Control of adipose tissue inflammation through TRB1

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Running title: TRB1 co-activates pro-inflammatory genes

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Objective: Based on its role as an energy storage compartment and endocrine organ, white adipose tissue (WAT) fulfils a critical function in the maintenance of whole-body energy homeostasis. Indeed, WAT dysfunction is connected to obesity-related type 2 diabetes triggered at least partly by an inflammatory response in adipocytes. The pseudokinase tribbles (TRB) 3 has been identified by us and others as a critical regulator of hepatic glucose homeostasis in type 2 diabetes and WAT lipid homeostasis. Therefore, this study aimed to test the hypothesis that the TRB gene family fulfils broader functions in the integration of metabolic and inflammatory pathways in various tissues.

Research Design and Methods: To determine the role of TRB family members for WAT function, we profiled the expression patterns of TRB1-3 under healthy and metabolic stress conditions. The differentially expressed TRB1 was functionally characterized in loss-of-function animal and primary adipocyte models.

Results: Here we show that the expression of TRB1 was specifically up-regulated during acute and chronic inflammation in WAT of mice. Deficiency of TRB1 was found to impair cytokine gene expression in white adipocytes and to protect against high-fat diet-induced obesity. In adipocytes, TRB1 served as a nuclear transcriptional co-activator for the NFκB subunit RelA, thereby promoting the induction of pro-inflammatory cytokines in these cells.

Conclusions: As inflammation is typically seen in sepsis, insulin resistance and obesity-related type 2 diabetes, the dual role of TRB1 as both a target and a (co)-activator of inflammatory signaling might provide a molecular rationale for the amplification of pro-inflammatory responses in WAT in these subjects.
Adipose tissue can be subdivided into two distinct categories, white and brown. Whereas brown adipose tissue (BAT) dissipates energy as heat, white adipose tissue (WAT) is specialized in the storage of chemical energy such as triglycerides (1), thereby providing a reserve storage compartment for excess nutrients as a protective measure against starvation (2). In addition, white adipocytes also serve as critical endocrine cells, secreting a variety of adipokines to control energy intake and expenditure at the systemic level (3). Importantly, under both acute and chronic metabolic stress as exemplified by sepsis and obesity-related type 2 diabetes, respectively, impaired WAT function is characterized by WAT tissue inflammation and an increased release of pro-inflammatory cytokines from this tissue as well as dysregulation of the energy balance (4). Indeed, sepsis induces the metabolic rate by 30-60% in humans (5), and obesity has been linked to enhanced macrophage recruitment and T cell receptor rearrangements in WAT (6; 7). Additionally, pro-inflammatory cytokine expression is activated in adipocytes per se in response to inflammatory cues including bacterial lipopolysaccharide (LPS) (8). Interestingly, recent evidence suggests that chronically elevated levels of LPS resulting from alterations in the gut microbiota during obesity, contribute to obesity-related insulin resistance (9), promoting the idea that common molecular pathways may underlie the pro-inflammatory responses in adipocytes and WAT under both acute and chronic metabolic disturbances (10).

The tribbles (TRB) protein family consists of three related serine/threonine kinase-like proteins, TRB1, 2, and 3, which have been highly conserved throughout evolution and serve as critical regulators of cell cycle progression during development in Drosophila and Xenopus (11). In mammals, TRB3 was originally identified as a critical checkpoint in hepatic glucose homeostasis during fasting and type 2 diabetes, mediated through its regulatory function on Akt/protein kinase B in the insulin signaling pathway (12). Consistently, subsequent studies have proposed TRB3 as a determinant of insulin sensitivity in liver, skeletal muscle and WAT (13-15), and of lipolysis in WAT by triggering the degradation of acetyl-CoA carboxylase via association with an E3 ubiquitin ligase (16). Interestingly, both TRB2 and TRB3 were found to suppress white adipocyte differentiation in an insulin-/peroxisome proliferator-activated receptor (PPAR)γ-dependent manner (17; 18). Apart from hormonal pathway control, all three tribbles family members have been implicated in the activation of macrophages, and, particularly TRB3, in the cellular endoplasmatic reticulum stress response (19-22), overall supporting the hypothesis that the TRB family may fulfill broader but largely unexplored functions in the integration of metabolic and inflammatory pathways in various tissues.

**RESEARCH DESIGN AND METHODS**

**Recombinant adenoviruses.** Adenoviruses expressing a TRB1-specific or non-specific shRNA under the control of the U6 promoter were cloned as described previously (23; 24). Viruses were purified by the cesium chloride method and dialyzed against phosphate-buffered-saline buffer containing 10% glycerol prior to use.

