Hepatocyte TRAF3 promotes insulin resistance and type 2 diabetes in mice with obesity

Zheng Chen1,3, Mark J. Canet1, Liang Sheng1, Lin Jiang1, Yi Xiong1, Lei Yin1, Liangyou Rui1,2,*.

ABSTRACT

Objective: Metabolic inflammation is believed to promote insulin resistance and type 2 diabetes progression in obesity. TRAF3, a cytoplasmic signaling protein, has been known to mediate/modulate cytokine signaling in immune cells. The goal is to define the metabolic function of hepatic TRAF3 in the setting of obesity.

Methods: Hepatocyte-specific TRAF3 knockout mice were generated using the loxp/albumin-cre system. Liver TRAF3 was deleted in adult mice via Cre adenoviral infection. Both high fat diet-induced and genetic obesity were examined. TRAF3 levels and insulin signaling were measured by immunoblotting. Hepatocyte-specific deletion of TRAF3 decreased hepatic insulin sensitivity, insulin resistance, glucose intolerance, and hepatic steatosis in mice with either high fat diet-induced obesity or genetic obesity (ob/ob); conversely, in lean mice, adenovirus-mediated overexpression of TRAF3 in the liver induced hyperinsulinemia, insulin resistance, and glucose intolerance. Deletion of TRAF3 enhanced the ability of insulin to stimulate phosphorylation of Akt in hepatocytes, whereas overexpression of TRAF3 suppressed insulin signaling.

Conclusions: Glucose increases the levels of hepatic TRAF3. TRAF3 in turn promotes hyperglycemia through increasing hepatic glucose production, thus forming a glucose-TRAF3 reinforcement loop in the liver. This positive feedback loop may drive the progression of type 2 diabetes and nonalcoholic fatty liver disease in obesity.

Keywords TRAF3; Obesity; Insulin; Liver; Inflammation; Diabetes; Gluconeogenesis

1. INTRODUCTION

Obesity is a primary risk factor for the development of insulin resistance, type 2 diabetes, and cardiovascular diseases [1]. Prevalence rates of obesity and obesity-associated metabolic diseases are increasing rapidly, posing a huge challenge to the society. Obesity is associated with chronic, low grade inflammation in metabolically active tissues, particularly in adipose tissue and the liver [2–5]. The liver produces glucose from glycogen, pyruvate, lactate, and amino acids through hepatic gluconeogenesis. During periods of fasting and starvation, glucose generated by the liver serves as a critical metabolic fuel for extrahepatic tissues, particularly the brain and red blood cells [6]. In the postprandial states, food-derived glucose stimulates pancreatic β cells to secret insulin which suppresses hepatic gluconeogenesis and hepatic glucose production, thus preventing hyperglycemia [6]. Obesity is associated with hepatic insulin resistance and nonalcoholic fatty liver disease (NAFLD) [7]. Liver insulin resistance contributes to increased hepatic glucose production, hyperglycemia, and glucose intolerance in obese states [8]. Numerous pro-inflammatory mediators, including various cytokines, damage-associated molecular patterns (DAMPs), and pathogen-associated molecular patterns (PAMPs), are believed to promote insulin resistance in obesity [2–8,9]; however, the intracellular signaling pathways, which link these factors to insulin resistance, remain largely unknown.

We recently identified TNF receptor-associated factor 3 (TRAF3) in myeloid cells as an important mediator of high fat diet (HFD)-induced insulin resistance in mice [10]. Myeloid cell-specific deletion of TRAF3 attenuates insulin resistance and hepatic steatosis in mice with either HFD-induced obesity or genetic obesity (ob/ob) [10]. TRAF3 is a ubiquitously expressed cytoplasmic adapter protein which mediates/modulates cytokine, DAMP, and PAMP signaling [11–16]. In B lymphocytes and myeloid cells, TRAF3 inhibits NF-κB-inducing kinase (NIK) and the NIK-activated noncanonical NF-κB pathway by...
promoting ubiquitination and degradation of NIK [17]. A subset of cytokines stimulates ubiquitination and degradation of TRAF3, thus activating NIK and the noncanonical NF-κB pathway [11]. In myeloid cells, TRAF3 also binds to and activates TBK1 and IKK in response to cytokines, DAMPs, and/or PAMPs; TBK1/IKK in turn activate the IRF3/ IFN-β pathways, thus stimulating the expression of type 1 interferons and IL-10 [12–14]. Furthermore, TRAF3 is able to inhibit the canonical NF-κB and the MAPK pathways under specific cellular contexts [18–23]. In immune cells, TRAF3 is able to regulate the expression and secretion of either pro-inflammatory or anti-inflammatory cytokines, depending on body weight and adiposity [10]. However, the function of TRAF3 in metabolic tissues remains unclear.

