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Hepatic Phosphoserine Aminotransferase 1 Regulates Insulin Sensitivity in Mice via Tribbles Homolog 3

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METABOLISM

Phosphoserine aminotransferase 1 (PSAT1) is an enzyme participating in serine synthesis. A role of PSAT1 in the regulation of insulin sensitivity, however, is unknown. In this study, 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, overexpression of PSAT1 by adenovirus expressing 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, overexpression 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 adenoviruses expressing small hairpin RNA against TRB3-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 nonessential amino acid serine may play an important role in regulating insulin sensitivity.

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 patients with T2D (2–4). Previous studies have demonstrated that essential amino acids, particularly branched-chain amino acids, 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, nonessential 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 these studies, the role that nonessential 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 3-phosphohydroxypyruvate 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, as a by-product of PSAT1 catalyzed reaction (14), stimulates insulin secretion (20). However, it is currently unknown whether PSAT1 participates in the regulation

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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 a novel function in the regulation of insulin sensitivity. The aim of our current study was to investigate this possibility and elucidate underlying mechanisms.

In this study, we show 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 C57BL/6J wild-type (WT) mice were obtained from Shanghai Laboratory Animal Co., Ltd. (Shanghai, China). Leptin receptor–deficient (*db/db*) mice were kindly provided by Dr. Xiang Gao (Nanjing University). Male C57BL/6J WT mice were fed for 16 weeks an HFD (Research Diets, Inc., New Brunswick, NJ) or chow diet, which has been previously used as control for HFD (23,24). Adenoviruses were injected in to mice under HFD or chow diet for 14 weeks, and then these mice were fed with an HFD or chow diet continuously before sacrifice. Eight- to 10-week-old mice were maintained on a 12-h 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 Institutes for Biological Sciences, 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 Shanghai Institutes for Biological Sciences, CAS) were maintained in DMEM containing 25 mmol/L glucose (Gibco, Invitrogen, Carlsbad, CA), 10% FBS, and 50 mg/mL penicillin and streptomycin, in an environment with 5% CO_2/95% air at 37°C. Control (complete amino acid), (−) ser (serine-deficient), and 2× ser (serine-supplemented) medium were prepared from amino acid–free DMEM (Invitrogen) by adding back all of the amino acids contained in regular DMEM, without serine only, or double the serine amount, respectively. Double-stranded small interfering RNA (siRNA) targeting human phosphoglycerate dehydrogenase (PGDH) or L-phosphoserine phosphatase (PSPH) were purchased from GenePharma (Shanghai, China). The siRNA sequence specific for human PGDH was 5′-GGGAG GAAAUUGCUGUUCATT-3′ and for human PSPH was 5′-GGAGUAUUGUAGAGCAUGUTT-3′. HepG2 cells were

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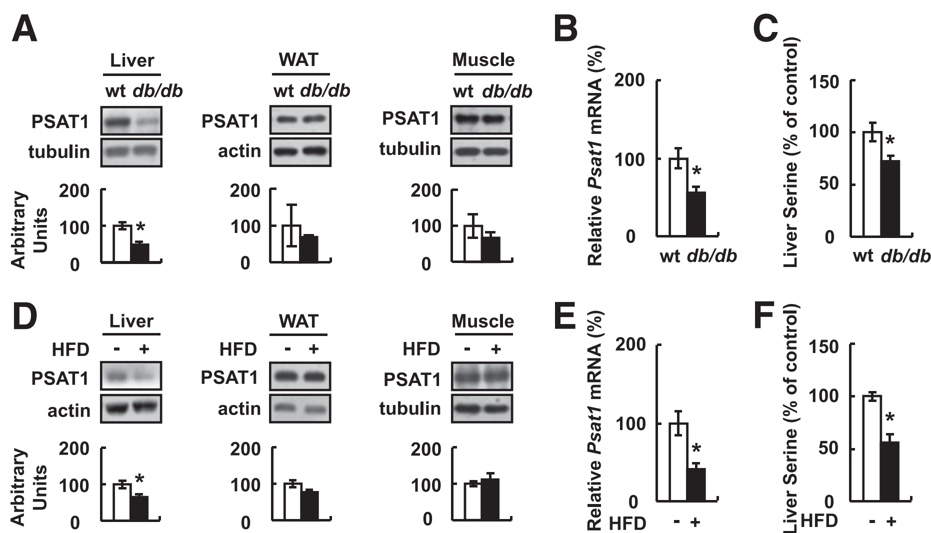

Figure 1—Hepatic PSAT1 expression is reduced under insulin-resistant conditions. PSAT1 expression was analyzed in the livers, WAT, and muscle, and liver serine levels were measured in wild-type (wt) and *db/db* mice in *A*–*C* or WT mice fed a control diet (− HFD) or HFD (+ HFD) for 16 weeks in *D*–*F*. Data were obtained with mice described above (n = 10–14 mice/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.

