAT-tg mice, as indicated by respective fat pad weights of 140 mg versus 10 mg at 30 weeks. Importantly, acetyl- and malonyl-CoA stores were not depleted in the cub/cub WAT, and palmitate and glucose oxidation were only modestly affected (<2-fold) compared with the 20-fold effect seen in SSAT-tg WAT. Taken together, the studies establish that the mechanistic linkage between polyamines and fat metabolism seen in SSAT-tg mice is not attributable to hairlessness.

As noted above, heterogeneity of the SSAT-ko phenotype presented a challenge to the interpretation of our findings. Thus, the high fat diet-induced obesity strategy was employed to exaggerate the differences between the transgenic, wild-type, and deleted phenotypes. Under this diet, the SSAT-ko mice were found to be uniformly distinct from SSAT-wt mice, which likewise were statistically different from the SSAT-tg mice (Fig. 5). The findings confirm indications that SSAT-ko mice are prone to fat accumulation as first noted in 20% of SSAT-ko on chow (Fig. 3) despite the fact that they consume comparable amounts of food. As noted above, the SSAT-ko mice on chow also show increases in acetyl- and malonyl-CoA pools, which, on a correlative basis, supports our initial tenet, namely that SSAT-induced changes in these fatty acid precursors modulates fat accumulation. In SSAT-ko mice, the heightened availability of acetyl- and malonyl-CoA favors fat accumulation, whereas in SSAT-tg mice, their depletion favors fat oxidation (Fig. 6C). It is relevant that, although the mean SSAT-wt mouse weight rose from 30.3 g on chow to 40.3 g on the high fat diet, the SSAT-tg mice completely resisted weight gain, an observation that may have relevance to the development of antiobesity strategies. The very recent report by the Finnish group (33) that SSAT-ko mice show signs of insulin resistance is consistent with our observation that they are prone to fat accumulation.

Genetically lean or obese mice are not uncommon. In a recent review, Reitman (30) discusses the metabolic implications of ~40 genetically lean mice, and none of them involve polyamine metabolism. Another unique aspect of the mice reported here is that divergent expression of a singular gene leads to opposite phenotypes with SSAT-tg tending to lose fat and SSAT-ko tending to accumulate fat. In addition, we believe that we may have identified the metabolic basis for these phenotypes, namely SSAT modulation of acetyl- and malonyl-CoA. In considering the various lean mice reported previously (30), we were struck by the similarity of the SSAT-tg mice to those in which acetyl-CoA carboxylase 2 has been genetically deleted (32, 34). Acetyl-CoA carboxylase 2 is an isoform enzyme that catalyzes the ATP-dependent carboxylation of acetyl-CoA to malonyl-CoA. As with the SSAT-tg mice, the acetyl-CoA carboxylase 2-deleted mice were leaner, consumed more food, and were more resistant to obesity on a high fat diet than wild-type mice. In further similarity to SSAT-tg mice, they displayed a huge (~8-fold) increase in palmitate oxidation in primary adipocytes. We attribute the increased fatty acid oxidation to compartmentalized relief of carnitine palmitoyltransferase 1 inhibition by the absence of acetyl-CoA carboxylase 2-generated malonyl-CoA. In the SSAT-tg mice, increased fatty acid oxidation can also be attributed to relief of malonyl-CoA inhibition of carnitine palmitoyltransferase 1 due to SSAT-mediated diversion of acetyl-CoA to palmitoyl acetylation (Fig. 6A). The centrality of acetyl-CoA carboxylase, acetyl-CoA, and malonyl-CoA in regulating fatty acid oxidation is further indicated by a recent report in which Qi et al. (35) showed that transgenic overexpression of Tribbles 3 (TRB3), the pseudokinase that regulates degradation of the acetyl-CoA carboxylase protein, protects mice from diet-induced obesity.
In conclusion, we present data consistent with the idea that SSAT-tg mice have a low body fat mass due to the diversion of acetyl-CoA from malonyl-CoA and fatty acid biosynthesis to polyamine acetylation (Fig. 6A). We propose that lowering of malonyl-CoA and acetyl-CoA actively contributes to the phenotype by favoring glucose and fatty acid oxidation. In the absence of polyamine acetylation, SSAT-ko mice tend to accumulate body fat via the increased availability of acetyl-CoA and malonyl-CoA for fatty acid synthesis and down-regulation of fatty acid oxidation (Fig. 6C). Although the above metabolic dynamics were documented in WAT, we hypothesize that edge been previously reported. It is interesting to consider that metabolic flux, they are negatively regulated by pathway end products Spd and Spm at the level of protein translation (36) and, hence, may not be suitable for maintaining metabolic flux through the pathway by facilitating their polyamine moieties out of the pathway by diverting polyamine moieties out of the pathway by facilitating their export out of the cell. Although one might intuitively expect that the biosynthetic enzymes be more effective in controlling flux, they are negatively regulated by pathway end products Spd and Spm at the level of protein translation (36) and, hence, may not be suitable for maintaining metabolic flux through the pathway. Our findings suggest that altered SSAT expression can affect fat metabolism. It now becomes of interest to determine whether changes in fat metabolism can affect SSAT expression and polyamine flux and, if so, whether feedback mechanisms possibly involving peroxisome proliferator-activated receptor-γ (37–39) are involved.

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