In this study, we demonstrate that TRAF3 levels in the liver are aberrantly higher in obese mice, presumably due to hyperglycemia. We generated and metabolically characterized mice with hepatocyte-specific deletion of TRAF3. Hepatocyte-specific TRAF3 knockout mice are protected against obesity-associated insulin resistance, glucose intolerance, and hepatic steatosis. We defined a glucose-TRAF3 reinforcement loop in the liver and examined its contribution to the progression of type 2 diabetes and NAFLD in obesity.

2. MATERIAL AND METHODS

2.1. Mice

Animal experiments were conducted following the protocols approved by the University Committee on the Use and Care of Animals (UCUCA). TRAF3f/f mice were provided by Dr. Robert Brink (Garvan Institute of Medical Research, Australia). TRAF3f/f mice were crossed with albumin-cre mice (Jackson laboratory, Bar Harbor, ME) to obtain HKO mice (in C57BL/6 genetic background). HKO mice were crossed with ob/ob mice (Jackson laboratory, Bar Harbor, ME) to obtain DKO mice. Mice were housed on a 12 h light–dark cycle in the Unit for Laboratory Animal Medicine at the University of Michigan, and fed ad libitum either a normal chow diet (9% fat in calories; TestDiet, St. Louis, MO) or a HFD (60% fat in calories; Research Diets, New Brunswick, NJ).

2.2. Adenoviral infection and streptozotocin (STZ) treatments

TRAF3f/f and ob/ob;TRAF3f/f male mice were infected with GFP or Cre adenoviruses (1 × 1011 viral particles/mouse) via tail vein injection. WT C57BL/6 male mice were infected with TRAF3 or GFP adenoviruses at 3.8 × 1011 particles/mouse. STZ was dissolved in 0.1 M citrate buffer (pH 4.5). WT C57BL/6 male mice (10 weeks) were intravenously injected with STZ at 100 mg/kg body weight, and then at 80 mg/kg one week later. The liver was harvested 4 weeks after STZ injection.

2.3. Blood chemistry and glucose (GTT), insulin (ITT), pyruvate (PTT), and lactate (LTT) tolerance tests

Blood samples were collected from tail veins. Plasma insulin was measured using mouse insulin ELISA kits (CRYSTAL CHEM, Downers Grove, IL). For GTTs, PTTs, and LTTs, mice were fasted overnight (Figures 2–4) or 6 h (Figure 7) and intraperitoneally injected with a glucose, pyruvate, or lactate. For ITTs, mice were fasted for 6 h and intraperitoneally injected with human insulin. Blood glucose was measured 0, 15, 30, 60, and 120 min after injection.

2.4. Immunoprecipitation, immunoblotting, and staining

Tissues or cells were homogenized in a buffer (50 mM Tris HCl, pH 7.5, 1.0% NP-40, 150 mM NaCl, 2 mM EGTA, 1 mM Na2VO4, 100 mM NaF, 10 mM Na2HPO4, 1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin). Tissue/cell extracts were immunoblotted with antibodies against TRAF3 (Santa Cruz, sc-947; Santa Cruz, sc-949), tubulin (Santa Cruz, sc-5286), phospho-Akt pThr308 (Cell Signaling, #4056; Santa Cruz, sc-16646-R), phospho-Akt pSer473 (Cell Signaling, #4060), or Akt (Cell signaling, #4961). Liver paraffin sections were subjected to H&E staining. Liver frozen sections were stained with Oil red O (0.5% in propylene glycol) & Hematoxylin (0.6% weight).

2.5. Liver triacylglycerol (TAG) levels

Liver samples were homogenized in 1% acetic acid, and lipids were extracted using 80% chloroform/Methanol (2:1). The organic fractions were dried in chemical hood, resuspended in 3 M KOH, incubated at 70 °C for 1 h, mixed with MgCl2 (0.75 M), and centrifuged. The aqueous fractions were used to measure TAG levels using Free Glycerol Reagent (Sigma).

