or preventing cortical membrane reorganization until cytokinesis is completed. Interactions between the actin ring and the spindle midzone and midbody have also been observed in metazoan eukaryotes (*2*, *31*, *32*), and microtubules are continuously required to maintain the position of the cleavage furrow and keep nuclei apart during cytokinesis in mammalian cells (*33*), suggesting that a similar mechanism to the one we have described in fission yeast may operate in other eukaryotes.

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Supporting Online Material

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TRB3: A *tribbles* Homolog That Inhibits Akt/PKB Activation by Insulin in Liver

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Insulin resistance is a major hallmark in the development of type II diabetes, which is characterized by the failure of insulin to promote glucose uptake in muscle and to suppress glucose production in liver. The serine-threonine kinase Akt (PKB) is a principal target of insulin signaling that inhibits hepatic glucose output when glucose is available from food. Here we show that TRB3, a mammalian homolog of *Drosophila tribbles*, functions as a negative modulator of Akt. TRB3 expression is induced in liver under fasting conditions, and TRB3 disrupts insulin signaling by binding directly to Akt and blocking activation of the kinase. Amounts of TRB3 RNA and protein were increased in livers of *db/db* diabetic mice compared with those in wild-type mice. Hepatic overexpression of TRB3 in amounts comparable to those in *db/db* mice promoted hyperglycemia and glucose intolerance. Our results suggest that, by interfering with Akt activation, TRB3 contributes to insulin resistance in individuals with susceptibility to type II diabetes.

Under physiological conditions, binding of insulin to its receptor triggers the activation of a phospholipid-dependent kinase cascade that culminates in phosphorylation of the Ser-Thr kinase Akt (also called PKB) (*1*). The Akt family of Ser-Thr kinases consists of three highly related family members: Akt1, Akt2, and Akt3. After their recruitment to the plasma membrane in response to growth factor stimulation, all three isoforms are phosphorylated at two conserved residues corresponding to Thr^{308} within the active loop and Ser^{473} in the regulatory domain of Akt1 (*1*). Akt activity appears to be important for glucose homeostasis; mice with a knockout of the Akt2 gene exhibit insulin resistance and glucose intolerance due to elevated hepatic glucose output and reduced glucose uptake in skeletal muscle (*2*).

Akt activity is attenuated in part by lipid phosphatases such as PTEN and SHIP, which act upstream of Akt to block its recruitment to the membrane (*3*–*6*). Akt also appears to be directly dephosphorylated by the Ser-Thr phosphatase PP2A in response to certain stimuli such as hyperosmotic stress (*7*). A protein inhibitor, referred to as COOH-terminal Akt modulatory protein (CTMP), has been shown to inhibit Akt activity by binding to its COOH-terminal regulatory domain (*8*), although its effect on insulin signaling has not been determined.

To identify additional proteins that modulate Akt activity, we used a yeast two-hybrid assay to screen for proteins from a preadipocyte F422A cDNA library that interacted with a GAL4-Akt ΔPH construct lacking the NH_2-terminal pleckstrin homology domain (amino acids 1 to 145) of Akt1. Twenty-five independent transformants from a screen of 2×10^6 cDNAs encoded a 354–amino acid protein previously identified as neuronal cell death–inducible putative protein kinase (NIPK) (*9*) and more recently designated in the GenBank database as TRB3. TRB3 and its related family members TRB1 and TRB2 share 45% sequence similarity overall and bear strong resemblance to *tribbles*, a *Drosophila* protein that inhibits mitosis early in development by binding to the CDC25 homolog String and promoting its ubiquitination and proteasome-mediated degradation (*10*–*13*). Like *tribbles*, TRB family members have a truncated kinase domain that lacks an adenosine 5′-triphosphate binding site (GXGXXG) and contains a variant catalytic core motif (TRB3: amino acids 175 to 182; LRDLKLRR versus consensus: HRDLKPEN). Correspondingly, *tribbles* and its mammalian counterparts lack detectable kinase activity by in vitro kinase assay (*13*, *14*).

