Mutation of mouse Samd4 causes leanness, myopathy, uncoupled mitochondrial respiration, and dysregulated mTORC1 signaling

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Sterile alpha motif domain containing protein 4 (Samd4; also called Smaug1) is a mammalian homolog of Drosophila Smaug, a translational repressor in fly embryos (1, 2). Sterile alpha motifs (SAM) have been shown to directly bind mRNA with stem-loop structures known as Smaug recognition elements (SRE) having the consensus sequence CNGG or CNGGN (3–5). Several studies have investigated the function of Samd4 in cultured mammalian cells. For example, when ectopically expressed in mammalian cells, Samd4 formed cytoplasmic granules containing polyadenylated RNA and markers of stress granules (6). Samd4 was also reportedly detected in neuronal dendrites, within mRNA-silencing foci that disassembled in response to the neurotransmitter N-methyl-D-aspartate (7). Recently, Samd4 overexpression was shown to suppress hallmarks of myotonic dystrophy-1 (DM1; Online Mendelian Inheritance in Man no. 160900) in a Drosophila model of the disease and in myoblasts from DM1 patients (8). However, the physiological role of Samd4 in mammals remains unknown.

Here, we describe an N-ethyl-N-nitosourea (ENU)-induced phenotype identified because homozygous mice are exceptionally thin and, therefore, named supermodel. The supermodel phenotype was attributed to a missense mutation of Samd4. Our data indicate that Samd4 is required for metabolic homeostasis and mechanistic target of rapamycin complex 1 (mTORC1) signaling.

Results

The supermodel Phenotype. We observed a mouse with a markedly lean body and thoracic kyphosis among the third generation (G3) of C57BL/6J mice carrying mutations induced by ENU (Fig. L4). The recessive phenotype, named supermodel (spmd), was transmitted in Mendelian fashion equally to male and female offspring, suggesting that an autosomal loss-of-function mutation was responsible. Both male and female homozygotes were highly infertile. Mutant mice had a statistically shortened life span compared with control littersmates, with deaths beginning at approximately 3 mo of age (Fig. 1B).

Male and female homozygous spmd mice fed standard rodent chow exhibited lower body weight, body mass index, and body length relative to wild-type littersmates (Fig. 1C and Table S1). When challenged with a high fat diet (HFD), wild-type mice increased their initial body weight 1.5-fold (males; \( P < 0.01 \)) or 1.8-fold (females; \( P < 0.01 \)) over a 6-mo period, whereas spmd homozygotes increased it by only 1.3-fold (males; \( P = 0.028 \)) or 1.1-fold (females; \( P = 0.10 \)) (Fig. 1C), indicating elevated resistance to HFD-induced obesity in spmd mice. Circulating levels of cholesterol and high-density lipoprotein (HDL) were reduced, whereas low-density lipoprotein (LDL) was elevated in spmd compared with wild-type mice (Table S2). Pathology analysis of liver sections suggested steatosis in 1 of 4 homozygous spmd mice.

We investigated the weight difference between spmd and wild-type mice by using computed tomography (CT), which indicated that spmd mice had reduced fat and muscle tissue (Fig. 1D). The reductions in spmd mice of both fat and muscle volume were proportionate to the overall reduction in whole body volume (Fig. S1A). However, the proportion of total fat volume corresponding to visceral fat was decreased, whereas the proportion


The authors declare no conflict of interest.

Data deposition: The mutant mouse strain reported in this paper has been deposited in the Mouse Genome Informatics database (accession no. 5547989), and the Mutant Mouse Regional Resource Center repository (ID# 37155-MU).


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Significance

Whereas many heritable obesity phenotypes are known, lean phenotypes are comparatively uncommon. Yet they can reveal critical checkpoints regulating energy balance. During a large-scale random germ-line mutagenesis project, we identified mice with a lean phenotype, myopathy, excessive energy expenditure despite diminished cage activity, and impaired glucose tolerance. This phenotype, termed “supermodel,” was strictly recessive and was ascribed to a missense mutation in Sterile alpha motif domain containing protein 4 (Samd4), a gene encoding an RNA-binding protein with no previously known function in mammals. This study provides evidence that Samd4 modulates the activities of the mechanistic target of rapamycin complex 1, a master regulator of metabolism.
of total fat volume corresponding to s.c. fat was increased, indicating an alteration in fat distribution in spmd mice (Fig. S1B). No change in bone mineral density or bone mineral content was observed in spmd mice (Fig. S1C).

We collected adipose tissues from 15-wk-old male and female spmd and wild-type littermates. Consistent with the lean phenotype of mutants, the weights of epigonadal white adipose tissue (eWAT) and inguinal WAT (iWAT) from notype of mutants, the weights of epigonadal white adipose tissue and wild-type littermates. Consistent with the lean phenotype, the weights of spmd homozygotes during the period of monitoring (Right). Mouse numbers at start of experiment: regular chow, n = 6 male and 7 female WT mice, n = 10 male and 6 female spmd mice; HFD, n = 13 male and 11 female WT mice, n = 8 male and 5 female spmd mice; WT littermates (n = 3) determined by CT analysis. Representative photographs of eWAT and iWAT from 9-wk-old male mice. Weights of eWAT and iWAT normalized to body weight in 16-wk-old mice (n = 4 male and 4 female WT mice, 4 male and 5 female homozygous spmd mice). Data in C, D, and F represent means ± SD. P values were determined by Student’s t test unless indicated.

