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Genetic Deletion of Trb3, the Mammalian *tribbles* Homolog, Displays Normal Hepatic Insulin Signaling and Glucose Homeostasis

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Running title: Normal glucose homeostasis in *Trib3*$^{-/-}$ mice

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Abstract

Trb3, a mammalian homologue of *Drosophila tribbles*, was proposed as a suppressor of Akt activity, predominantly in conditions of fasting and diabetes. Given these prior studies, we sought to determine whether Trb3 plays a major role in modulating hepatic insulin sensitivity. To answer this question, we produced mice in which a *lacZ* reporter was knocked into the locus containing the gene (*Trib3*), resulting in a *Trib3* null animal. *Trib3* expression analyses demonstrated that the *Trib3* is expressed in liver, adipose tissues, as well as heart, kidney, lung, skin, small intestine, and stomach, and denervated, but not normal, skeletal muscle. *Trib3*$^{-/-}$ mice are essentially identical to their wild type littermates in overall appearance and body composition. Phenotypic analysis of *Trib3*$^{-/-}$ mice did not detect any alteration in serum glucose, insulin, or lipid levels, glucose or insulin tolerance, or energy metabolism. Studies in *Trib3*$^{-/-}$ hepatocytes revealed normal Akt and Gsk3β phosphorylation patterns, glycogen levels, and expressions of key regulatory gluconeogenic and glycolytic genes. These data demonstrate that deletion of *Trib3* has minimal effect on insulin-induced Akt activation in hepatic tissue, and as such question any non-redundant role for Trb3 in the maintenance of glucose and energy homeostasis in mice.

Introduction

The Akt/phosphatidylinositol 3-kinase (PI3K) pathway is critically involved in the regulation of glucose and lipid metabolism in peripheral tissues, muscle and adipose tissue, as well as in regulating glucose output from the liver. This pathway is an evolutionarily conserved signaling cassette that functions in mammals to transduce survival signals in response to growth factor stimulation and nutrient availability. The binding of insulin to its tyrosine kinase receptor leads to the rapid recruitment and activation of the lipid kinase PI3K, resulting in the generation of phosphatidylinositol 3,4-bisphosphate (PtdIns(3,4)P_2) and phosphatidylinositol 3,4,5-triphosphate (PtdIns(3,4,5)P_3). The importance of this pathway in the liver to glucose homeostasis, and by inference to the hyperglycemia associated with obesity and type 2 diabetes, was recently demonstrated in studies in mice with hepatic over expression of constitutively active Akt (1). These mutant mice demonstrated reduced expression of key gluconeogenic enzymes, phosphoenolpyruvate carboxylase 1 (PEPCK) and glucose-6-phosphatase (G6P), resulting in a phenotype of marked hypoglycemia, hypoinsulinemia, hypertriglyceridemia, and subsequent hepatomegaly. As activation of this pathway also tightly regulates glycogen synthesis, via activation of glycogen synthase kinase 3 (GSK3) (2; 3), it is not surprising that hepatic constitutive activation of Akt caused a greater than 20 fold increase in hepatic glycogen content (1). These data indicate that liver Akt is a key mediator of insulin signaling in the regulation of hepatic glucose output and that as such proteins which modulate Akt activity should have significant effects on glucose homeostasis. Recent studies identified Trb3, a mammalian homologue of *Drosophila tribbles (*tribbles 3; Trb3; neuronal cell death-inducible putative protein kinase; NIPK*)*, as a binding partner for Akt by a two hybrid screening protocol (4; 5). Subsequent physiological studies proposed that Trb3 inhibits insulin signaling by directly binding to Akt and blocking its activation, predominantly in liver (4). Trb3 was shown to bind to Akt and block phosphorylation, but more importantly, it was up-regulated by fasting and diabetes and was proposed to play a major role in hepatic insulin resistance. Given these previous studies demonstrating that Trb3 is an important regulator of Akt activity, we asked whether the Trb3 protein is required for Akt modulation of glucose homeostasis. As the initial step to investigate this possibility, we generated mice with a target deletion of the gene, which encodes the Trb3 protein, *Trib3*. Here we report that insulin-induced activation of liver Akt, as well as whole body glucose and energy homeostasis, is not altered in Trb3 deficient mice.