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) according to the manufacturer's instructions. Adenovirus expressing scrambled or short hairpin RNA directed against the coding region for mouse PSAT1 or TRB3 was generated using the BLOCK-iT Adenoviral RNAi Expression System (Invitrogen) according to the manufacturer's instructions. The scrambled sequence is 5′-TTCTCCGAACGTGTCACGT-3′. The short hairpin RNA 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 plaque-forming units/well in a 12-well plate or via a tail vein injection using 5×10^8 plaque-forming units per mouse.

Blood Glucose, Serum Insulin, Glucose Tolerance Test, Insulin Tolerance Test, and HOMA of Insulin Resistance Index

Levels of blood glucose and serum insulin were measured using a Glucometer Elite monitor and a Mercodia Ultrasensitive Rat Insulin ELISA kit (ALPCO Diagnostics,

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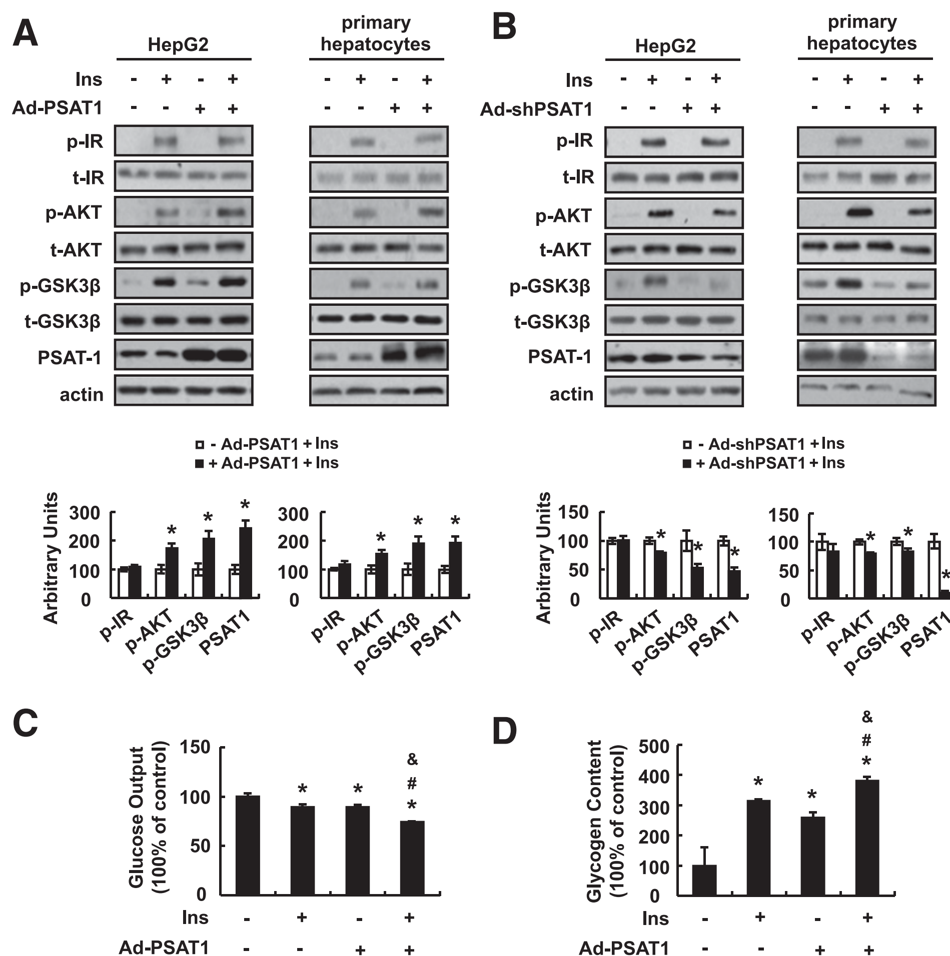

Figure 2—PSAT1 regulates insulin sensitivity in vitro. *A* and *B*: Cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) for 48 h in *A* or Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (− Ad-shPSAT1) for 72 h in *B*; both cases were followed with (+ Ins) or without (− Ins) 100 nmol/L insulin 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 SNK test for the effects of Ad-PSAT1 or Ad-shPSAT1 versus corresponding control following insulin stimulation (**P* < 0.05) in *A* and *B* and any group versus the − Ad-PSAT1 group without insulin stimulation (**P* < 0.05), with versus without insulin stimulation in the + 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-GSK3β (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.