2.6. Primary hepatocyte cultures

Primary hepatocyte cultures were prepared by liver-perfusion with type II collagenase (Worthington Biochem, Lakewood, NJ) and grown on collagen-coated plates as described previously [24]. For glucose stimulation experiments, hepatocytes were incubated overnight in serum-free DMEM supplemented with 0, 25, or 100 mM glucose.

2.7. Quantitative real time RT-PCR (qPCR)

Total RNAs were extracted using TRIzol reagent (Invitrogen life technologies, Carlsbad, CA). The first-strand cDNAs were synthesized using random primers and M-MLV reverse transcriptase (Promega, Madison, WI). qPCR was performed using Absolute QPCR SYBR Mix (Thermo Fisher Scientific, UK) and Mx3000P real time PCR system (Stratagene, La Jolla, CA). qPCR primers were: TRAF3 forward: 5′-TGAGCTGGAAAGCAGTAACA-3′, reverse: 5′-AGATCGACACCCCGTG-TAG-3′; IL-1β forward: 5′-GGCTTTGGCCTCAAGGAAGATC-3′, reverse: 5′-GGAGAAGCAGAATCTGATGAG-3′; IL-6 forward: 5′-ACGCGAGCTTCTACA-3′, reverse: 5′-GGTCTTACACTCACT-3′; TNFz forward: 5′-CATCCTTCTCAAAGCTGATGCAAA-3′, reverse: 5′-TGGAAGAACACATACTGCTACCCG-3′, reverse: 5′-GCGCATTACCTACCCGAGGAAAGC-3′.

2.8. Statistical analysis

Data were presented as means ± SEM. Differences between groups were analyzed by two-tailed Student’s t tests. P < 0.05 was considered statistically significant.

3. RESULTS

3.1. TRAF3 levels in the liver are regulated by the metabolic state and are aberrantly higher in obese mice

To determine whether the metabolic state affects the expression of TRAF3 in the liver, male mice were fasted for 24 h and refed a normal chow diet for 3 h. The levels of TRAF3 mRNA and protein in the liver were measured by qPCR and immunoblotting, respectively. Fasting (24 h) lowered the levels of both TRAF3 mRNA and protein, and refeeding largely reversed fasting-induced reduction of TRAF3 in the liver (Figure 1A–B). Liver TRAF3 levels appear to be regulated by nutritional signals.

To determine whether overnutrition increases TRAF3 levels in the liver, C57BL/6 male mice were fed a HFD for 10 weeks, and liver TRAF3 protein levels were measured by immunoblotting. Mice fed a HFD...
Figure 1: TRAF3 levels in the liver are aberrantly higher in obese mice. A. C57BL/6 male mice (8–9 weeks) were fasted for 24 h and then refed for 3 h. Liver TRAF3 mRNA abundance was measured by qPCR and normalized to β-actin expression. Randomly-fed: n = 8, fasted: n = 8, refed: n = 8. B. C57BL/6 male mice were fasted and refed as described above. Liver extracts were immunoblotted with antibodies against TRAF3 or tubulin. C. C57BL/6 male mice (7–8 weeks) were fed a chow diet or HFD for 10 weeks. WT and ob/ob mice (13 weeks) were fed a chow diet. Liver extracts were immunoblotted with antibodies against TRAF3 or tubulin. D. C57BL/6 male mice (10 weeks) were injected with STZ. Liver extracts were prepared 4 weeks after STZ treatments and immunoblotted with antibodies against TRAF3 or tubulin. E. Primary hepatocytes were prepared from C57BL6 mice (8–9 weeks) and grown overnight in serum-free DMEM supplemented with 0, 25 or 100 mM G-glucose. Cell extracts were immunoblotted with the indicated antibodies. *p < 0.05.