Research Design and Methods

Target deletion of Trib3 gene in mice — Mice were generated using bacterial artificial chromosome (BAC)-based targeting vectors. In brief, we modified a BAC containing the *Trib3* coding sequence by replacing the 6.2-kb coding region, extending from the initiation to the termination codon, with the β-galactosidase (*β-gal*) reporter gene followed by a neomycin

cassette. The targeting vector was linearized and electroporated to mouse embryonic stem (ES) cells. The targeted ES cells were identified with the loss of native allele (LONA) assay as described previously (6). After germ line transmission was established, F1 heterozygous mice were bred together to generate F2 *Trib3*$^{+/+}$ (WT) and *Trib3*$^{-/-}$ mice. We performed all the experiments with the F2 generation of male mice at 8-12 weeks of age unless noted otherwise. All animal procedures were conducted in compliance with protocols approved by the Regeneron Institutional Animal Care and Use Committee. Mice were housed under 12 h of light per day in a temperature-controlled environment and had free access to standard chow diet (#5001; Purina, St Louis, MO).

Expression analysis — For Northern blot multi-tissue analysis we generated and labeled specific *Trib1*, *Trib2*, and *Trib3* probes and hybridized against pre-made mouse polyA+ RNA Northern blot membranes (Origene Technologies, Rockville, MD) as outlined previously (7). Analysis for specific deletion of *Trib3 mRNA* was conducted on liver total RNA isolated using Trizol reagent (Life Technologies, CA) taken from 8-week-old mice that were fasted for 24 h. We loaded 10 μg per lane of total RNA on a gel, transferred to a nylon membrane and probed with ^{32}P-labeled *Trib3* probe. These same filters were stripped of the probe and re-blotting for *Gapd*. To assess for any changes in expression of *Trib1*, *Trib2*, and *Trib3* mRNA in *Trib3*$^{-/-}$ mice, tissue was taken from liver, white and brown adipose tissue and skeletal muscle (gastrocnemius) and analyzed in separate reactions using the Taqman real-time PCR chemistry and detection system (Applied Biosystems, Foster City, California) with the primers pairs and labeled probes (sequences available upon request). mRNA levels were calculated from a standard curve, and normalized to a housekeeping reference (*Gapd*). Data were expressed as the mean ± SEM for 5 separate samples run in triplicate. For analysis of hepatic gene expression profile, we used Taqman real-time PCR chemistry and detection system as described above and probes as outlined previously (7). Relative mRNA levels were calculated using a standard curve, with the PCR product for each primer pair normalized to a housekeeping reference (*Gapd*), then, to the mean value of WT mice. Data were expressed as the mean ± SEM for 6 separate samples run in triplicate. To perform β-gal analysis in denervated skeletal muscle, the right sciatic nerve was isolated in the mid-thigh region and cut leading to denervation of the lower limb muscles. Two weeks later, the tibialis anterior (TA) muscle of both limbs was isolated from ad-lib fed mice under isoflurane (2-2.5%) anesthesia.

Insulin signaling analysis — Mice were fasted overnight and injected i.v. with a bolus of insulin (5 U/kg). After 2 or 30 min, mice were sacrificed and tissues collected. Liver detergent extracts were prepared in buffer containing 50 mM Hepes (pH7.4), 1% NP-40, 150 mM NaCl, 1 mM EDTA, 30 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.5 mM sodium orthovanadate, 5 μg/mL

aprotinin, 5 μg/mL leupeptin, and 1 mM PMSF. For Western blotting, equal amounts of protein (50 μg) were resolved by SDS-PAGE on 4-12% precast gels (Invitrogen, Carlsbad, CA) and transferred to nitrocellulose membranes. We probed the membranes with anti-Trb3 (EMD Biosciences, Darmstadt, Germany), Akt, phospho-specific Akt (Ser^{473}), phospho-specific Gsk3α/β ($Ser^{21/9}$) (Cell Signaling Technology, Beverly, MA), or Gsk3β (Santa Cruz Biotechnology, Santa Cruz, CA) antibodies. Bound antibodies were detected with horseradish peroxidase-coupled antibodies against rabbit or mouse IgG (Bio-Rad Laboratories, Hercules, CA) using the ECL detection system (Amersham Biosciences, Buckinghamshire, UK). Akt phosphorylation was quantitated with pre-optimized phospho-ELISA for Akt (Biosources, Camarillo, CA).

