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TRB3 suppresses adipocyte differentiation by negatively regulating PPARγ transcriptional activity[S]

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Abstract **In the course of an effort to identify the regulators for peroxisome proliferator-activated receptor γ (PPARγ)-dependent perilipin gene expression, we found that *tribbles* homolog 3 (TRB3), containing a single kinase domain without enzymatic activity, downregulates PPARγ transcriptional activities by protein-protein interaction. We examined the role that TRB3 plays in adipocyte differentiation in 3T3-L1 cells. TRB3 gene and protein expression was increased during adipocyte differentiation concomitantly with an increase in the mRNA levels of CCAAT/enhancer binding protein homologous protein. The physical interaction between TRB3 and PPARγ was also verified in 3T3-L1 adipocytes. Forced TRB3 expression in 3T3-L1 cells decreased the mRNA levels of PPARγ-target genes and intracellular triglyceride levels, whereas knockdown of TRB3 expression by RNA interference increased them. TRB3 also inhibits PPARγ-dependent adipocyte differentiation in lentivirus-mediated PPARγ-expressing 3T3-L1 cells.** **These results provide evidence that TRB3 acts as a potent negative regulator of PPARγ, a master regulator of adipocyte differentiation, and tightly controls adipogenesis.**—Takahashi, Y., N. Ohoka, H. Hayashi, and R. Sato. **TRB3 suppresses adipocyte differentiation by negatively regulating PPARγ transcriptional activity.** ***J. Lipid Res.*** **2008.** 49: **880–892.**

Supplementary key words *tribbles* homolog 3 • perilipin • triglyceride • 3T3-L1 • peroxisome proliferator-activated receptor γ

Obesity is a major risk factor in the development of type II diabetes. In recent years, adipocytes have been shown to play an important role in the generation of the secreted hormones and cytokines involved in metabolic activities (1). Abnormally and/or excessively differentiated adipocytes may help induce the metabolic syndrome by producing insulin-resistant hormones and cytokines (e.g., tumor necrosis factor-α) (2, 3). The differentiation of preadipocytes into adipocytes is regulated by an elaborate network of transcription factors that control the expression of hundreds of genes responsible for establishing the mature adipocyte phenotype. It is now well accepted that both peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding proteins (C/EBPs) function as critical regulators of adipogenesis in the complex transcriptional cascade (4, 5). PPARγ, a master regulator of adipogenesis, induces the expression of adipocyte-specific genes through the binding of PPARγ-retinoid X receptor-α (RXRα) heterodimers to a PPAR-response element (PPRE) (6, 7), resulting in the promotion of intracellular fat storage. PPARγ and RXRα belong to the nuclear receptor superfamily and share a high degree of homology in the N terminus with a ligand-independent transcriptional activation function (AF)-1 domain and in the C terminus with a ligand-dependent transcriptional AF-2 domain (8). A number of investigations have demonstrated that C/EBPβ along with C/EBPδ directly induces the expression of PPARγ2, an isoform of PPARγ expressed exclusively in adipocytes (9). In turn, PPARγ activates the transcription of the C/EBPα gene (9).

Another signaling pathway that stimulates adipogenesis is mediated by sterol-regulatory element binding protein-1c (SREBP-1c) (10). SREBP-1c is a basic helix-loop-helix protein abundantly expressed in adipose tissue and liver (11), and its gene expression is induced by insulin (12, 13). SREBP-1c has been shown to increase fatty acid and triglyceride synthesis, and this has been attributed in part to the influence on PPARγ activity exerted by inducing its gene expression and producing as yet unidentified endogenous ligands (14, 15).

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Abbreviations: AF, activation function; C/EBP, CCAAT/enhancer binding protein; CHOP, C/EBP homologous protein; ER, endoplasmic reticulum; GFP, green fluorescent protein; PPAR, peroxisome proliferator-activated receptor; PPRE, PPAR-response element; RXR, retinoid X receptor; shRNA, short hairpin RNA; SREBP, sterol-regulatory element binding protein; TRB3, *tribbles* homolog 3.

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[S] The online version of this article (available at http://www.jlr.org) contains supplementary data in the form of four figures.

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tribbles homolog 3 (TRB3), identified as a mammalian homolog of *Drosophilatribbles*, functions as a negative regulator of the serine-threonine kinase Akt in the liver and skeletal muscle (16, 17). In the liver, TRB3 is induced under stressful conditions, such as fasting, endoplasmic reticulum (ER) stress, and nutrient starvation (18, 19). In *db/db* mice, a rodent model of type II diabetes, increased hepatic expression of TRB3 results in hyperglycemia and insulin resistance (16). On the other hand, transgenic mice that overexpress TRB3 in adipose tissue are resistant to diet-induced obesity and display enhanced insulin sensitivity (20). Thus, the functions of TRB3 are various and different in distinct tissues.

In this study, we show that TRB3 reduces PPARγ-dependent perilipin gene expression. We hypothesized that TRB3 could be involved in the regulation of adipogenesis that is governed predominantly by PPARγ and investigated TRB3 functions during adipocyte differentiation. TRB3 gene expression is augmented along with the differentiation of 3T3-L1 cultured cells, a commonly used model of adipocyte differentiation. TRB3 overexpression or knockdown in 3T3-L1 cells affects adipocyte differentiation and triglyceride accumulation in the cells. These results indicate that TRB3 plays a critical role in adipogenesis and could be a novel therapeutic target for preventing the abnormal adipocity that is involved in metabolic disorders.

MATERIALS AND METHODS

Materials

Insulin, 3-isobutyl-1-methylxanthine, dexamethazone, pioglitazone, anti-perilipin antibody, and anti-human TRB3 antibody were obtained as described previously (18, 21). Troglitazone was kindly provided by Dr. R. M. Evans (Salk Institute). Taq-Man probes for mouse TRB3, aP2, perilipin, adiponectin, and SREBP-1 were from Applied Biosystems. Anti-Flag and anti-Myc monoclonal antibodies were from Sigma. Anti-mouse TRB3 antibodies were from Calbiochem and Santa Cruz. Anti-actin and anti-green fluorescent protein (GFP) antibodies were from Chemicon and Abcam, respectively. Anti-Akt and anti-phospho-Akt (serine-473, threonine-308) were from Cell Signaling. The protease inhibitor cocktail was from Sigma.

Cell culture

HEK293 and 293-T cells were cultured in DMEM with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin. 3T3-L1 fibroblasts (obtained from the Health Science Research Resources Bank, Osaka, Japan) were cultured and differentiated into adipocytes as described previously (21). Plat-E cells (22) were cultured in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, 10 μg/ml blasticidin, and 1 μg/ml puromycin. 3T3-L1/Tet-Off/TRB3 stable cells were cultured in DMEM with 10% Tet System-approved FBS (Clontech), 100 U/ml penicillin, 100 μg/ml streptomycin, 600 μg/ml G418, 400 μg/ml hygromycin, and 1 μg/ml doxycycline.

Plasmid constructs

Expression retroviral plasmids for TRB3 and TRB3ΔAkt (lacking amino acids 239–266) were constructed by inserting fragments coding for human TRB3 into pMXs-IRES-GFP (23). To generate an expression plasmid for mouse TRB3, a PCR fragment encoding mouse TRB3 obtained by RT-PCR using total white adipose tissue RNA from a BALB/c mouse was inserted into p3xFLAG-CMV (Sigma). An expression plasmid for Flag-tagged TRB3 was constructed by inserting a fragment coding for Flag-tagged mouse TRB3 into pBI-EGFP (Clontech). An expression lentiviral plasmid for Flag-tagged TRB3 and PPARγ was constructed by inserting a fragment coding for Flag-tagged TRB3 and PPARγ, respectively, into CSII-EF-MCS-IRES2-Venus (RIKEN). Expression lentiviral plasmids for short hairpin RNA (shRNA) of mouse TRB3 or control were constructed by recombining CS-RfA-EG (RIKEN) with pENTR4-H1 (RIKEN) inserted by oligonucleotide DNA for shRNA expression. The target sequences were as follows: TRB3, 5′-CGAGTGAGAG-ATGAGCCTG-3′; control (Scramble II Duplex from Dharmacon), 5′-GCGCGCTTTGTAGGATTCG-3′. The target for TRB3 has been used previously and confirmed to be effective (16, 20). Expression and reporter plasmids [pCMX-GAL4-PPARγ AF2 and p(PPRE)$_3$-tk-Luc] were kindly provided by Dr. R. M. Evans (Salk Institute). An expression plasmid (pSVSPORT-PPARγ) was kindly provided by Dr. M. Imagawa (Nagoya City University). Expression plasmids for the TRB3 mutants (pcDNA3.1-Flag-TRB3, pCMV5-Myc-TRB3, and pCMV5-Flag-TRB3) were constructed as described previously (18). Other expression plasmids (pCMV5-PPARγ, pCMV5-RXRα, pCMV5-PPARγ DN, replacing lysine-497 and glutamate-499 with alanine, pCMV5-PPARγΔA/B lacking amino acids 1–137, pCMV5-PPARγΔE/F lacking amino acids 311–505, pCMV5-GAL4-PPARγ, and pCMV5-GAL4-RXRα) were generated using PCR products. All constructs were verified by sequencing.

