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TRB2, a Mouse Tribbles Ortholog, Suppresses Adipocyte Differentiation by Inhibiting AKT and C/EBPβ*[S]

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Adipocyte differentiation is regulated by a complex array of extracelluar signals, intracellular mediators and transcription factors. Here we describe suppression of adipocyte differentiation by TRBs, mammalian orthologs of *Drosophila* Tribbles. Whereas all the three TRBs were expressed in 3T3-L1 preadipocytes, TRB2 and TRB3, but not TRB1, were immediately down-regulated by differentiation stimuli. Forced expression of TRB2 and TRB3 inhibited adipocyte differentiation at an early stage. Akt activation is a key event in adipogenesis and was severely inhibited by TRB3 in 3T3-L1 cells. However, the inhibition by TRB2 was mild compared with severe inhibition by TRB3, though TRB2 suppressed adipogenesis as strongly as TRB3. Interestingly, TRB2 but not TRB3 reduced the level of C/EBPβ, a transcription factor required for an early stage of adipogenesis, through a proteasome-dependent mechanism. Furthermore, knockdown of endogenous TRB2 by siRNA allowed 3T3-L1 cells to differentiate without full differentiation stimuli. These results suggest that inhibition of Akt activation in combination with degradation of C/EBPβ is the basis for the strong inhibitory effect of TRB2 on adipogenesis.

Adipocytes play a key role for energy homeostasis by storing and releasing surplus energy. Adipose tissues are categorized into two functionally different types: white adipose tissue (WAT)[3] stores excess energy as triglycerides and releases fatty acids in response to energy needs (1), while brown adipose tissue (BAT) releases energy as heat by uncoupling oxidative phosphorylation from the respiratory chain (2). In addition, adipose tissues form an endocrine system by secreting adipokines that regulate energy homeostasis (3). Alterations in adipose tissues, either excess or deficiency, are associated with metabolic disorders (4).

In the past decade, genetic studies using knock-out mice have identified several key transcription factors that regulate adipocyte development. CCAAT/enhancer-binding proteins (C/EBPs) belong to the large family of bZIP transcription factors. C/EBPα knock-out mice show reduced fat accumulation in both WAT and BAT (5). Whereas adipogenesis is only slightly impaired in mice deficient for either C/EBPβ or C/EBPδ, C/EBPβ/δ double knock-out mice show severe defects in BAT development (6). Peroxisome proliferator-activated receptor γ (PPARγ), a member of nuclear hormone receptors superfamily, is also known to be required for adipocyte differentiation (7). Besides genetic evidences *in vivo*, these transcription factors have been characterized *in vitro* by using cultured mouse embryonic fibroblasts (MEFs) and established cell lines such as 3T3-L1 and 3T3-F442A (8, 9). In the adipocyte differentiation *in vitro*, expression of C/EBPβ and C/EBPδ is immediately up-regulated in response to differentiation stimuli (10). Upon induction of differentiation, preadipocytes undergo synchronous cell division, which is termed mitotic clonal expansion (MCE). MCE is required for subsequent adipose conversion and is a C/EBPβ-dependent process (11). After several rounds of cell cycle, expression of C/EBPα and PPARγ is induced, which then stimulates the expression of adipogenic genes (10).

In addition to transcriptional networks, sets of signal cascades have been reported to modulate the adipogenic program. Mitogen-activated protein (MAP) kinases, ERK, p38 and JNK, coordinate adipogenesis positively or negatively (12). Wnt-10b is expressed in preadipocytes, and the Wnt/β-catenin pathway negatively regulates early adipogenesis (13). Insulin and IGF signals are known to affect adipogenesis (14), and mice deficient for IRSs or Akt, components of the insulin-signal cascade, show impaired adipogenesis (15–17). Thus, adipogenesis is a complex process that is coordinated by positive and negative signals.

In this study we describe roles of mammalian orthologs of Tribbles in adipogenesis. Tribbles was originally described in *Drosophila* as a regulator of Sting/CDC25 and three mammalian orthologs were subsequently found. They share a conserved motif highly homologous to serine/threonine kinases; however they lack key residues required for kinase activity (18–20). In *Drosophila* oogenesis, Tribbles acts as a negative regulator for Slbo, a *Drosophila* ortholog of mammalian C/EBPs (21). Mouse TRB3 has been reported to be an Akt-binding protein and inhibit the activation of Akt by IGF in hepatocytes (22). Human TRB1 and TRB3 associate with MAP kinase kinase and modulate MAP kinase cascades in a dose-dependent mechanism (23). TRB2 was originally identified as a phosphorylated

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[3] The abbreviations used are: WAT, white adipose tissue; BAT, brown adipose tissue; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; Dex, dexamethasone; MCE, mitotic clonal expansion; PPARγ, peroxisome proliferator-activated receptor γ; Mix, methyl-isobutyl-xanthine.

protein induced by thyroid hormone in dog thyroid cells (24). Overexpression of TRB2 also inhibited Akt activation (22), while its biological function still remains unknown. Here we show that TRB2 and TRB3 exhibit anti-adipogenic effects through distinct mechanisms, *i.e.* both TRB2 and TRB3 inhibit Akt activation and TRB2 promotes proteasome-dependent degradation of C/EBPβ.

EXPERIMENTAL PROCEDURES

Cell Culture and Differentiation—3T3-L1 preadipocytes were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine serum until confluence and maintained in the same medium for another 2 days. Adipogenesis were induced by a protocol previously described (25), in brief, cells were then induced to differentiate in DMEM containing 10% fetal bovine serum (FBS), 0.5 mM methyl-isobutylxanthine (Mix), 1 μM dexamethasone (Dex), and 5 μg/ml insulin. After 2 days, the medium was switched to DMEM containing 10% FBS and 5 μg/ml insulin. Two days later, cells were shifted to DMEM containing 10% FBS. Eight days after the addition of MDI, cells were fixed with 4% formaldehyde and stained with Oil-Red-O.