ACC protein analysis — Mice were fasted 24 h, sacrificed, and tissues collected. Preparation of protein extracts and SDS-PAGE were performed as described above. The membranes were probed with an anti-ACC1/2 (Cell Signaling Technology) antibody. Bound antibodies were detected with the system described above. We measured the intensity of the bands by scanning densitometry of the autoradiograms with NIH ImageJ software (version 1.36x; NIH).

Glycogen content assays — Mice were fasted 24 h, sacrificed, and liver collected. Liver samples were dissolved in NaOH and incubated at 65°C for 2 h. Glycogen was precipitated in ethanol and digested with amyloglucosidase (Sigma-Aldrich, St. Louis, MS). Glucose concentration was determined using the Trinder glucose assay kit (Sigma-Aldrich).

Blood analyses — Following 4 h fasting, mice were anesthetized with isoflurane. We collected orbital blood and analyzed serum for triglycerides and cholesterol with the Bayer 1650 blood chemistry analyzer (Bayer, Tarrytown, NY). Non-esterified free fatty acids (NEFA's) were analyzed with a diagnostic kit (WAKO, Richmond, VA). We measured insulin and leptin levels using ELISA (LincoPlex; Linco, St. Charles, MO). 4-h fasting blood glucose levels were measured with Accu-check (Roche Diagnostics, Nutley, NJ) without anesthesia.

Metabolic tests — For glucose tolerance tests, mice were fasted overnight and administered a bolus glucose (2 g/kg) by oral gavage. For insulin tolerance tests, mice were fasted for 4 h and injected i.p. with a bolus insulin (0.75 U/kg). For pyruvate tolerance tests, mice were fasted for 18 h and injected i.p. with a bolus sodium pyruvate (2 g/kg). We collected blood from tail vein at 0, 30, 60 and 120 min and measured glucose levels using Accu-check. Metabolic parameters were measured using an Oxymax open circuit indirect calorimetry system (Columbus Instruments International Corp, Columbus, OH) as described previously (7). Briefly, mice were individually placed in the calorimeter cages, and data collected on gas exchanges and activity for 48 h. Oxygen consumption (mL/kg) and carbon dioxide production (mL/kg) by

each animal were measured at one hour intervals. Basal metabolic rate (mL/kg/h) represents the mean oxygen consumption rate at rest during the light cycle. Respiratory quotient (RQ) was calculated as the ratio between CO_2 production rate (VCO_2) over oxygen consumption rate (VO_2). Energy expenditure (Kcal/kg/h) was calculated as the product of the caloric value of oxygen (= 3.815 + 1.232 x RQ) and the volume of consumed oxygen. We assessed body composition with the use of dual emission X ray absorption (PIXImus; Lunar, Madison, WI).

Statistical analysis — We present all values as the mean ± standard error of mean (SEM). For comparison of means, we performed unpaired nonparametric Student's T-test or ANOVA, where appropriate, using Prism software (GraphPad, San Diego, CA). We selected a threshold for statistical significance of P <0.05. When a significant F ratio was obtained with ANOVA, post hoc analysis was conducted between groups using a multiple comparison procedure with Bonferroni/Dunn post hoc comparison.

Results

Targeted disruption of the *Trib3* locus

The recently described VelociGene® technology was used to delete the entire *Trib3* coding region (Figure 1A). The correct gene targeting in F1D4 ES cells was confirmed by LONA assay (6), and mice derived from two independent ES clones were initially bred to C57Bl/6 females to generate F1 offspring. F1 heterozygote breeders were set up to generate F2 litters, and all genotypes were born at the predicted Mendelian birth ratio (Figure 1B). Cohorts of *Trib3*$^{-/-}$ mice reached all developmental milestones over the next 6-8 weeks and showed no abnormalities in growth thereafter (data not shown).