Retrovirus infection

Plat-E cells were transfected with retroviral plasmids by the Chen-Okayama method (24), and after 48 h of transfection, the culture medium containing retroviruses was collected and filtered. 3T3-L1 cells were infected with the medium supplemented with 10 μg/ml polybrene by a centrifugation method (2,500 rpm, 60 min). After 8 h of infection, the cells were refed with a fresh culture medium (DMEM with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin).

Lentivirus infection

293-T cells were transfected with an expression lentiviral plasmid together with a packaging (pCAG-HIVgp) and a VSV-G-/Rev-expressing (pCMV-VSV-G-RSV-Rev) plasmid by the Chen-Okayama method (24). After 60 h of transfection, the medium containing lentiviruses was collected and filtered. 3T3-L1 cells were infected with the medium supplemented with 10 μg/ml polybrene by a centrifugation method (2,500 rpm, 90 min). The cells were then refed with a fresh culture medium (DMEM with 10% calf serum, 100 U/ml penicillin, and 100 μg/ml streptomycin).

Stable cell lines

3T3-L1 preadipocytes transfected with the tetracycline-controlled transactivator-expressing plasmid (pTet-Off; Clontech) using Lipofectamine 2000 reagent (Invitrogen) were selected in the presence of 600 μg/ml G418 for 14 days. An individual clone was transfected with the pTRE-luc reporter plasmid using Lipofectamine 2000 reagent, and after 48 h of transfection, luciferase activities were measured and 3T3-L1/Tet-Off stable cells were established. 3T3-L1/Tet-Off cells were transfected with either the mock (pBI-EGFP) or the Flag-tagged (pBI-EGFP-TRB3) mouse TRB3-expressing plasmid containing the TRE promoter together with a pcDNA3.1/Hygro vector (Invitrogen) using Lipofectamine 2000 reagent. Cells were selected in the presence of 400 μg/ml hygromycin for 7 days, Flag-TRB3 protein levels in the absence of doxycycline were

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determined by Western blot analysis, and 3T3-L1/Tet-Off/TRB3 stable cells were established.

Small interfering RNA experiments

The small interfering RNAs for mouse TRB3 (5′-CGAGUGAGAGAUGAGCCUG-3′, the sequence used in lentiviral experiments) and control (5′-GCGCGCUUUGUAGGAUUCG-3′, the sequence of Scramble II Duplex by Dharmacon) were transfected into 3T3-L1 preadipocytes using Lipofectamine RNA interference MAX reagent (Invitrogen) according to the manufacturer's instructions. Three days after transfection, the 3T3-L1 cells were subjected to adipocyte differentiation.

Northern blot analysis and real-time RT-PCR

3T3-L1 cells differentiated into adipocytes were harvested, and total RNA was extracted using an RNA preparation kit (ISOGEN; Nippon Gene Corp.). Northern blotting was performed as described previously (21). TRB3 and C/EBP homologous protein (CHOP)-radiolabeled hybridization probes were generated using the individual cDNA fragments. Reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). Fluorescent real-time PCR was performed on an ABI PRISM 7000 system using Taq-Man Gene Expression Assays (Applied Biosystems) or SYBR Green PCR Master Mix (Applied Biosystems) with the primers as follows: C/EBPα, 5′-GCGCAAGAGCCGAGATAAAG-3′ (sense), 5′-CGGTCATTGTCACTGGTCAACT-3′ (antisense); C/EBPβ, 5′-CGCCTTTAGACCCATGGAAG-3′ (sense), 5′-CCCGTAGGCCAGGCAGT-3′ (antisense); C/EBPδ, 5′-CTCCCGCACACAACATACTG-3′ (sense), 5′-CTTCGGCAACCACCTAAAAG-3′ (antisense). S17 rRNA protein transcript was used as an internal control to normalize the mRNA levels of each gene.

Immunoprecipitation and Western blot analysis

Total cellular proteins were solubilized in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, pH 8.0, and a protease inhibitor cocktail). Immunoprecipitation and Western blotting were performed as described previously (25, 26). The signals on the membrane were quantified with an LAS1000 lumino image analyzer (FujiFilm).

Reporter assays

3T3-L1 preadipocytes were transfected with luciferase reporter and expression plasmids by Lipofectamine 2000 reagent, and after 24 h of transfection, they were refed with a fresh medium containing 10 μM troglitazone. Luciferase assays were carried out as described previously (26, 27).

Purification of recombinant proteins and in vitro binding assay

Glutathione *S*-transferase-tagged Flag-TRB3 and Myc-PPARγ were expressed in *Escherichia coli* BL21 and affinity-purified with glutathione-Sepharose 4B (Amersham Bioscience). Recombinant Flag-TRB3 and Myc-PPARγ proteins were cleaved from the glutathione *S*-transferase fusion proteins with PreScission Protease (GE Healthcare). The purity and concentration of the recombinant protein were estimated by Coomassie Brilliant Blue staining.

In an in vitro binding assay, protein-protein complexes were mixed in RIPA buffer containing 10 mg/ml BSA and subjected to immunoprecipitation with anti-Flag antibodies.

Oil Red O staining

When differentiated into adipocytes, 3T3-L1 cells were cultured with differentiation medium containing 10 μM pioglitazone for the first 2 days and then refed with an adipocyte growing medium. The cells were refed with a fresh medium every 2 days and, after harvest, fixed with 4% paraformaldehyde/PBS and stained with Oil Red O solution [0.5% Oil Red O-2-propanol: milliQ, 3:2 (v/v)] for 1 h at room temperature.

Lipid analysis

3T3-L1 cells were washed with PBS, and lipids were extracted by hexane-2-propanol (3:2, v/v). The amounts of intracellular triglyceride were determined by the Triglyceride E-test from Wako and normalized to the amounts of total cellular protein determined by BCA protein assay (Pierce) according to each manufacturer's instructions.

Statistical analysis

The results obtained in this study are presented as means ± SD and were evaluated with Student's *t*-test for the two groups.

RESULTS

TRB3 suppresses PPARγ transcriptional activities

In our previous report, we demonstrated that the genetic expression of perilipin, a lipid droplet surface protein in adipocytes, is controlled by PPARγ (21). The mouse perilipin gene promoter (~2.0 kb) contains a functional PPRE. We sought modulators for PPARγ-dependent perilipin gene expression among a number of transcription factors and regulatory proteins and found that TRB3 can suppress perilipin promoter activity. We performed luciferase assays using reporter genes containing the mouse perilipin promoter (~2.0 kb, pPlin-2.0) or three tandem repeats of the consensus PPRE [p(PPRE)$_3$-tk]. The promoter activities were stimulated when PPARγ and RXRα were expressed in 3T3-L1 preadipocytes but suppressed when TRB3 was coexpressed in the presence or absence of troglitazone, a potent agonist of PPARγ (**Fig. 1A**).

We next used a heterologous Gal-4 system, with expression plasmids encoding a full-length PPARγ or an AF-2 ligand binding domain of PPARγ coupled to the DNA binding domain of yeast Gal-4, to determine the direct effect of TRB3 on PPARγ transcriptional activities. In the presence of troglitazone, both promoter activities were increased (Fig. 1B, left, middle). TRB3 suppressed the transcriptional activities of full-length PPARγ more potently than those of the AF-2 domain (Fig. 1B, left, middle). On the other hand, the transcriptional activities of full-length RXRα were not affected by TRB3 (Fig. 1B, right).