Retroviral experiments were performed as follows. PLATE cells were transfected with 4 μg of pMX vectors at 60% confluence. After 2 days, the retroviral supernatant was centrifuged and resuspended in fresh medium containing 8 μg of polybrene. 3T3-L1 cells were plated prior to infection and then incubated with viral medium for 24 h. Infected cells were propagated for each experiment. In experiments using C/EBPβ or PPARγ, control or TRB-expressing cells were sorted by a FACS vantage and then infected with virus for expression of C/EBPβ or PPARγ. Cells were induced to adipogenesis with MDI and troglitarizone. MEFs were prepared form E 14.5 mouse embryo by standard protocol. MEFs were infected with virus and induced to differentiate as described above.

Plasmids Construction—Mouse TRB1, TRB2, and TRB3 cDNAs were amplified by PCR with the sets of primers TN068 (5′-GGATCCGAATGCGGGTCGGTCCCGTG-3′) and TN049 (5′-AGAGAGGCGACACCACATTG-3′), TN069 (5′-GGATCCGAATGAACATACACAGGTC-3′), and TN045 (5′-CCCATGCTACGTGTTCCGTC-3′), TN070 (5′-GGATCCGAATGCGAGCTACACCTCTG-3′), and TN047 (AGGCACAGGAACGAATAAGG-3′), respectively. In addition to full-length TRB2, we obtained one clone that was alternatively spliced and had a premature termination codon at the 242 amino acid position. We used this construct as a C-terminal-truncated TRB2 (TRB2ΔC). To construct FLAG-tagged TRBs, BamHI-NotI-digested fragments were cloned into the BamHI-NotI sites of the pCDNA3-FLAG vector. To generate retroviral vectors expressing FLAG-tagged TRBs, HindIII-NotI fragments were subcloned into pBluescript, and then SalI-NotI-digested fragments were cloned into the XhoI-NotI-linearized pMX vector. C/EBPβ expressing construct used for immunoprecipitation experiments was generated as follows. The coding region of C/EBPβ was amplified by PCR with sets of primers TN099 (5′-GAATTCATGCACCGCCTGCTGGCCTG-3′) TN198 (5′-CTAGCAGTGGCCCGCCGAGGC-3′) and then cloned into the pCDNA3 vector.

Northern Blot and Quantitative PCR Analysis—Total RNA was prepared from cells with TRIzol reagent (Invitrogen). 10 μg of each RNA samples was loaded on a 1% agarose gel containing 5.5% formaldehyde and resolved by electrophoresis. RNA was transferred to a nylon membrane and then hybridized with dioxygenin (DIG)-labeled antisense probe. After washing and blocking, membrane was incubated with AP-conjugated anti-DIG antibodies, and the signal was detected by CDP-star. For quantitative PCR, total RNA was prepared from cells with TRIzol. After DNase treatment, cDNA was generated and analyzed by Light Cycler (Roche Applied Sciences) according to the manufacturer's protocol. GAPDH was used as an internal standard.

BrdU Labeling—Cells were plated on glass coverslips and cultured until confluence. Confluent cells were maintained for another 2 days, induced to differentiate with MDI for 12 h, and then labeled for 12 h with 10 μM BrdU. Cells were washed with phosphate-buffered saline and fixed in 70% ethanol for 20 min. After denaturation (2 N HCl for 20 min) and neutralization (0.1 M sodium borate, pH 8.5), cells were incubated with anti-BrdU monoclonal antibody and subsequently probed with FITC-conjugated secondary antibody. A coverslip was mounted on a slide glass with GEL/MOUNT containing Hoechst 33342 and analyzed by microscopy. Two independent experiments were performed. To examine the percentage of BrdU positive cells, four fields (more than 1000 cells) were counted.

Immunoprecipitation and Western Blot Analysis—Cells were washed with cold phosphate-buffered saline and then solubilized in lysis buffer (50 mM Tris-HCl, 2 mM EDTA, 100 mM NaCl, 50 mM NaF, 30 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin, 0.075 units/ml aprotinin, 1 mM dithiothreitol, and 1% Triton X-100). Extracts were centrifuged at 15,000 rpm for 30 min at 4 °C. Protein concentrations were determined by the Bio-Rad protein assay. The supernatants were incubated at 4 °C overnight with protein G-Sepharose beads bounded with anti-FLAG (M2, Sigma) antibody. Immunoprecipitates were washed with lysis buffer and boiled in 1× SDS-PAGE sample buffer. Proteins were resolved by SDS-PAGE and transferred to a nylon membrane. After blocking procedure, proteins were immunoblotted with anti-Akt (Cell Signaling), anti-phospho-Akt (Thr^{308}, Ser^{437}, Cell Signaling), anti-C/EBPβ (C-19, Santa Cruz Biotechnology), and anti-FLAG antibodies and detected by enhanced chemiluminescence (PerkinElmer Life Sciences). Experiments were performed independently for three times, and the levels of Akt phosphorylation and LAP protein were quantified by NIH Image.

RNA Interference—Expression virus vectors for luciferase and TRB2 shRNA were generated as follows. Target sequence, Luc (5′-GGATGAAGAGAGGACTCGT-3′) or TRB2 (5′-GGATGAAGAGAGGACTCGT-3′), was ligated with the linearized pSIREN-RetroQ vector (BD) as described by the manufacturer. Retroviral medium was prepared as described above.

RESULTS

Mouse Tribbles Orthologs Are Differentially Regulated during Adipogenesis—It has been shown that insulin and IGF are key molecules in early adipogenesis (14). Whereas TRB3 was pre-