To clarify tissue specific expression pattern of *Trib3* and the other *Trib* isoforms (*Trib1* and *2*) in mice, we performed Northern blot analysis using pre-made mouse polyA+ RNA blots, which include 12 major tissues (brain, heart, kidney, liver, lung, muscle, skin, small intestine, spleen, stomach, testis, thymus) (Figure 2A). *Trib3* expression was the highest in liver and also detected in heart, kidney, lung, skin, small intestine, and stomach. In agreement with qRT-PCR data (Figure 2B), *Trib3* expression was not detected in skeletal muscle. High levels of *Trib1* expression were observed in liver and skin, whereas *Trib2* did not appear to be expressed in liver. Neither *Trib1* nor *Trib2* was expressed in skeletal muscle. These *Trib1* and *Trib2* expression patterns were in agreement with qRT-PCR data for *Trib1* and *Trib2* in insulin sensitive tissues (Figure 2C and D). The qRT-PCR analysis also revealed abundant expression of all *Trib* isoforms in white adipose tissue. *Trib1* and *Trib2* mRNA levels in insulin sensitive tissues were similar between *Trib3*$^{-/-}$ and WT mice, indicative of no compensatory induction with deletion of the *Trib3* gene. Unfortunately the insertion of the *lacZ* reporter gene into the *Trib3* locus did not give a robust or reproducible staining by whole mount or in tissue sections by β-gal staining. However when mice were fasted for 24 h, we observed *lacZ* expression in

liver, pancreas, and the intestinal tract (data not shown). No specific staining could be detected with other tissues, probably due to lower sensitivity of this method compared to qRT-PCR.

As *Trib3* expression was reported to be up-regulated in response to nutrient starvation and stress conditions (8-10), we conducted a detailed analysis of *Trib3* mRNA and protein in *Trib3*$^{-/-}$ and WT littermates under these conditions. Northern blot analysis (Figure 1C) with *Trib3* specific probes as well as Western blot analysis (Figure 1D) of liver protein extracts with Trb3 specific antibodies confirmed absence of the *Trib3* gene product. To confirm lack of *Trib3* expression in other tissues, we measured *Trib3* mRNA levels in insulin sensitive tissues (liver, white and brown adipose tissue and skeletal muscle) by qRT-PCR. Consistent with previous reports (4; 11), we detected *Trib3* expression in liver and adipose tissues but very low levels in skeletal muscle of WT animals. In *Trib3*$^{-/-}$ mice, *Trib3* mRNA was absent in all the tissues examined, confirming global deletion of *Trib3* gene (Figure 2B). To determine the effect of Trb3 loss in other settings where Akt has been shown to be important, we denervated skeletal muscle of *Trib3*$^{-/-}$ mice. Denervation surgery was performed on the right hind limb of *Trib3*$^{-/-}$ and WT mice, leaving the left hind limb unperturbed; the tibialis anterior (TA) muscle of both sides were analyzed 14 days later. The 14-day denervation caused atrophy in the right TA muscle in both groups. No difference in % atrophy was detected, despite the fact, in *Trib3*$^{-/-}$ mice, we observed strong *lacZ* expression on a sub-population of fast fibers of denervated TA muscle; we did not observe expression in non-denervated muscle (Supplementary Figure 1).

Body composition and serum chemistry of *Trib3*$^{-/-}$ mice

Trib3$^{-/-}$ mice were analyzed until 25 weeks of age and no difference in appearance, body weight, and body composition in comparison to their WT littermates was observed (Table 1). Extensive analyses of 4-h fasting whole blood and serum at 8 and 25 weeks of age showed no difference in glucose, triglycerides, cholesterol, NEFA, insulin and leptin levels between *Trib3*$^{-/-}$ and WT male mice. In addition, 24-h fasting and post-prandial blood glucose levels were normal in *Trib3*$^{-/-}$ male mice. We obtained similar results in a cohort of female mice (data not shown).

Hepatic insulin signaling in *Trib3*$^{-/-}$ mice

A potential role for Trb3 to modulate hepatic insulin signaling was suggested by a two hybrid screening experiment using Akt as a bait, which resulted in the cloning of Trb3 and differential expression studies, followed shortly by *Trib3* RNAi knockdown studies (4; 5). To determine if an increase in Akt phosphorylation in response to insulin in HepG2 cells is demonstratable in *Trib3*$^{-/-}$ mice, we injected a bolus of insulin and collected liver tissue at 2 or 30 min post intravenous injection. Western blot analysis of equal amounts of liver tissue lysates with phosphospecific antibodies to Akt (Ser^{473}) did not detect any basally phosphorylated Akt in either group (Figure 3A). At both 2 min and 30 min after an insulin injection, Akt activation

was equal for *Trib3*$^{-/-}$ and WT mice. Analysis by ELISA to measure levels of insulin-induced Akt phosphorylation at Thr^{308} also failed to find any difference between *Trib3*$^{-/-}$ and WT liver (Figure 3B). Previously reported *in vitro Trib3* knockdown experiments (4) demonstrate an increase in insulin-induced phosphorylation of Gsk3β, an Akt substrate. Analysis of specific Gsk3β phosphorylation using phosphospecific antibodies to Gsk3β (Ser^{9}) and Gsk3α (Ser^{21}) found no differences in insulin-induced Gsk3β phosphorylation between *Trib3*$^{-/-}$ and WT liver. These data suggest that complete deletion of *Trib3* does not effect insulin-induced activation of Akt or specific downstream substrates *in vivo*.