A Mutation of Samd4 Causes the spmd Phenotype. To identify the mutation responsible for the spmd phenotype, F1 mice produced by intracytoplasmic injection of sperm from spmd homozygotes into eggs from C57BL/10J mice were intercrossed, and a total of 43 F2 offspring (10 with lean phenotype and 33 with normal body size) were used for genetic mapping by bulk segregation analysis (BSA). We detected peak linkage of the phenotype (LOD 5.41) with a single nucleotide polymorphism (SNP) at 55054219 bp on chromosome 14 (Fig. 3A), within a critical region bounded by SNPs at 29705069 and 76795502 bp (National Center for Biotechnology Information m38). Whole genome SOLiD sequencing of coding exons and splice junctions identified a single mutation within the critical region, an A to C transversion at 47016337 bp on chromosome 14 within Samd4 (Fig. 3B). The mutation occurs at 994 bp within the Samd4 transcript (Vega OTT000000057924) and leads to a histidine-to-proline substitution at amino acid 86 of the 711-aa Samd4 isoform 1 and the 623-aa Samd4 isoform 2. Expression of Samd4 was detected in both muscle and fat tissue of wild-type mice, with 9- to 10-fold higher levels in muscle than in eWAT (Fig. S4A). Samd4 is one of two mammalian homologs of Drosophila Smaug, which functions to repress translation of developmental regulators such as nanos in fly embryos (1, 9). The second mammalian Smaug homolog is Samd4B; it reportedly repressed transcription in a SAM domain-dependent manner upon overexpression in HEK293 cells (10).

Human and mouse Samd4 share sequence similarity with Smaug within two N-terminal regions designated Smaug similarity region (SSR) 1 and SSR2, and in the RNA-binding SAM domain (3, 4). The spmd mutation affects a histidine within SSR2 (Fig. 3C) and is predicted to be probably damaging by PolyPhen-2 (score 1.000). Intact expression of mutant Samd4 protein was detected in heart lysates from homozygous spmd mice, indicating that the mutation does not abrogate protein expression (Fig. S4B).

Genotyping of individual mice from mapping crosses confirmed homozygosity of the Samd4 mutation in each mouse with the lean phenotype, but none of the mice with normal body size. A BAC containing C57BL/6J genomic DNA encompassing the Samd4 5’ UTR through the third exon was modified by replacing exon sequences downstream from the start codon with a Samd4-GFP cDNA construct encoding Samd4 isoform 2 (Fig. S5A). Sequencing identified no mutations in other genes contained by the modified BAC, which were LOC102638347 and a partial
sequence of Cgrrf1, both genes of unknown function. Transgenic expression of the modified BAC in homozygous spmd mice (Fig. S5 B and C) significantly increased their body size and weight (Fig. S5 D and E); more complete rescue of the phenotype may result from transgenic expression of both Samd4 isoforms 1 and 2.

We also performed CT analysis to determine whether fat or muscle tissue might be improved preferentially by transgenic Samd4 expression. Fat volume and muscle volume relative to total body volume in transgenic spmd homozygotes were similar to those of wild-type mice, indicating that transgene expression rescued both fat and muscle, increasing them in their normal proportions relative to total body volume (Fig. S5F). The alteration in visceral and s.c. fat distribution observed in homozygous spmd mice was corrected by the transgene (Fig. S5G). These data demonstrate that the spmd phenotype is caused by the H86P mutation of Samd4.

**Altered Glucose Metabolism in spmd Mice.** The effects of the spmd mutation on adipose and muscle tissue led us to examine metabolic parameters in spmd mice. Spmd homozygotes 10 to 12 wk of age had reduced fasting glucose and insulin levels compared with wild-type mice (Fig. 4 A and B). However, i.p. glucose tolerance tests showed delayed clearance of glucose from the blood of spmd mice compared with wild-type mice (Fig. 4C). These data demonstrate that transgene expression rescued both fat and muscle, increasing them in their normal proportions relative to total body volume (Fig. S5F). The alteration in visceral and s.c. fat distribution observed in homozygous spmd mice was corrected by the transgene (Fig. S5G). These data demonstrate that the spmd phenotype is caused by the H86P mutation of Samd4.

**Uncoupled Respiration in WAT and Skeletal Muscle of spmd Mice.** To understand the nature of the energy imbalance that leads to leanness in spmd mice, we examined their energy expenditure and caloric intake. We assessed energy expenditure in vivo by metabolic cage studies of 10- to 12-wk-old male homozygous spmd and wild-type mice. Spmd mice had significantly enhanced oxygen consumption and carbon dioxide production over a 72-h period, indicative of elevated energy expenditure (Fig. 5A). Paradoxically, spmd mice were hypoactive, as shown by decreased ambulatory movement and exploratory rearing/jumping relative to wild-type mice, especially during the dark period of the light cycle (Fig. 5B). We found that caloric intake normalized to body weight was similar between spmd and wild-type mice (Fig. 5C). Thus, spmd mice consumed a similar number of calories as wild-type mice, but expended a dramatically greater amount of energy despite diminished mobility. The disparity between activity and energy expenditure led us to hypothesize that mitochondrial respiration may be uncoupled from ATP generation in spmd mice. We tested the expression of mitochondrial uncoupling genes and brown fat markers in WAT by quantitative PCR. Ucp1 and Ucp3 are uncoupling genes and markers of brown fat, and both showed significant increases in expression in spmd compared with wild-type eWAT (Fig. 5D). In addition, brown fat markers Prdm16 and Cidea, and uncoupling gene Ucp2, were elevated in spmd eWAT (Fig. 5D). These data suggest a switch by spmd eWAT to a brown-like adipocyte identity characterized by the expression of thermogenic genes. However, Pragc1a, encoding PGC-1α, and Pdnm16, key transcriptional regulators of brown adipocyte development, were expressed at wild-type levels in spmd eWAT.