Finally, we examined the effect of TRB3 mutants (**Fig. 2C**, right) on PPARγ transcriptional activities using pPlin-2.0. TRB3ΔN (amino acids 128–358) as well as wild-type TRB3 suppressed PPARγ transcriptional activities in the presence or absence of troglitazone, whereas neither TRB3ΔC (amino acids 1–282) nor TRB3ΔAkt lacking the Akt binding domain (amino acids 239–265) had any effect on PPARγ transcriptional activities (Fig. 1C). We confirmed that PPARγ protein levels were not affected by TRB3 (see supplementary Fig. I).

These results clearly indicate that TRB3 suppresses PPARγ transcriptional activities and that the C-terminal half of TRB3 is required to exert its inhibitory effects.

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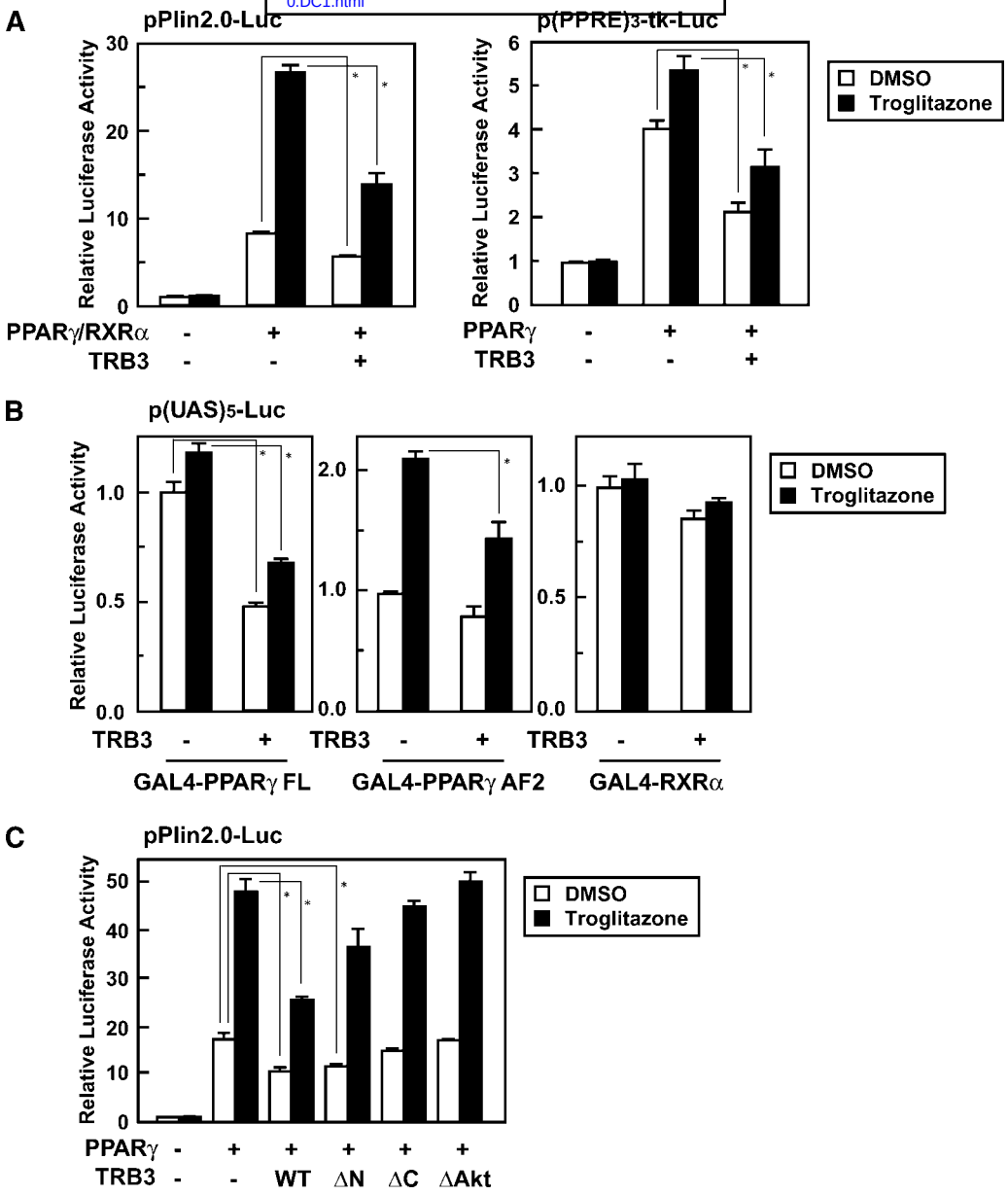

Fig. 1. Effects of *tribbles* homolog 3 (TRB3) on the transcription activity of peroxisome proliferator-activated receptor γ (PPARγ). A: 3T3-L1 preadipocytes were transfected with expression plasmids for PPARγ, retinoid X receptor-α (RXRα), or TRB3 and a reporter plasmid with the perilipin promoter (left) or the PPAR-response element $(PPRE)_3$-tk promoter (right) together with pCMV-β-gal. After 24 h of transfection, the cells were refed with a medium containing 10 μM troglitazone and incubated for 24 h. Luciferase activities were normalized to β-galactosidase activities and considered as 1 in the absence of PPARγ, RXRα, TRB3, and troglitazone. B: 3T3-L1 preadipocytes were transfected with expression plasmids for GAL4-PPARγ-FL (full length; left), GAL4-PPARγ-AF2 (activation function-2; middle), or GAL4-RXRα (right) together with expression plasmids for TRB3 and pCMV-β-gal. The cells were then processed as described for A. C: 3T3-L1 preadipocytes were transfected with expression plasmids for PPARγ, RXRα, or various TRB3 mutants (see Fig. 2C, right) and a reporter plasmid with the perilipin promoter together with pCMV-β-gal. The cells were then processed as described for A. WT, wild type. All luciferase assays were performed in triplicate. The data represent means ± SD. * $P < 0.01$.

TRB3 interacts with PPARγ

The results described above prompted us to test the hypothesis that TRB3 suppresses PPARγ transcriptional activities as a result of a protein-protein interaction. We first analyzed the interaction by a coimmunoprecipitation method using HEK293 cells transiently transfected with expression plasmids for these proteins. When cells expressed both Myc-TRB3 and Flag-PPARγ, Myc-TRB3 was recovered in the immunoprecipitates with an anti-Flag antibody (Fig. 2A, lane 3), indicating that TRB3 associates with PPARγ. On the other hand, TRB3 did not interact with RXRα, a heterodimer partner of PPARγ (Fig. 2A, lane 4). To investigate whether TRB3 interacts with PPARγ directly, we next performed an in vitro binding