To evaluate the association between Akt1 and TRB3, we performed mammalian two-

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hybrid assays in 293T cells. Consistent with results from the yeast two-hybrid screen, a GAL4-Akt construct showed no transcriptional activity on its own, but strongly induced GAL4 luciferase reporter activity when cotransfected with TRB3-VP16 expression vector (Fig. 1A). In vitro–translated ^{35}S-labeled TRB3 also interacted with baculovirus-expressed glutathione *S*-transferase (GST)–Akt1 in vitro (Fig. 1B).

To determine whether endogenous TRB3 and Akt are associated in vivo, we generated a polyclonal antiserum to the NH_2-terminal 145 amino acids of TRB3, which are not conserved in TRB1 or TRB2. In Western blot assays of HepG2 hepatocyte extracts, antiserum to TRB3 (anti-TRB3) recognized a single band of 45 kD, which is consistent with the predicted molecular size of the protein (Fig. 1C). Immunoprecipitation of proteins from HepG2 whole-cell extracts with anti-TRB3 revealed a 60-kD band that interacted with anti-Akt (Fig. 1C). No Akt was detected in immunoprecipitates prepared with preimmune serum or with anti-TRB3 that was blocked by incubation with recombinant TRB3 protein. Confirming the association noted with endogenous proteins, Flag-tagged TRB3 was recovered from immunoprecipitates of hemagglutinin (HA)–tagged Akt1 in transfected cells (fig. S1). TRB3 was also detected in immunoprecipitates of Akt2, suggesting a more general involvement of TRB3 in regulating cellular Akt activity (*14*).

In experiments to characterize a relevant Akt interaction domain in TRB3, we identified a variant TRB3 cDNA in the GenBank database that lacks 26 amino acids (amino acids 239 to 265) within the conserved kinase domain. Compared with the wild-type protein, the ΔTRB3 (Δ) polypeptide interacted more weakly with Akt in coimmunoprecipitation assays (fig. S1). TRB2 was also found to interact with overexpressed Akt in coimmunoprecipitation assays (*14*).

Phosphorylation of Akt1 at Thr^{308} and Ser^{473} is tightly correlated with its activation (*1*). To determine whether the association with TRB3 modulates Akt activity, we monitored Akt phosphorylation in response to growth factor stimuli. Treatment of human embryonic kidney 293 (HEK293) cells with insulin-like growth factor (IGF1) induced phosphorylation of Akt at Thr^{308} and Ser^{473} within 15 min (fig. S2 and Fig. 2A). Expression of TRB3 inhibited Akt phosphorylation at both sites without altering total amounts of Akt protein. TRB2 was similarly effective at blocking Akt phosphorylation (*14*). Overexpression of TRB3 reduced Akt activity, as determined by in vitro kinase assays with a peptide substrate corresponding to the consensus sequence recognized by Akt (Fig. 2B).