Glucose metabolism in *Trib3*$^{-/-}$ mice

Previous studies of *Trib3* expression pattern and in particular those where *Trib3* is over-expressed, showed a significant impairment in glucose tolerance and a decrease in liver glycogen content (5). This would suggest a role for Trb3 in impairing hepatic glucose regulation. Elevated hepatic glucose production is known to be a major factor in the hyperglycemia associated with type 2 diabetes. Analysis of glucose and insulin levels in both fasting and fed condition did not reveal any difference in these parameters between genotypes (Table 1 and data not shown). In order to uncover functions of Trb3 in liver that could not be detected by insulin signaling studies, we stressed mice by 24-h fasting, sacrificed animals, and harvested the liver. Analysis of specific genes involved in hepatic glucose production (*Pepck*, *G6p, Pgc1α* and *Glucokinase*; Figure 4A) by qRT-PCR failed to detect any differential expression pattern that would be indicative of an alteration in gluconeogenesis. 24-fasted liver glycogen content was also identical between genotypes (Figure 4B). Pyruvate is a precursor for gluconeogenesis, and pyruvate tolerance tests have been used by others to detect subtle abnormalities in hepatic glucose production (12). Glucose response of mice challenged by the bolus administration of pyruvate (2 g/kg) was indistinguishable between *Trib3*$^{-/-}$ and WT mice (Figure 4C). Furthermore, in response to the bolus administration of glucose (2 g/kg) or insulin (0.75 U/kg), *Trib3*$^{-/-}$ mice showed normal glucose homeostasis and insulin sensitivity (Figure 5A and B). No compensatory changes were observed in the counter regulatory hormone glucagon. These physiological results suggest normal hepatic insulin sensitivity, and that Trb3 does not play a pivotal role in the maintenance of glucose homeostasis in mice.

Lipid metabolism in *Trib3*$^{-/-}$ mice

Qi et al. recently reported that Trb3 mediates degradation of Acetyl CoA Carboxylase (ACC) in adipose tissue by promoting interactions of E3 ubiquitin ligase COP1 to ACC (11). ACC is an enzyme, which catalyzes the formation of malonyl-CoA from acetyl-CoA, therefore, serving as a critical regulator of fat storage and utilization. To examine if ACC levels were altered, we conducted a Western blot analysis on tissues from *Trib3*$^{-/-}$ and WT mice using an anti-ACC1/2 antibody. In all tissues analyzed (WAT, BAT, liver and skeletal

muscle), we could not detect any change in ACC protein level (Figure 3C). Additionally, expression levels of genes known to be involved in the regulation of fatty acid oxidation (*Ppar*α, *Pgc1*α and *Ucp1*) were also unaltered in *Trib3*$^{-/-}$ mice (data not shown). Combined with the physiological data — normal fat mass and serum lipid levels (Table 1) — these results strongly suggest normal lipid metabolism in *Trib3*$^{-/-}$ mice.

Energy metabolism in *Trib3*$^{-/-}$ mice

Roles of Trb3 in the control of energy homeostasis have not been well understood. Therefore, we examined metabolic parameters of *Trib3*$^{-/-}$ mice with the use of indirect calorimetry. We placed mice in individual metabolic chambers for 48 h and monitored gas exchange, activity, and consumptions of food and water. Basal metabolic rates or resting oxygen consumption did not differ between *Trib3*$^{-/-}$ and WT mice (Figure 5C). Respiratory quotient (RQ) provides a measure of fuel substrate preference (carbohydrate vs. lipid), and an RQ of greater than 0.9 indicates oxidization of carbohydrate to fulfill energy needs. *Trib3*$^{-/-}$ and WT mice displayed indistinguishable RQ (Figure 5D). Energy expenditure or heat production was also similar between *Trib3*$^{-/-}$ and WT mice (Figure 5E). Physical activity levels of *Trib3*$^{-/-}$ mice were ~47% higher than those of WT mice, however, the difference did not reach statistical significance (P =0.063) (Figure 5F). We also monitored water and food intake and found no difference between *Trib3*$^{-/-}$ and WT mice (data not shown). These data indicate that Trb3 does not have a major impact on energy homeostasis in mice.