3.2. Deletion of liver TRAF3 in adult obese mice attenuates insulin resistance, hyperglycemia, and glucose intolerance

To determine whether aberrant levels of hepatic TRAF3 contribute to metabolic disorders in obesity, we generated and metabolically characterized mice with liver-specific deletion of TRAF3. TRAF3−/− mice, in which the TRAF3 gene is flanked by two loxp sites, have been described previously [10,26]. TRAF3−/− male mice were fed a HFD for 8 weeks to induce obesity. To delete TRAF3 specifically in the liver, obese TRAF3−/− mice were infected with Cre adenoviruses via tail vein injection, and green fluorescent protein (GFP) adenoviruses were used as control. Cre adenoviral infection is commonly used to generate liver-specific deletion of target genes [27]. Liver TRAF3 protein levels were ~81% lower in the Cre group than in the GFP group (Figure 2A). Body weight was similar between the GFP (30.3 ± 1.2 g, n = 12) and the Cre (29.8 ± 0.9 g, n = 11) groups. Both overnight fasting blood glucose and insulin levels were significantly lower in the Cre group (Figure 2B). To further analyze insulin sensitivity and glucose metabolism, we performed glucose tolerance (GTT), insulin tolerance (ITT), and pyruvate tolerance (PTT) tests on mice 10–21 days after infection. Liver-specific deletion of TRAF3 significantly improved glucose intolerance and insulin resistance (Figure 2C–D) and reduced hepatic gluconeogenesis in these obese mice (Figure 2E). Blood glucose increased 15 min after insulin injection (Figure 2F), presumably due to a combination of insulin resistance and stress response to injection. To determine whether acute deletion of liver TRAF3 similarly improves insulin resistance and glucose intolerance in mice with genetic obesity, TRA3−/− mice were crossed with ob/ob mice to generate ob/ob;TRAF3−/− double mutant mice. Adult ob/ob;TRAF3−/− male mice (8–9 weeks) were infected with Cre adenoviruses via tail vein injection to delete
TRAF3 specifically in the liver. Deletion of liver TRAF3 significantly decreased overnight fasting blood glucose and insulin (Figure 2F), and markedly improved glucose intolerance and insulin resistance (Figure 2G–H) in ob/ob;TRAF3f/f mice. Together, these data indicate that inhibition of liver TRAF3 is sufficient to improve insulin resistance and glucose intolerance in both dietary and genetic obesity.

3.3. Embryonic onset, hepatocyte-specific deletion of TRAF3 protects against obesity-associated insulin resistance and glucose intolerance

To determine whether TRAF3 deficiency in hepatocytes is able to prevent obesity-induced insulin resistance, hepatocyte-specific TRAF3 knockout (HKO) mice were generated by crossing TRAF3f/f mice with albumin-Cre transgenic mice. TRAF3 was deleted specifically in the liver but not in skeletal muscle and adipose tissue (Supplemental Fig. 1). Blood glucose, plasma insulin, GTT, and ITT were similar between HKO and TRAF3f/f (control) mice fed a normal chow diet (data not shown), indicating that hepatocyte TRAF3 is dispensable under normal conditions. HKO and TRAF3f/f male mice (7~8 weeks) were fed a HFD, and body weight was similar between these two groups (Figure 3A). Overnight fasting insulin levels were significantly lower in HKO mice fed a HFD for 10 weeks (Figure 3B). Obese HKO mice displayed improvement in glucose intolerance and insulin resistance relative to TRAF3f/f mice (Figure 3C–D).

To determine whether embryonic onset, hepatocyte-specific deletion of TRAF3 also prevents insulin resistance and glucose intolerance in ob/ob mice, HKO mice were crossed with ob/+ mice to generate leptin/TRAF3 double mutant mice (DKO) (genotype: ob/ob;TRAF3f/f;albumin-Cre+). ob/ob;TRAF3f/f (designated as ob/ob) mice were used as control. Body weight was comparable between DKO and ob/ob mice (Figure 4A), but overnight fasting blood glucose and insulin levels were significantly lower in DKO male mice (Figure 4B). Deletion of hepatic
TRAF3 is involved in NAFLD development, TRAF3f/f male mice (7–8 weeks) were fed a HFD. A. Growth curves. B. Overnight-fasted blood glucose and insulin. C. GTTs (glucose: 2 g/kg body weight). D. ITTs (insulin: 1 unit/kg). E–D: mice fed a HFD for 10–12 weeks. *p < 0.05.