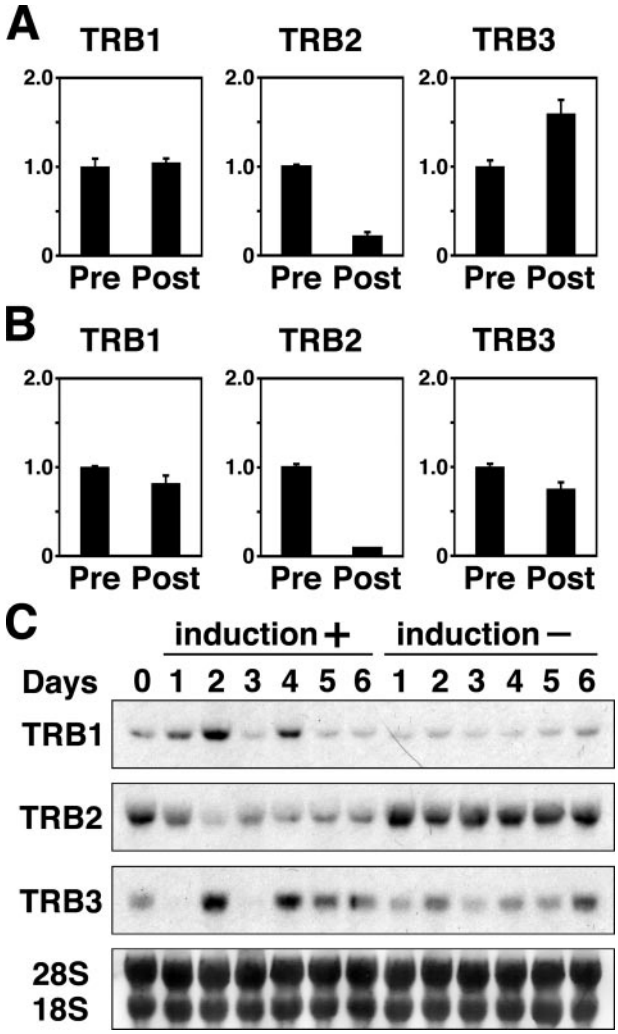

FIGURE 1. **Expression of TRBs during differentiation.** *A*, 3T3-L1 preadipocytes were induced to differentiate as described under "Experimental Procedures." Total RNA was extracted from cells prior to induction (*Pre*) or 6 days after induction (*Post*) and then subjected to quantitative PCR analysis. *B*, expression of TRBs in MEFs were determined by quantitative PCR as described in *A*. *C*, 3T3-L1 cells were grown to confluence and cultured for another 2 days (*Day 0*). Cells were induced to differentiate (*induction* +) or cultured in the medium without differentiation stimuli. Total RNA was extracted at the indicated days and then analyzed by Northern blot.

viously shown to modulate Akt-mediated insulin signaling (22), it remained unclear whether TRB3 is integrated in the adipogenic program. To investigate a possible involvement of TRB3 in adipogenesis, we first examined the expression of TRB3, together with two other mouse Tribbles orthologs, TRB1 and TRB2, during adipocyte differentiation. 3T3-L1 preadipocytes and mouse embryonic fibroblasts (MEFs) were induced to differentiate with Mix, Dex, and insulin (MDI) and then analyzed by quantitative PCR. As shown in Fig. 1*A*, TRB2 but not TRB1 or TRB3 was reduced in differentiated 3T3-L1 cells as compared with undifferentiated cells. TRB2 was also decreased in MEF cells that were cultured with MDI (Fig. 1*B*). To understand the regulatory mechanism of TRB expression during adipogenesis, we examined the mRNA levels of TRBs at various points. TRB3 was expressed in 3T3-L1 preadipocytes and disappeared 1 day after MDI treatment (Fig. 1*C*, *day 1*). Interestingly, however, TRB3 expression was oscillated by medium change; the expression was restored and reached a level higher than undifferentiated cells next day (Fig. 1*C*, *day 2*). It was reduced again when fresh medium was added at day 3 and increased again in the following day (Fig. 1*C*, *days 3* and *4*). As compared with TRB3, TRB1 and TRB2 showed distinct expression patterns. TRB1 was increased at *day 2* and *day 4*, whereas TRB2 was reduced by differentiation stimuli, and its expression remained low throughout the differentiation process. TRB3 expression was also oscillated in the absence of MDI; however the extent was smaller than that in differentiating cells, whereas TRB1 and TRB2 were not altered.

Enforced Expression of TRB2 or TRB3 Inhibits Differentiation of 3T3-L1 Preadipocytes—To investigate whether the expression of TRB2 and TRB3 affects adipocyte differentiation, we created cells stably expressing TRB2 or TRB3. 3T3-L1 preadipocytes were infected with virus carrying the cDNA for TRB2 or TRB3. Constitutive expression of TRBs did not affect the growth of these cells before differentiation (data not shown). Infected cells were grown to confluence and then induced to differentiate with MDI. Eight days after induction, control cells harbored lipid droplets as visualized by Oil-Red-O staining, whereas lipid accumulation was severely impaired in cells expressing TRB2 or TRB3 (Fig. 2*A*). Consistent with the defects in adipogenesis, expression of aP2, an adipogenic gene encoding a fatty acid-binding protein, was reduced in both TRB2- and TRB3-expressing cells (Fig. 2*B*). Furthermore, C/EBPα and PPARγ, transcription factors required for adipogenesis, were also reduced in those TRB-expressing cells. In contrast, expression of C/EBPβ, an early inducible gene during adipogenesis *in vitro*, was unaffected by enforced expression of TRB2 and TRB3 (Fig. 2*C*). These results indicate that constitutive expression of TRB2 or TRB3 inhibits the adipogenic program at an early step. To further confirm the effects of TRB2 and TRB3 in different cells, we expressed TRB2 and TRB3 in MEFs and examined their effect on adipogenesis. As shown in Fig. 2*D*, the level of aP2 mRNA was significantly reduced in MEFs infected with TRB2- or TRB3- expression virus as compared with control virus. Thus, TRB2 and TRB3 have anti-adipogenic function in mammalian cells.

Peng *et al.* (16) previously showed that the induction of C/EBPα and PPARγ expression was defective in MEFs prepared from Akt1/2 double knock-out mice. Because TRB3 has been reported to function as an inhibitor for Akt (22) and TRB-expressing cells exhibited defects similar to Akt1/2 knock-out MEFs, we examined the possibility that TRBs inhibit adipogenesis via Akt. To this end, we analyzed the phosphorylation of Akt at Thr^{308} and Ser^{473}, indicative of Akt activation (26). After confluence, virus-infected cells were cultured in the presence or absence of MDI and then subjected to Western blot analysis. As shown in Fig. 3, Akt was phosphorylated in response to MDI in control cells. The phosphorylation of Akt was severely inhibited in cells expressing TRB3, suggesting that TRB3 blocks the adipocyte differentiation by inhibiting Akt signaling. In contrast, TRB2 had a modest effect on the Akt activation, while it suppressed adipogenesis as strongly as TRB3, suggesting that TRB2 blocks adipocyte differentiation by a mechanism distinct from TRB3.

