Hepatic phosphoserine aminotransferase 1 (PSAT1) regulates insulin sensitivity in mice via tribbles homolog 3 (TRB3)

Junjie Yu, Fei Xiao, Yajie Guo, Jiali Deng, Bin Liu, Qian Zhang, Kai Li, Chunxia Wang, Shanghai Chen and Feifan Guo*

Key Laboratory of Nutrition and Metabolism, Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences, the Graduate School of the Chinese Academy of Sciences

320 Yueyang Road, Shanghai, China 200031

Contact Information

* Correspondence should be addressed to Feifan Guo; E-mail: ffguo@sibs.ac.cn

Address: 320 Yueyang Road, Shanghai, China 200031

telephone number: 86-21-54920250 fax number: 86-21-54920291

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Abstract

Phosphoserine aminotransferase 1 (PSAT1) is an enzyme participating in serine synthesis. A role of PSAT1 in the regulation of insulin sensitivity, however, is unknown. Here, we showed that hepatic PSAT1 expression and liver serine levels are reduced in genetically engineered leptin receptor-deficient (db/db) mice and high-fat diet (HFD)-induced diabetic mice. Additionally, over-expression of PSAT1 by adenovirus expressing PSAT1 (Ad-PSAT1) improved insulin signaling and insulin sensitivity in vitro and in vivo under normal conditions. Opposite effects were observed when PSAT1 was knocked down by adenovirus expressing small hairpin RNA specific for PSAT1 (Ad-shPSAT1). Importantly, over-expression of PSAT1 also significantly ameliorated insulin resistance in diabetic mice. In addition, PSAT1 inhibited the expression of hepatic tribbles homolog 3 (TRB3) in vitro and in vivo, and Ad-shTRB3-mediated inhibition of TRB3 reversed the attenuated insulin sensitivity in Ad-shPSAT1 mice. Interestingly, we found that serine mediates PSAT1 regulation of TRB3 expression and insulin signaling in vitro. These results identify a novel function for hepatic PSAT1 in regulating insulin sensitivity and provide important insights in targeting PSAT1 for treating insulin resistance and type 2 diabetes. Our results also suggest that non-essential amino acid serine may play an important role in regulating insulin sensitivity.

Introduction
Insulin resistance, in which insulin fails to effectively activate its downstream signaling pathways, is a common feature of type 2 diabetes (T2D) (1). Nutrients are important contributing factors to the development of insulin resistance in T2D patients (2-4). Previous studies have demonstrated that essential amino acids, particularly branched-chain amino acids (BCAAs), are closely related to the development and prediction of insulin resistance in both human and animal models (5-7). Other studies have shown that, however, non-essential amino acids might also be related to the development of insulin resistance. For example, high serum glycine levels are associated with decreased risk of T2D in humans (8) and glutamine supplementation attenuates high-fat diet (HFD)-induced insulin resistance in rats (9). Despite of these studies, the role that non-essential amino acids and the enzymes related to their synthesis play in the regulation of insulin sensitivity remains unclear.

Phosphoserine aminotransferase 1 (PSAT1), an enzyme involved in serine biosynthesis that was first purified from the brains of sheep, is expressed at high levels in many tissues, including liver (10). Three steps are involved in serine synthesis (11) and PSAT1 is involved in the second step catalyzing the conversion of 3-phosphohydroxypyruvate (PHP) to form L-phosphoserine (11; 12). Serine contributes to the regulation of several physiological processes (13-16) and, as one of a key enzymes in serine synthesis, PSAT1 has some important metabolic functions (17-19). For example, PSAT1 deficiency results in intractable seizures and acquired microcephaly (17), while increased expression of PSAT1 is associated with the development of colorectal cancer (18).
Several lines of evidence indicate that PSAT1 has a role in controlling blood glucose. For example, PSAT1 regulates serine synthesis (19) and serine reportedly regulates glycogen synthesis (16). In addition, α-Ketoglutaric acid (α-KG), as a byproduct of PSAT1 catalyzed reaction (14), stimulates insulin secretion (20). However, it is currently unknown whether PSAT1 participates in the regulation of insulin sensitivity, another important aspect of maintaining glucose homeostasis (1). Previous studies have shown that PSAT1 expression and serine biosynthesis are increased under protein-restricted diet in liver of rats (21), a condition demonstrated to be beneficial for improving insulin sensitivity (22), which suggests that PSAT1 might have novel function in the regulation of insulin sensitivity. The aim of our current study was to investigate this possibility and elucidate underlying mechanisms.

Here, we showed that hepatic PSAT1 regulates insulin sensitivity in vitro and in vivo under normal and insulin-resistant conditions. Furthermore, the effects of PSAT1 were mediated by inhibition of tribbles homolog 3 (TRB3). In addition, PSAT1 regulated TRB3 expression in a serine-dependent manner and decreased liver serine levels might contribute to insulin resistance in vivo.

Research Design and Methods

Animals and treatment

Male C57 BL/6J wild-type (WT) mice were obtained from Shanghai Laboratory Animal Co., Ltd. (SLAC, Shanghai, China). Leptin receptor-deficient (db/db) mice were kindly provided by Dr. Xiang Gao, Nanjing University, China. Male C57 BL/6J
WT mice were fed for 16 weeks a high-fat diet (HFD, Research Diets, Inc. New Brunswick, NJ, USA) or chow diet, which has been previously used as control for HFD (23; 24). Adenoviruses were injected to mice under HFD or chow diet for 14 weeks and then these mice were fed with a HFD or chow diet continuously before sacrifice. Eight- to ten-week-old mice were maintained on a 12-hr light/dark cycle at 25 °C and provided free access to commercial rodent chow and tap water prior to initiation of the experiments. Food intake and body weight were measured daily. Animal experiments were conducted in accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute for Nutritional Sciences, Shanghai Institute for Biological Sciences (Sibs), Chinese Academy of Sciences (CAS).

**Primary mouse hepatocyte isolation, cell culture and treatments**

Primary mouse hepatocytes were prepared by collagenase perfusion as previously described (25). HepG2 and Hep1-6 cells (Cell Centre of Sibs, CAS, China) were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mmol/L glucose (Gibco, Invitrogen, Carlsbad, CA, USA), 10 % fetal bovine serum (FBS) and 50 mg/mL penicillin and streptomycin, in an environment with 5% CO₂–95% air at 37°C. Control (complete amino acid), (-) ser (serine-deficient), 2 x ser (serine-supplemented) medium were prepared from amino acid-free DMEM (Invitrogen, Carlsbad, CA, USA) by adding back all the amino acids contained in regular DMEM, without serine only, or double serine amount, respectively.
Double-stranded siRNA targeting human phosphoglycerate dehydrogenase (PGDH) or L-phosphoserine phosphatase (PSPH) were purchased from GenePharma (Shanghai, China). The siRNA sequence specific for human PGDH: 5’-GGGAGGAAAUUGCUGUUCATT-3’, and for human PSPH: 5’-GGAGUAUUGUAGAGCAUGUTT-3’. HepG2 cells were transfected with siRNA using X-tremeGene siRNA Transfection Reagent (Roche Diagnostics, Mannheim, Germany).