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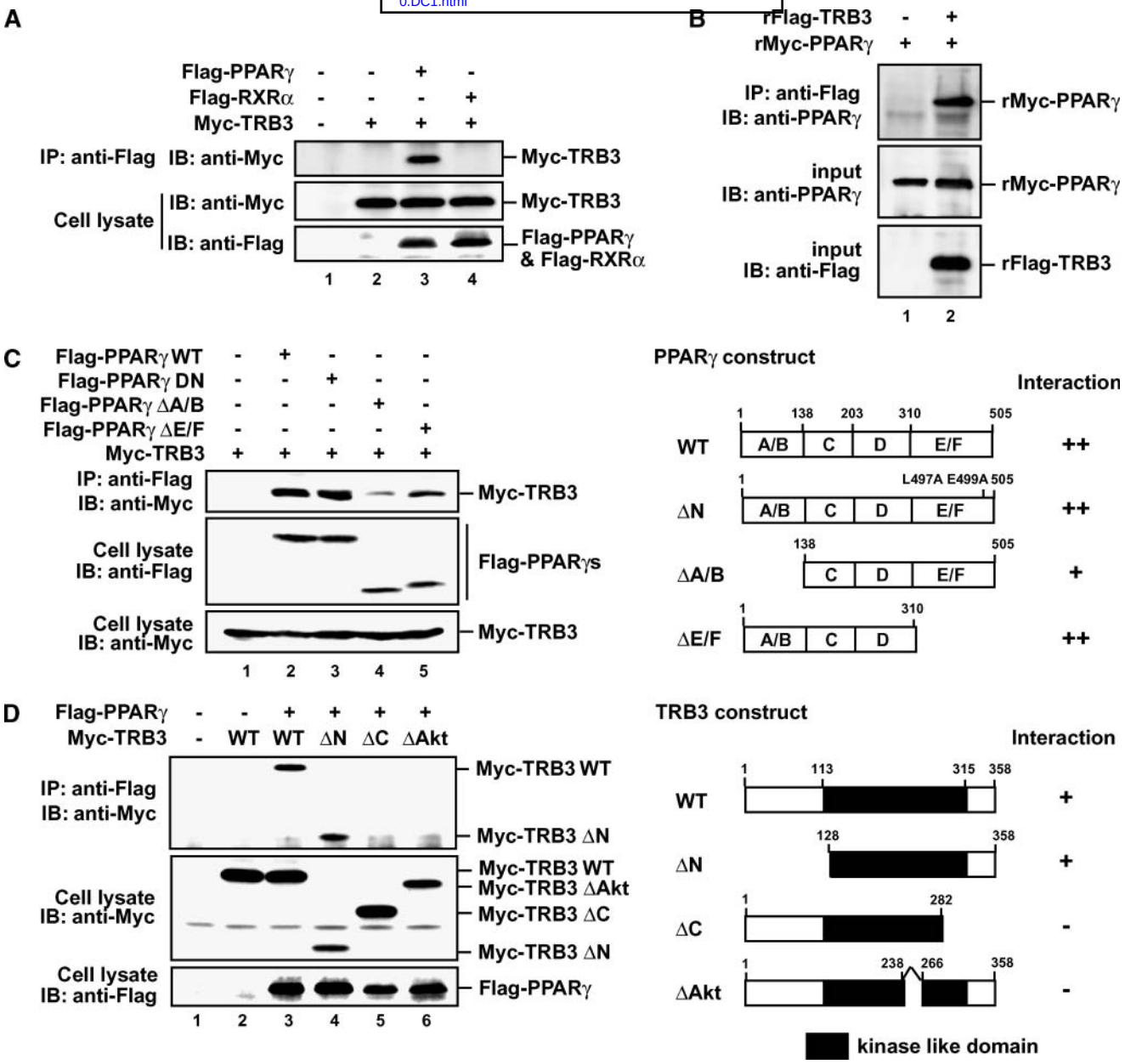

Fig. 2. Interaction between TRB3 and PPARγ. A: HEK293 cells were transfected with an expression plasmid for Flag-PPARγ or Flag-RXRα together with Myc-TRB3. The cells were harvested after 48 h of transfection, and total cell lysates were subjected to immunoprecipitation using anti-Flag antibodies. Both lysates and precipitates were subjected to SDS-PAGE and Western blot analysis with anti-Myc and anti-Flag antibodies. B: Recombinant Flag-TRB3 (rFlag-TRB3; 0.1 μg) and Myc-PPARγ (rMyc-PPARγ; 0.1 μg) were subjected to in vitro binding assay, as described in Materials and Methods. The protein-protein complexes were immunoprecipitated with anti-Flag antibodies and immunoblotted with anti-PPARγ antibodies. The level of each protein was assessed by immunoblotting with a 2% input of anti-Flag and anti-PPARγ antibodies. C, D: HEK293 cells transfected with the indicated constructs (PPARγ mutants and TRB3 mutants) were harvested after 48 h of transfection, and total cell lysates were subjected to immunoprecipitation using anti-Flag antibodies. The results shown in B and C are summarized. ++, tight interaction; +, interaction; −, no interaction. IB, immunoblot; IP, immunoprecipitate; WT, wild type.

assay using recombinant purified proteins, and direct interaction was observed (Fig. 2B, lane 2).

To identify the domain of PPARγ required for the interaction with TRB3, we generated various deletion constructs of PPARγ. We generated a dominant negative form of PPARγ containing substitutions of lysine-497 and glutamate-499 with alanine, PPARγΔA/B (amino acids 138–505) lacking the A/B region that contains the constitutive transcriptional activation domain, AF1, and PPARγΔE/F (amino acids 1–310) lacking the E/F region with the ligand-dependent transcriptional activation domain, AF2 (Fig. 2C, right). Although all PPARγ mutants interacted with full-length TRB3, PPARγΔA/B had only a weak interaction with TRB3 (Fig. 2C, left).

Next, we used various deletion constructs of TRB3, as shown in Fig. 2D, right, to identify the domain required

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for the interaction with PPARγ. Although TRB3ΔN (amino acids 128–358) interacted with PPARγ, deletion of either the Akt binding domain (amino acids 239–265) or the C-terminal 76 amino acid residues (ΔAkt and ΔC), which cannot suppress the transcriptional activities of PPARγ (Fig. 1C), abolished the interaction (Fig. 2D, left), suggesting that the direct interaction between TRB3 and PPARγ is essential for the suppression of PPARγ transcriptional activities.

TRB3 mRNA levels increase during adipocyte differentiation in 3T3-L1 cells

To verify that TRB3 is involved in the adipocyte differentiation accompanying PPARγ, we first examined daily changes in the levels of TRB3 mRNA and protein during the differentiation of 3T3-L1 cells. Real-time RT-PCR (**Fig. 3A**, left) as well as Northern blot analysis (see supplementary Fig. IIA) revealed that TRB3 mRNA levels decreased during the first 2 days after differentiation and then increased drastically. In terms of other *tribbles* family members, TRB1 was not detectable (data not shown), but TRB2 mRNA levels decreased during differentiation (see supplementary Fig. IIA). During the initial 48 h, TRB3 mRNA levels declined for the first 24 h, then gradually returned to the 0 h level at 24 h, and again declined until 48 h (see supplementary Fig. IIB). After the downregulation in early stage, TRB3 mRNA levels were induced for the next several days until day 14 (Fig. 3D). On the other hand, the mRNA levels of C/EBPβ and C/EBPδ, both of which are key regulators in the early stage of adipocyte