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**Fig. 2.** Adipocyte defects and myopathy in spmd mice. (A) Sections from eWAT of 8- to 10-wk-old WT and homozygous spmd mice were examined by bright field (Upper) and fluorescence microscopy (Lower). (B) Hind limb muscle sections from 8- to 10-wk-old WT and homozygous spmd mice. (Top) H&E staining of gastrocnemii. Green arrowhead indicates myofibers with irregular shapes. Metachromatic ATPase staining (Middle), and myosin (Type I, slow) staining (Bottom). Filled triangles indicate soleus (Sol); open triangles indicate gastrocnemius (Gast). (Scale bars: 60 μm.)

**Fig. 3.** Identification of the spmd mutation in Samd4. (A) Chromosomal mapping of the spmd mutation by BSA. LOD, logarithm of odds score. (B) DNA sequence chromatogram of the mutated nucleotide in Samd4. (C) Domain structure of Samd4. The spmd mutation is indicated in red. SAM, sterile alpha motif; SSR, Smaug similarity region.
It may be that PGC-1α and PRDM16 are controlled at the posttranscriptional level in spmd eWAT, as has been shown for PRDM16 induced by PPARγ agonists (11). Up-regulation of Ucp2, Cidea, and Prdm16 expression were observed in spmd skeletal muscle (Fig. 5E). Together, these data strongly suggest that mitochondrial uncoupling results in increased energy expenditure by spmd mice.

Samd4 Is Necessary for mTORC1 Signaling in Vivo, Is Phosphorylated by Akt, and Interacts with 14-3-3 Proteins In Vitro. mTORC1 is a key regulator of cell growth and metabolism, affecting the activities of signaling pathways involved in nutrient uptake, protein and lipid synthesis, energy expenditure, and autophagy (12). Raptor is an mTORC1 component that functions as a scaffold protein regulating substrate binding to the complex. Spmd mice phenocopied muscle- and adipose-specific Raptor mutants in many aspects, such as reduced adipose tissue and skeletal muscle mass, muscular dystrophy, resistance to HFD-induced obesity, and elevated oxidative respiration due to mitochondrial uncoupling in adipose tissue (13, 14). We therefore hypothesized that Samd4 may be necessary for mTORC1 signaling. In support of this hypothesis, we found that the mTORC1 target eIF4E binding protein 1 (4E-BP1), and also S6, a substrate of the mTORC1 kinase 1 (S6K1), were hypophosphorylated in both muscle and adipose tissues from homozygous spmd mice compared with wild-type mice (Fig. 6A).

We sought to identify Samd4-interacting proteins as a means to understand the mechanism of Samd4 function. We used C2C12 myoblast cells stably expressing Flag-tagged wild-type Samd4 or Samd4-H86P for quantitative mass spectrometric analysis following immunoprecipitation with Flag antibody. Wild-type Samd4 and Samd4-H86P immunoprecipitates contained, respectively, 220 and 212 putative interactors (Dataset S1). Samd4 itself was among the proteins with the highest spectral counts, indicating effective pull down of the target protein complex. None of the known mTORC1 complex components were found in either wild-type or mutant Samd4 immunoprecipitates. However, among wild-type Samd4 interactors were six of seven mammalian 14-3-3 protein isoforms, which were represented with high spectral counts, ε and ζ being the most abundant. Three of the isoforms (η, 0, β) were completely absent, and three were present in greatly reduced amounts (ε, ζ, γ) in the Samd4-H86P immunoprecipitate.
The 14-3-3 proteins propagate signaling in numerous pathways by binding to phosphorylated serine residues within the consensus sequences (R/K)XX(pS/pT)XP (mode I) and RX(P/Y)X(pS)XP (mode II), with mode I sequences more prevalent among reported 14-3-3 binding proteins (15, 16). Two putative 14-3-3 binding motifs were identified in Samd4 that conformed to the mode I motif (RSV(pS)LT, aa 251–265; KTR(pS)LP, aa 655–660) (17). By immunoprecipitation and immunoblotting, we confirmed an interaction between Samd4 and 14-3-3 in Flag-Samd4-expressing C2C12 cells and in 293T cells overexpressing Samd4 and 14-3-3 (Fig. 6B). Moreover, we showed that Samd4 is phosphorylated on serine(s) within a 14-3-3 binding motif (Fig. 6C). Consistent with the mass spectrometry data, Flag-Samd4-H86P failed to pull down 14-3-3 proteins (Fig. 6B), and was not phosphorylated on serine(s) within any 14-3-3 binding motif (Fig. 6C). These data suggest that 14-3-3 proteins exist in a complex with phosphorylated Samd4, but not Samd4-H86P.

The serine/threonine kinase Akt/PKB phosphorylates sites within a consensus sequence similar to the 14-3-3 binding motif (RXRX[X/S/T]T), and Samd4 contains such a motif (RGRSDS; aa 163–168) (17). We therefore tested whether Akt is capable of phosphorylating Samd4 in vitro. Incubation of purified recombinant GST-Samd4 or GST-Samd4-H86P with ATP and activated Akt (myr-Akt1-HA) immunoprecipitated from 293T cells resulted in accumulation of phosphate groups on Akt phosphorylation sites in wild-type Samd4 (Fig. 6D). However, Akt failed to phosphorylate Samd4-H86P, and the negative control protein 4E-BP1 (18). Thus, Samd4 is subject to phosphorylation by Akt in vitro. The spmd mutation, which lies outside putative Akt phosphorylation site consensus sequences, may disrupt the conformation of Samd4 and, thereby, prevent phosphorylation.