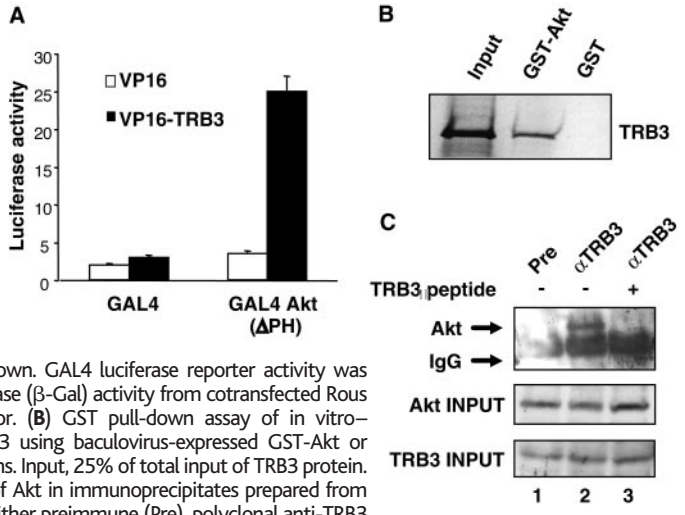

Fig. 1. Association of TRB3 with Akt. (**A**) Mammalian two-hybrid assay of 293T cells transfected with GAL4-Akt ΔPH construct lacking the NH_2-terminal pleckstrin homology domain of Akt (amino acids 1 to 145) and either TRB3-VP16 or control VP16 expression vector. Effect of GAL4 DNA binding domain construct alone (GAL4) on reporter activity is also shown. GAL4 luciferase reporter activity was normalized to β-galactosidase (β-Gal) activity from cotransfected Rous sarcoma virus–β-Gal vector. (**B**) GST pull-down assay of in vitro–translated ^{35}S-labeled TRB3 using baculovirus-expressed GST-Akt or control GST-Sepharose resins. Input, 25% of total input of TRB3 protein. (**C**) Western blot analysis of Akt in immunoprecipitates prepared from HepG2 cell extracts using either preimmune (Pre), polyclonal anti-TRB3 (αTRB3), or anti-TRB3 blocked with TRB3 polypeptide (amino acids 1 to 145). Recovery of 60-kD Akt-immunoreactive band from each immunoprecipitate is shown. Input levels of TRB3 and Akt are indicated.