Figure 3: Embryonic onset, hepatocyte-specific deletion of TRAF3 prevents HFD-induced insulin resistance and glucose intolerance. HKO (n = 11) and TRAF3f/f (n = 11–12) male mice (7–8 weeks) were fed a HFD. A. Growth curves. B. Overnight-fasted blood glucose and insulin. C. GTTs (glucose: 2 g/kg body weight). D. ITTs (insulin: 1 unit/kg). E–D: mice fed a HFD for 10–12 weeks. *p < 0.05.

TRAF3 markedly improved glucose tolerance and insulin resistance in DKO male mice (Figure 4C–D). Pyruvate treatment in ob/ob mice was lethal, so we performed lactate tolerance tests (LTT) instead to estimate hepatic gluconeogenesis. Hepatic gluconeogenesis was lower in DKO male mice (Figure 4E). Similar to male DKO mice, embryonic onset, hepatocyte-specific deletion of TRAF3 also markedly improved glucose intolerance and insulin resistance (Figure 4F–G) and decreased hepatic gluconeogenesis (Figure 4H) in female DKO mice. Therefore, early inactivation of hepatic TRAF3 has a protective effect against obesity-induced insulin resistance and glucose intolerance.

3.4 Deletion of hepatic TRAF3 ameliorates obesity-associated hepatic steatosis

Obesity is a risk factor for NAFLD [7]. To determine whether hepatic TRAF3 is involved in NAFLD development, TRAF3f/fl male mice (7 weeks) were fed a HFD for 8 weeks to induce obesity and NAFLD, and then infected with Cre adenoviruses for 30 days to delete TRAF3 in the liver. Lipid droplets (LDs) were visualized by H&E staining of liver sections, and liver triacylglycerol (TAG) content were measured chemically. Numerous LDs were detected in the livers of GFP adenovirus-infected control mice, and deletion of liver TRAF3 markedly decreased LD abundance in the livers of Cre adenovirus-infected mice (Figure 5A). Liver TAG levels were lower in the Cre than in the GFP groups (data not shown). To determine whether deletion of hepatocyte TRAF3 ameliorates hepatic steatosis in mice with genetic obesity, we measured liver lipid content in DKO and ob/ob;TRAF3f/fl male mice (11 weeks). LDs were visualized by Oil red O staining of liver sections. A large number of LDs were detected in the livers of ob/ob;TRAF3f/fl mice, and hepatocyte-specific deletion of TRAF3 markedly reduced LD content in the livers of DKO mice (Figure 5B). In agreement, liver TAG levels were significantly lower in DKO than in ob/ob;TRAF3f/fl mice (Figure 5C). These data suggest that aberrant hepatic TRAF3 levels contribute to NAFLD development.

To determine whether inflammation is involved in mediating TRAF3 action in obesity, we examined the effect of hepatocyte-specific deletion of TRAF3 on the expression of various cytokines in the liver. HKO and TRAF3fl (control) male mice were fed a HFD for 12 weeks, and the mRNA abundance of the cytokines were quantified by qPCR. Expression of pro-inflammatory cytokine interleukin (IL)-1β was significantly higher in HKO than in TRAF3fl mice (Figure 5D). The expression of IL-6 and TNFα was also higher in DKO than in ob/ob;TRAF3f/fl mice (Figure 5D). These data suggest that hepatic TRAF3 has anti-inflammatory activity in the setting of obesity.

3.5 Hepatic TRAF3 negatively regulates insulin signaling in the liver

To examine the effect of hepatocyte-specific deletion of TRAF3 on insulin signal transduction, HKO and TRAF3f/fl mice were fed a HFD for 10–12 weeks, fasted for 20–24 h, and stimulated with insulin (2 units/kg body weight) for 5 min. Insulin-stimulated phosphorylation of
Akt (both pThr308 and pSer473) in the liver, and hepatocyte-specific deletion of TRAF3 further increased insulin-stimulated phosphorylation of hepatic Akt in HKO mice (Figure 6A). To determine whether hepatocyte-specific deletion of TRAF3 improves insulin signaling in ob/ob mice, DKO and ob/ob;TRAF3<sup>f/f</sup> mice were fasted for 20–24 h and injected with insulin (4 units/kg body weight), and insulin-stimulated phosphorylation of Akt was examined in the liver 5 min later. Insulin also stimulated phosphorylation of hepatic Akt in ob/ob;TRAF3<sup>f/f</sup> mice, and hepatocyte-specific deletion of TRAF3 further increased insulin-stimulated phosphorylation of hepatic Akt in DKO mice (pSer473) (Figure 6B). To further verify inhibition of insulin signaling by endogenous TRAF3, primary hepatocytes were prepared from HKO and TRAF3<sup>f/f</sup> mice and stimulated with insulin. Deletion of TRAF3 increased the ability of insulin to stimulate phosphorylation of Akt in cultured hepatocytes (Figure 6C). These results suggest that TRAF3 cell-autonomously inhibits insulin signal transduction in hepatocytes.