**Discussion**

The striking resemblance of the spmd phenotype to the phenotypes of muscle- and adipose-specific Raptor mutants (13, 14), together with significantly diminished levels of phosphorylation on 4E-BP1 and S6 in spmd tissues, provides strong evidence that Samd4 plays an important role in metabolic regulation in conjunction with mTORC1 in vivo. Samd4 coimmunoprecipitated with all 14-3-3 isoforms in the muscle progenitor cell line C2C12, suggesting that Samd4 may mediate its effects through interactions with 14-3-3 proteins. The 14-3-3 proteins have been shown to play a positive role in mTORC1 signaling through sequestration of the mTOR inhibitor PRAS40 (19), and through binding and inhibition of TSC2, a negative regulator of mTORC1 (20). Similarly, Akt activates mTORC1 by mediating the inhibitory phosphorylation of PRAS40 and TSC2 (21, 22). Akt also phosphorylates Samd4, supporting the hypothesis that Samd4, 14-3-3, PRAS40, and TSC2 function together in a regulatory network downstream from Akt that activates mTORC1.

SAMD4 is known as a translational repressor that binds directly to RNA. In *Drosophila*, Smaug inhibits translation by blocking the formation of a competent translation initiation complex (9, 23), by promoting deadenylation of target mRNAs leading to their destabilization and degradation (24–26), and by microRNA-independent recruitment of Argonaute 1 (Ago1) to target mRNAs (27). RNA immunoprecipitation combined with microarray analysis demonstrated that Smaug binds directly to numerous sites in various mRNA species by using the indicated antibodies. (8) Flag immunoblots (Upper) were reprobed with either 14-3-3 or Myc antibodies (Lower). Lanes 1 and 4 contain 10% of the IPs from C2C12 cells; lanes 1 and 2 contain 100% of the IPs from 293T cells. (C) 50% of Flag immunoprecipitates from C2C12 cells were immunoblotted using Flag antibody (Upper). The remaining 50% of the same immunoprecipitates were also immunoblotted with p-Ser-14-3-3 binding motif antibody (Lower). (D) In vitro kinase assay. Five micrograms of purified recombinant proteins visualized by Coomassie blue staining (Left). Recombinant proteins were incubated for 30 min with ATP and HA-tagged activated Akt immunoprecipitated from 293T cells. Reaction mixtures were immunoblotted with phospho-Akt substrate and HA antibodies (Right).
embryonic fly RNAs, including those encoding glycolytic enzymes, and components of the proteasome regulatory particle, the TRiC/CCT chaperonin, and lipid droplets (28). In total, Smaug has been shown to destabilize ~1,000 RNAs in fly embryos (2). Human Samd4 has been shown to both repress and promote translation under conditions of overexpression (6, 8).

Whether the metabolic regulatory role of Samd4 involves RNA binding and translational repression remains unknown. Nonetheless, it is notable that mTORC1 is a primary regulator of translation, acting through S6K1 and 4E-BP1, key translational regulators that promote protein synthesis when phosphorylated by mTORC1 (29). Global analysis of the targets of acute translational regulation by mTORC1 in mouse embryonic fibroblasts demonstrated wide variation in their levels of translational inhibition, indicating that the targets of mTORC1 are differentially regulated, although the mechanisms that mediate this fine tuning are not known (30). Our findings raise the possibility that RNA binding and translational regulation by Samd4 represent one mechanism for modulating mTORC1-dependent translation.

The energy-wasting diastase and inanimation of supermodel homoyzogotes leads us to hypothesize that dysregulation of Samd4 and/or associated proteins may occur in chronic diseases of infectious and neoplastic etiology, where identical metabolic problems are commonly observed. Because Samd4 is expressed much more prominently in muscle than in fat, it is also plausible to consider that some of the effects of Samd4 mutation are not cell intrinsic, but mediated by unknown molecules that communicate between these two cell types. If extracellular signals influence Samd4, they might consequently influence metabolic activity.

Materials and Methods

Detailed materials and methods are described in SI Materials and Methods.

Mice and in Vivo Analyses. Mice were fed standard chow (Teklad 2016 Harlan) or 60 kcal % high-fat diet (Research Diet) and maintained at the University of Texas Southwestern Medical Center in accordance with institutionally approved protocols. The spmd strain (C57BL/6J-Samd4(Δt68)) (Mouse Genome Informatics ID 5547998) was generated by ENU mutagenesis, is described at http://mutagenetix.utsouthwestern.edu, and is available from the Mutant Mouse Regional Resource Center. CT scans; metabolic profiling; glucose, insulin, and arginine tolerance tests; and hyperglycemic clamp are described in SI Materials and Methods.

Expression of GST-Tagged Fusion Proteins and in Vitro Kinase Assay. GST, GST-Samd4, GST-Samd4-H86P, and MBP-4E-BP1 were expressed and purified from BL21-CodonPlus (DE3)-RIPL competent cells (Agilent) by using standard procedures. For in vitro kinase assays, transiently expressed HA-tagged Akt1 was immunoprecipitated from 293T cells and incubated with ATP and the purified recombinant proteins as described in SI Materials and Methods.