**Generation and administration of recombinant adenoviruses**

Recombinant adenovirus expressing human PSAT1 was generated using the AdEasy™ Adenoviral Vector System (Qbiogene, Irvine, CA, USA) according to the manufacturer’s instructions. Adenovirus expressing scrambled or small hairpin RNA (shRNA) directed against the coding region for mouse PSAT1 or TRB3 was generated using the BLOCK-iT™ Adenoviral RNAi Expression System (Invitrogen, Carlsbad, CA, USA) according to the manufacturer’s instructions. The scrambled sequence is 5’-TTCTCCGAACGTGTCACGT-3’. The shRNA sequence for mouse PSAT1 is 5’-GCATCAGTGTGCTCGAAATGA-3’ and for mouse TRB3 is 5’-GGAACCTTCAGAGCGACTTGT-3’. High-titer stocks of amplified recombinant adenoviruses were purified as previously described (7). Viruses were diluted in PBS and administered at a dose of $10^7$ pfu/well in a 12-well plate or via a tail vein injection using $5*10^8$ pfu per mouse.
Blood glucose, serum insulin, glucose tolerance test (GTT), insulin tolerance test (ITT) and homeostasis model assessment-insulin resistance (HOMA-IR) index

Levels of blood glucose and serum insulin were measured using a Glucometer Elite monitor and a Mercodia Ultrasensitive Rat Insulin ELISA kit (ALPCO Diagnostic, Salem, NH, USA), respectively. The GTTs and ITTs were performed by administering an intraperitoneal (IP) injection of 2 g/kg glucose following overnight fasting and 0.75 U/kg insulin after 4 h of fasting, respectively. The HOMA-IR index was calculated according to the following formula: [fasting glucose levels (mmol/L)] x [fasting serum insulin (µU/mL)]/ 22.5. For each adenovirus injection experiment, blood glucose was examined daily. Until differences were observed between experimental group and control group, GTT and ITT were performed, therefore may resulting in different measurement time in different animal experiments as indicated.

Measurement of serum and liver serine levels

Tissue and serum samples were prepared as previously described (26). Amino acid levels were determined by standard ion exchange chromatography using a Beckman 6300 automated amino acid analyzer.

In vivo insulin signaling assay

Mice were fasted for 6 h prior to insulin injection as previously described (25). Sections of liver were excised from anesthetized live mice and kept as untreated controls. Three minutes after injection with insulin at a dose of 2 U/kg in WT or 5
U/kg in db/db mice via the portal vein, pieces of liver section were excised for western blot analysis.

**Western blot analysis**

Western blot analysis was performed as previously described (7). Primary antibodies [anti-p-IR (tyr1150/1151), anti-IR, anti-p-IRS1 (tyrosine 612/608-human/mouse), anti-IRS1, anti-p-PDK1 (ser241), anti-PDK1, anti-p-AKT (ser473), anti-AKT, anti-p-GSK3β (ser 9), anti-GSK3β, anti-p-FOXO1 (S256) and anti-FOXO1 (all from Cell Signaling Technology, Beverly, MA, USA)], anti-PSAT1 and anti-TRB3 (both from Santa Cruz Biotechnology Inc, Santa Cruz, CA, USA) were incubated overnight at 4 °C and specific proteins were visualized by ECL Plus (Amersham Biosciences Inc, Amersham, Buckinghamshire, UK). Band intensities were measured using Quantity One (Bio-Rad Laboratories, Hercules, CA, USA) and normalized to total protein, actin or tubulin.

**RNA isolation and relative quantitative RT-PCR**

*Psat1* mRNA levels were examined by RT-PCR as previously described (27). The sequences of primers used to specifically detect the mouse PSAT1 were as follows: sense primer, 5’- ACGCCAAAGGAGACGAAGCT-3’; and antisense primer: 5’-ATGTTGAGTTCTACCGCCTTGTC-3’.

**Statistics**
All data are expressed as mean ± SEM. Significant differences were assessed either by two-tailed student *t-test* or one-way ANOVA followed by the Student-Newman-Keuls test. P < 0.05 was considered statistically significant.

**Results**

**PSAT1 expression is decreased in livers of diabetic mice**

To investigate whether PSAT1 is involved in the regulation of insulin sensitivity, we examined hepatic PSAT1 expression in insulin-resistant mice. Animal models of insulin resistance are divided into two categories: genetically models such as leptin receptor-deficient (*db/db*) mice (28) and nutritionally induced models such as that induced by HFD (29). Evidence demonstrating insulin resistance in these mice used in current study is summarized in Table S1. Surprisingly, PSAT1 protein was lower in the livers, but not other tissues examined including white adipose tissue (WAT) and muscle, of *db/db* mice compared with control mice (Fig. 1A). Consistently, hepatic *Psat1* mRNA and liver serine levels were also decreased in *db/db* mice (Fig. 1B and 1C). Similar results were obtained in mice under HFD for 16 weeks compared with their relevant control mice (Fig. 1D-F). Interestingly, inflammation might contribute to the decreased PSAT1 expression in these mice as demonstrated by the inhibitory effects of tumor necrosis factor α (TNFα) on PSAT1 expression in HepG2 cells compared with control vehicle (Fig. S1).

**PSAT1 regulates insulin sensitivity in vitro**
To confirm the role of PSAT1 in insulin sensitivity, we infected the human hepatocellular liver carcinoma HepG2 cells and primary cultured mouse hepatocytes with adenovirus expressing PSAT1 (Ad-PSAT1) or control green fluorescent protein (Ad-GFP) and examined changes of insulin signaling pathway in these cells. Over-expression of PSAT1 increased insulin-stimulated phosphorylation of protein kinase B (AKT) (ser473) and glycogen synthase kinase 3 \( \beta \) (GSK3\( \beta \)) (ser 9) in both cell lines compared with control cells (Fig. 2A). Consistently, insulin-stimulated phosphorylation of AKT and GSK3\( \beta \) were impaired when endogenous PSAT1 protein levels were reduced by adenovirus expressing small hairpin RNA specific for PSAT1 (Ad-shPSAT1) compared with control cells infected with scrambled adenovirus (Ad-scrambled) (Fig. 2B). Insulin-stimulated phosphorylation of insulin receptor (IR) (tyr1150/1151), insulin receptor substrate 1 (IRS1) (tyrosine 612/608-human/mouse) and phosphoinositide-dependent protein kinase 1 (PDK1) (ser241), however, were not affected by Ad-PSAT1 or Ad-shPSAT1 (Fig. 2A and 2B; Fig. S2A and S2B). In addition, over-expression of PSAT1 decreased glucose output and increased glycogen content in the presence or absence of insulin (Fig. 2C and 2D).