Salem, NH), respectively. The glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs) were performed by administering an intraperitoneal injection of 2 g/kg glucose following overnight fasting and 0.75 units/kg insulin after 4 h of fasting, respectively. The HOMA of insulin resistance (HOMA-IR) index was calculated according to the following formula: (fasting glucose levels [mmol/L]) × (fasting serum insulin [μU/mL])/22.5. For each adenovirus injection experiment, blood glucose was examined daily. Until differences were observed between the experimental group and control group, GTT and ITT were performed and therefore may result in different measurement times 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 units/kg in WT or 5 units/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–phosphorylated insulin receptor [p-IR; tyr1150/1151], anti-IR, anti-p–insulin receptor substrate 1 [IRS1; tyrosine 612/608-human/mouse], anti-IRS1, anti-p–phosphoinositide-dependent protein kinase 1 [PDK1; ser241], anti-PDK1, anti-p–protein kinase B [AKT; ser473], anti-AKT, anti-p–glycogen synthase kinase 3β [GSK3β; ser9], anti-GSK3β, anti-p–forkhead box O1 [FOXO1; S256], and anti-FOXO1 [all from Cell Signaling Technology, Beverly, MA]), anti-PSAT1, and anti-TRB3 (both from Santa Cruz Biotechnology, Santa Cruz, CA) were incubated overnight at 4°C, and specific proteins were visualized by ECL Plus (Amersham Biosciences, Buckinghamshire, U.K.). Band intensities were measured using Quantity One (Bio-Rad, Hercules, CA) 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′.

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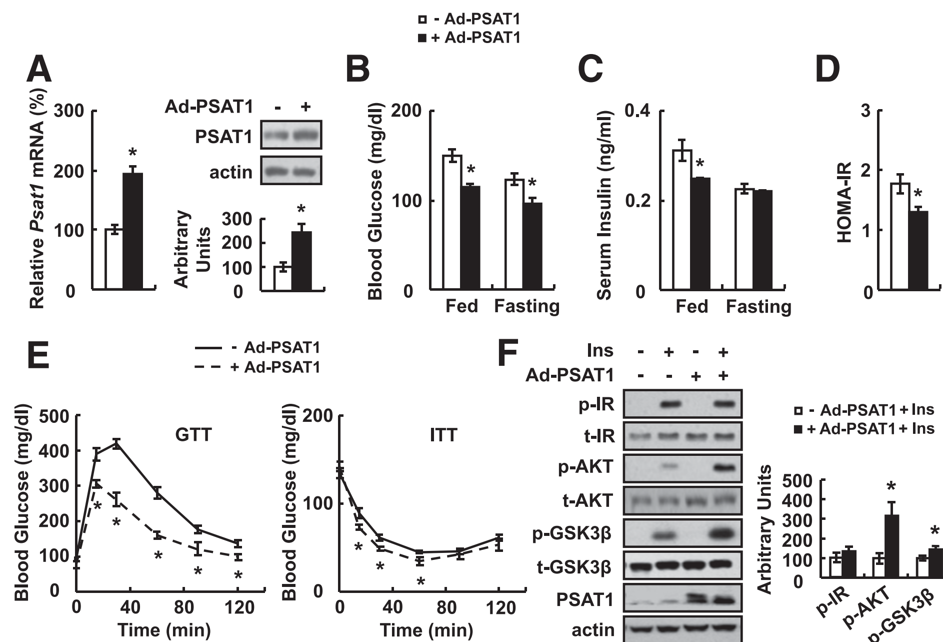

Figure 3—Overexpression of PSAT1 improves insulin sensitivity in WT mice. Male C57BL/6J WT mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) via tail vein injection, followed by examination of PSAT1 expression in liver at day 7 in *A*, measurement of fed blood glucose and serum insulin levels at day 7 or fasting blood glucose and serum insulin levels at day 3 in *B* and *C*, calculating HOMA-IR index in *D*, performance of GTTs and ITTs at days 3 or 5 in *E*, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 7 in *F*. Data were obtained with mice described above (n = 10–14 mice/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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), t-GSK3β, and PSAT1 protein (left, Western blot; right, quantitative measurements of p-IR, p-AKT, and p-GSK3β protein relative to their total protein).

Statistics

All data are expressed as means ± SEMs. Significant differences were assessed either by two-tailed Student *t* test or one-way ANOVA followed by the Student-Newman-Keuls (SNK) test. A *P* value <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: genetic 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 the current study is summarized in Supplementary Table 1. 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. 1*A*). Consistently, hepatic *Psat1* mRNA and liver serine levels were also decreased in *db/db* mice (Fig. 1*B* and 1*C*). Similar results were obtained in mice under HFD for 16 weeks compared with their relevant control mice (Fig. 1*D–F*). Interestingly, inflammation might contribute to the decreased PSAT1 expression in these mice as demonstrated by the inhibitory effects of tumor necrosis factor-α (TNF-α) on PSAT1 expression in HepG2 cells compared with control vehicle (Supplementary Fig. 1).