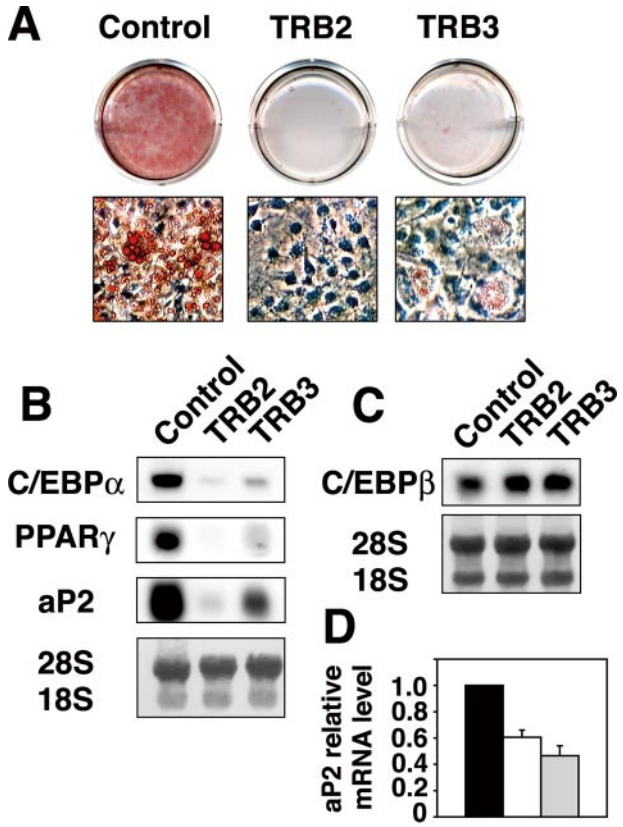

FIGURE 2. **Effect of TRB expression on adipogenesis.** *A*, 3T3-L1 preadipocytes were infected with control virus or virus containing a cDNA for TRB2 or TRB3. Eight days after induction, cells were stained with Oil-Red-O to visualize the lipid droplets. Stained plates (*upper panel*) and microscopic images (*lower panel*) are shown. *B*, five days following induction, total RNA was extracted from cells and then subjected to Northern blot analysis with probes as indicated. *C*, total RNA was extracted from cells 24 h after induction and the expression of C/EBPβ was examined by Northern blotting. *D*, MEFs were infected with control virus or virus expressing TRB2 or TRB3 and then induced to differentiate. The amount aP2 mRNA in control cells (*black*), TRB2-expressing cells (*white*), TRB3-expressing cells (*gray*) was determined by quantitative PCR.

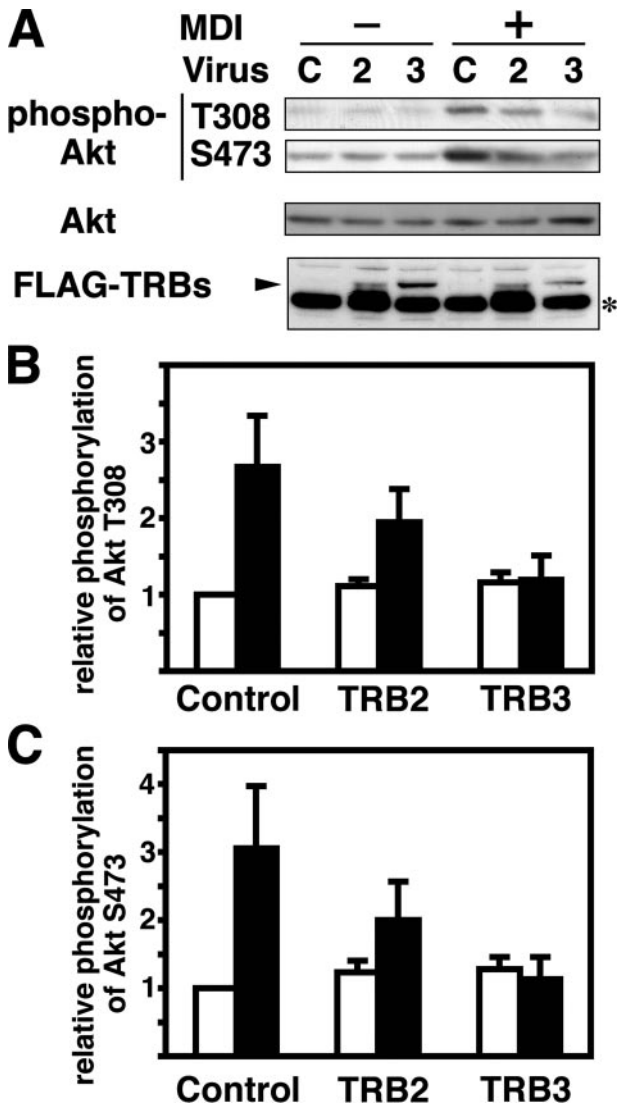

FIGURE 3. **Effects of TRB expression on Akt activation following MDI stimulation.** 3T3-L1 cells infected with control (*C*), TRB2 (*2*), or TRB3 (*3*) virus were cultured until confluence. Two days after confluence, cells were cultured in the absence or presence of MDI and incubated for 1 h. Total cell lysates were prepared and subjected to Western blotting analysis. Phosphorylated Akt was detected with specific antibodies against phospho-Thr^{308} (*T308*) and phospho-Ser^{473} (*S473*). Akt and FLAG-TRBs were detected with anti-Akt and anti-FLAG antibodies (*A*). *Asterisk* indicates nonspecific band (*B* and *C*), three independent experiments were performed, and phosphorylation of Akt was quantified by NIH image. *Open bars*, absence of MDI; *filled bars*, presence of MDI.