Discussion

The PI3K/Akt pathway primarily mediates insulin's anabolic effects on the synthesis and storage of proteins, carbohydrates and lipids. As resistance to insulin's effects is one of the earliest hallmarks of type 2 diabetes, the identification and potential inhibition of negative regulators of this pathway is of great therapeutic interest. In this respect genetic deletion studies in rodents have been able to provide in-depth insight into the signaling intermediates and the involvement of specific physiological process, particularly with complex hormones such as insulin. From detailed *in vivo* and biochemical studies outlined here on Trb3 deficient mice, however, we could find no evidence to support the theory that Trb3 plays a major role in regulating hepatic insulin sensitivity and glucose homeostasis.

Trb3 was initially proposed to negatively regulate Akt activity and insulin action (4) and as such an extensive tissue specific expression pattern analysis was of great interest. *Trib3* expression analysis by Northern blotting and qRT-PCR revealed its expression in liver, adipose tissues as well as heart, kidney, lung, skin, small intestine, and stomach. We also performed the analysis utilizing the β-gal reporter gene placed in the endogenous *Trib3* locus. In skeletal muscle, *lacZ* staining was absent in tissues from fasted *Trib3*$^{-/-}$ mice but present in denervated TA fibers. These results indicate the possibility that Trb3 is induced by distinct stresses in different tissues. The data

also demonstrate the surprising finding that Trb3 is not universally expressed even in a discrete cell type, under a particular condition. This finding helps to suggest that Trb3 is not an obligate regulator of the Akt signaling pathway and leaves open the possibility that Trb3 serves a role in particular cells. However, when global homeostasis is assessed, whatever discrete role Trb3 might play was shown to be inadequate to induce a measurable global perturbation.

Insulin's actions on the hepatic glucose production are primarily mediated by PI3K, which in turn activates several downstream targets such as Akt and atypical forms of protein kinase C (PKC). Akt activation has been documented to suppress gluconeogenesis, predominantly through FoxO1 regulated expression of G6P (14; 15), as well Gsk3β activation of glycogen synthesis (16). Recent tissue specific deletion studies have elegantly demonstrated that PI3K pathway activation of PKC λ/ξ isoforms is more important for regulating insulin's actions on lipid metabolism (17), and as such, specific inhibitors of Akt, such as Trb3, may be confined to modulating glucose metabolism. Previously reported *in vitro* studies in HepG2 cells with RNAi knockdown of *Trib3* demonstrated an enhanced insulin-induced Akt and Gsk3β phosphorylation (4). Our *in vivo* analysis of hepatic Akt and Gsk3β activation in *Trib3*$^{-/-}$ mice after insulin stimulation however is not consistent with those findings. We found no increases in levels of phosphorylation at two major phosphorylation sites of Akt (Ser^{473} and Thr^{308}) and phosphorylation sites of Gsk3α and β (Ser^{21} and Ser^{9}, respectively) in the liver of *Trib3*$^{-/-}$ mice. It should also be noted that other *in vitro* studies in rat primary hepatocyte cultures with Trb3 over-expression also could not demonstrate a decrease in insulin-induced phosphorylation of Akt and its substrates, Gsk3α/β and 4e-bp1 (18).

Consistent with the unchanged Akt activation in response to insulin are our physiological findings in *Trib3*$^{-/-}$ mice. Fed or fasted levels of serum glucose and insulin levels were similar for each genotype. No alteration could also be found in a glucose, insulin, or pyruvate tolerance test in *Trib3*$^{-/-}$ mice, and no evidence could be found for altered secretion of insulin in response to a glucose bolus. Once again these results are in conflict with the report by Koo et al., where knockdown of hepatic *Trib3* expression improved glucose tolerance in mice (5). It is possible that the discrepancies may have arisen from differences in the model and test conditions, such as durations and sites of Trb3 inactivation; for example *Trib3*$^{-/-}$ mice lacked Trb3 globally from gestation, whereas hepatic *Trib3* knockdown studies are of short duration, in mice after the age of 7 weeks. Alternatively, reduction of Trb3 solely in liver may improve glucose tolerance. These are however relatively ineffectual answers given the data from similar genetic deletion studies of other intermediates in or regulators of the insulin-signaling pathway.