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 the insulin signaling pathway in these cells. Overexpression of PSAT1 increased insulin-stimulated phosphorylation of AKT (ser473) and GSK3β (ser9) in both cell lines compared with control cells (Fig. 2*A*). Consistently, insulin-stimulated phosphorylation of AKT and GSK3β 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. 2*B*). Insulin-stimulated phosphorylation of IR (tyr1150/1151), IRS1 (tyrosine 612/608-human/mouse), and PDK1 (ser241), however, was not affected by Ad-PSAT1 or Ad-shPSAT1 (Fig. 2*A* and *B* and Supplementary Fig. 2). In addition, overexpression of PSAT1 decreased glucose output and increased glycogen content in the presence or absence of insulin (Fig. 2*C* and *D*).

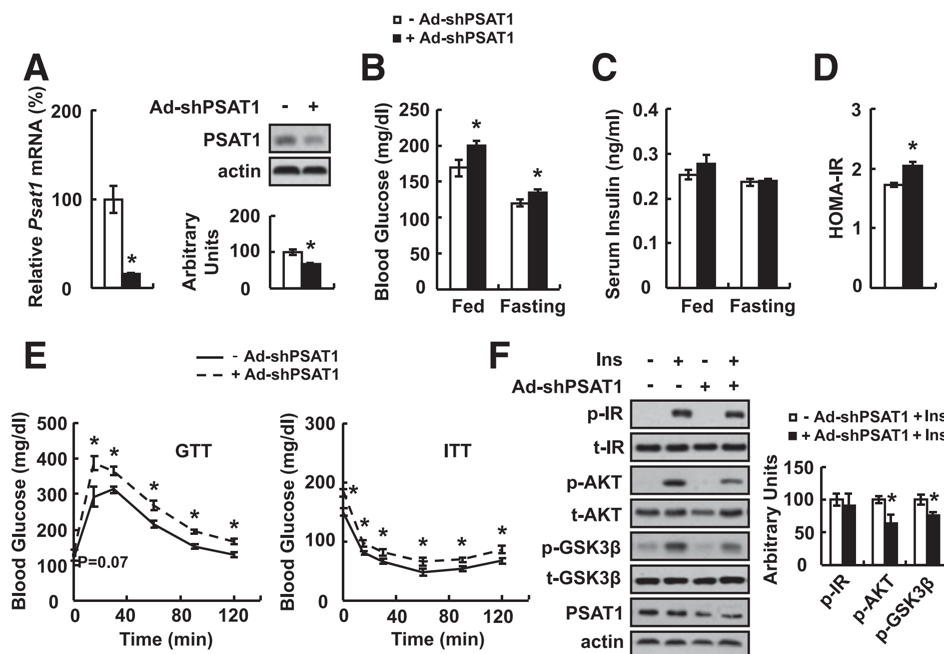

Figure 4—Knockdown of PSAT1 attenuates insulin sensitivity in WT mice. Male C57BL/6J WT mice were injected with Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (− Ad-shPSAT1) via tail vein injection, followed by examination of PSAT1 expression in liver at day 13 in *A*, measurement of fed blood glucose and serum insulin levels at day 13 or fasting blood glucose and serum insulin levels at day 9 in *B* and *C*, calculating HOMA-IR index in *D*, performance of GTTs and ITTs at days 9 or 11 in *E*, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 13 in *F*. Data were obtained with mice described above (n = 10–14 mice/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 (top right, Western blot; left and bottom right, 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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), t-GSK3β, and PSAT1 protein (left, Western blot; right, quantitative measurements of p-IR, p-AKT, and p-GSK3β protein relative to their total protein).

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Overexpression of PSAT1 by Ad-PSAT1 Improves Insulin Sensitivity In Vivo

To explore the role of PSAT1 in insulin sensitivity in vivo, we injected male C57BL/6J 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. 3*A*). Although body weight or food intake was not altered by Ad-PSAT1 (Supplementary Fig. 3*A* and *B*), Ad-PSAT1 significantly decreased blood glucose levels in mice under both fed and fasting conditions (Fig. 3*B*). Fasting serum insulin levels remained unchanged; however, fed serum insulin levels were significantly decreased in Ad-PSAT1 mice (Fig. 3*C*). Furthermore, the HOMA-IR index was decreased in these mice (Fig. 3*D*). 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 GTTs and ITTs, respectively (Fig. 3*E*). Additionally, insulin-stimulated phosphorylation of AKT and GSK3β, but not IR, IRS1, or PDK1, was increased in the livers of these mice (Fig. 3*F* and Supplementary Fig. 3*C*).