**Over-expression of PSAT1 by Ad-PSAT1 improves insulin sensitivity in vivo**

To explore the role of PSAT1 in insulin sensitivity in vivo, we injected male C57 BL/6J wild-type (WT) mice with Ad-PSAT1 or Ad-GFP via tail vein injection. As a result, increased Psat1 mRNA and protein levels were observed in the livers of
Ad-PSAT1 mice compared with control mice (Fig. 3A). Although body weight or food intake was not altered by Ad-PSAT1 (Fig. S3A and S3B), Ad-PSAT1 significantly decreased blood glucose levels in mice under both fed and fasting conditions (Fig. 3B). Fasting serum insulin levels remained unchanged, however, fed serum insulin levels were significantly decreased in Ad-PSAT1 mice (Fig. 3C). Furthermore, the HOMA-IR index was decreased in these mice (Fig. 3D). Blood glucose levels were decreased much more quickly following challenging to glucose or insulin in Ad-PSAT1 mice compared with control mice as measured by glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs), respectively (Fig. 3E). Additionally, insulin-stimulated phosphorylation of AKT and GSK3β, but not IR, IRS1 or PDK1, were increased in the livers of these mice (Fig. 3F and Fig. S3C).

Hepatic genes involved in glucose and lipid metabolism were also examined. Expression of the gluconeogenesis genes phosphoenolpyruvate carboxykinase (Pepck) and the glycogenolysis gene phosphorylase, glycogen, liver (Pygl) (30) were decreased, while the glycogen synthesis gene glycogen synthase 2 (Gys2) (31) were increased, in the livers of Ad-PSAT1 mice compared with control mice (Fig. S3D). Furthermore, we investigated the effects of PSAT1 on expression of genes related to lipogenesis [fatty acid synthase (Fas) and stearol-CoA desaturase 1 (Scd1)] and β-oxidation [peroxisome proliferator activated receptor alpha (Ppar α) and carnitine palmitoyltransferase 1 (Cpt1)] (32) and no significant effects of PSAT1 on expression of these genes were observed (Fig. S3D). In addition, no changes in PSAT1
expression and insulin signaling were observed in WAT and muscle of Ad-PSAT1 mice (Fig. S3E-H).

**Knockdown of PSAT1 by Ad-shPSAT1 impairs insulin sensitivity in vivo**

The above results raise the possibility that knockdown of PSAT1 might attenuate insulin sensitivity. To test this possibility, we injected male C57 BL/6J WT mice with Ad-shPSAT1 or Ad-scrambled. Both mRNA and protein levels of PSAT1 were significantly decreased in the livers of Ad-shPSAT1 mice (Fig. 4A). Body weight or food intake, however, was not altered by Ad-shPSAT1 (Fig. S4A and S4B). Fed and fasting blood glucose levels were significantly elevated in Ad-shPSAT1 mice compared with Ad-scrambled mice (Fig. 4B). As a result, although fed and fasting serum insulin levels remained unchanged, HOMA-IR index was increased in Ad-shPSAT1 mice (Fig. 4C and 4D). Consistent with these changes, Ad-shPSAT1 mice exhibited decreased glucose tolerance and clearance as measured by GTTs and ITTs, respectively (Fig. 4E). Insulin-stimulated phosphorylation of AKT and GSK3β, but not IR, IRS1 and PDK1, were also greatly impaired in the livers of mice injected with Ad-shPSAT1 (Fig. 4F and Fig. S4C). Expression of *Pepck, Pygl, Pparα* and *Cpt1* were increased and *Gys2* expression was decreased in the livers of Ad-shPSAT1 mice (Fig. S4D). Again, no changes in PSAT1 expression and insulin signaling were observed in WAT and muscle of Ad-shPSAT1 mice (Fig. S4E-H).

**Over-expression of PSAT1 ameliorates insulin resistance in diabetic mice**
To determine whether decreased PSAT1 expression contributes to insulin resistance in \( db/db \) mice, we injected them with Ad-PSAT1 or Ad-GFP and examined whether over-expression of PSAT1 could reverse insulin resistance in these mice. As expected, PSAT1 expression was increased in the livers of Ad-PSAT1 mice compared with control mice, without affecting their body weight or food intake (Fig. 5A-C). Over-expression of PSAT1 in \( db/db \) mice also significantly decreased levels of fed and fasting blood glucose and fed serum insulin, as well as HOMA-IR index, except for levels of fasting serum insulin (Fig. 5D-F). In Ad-PSAT1 \( db/db \) mice, GTT and ITT demonstrated improved glucose clearance and insulin sensitivity (Fig. 5G). Insulin-stimulated phosphorylation of AKT and GSK3\( \beta \), but not IR, were also greatly increased in the livers of \( db/db \) mice injected with Ad-PSAT1 (Fig. 5H).

To further validate our hypothesis in HFD-induced insulin resistance, we injected Ad-PSAT1 or Ad-GFP into mice under HFD or chow diet for 14 weeks and then allowed these mice to continuously feeding on their diet until they were sacrificed. PSAT1 expression was significantly elevated in the livers of Ad-PSAT1 mice compared with control Ad-GFP mice (Fig. 6A). Ad-PSAT1 injection did not change body weight or food intake in HFD mice (Fig. 6B and 6C). Consistent with previous reports (29), a HFD resulted in significantly decreased insulin sensitivity, as demonstrated by the increased blood glucose and serum insulin levels (under both fed and fasting conditions) and HOMA-IR index, as well as decreased glucose, insulin tolerance and insulin signaling in the liver compared with mice maintained on a control diet (Fig. 6D-H). The above parameters demonstrating the impaired insulin
sensitivity in HFD mice, however, was largely reversed by Ad-PSAT1 injection compared with mice injected with Ad-GFP except for serum insulin levels (Fig. 6D-H).