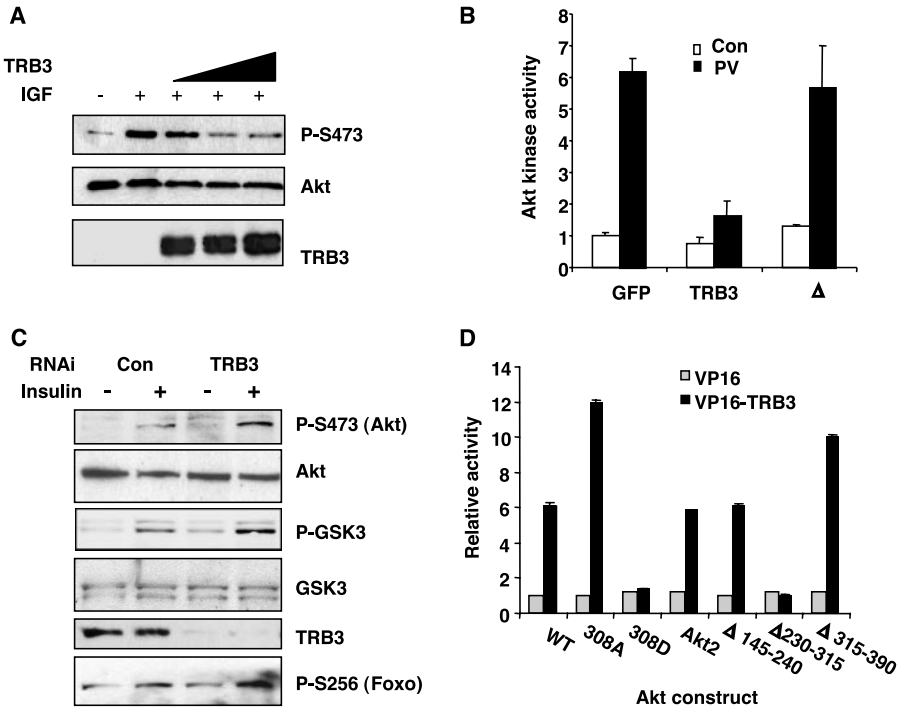

Fig. 2. Inhibition of growth factor–dependent phosphorylation of Akt by TRB3. (**A**) Western blot analysis of phospho (Ser^{473}) Akt, total Akt, and TRB3 protein levels in HEK293 cells transfected with HA-tagged Akt1 expression vector plus Flag-tagged TRB3 vector. Cells were treated either with IGF1 (100 nM) or left untreated for 30 min. Immunoprecipitates of Akt were prepared with anti-HA, and Western blot assays were done with antibody specific to phospho (Ser^{473}) as well as anti-HA to visualize total amounts of Akt. Amounts of Flag-tagged TRB3 are shown. Cells were transfected with increasing amounts of TRB3 expression vector as indicated. (**B**) Inhibition of Akt kinase activity by TRB3. In vitro kinase assays were done with Akt immunoprecipitates prepared from control or pervanadate-treated HEK293 cells transfected with HA-Akt1 and either control vector (GFP), wild-type TRB3, or ΔTRB3. Kinase assays were performed with a consensus Akt peptide substrate. Treatment with sodium pervanadate (PV: 100 μM, 15 min) or vehicle is indicated. (**C**) Disrupting TRB3 expression potentiates insulin signaling through Akt in hepatocytes. Western blot assays of phospho (Ser^{473}) and total Akt levels as well as phospho (Ser^{9}) and total GSK-3β, and phospho (Ser^{256}) Foxo levels in control and insulin-treated HepG2 cells transfected with wild-type (TRB3) or mutant (Con) TRB3 RNAi oligonucleotides. Total levels of TRB3 in HepG2 cells are also shown. (**D**) Interaction of TRB3 with unphosphorylated Akt. Mammalian two-hybrid assay of 293T cells transfected with GAL4-Akt constructs and either TRB3-VP16 or control VP16 expression vector. Effect of mutating Thr^{308} in Akt to alanine or aspartate on the two-hybrid interaction is shown. Constructs expressing truncated Akt polypeptides (Δ145-240, Δ230-315, Δ315-390) are indicated. Comparable association of Akt1 (WT) and Akt2 with TRB3 is also shown.