TRB1 shRNA for: 5’-CACCGGGCTATGGTCACCAATCGAAATTCTCGAGTCACATAGGCC-3’

TRB1 shRNA rev: 5’-AAAAGGGCTATGGTCACCAAAATTTTGATTTCGAGTCACATAGGCC-3’

**Animal experiments.** Male 8-12 week old C57Bl6, db/db, ob/ob, TLR4, TNFR1/2, JNK, p50, and TNFR1/IL-1β receptor knockout
mice were obtained from Charles River Laboratories (Brussels, BEL). Mice with TRB1 haploinsufficiency have been described before (19). All animals were maintained on a 12 h light-dark cycle with regular unrestricted diet. For LPS experiments, animals were fasted for 18 h with free access to water and injected with 20 mg/kg body weight LPS. In each experiment, three to seven animals received identical treatments and were analyzed 2.5 h after LPS administration. For tumor induction in the cachexia model, 1.5x10^6 Colon 26 (C26) cells (25) in PBS were injected subcutaneously into 10-week-old CD2F1 mice (Charles River Laboratories, Brussels, BEL). Control mice were injected with heat-inactivated C26 cells. Subcutaneous implantation of C26 cells promoted severe reduction of body weight, as well as skeletal muscle and adipose tissue mass. In addition, C26 mice displayed reduced levels of serum TG and substantial hepatic steatosis as described (26). In high-fat diet experiments, TRB1^+/− mice and wild-type littermates were either fed a standard chow diet (10% energy from fat, Research diets D12450B, New Brunswick, USA) or a high-fat diet (60% energy from fat, Research diets D12492) for a period of 13 weeks. Insulin and glucose tolerance tests were performed as described previously (27). Organs including liver, epididymal fat pads, and gastrocnemius muscles were collected after the corresponding time period, snap-frozen and used for further analysis. Total body fat content was determined by an Echo MRI body composition analyzer (Echo Medical Systems, Houston, TX). Animal handling and experimentation was done in accordance with NIH guidelines and approved by local authorities.

**Blood metabolites.** Serum levels of glucose, insulin, TG, cholesterol and cytokines were determined using an automatic glucose monitor (One Touch, Lifescan, Neckargemünd, DEU) or commercial kits (MP Biomedicals, Orangeburg, NY; Merckodia, Uppsala, SE; Sigma, Munich, DEU; RANDOX, WAKO, Neuss, DEU, Millipore, Schwalbach, DEU).

**Cell culture and transient transfection assays.** 3T3-L1 pre-adipocytes and HEK293 cells were transfected using Lipofectamine (Invitrogen, Karlsruhe, DEU) reagent according to the manufacturer’s instructions. Cell extracts were prepared 48 h after transfection, and luciferase assays were performed as described (23), normalizing to the activity from co-transfected β-galactosidase expression plasmid. Primary stromal-vascular fractions were isolated from mouse epididymal fat depots, cultured, and differentiated into mature primary adipocytes (essentially) as described (28). Cells were infected with recombinant adenoviruses at a multiplicity of infection of 1000 and stimulated with 1.5 ng/ml TNFα (Biomol, Hamburg, DEU). Cytokine-conditioned medium (CM) was obtained by stimulating RAW264.7 mouse macrophages with 100 ng/ml LPS for 3.5 h and collecting the medium. Medium harvested from RAW264.7 cells not exposed to LPS was used as the control in all experiments (M).

Analysis of the macrophage-derived CM and M was done by MILLIPLEX™ MAP-Kit against mouse cytokines and chemokines (Millipore, Schwalbach, DEU).

3T3-L1 and primary adipocytes were treated with pharmacological inhibitors (50 µM Parthenolide, 50 µM SP600125, 50 µM PD98059, 10 µM SB202190, Calbiochem, Darmstadt, DEU) 30 min prior to stimulation with CM or M. Cells were lysed in QIAzol (QIAGen, Hilden, DEU) and RNA was isolated according to standard procedures. Cell separation studies of adipose tissue macrophages (ATM) from epididymal fat pads of LPS- or PBS-injected mice were done by magnetic immunoaffinity isolation using anti-CD11b antibodies, conjugated to magnetic beads (MACS Cell Separation...
System; Miltenyi Biotec). Following isolation of ATMs from the SVF using positive selection columns (MS columns; Miltenyi Biotec), the remaining cells were eluted as the SVF fraction. For the analysis of mRNA expression levels, eluted cells (CD11b-positive (†) and SVF) and the floating adipocyte fraction were resuspended in QIAzol reagent.

**Quantitative Taqman RT-PCR.** Total RNA was extracted from homogenized mouse WAT or cell lysates using QIAzol and the RNasy (Qiagen, Hilden, DEU) kit. cDNA was prepared by reverse transcription using Superscript II (Invitrogen, Karlsruhe, DEU) and Oligo dT primer (Fermentas, St. Leon-Rot, DEU). cDNAs were amplified using assay-on-demand kits and an ABIPRISM 7300 Sequence detector (Applied Biosystems, Darmstadt, DEU). RNA expression data was quantified according to the ∆Ct method as described (29), and normalized to levels of TATA-box binding protein (TBP) RNA.