Transfection and Immunoprecipitation. C2C12 myoblasts and 293T cells were transfected with the indicated plasmids using Lipofectamine LTX with Plus Reagents (Invitrogen Life Technologies) or TransIT-LT1 (Mirus Bio), respectively, according to the manufacturer’s instructions. Immunoprecipitations were carried out by using standard procedures, as described in SI Materials and Methods.

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Supporting Information

Chen et al. 10.1073/pnas.1406511111

SI Materials and Methods

Mice. C57BL/6J mice purchased from The Jackson Laboratory were mutagenized with N-ethyl-N-nitrosourea (ENU) as described (1). Mutagenized G0 males were bred to C57BL/6J females, and the resulting G1 males were crossed to C57BL/6J females to produce G2 mice. G2 females were backcrossed to their G1 sires to yield G3 mice.

For genotyping, Samd4 genomic DNA amplified across the spmd mutation site was sequenced by using an ABI 3730 XL capillary sequencer. The following primers were used for PCR: 5′-TGACCAGATCCATCCACAGTTGAG-3′, 5′-TCCATAATCC-ACAGAGTCCGAGG-3′. The following primer was used for sequencing: 5′-CACAGTTGGAACATTAGCCAG-3′.

Body weights and survival rates were recorded once per week. Gastrocnemius and soleus muscles, epigonadal white adipose tissue (eWAT), and heart were dissected from mice, and homogenized and lysed in RIPA buffer. Proteins were separated by SDS/PAGE, transferred to membranes, and immunoblotted by using the indicated antibodies.

In Vivo Computed Tomography Scans. Mice were anesthetized by 1% isoflurane inhalation and positioned in the mouse bed. The whole body of each mouse (from nose to start of tail) was scanned at an isotropic voxel size of 93 μm (80 kV, 450 μA, and 100 ms integration time) by using the eXplore Locus micro-computed tomography (CT) scanner (GE Healthcare). Each scan took approximately 15 min, and the mice showed no sign of discomfort during the procedure. Three-dimensional images were reconstructed from 2D grayscale images by using Microview Software advanced fat analysis tool. For determination of total adipose tissue volume, a region of interest (ROI) was drawn around the body of the animal and then a histogram of grayscale values was created for the selected ROI. Quantitation of fat, muscle, and bone volumes was performed by applying standard grayscale thresholds for each tissue type (adipose: upper threshold 70, lower threshold 400; muscle: upper threshold 300, lower threshold 70, bone: upper threshold 4000, lower threshold 300). The abdominal muscular wall was used as the differentiation line to separate visceral adipose tissue from s.c. adipose tissue. Contour lines were drawn around the viscera, and a 3D ROI was generated. Visceral fat volume was determined from the corresponding histogram by using the thresholds listed above. s.c. fat volume was determined by subtracting visceral fat volume from total body fat volume.

Indirect Calorimetry. In vivo metabolic profiling was assessed by using the Oxymax/CLAMS monitoring system (Columbus Instruments). Monitoring occurred over 72 h under a normal 12:12 h light-dark cycle. Measurements were taken at an interval of either 12 min for energy parameters or 72 min for activity parameters. Each mouse was subjected to 24-h acclimation before the experiment with free access to water and food in the individual chamber.

Measurement of Circulating Lipoproteins. Mice (n = 4 per group) were fasted for 5 h before submandibular bleeding. Serum levels of triglyceride, cholesterol, HDL, LDL, and very low-density lipoprotein (VLDL) were measured by using the Vitros 250 system (GMI).

i.p. Glucose Tolerance, Insulin Tolerance, and Arginine Tolerance Tests, and Hyperglycemic Clamps. Glucose tolerance tests were initiated by injection with glucose (1.5 g/kg, i.p.) after a 16-h fast. Blood was collected from the tail vein for measurement of glucose with the AlphaTRAK glucometer and test strips. Insulin and arginine tolerance tests were performed as described (2). Insulin tolerance tests were initiated by injection with human insulin (0.75 mU/kg, i.p.) after a 3-h fast. Arginine tolerance tests were initiated by injection of l-arginine (1 mg/g, i.p.) after a 6-h fast. Hyperglycemic clamps were performed as described (3).

Bulk Segregation Analysis and BAC Transgenesis. Bulk segregation analysis was performed as described (4) using mice grouped based on phenotype (lean vs. normal body size). SOLiD 4 sequencing was performed according to manufacturer instructions (Applied Biosystems), and data was analyzed as described (5). The effect of the spmd mutation was predicted by using the HumDiv-trained PolyPhen-2 server (version 2.2.2) (6).

To generate the modified BAC, the cDNA sequence from the beginning of Samd4v2 to the end of the kanamyacin selection marker was recovered from pEGFP-N3-Samd4v2. The sequence was inserted into RP23-178L4 (BACPAC Resources Center) by homologous recombination, replacing exon sequences from the Samd4 start codon through exon 3, to obtain a modified BAC containing the Samd4v2 cDNA with intact upstream regulatory sequences (Fig. S5). The sequence of the BAC was verified by DNA sequencing.

Transgenesis was performed by injecting the modified BAC into the male pronucleus of fertilized C57BL/6J oocytes (University of Texas Southwestern Transgenesis Core Facility). Embryos were transferred to pseudopregnant CD1 females, which gave birth to 32 pups. Two transgenic founders were identified by PCR genotyping using primers 115 (5′-CTTGGAAACGAGGTCGTCTTCG-3′), 117 (5′-CTTTATCTTGGATTCCTGGTGGC-3′), 172 (5′-GGATACTCATGGTGGATCTTCG-3′), 119 (5′-CTCTTCCAATAGCTGAAATGCC-3′), and 116 (5′-AGGGCGGGAGACTGGA-TTCC-3′) (Fig. S5).