3.6. Liver-specific overexpression of recombinant TRAF3 induces insulin resistance and glucose intolerance in lean mice

To further determine whether aberrant overexpression of TRAF3 in the liver is a causal factor for insulin resistance, C57BL/6 male mice (8–9 weeks) were infected with TRAF3 or GFP (control) adenoviruses via tail vein injection. Liver-specific overexpression of TRAF3 did not alter body weight and blood glucose levels, but significantly increased insulin sensitivity (Figure 7A). To further analyze insulin sensitivity and glucose metabolism, we performed GTT, ITT, and PTT. Liver-specific overexpression of TRAF3 induced glucose intolerance and insulin resistance (Figure 7B–C) and increased hepatic gluconeogenesis (Figure 7D). Fourteen days after infection, mice were fasted overnight and injected with insulin (0.75 U/kg body weight). Liver extracts were prepared 5 min after insulin stimulation and immunoblotted with antibodies against phospho-Akt, Akt, or TRAF3. Insulin robustly stimulated phosphorylation of Akt (pThr308 and pSer473), and overexpression of TRAF3 suppressed insulin-stimulated phosphorylation of Akt, suggesting that TRAF3 is a causal factor for insulin resistance in the liver.
phosphorylation of Akt (Figure 7E–F). TRAF3 was overexpressed specifically in the liver but not in the skeletal muscle, adipose tissue and brain (Figure 7E and data not shown). These data further support the concept that elevated levels of hepatic TRAF3 is a causal factor for insulin resistance and glucose intolerance in obesity.

4. DISCUSSION

Metabolic inflammation has been extensively examined in adipose tissue, but liver inflammation and its contribution to glucose metabolism are not fully understood. In the current study, we identified hepatic TRAF3 as a new linker between inflammation, insulin resistance, and glucose intolerance in obesity. In addition to mediating immune responses, TRAF3 may also act as a metabolic regulator in the setting of obesity and is involved in type 2 diabetes and NAFLD progression.

We have provided several lines of evidence that support a critical metabolic function of hepatic TRAF3. First, the levels of hepatic TRAF3 were regulated by the metabolic state. Liver TRAF3 levels were lower in the fasted state, and increased after refeeding. Second, glucose, the principal metabolic fuel, directly increased TRAF3 levels in hepatocytes, so hepatic TRAF3 may be a component of the nutrient sensing system. Third, liver TRAF3 levels were aberrantly higher in mice with either HFD-induced obesity or genetic (ob/ob) obesity. Hyperglycemia is likely to be an important stimulator which increases liver TRAF3 levels in obesity. Forth, deletion of hepatocyte TRAF3 ameliorated hyperinsulinemia, glucose intolerance, hepatic steatosis, and insulin resistance in mice with either HFD-induced obesity or genetic obesity. Fifth, overexpression of recombinant TRAF3 in the liver impaired insulin signaling and the ability of insulin to reduce blood glucose, leading to hyperinsulinemia and glucose intolerance in lean mice. Together, these data provide powerful evidence supporting that aberrant expression of hepatic TRAF3 is an important causal factor for insulin resistance, type 2 diabetes, and NAFLD in obesity.