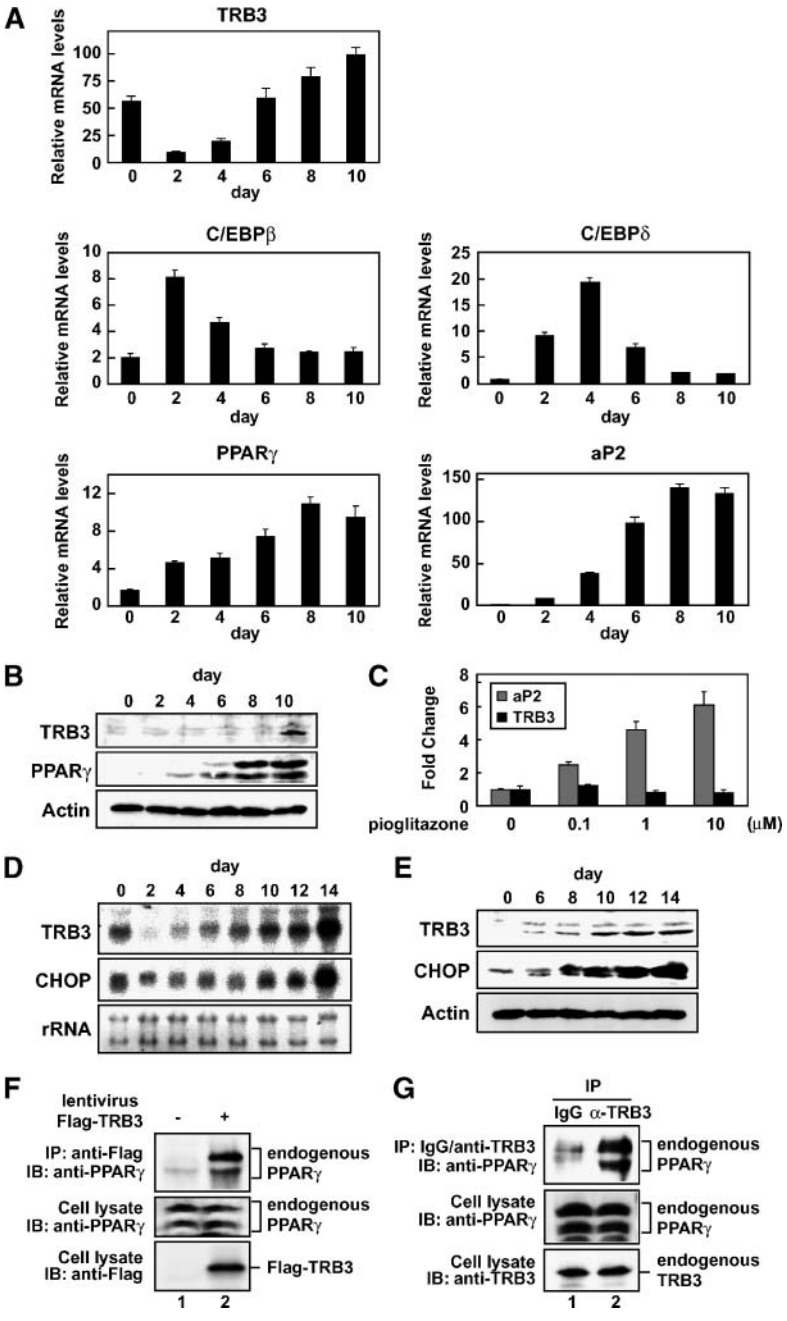

Fig. 3. Changes in TRB3 mRNA and protein expression during adipocyte differentiation in 3T3-L1 cells. Total RNA was isolated from 3T3-L1 cells on the indicated days after treatment with a differentiation medium (see Materials and Methods). A: Relative mRNA levels of TRB3, CCAAT/enhancer binding protein (C/EBP)β, C/EBPδ, PPARγ, and aP2 were determined by real-time RT-PCR after being normalized to S17 mRNA. The data represent means ± SD. B: 3T3-L1 cells were harvested on the indicated days during adipocyte differentiation, and whole cell lysates were subjected to SDS-PAGE and Western blot analysis with anti-TRB3, anti-PPARγ, and anti-actin antibodies. C: Pioglitazone was added to the medium on day 2. 3T3-L1 cells were harvested on day 3, and the relative mRNA levels of aP2 and TRB3 were determined by real-time RT-PCR after being normalized to S17 mRNA. Relative mRNA levels of aP2 and TRB3 in the absence of pioglitazone are considered as 1. The data represent means ± SD. D: Total RNA obtained on the indicated days was subjected to electrophoresis and blot hybridization with the indicated ^{32}P-labeled probes. E: Whole cell lysates were subjected to SDS-PAGE and Western blot analysis with anti-TRB3, anti-actin, and anti-C/EBP homologous protein (CHOP) antibodies. F: 3T3-L1 cells were infected with either an empty (mock) or human TRB3 expressing lentiviral vector at 4 days after differentiation into adipocytes. The cells were harvested after 4 days of infection, and total cell lysates were subjected to immunoprecipitation using anti-Flag antibodies. Both lysates and precipitates were subjected to SDS-PAGE and Western blot analysis with anti-Flag and anti-PPARγ antibodies. G: 3T3-L1 cells were harvested at 10 days after differentiation into adipocytes, and whole cell lysates were subjected to immunoprecipitation using anti-TRB3 antibodies. Both the lysates and precipitates were subjected to SDS-PAGE and Western blot analysis with anti-TRB3 and anti-PPARγ antibodies. All of the values given are averages of data from three experiments performed in triplicate. IB, immunoblot; IP, immunoprecipitate.

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differentiation, decreased gradually after being transiently induced (day 2 and day 4, respectively), and their expression patterns were quite different from that of TRB3 (Fig. 3A). The mRNA levels of PPARγ and aP2, a PPARγ target gene (28), increased gradually and reached a peak on day 8 (Fig. 3A; see supplementary Fig. IIA). We further confirmed TRB3 protein expression during differentiation with Western blotting. A commercial antibody against mouse TRB3 recognized endogenous TRB3 protein on day 10 (Fig. 3B). Although mRNA levels of TRB3 were relatively high on day 0, its protein levels were not correspondingly high. The reason is still unclear, but it can be speculated that TRB3 expression is regulated either translationally or posttranslationally.

Because TRB3 gene expression is induced during differentiation and it has been reported that the TRB3 promoter contains a PPRE and is activated by PPARα in the liver (29), we next examined whether TRB3 gene expression in adipocytes is augmented by PPARγ, a member of the PPAR family expressed exclusively in adipocytes. Treatment of 3T3-L1 cells with pioglitazone, a potent agonist of PPARγ, unexpectedly failed to stimulate TRB3 gene expression during adipocyte differentiation, unlike aP2 gene expression, which was stimulated (Fig. 3C). These results indicate that the expression of TRB3 is accelerated during adipocyte differentiation in a PPARγ-independent manner. As our previous study had shown that TRB3 expression is induced during ER stress in a CHOP-dependent manner, CHOP mRNA and protein expression during adipocyte differentiation was examined. As shown in Fig. 3D, E, the induction pattern of CHOP mRNA and protein closely resembles that of TRB3.

Because endogenous TRB3 and PPARγ proteins coexist at the late stages of differentiation, as shown in Fig. 3B, we then performed immunoprecipitation to verify whether TRB3 interacts physically with endogenous PPARγ in 3T3-L1 adipocytes. First, we performed immunoprecipitation of the total lysates of 3T3-L1 cells expressing the lentivirus-mediated Flag-TRB3. When cells were immunoprecipitated with anti-Flag antibody, endogenous PPARγ was discovered in the immunoprecipitation (Fig. 3F). Moreover, PPARγ was coimmunoprecipitatable with TRB3 using anti-TRB3 antibodies from the lysates of 3T3-L1 cells after 10 days of differentiation (Fig. 3G), indicating that endogenous TRB3 and PPARγ interacted physically in 3T3-L1 adipocytes.

These findings suggest that both TRB3 and PPARγ coexist in the differentiating 3T3-L1 cells and thus would have the opportunity to communicate with each other, thereby affecting PPARγ target gene expression during adipogenesis.

TRB3 overexpression prevents adipocyte differentiation in 3T3-L1 cells

To further investigate the function of TRB3 in adipocyte differentiation, we next overexpressed TRB3 in 3T3-L1 preadipocytes by retroviral infection. At 7 days after infection, the cells were subjected to differentiation into adipocytes, and TRB3 protein expression on day 6 was confirmed by Western blot analysis (**Fig. 4A**). The protein expression of perilipin was reduced by TRB3 overexpression, consistent with the results in Fig. 1 (Fig. 4A). We assessed the degree of adipocyte differentiation in the cells on day 8 by Oil Red O staining and found a reduced interacellular lipid accumulation in the TRB3-expressing cells compared with the controls (Fig. 4B). This was further confirmed by direct determination of the intracellular triglyceride content on days 6 and 9 (Fig. 4C). On the other hand, TRB3ΔAkt, which did not interact with PPARγ and failed to suppress perilipin promoter activities (Figs. 1C, 2D), did not have any effect on either perilipin protein expression or intracellular lipid accumulation (Fig. 4A, B). These results clearly indicate that TRB3 overexpression delays or prevents adipocyte differentiation.

When the expression of several genes induced during adipocyte differentiation was compared in these two cells, we found that the mRNA levels of aP2, perilipin, adiponectin, and C/EBPα, which are all PPARγ target genes (9, 21, 28, 30), were reduced in the TRB3-expressing cells (Fig. 4D). On the other hand, mRNA expression of SREBP-1, which is thought to act at an early stage of differentiation before PPARγ activation (14), was hardly affected by TRB3 overexpression throughout the course of differentiation (Fig. 4D). PPARγ gene expression was reduced in the TRB3-expressing cells only on days 2 and 4, and the decrease was marginal compared with the large decline in PPARγ target genes (Fig. 4D). Gene expression of C/EBPβ and C/EBPδ, which are required for an early stage of adipogenesis and are not under the control of PPARγ, was scarcely affected by the overexpressed TRB3 (Fig. 4D). These findings indicate that TRB3 functions as a negative regulator of adipocyte differentiation as a result of the suppression of PPARγ target gene expression.

To more completely verify TRB3 function in adipocyte differentiation, we next established a stable cell line that can be made to induce TRB3 expression for a shorter period by the use of the Tet-Off system. Removing doxycycline from the medium on day −5 enabled the cells to express exogenous Flag-TRB3 on day 0 (see supplementary Fig. IIIA). When doxycycline was added to the medium, TRB3 protein expression was suppressed completely. When both the TRB3-infected and mock-infected cells were differentiated in the absence of doxycycline, TRB3 overexpression reduced the intracellular lipid accumulation (see supplementary Fig. IIIB, C). In addition, forced TRB3 expression also suppressed the gene expression of aP2, perilipin, and adiponectin, but not SREBP-1 (see supplementary Fig. IIID). These findings are completely consistent with those observed in cells infected with the retroviral TRB3 expression vector. Therefore, we conclude that the TRB3 highly expressed in 3T3-L1 cells delays or inhibits adipocyte differentiation.