Hepatic genes involved in glucose and lipid metabolism were also examined. Expression of the gluconeogenesis gene *Pepck* and the glycogenolysis gene phosphorylase, glycogen, and liver (*Pygl*) (30) were decreased, while the glycogen synthesis gene glycogen synthase 2 (*Gys2*) (31) was increased, in the livers of Ad-PSAT1 mice compared with control mice (Supplementary Fig. 3*D*). Furthermore, we investigated the effects of PSAT1 on expression of genes related to lipogenesis (fatty acid synthase and stearol-CoA desaturase 1) and β-oxidation (peroxisome proliferator–activated receptor α [*Pparα*] and carnitine palmitoyltransferase 1 [*Cpt1*]) (32), and no significant effects of PSAT1 on expression of these genes were observed (Supplementary Fig. 3*D*). In addition, no changes in PSAT1 expression and insulin signaling were observed in WAT and muscle of Ad-PSAT1 mice (Supplementary Fig. 3*E–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 C57BL/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. 4*A*). Body weight or food intake, however, was not altered by Ad-shPSAT1 (Supplementary Fig. 4*A* and *B*). Fed and fasting blood glucose levels were significantly elevated in Ad-shPSAT1 mice compared with Ad-scrambled mice (Fig. 4*B*). As a result, although fed and fasting serum insulin levels remained unchanged, HOMA-IR index was increased in Ad-shPSAT1 mice (Fig.

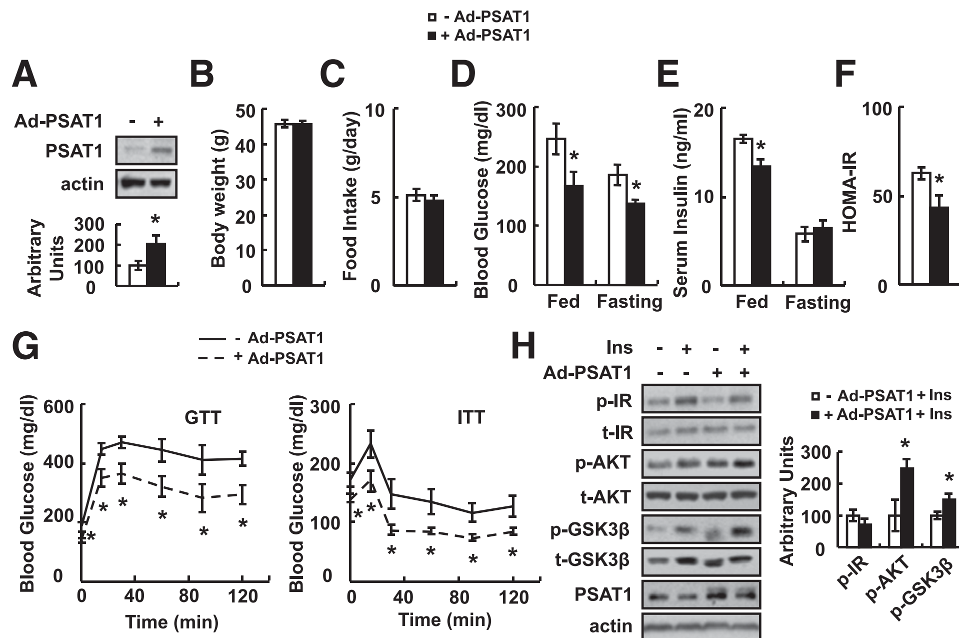

Figure 5—Overexpression of PSAT1 ameliorates insulin resistance in *db/db* mice. Male C57BL/6J *db/db* mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) via tail vein injection, followed by examination of hepatic PSAT1 expression at day 11 in *A*, measurement of body weight and food intake 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 day 7 in *D* and *E*, calculating HOMA-IR index in *F*, performance of GTTs and ITTs at days 7 and 5 in *G*, respectively, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 5 units/kg insulin stimulation for 3 min at day 11 in *H*. Data were obtained with mice described above (n = 10–14 mice/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 (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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), t-GSK3β, and PSAT1 protein (left, Western blot; right, quantitative measurements of p-IR, p-AKT, and p-GSK3β protein relative to their total protein).

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4C and D). 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, was also greatly impaired in the livers of mice injected with Ad-shPSAT1 (Fig. 4F and Supplementary Fig. 4C). Expression of *Pepck*, *Pygl*, *Pparα*, and *Cpt1* were increased and *Gys2* expression was decreased in the livers of Ad-shPSAT1 mice (Supplementary Fig. 4D). Again, no changes in PSAT1 expression and insulin signaling were observed in WAT and muscle of Ad-shPSAT1 mice (Supplementary Fig. 4E–H).

Overexpression 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 overexpression 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). Overexpression 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 GSK3β, but not IR, was 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 feed on their diet until they were killed. 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 C). Consistent with previous reports (29), an 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, were largely reversed by Ad-PSAT1 injection compared with mice injected with Ad-GFP, except for serum insulin levels (Fig. 6D–H).