We used two approaches to delete hepatocyte TRAF3 in mice. In the first approach, TRAF3+ mice were fed a HFD to induce obesity and insulin resistance, and then infected with Cre adenoviruses to conditionally delete liver TRAF3 in obese mice. This approach addresses a concern about potential developmental compensations as observed in other animal models. The results demonstrate that inhibition of liver TRAF3 has a curative effect against insulin resistance, glucose intolerance, and hepatic steatosis in obese mice. Cre adenoviral infection may cause liver inflammation, thus complicating...
data interpretation; therefore, GFP adenoviral infection was used as control to address this concern. In the second approach, HKO mice were generated by crossing TRAF3fl/fl mice with albumin-Cre drivers. The results demonstrate that early inactivation of liver TRAF3 has a preventive effect against the development of insulin resistance and type 2 diabetes. These two approaches complemented each other, and provided convincing evidence to support the concept that aberrantly-high levels of hepatic TRAF3 serve as a negative regulator of NIK. We previously reported that inhibition of NIK enhances the ability of glucagon to stimulate gluconeogenesis in hepatocytes, thus coupling inflammation to glucose counterregulation in obesity [28]. NIK activation does not affect insulin signaling [28], so NIK is unlikely to mediate TRAF3 action in the liver. TRAF3 binds to TRAF2 [29,30], and deletion of TRAF2 in hepatocytes attenuates HFD-induced hyperglycemia [31]. TRAF2 is able to enhance glucagon response, but it does not affect insulin action in the liver [31]; thus, TRAF2 is also unlikely to mediate the metabolic action of hepatic TRAF3. Myeloid cell TRAF3 suppresses inflammation in lean mice, but it switches its mode of action from anti-inflammatory to pro-inflammatory effect in obesity. In agreement with this, deletion of hepatocyte TRAF3 decreased the expression of pro-inflammatory cytokines in mice with either HFD-induced or genetic obesity [10]. In contrast, hepatic TRAF3 appears to retain its anti-inflammatory effect in obesity. In agreement with this, deletion of hepatocyte TRAF3 decreased the expression of pro-inflammatory cytokines in mice with either HFD-induced or genetic obesity. Thus, hepatic TRAF3 action on glucose and lipid metabolism cannot be explained by its regulation of liver inflammation in obesity. We observed that glucose stimulation directly increased TRAF3 levels in primary hepatocytes. Hyperglycemia was associated with an increase in hepatic TRAF3 levels. It will be interesting to determine whether ChREBP, a glucose sensor, mediates the effect of glucose on TRAF3 levels. Elevated levels of hepatic TRAF3 in turn promoted hyperglycemia by increasing hepatic gluconeogenesis, thus forming a glucose-TRAF3 reinforcement loop in the liver. The glucose-TRAF3 positive feedback loop may be further activated and/or amplified by additional obesity-associated factors, and may play an important role in driving type 2 diabetes and NAFLD progression.

**Figure 6: TRAF3 negatively regulates insulin signaling in the liver.**

A. TRAF3fl/fl and HKO male mice (7–8 weeks) were fed a HFD for 10–12 weeks, fasted for 20–24 h, and injected with insulin (2 units/kg body weight) via inferior vena. Liver extracts were prepared 5 min after insulin stimulation and immunoblotted with antibodies against phospho-Akt (pThr308 and pSer473) or Akt. Insulin-stimulated phosphorylation of Akt was quantified and normalized to total Akt levels. B. DKO and ob/ob/TRAf3fl/fl male mice (10 weeks) were fasted overnighted and stimulated with insulin (4 units/kg) for 5 min. Liver extracts were immunoblotted with antibodies against phospho-Akt (pSer473) or Akt. Phosphorylation of Akt was quantified and normalized to total Akt levels. C. Primary hepatocytes were prepared from TRAF3fl/fl and HKO male mice (8–9 weeks) and stimulated with 100 nM insulin for the indicated time. Cell extracts were immunoblotted with antibodies against phospho-Akt (pSer473) or Akt. *p < 0.05.
5. CONCLUSIONS

We have identified hepatic TRAF3 as a new metabolic regulator in obese mice. Liver TRAF3 levels are abnormally higher in obesity, presumably due to hyperglycemia. Hepatocyte-specific deletion of TRAF3 attenuates insulin resistance, glucose intolerance, and hepatic steatosis in obese mice. Obesity-associated factors trigger and/or amplify a glucose-TRAF3 reinforcement loop in the liver, which is likely to drive type 2 diabetes and NAFLD progression. Therefore, disrupting this glucose-TRAF3 positive feedback loop may serve as a new strategy to treat type 2 diabetes and NAFLD.

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CONFLICT OF INTEREST

None declared.
APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2015.09.013.

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