**Protein analysis.** Protein was extracted from frozen organ samples or cultured adipocytes in 2x SDS-8 M Urea cell lysis buffer, and 20-30 µg of protein were loaded onto 10% SDS-polyacrylamide gels and blotted onto nitrocellulose membranes. Western blot assays were performed as described (23) using antibodies specific for TRB1 (30), RelA, P300, β-actin (Santa Cruz, Heidelberg, DEU), or valosin-containing protein (VCP) (Abcam, Cambridge, UK).

**Chromatin immunoprecipitation assay.** 3T3-L1 pre-adipocytes were transfected with a plasmid encoding FLAG-tagged TRB1, stimulated with CM or M for 6 h, fixed with formaldehyde 48 h after transfection, and ChIP assays were performed as described (31) using FLAG-specific antibodies (Upstate, Lake Placid, NY), or non-specific anti-HA antibody (Santa Cruz, Heidelberg, DEU). Precipitated DNA fragments were analyzed by PCR amplification as described above using primers directed against the IL6, IL-1β, and TNFα promoters. Primers against cytokine promoter regions lacking a RelA recognition site were used as negative controls as described (32). Primer sequences:
mIL6_for: TGTGTGTCGTCTGTCATTGCG; mIL6_rev: AGCTACAGACATCCCCAGTCTC; mIL6_A_for (without NF-κB): CCTACTTTCAACGCTGGAATC; mIL6_A_rev (without NF-κB) TCAAGTCTTCTAGGGCTGATT; mIL-1b_for: TGGCCCATTTCAACCAGG; mIL-1b_rev: TGCTACCCTGAATAATTCTATCCC; mIL-1b_for (without NF-κB): CCCAAGGAAAATTCTACAGC; mIL-1b_rev (without NF-κB): ACCACTGCAGGGTTTGTTGTC; mTNFalpha_for: CCCCCCGATGGGAGAAACCAGAGA; mTNFalpha_rev: GCTAGTCCTTGCTCTCGCTG

**Plasmids.** Wild-type or mutated NFκB reporter plasmids have been described previously (32). FLAG-TRB1 expression vector was generated by PCR-based standard procedures and cloned into pcDNA3.1 (Invitrogen, Karlsruhe, DEU) using standard protocols.

**GST-Pulldown Assay.** GST fusion proteins (pGEX5.1, pGEX5.1_GST_p65, pGEX5.1_GST_p65 RHD_1-305; pGEX5.1_GST_p65_TA_441-551 (32)) were produced in BL21 cells and affinity purified using glutathione sepharose (Amersham Biosciences, Darmstadt, DEU). In vitro transcription/translation was performed using the TNT T7/T3 quick coupled transcription/translation system (Promega, Mannheim, DEU) according to the manufacturer's instructions GST and in vitro translated proteins were incubated at 4°C overnight. After extensive washing, GST-precipitated proteins were separated by SDS-PAGE and detected by autoradiography.

**Immunoprecipitation.** HEK293 cells were cotransfected with a RelA expression vector plus Flag-TRB1 or an empty Flag vector. Subsequently, cells were lysed, centrifuged and the supernatant was incubated with anti-FLAG M2 Agarose (Sigma, Munich, DEU) for 2 h. The immunoprecipitates were
subsequently analyzed by Western blot as described.

Statistical Analysis—Statistical analyses were performed using a 2-way analysis of variance (ANOVA) with Bonferroni-adjusted post-tests, or Student t-test in one-factorial designs, respectively. The significance level was at p = 0.05.

RESULTS

TRB1 expression in WAT is elevated in acute and chronic inflammation. To initially explore the expression profiles of TRB family members under conditions of metabolic and particularly WAT dysfunction, we extracted total RNA from various tissues of wild-type or db/db mice, the latter representing a standard model for obesity and the Metabolic Syndrome (33). Quantitative PCR analysis confirmed the previously reported up-regulation of TRB3 expression in livers of obese mice (12), thereby verifying the experimental system (data not shown). In contrast, hepatic mRNA levels of TRB1 and TRB2 remained unchanged under these conditions (data not shown).

Intriguingly, TRB1 mRNA expression was found to be significantly elevated in WAT of db/db animals as compared to wild-type controls (Fig. 1A). In contrast, TRB2 and TRB3 showed no difference in their WAT expression levels (Fig. 1A), suggesting a specific impact of obesity-related conditions on TRB1 adipose tissue expression. Indeed, correlating with increasing, age-dependent overweight condition a higher expression of WAT TRB1 levels was also observed in a second, early-onset obesity mouse model (Sup. Fig. 1A available in the online appendix at http://diabetes.diabetesjournals.org).