TRB3 knockdown stimulates adipocyte differentiation in 3T3-L1 cells

We subsequently examined the functions of endogenous TRB3 in adipocyte differentiation by RNA interference. First, we modified 3T3-L1 cells through lentiviral infection with a lentiviral vector expressing shRNA to

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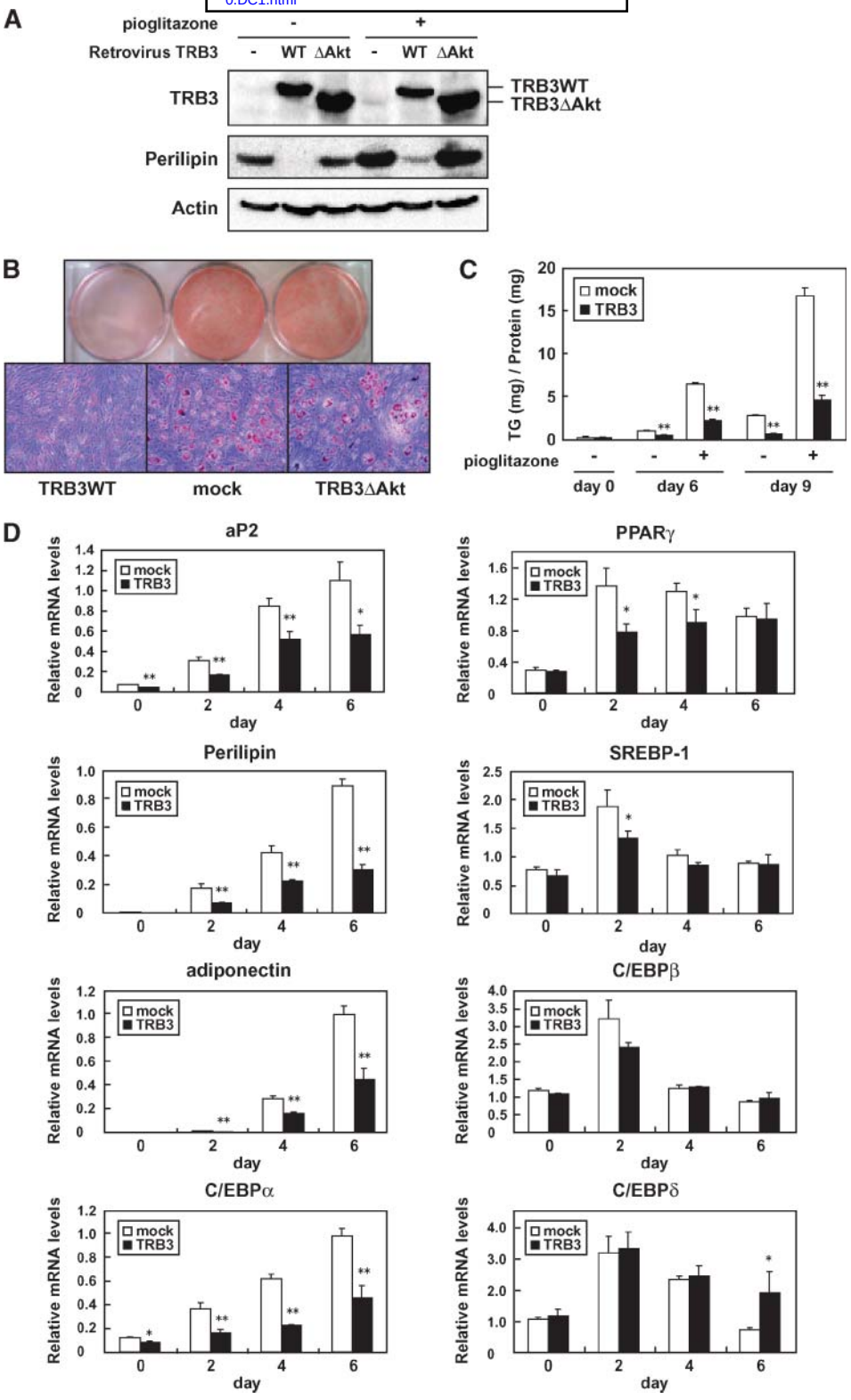

Fig. 4. TRB3 overexpression by retroviral infection in 3T3-L1 cells during adipocyte differentiation. 3T3-L1 cells infected with an empty (mock), human TRB3, or TRB3ΔAkt-expressing retroviral vector differentiated into adipocytes at 7 days after infection. A: On day 6, the cells were harvested and whole cell lysates were subjected to SDS-PAGE and Western blot analysis with anti-human TRB3, anti-perilipin, and anti-actin antibodies. B: Oil Red O staining of the cells was performed on day 8. C: The amounts of intracellular triacylglyceride (TG) were quantified on days 0, 6, and 9. The data represent means ± SD. D: The cells were harvested on the indicated days, and relative mRNA levels of aP2, perilipin, adiponectin, C/EBPα, PPARγ, sterol-regulatory element binding protein-1 (SREBP-1), C/EBPβ, and C/EBPδ were determined by real-time RT-PCR after being normalized to S17 mRNA. The data represent means ± SD. All of the values given are averages of data from three experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

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achieve long-term knockdown of TRB3 expression. On day 0, the mRNA level of TRB3 was reduced by >60% in the cells infected with a lentiviral vector for TRB3-specific shRNA (**Fig. 5A**, left). The knockdown effect of TRB3 on the protein levels was also confirmed by Western blot analysis (Fig. 5A, right). These cells were differentiated into adipocytes, and on day 10, the greater amount of intracellular lipid accumulation in the TRB3 knockdown cells was confirmed by Oil Red O staining (Fig. 5B) or by direct measurement of the intracellular triglyceride content (Fig. 5C). On day 6, when TRB3 shRNA was still effective and TRB3 gene expression was suppressed significantly in the presence or absence of a potent PPARγ agonist, pioglitazone (Fig. 5D), the expression of PPARγ target genes was stimulated in the TRB3 knockdown cells (Fig. 5D). Moreover, when TRB3 gene expression in 3T3-L1 cells was knocked down by double-stranded TRB3-specific RNA oligonucleotides, similar results were obtained (see supplementary Fig. IVA–C). These data clearly show that endogenous TRB3 acts as a negative regulator of adipocyte differentiation through the suppression of PPARγ target gene expression.