TRB2 Reduces the Level of LAP Protein through a Proteasome-dependent Mechanism—It was previously reported that in *Drosophila,* Tribbles associates with Slbo, a protein homologous to mammalian C/EBP proteins, and promotes its degradation through a proteasome-dependent manner (21). To investigate whether mammalian Tribbles orthologs have similar function, we examined the level of C/EBP proteins during adipogenesis in cells expressing TRB2 or TRB3. As shown in Fig. 2, *B* and *C*, the C/EBPβ mRNA level was unchanged by enforced expression of TRBs, but the induction of C/EBPα was impaired in cells expressing TRBs. Thus, we examined the level of C/EBPβ protein in cells expressing either TRB2 or TRB3. Control and TRB-expressing cells were induced to differentiate with MDI and then subjected to Western blot analysis. It has been shown that C/EBPβ isoforms, LAP and LIP, are produced through an alternative translation or through a proteolytic cleavage (27–29). LAP possesses the DNA binding and trans-activating domains, whereas LIP lacks the trans-activating domain and acts as an inhibitor (28). The levels of both C/EBPβ isoforms in TRB3-expressing cells were similar to those in control cells, whereas LAP was significantly reduced in TRB2-expressing cells (Fig. 4*A*). Interestingly, LIP, an inhibitory isoform of C/EBPβ, was found at similar levels in both control and TRB2-expressing cells. To test if the reduction of LAP by TRB2 is proteasome dependent, we examined the effect of a proteasome inhibitor, MG132. As shown in Fig. 4*B*, the LAP protein level was recovered when TRB2-expressing cells were treated with MG132, indicating that the degradation of LAP is promoted in TRB2-expressing cells. These results strongly suggest that TRB2 inhibits adipogenesis by down-regulating the protein level of the active C/EBPβ isoform LAP. To test this idea, we examined MCE that is a C/EBPβ-dependent process in early adipogenesis. Control and TRB2-expressing cells were induced to differentiate and cell-cycle progression was monitored by BrdU incorporation. Control cells entered cell cycle and incorporated BrdU by differentiation stimuli. Expression of TRB2 resulted in

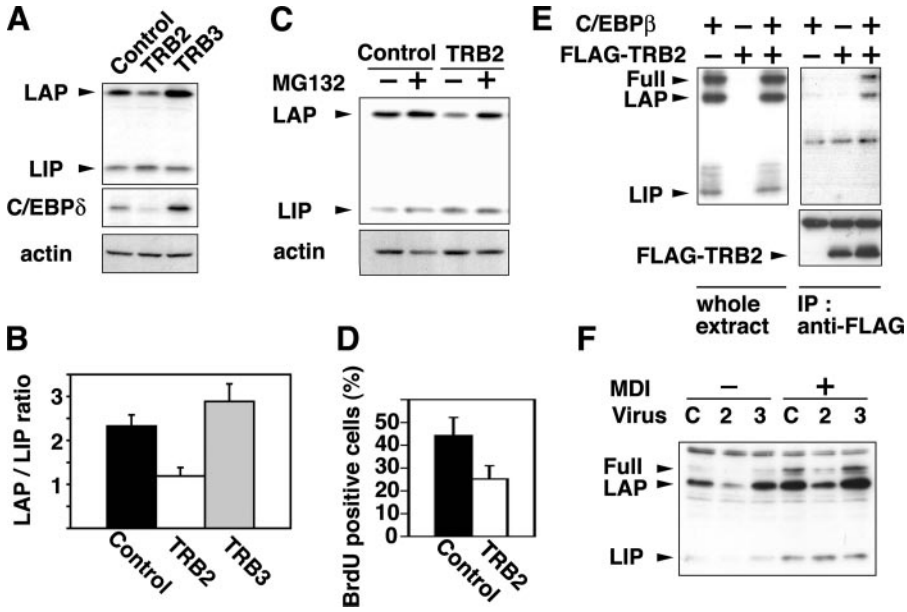

FIGURE 4. **Functional and physical interaction between TRB2 and C/EBPβ.** *A*, control or TRBs-infected cells were induced to differentiate with MDI for 24 h. Extracts prepared from cells were resolved by SDS-PAGE and immunoblotted with anti-C/EBPβ, anti-C/EBPδ, and anti-actin antibodies. *B*, three independent experiments were performed as described in *A* and C/EBPβ LAP/LIP ratio was quantified. *C*, eighteen hours after induction, control or TRB2-expressing cells were cultured in the absence or presence of MG132 for 6 h. The amount of C/EBPβ protein was determined by Western blotting. *D*, control or TRB2-expressing cells were induced to differentiate. Eighteen hours after induction, cells were labeled with BrdU for 6 h and then fixed as described under "Experimental Procedures." Percentage of BrdU-positive cells are represented. *E*, HEK293 cells were transiently transfected with pCDNA3-FLAG-TRB2 and pCDNA3-C/EBPβ as indicated. Extracts prepared from transfected cells were subjected to immunoprecipitation with anti-FLAG antibody. The immunocomplex was separated by SDS-PAGE and immunoblotted with antibodies against C/EBPβ and FLAG epitope. *F*, control or TRB -infected cells were cultured in the absence or presence of MDI for 6 h. Extracts prepared from cells were resolved by SDS-PAGE and immunoblotted with anti-C/EBPβ antibody.

reduction of BrdU positive cells compared with control cells (Fig. 4*C*). Thus, enforced expression of TRB2 impairs a C/EBPβ-dependent process during adipogenesis. If insufficiency of C/EBPβ results in defective adipogenesis in TRB2-expressing cells, enforced expression of C/EBPβ would suppress the TRB2-dependent defects. To examine this hypothesis, we created cells exogenously expressing C/EBPβ and induced differentiation. Overexpression of C/EBPβ enhanced adipogenesis in control cells and TRB2-expressing cells could differentiate to adipocytes by expression of exogenous C/EBPβ (supplemental Fig. S1). These results support the model that C/EBPβ is a target of TRB2.

TRB2 Physically Interacts with C/EBPβ Isoform LAP, but Not with LIP—Given the analogous function of Tribbles and TRB2 in the regulation of C/EBP protein, we next examined the physical interaction between TRB2 and C/EBPβ in mammalian cells. HEK293 cells were transiently transfected with FLAG-TRB2 and C/EBPβ. Extracts prepared from transfected cells were analyzed by immunoprecipitation. Full-length C/EBPβ (38-kDa), LAP (35-kDa), and LIP (21-kDa) proteins were produced by the C/EBPβ expression vector in HEK293 cells. Both full-length C/EBPβ and LAP proteins were co-immunoprecipitated with FLAG-TRB2, whereas LIP was not detected in the immunocomplex (Fig. 4*D*). In 3T3-L1 cells, full-length C/EBPβ and LAP were reduced by TRB2, whereas LIP was not affected by TRB2 (Fig. 4*E*). Taken together, the physical interaction between TRB2 and the active C/EBPβ isoform is correlated with the reduction of C/EBPβ protein.