Expression was however not affected by late-onset obesity as associated with high fat-diet (HFD) feeding of adult wild-type mice (Sup. Fig. 1C). Furthermore, TRB1 mRNA levels were found to be elevated in WAT of tumor-bearing, cachectic mice, while TRB2 and TRB3 again remained unchanged (Fig. 1B). Despite contrary states of energy availability, obesity actually shares many phenotypic features with severe wasting conditions such as cancer cachexia, including insulin resistance, hepatic steatosis, and particularly chronic inflammation (34). To test the hypothesis that pro-inflammatory conditions represent a common trigger for TRB1 expression in WAT, we provoked extensive pro-inflammatory cytokine production and signaling in mice by injecting sub-lethal doses of LPS. As shown in figure 1C and D, LPS treatment stimulated TRB1 mRNA and protein expression in WAT as compared with controls (Fig. 1C and 1D), demonstrating that the expression of TRB1 in WAT is specifically induced by pro-inflammatory conditions, thereby representing a distinguishing feature from other TRB family members.

TRB1 expression is under the control of cytokine signaling in an adipocyte-autonomous manner.

WAT is composed of a variety of different cell types, including mature adipocytes and the so-called stromal-vascular fraction (SVF), comprising macrophages, endothelial cells, and corresponding progenitors (1). Cell separation studies using WAT explants from LPS-treated or non-treated wild-type mice demonstrated that TRB1 mRNA expression was specifically induced by LPS in whole WAT depots, mature adipocytes and in the SVF but not in CD11b+ macrophage-enriched cellular fractions (Fig. 2A), indicating that adipocytes indeed represent the major site of TRB1 regulation in response to pro-inflammatory signaling in WAT. To specifically explore potential signaling pathways involved in TRB1 induction under pro-inflammatory conditions in adipocytes, we isolated the SVF from WAT depots of wild-type and toll-like receptor (TLR) 4 knockout mice, the latter deficient in the cellular LPS receptor (35). Isolated SVF were
differentiated into mature, primary adipocytes (data not shown) and treated either with cytokine-enriched conditioned medium (CM) from LPS-treated macrophages or LPS (Sup. Fig. 2A). Whereas CM efficiently stimulated TRB1 expression in both primary wild-type and TLR4 KO adipocytes, heat-inactivated CM (Sup. Fig. 2B) and LPS treatment had no effect in either cell type (Fig. 2B), supporting the hypothesis that not LPS/TLR4 signaling per se is responsible for the induction of TRB1 under pro-inflammatory conditions but most likely LPS-triggered cytokines such as tumor necrosis factor (TNF) α and interleukins. Interestingly, ablation of either TNF receptors (TNFR) 1 and 2 or TNFR1 and interleukin 1-β receptor in primary adipocytes had no effect on TRB1 mRNA induction in response to CM treatment (Sup. Fig. 2C), arguing that the induction of TRB1 expression in WAT is driven by multiple pro-inflammatory mediators in a combinatorial manner. In this respect, the activator protein (AP) 1 and nuclear factor (NF) κB transcriptional complexes represent common integration sites for divergent upstream pro-inflammatory signaling pathways in various cell types (10). Whereas the CM-dependent TRB1 induction in primary adipocytes was not affected by genetic deficiency for the AP1 upstream kinase jun N-terminal kinase (JNK) 1 (Sup. Fig. 1D), knockout of the NFκB subunit p50 significantly but incompletely impaired TRB1 gene expression upon cytokine stimulation in primary adipocytes (Fig. 2C), suggesting that the NFκB transcriptional complex may represent a critical checkpoint for TRB1 gene regulation in these cells, and that other NFκB components apart from p50 are essential in this context. To test this hypothesis directly, we treated mature adipocytes derived from differentiated 3T3-L1 pre-adipocytes with various inhibitors for specific intracellular signaling pathways upon CM exposure. Inhibitors of p38 (SB202190), extracellular signal-regulated kinase (ERK) (PD98059), and protein kinase A pathways elicited no effect on CM-triggered TRB1 mRNA levels (Fig. 2D and data not shown). However, specific pharmacological inhibition of the NFκB signaling axis by Parthenolide as well as the non-isooform specific JNK inhibitor SP600125 (36) completely eliminated the effect of CM on TRB1 mRNA levels in differentiated 3T3-L1 adipocytes as well as in SVF-derived primary adipocytes from wild-type mice (Fig. 2D and 2E, Sup. Fig. 2E), thereby demonstrating that the cell autonomous induction of TRB1 in white adipocytes under pro-inflammatory conditions is determined in a combinatorial fashion by distinct pro-inflammatory axes, including NFκB and JNK signaling.