The effects of PSAT1 on insulin sensitivity are mediated by TRB3

TRB3 is a pseudokinase that impairs insulin signaling via binding directly to AKT and inhibiting its activation (33). Furthermore, serine deprivation induces expression of activating transcription factor 4 (ATF4) (34), which has been shown to stimulate TRB3 expression (35). Based on these, we hypothesized that PSAT1, as a key enzyme regulating serine biosynthesis (13), may regulate insulin sensitivity via TRB3. As predicted, we found that TRB3 protein was significantly decreased by Ad-PSAT1 and increased by Ad-shPSAT1, respectively, in vitro and in vivo (Fig. 7A and 7B). Meanwhile, elevated TRB3 expression was also found in the liver, but not WAT or muscle, of \( db/db \) mice or HFD mice compared with their relevant control mice (Fig. 7C, Fig. S5A and S5B). The possible involvement of TRB3 in PSAT1-regulated insulin signaling was then investigated in non-immunogenic murine hepatocellular carcinoma Hep1-6 cells, a cell type commonly used for studying the insulin signaling pathway (36), infected with Ad-shPSAT1 and adenoviruses expressing small hairpin RNA against TRB3 (Ad-shTRB3) or Ad-scrambled. As predicted, knockdown of TRB3 significantly reversed the inhibitory effect of Ad-shPSAT1 on insulin-stimulated phosphorylation of AKT and GSK3\( \beta \), but not IR, compared with control cells (Fig. 7D).
To gain further insights into the importance of TRB3 in regulating insulin sensitivity by PSAT1 in vivo, we injected mice with Ad-shTRB3 and examined its effects on the impaired insulin sensitivity by Ad-shPSAT1. Functional validation of Ad-shTRB3 was demonstrated by its ability to reduce TRB3 levels in liver (Fig. 7E). Ad-shTRB3 significantly decreased fasting blood glucose and insulin levels in Ad-shPSAT1 mice, however, it had no effect on the increase of fed blood glucose and insulin levels by caused by knockdown of PSAT1 (Fig. 7F and 7G). The HOMA-IR index was decreased following administration of Ad-shTRB3 in Ad-shPSAT1 mice (Fig. 7H). Moreover, glucose clearance and insulin sensitivity, which were attenuated by knocking down PSAT1, were also markedly reversed by Ad-shTRB3 (Fig. 7I and 7J). Previous studies have shown that TRB3 expression is inhibited by activation of Forkhead box O1 (FOXO1) (37). However, FOXO1 is unlikely to mediate PSAT1-induced inhibition of TRB3, because we found that hepatic FOXO1 was inactivated (as demonstrated by increased phosphorylation) by Ad-PSAT1 and activated (as demonstrated by decreased phosphorylation) by Ad-shPSAT1 in vitro and in vivo (Fig. S6A and S6B).

PSAT1 regulates TRB3 expression via altered serine levels in HepG2 cells

PSAT1 is an enzyme that participates in serine synthesis pathway and increases serine levels (19), suggesting that PSAT1 might regulate TRB3 expression via altered serine levels. Although serum serine levels remained unchanged, liver serine levels were significantly increased in Ad-PSAT1 mice and decreased in Ad-shPSAT1 mice.
compared with their respective control mice (Fig. 8A and 8B). To investigate the role of serine in mediating PSAT1-regulated TRB3 expression, we either: a) infected HepG2 cells with Ad-PSAT1, which is supposed to increase serine levels, and then incubated them with a serine-deficient medium for 24 hours; or b) infected HepG2 cells with Ad-shPSAT1, which is supposed to decrease serine levels, and then incubated them with a serine-supplemented (2-fold increase in serine levels) medium for 24 hours. Serine deficiency stimulated TRB3 expression and also reversed the Ad-PSAT1-induced inhibition of TRB3 expression (Fig. 8C). Although serine supplementation had no effect on TRB3 expression under basal condition, it inhibited Ad-shPSAT1’s stimulation of TRB3 expression (Fig. 8D). Furthermore, serine supplementation reversed Ad-shPSAT1-mediated inhibition of AKT and GSK3β phosphorylation, but not IR phosphorylation, following insulin stimulation (Fig. 8E). However, the other two enzymes involved in serine synthesis, including 3-phosphoglycerate dehydrogenase (3-PGDH) and L-phosphoserine phosphatase (PSPH) (12), did not have similar effects as PSAT1 on insulin signaling in HepG2 cells when their expression was knocked down by small interfering RNA directed against each of them (Fig. S7A-D).

Discussion
While PSAT1 knock out homozygous embryos are known to exhibit growth retardation, exencephaly and craniofacial abnormalities (38), the tissue specific functions of PSAT1 are poorly understood. In this study, we found that hepatic PSAT1 expression is decreased in genetically engineered diabetic $db/db$ mice and
HFD-induced diabetic mice. Furthermore, over-expression or knock down of PSAT1 improved or impaired insulin signaling and insulin sensitivity under normal conditions, respectively. In addition, over-expression of PSAT1 ameliorated insulin resistance in diabetic mice. To our knowledge, our results are the first time to demonstrate the important role of PSAT1 in the regulation of insulin sensitivity and the development of insulin resistance. One potential explanation for decreased insulin sensitivity in \( db/db \) and HFD-fed mice is increased levels of inflammation factors, such as TNFα (39; 40), and we speculated that the decreased hepatic PSAT1 expression might be caused by elevated TNFα. Further research will be necessary to validate this finding.

Although various tissues in mice can influence systematic insulin sensitivity, we suggest that the improved or impaired insulin sensitivity observed in Ad-PSAT1 or Ad-shPSAT1 mice, respectively, is specifically caused by altered PSAT1 expression in the liver and not by secondary effects from other tissues. Consistent with this possibility, we found that insulin-stimulated AKT and GSK3β phosphorylation was directly regulated by over-expression or knock down of PSAT1 in HepG2 cells and primary hepatocytes. Furthermore, insulin signaling was unaffected in the muscle and WAT of mice injected with Ad-PSAT1 or Ad-shPSAT1.

Both fed and fasting blood glucose levels were decreased in Ad-PSAT1 mice. Our results showed that over-expression of PSAT1 may decrease blood glucose levels by promoting glycogen synthesis and inhibiting gluconeogenesis and glycogenolysis. In support of this notion, over-expression of PSAT1 significantly inhibited glucose
output and increased glycogen content in primary hepatocytes. Although most of the genes related to lipid metabolism were not affected by PAST1, expression of genes related to β-oxidation was modestly increased by Ad-shPSAT1, suggesting a possible role of PSAT1 in lipid metabolism, which requires further investigation.

The insulin signaling pathway is activated when insulin binds to an insulin receptor, resulting in phosphorylation of IRS1 and consequent activation of phosphatidylinositol 3-kinase (PI3K)/PDK1/AKT and GSK3β, key components of insulin signaling (1; 41). In this study, we found that over-expression or knockdown of PSAT1 affects insulin-stimulated phosphorylation of AKT and GSK3β, but not IR, IRS1, or PDK1, suggesting that PSAT1 is likely to regulate insulin sensitivity via intracellular signaling pathways that directly inhibit phosphorylation of AKT. TRB3 is a well-known AKT inhibitor, binding directly to AKT and blocking AKT phosphorylation (33). While TRB3-deletion mice exhibit normal insulin signaling and glucose homeostasis (42), adenovirus-mediated over-expression or knockdown of TRB3 in mice results in improved or impaired insulin sensitivity, respectively (33; 43), suggesting that hepatic TRB3 is critical for regulation of insulin sensitivity. Consistent with previous findings, we demonstrated that TRB3 is required for PSAT1-regulated insulin sensitivity. Our current study, however, could not exclude the involvement of TRB3-independent pathways in PSAT1 regulation of insulin sensitivity, which requires future study.

Here, we provided evidence showing that serine is involved in the regulation of TRB3 by PSAT1. Serine is a non-essential amino acid, but is indispensable in the
synthesis of proteins, sphingolipids, other amino acids and nucleotides (34). Recent studies have shown that the serine synthesis pathway is involved in the development of breast cancer, tumorigenesis, cancer cell proliferation (14; 15; 44) and in the glycogen synthesis process (16). A previous study has also shown that TRB3 expression can be induced by amino acid deprivation (45). Whether serine deficiency also increases TRB3 expression is currently unclear; however, we have now demonstrated that serine levels directly regulate TRB3 expression in vitro.