Cells and Transfection. C2C12 myoblasts (ATCC CRL-1772) and 293T cells were cultured in high glucose DMEM (Gibco Life Technologies) containing 10% (vol/vol) FBS (HyClone), 10,000 units/mL penicillin, and 10,000 μg/mL streptomycin at 37 °C in 5% CO2. For establishment of stable cell lines, either pCMV-tag2B-Samd4v1 or pCMV-tag2B-Samd4v1-H86P was transfected into C2C12 myoblasts by using Lipofectamine LTX with Plus Reagents (Invitrogen Life Technologies) according to the manufacturer’s instructions. After 24 h, 800 μg/mL Geneticin (Gibco Life Technologies) was added to the medium for 2 wk to select the cells expressing Samd4 constructs. Limiting dilution assays in medium containing 800 μg/mL Geneticin were used to obtain the monoclonal cell lines 14A2 and 1C11, expressing wild-type Samd4 or Samd4-H86P cDNAs, respectively. The cell lines 14A2 and 1C11 were maintained in DMEM containing 10% (vol/vol) FBS and 600 μg/mL Geneticin.

Immunoprecipitation Assays. Cells were lysed for immunoprecipitations 24 h after transient transfections. C2C12 or 293T cells were harvested in cold CHAPS lysis buffer [0.5% (wt/vol) CHAPS, 40 mM Hepes at pH 7.4, 120 mM NaCl, and 1 mM EDTA] supplemented with Roche Complete Protease Inhibitor; 4× more Flag-Samd4-H86P-expressing cells were used than Flag-Samd4-expressing cells because of the higher expression
level of Flag-Samd4. The cleared lysates were incubated with either anti-Flag M2 magnetic beads diluted 1:50 (Sigma-Aldrich), anti-c-Myc agarose beads (Sigma-Aldrich), or mouse IgG magnetic beads (Cell Signaling Technology) on a rotator at 4 °C overnight. After three washes in cold lysis buffer, proteins were eluted in 6x Laemmli SDS sample buffer, fractionated by SDS/PAGE, and transferred to a membrane. Immunoblottting was performed by using the indicated antibodies.

For detection of Samd4-GFP in transgenic mice, hearts were dissected from mice and homogenized and lysed in cold RIPA buffer supplemented with Roche Complete Protease Inhibitor. The cleared lysates were incubated with anti-GFP mAb magnetic beads (MBL International) on a rotator at 4 °C overnight. After three washes in cold lysis buffer, proteins were eluted in 6x Laemmli SDS sample buffer, fractionated by SDS/PAGE, and transferred to a membrane. Immunoblottting was performed by using GFP antibody (Abcam).

Expression of GST-Tagged Fusion Proteins and in Vitro Kinase Assay. BL21-CodonPlus (DE3)-RIPL competent cells (Agilent) were transformed with pGEX6P1, pGEX6P1-Samd4, or pGEX6P1-Samd4-H86P. BL21 cultures were grown at 37 °C to log phase, after which 0.2 mM IPTG was added and the cultures were induced to express the proteins at 25 °C. Bacteria were harvested after overnight induction, sonicated on ice, and lysed in PBS lysis buffer containing 1% (vol/vol) Triton X-100, 10% (vol/vol) glycerol, 1 mM DTT, and 1 mM PMSF. The cleared lysates were incubated with Glutathione Sepharose 4B (GE Life Technologies) on a rotator at 4 °C overnight. Recombinant proteins were eluted in kinase buffer (25 mM Tris at pH 7.4, 10 mM MgCl$_2$, and 1 mM DTT) supplemented with 10 mM reduced glutathione. Expression of GST, GST-Samd4, and GST-Samd4-H86P was analyzed by SDS/PAGE followed by Coomassie Blue staining and immunoblottting.

myr-Akt1-HA plasmid (a gift from Scott Summers, Duke University, Durham, NC) was transfected into 293T cells. After 24 h, cells were harvested and lysed in PBS lysis buffer containing 0.5% (vol/vol) Nonidet P-40, 50 mM Tris at pH 7.4, 150 mM NaCl, 1 mM EDTA, and 10% (vol/vol) glycerol supplemented with Roche Complete Protease Inhibitor and PhosSTOP. Cleared lysates were incubated with anti-HA magnetic beads (Pierce) on a rotator at 4 °C overnight. After incubation, beads were sequentially washed twice by lysis buffer, twice by high salt wash buffer [5x PBS, 0.5% (vol/vol) Nonidet P-40, and 0.1% (wt/vol) SDS] and twice by kinase buffer. For nonradioactive in vitro kinase assay, recombinant proteins were incubated with equal amounts of Akt1 immunoprecipitate in reaction buffer (50 mM Tris at pH 7.4, 10 mM MgCl$_2$, 1 mM DTT, and 5 mM ATP) at 30 °C for 30 min. The reaction was stopped by adding 6x Laemmli SDS sample buffer, followed by SDS/PAGE, membrane transfer, and immunoblotting with the indicated antibodies.