**RB1 controls cytokine gene expression in WAT.** The data thus far established TRB1 as a novel output gene of the pro-inflammatory pathway in WAT, prompting us to investigate the functional relevance of these findings in an in vivo setting. Due to the high perinatal mortality of homozygous TRB1 knockout mice on the C57BL6 background strain (unpublished data), we studied mice with haploinsufficiency for TRB1 (19), displaying an approximately 50% reduction in whole-body TRB1 mRNA levels (Sup. Fig. 3A). Notably, both TRB2 and TRB3 mRNA levels in WAT of these mice were substantially lower as compared to TRB1 and not affected by TRB1 deficiency (Sup. Fig. 3A). Under basal conditions, TRB1 haploinsufficiency slightly increased food consumption (Sup. Fig. 3B) but had no effect on body weight, total body fat content as determined by magnetic resonance technology (Fig. 4A and 4B), blood glucose levels (Sup. Fig. 3C), serum insulin levels (Sup. Fig. 3D), or serum cholesterol (Sup. Fig. 3E) as well as non-esterified fatty acid (NEFA) (Sup. Fig. 3F) and triglyceride levels (Sup. Fig. 3G) as compared with wild-type littermates. In WAT, key genes in glucose- and lipid-
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regulatory pathways, including glucose transporter (Glut) 4, acetyl-carboxylase (ACC) 2, fatty acid synthase (FAS), and fatty acid-binding protein (FABP) 4, were not different when comparing wild-type and TRB1 haploinsufficient animals (Sup. Fig. 3H). In contrast, WAT mRNA expression of interleukin (IL)-1β, TNFα, and plasminogen activator inhibitor (PAI) 1 was significantly reduced in TRB1 haploinsufficient mice under basal and/or LPS-stimulated conditions (Fig. 3A), supporting the hypothesis that TRB1 is specifically required for the execution of the pro-inflammatory program in WAT.

Given the variable impact of TRB1 haploinsufficiency on WAT cytokine gene expression (Fig. 3A), which can most likely be explained by differential effects of TRB1 on gene expression in different WAT cell types including macrophages (19), we next sought to determine the cell autonomous role of TRB1 in pro-inflammatory responses specifically in adipocytes. To this end, we infected 3T3-L1-derived mature adipocytes with an adenovirus carrying a TRB1-specific or a non-specific control shRNA. TRB1-specific shRNA treatment substantially reduced TRB1 protein and mRNA expression in these cells (Sup. Fig. 4A and Sup. Fig. 4B), but had no influence on TRB2 and TRB3 expression levels (Sup. Fig. 4B), demonstrating the specificity of the shRNA knockdown strategy. In consistence with the results from TRB1 haploinsufficient mice (Fig. 3A), TRB1 deficiency impaired IL-6 and IL-1β gene expression (Sup. Fig. 4C) and release from these cells in response to pro-inflammatory stimulation (Sup. Fig. 4D), but exerted no discernable effect on metabolic gene expression (Sup. Fig. 4E).

To verify these findings in a primary cell system, we employed differentiated SVF-derived mature adipocytes from wild-type mice infected with an adenovirus carrying a TRB1-specific or a non-specific control shRNA as above. Adenoviral shRNA delivery substantially impaired TRB1 but not TRB2 and TRB3 expression in these primary cells (Fig. 3B and Sup. Fig. 4F). Recapitulating the results in TRB1-deficient mice and 3T3-L1-derived adipocytes, loss of TRB1 in fully differentiated mature adipocytes had no major influence on the expression of genes involved in either glucose metabolism, lipogenesis, or fatty acid oxidation as demonstrated by quantitative PCR analysis (Sup. Fig. 4G). However, TRB1 deficiency substantially blunted the cytokine-triggered induction of pro-inflammatory genes, including IL-6, IL-1β, IFN-β, TNFα, and PAI-1 (Fig. 3C), also reflected by a significantly impaired release of individual pro-inflammatory cytokines from these primary cells upon cytokine exposure as determined by ELISA (Fig. 3D).

Together with the in vivo data, these results demonstrated that TRB1 action is specifically required for the cell autonomous cytokine production and release by white adipocytes during inflammation.

TRB1 haploinsufficiency protects against high fat diet-induced obesity. The critical role of TRB1 in the execution of WAT cytokine gene expression during acute inflammation prompted us to assess the potential impact of TRB1 on chronic, low-grade inflammatory conditions as associated with adiposity and other components of the Metabolic Syndrome. To this end, heterozygous TRB1 knockout mice and wild-type littermates were placed on a HFD or a control diet, with 60% or 10% calories from fat respectively, for 13 weeks. Wild-type mice on the HFD showed a significant body weight as well as fat mass gain throughout the experimental period (Fig. 4A and B), associated with the increased expression of pro-inflammatory markers in WAT as reported (Fig. 4E, (37)). Under HFD conditions, TRB1 heterozygosity had no effect on plasma glucose (Sup. Fig. 3C), serum insulin (Sup. Fig. 3D), or serum
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triglyceride levels (Sup. Fig. 3G), as compared with wild-type controls. In contrast, weight gain and adiposity were almost completely prevented in TRB1+/- mice after 13 weeks on HFD, remaining at body weight and body fat mass levels of wild-type mice on control diet (Fig. 4A and B). Consistent with the lean phenotype and improvements in body composition, HFD-induced glucose intolerance tended to be improved in TRB1+/- mice (Fig. 4C), and TRB1+/- mice exhibited a trend towards increased blood glucose clearance after exogenous insulin administration, indicative of improved systemic insulin sensitivity (Fig. 4D). Moreover, TRB1 haploinsufficiency inhibited the HFD-mediated increase in pro-inflammatory gene expression in WAT of these animals (Fig. 4E), overall demonstrating the specific regulatory function of TRB1 for obesity-induced pro-inflammatory and metabolic programs under conditions of chronic energy excess in vivo.