To uncover the molecular feature of TRB2, we made a C-terminally truncated TRB2 (TRB2ΔC) that partially lacks the conserved kinase-like domain (Fig. 5*A*). 3T3-L1 cells were infected with the TRB2ΔC expression virus vector and then induced to differentiate with MDI. Although cells expressing full-length TRB2 failed to differentiate, TRB2ΔC-expressing cells as well as control cells were converted into adipocytes and accumulated lipid droplets (Fig. 5*B*). Furthermore, enforced expression of TRB2ΔC did not affect expression of adipogenic genes and the level of C/EBPβ protein (Fig. 5, *C*, *D*, and *E*). We next examined the association of C/EBPβ with tuncated-TRB2 and found that TRB2ΔC physically interacted with LAP (Fig. 5*F*). Thus, the C-terminal region is important for TRB2 function rather than the association with C/EBPβ.

PPARγ Rescues the Defects of Adipogenesis in Cells Expressing TRB2 or TRB3—The induction of C/EBPα and PPARγ are dependent on C/EBPβ and C/EBPδ in cultured cells (7). As shown in Fig. 4*A*, TRB2 reduced the level of C/EBPδ as well as C/EBPβ protein. If TRB2 inhibits adipogenesis by degrading C/EBPβ and C/EBPδ, PPARγ could suppress the defects of TRB2-expressing cells. To test this hypothesis, we created cells expressing both TRB2 and PPARγ and then induced to differentiate. As shown in Fig. 6, cells expressing TRB2 and PPARγ rarely differentiated to adipocytes in the absence of a PPARγ ligand, troglitazone, whereas they could differentiate in the presence of troglitazone. However lipid droplets in TRB2-expressing cells were smaller than those in control cells. We next examined the effects of PPARγ on adipogenesis in cells expressing TRB3. It has been shown that expression of PPARγ is defective in Akt deficient MEFs and PPARγ rescues the defects of adipogenesis (16). If TRB3 inhibits adipogenesis by attenuating Akt activation, PPARγ also should rescue the defects of TRB3-expressing cells. Predictably, TRB3-expressing cells as well as control cells differentiated to adipocytes by activation of PPARγ (Fig. 6) These results indicate that TRB2 and TRB3 inhibit events prior to PPARγ-dependent processes, however the effect of PPARγ was partial in TRB2-expressing cells, suggesting that TRB2 may inhibits a PPARγ-dependent process as well (See "Discussion").

Induction of Adipogenesis by TRB2 Knockdown in Response to a Low Concentration of Dexamethasone—To clarify the role of endogenous TRB2 in adipocyte differentiation, we knocked down the expression of TRB2 in 3T3-L1 cells with retrovirus expressing shRNA. The mRNA level of endogenous TRB2 was significantly reduced in cells expressing TRB2 shRNA as com-

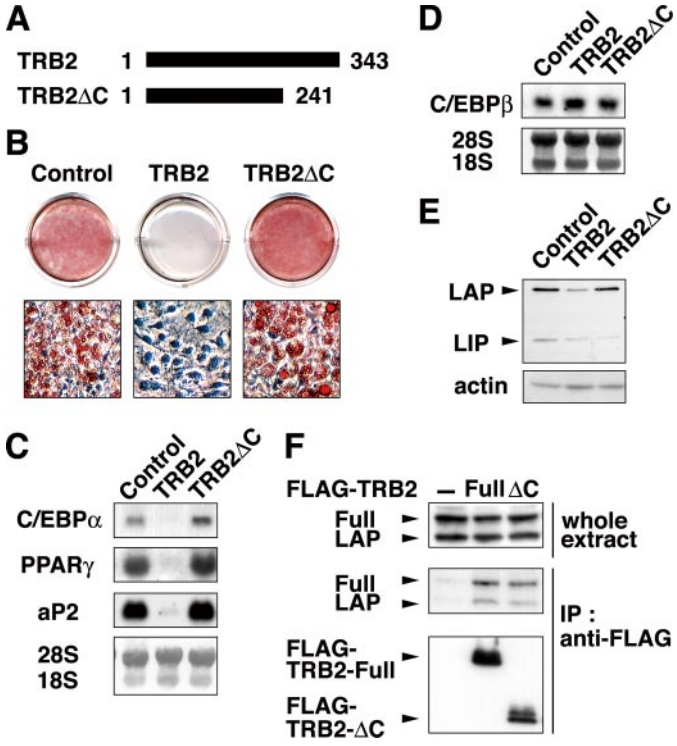

FIGURE 5. **Analyses of C-terminal-truncated TRB2.** *A*, *schematic* of full-length TRB2 and C-terminal-truncated TRB2 (TRB2ΔC). 3T3-L1 cells were infected with virus expressing TRB2 or TRB2ΔC and then induced to differentiate with MDI. *B*, eight days after induction, lipids were stained by Oil-Red-O. *C*, five days following induction, expression of adipogenic genes was examined by Northern blotting. *D* and *E*, twenty-four hours after MDI stimulation, the level of C/EBPβ mRNA and protein was examined by Northern blotting (*D*) and Western blotting (*E*). *F*, HEK293 cells were transfected with pCDNA3-C/EBPβ and expression vectors for FLAG-tagged TRB2. The association of C/EBPβ with TRB2 was examined by immunoprecipitation assay as described in Fig. 4*E*.