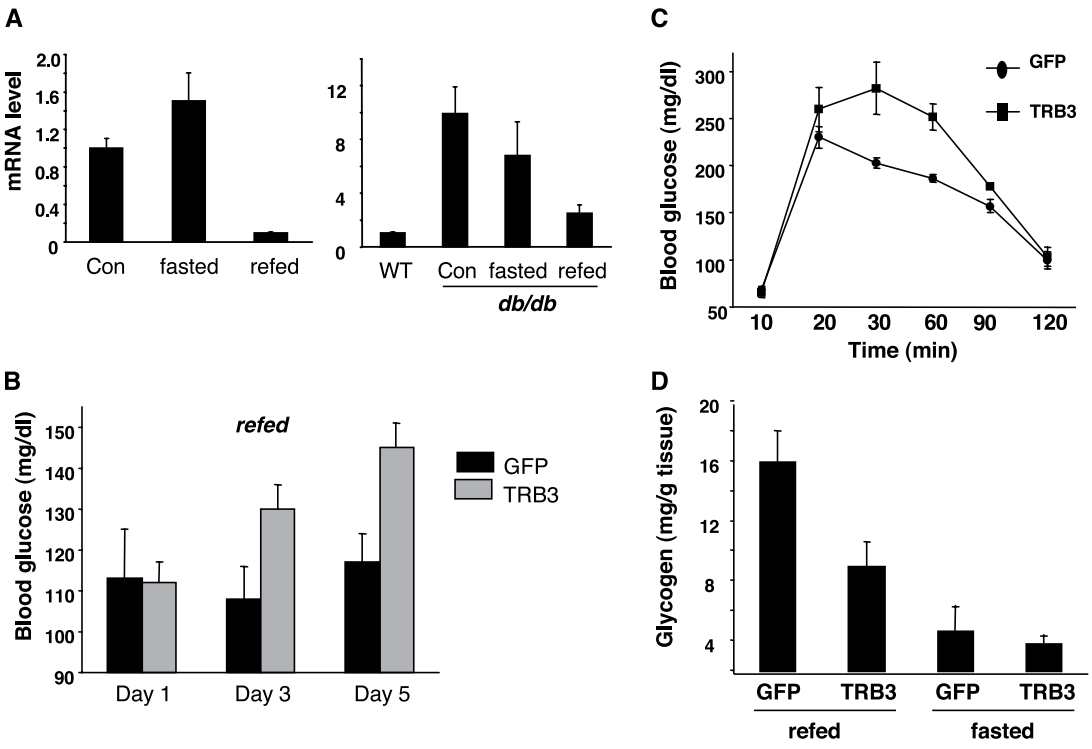

Fig. 3. Hepatic glucose output promoted by TRB3. (**A**) (Left) Quantitative PCR analysis of TRB3 transcripts in livers of control (ad libitum feeding), fasted (24 hours), or refed adult male mice ($n = 3$). Relative TRB3 expression was normalized to that of 36B4 ribosomal protein RNA in each sample. Error bars show standard errors. (Right) TRB3 hepatic RNA levels in *db/db* diabetic mice under control, fasted, or refed conditions as described above. WT, amount of TRB3 RNA in control wild-type mice is shown for comparison. (**B**) Effect of acute TRB3 overexpression in liver on glucose homeostasis. Six-week-old C57Bl6 male mice ($n = 7$ per group) were infected with control GFP or TRB3-expressing adenovirus by tail vein injection, and glucose concentrations were monitored under refed conditions at the times shown after infection. (**C**) Glucose tolerance test of mice infected with control GFP or TRB3 adenovirus. Mice were injected intraperitoneally with glucose (2 g/kg), and blood glucose concentrations were monitored at the intervals indicated. (**D**) Liver glycogen content in mice infected with control GFP or TRB3 adenovirus ($n = 4$ per group) under refed or fasted conditions.

The ΔTRB3 polypeptide did not inhibit Akt kinase activity. Thus unlike *tribbles*, which inhibits CDC25 and C/EBP in *Drosophila* by triggering ubiquitin-mediated proteolysis of these proteins (*10–12*), TRB3 appears to block Akt activity by disrupting its phosphorylation without reducing the abundance of the protein.

To determine whether the inhibitory action of TRB3 on Akt is physiologically relevant, we disrupted endogenous TRB3 expression in hepatocytes by RNA interference (RNAi). In transient transfection assays, a 21–base pair (bp) TRB3 RNA duplex oligonucleotide reduced the abundance of both endogenous TRB3 and overexpressed Flag-TRB3 protein in a dosage-dependent manner (Fig. 2C, fig. S3). RNAi-mediated knockdown of TRB3 in HepG2 cells potentiated Akt phosphorylation at Thr^{308} and Ser^{473} in response to growth factor signaling (Fig. 2C, fig. S4). Correspondingly, disruption of TRB3 expression also enhanced phosphorylation of Akt substrates such as GSK3β (Ser^{9}) and Foxo (Ser^{256}) in response to insulin and pervanadate, a potent activator of Akt (*8*). The effects of TRB3 RNAi appeared to be specific because cotransfection of a mouse TRB3 expression vector, not recognized by the human TRB3 RNAi oligonucleotide used in this study, reversed this phenotype (fig. S5).

To determine the mechanism by which TRB3 may inhibit Akt activity, we performed mammalian two-hybrid assays using TRB3-VP16 expression plasmid and mutant GAL4 Akt constructs. Relative to the wild-type protein, phosphorylation-defective T308A mutant Akt appeared to associate more efficiently with TRB3 (Fig. 2D). By contrast, substitution of Thr^{308} with Asp to mimic Thr^{308} phosphorylation strongly inhibited the interaction between TRB3 and Akt (Fig. 2D), suggesting that TRB3 preferentially binds to the unphosphorylated form of the kinase. Indeed, assays with a series of truncated Akt expression vectors revealed that amino acids 240 to 315 in Akt1 are essential for this association, indicating that TRB3 may block Akt activation by binding directly to and masking the Thr^{308} phosphorylation site (Fig. 2D). Consistent with results from coimmunoprecipitation studies, a GAL4 Akt2 construct interacted with TRB3 in a manner similar to Akt1, suggesting that TRB3 may perform a general role in regulating cellular Akt activity.