TRB1 controls cytokine gene expression in adipocytes via direct promoter recruitment. Finally, we sought to define the molecular mechanism of the regulatory function of TRB1 in adipocyte inflammation. To this end, we performed cellular fractionation studies to determine the principal localization of TRB1 within primary white adipocytes. In agreement with previous reports (38), TRB1 protein was exclusively detected in the adipocyte nuclear fraction (Fig. 5A), suggesting that TRB1 exerts its pro-inflammatory role on cytokine gene expression mainly through nuclear functions. Consistent with this notion, in chromatin immunoprecipitation assays of 3T3-L1 pre-adipocytes TRB1 but not negative control precipitates efficiently recovered endogenous IL-6, IL-1β, and TNFα promoter fragments, carrying critical inflammation-responsive regulatory DNA elements (Fig. 5B, left). Interestingly, TRB1 was found to be further enriched on these promoter sites upon CM stimulation as compared to the basal state (Fig. 5B, left). On the contrary, no or reduced TRB1 promoter association was observed with IL-6 and IL-1β promoter regions, respectively, which lacked recognition elements for pro-inflammatory activator complexes, including the NFκB subunit RelA (Fig. 5B, right), demonstrating the specificity of the observed effects. Taken together, these results showed that TRB1 is directly recruited to cytokine gene promoters in the nucleus and particularly directed to NFκB/RelA recognition site-containing promoter regions. To test whether TRB1 can indeed functionally modulate RelA-dependent gene transcription, we employed promoter reporter constructs harboring isolated wild-type or mutated RelA binding sites in transient transfection assays of 3T3-L1 pre-adipocytes. Co-transfection of a TRB1 cDNA expression plasmid co-activated RelA-driven wild-type promoter activity by 1.5-fold as compared with controls (Fig. 5C). Furthermore, shRNA-mediated knockdown of TRB1 in these pre-adipocytes significantly impaired RelA-dependent promoter activity but had no effect on mutated promoter function (Fig. 5D), demonstrating the requirement of endogenous TRB1 for full RelA transcriptional activity in this context.

Overall, these data supported the hypothesis that TRB1 acts as a direct transcriptional co-activator for NFκB/RelA in the control of cytokine gene expression in white (pre-) adipocytes. Indeed, GST-tagged RelA but not GST alone recovered in vitro translated TRB1 in GST pulldown assays via its transactivator (TA) domain (Fig. 5E), and TRB1 was found to bind to RelA in cellular co-immunoprecipitation assays (Fig. 5F). Taken together, these results underline the notion that TRB1 can affect RelA transcriptional activity via direct physical interaction upon promoter recruitment.

**DISCUSSION**
Our results suggest a novel nuclear co-activator function of TRB1 on pro-inflammatory cytokine promoters in white adipocytes, mediated by its recruitment to NFκB DNA recognition sites. Also, given the discovery that TRB1 gene expression is induced via pro-inflammatory pathways in these cells, our data are consistent with a model in which TRB1 acts as both a target and effector of pro-inflammatory signaling in adipocytes via its direct physical interaction with the RelA subunit of NFκB.