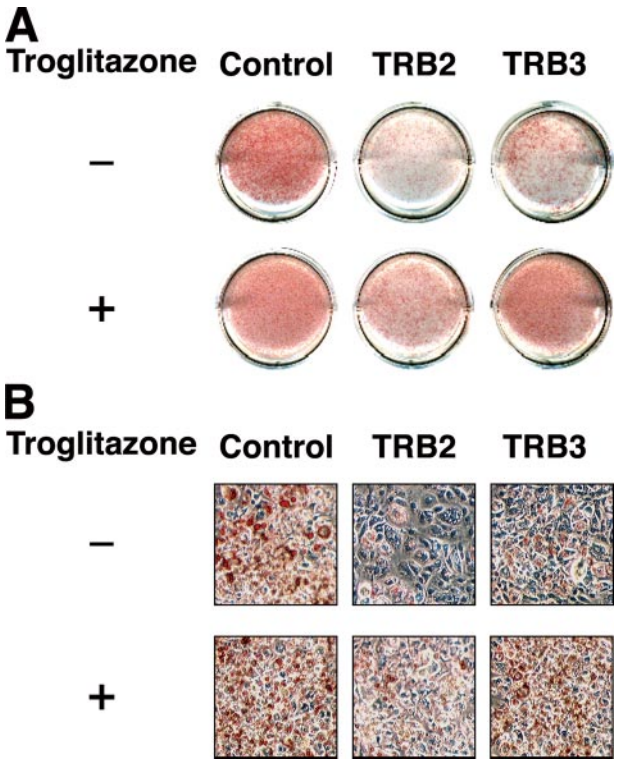

FIGURE 6. **Effect of PPARγ activation on adipogenesis in TRB-expressing cells.** Control or TRB-expressing cells were infected with virus containing a cDNA for PPARγ. Cells were induced to differentiate with MDI in the absence (*upper panel*) or presence (*lower panel*) of PPARγ ligand, troglitarizone. Eight days after induction, lipids were stained by Oil-Red-O. Stained plates (*A*) and microscopic images (*B*) are shown.

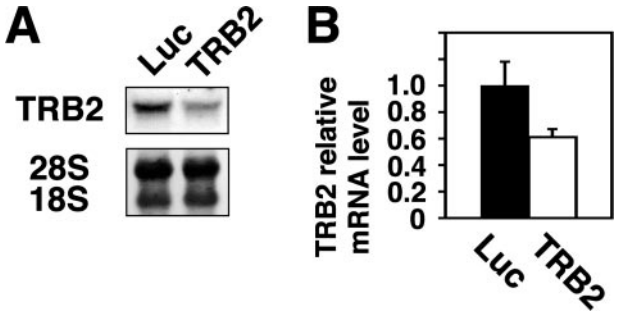

FIGURE 7. **Effects of TRB2 siRNA 3T3-L1 preadipocytes were infected with virus expressing shRNA for luciferase or TRB2.** *A*, total RNA was prepared from infected cells and the level of TRB2 mRNA was determined by Northern blotting. *B*, cDNA was prepared from control and TRB2-knockdown cells, and then relative expression level of TRB2 was measured by quantitative PCR. GAPDH was used as an internal standard.

pared with control cells (Fig. 7, *A* and *B*). TRB2 knockdown cells showed no obvious defects in growth (data not shown). As TRB2 reduced the levels of active isoforms of C/EBPβ, knockdown of TRB2 may activate the adipogenic program more efficiently. To test this hypothesis, we examined whether TRB2 knockdown cells undergo adipogenesis by a low concentration of differentiation stimuli, MDI. However, we did not observe adipogenesis in both control and TRB2-knockdown cells in the presence of 1/100 of the normal concentration of MDI. Because Mix, Dex, and insulin are required for various processes in adipogenesis, it is possible that knockdown of TRB2 failed to complement the reduction of all three inducers. It has been reported that Pref-1, an anti-adipogenic factor, is downregulated in response to Dex and decrease of Pref-1 by antisense RNA allowed cells differentiate in a low concentration of Dex (30). If endogenous TRB2 has a negative role in an adipogenic process in response to specific agent, TRB2 knockdown could complement the reduction of such agent. To test whether TRB2 is down-regulated in response to a specific agent, we examined effects of each inducer on TRB2 expression and found that the TRB2 level was reduced by Dex (Fig. 8*A*). We then examined adipocyte differentiation in a low concentration of Dex. 3T3-L1 cells were converted to adipocyte in standard differential medium containing 1 μM Dex, but they rarely differentiated in the presence of 1 nM Dex. We found that upon stimulation with 1 nM Dex TRB2-knockdown cells accumulated more lipids than control cells infected with the expression vector of Luciferase shRNA (Fig. 8*B*). Consistent with this observation, the expression of aP2 was enhanced in TRB2-knockdown cells as compared with control cells (Fig. 8, *C* and *D*). Furthermore, in TRB2-knockdown cells, the level of C/EBPβ LAP protein was increased without altering its transcripts (Fig. 8, *E* and *F*). Again, these results indicate that TRB2 acts as an inhibitor for C/EBPβ protein and adipogenesis.

DISCUSSION

The Tribbles family is characterized by the evolutionally conserved kinase-like domain highly homologous to serine/threo-

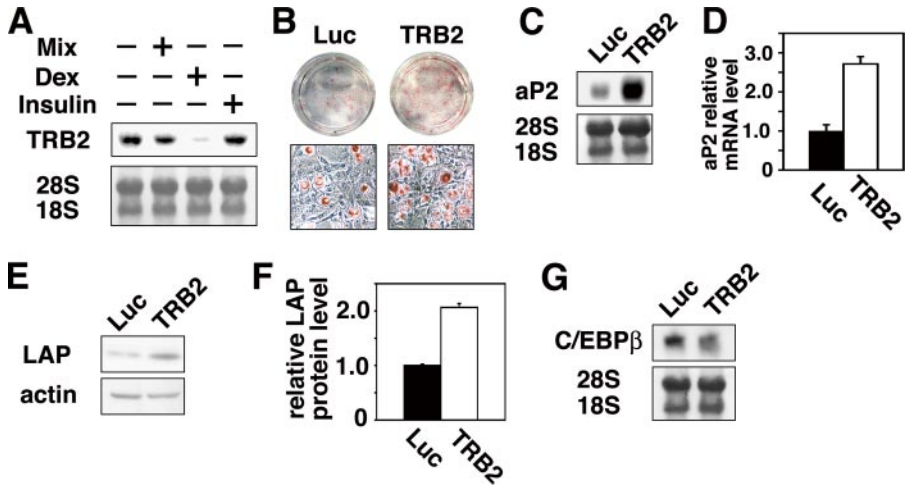

FIGURE 8. **Effect of TRB2 knockdown on the adipogenic program.** *A*, two days after confluence, 3T3-L1 cells were shifted to the medium containing Mix, Dex, or insulin and cultured for 24 h. After RNA preparation, the expression of TRB2 was examined by Northern blotting. *B–D*, control or TRB2-knockdown cells were stimulated with Mix, insulin, and 1 nM Dex. After 2 days, cells were cultured by standard differentiation method. Eight days following induction, plates were stained with Oil-Red-O (*B*), the expression of aP2 was determined by Northern blotting (*C*), and relative expression level of aP2 was measured by quantitative PCR (*D*). *E* and *F*, cells were treated with Mix, insulin and 1 nM Dex for 6 h. The level of C/EBPβ LAP protein was examined by Western blotting (*E*) and quantified by NIH Image (*F*). *G*, total mRNA was prepared from cells as described above and C/EBPβ mRNA was examined by Northern blotting.