The ability of endogenous TRB3 to associate with Akt and to regulate its activity in cultured hepatocytes prompted us to evaluate TRB3 expression in mouse liver under fasting or feeding conditions. In quantitative polymerase chain reaction (PCR) assays, amounts of hepatic TRB3 RNA and protein were 10- to 20-fold higher in organs from fasted animals than in livers from animals that had been refed (Fig. 3A, left; fig S6). Amounts of TRB3 RNA and protein were also increased 10-fold in livers from *db/db* diabetic mice compared with those in livers from wild-type mice (Fig. 3A, right; fig. S6). In *db/db* mice, the change in TRB3 expression was smaller during the fasting-to-feeding transition than that in wild-type mice (Fig. 3A, right). Amounts of TRB1 and TRB2 transcripts did not vary with nutritional status (*14*). Supporting a role for counter-regulatory hormones in stimulating TRB3 expression under fasting conditions, levels of TRB3 RNA were induced two- to threefold in FAO hepatocytes after treatment with either glucocorticoids or cyclic AMP agonist (fig. S7). Taken together, these results suggest that TRB3 could inhibit Akt specifically during fasting, and that inappropriate expression of TRB3 in diabetes may contribute to insulin resistance by blocking Akt activity in the fed state. Indeed, Akt was readily detected in immunoprecipitates of TRB3 prepared from whole-liver extracts from fasted *db*/*db* mice, demonstrating that the TRB3:Akt complex is present in liver under fasting conditions (fig. S8).

To examine the role of TRB3 in glucose homeostasis in vivo, we infected adult male C57Bl6 mice ($n = 7$ per group) with TRB3 or control green fluorescent protein (GFP)

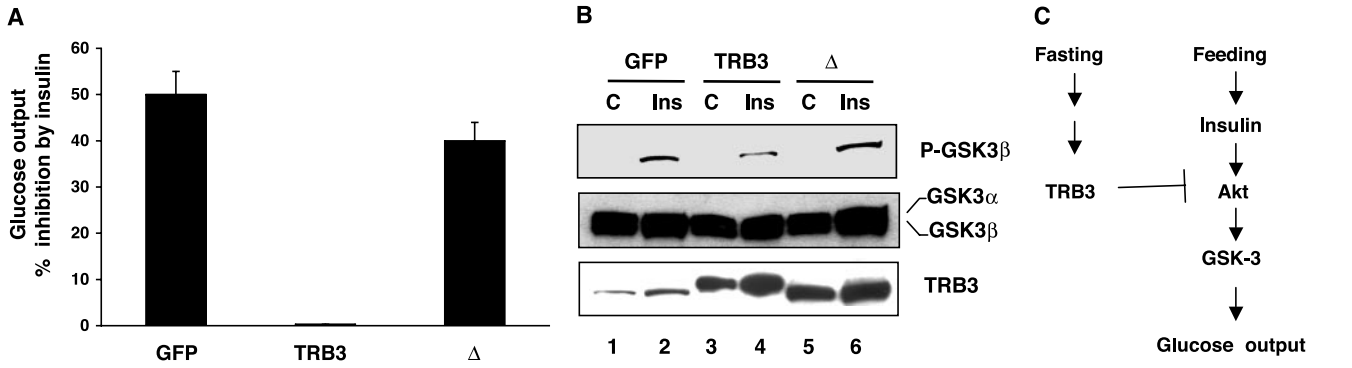

Fig. 4. Blockade of insulin action by TRB3 in cultured hepatocytes. (**A**) Glucose-output assay of FAO hepatoma cells infected with wild-type TRB3, interaction-defective ΔTRB3 (Δ), or control (GFP) adenovirus. Cells were treated with insulin (10^{-8} M) or left untreated for 6 hours. Relative inhibition of glucose output by insulin is indicated. (**B**) Western blot assay showing effect of control GFP, wild-type TRB3, and mutant ΔTRB3 adenoviruses on phosphorylation of GSK-3β at Ser^9 in FAO cells. Treatment with insulin or vehicle (C) is indicated. Total amounts of GSK-3 (α+β) and TRB3 polypeptides are shown. (**C**) Proposed model for TRB3 action in liver. TRB3 expression is induced under fasting conditions where it blocks insulin action by binding to Akt. Loss of Akt activity enhances glucose output from the liver in part by suppressing insulin-dependent phosphorylation of GSK-3 by Akt.