The role of essential amino acids in insulin sensitivity has been recognized in humans and various animal models recently (5-7). Although some studies have indicated the possible involvement of non-essential amino acids in this pathological change (8; 9), their role in the regulation of insulin sensitivity remains poorly understood. In this study, we found that levels of non-essential amino acid serine were decreased in livers of \textit{db/db} and HFD mice, suggesting that the decreased serine levels, possibly mediated by decreased expression of PSAT1, could be an underlying cause of insulin resistance in vivo. This claim is further supported by our in vitro study, which showed that altered serine levels affected TRB3 expression and reversed PSAT1-regulated insulin signaling. Furthermore, serine biosynthesis takes place mainly in the liver and kidneys, which can be regulated under protein restriction or fasting conditions (13). Here, we observed that liver but not serum serine levels were changed by Ad-PSAT1. This finding suggests that liver serine has a specific role in the regulation of insulin sensitivity. Therefore, we cannot eliminate the involvement
of non-essential amino acids from insulin sensitivity regulation, and their role in this process requires further investigation.

In conclusion, as described in our working model (Fig. 8F), we observed that PSAT1 regulates insulin sensitivity and decreased hepatic PSAT1 expression contributes to insulin resistance in both \( db/db \) and HFD mice. Furthermore, we demonstrated that TRB3 is required for the regulation of insulin sensitivity by PSAT1. Additionally, PSAT1 was found to regulate TRB3 via altered serine levels in vitro, and that liver serine levels decreased in both \( db/db \) and HFD-fed mice. Taken together, our results indicate a novel function for hepatic PSAT1 in the regulation of insulin sensitivity. Our study provides new insights into the molecular mechanisms of insulin resistance, and we suggest that PSAT1 could be a possible novel drug target for treating insulin resistance, an important factor in the increased fasting blood glucose exhibited by T2D patients. Our results also show that, in the liver, the non-essential amino acid serine may have an important function in regulating insulin sensitivity. Because PSAT1 is expressed in many tissues besides the liver, its potential role in the regulation of insulin sensitivity throughout the body should be explored in future research.

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Junjie Yu researched data, wrote, reviewed and edited the manuscript. Fei Xiao, Yajie Guo, Kai Li, Jiali Deng, Qian Zhang researched data. Fei Xiao and Chunxia Wang, contributed to discussion. Bin Liu and Shanghai Chen provided research material. Feifan Guo directed the project, contributed to discussion and wrote, reviewed, and edited the manuscript. Feifan Guo is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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Figure Legends

Fig. 1 Hepatic PSAT1 expression is reduced under insulin-resistant conditions

PSAT1 expression was analyzed in the livers, white adipose tissue (WAT) and muscle, and liver serine levels were measured, in wild-type (wt) and leptin receptor-deficient (db/db) mice in A-C, or WT mice fed a control (- HFD) or HFD (+ HFD) for 16 weeks in D-F. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student t-test for the effects of db/db or HFD mice versus control mice (*: p < 0.05). (A and D) PSAT1 protein expression (top, western blot; bottom, quantitative measurement of PSAT1 protein relative to tubulin or actin); (B and E) Hepatic Psat1 mRNA level; (C and F) Liver serine level.

Fig. 2 PSAT1 regulates insulin sensitivity in vitro

(A and B) Cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (-Ad-PSAT1) for 48 h in A, or Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (-Ad-shPSAT1) for 72 h in B; both cases were followed with (+ Ins) or without (- Ins) 100 nM insulin stimulation for 20 min; (C and D) Primary mouse hepatocytes were
infected with Ad-PSAT1 (+ Ad-PSAT1) or without Ad-PSAT1 (- Ad-PSAT1) for 24 h. The cells were then treated with (+ Ins) or without (- Ins) 100 nmol/L insulin for another 24 h, followed by the measurement of glucose production or glycogen content. Data were obtained with at least three independent in vitro experiments and are presented as means ± SEMs. Statistical significance was calculated using the one-way ANOVA followed by the Student-Newman-Keuls (SNK) test for the effects of Ad-PSAT1 or Ad-shPSAT1 versus corresponding control following insulin stimulation (*: p < 0.05) in A and B, any group versus the – Ad-PSAT1 group without insulin stimulation (*: p<0.05), with versus without insulin stimulation in + Ad-PSAT1 group (#: p<0.05), or Ad-PSAT1 versus the control group after insulin stimulation (&: p<0.05) in C and D. (A and B) p-IR (tyr1150/1151), p-AKT (ser473), p-GSK3 β (ser9) and PSAT1 protein (top, western blot; bottom, quantitative measurements of p-IR, p-AKT, p-GSK3β and PSAT1 protein relative to their total protein or actin); (C) Glucose output assay; (D) Glycogen content.

**Fig. 3 Over-expression of PSAT1 improves insulin sensitivity in wild-type (WT) mice**

Male C57 BL/6J WT mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) via tail-vein injection, followed by examination of PSAT1 expression in liver at day 7 in A, measurement of fed blood glucose and serum insulin levels at day 7 or fasting blood glucose and serum insulin levels at 3 in B and C, calculating HOMA-IR index in D, performance of glucose tolerance tests (GTTs) and insulin
tolerance tests (ITTs) at day 3 or 5 in E, and examination of insulin signaling in liver before (- Ins) and after (+ Ins) 2 U/kg insulin stimulation for 3 min at day 7 in F. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student t-test for the effects of the Ad-PSAT1 versus the control group (*: p<0.05). (A) Psat1 mRNA and protein (top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin); (B) Blood glucose levels; (C) Serum insulin levels; (D) HOMA-IR index; (E) GTT and ITT; (F) p-IR (tyr1150/1151), p-AKT (ser473), p-GSK3β (ser9) and PSAT1 protein (left, western blot; right, quantitative measurements of p-AKT and p-GSK3β protein relative to their total protein).

**Fig. 4 Knockdown PSAT1 attenuates insulin sensitivity in wild-type (WT) mice**

Male C57 BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) via tail vein injection, followed by examination of PSAT1 expression in liver at day 13 in A, measurement of fed blood glucose and serum insulin levels at day 13 or fasting blood glucose and serum insulin levels at 9 in B and C, calculating HOMA-IR index in D, performance of glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) at day 9 or 11 in E, and examination of insulin signaling in liver before (- Ins) and after (+ Ins) 2 U/kg insulin stimulation for 3 min at day 13 in F. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs. Statistical significance was calculated
using the two-tailed student \( t \)-test for the effects of Ad-shPSAT1 versus the control group (*: \( p < 0.05 \)). (A) \( Psat1 \) mRNA and protein (\( \text{top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin} \)); (B) Blood glucose levels; (C) Serum insulin levels; (D) HOMA-IR index; (E) GTT and ITT; (F) p-IR (tyr1150/1151), p-AKT (ser473), p-GSK3\( \beta \) (ser9) and PSAT1 protein (\( \text{left, western blot; right, quantitative measurements of p-IR, p-AKT and p-GSK3} \)\( \beta \) protein relative to their total protein).