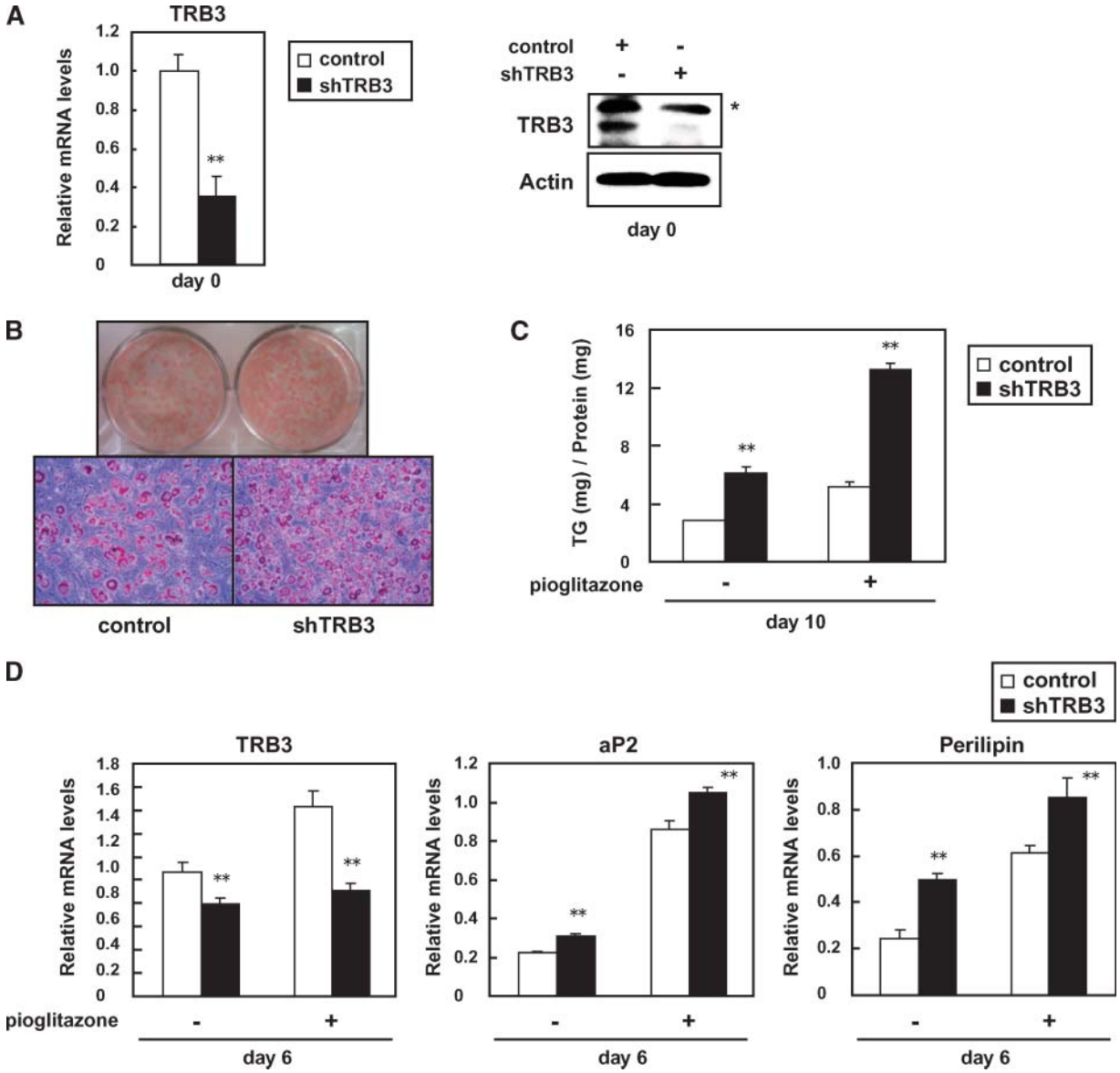

Fig. 5. Effect of the knockdown of endogenous TRB3 by lentiviral infection during adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells differentiated into adipocytes at 7 days after being infected with a lentiviral vector expressing short hairpin RNA (shRNA) for TRB3 or control shRNA. A: Knockdown efficiency of TRB3 expression levels in 3T3-L1 cells on day 0 was quantified by real-time RT-PCR or Western blot analysis with anti-TRB3 and anti-actin antibodies. The asterisks in the top panel represent nonspecific bands. B: Oil Red O staining of the cells was performed on day 10. C: The amounts of intracellular triglyceride (TG) were quantified on day 10. The data represent means ± SD. The cells were incubated with or without 10 μM pioglitazone from day 0 to day 2. D: 3T3-L1 cells were harvested on day 6, and relative mRNA levels of TRB3, aP2, and perilipin were determined by real-time RT-PCR after being normalized to S17 mRNA. The data represent means ± SD. All of the values given are averages of data from three experiments performed in triplicate. ** $P < 0.01$.

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TRB3 blocks PPARγ-mediated adipocyte differentiation in 3T3-L1 cells

Because TRB3 has been reported to inhibit Akt activity (16, 17), it is possible that the inhibitory effect of TRB3 on adipocyte differentiation might be mediated by Akt inactivation. To verify this issue, we assessed insulin-stimulated Akt activity in TRB3-overexpressing 3T3-L1 cells. TRB3 scarcely affected the phosphorylation of Akt at serine-473 but decreased the phosphorylation of Akt at threonine-308 (**Fig. 6A**). This suggests that the inhibitory effect on adipocyte differentiation is mediated, at least partially, by Akt inactivation.

Next, we generated PPARγ-overexpressing 3T3-L1 cells by lentiviral infection to examine whether TRB3 could suppress PPARγ-dependent adipocyte differentiation. PPARγ-expressing 3T3-L1 cells differentiated into adipocytes without hormonal stimulation, as is the case with PPARγ-expressing NIH-3T3 cells (5) (Fig. 6B). When coinfected with TRB3, triglyceride accumulation was reduced in both the absence and presence of troglitazone (Fig. 6B) and mRNA levels of aP2, perilipin, and adiponectin were all decreased (Fig. 6C), whereas the expression of PPARγ was not affected (Fig. 6C, D). These results clearly demonstrate that the inhibitory effect of TRB3 on adipogenesis is certainly mediated by PPARγ inactivation, independent of the upstream signaling pathway, including Akt.

DISCUSSION

The current study clearly demonstrates that TRB3 inhibits adipocyte differentiation by impairing PPARγ transcriptional activity, indicating that TRB3 functions as a novel antiadipogenic factor. It is noteworthy that TRB3 governs adipogenesis by suppressing the activity of PPARγ, a master regulator of adipocyte differentiation, through protein-protein interaction. Based on the results obtained using the heterologous Gal-4 system, the interaction directly suppressed PPARγ transcriptional activity, not its DNA binding activity. It is likely that the recruitment of certain coactivators required for the transactivation of PPARγ during adipogenesis is blocked by the TRB3 association. Considering that the expression of TRB3 is induced during differentiation (Fig. 3) and has an inhibitory effect on adipogenesis in PPARγ-dependent differentiated 3T3-L1 cells (Fig. 6), it is suggested that TRB3 is active in the abnormally and excessively differentiated adipocytes that are found in obesity and metabolic syndrome.

There are many antiadipogenic factors reported, such as GATA factors, KLF2, KLF7, CHOP, Pref-1, and Wnt10b (31, 32). The expression of all of these factors decreases at an early stage of differentiation. Because mRNA levels of TRB3 are decreased, as are those of other antiadipogenic factors (Fig. 3A, D; see supplementary Fig. IIB), the decline in TRB3 at an early stage is expected to contribute to an acceleration of differentiation. Also, it makes sense that TRB3 induced at the later stage of differentiation works as a brake on adipogenesis by inhibiting PPARγ for the purpose of preventing excessive progression. Overall, TRB3 would function as a negative regulator of adipogenesis throughout differentiation; however, its physiological significance is likely to be different between the early and the later stages of differentiation.

As TRB3 was originally discovered as an inhibitory factor for Akt in the insulin signaling pathway, one can speculate that TRB3 represses the adipocyte differentiation initiated by insulin activity. We show here that TRB3 mRNA levels at the beginning of differentiation are relatively high (Fig. 3A) and confirm that Akt activity at threonine-308 was partially impaired in TRB3-overexpressing 3T3-L1 cells (Fig. 6A). Recently, on the other hand, it was reported that TRB3 blocks C/EBPβ transcriptional activities (33). Because the expression of TRB3 is inversely correlated with that of C/EBPβ at the early stage of differentiation, the decline in TRB3 could be favorable to the activation of C/EBPβ and the subsequent progression of adipogenesis. In this report, TRB3 did not affect Akt activity during differentiation in 3T3-L1 cells, but it is likely that TRB3 is an antiadipogenic factor at the time of the onset of adipogenesis. Indeed, when TRB3 was overexpressed in 3T3-L1 cells by retroviral infection, the gene expression of C/EBPα was suppressed on days 0 and 2, before the induction of PPARγ gene expression (Fig. 4). This suggests that TRB3 blocks the signaling pathway upstream of PPARγ and depresses C/EBPα gene expression, thereafter diminishing the gene expression of PPARγ, a C/EBPα target gene, on days 2 and 4. However, overexpression of TRB3 did not lead to significant changes in the gene expression of SREBP-1 (Fig. 4), suggesting that Akt activity was not so impaired by TRB3 during differentiation. Considering that TRB3 blocks PPARγ-dependent adipocyte differentiation (Fig. 6), it is suggested that TRB3 functions not only at the early stage of differentiation by inhibiting C/EBPβ but also at the later stage of differentiation by inhibiting PPARγ transcriptional activities; this is also supported by the fact that TRB3 is induced right after the activation of PPARγ (Fig. 3).

It has also been reported that TRB3 promotes acetyl-coenzyme A carboxylase 1 ubiquitination through an association with the E3 ubiquitin ligase, COP1, and stimulates its degradation, reducing triglyceride synthesis in adipocytes (20). Transgenic mice expressing TRB3 in adipose tissue are lean and protected from diet-induced obesity. Because TRB3 expression in these mice is under the control of the aP2 gene promoter, thereby being fully stimulated in late adipogenesis, the observed phenotype does not allow us to evaluate TRB3 function during adipocyte differentiation, but it does suggest that TRB3 works as an antiadipogenic factor in the later stages of differentiation after PPARγ is activated.