**Immunohistochemistry and Metachromatic ATPase Staining.** Tissues for routine histology, special stains, and immunohistochemistry were harvested from anesthetized mice and fixed or cryoembedded according to standard procedures (7, 8) with modifications for tissue type and stains (9–11). Samples of distal hind limb muscle, including gastrocnemius, plantaris, soleus, extensor digitorum longus, and tibialis anterior, were either immersion fixed in 1% (wt/vol) periodic acid/10% neutral-buffered formalin (NBF) (9) for 48 h before storage in 70% (vol/vol) ethanol, or cryoembedded in gum tragacanth without fixation. Fixed muscle groups were paraffin processed and serial sections were prepared with H&E, as well as Periodic acid-Schiff stain for glycogen. Fiber typing in serial frozen sections made from cryoembds of unfixed muscle groups was performed by a metachromatic dye-ATPase method conducted at pH 4.4 for slow fiber-type specificity (10, 11). Type I myosin (slow) immunohistochemistry was performed on fresh muscle cryosections without antigen retrieval by using a monoclonal myosin (skeletal, slow) antibody (Sigma-Aldrich; clone NOQ7.5.4D) at a dilution of 1:16,000 overnight. Bound primary was detected with peroxidase-conjugated goat anti-mouse secondary antibody (Sigma-Aldrich; A8924), and color developed with DAB chromogen from DAKO. Adipose tissues were immersion fixed for 48 h in 10% NBF and stored briefly in 50% (vol/vol) ethanol. Subsequent paraffin processing and embedding were carried out and sections were prepared with H&E.

**Antibodies.** phospho-4E-BP1, 4E-BP1, S6, phospho-S6, 14–3–3 (pan), phospho-Ser-14–3–3 binding motif, and phospho-Akt substrate antibodies were from Cell Signaling Technology. β-actin, Flag, and Myc antibodies were from Sigma-Aldrich. Samd4 antibody (ARP3707_P050) was from Aviva Systems Biology. HA antibody was from Pierce. GFP antibody was from Abcam.

**Real-Time Quantitative RT-PCR.** Total RNA was isolated from tissues with TRizol reagent (Invitrogen) and used for cDNA synthesis with the SuperScript III system (Invitrogen). Real-time PCR was performed by using the StepOnePlus Real-Time PCR System (Life Technologies) with DyNAmo SYBR Green qPCR kit (Finnzymes). The mRNA expression levels relative to 18S rRNA expression were calculated by ΔΔCt methods. Expression levels of mitochondrial uncoupling genes and brown fat markers were analyzed by real-time quantitative PCR by using the following primers:

<table>
<thead>
<tr>
<th>Mouse genes</th>
<th>qRT-PCR primers</th>
</tr>
</thead>
<tbody>
<tr>
<td>18s</td>
<td>Fw: CGCGTTTCATATTGTTGCT; Rv: TCCTTCTCGAATCCGACT</td>
</tr>
<tr>
<td>Samd4</td>
<td>Fw: AAGTACCCTGCCCTCTCCTCC; Rv: CTGGAGCGCTGAGGCTG</td>
</tr>
<tr>
<td>PPARα</td>
<td>Fw: AGCGAGTACCTGACGCTGTTT; Rv: GCCCTTGACCTTTCATAGT</td>
</tr>
<tr>
<td>PPARγ</td>
<td>Fw: CTGTAAGCCGAGCGCTTGAGG; Rv: GCCATCTCTGGACTC</td>
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<tr>
<td>Ucp1</td>
<td>Fw: GCCCTTTGAAACACAAA; Rv: GTGCGCTTCTCTCTGAGT</td>
</tr>
<tr>
<td>Cidea</td>
<td>Fw: GCCGCGACGACAGACACTATC; Rv: CTTTCTCTGCCCTCCCTC</td>
</tr>
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<td>Ucp2</td>
<td>Fw: GCCGCGACGACAGACACTATC; Rv: CTTTCTCTGCCCTCCCTC</td>
</tr>
<tr>
<td>Ucp3</td>
<td>Fw: AAGGAGTACCGACGCTGAGT; Rv: AGGGATTCGACCACCTCTTCT</td>
</tr>
<tr>
<td>Myostatin</td>
<td>Fw: CATGACGATGAAAGGAGAT; Rv: TGGACTTCATACCCATTCCT</td>
</tr>
<tr>
<td>Fabp4</td>
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</tr>
<tr>
<td>Eif4ebp1</td>
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</tr>
<tr>
<td>Foxo1</td>
<td>Fw: AAGTCCGAGGAGAGGAACG; Rv: AGGAGTACCGACGCTGAGT</td>
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<tr>
<td>Eif4g1</td>
<td>Fw: AACTTGCGAGCGAGAATCTC; Rv: TTGCTCAGGCTCTTCTG</td>
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<td>Fgl21</td>
<td>Fw: TGGGCTGCTCATCAAGC; Rv: CACCCAGGATTGGAAGACC</td>
</tr>
<tr>
<td>Pgc1α</td>
<td>Fw: ATGGTGCGCTCCTCTGCTC; Rv: ATTTGACCTTCCTACCCCT</td>
</tr>
<tr>
<td>Prdm16</td>
<td>Fw: AAGGCGCGACGACGCTGAGT; Rv: AGGGATTCGACCACCTCTTCT</td>
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</table>

Chen et al. www.pnas.org/cgi/content/short/1406511111

2 of 6

Fig. S1. Reduced fat and muscle volume in spmd mice. CT analysis of 8-wk-old male homozygous spmd (n = 3) and WT littermates (n = 3). (A) Fat and muscle volume relative to total body volume. (B) Visceral fat volume and s.c. fat volume relative to total fat volume. (C) Bone mineral density and bone mineral content. (D) Weight of iBAT normalized to body weight in 16-wk-old mice (n = 4 male and 4 female WT mice, 4 male and 5 female homozygous spmd mice) and representative photographs of iBAT from 9-wk-old male and female mice. Data represent means ± SD. P values were determined by Student t test.
Fig. S2. Adipocyte defects in spmd mice. Sections from iWAT and iBAT of 8- to 10-wk-old WT and homozygous spmd mice were examined by bright field (Left) and fluorescence microscopy (Right). (Scale bars: 60 μm.)