**Fig. 5 Over-expression of PSAT1 ameliorates insulin resistance in \( db/db \) mice**

Male C57 BL/6J \( db/db \) mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) via tail-vein injection, followed by examination of hepatic PSAT1 expression at day 11 in A, measurement of food intake and body weight in B and C, detection of fed blood glucose and serum insulin levels at day 9 or fasting blood glucose and serum insulin levels at 7 in D and E, calculating HOMA-IR index in F, performance of glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) at day 7 and 5 in G, respectively, and examination of insulin signaling in liver before (- Ins) and after (+ Ins) 5 U/kg insulin stimulation for 3 min at day 11 in H. Data were obtained with mice described above (\( n = 10-14 \) mice per group) and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student \( t \)-test for the effects of Ad-PSAT1 versus the control group (*: \( p < 0.05 \)). (A) PSAT1 protein (\( \text{top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin} \)); (B) Body weight; (C) Food intake; (D) Blood glucose levels; (E)
Serum insulin levels; (F) HOMA-IR index; (G) GTT and ITT; (H) p-IR (tyr1150/1151), p-AKT (ser473), p-GSK3β (ser9) and PSAT1 protein (left, western blot; right, quantitative measurements of p-IR, p-AKT and p-GSK3β protein relative to their total protein).

**Fig. 6 Over-expression of PSAT1 ameliorates insulin resistance in mice under HFD**

Male C57 BL/6J wild-type mice were fed a control (-HFD) or HFD diet (+HFD) for 14 weeks, and then injected with Ad-PSAT1 (+Ad-PSAT1) or Ad-GFP (-Ad-PSAT1) via tail-vein injection, followed by examination of hepatic PSAT1 expression at day 11 in A, measurement of food intake and body weight in B and C, detection of fed blood glucose and serum insulin levels at day 9 or fasting blood glucose and serum insulin levels at 7 in D and E, calculating HOMA-IR index in F, performance of glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) at day 7 and 5 in G, respectively, and examination of insulin signaling in liver before (-Ins) and after (+Ins) 2 U/kg insulin stimulation for 3 min at day 11 in H. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs.

Statistical significance was calculated using one-way ANOVA followed by the Student-Newman-Keuls (SNK) test for the effects of any group versus -Ad-PSAT1 under control diet (*: p < 0.05) or +Ad-PSAT1 versus -Ad-PSAT1 under HFD (‡: p < 0.05). (A) PSAT1 protein (top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin); (B) Body weight; (C) Food intake; (D) Blood
glucose levels; (E) Serum insulin levels; (F) HOMA-IR index; (G) GTT and ITT; (H) p-IR (tyr1150/1151), p-AKT (ser473), p-GSK3 β (ser9) and PSAT1 protein (left, western blot; right, quantitative measurements of p-IR, p-AKT and p-GSK3 β protein relative to their total protein).

**Fig. 7 PSAT1 regulates insulin sensitivity via TRB3**

(A) HepG2 cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) for 48 h or male C57 BL/6J wild-type (WT) mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) via tail-vein injection, followed by examination of TRB3 in liver at day 7; (B) HepG2 cells were exposed to Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) for 72 h or male C57 BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) via tail vein injection, followed by examination of TRB3 in liver at day 13; (C) TRB3 expression was analyzed in the livers of WT and db/db mice, or WT mice fed a control (- HFD) or HFD (+ HFD) for 16 weeks; (D) Hep1-6 cells were infected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1), and Ad-shTRB3 (+ Ad-shTRB3) or Ad-scrambled (- Ad-shTRB3) for 72 h, followed with 10 nM insulin stimulation for 3 min; (E-J) Male C57BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) and Ad-shTRB3 (+ Ad-shTRB3) or Ad-scrambled (- Ad-shTRB3) via tail-vein injection, followed by examination of PSAT1 and TRB3 expression in liver at day 14 in E, measurement of fed blood glucose and serum insulin levels at day 12 and fasting
blood glucose and serum insulin levels at day 8 in F and G, calculating HOMA-IR index in H, performance of glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) at day 8 or 10 in I, and examination of insulin signaling in liver before (- Ins) and after (+ Ins) 2 U/kg insulin stimulation for 3 min at day 8 in J. Data were obtained with mice described above (n = 10-14 mice per group) or at least three independent in vitro experiments and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student t-test for the effects of the Ad-PSAT1 or Ad-shPSAT1 versus the control group (*: p < 0.05) in A and B, or the effects of db/db or HFD mice versus control mice in C, or using the one-way ANOVA followed by the Student-Newman-Keuls (SNK) test for the effects of any group versus without Ad-shPSAT1 and Ad-shTRB3 (*: p < 0.05), or with versus without Ad-shTRB3 in + Ad-shPSAT1 group (#: p < 0.05) in C-G. (A-C and E) TRB3 and PSAT1 protein (top, western blot; bottom, quantitative measurements of TRB3 protein relative to actin or tubulin); (D) p-IR (tyr1150/1151), p-AKT (ser473), p-GSK3β (ser9), PSAT1 and TRB3 protein (left, western blot; right, quantitative measurements of p-IR, p-AKT and p-GSK3β protein relative to their total protein); (F) Blood glucose levels; (G) Serum insulin levels; (H) HOMA-IR index; (I) GTT and ITT; (J) p-IR (tyr1150/1151), p-AKT (ser473) and p-GSK3β (ser9) protein (left, western blot; right, quantitative measurements of p-IR, p-AKT and p-GSK3β protein relative to their total protein).

**Fig. 8 PSAT1 regulates TRB3 expression via serine in HepG2 cells**
(A) Male C57 BL/6J wild-type (WT) mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) via tail vein injection, followed by examination of serum and liver serine levels at day 7; (B) Male C57 BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) via tail-vein injection, followed by examination of serum and liver serine levels at day 13; (C) HepG2 cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) for 24 h, and then incubated with (+ Ser) or without serine (- Ser) medium for another 24 h; (D and E) HepG2 cells were infected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) for 48 h, and then incubated with control (+ Ser) or serine supplemented (2 x Ser) medium for another 24 h, followed without insulin stimulation in D or with 100 nM insulin stimulation for 20 min in E. Data were obtained with mice described above (n = 10-14 mice per group) or at least three independent in vitro experiments and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student $t$-test for the effects of Ad-PSAT1 or Ad-shPSAT1 versus control group (*: p < 0.05) in A and B, or using one-way ANOVA followed by the Student-Newman-Keuls (SNK) test for the effects of any group versus - Ad-PSAT1 or - Ad-shPSAT1 in control medium (*: p < 0.05) in C-E, serine deficiency versus control medium under Ad-PSAT1 infection (#: p < 0.05) in C, or serine supplementation versus control medium under Ad-shPSAT1 infection (#: p < 0.05) in D and E. (A and B) Serum and liver serine levels; (C and D) TRB3 protein (left, western blot; right, quantitative measurement of TRB3 protein relative to actin); (E) p-IR (tyr1150/1151), p-AKT (ser473) and p-GSK3 β (ser9)
protein (*left*, western blot; *right*, quantitative measurements of p-IR, p-AKT and p-GSK3β protein relative to their total protein); (F) Working model.
Fig. 1