As far as PPARγ is concerned, there were no changes in the exogenous PPARγ protein level by the enforced expression of TRB3 (Fig. 6D; see supplementary Fig. I), suggesting that their interaction results in a suppression of PPARγ transcriptional activity, not in increased degradation. Therefore, it is evident that TRB3 is a multifunctional protein that represses adipocyte differentiation and diminishes triglyceride accumulation in adipocytes.

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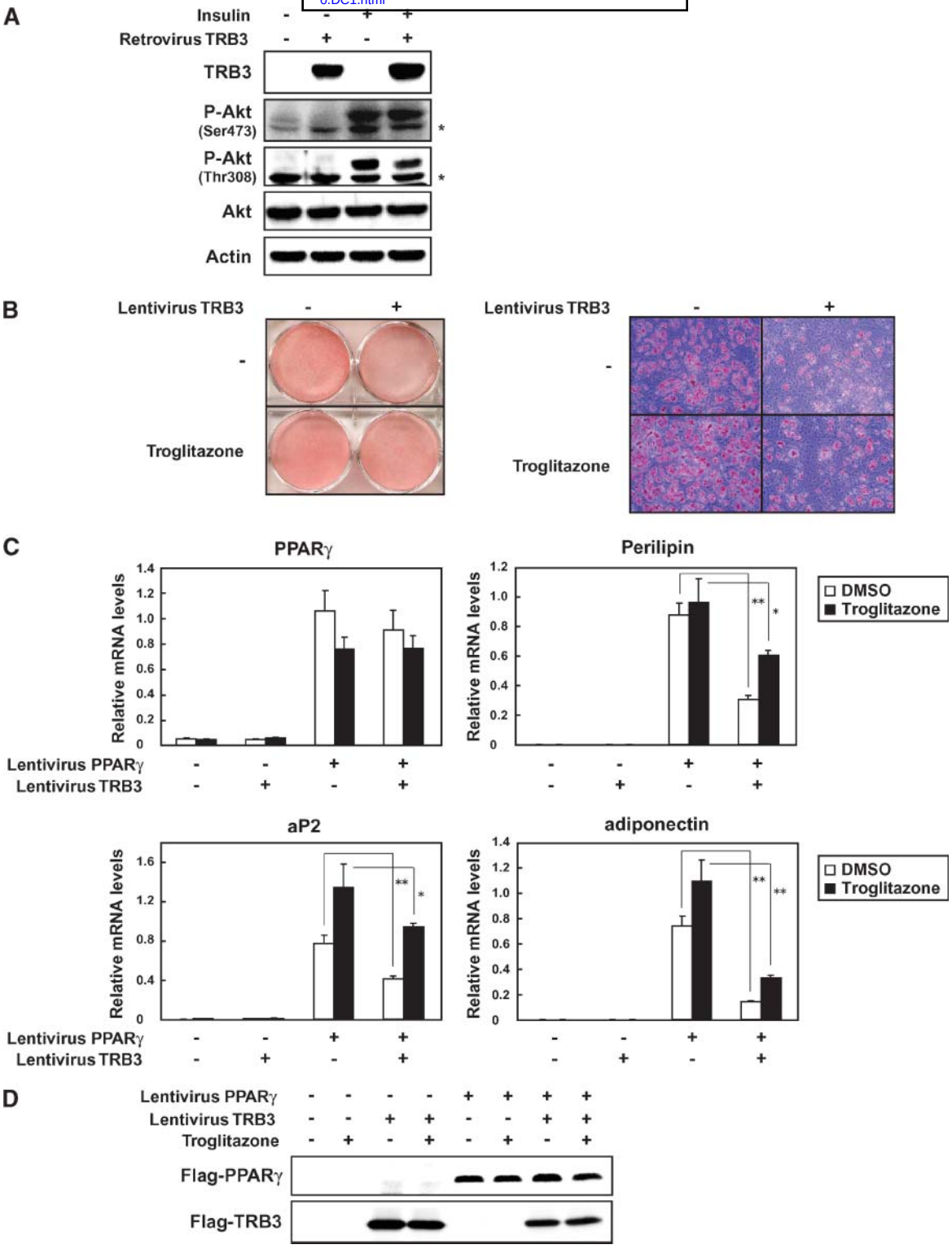

Fig. 6. TRB3 inhibits PPARγ-dependent adipocyte differentiation in 3T3-L1 cells. A: 3T3-L1 cells were infected with a retroviral vector expressing TRB3, and 7 days after infection, the cells were serum-starved for 4 h and then treated with 1 μg/ml insulin for 2 h. The cells were harvested, and whole cell lysates were subjected to SDS-PAGE and Western blot analysis with anti-human TRB3, anti-phospho-Akt (at serine-473 and threonine-308), anti-Akt, and anti-actin antibodies. The asterisks represent nonspecific bands. B: 3T3-L1 cells were infected with PPARγ-expressing lentiviral vector together with either mock or TRB3-expressing lentiviral vector. After 3 days of infection, the cells were cultured with or without 10 μM troglitazone for 9 days. Then, cells were harvested and Oil Red O staining was performed. C, D: 3T3-L1 cells were infected with PPARγ-expressing lentiviral vector together with either mock or TRB3-expressing lentiviral vector. After 3 days of infection, the cells were cultured with or without 10 μM troglitazone for 16 h. Then, the cells were harvested and the mRNA levels of PPARγ, aP2, perilipin, and adiponectin were quantified by real-time RT-PCR (C) and protein levels of PPARγ and TRB3 were determined by Western blot analysis with anti-Flag antibody (D). The data represent means ± SD. All of the values given are averages of data from three experiments performed in triplicate. * $P < 0.05$, ** $P < 0.01$.

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We previously showed that ER stress induces TRB3 gene expression (18). Obesity causes ER stress, and this impairs insulin signaling by excessive serine phosphorylation of insulin receptor substrate-1 (34). On the other hand, a reduction of ER stress by chemical chaperones restores insulin sensitivity in the liver, skeletal muscle, and adipose tissue of diabetic *ob/ob* mice (35). Thus, TRB3 induced by ER stress might prevent adipocytes from enhancing further excessive differentiation and accumulating more triglyceride. Indeed, transgenic mice expressing TRB3 in adipose tissue are resistant to diet-induced obesity and have improved insulin sensitivity (20). However, in the liver, overexpression of TRB3 results in hyperglycemia and glucose intolerance, and knockdown of TRB3 improves glucose tolerance, indicating that TRB3 helps trigger type II diabetes (16). Therefore, it can be speculated that TRB3 functions differently in distinct tissues.

Recently, TRB3-deficient mice were generated and displayed no evident abnormalities (36). This implies that TRB3 does not have a major role in energy homeostasis, at least under normal nutrient conditions. Although the reason why TRB3-deficient mice display normal energy homeostasis is inexplicable at present, it is speculated that other genes compensate for the lack of TRB3, or that TRB3 is only functional when induced by various specific stresses. The role of TRB3 may become clear when TRB3-deficient mice are fed a high-fat diet or crossed with certain disease models, such as *ob/ob* mice.

We show here that TRB3 interacts with the ligand-independent AF-1 and ligand-dependent AF-2 domains of PPARγ (Fig. 2) and thereby impairs its transcriptional activities. The suppressed transcriptional activity of PPARγ attributable to the interaction with TRB3 was clearly observed when the luciferase assays were carried out in 3T3-L1 preadipocytes rather than in HEK293, HepG2, and HeLa cells (data not shown). Although several different tissues, including hepatocytes, express PPARγ, it is possible that the TRB3-mediated suppression of PPARγ transcriptional activities might occur exclusively in the adipocytes in which PPARγ2, an adipocyte-specific isoform, and its essential cofactors are coexpressed. This might be another example of the cell-specific TRB3 function discussed above. Further study will be necessary to resolve the underlying diversity of TRB3 functions.

In the current study, we elucidated the roles of TRB3 in preadipocytes and adipocytes during differentiation. At present, little is known about the precise functions of TRB3 in hypertrophic adipose cells in obesity. Because TRB3 and PPARγ coexist in mature adipocytes and mouse white adipose tissues (data not shown), TRB3 is likely to modulate the character of adipose cells by modifying PPARγ activities. Thus, TRB3 is potentially an intriguing therapeutic target for the treatment of insulin resistance and obesity.

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