Fig. S3. Increased glycogen content of spmd muscle. Sections from gastrocnemius muscles of 12-wk-old WT and homozygous spmd mice were stained with H&E (Upper) and Periodic Acid-Schiff (PAS) stains (Lower). Green arrowheads indicate muscle fibers with centralized nuclei. (Scale bars: 60 μm.)
Fig. S4. Levels of Samd4 mRNA and protein expression. (A) Expression of Samd4 in muscle and adipose tissue of WT mice (n = 4). Expression levels were normalized to 18S rRNA expression. Data represent means ± SD. *P value was determined by Student t test. (B) Lysates of heart tissue from WT and homozygous spmd mice were immunoblotted by using Samd4 or GAPDH antibodies. Samd4 isoform 1 (upper band) and isoform 2 (lower band) were detected.

Fig. S5. Partial rescue of spmd phenotype by transgenic expression of wild-type Samd4. (A) Construction of the modified BAC containing Samd4 isoform 2 cDNA. Organization of Samd4 genomic DNA encompassed by the original BAC, RP23-178L4 (Upper). A cDNA construct encoding GFP-tagged Samd4 isoform 2 (Lower) was used to replace exon sequences downstream from the start codon in the BAC. Positions of primers used for genotyping are indicated. (B) PCR genotyping of transgenic mice using the primers indicated in A. (C) Protein expression of Samd4-GFP in transgenic mice. Lysates of heart tissue from a WT mouse, Samd4/spmd mouse, and Samd4/spmd; Tg(Samd4) mouse were subject to immunoprecipitation by using anti-GFP-coupled beads. Immunoprecipitates were immunoblotted by using GFP antibody. (D) A Samd4/spmd mouse (Left) and a Samd4/spmd; Tg(Samd4) mouse (Right) at 8 wk of age. (E) Body weights of 8-wk-old WT, Samd4/spmd, and Samd4/spmd; Tg(Samd4) mice (*P value was determined by Student t test. (F and G) CT analysis of 10-wk-old male Samd4/spmd (n = 3), Samd4/spmd; Tg(Samd4) (n = 5), and WT mice (n = 3). (F) Fat and muscle volume relative to total body volume. (G) Visceral fat volume and s.c. fat volume relative to total fat volume. Data represent means ± SD. *P values were determined by Student t test.
Fig. 56. Increased insulin sensitivity of spmd mice. (A) Ratio of glucose infusion rate to plasma insulin concentration at the indicated times during hyperglycemic clamp in male WT (n = 8) and homozygous spmd mice (n = 5). For 0, 5, 10, 15, 30, 60, 90, and 120 min, respectively, P = 0.034, 0.0072, 0.11, 0.13, 0.73, 0.37, 0.042, and 0.17. (B) Arginine tolerance test. Serum insulin was measured at the indicated times after i.p. arginine injection in WT (n = 7) and homozygous spmd mice (n = 9). For 0, 5, and 15 min, respectively, P = 0.0025, 0.0006, and 0.31. Data in A and B represent means ± SEM. P values were determined by Student t test.

Table S1. Body length and body mass index of 9-wk-old mice

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Anal-nasal length, cm</th>
<th>Body mass index, kg/m²</th>
</tr>
</thead>
<tbody>
<tr>
<td>♂ +/+ (n = 7)</td>
<td>9.96 ± 0.11</td>
<td>2.46 ± 0.13</td>
</tr>
<tr>
<td>♂ spmd/spmd (n = 7)</td>
<td>9.21 ± 0.20</td>
<td>1.75 ± 0.15</td>
</tr>
<tr>
<td>♀ +/+ (n = 10)</td>
<td>9.44 ± 0.21</td>
<td>2.08 ± 0.10</td>
</tr>
<tr>
<td>♀ spmd/spmd (n = 8)</td>
<td>8.91 ± 0.16</td>
<td>1.71 ± 0.16</td>
</tr>
</tbody>
</table>

Data represent mean ± SD. For all differences between +/+ and spmd/spmd, P < 0.0001, determined by the Student t test.

Table S2. Circulating lipoproteins in 10-wk-old female mice

<table>
<thead>
<tr>
<th>Lipoprotein</th>
<th>+/+ (n = 4)</th>
<th>spmd/spmd (n = 4)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cholesterol, mg/dL</td>
<td>92 ± 7.8</td>
<td>77.8 ± 5.7</td>
<td>0.026</td>
</tr>
<tr>
<td>Triglycerides, mg/dL</td>
<td>92.8 ± 7.0</td>
<td>88.3 ± 9.6</td>
<td>0.48</td>
</tr>
<tr>
<td>HDL, mg/dL</td>
<td>73.8 ± 6.7</td>
<td>57.3 ± 5.9</td>
<td>0.010</td>
</tr>
<tr>
<td>LDL, mg/dL</td>
<td>0.3 ± 0.5</td>
<td>2.8 ± 1.7</td>
<td>0.031</td>
</tr>
<tr>
<td>VLDL, mg/dL</td>
<td>18.3 ± 1.3</td>
<td>17.8 ± 2.1</td>
<td>0.69</td>
</tr>
</tbody>
</table>

Data represent mean ± SD. P values were determined by the Student t test. VLDL, very low density lipoprotein.

Other Supporting Information Files

Dataset 1 (XLSX)