A

Liver
wt db/db
PSAT1
actin
tubulin

WAT
wt db/db
PSAT1
actin
tubulin

Muscle
wt db/db
PSAT1
actin
tubulin

Relative Psat1 mRNA (%)

wt db/db

Liver Serine (% of control)

wt db/db

B

Muscle WAT
wt db/db
PSAT1
actin
tubulin

Relative Psat1 mRNA (%)

HFD - +

Liver Serine (% of control)

HFD - +

C

D

Liver
HFD - +
PSAT1
actin

WAT
HFD - +
PSAT1
actin
tubulin

Muscle
HFD - +
PSAT1
actin
tubulin

Relative Psat1 mRNA (%)

HFD - +

Liver Serine (% of control)

HFD - +

E

F
### Fig. 2

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|          | -     | +                  |
| Ad-shPSAT1 | -     | +                  |
| p-IR     |       |                    |
| t-IR     |       |                    |
| p-AKT    |       |                    |
| t-AKT    |       |                    |
| p-GSK3β  |       |                    |
| t-GSK3β  |       |                    |
| PSAT-1   |       |                    |
| actin    |       |                    |

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**Graphs**

- Ad-PSAT1 + Ins
- + Ad-PSAT1 + Ins

**Graphs**

- Ad-shPSAT1 + Ins
- + Ad-shPSAT1 + Ins

**Arbitrary Units**

- p-IR
- p-AKT
- p-GSK3β
- PSAT-1
Glucose Output (100% of control)

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Glycogen Content (100% of control)

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Fig. 2
Figure 3

A. Relative Psat1 mRNA (%)

B. Blood Glucose (mg/dl)

C. Serum Insulin (ng/ml)

D. HOMA-IR

E. GTT

F. ITT

- Ad-PSAT1
- Ad-PSAT1 + Ins
- + Ad-PSAT1
- + Ad-PSAT1 + Ins

Relative Psat1 mRNA (%)

Blood Glucose (mg/dl)

Serum Insulin (ng/ml)

HOMA-IR

GTT

ITT

Blood Glucose (mg/dl)

Time (min)

Blood Glucose (mg/dl)

Time (min)

Blood Glucose (mg/dl)

Time (min)
Fig. 5

A. Ad-PSAT1

B. PSAT1

C. actin

D. fed, fasting, blood glucose (mg/dl)

E. fed, fasting, serum insulin (ng/ml)

F. fed, fasting, HOMA-IR

G. GTT, ITT, blood glucose (mg/dl)

H. Ins, Ad-PSAT1, p-IR, t-IR, p-AKT, t-AKT, p-GSK3β, t-GSK3β, PSAT1, actin
**Fig. 6**

A. Western blot analysis of PSAT1 and actin in mice fed a high-fat diet (HFD) with or without Ad-PSAT1.

B. Body weight (g) of mice fed a high-fat diet (HFD) with or without Ad-PSAT1.

C. Food intake (g/day) of mice fed a high-fat diet (HFD) with or without Ad-PSAT1.

D. Blood glucose (mg/dl) of mice fed a high-fat diet (HFD) with or without Ad-PSAT1.

E. Serum insulin (ng/ml) of mice fed a high-fat diet (HFD) with or without Ad-PSAT1.

F. HOMA-IR of mice fed a high-fat diet (HFD) with or without Ad-PSAT1.

G. Blood glucose (mg/dl) over time in a glucose tolerance test (GTT) and insulin tolerance test (ITT).

H. Western blot analysis of insulin (Ins), p-IR, t-IR, p-AKT, t-AKT, p-GSK3β, and t-GSK3β in mice fed a high-fat diet (HFD) with or without Ad-PSAT1.
**Fig. 7**

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- Ad-PSAT1
- + Ad-PSAT1

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- Ad-shPSAT1
- + Ad-shPSAT1

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- Ad-PSAT1
- + Ad-PSAT1

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- Ad-shPSAT1
- + Ad-shPSAT1

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- Ad-PSAT1
- + Ad-PSAT1

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- Ad-shPSAT1
- + Ad-shPSAT1

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- Ad-shPSAT1
- Ad-shTRB3

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- Ad-shPSAT1
- Ad-shTRB3

---

Diabetes
Fed Fasting Blood Glucose (mg/dl)

Fed Fasting Serum Insulin (ng/ml)

HOMA-IR

Blood Glucose (mg/dl)

Time (min)

GTT

ITT

Ad-shPSAT1 - + +
Ad-shTRB3 - - +
PSAT1
TRB3
actin

Arbitrary Units

Ad-shPSAT1 - Ad-shTRB3
+ Ad-shPSAT1 - Ad-shTRB3
+ Ad-shPSAT1 + Ad-shTRB3

Ad-shPSAT1 - - +
Ad-shPSAT1 - + +
Ad-shPSAT1 + + +
Ad-shTRB3 - - +
Ad-shTRB3 - + +
Ad-shTRB3 + + +

Blood Glucose (mg/dl)

Time (min)

Arbitrary Units

p-IR
t-IR
p-AKT
t-AKT
p-GSK3β
t-GSK3β

Fig. 7
Supplementary data

Figure Legends

Fig. S1 PSAT1 expression is decreased in TNF α treated-HepG2 cells

HepG2 cells were treated with 40 ng/ml TNFα (+ TNFα, Sigma) or without (- TNFα) for 24 h, followed by examination of PSAT expression. Means ± SEMs shown are representative of at least three independent in vitro experiments. Statistical significance was calculated using the two-tailed student t-test for the effects of with versus without TNFα (*: p < 0.05). PSAT1 protein (top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin).

Fig. S2 PSAT1 has no effect on insulin-stimulated phosphorylation of IRS1 and PDK1 in vitro

(A and B) Cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) for 48 h in A, or Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) for 72 h in B; both cases were followed with (+ Ins) or without (- Ins) 100 nM insulin stimulation for 20 min. Data were obtained with at least three independent in vitro experiments and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student t-test for the effects of Ad-PSAT1 or Ad-shPSAT1 versus corresponding control following insulin stimulation (*: p < 0.05). (A and B) p-IRS1 (tyrosine 612/608-human/mouse) and p-PDK1 (ser241) (top, western blot; bottom, quantitative measurements of p-IRS1, p-PDK1 relative to their total protein).
Fig. S3 Various metabolic effects of PSAT1 over-expression in wild-type (WT) mice

Male C57 BL/6J WT mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) for 7 days, followed by measurement of body weight and food intake in A and B; examination of insulin signaling before (- Ins) and after (+ Ins) 2 U/kg insulin stimulation for 3 min in liver, 4 min in white adipose tissue (WAT) and 5 min in muscle at day 7 in C, F and H; detection of glucose and lipid metabolism genes in D; examination of PSAT1 expression in WAT and muscle in E and G. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student t-test for the effects of Ad-PSAT1 versus corresponding control without insulin treatment in A, B, D, E and G, or with insulin treatment in C, F and H (*: p < 0.05). (A) Body weight change; (B) Food intake; (C) p-IRS1 (tyrosine 612/608-human/mouse) and p-PDK1 (ser241) (top, western blot; bottom, quantitative measurements of p-IRS1, p-PDK1 relative to their total protein); (D) mRNA levels of genes related to glucose and lipid metabolism. The sequences of primers were listed in Table S2; (E and G) PSAT1 protein (top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin or tubulin); (F and H) p-IR (tyr1150/1151), p-AKT (ser473) and p-GSK3β (ser9) protein (top, western blot; bottom, quantitative measurements of p-IR, p-AKT, p-GSK3β relative to their total protein).