Many genetic studies have illustrated the direct action of insulin on liver physiological function, particularly its critical role in the control of gluconeogenesis. Liver specific deletion of the insulin receptor (LIRKO) yields mice that develop marked insulin resistance and glucose

intolerance, accompanied by a failure of insulin to suppress hepatic glucose production and to regulate hepatic gene expression (19). Conversely, hepatic over-expression of constitutively active Akt induces a marked hypoglycemia, hypoinsulinemia and hypertriglyceridemia and concomitant changes in gluconeogenic genes (1). More pertinent to this discussion are results from the ablation of the phosphoinositide phosphatases, SHIP2 and PTEN, both negative regulators of the PI3K pathway. Although SHIP2 deficient mice do not display changes in basal or fasted glucose, insulin or tolerance tests they do show significant changes in energy expenditure, enhanced insulin stimulated Akt activity in liver and reduced circulating triglycerides, NEFA and cholesterol (20). While little physiological information is available from the global deletion of PTEN as it is embryonically lethal, hepatic specific deletion studies suggest a significant role in insulin signaling and metabolic regulation (21). In particular, the hepatic gluconeogenic enzymes, G6P and PEPCK, were significantly reduced and this correlated with liver glycogen levels and Gsk3β activity. None of the above parameters were modified in Trb3 deficient mice.

In summary, by global deletion of *Trib3* gene in mice, this study directly asked the question as to whether inhibition of Trb3 would alter glucose or energy homeostasis *in vivo*. Although *Trib3* expression was observed in liver at nutrient starvation as reported by others, whole-body ablation of Trb3 did not affect liver insulin sensitivity or overall metabolism. Based on these results, we conclude that loss of Trb3 is not sufficient to alter the maintenance of glucose and energy homeostasis in mice, and cast doubt on the use of Trb3 as a therapeutic target for type 2 diabetes. It is still possible that Trb3 is important in other contexts, such as more prolonged or different stress conditions. Challenge studies, such as high-fat feeding and induction of hypoxia, as well as crossing of *Trib3*$^{-/-}$ mice with other gene knockout or disease models would be necessary to unveil these possible roles of Trb3.

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Table 1. Serum chemistry & body composition parameters of 2-month-old male WT and *Trib3*$^{-/-}$ mice

Genotype	WT (n=8)	*Trib3*$^{-/-}$ (n=10)
Glucose (mg/dL)	142 ± 9	150 ± 8
Triglycerides (mg/dL)	75 ± 4	71 ± 3
Cholesterol (mg/dL)	82 ± 4	74 ± 2
NEFA (mEQ/L)	0.58 ± 0.02	0.57 ± 0.02
Insulin (ng/mL)	0.43 ± 0.10	0.40 ± 0.08
Leptin (ng/mL)	0.92 ± 0.08	0.87 ± 0.04
Body weight (g)	23.8 ± 0.7	22.1 ± 0.7
% Fat free mass	73.9 ± 0.7	73.5 ± 0.7
% Fat mass	18.7 ± 0.4	18.7 ± 0.5

Data are means ± SEM. *P* values were calculated by Student's T-test. None of the data were significantly different between WT and *Trib3*$^{-/-}$ mice. For serum chemistry, retro-orbital blood was collected after 4-h fasting under isoflurane (2-2.5%) anesthesia. 4-h fasting glucose levels were measured with tail-bleed without anesthesia.

Figures can be found in an online appendix at http://dx.doi.org/10.2337/db06-1448.

Figure 1. Generation of *Trib3*$^{-/-}$ mice. (A) Schematic diagram of the murine wild-type *Trib3* allele, and the targeting vector used to generate a null *Trib3* allele by precise substitution of the *lacZ* reporter gene as well as a neo selectable marker. Yellow boxes: homology boxes; red boxes: coding exons. **(B)** PCR assays with probes for *Trib3* or inserted *lacZ*, as described in the methods section, distinguish and identify the wild-type and null *Trib3* alleles found in WT (*Trib3*$^{+/+}$), Het (*Trib3*$^{+/-}$) and KO (*Trib3*$^{-/-}$) mice. **(C)** Northern blot analysis of total RNA and **(D)** Western blot analysis of total protein lysates, both prepared from liver tissue of mice fasted for 24 h. *Trib3* probe detects the 1970-bp fragment, and Trb3 antibody a 45kDa single protein band in WT but not in the *Trib3*$^{-/-}$ mice.