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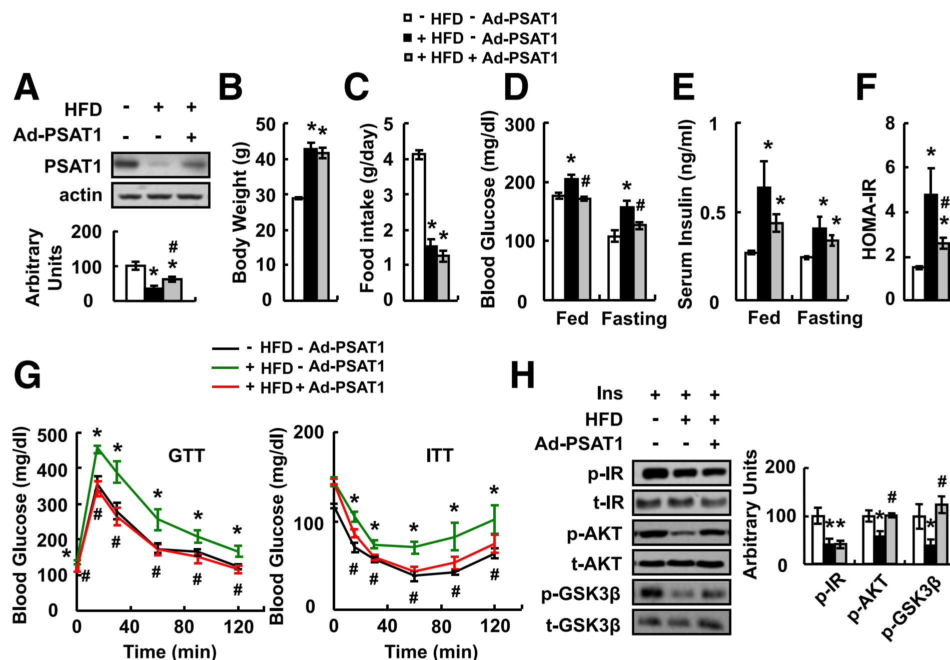

Figure 6—Overexpression of PSAT1 ameliorates insulin resistance in mice under HFD. Male C57BL/6J WT mice were fed a control (− HFD) or HFD diet (+ HFD) for 14 weeks and then injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) via tail vein injection, followed by examination of hepatic PSAT1 expression at day 11 in *A*, measurement of body weight and food intake 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 day 7 in *D* and *E*, calculating HOMA-IR index in *F*, performance of GTTs and ITTs at days 7 and 5 in *G*, respectively, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 11 in *H*. Data were obtained with mice described above (n = 10–14 mice/group) and are presented as means ± SEMs. Statistical significance was calculated using one-way ANOVA followed by the 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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), and t-GSK3β protein (left, Western blot; right, quantitative measurements of p-IR, p-AKT, and p-GSK3β protein relative to their total protein).