Fig. S4 Various metabolic effects of PSAT1 knock down in wild-type (WT) mice
Male C57 BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) for 13 days, followed by measurement of body weight and food intake in A and B; examination of insulin signaling before (- Ins) and after (+ Ins) 2 U/kg insulin stimulation for 3 min in liver, 4 min in WAT and 5 min in muscle at day 13 in C, F and H; detection of glucose and lipid metabolism gene in D; examination of PSAT1 expression in white adipose tissue (WAT) and muscle in E and G. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student \( t \)-test for the effects of Ad-shPSAT1 versus corresponding control without insulin treatment in A, B, D, E and G, or with insulin stimulation in C, F and H (*: p < 0.05). (A) Body weight change; (B) Food intake; (C) p-IRS1 (tyrosine 612/608-human/mouse) and p-PDK1 (ser241) (top, western blot; bottom, quantitative measurements of p-IRS1, p-PDK1 relative to their total protein); (D) mRNA levels of genes related to glucose and lipid metabolism. The sequences of primers were listed in Table S2; (E and G) PSAT1 protein (top, western blot; bottom, quantitative measurement of PSAT1 protein relative to actin or tubulin); (F and H) p-IR (tyr1150/1151), p-AKT (ser473) and p-GSK3 \( \beta \) (ser9) protein (top, western blot; bottom, quantitative measurements of p-IR, p-AKT, p-GSK3\( \beta \) relative to their total protein).

**Fig. S5** No differences in TRB3 expression are observed in white adipose tissue (WAT) and muscle of insulin-resistant mice
TRB3 expression were analyzed in WAT and muscle of wild-type (wt) and \(db/db\) mice in A, or WT mice fed a control (- HFD) or HFD (+ HFD) for 16 weeks in B. Data were obtained with mice described above (n = 10-14 mice per group) and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student \(t\)-test for the effects of \(db/db\) or HFD mice versus their relevant control mice (*: \(p < 0.05\)). (A and B) TRB3 protein (top, western blot; bottom, quantitative measurement of TRB3 protein relative to tubulin or actin).

Fig. S6 Hepatic FOXO1 phosphorylation is increased by Ad-PSAT1 and decreased by Ad-shPSAT1 in vitro and in vivo

(A) HepG2 cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) for 48 h or male C57 BL/6J wild-type (WT) mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (- Ad-PSAT1) via tail-vein injection, followed by examination of FOXO1 in liver at day 7; (B) HepG2 cells were exposed to Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) for 72 h or male C57 BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (- Ad-shPSAT1) via tail vein injection, followed by examination of FOXO1 in liver at day 13. Data were obtained with mice described above (n = 10-14 mice per group) or at least three independent in vitro experiments and are presented as means ± SEMs. Statistical significance was calculated using the two-tailed student \(t\)-test for the effects of the Ad-PSAT1 or Ad-shPSAT1 versus the control group (*: \(p < 0.05\)). (A and B)
p-FOXO1 and FOXO1 protein (*top*, western blot; *bottom*, quantitative measurements of p-FOXO1 protein relative to total FOXO1)

**Fig. S7 Insulin signaling is not inhibited in PGDH RNAi or PSPH RNAi-treated HepG2 cells**

HepG2 cells were treated with PGDH siRNA (+ PGDH RNAi) or control reagent (-PGDH RNAi) for 48 h in A, PSPH siRNA (+ PSPH RNAi) or control reagent (-PSPH RNAi) for 48 h in B, both cases were followed with (+ Ins) or without (- Ins) 100 nM insulin stimulation for 20 min. Means ± SEMs shown are representative of at least three independent in vitro experiments. Statistical significance was calculated using the two-tailed student *t*-test for the effects of PGDH RNAi or PSPH RNAi versus corresponding control following insulin stimulation (*: p < 0.05). (A) *Pgdh* mRNA; (B and D) p-IR (tyr1150/1151), p-AKT (ser473) and p-GSK3β (ser9) protein (*left*, western blot; *right*, quantitative measurements of p-IR, p-AKT, p-GSK3β relative to their total protein); (C) *Psph* mRNA.
Fig. S1

Diabetes

![Graph showing TNFα and PSAT-1 levels with arbitrary units on the y-axis and TNFα - and + conditions on the x-axis.](image-url)
Fig. S2
A. Body Weight Change (%)
B. Food Intake (g/day)

C. Liver
- Ins - + - +
Ad-shPSAT1 - - + +

Y-p-IRS1

p-PDK1
t-PDK1

D. Relative mRNA level (%)

E. WAT
- Ad-shPSAT1 - +
Ad-shPSAT1 - +
PSAT1 actin

F. WAT
- Ins - + - +
Ad-shPSAT1 - - + +

p-IR
t-IR

p-AKT
t-AKT

p-GSK3β
t-GSK3β

G. Muscle
- Ad-shPSAT1 - +
Ad-shPSAT1 - +

p-IR
t-IR

p-AKT
t-AKT

p-GSK3β
t-GSK3β
Fig. S5

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Arbitrary Units
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## Metabolic parameters in different mice

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<th>db/db (8w)</th>
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<th>HFD (4w wt mice under HFD for 16w )</th>
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<td>Blood glucose (mg/dl)</td>
<td>162.3 ± 4.51</td>
<td>316.2 ± 18.57*</td>
<td>156.3 ± 6.82</td>
<td>200.7 ± 5.15*</td>
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<td>Serum insulin (ng/ml)</td>
<td>0.33 ± 0.01</td>
<td>14.42 ± 4.14*</td>
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<td>Serum glucagon (pg/ml)</td>
<td>194.17 ± 1.46</td>
<td>103.56 ± 20.08*</td>
<td>212.47 ± 26.92</td>
<td>135.87 ± 17.96*</td>
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<td>Serum TG (mg/dl)</td>
<td>19.56 ± 2.10</td>
<td>301.33 ± 53.57*</td>
<td>27.46 ± 8.41</td>
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<td>Serum TC (mM/l)</td>
<td>1.93 ± 0.06</td>
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<td>Serum FFA (mEq/l)</td>
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### List of oligonucleotide primer pairs used in RT-PCR analysis

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