A biological function for TRB1 has been reported only in a very limited number of studies. Thus far, TRB1 has been suggested to represent a biomarker for antibody-mediated allograft failure, expressed mainly in antigen-presenting cells and activated endothelial cells (39). In addition, TRB1 controls vascular smooth muscle cell proliferation and chemotaxis via regulation of MAP kinase activity (40), and has been shown to control NF-IL6-mediated gene expression in macrophages in an NFκB-independent manner (19). By acting as a positive mediator of inflammatory cues in adipocytes, the co-activator function of TRB1 for cytokine gene expression in these cells may thereby reflect a specific “adipose” aspect of a broader involvement of TRB1 in (pro-) inflammatory programs and pathologies at a systemic level. Interestingly, TRB1 haploinsufficiency resulted in no obvious metabolic phenotype under basal conditions (Fig. Sup. 3A-G), and TRB1 haplodeficiency did not seem to influence adipocyte differentiation per se as the body fat content was not different from wild-type animals under chow-fed conditions (Fig. 4B), thereby contrasting the essential requirements of both TRB2 and TRB3 for the adipogenic program (17; 18). However, TRB1 expression has been found to be elevated in human atherosclerotic arteries (40), and recent genome-wide association studies have pinpointed variants of the TRB1 locus as risk factors for hypertriglyceridemia and coronary artery disease (41-43), conditions tightly linked to a chronic inflammatory status (44). Indeed, during low-grade inflammatory conditions TRB1 heterozygosity protected against HFD-induced obesity, glucose intolerance as well as insulin resistance, and inhibited cytokine gene expression in WAT (Fig. 4). Rather than controlling metabolic pathways directly, it is tempting to speculate that the pro-inflammatory action of TRB1 in adipocytes contributes to the above mentioned (metabolic) pathologies by enhancing the release of circulating cytokines from WAT in response to a primary, pro-inflammatory hit, e.g. macrophage-derived cytokines. This notion is consistent with the induction of TRB1 expression in synovial fibroblasts upon IL-1β exposure (45) as well as with our data showing that the transcriptional activation of TRB1 in adipocytes does not rely on direct TLR4/LPS signaling (Fig. 2A) but rather on a complex cytokine cocktail as produced by tissue macrophages or stressed adipocytes per se (Fig. 2A). Given the whole body heterozygosity, the observed resistance against diet-induced obesity could be further enhanced by non-adipose tissue functions of TRB1, including increased uncoupled respiration in BAT, increased physical activity, and/or differences in intestinal absorption, which will be addressed in future studies.

Overall, TRB1 may serve as a functional receptor in the communication between metabolic (adipocytes) and immune cells (e.g. macrophages) in WAT, thereby amplifying WAT inflammation in response to activated WAT-associated immune reactions. As pro-inflammatory signaling is typically increased in sepsis, insulin resistance, and obesity-related type 2 diabetes (46), the cytokine-inducible TRB1 co-activator function in WAT might provide a molecular rationale for the amplification of systemic inflammation in these subjects.

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FIGURE LEGENDS

Figure 1: TRB1 expression in WAT is elevated in acute and chronic inflammation. A-C, Quantitative PCR analysis of TRB1, TRB2, or TRB3 mRNA levels in abdominal white adipose tissue (WAT) of wild-type (wt) and obese db/db mice (A), healthy control and tumor-bearing, cachectic (C26) CD2F1 mice (B), and control (PBS)- and lipopolysaccharide (LPS)-injected C57Bl6 mice (means ± SEM, n=5). (*); p≤0.05; (**); p≤0.01; (**); p≤0.001. D, Western blot of WAT extracts from two representative control (PBS)- or LPS-injected animals as in (C) using antibodies against TRB1 or valosin-containing protein (VCP). Relative amounts of TRB1 protein levels normalized to VCP protein levels shown. AU, arbitrary units.

Figure 2: TRB1 expression is under the control of cytokine signaling in an adipocyte-autonomous manner. A, Quantitative PCR analysis of TRB1 mRNA levels in WAT depots, mature adipocytes after separation from SVF, SVF or CD11b+-enriched cellular fractions from WAT depots. B-C, Quantitative PCR analysis of TRB1 mRNA levels in primary adipocytes derived from the stromal-vascular WAT fractions of wild-type (wt) and toll-like receptor 4 (B) or p50 (C) knockout (ko) mice. Cells were treated with LPS and LPS-conditioned (CM) or non-LPS-conditioned (M) macrophage supernatant as indicated. D-E, Quantitative PCR analysis of TRB1 mRNA levels in mature adipocytes derived from 3T3-L1 pre-adipocytes (D) or the stromal-vascular fraction of wild-type mice (E). Cells were pre-treated with inhibitors against p38 (SB202190), the extracellular signal-regulated kinase (ERK) (PD98059), and NFκB (Parthenolide) pathways and subsequently stimulated with LPS-conditioned (CM) or non-LPS-conditioned (M) macrophage supernatant as indicated (means ± SEM, n=5). (*); p≤0.05; (**); p≤0.01; (**); p≤0.001. n.s., non significant.

Figure 3: TRB1 controls cytokine gene expression in WAT. A, Quantitative PCR analysis of interleukin (IL)-1β, tumor necrosis factor (TNF) α, and plasminogen activator inhibitor (PAI) 1 mRNA levels in WAT of wild-type (wt) or TRB1 heterozygous knockout mice (TRB1 +/-) under basal or LPS-injected (20 mg/kg) conditions (means ± SEM, n=6-7). B, Western blot of extracts from primary mature adipocytes derived from the stromal-vascular WAT fraction of wild-type mice using antibodies against TRB1 or VCP. Cells were infected with a non-specific control (NC) or TRB1-specific shRNA adenovirus at a multiplicity of infection of 1000. C, Quantitative PCR analysis of IL-6, IL-1β, interferon (IFN) β, TNFα, and PAI-1 mRNA levels in primary mature adipocytes derived from the stromal-vascular WAT fraction of wild-type mice. Cells were infected with a non-specific control (NC) or TRB1-specific shRNA adenovirus at a multiplicity of infection of 1000 and exposed to TNFα for 6 h prior to harvesting. D, Release of
IL-6 and PAI-1 from the same cells as in (C) was measured by ELISA. (means ± SEM, n=3). (*); p≤0.05; (**) p≤0.01; (***) p≤0.001.