Figure 2. Analysis of *Trib1, 2 and 3* expression patterns. (A) Northern blot analysis of *Trib1, 2* and *3* expressions with mouse multi-tissue panel. qRT-PCR analysis of **(B)** *Trib3*, **(C)** *Trib1*, and **(D)** *Trib2* expression in liver, white adipose tissue (WAT), brown adipose tissue (BAT), and gastrocnemius muscle (GAST) in WT and *Trib3*$^{-/-}$ mice. Each data represents the mean ± SEM of n = 5 animals and was normalized to a housekeeping reference (*Gapd*). *Trib3* expression was not detected in any of these tissues in *Trib3*$^{-/-}$ mice.

Figure 3. Hepatic insulin sensitivity and lipid metabolism in *Trib3*$^{-/-}$ mice. (A-B) *Trib3*$^{-/-}$ and WT mice were anesthetized, and administered an i.v. bolus of insulin (5U/kg) or vehicle (-). Separate cohorts were sacrificed 2 or 30 minutes after injection from which tissues were removed and frozen in liquid nitrogen. After homogenization, equal amounts of liver lysates were assessed for activation of the downstream signaling molecule Akt and Gsk3 by SDS-PAGE with total and phospho-specific antibodies **(A)** and ELISA for total and phosphorylated Akt **(B)**. No difference could be observed in activation of Akt or Gsk3 at 2 or 30 minutes in either genotype. The data point represents the mean ± SEM of n = 6-8 animals and all measures were taken from mice between 10-12 weeks of age on a chow diet. **(C)** ACC protein levels in white adipose tissue (WAT), brown adipose tissue (BAT), liver and gastrocnemius muscle (GAST) were indistinguishable between *Trib3*$^{-/-}$ and WT mice. 24-h fasted animals (n = 3) were sacrificed to collect and snap-freeze tissues. After homogenization, equal amounts of lysates were assessed for ACC protein levels by Western blotting with an anti-ACC1/2 antibody. Representative blots are shown (left panel). The bar graphs represent mean ± SEM of arbitrary densitometric units (right panel).

Figure 4. Liver glucose metabolism in *Trib3*$^{-/-}$ mice. (A) Analysis of 24-h fasted liver gene expression. mRNA levels of genes involved in glucose metabolism, Glucose-6-phosphatase (G6p), Phosphoenolpyruvate carboxykinase (Pepck), Glucokinase, Fatty acid synthase (FAS), and peroxisome proliferator–activated receptor coactivator-1α (Pgc1α), and the forkhead transcription factor FoxO1, were measured by qRT-PCR. Each data represents

the mean ± SEM of *n* = 6 animals. Data was normalized to a housekeeping reference (*Gapd*), then to the mean WT value. None of the values were significantly different between *Trib3*$^{-/-}$ and WT mice. **(B)** Liver glycogen content after 24-h fasting was identical between genotypes. The determinations were performed in duplicate and expressed as means ± SEM (mg/mL glucose per mg protein). (C) i.p. pyruvate tolerance tests were performed after 18 h fast with 2g of sodium pyruvate per kg of body weight in in *Trib3*$^{-/-}$ and WT animals.

Figure 5 Glucose homeostasis of control and *Trib3*$^{-/-}$ mice. (A) Oral glucose tolerance tests (2g glucose/kg body weight) were performed after an overnight fast **(B**) and i.p. insulin tolerance tests in 4 h-fasted mice were performed with 0.75U of insulin per kg of body weight in *Trib3*$^{-/-}$ and WT animals. Indirect calorimetry failed to show any difference for each genotype in **(C)** Basal metabolic rate (BMR; calculated from the oxygen consumption, mL/kg/h), **(D)** Respiratory quotient (VCO_2/VO_2), **(E)** Energy Expenditure (EE; Kcal/kg body weight/h) or **(F)** Activity (counts/h). Each data point represents the mean ± SEM of *n* = 6-8 animals. All measures were taken from mice between 9-12 weeks of age on a chow diet.

Suppl. Figure 1. Analysis of the reporter gene *lacZ* in the *Trib3*$^{-/-}$ loci. *lacZ* expression was detected in denervated fibers of tibialis anterior but not in unperturbed fibers. H&E (left) and β-gal (right). 10X.