adenoviruses by tail vein injection and monitored blood glucose levels under fasting and refed conditions. In animals infected with TRB3 adenovirus, amounts of TRB3 RNA and protein in liver were 1.5- to 2-fold higher than those observed in *db/db* diabetic mice (fig. S9) (*14*). Expression of TRB3 had no effect on blood glucose levels in the fasted state (*14*). By contrast, concentrations of blood glucose levels were increased in the refed state (150 mg/dl versus 115 mg/dl; $n = 7$), indicating that TRB3 may interfere with acute effects of insulin on hepatic glucose release (Fig. 3B). Consistent with the notion that TRB3 contributes to insulin resistance, plasma insulin concentrations in the refed state were modestly increased in TRB3-infected mice (2.2 ng/ml) versus GFP control (1.6 ng/ml) mice (fig. S10). Indeed, TRB3-overexpressing mice displayed impaired glucose tolerance; after intraperitoneal glucose injection (2 g/kg), their blood glucose levels remained higher than those in control animals (Fig. 3C). Liver glycogen content in mice infected with TRB3 adenovirus (8 mg/g tissue; $n = 4$) was twofold lower under feeding conditions than in control mice (16 mg/g tissue; $n = 4$), suggesting that the hyperglycemic effects of TRB3 may in part reflect elevated hepatic glycogenolysis and/or reduced synthesis (Fig. 3D).

We also evaluated the role of TRB3 in insulin-dependent glucose metabolism. We used a glucose-output assay on rat FAO hepatoma cells infected with adenovirus constructs expressing either control GFP or TRB3. Glucose release from control cells infected with GFP adenovirus was inhibited 50% after treatment with insulin (10^{-8} M) (Fig. 4A). But the inhibitory effect of insulin on glucose output was almost completely blocked in cells infected with TRB3 adenovirus (Fig. 4A). An adenovirus construct expressing the ΔTRB3 polypeptide had no effect on insulin signaling.

Under physiological conditions, insulin inhibits glycogenolysis by promoting the Akt-dependent phosphorylation of GSK-3β at Ser^9. The dual importance of GSK-3 as a regulator of glucose production and as a bona fide substrate of Akt (*15–19*) prompted us to examine whether TRB3-mediated inhibition of Akt correspondingly reduced insulin-dependent phosphorylation of GSK-3. Compared with hepatocytes infected with control GFP adenovirus, cells infected with wild-type TRB3 adenovirus showed reduced insulin-dependent phosphorylation of GSK-3β on Ser^9 (Fig. 4B). But mutant ΔTRB3 adenovirus had no effect in this regard, indicating that TRB3 interferes with insulin effects on glucose output, at least in part, by blocking phosphorylation of relevant Akt substrates.

Taken together, our results suggest that TRB3 promotes glucose output from liver under fasting conditions by binding to and interfering with Akt phosphorylation in response to residual insulin signaling (Fig. 4C). Pathological overexpression of TRB3 in the fed state may therefore contribute to insulin resistance and promote hyperglycemia. Our results also provide a potential molecular explanation for the insulin resistance that has been observed under chronic fasting conditions and in response to counter-regulatory hormones like dexamethasone (*20–22*). In this regard, prolonged starvation has been shown to blunt the stimulatory effect of insulin on glycogen synthase activity (*23*). And glucocorticoids have been found to promote insulin resistance in adipocytes, in part, by reducing insulin-stimulated Akt activation (*24*). The observed expression of TRB3 in response to diabetes and fasting may explain these findings and establish TRB3 as an attractive drug target in the treatment of type II diabetes.

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Supporting Online Material

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