**Figure 4:** TRB1 haploinsufficiency protects against high fat diet-induced obesity. A-B, Body weight (A) and total body fat content as determined by magnetic resonance technology (B) in wild-type (wt) or TRB1 heterozygous knockout mice (TRB1^{+/−}) placed on a low-fat (10% calories from fat, LFD) or high-fat (60% calories from fat, HFD) diet for the indicated time points. C-D, Glucose (C) and insulin (D) tolerance tests in the same mice as in A-B. E, Quantitative PCR analysis of interleukin (IL)-1β, tumor necrosis factor (TNF) α, and plasminogen activator inhibitor (PAI) 1 mRNA levels in WAT of the same mice as in (A-B) after 13 weeks on a low- (LFD) or high-fat diet (HFD) (means ± SEM, n=3-4). (*), HFD wt vs. LFD wt; ($), TRB1^{+/−} HFD vs. wt HFD; (*); p≤0.05; (**) p≤0.01; (***) p≤0.001.

**Figure 5:** TRB1 controls cytokine gene expression in adipocytes via direct promoter recruitment. A, Representative Western blot of cytosolic or nuclear extracts from mature 3T3-L1-derived adipocytes using antibodies against p300, RelA, TRB1, β-actin or valosin-containing protein (VCP) as indicated. B, ChIP assay of 3T3-L1 pre-adipocytes transfected with a Flag-TRB1 cDNA using antibodies specific for Flag (αFlag) or non-specific IgG (αHA). Cells were treated with LPS-conditioned (CM) or non-LPS-conditioned (M) macrophage media as indicated. Precipitated fragments were analyzed by real-time PCR using IL6, IL1β, and TNFα promoter primers. Data show fold enrichment relative to control IgG. Left, primer pairs including NFκB sites; Right, primer pairs without NFκB sites (means ± SEM, n=4). C-D, Transient transfection assay of 3T3-L1 pre-adipocytes co-transfected with 3xNFκB-Luc (containing wild-type NFκB binding sites) (C+D) or 3x-NFκB-mut-Luc (containing mutated NFκB binding sites) (D) together with plasmids encoding RelA and TRB1 (C), or TRB1-specific or non-specific control shRNA constructs as indicated (means ± SEM, n=3). (*); p≤0.05; (**) p≤0.01; (***) p≤0.001. E, Pulldown assays performed with full-length RelA, the trans-activation domain (TA) or the Rel-homology domain (RHD) of RelA fused to GST and GST alone as a control. GST fusion proteins were incubated with in vitro translated full length TRB1. Bound proteins were resolved by SDS-Page and visualized by autoradiography. Input lanes represent 20% of the input. Schematic representations of TRB1-RelA interactions are shown. F, Co-immunoprecipitation of TRB1 and RelA from HEK293 cells transfected with Flag-TRB1 or an empty vector using anti-FLAG M2 antibody. Bound proteins were resolved by SDS-Page and subsequently detected by Western blot using RelA and FLAG M2 antibodies.
Figure 1

TRB1 co-activates pro-inflammatory genes
Figure 2

A. TRB1 co-activates pro-inflammatory genes

B. Primary adipocytes

C. Primary adipocytes

D. 3T3-L1 adipocytes

E. Primary adipocytes
Figure 3

A  

TRB1 co-activates pro-inflammatory genes  

IL-1β  

TNFα  

PAI-1  

B  

Primary adipocytes  

shNC  

shTRB1  

TRB1  

VCP  

C  

Primary adipocytes  

IL-6  

IL-1β  

IFN-β  

TNFα  

PAI-1  

D  

IL-6  

PAI-1
Figure 4

TRB1 co-activates pro-inflammatory genes
Figure 5

A. Cytosol/Nucleus
    - p300
    - RelA
    - TRB1
    - β-Actin
    - VCP

B. Fold enrichment
   - IL-6
   - IL-1β
   - TNFα
   - M
   - CM
   - α-Flag
   - α-HA

C. Relative luciferase activity
   - Flag
   - TRB1-Flag
   - RelA
   - RelA
   - RelA
   - RelA
   - RelA
   - RelA
   - RelA
   - RelA

D. Relative luciferase activity
   - 3xNF-κB
   - 3xNF-κB mut
   - shNC
   - shTRB1

E. Input GST RelA RelA-RelA-TAD RHD
   - TRB1-Flag

F. IP: anti-Flag
   - IB: anti-Flag
   - IB: anti-RelA