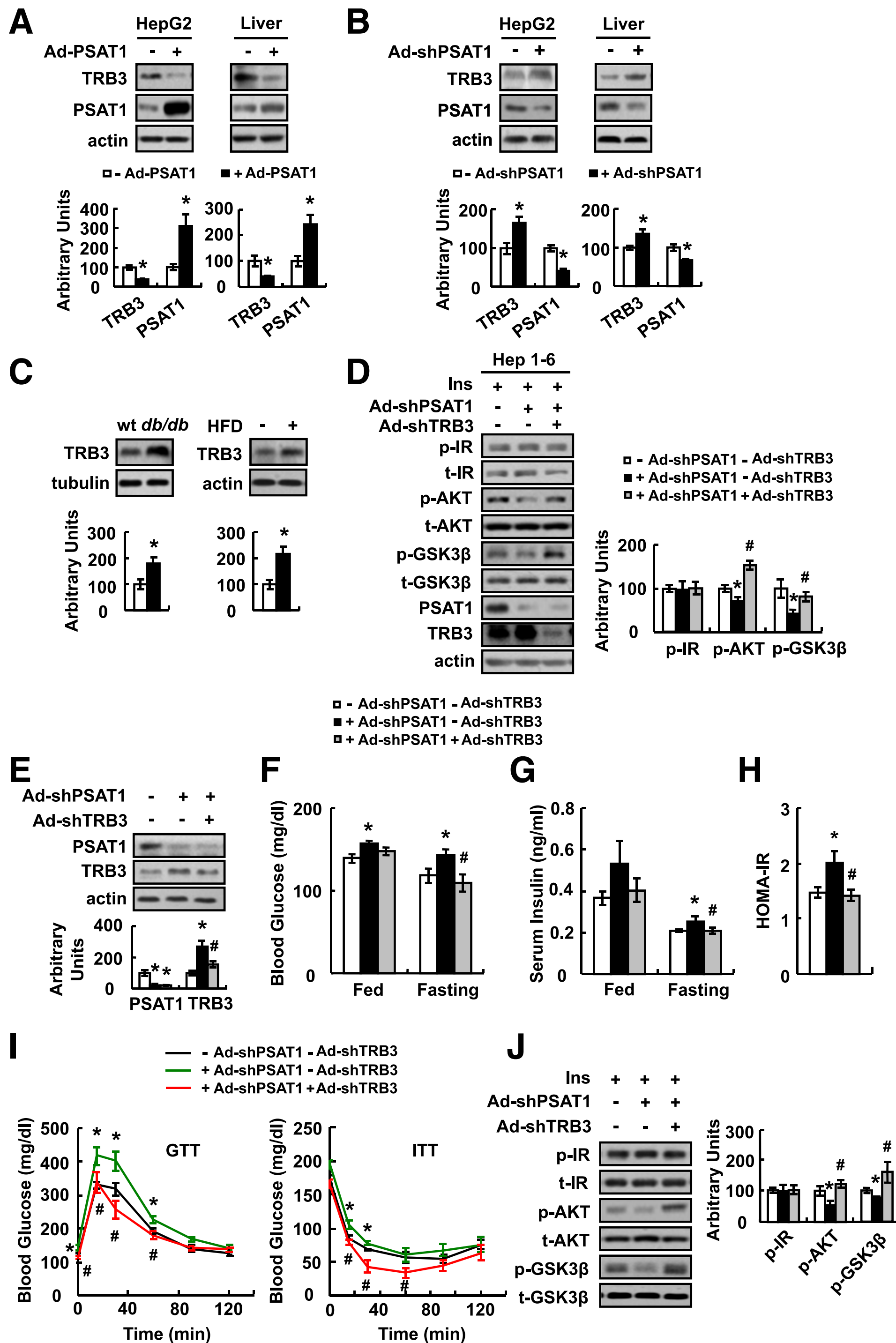

Figure 7—PSAT1 regulates insulin sensitivity via TRB3. *A*: HepG2 cells were infected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) for 48 h, or male C57BL/6J WT mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) via tail vein injection, followed by examination of TRB3 in liver at day 7. *B*: HepG2 cells were exposed to Ad-shPSAT1 (+ Ad-shPSAT1) or Ad-scrambled (− Ad-shPSAT1) for 72 h, or male C57BL/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 nmol/L 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 GTTs and ITTs at days 8 or 10 in *I*, and examination of insulin signaling in liver before (− Ins) and after (+ Ins) 2 units/kg insulin stimulation for 3 min at day 8 in *J*. Data were obtained with mice described above (n = 10–14 mice/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

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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 (34), which has been shown to stimulate TRB3 expression (35). Based on these findings, 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. 7*A* and *B*). 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. 7*C* and Supplementary Fig. 5). The possible involvement of TRB3 in PSAT1-regulated insulin signaling was then investigated in nonimmunogenic 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 GSK3β, but not IR, compared with control cells (Fig. 7*D*).

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. 7*E*). 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. 7*F* and *G*). The HOMA-IR index was decreased following administration of Ad-shTRB3 in Ad-shPSAT1 mice (Fig. 7*H*). Moreover, glucose clearance and insulin sensitivity, which were attenuated by knocking down PSAT1, were also markedly reversed by Ad-shTRB3 (Fig. 7*I* and *J*). Previous studies have shown that TRB3 expression is inhibited by activation of 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 (Supplementary Fig. 6).

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. 8*A* and *B*). To investigate the role of serine in mediating PSAT1-regulated TRB3 expression, we either *1*) infected HepG2 cells with Ad-PSAT1, which is supposed to increase serine levels, and then incubated them with a serine-deficient medium for 24 h or *2*) infected HepG2 cells with Ad-shPSAT1, which is supposed to decrease serine levels, and then incubated them with a serine-supplemented (twofold increase in serine levels) medium for 24 h. Serine deficiency stimulated TRB3 expression and also reversed the Ad-PSAT1–induced inhibition of TRB3 expression (Fig. 8*C*). Although serine supplementation had no effect on TRB3 expression under basal conditions, it inhibited Ad-shPSAT1 stimulation of TRB3 expression (Fig. 8*D*). Furthermore, serine supplementation reversed Ad-shPSAT1–mediated inhibition of AKT and GSK3β phosphorylation, but not IR phosphorylation, following insulin stimulation (Fig. 8*E*). However, the other two enzymes involved in serine synthesis, including 3-PGDH and PSPH (12), did not have similar effects as PSAT1 on insulin signaling in HepG2 cells when their expression was knocked down by siRNA directed against each of them (Supplementary Fig. 7).

DISCUSSION

While PSAT1 knockout 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, overexpression or knockdown of PSAT1 improved or impaired insulin signaling and insulin sensitivity under normal conditions, respectively. In addition, overexpression of PSAT1 ameliorated insulin resistance in diabetic mice. To our knowledge, our results are the first 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,

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(*$P < 0.05$) in *A* and *B*, the effects of *db/db* or HFD mice versus control mice in *C* (*$P < 0.05$), or using the one-way ANOVA followed by the 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 *D*–*J*. *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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), t-GSK3β, 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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), and t-GSK3β protein (left, Western blot; right, quantitative measurements of p-IR, p-AKT, and p-GSK3β protein relative to their total protein).

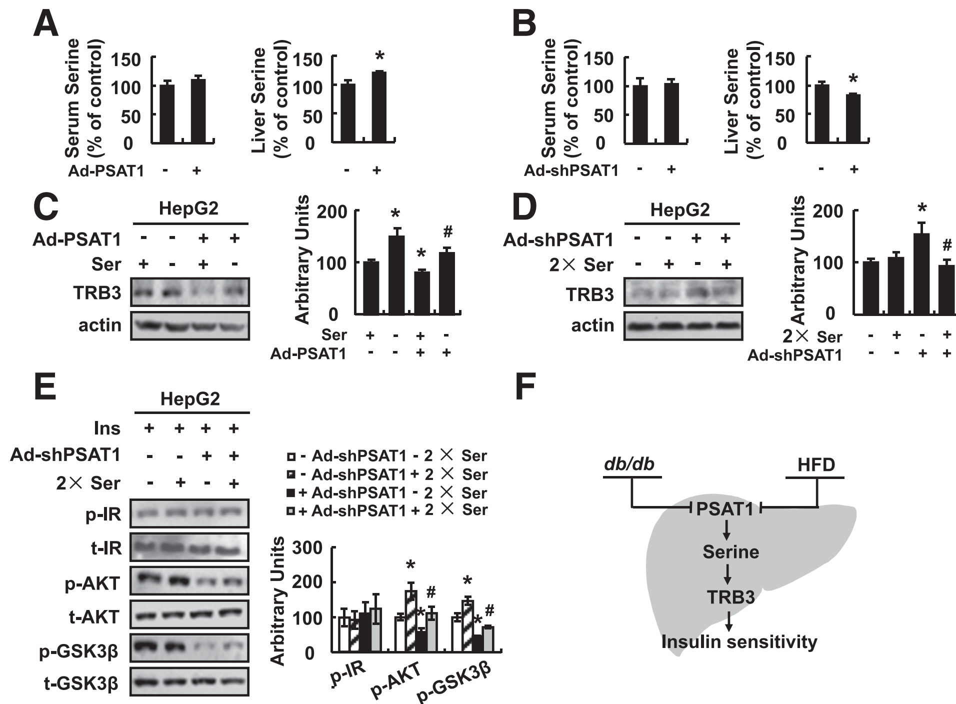

Figure 8—PSAT1 regulates TRB3 expression via serine in HepG2 cells. *A*: Male C57BL/6J WT mice were injected with Ad-PSAT1 (+ Ad-PSAT1) or Ad-GFP (− Ad-PSAT1) via tail vein injection, followed by examination of serum and liver serine levels at day 7. *B*: Male C57BL/6J WT mice were injected with Ad-shPSAT1 (+ 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 (2x Ser) medium for another 24 h, followed without insulin stimulation in *D* or with 100 nmol/L insulin stimulation for 20 min in *E*. Data were obtained with mice described above (n = 10–14 mice/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 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), t-IR, p-AKT (ser473), t-AKT, p-GSK3β (ser9), and t-GSK3β protein (left, Western blot; right, quantitative measurements of p-IR, p-AKT, and p-GSK3β protein relative to their total protein). *F*: Working model.

such as TNF-α (39,40), and we speculated that the decreased hepatic PSAT1 expression might be caused by elevated TNF-α. Further research will be necessary to validate this finding.

Although various tissues in mice can influence systemic 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 overexpression or knockdown 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 overexpression of PSAT1 may decrease blood glucose levels by promoting glycogen synthesis and inhibiting gluconeogenesis and glycogenolysis. In support of this notion, overexpression 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 PSAT1, 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 IR, resulting in phosphorylation of IRS1 and consequent activation of phosphatidylinositol 3-kinase/PDK1/AKT and GSK3β, key components of insulin signaling (1,41). In this study, we found that overexpression 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

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normal insulin signaling and glucose homeostasis (42), adenovirus-mediated overexpression 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.

In this study, we provided evidence showing that serine is involved in the regulation of TRB3 by PSAT1. Serine is a nonessential 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 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 nonessential 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 nonessential amino acid serine were decreased in livers of *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). In this study, 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 nonessential amino acids from insulin sensitivity regulation, and their role in this process requires further investigation.

In conclusion, as described in our working model (Fig. 8*F*), 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 patients with T2D. Our results also show that, in the liver, the nonessential 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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Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. J.Y. researched data and wrote, reviewed, and edited the manuscript. F.X. researched data and contributed to discussion. Y.G., J.D., Q.Z., and K.L. researched data. B.L. and S.C. provided research material. C.W. contributed to discussion. F.G. directed the project, contributed to discussion, and wrote, reviewed, and edited the manuscript. F.G. 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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