nine kinases. In *Drosophila,* Tribbles is the only member and plays a role in protein degradation. In mammals, three members have been found, and TRB3 is known to modulate signal cascades such as Akt and MAPK. Among the three members, we found that expression of TRB2 and TRB3 was immediately down-regulated in response to differentiation stimuli in 3T3-L1 preadipocyte, suggesting that they may have some role for adipogenesis. In fact, forced expression of TRB2 and TRB3 inhibited adipocyte differentiation in 3T3-L1 cells.

Because Akt mediates adipogenic signals (16) and TRB3 inhibits Akt signaling and glucose production in hepatocytes (22), we considered the possibility that the inhibitory effect of TRBs is mediated by Akt. This is indeed the case for TRB3; MDI induced phosphorylation of Akt at Thr^{308} and Ser^{473} and forced expression of TRB3 in 3T3-L1 cells blocked the Akt phosphorylation as well as adipocyte differentiation. FOXO1 is a forkhead transcription factor that inhibits adipogenesis and a differentiation inducer such as insulin transiently inactivates FOXO1 by Akt-dependent phosphorylation in early adipogenesis (31). These results support the model that TRB3 acts as an inhibitor for Akt in preadipocytes, and its down-regulation inhibits Foxo1 activity by permitting Akt-dependent phosphorylation.

The inhibitory effect of TRB2 was similar to that of TRB3, however, TRB2 only partially inhibited the Akt phosphorylation, suggesting that TRB2 inhibits adipogenesis via a mechanism distinct from the inhibition of Akt phosphorylation. We found that TRB2 specifically interacts with the active isoform of C/EBPβ LAP and reduces it through a proteasome-dependent manner. LAP is necessary and sufficient for MCE in adipocyte differentiation (11). In agreement with these observations, MCE is impaired in cells in which TRB2 is ectopically expressed. As *Drosophila* Tribbles promotes degradation of Slbo, a *Drosophila* C/EBP (21), these results suggest that TRB2 is a functional counterpart of *Drosophila* Tribbles. The kinase-like domains of *Drosophila* Tribbles and TRB proteins are highly conserved, whereas N- and C-terminal regions are variable. Our deletion analysis revealed that TRB2ΔC binds LAP, but fails to affect adipogenesis, suggesting that the C-terminal region is important for TRB2 functions rather than the association with C/EBPβ. As *Drosophila* Tribbles promotes degradation of Slbo through an ubiquitin-dependent mechanism (21), it is possible that C-terminal region of TRB2 associates with co-factors such as a ubiquitin ligase. Interestingly, SKIP3, an alternative name of TRB3, binds ATF4 and degrades it in human tumors (32). Recently, TRB3 was shown to associate with COP1, an E3 ubiquitin ligase, and promotes acetyl coenzyme A carboxylase (ACC1) degradation (33). Thus, TRBs may regulate protein stability in mammalian cells depending on cellular contexts. Interestingly, we observed that TRB1, a member of the mouse TRB family, also possesses an ability to reduce the C/EBPβ LAP protein level in 3T3-L1 cells in a manner similar to TRB2 (Supplemental Fig. 2). Enforced expression of TRB1 resulted in defective adipogenesis, suggesting that TRB1 negatively regulates adipogenesis. As TRB1 is transiently up-regulated during adipogenesis, it is possible that TRB1 functions as a negative feedback regulator for fine tuning of adipogenesis. TRB1 and TRB2 as well as TRB3 have a COP1-bindig motif (33), it is possible that all TRB family members may associate with COP1. However, TRB1 and TRB2 promote degradation of LAP, whereas TRB3 does not, suggesting that LAP is regulated by a mechanism distinct from COP1-dependent proteolysis.

Akt-deficient MEFs are unable to express PPARγ and adipogenic defects in Akt deficient MEFs are restored by PPARγ and troglitazone (16). We demonstrated that activation of PPARγ suppresses the defects of TRB2- and TRB3-expressing cells; however suppression of TRB2-expressing cells was partial, suggesting that TRB2 affects the PPARγ dependent process. PPARγ induces C/EBPα expression and then they cooperatively promote the adipogenic program (34). During revision of this manuscript, Keeshan *et al.* (35) reported that TRB2 promotes C/EBPα degradation in cancer cells. It is possible that TRB2 also promotes C/EBPα degradation in adipocytes and functions as an inhibitor for C/EBP family proteins. In fact, we observed that the level of C/EBPδ protein, which is a member of the C/EBP family and is involved in early adipogenesis, was also decreased in TRB2-expressing cells (Fig. 4*A*). Taken together, TRB2 is a protein that negatively regulates C/EBP family proteins through proteolysis.

Dex is required for 3T3-L1 preadipocytes to differentiate into adipocytes *in vitro*. We have demonstrated that TRB2

expression is repressed by Dex and TRB2-knockdown cells readily differentiate into adipocytes in the presence of 1 nM Dex, which is 1000-fold less than that in the normal differentiation condition. These results suggest that Dex plays a role for the repression of TRB2 during early adipogenesis. Pref-1, also known as Dlk, is expressed in preadipocytes and down-regulated in a Dex-dependent manner (30) and overexpression of Pref-1 inhibits adipocyte differentiation (36, 37). We have observed that Pref-1 is still expressed after MDI stimulation in TRB2-expressing cells,[4] raising the possibility that TRB2 regulates the expression of Pref-1.

It was recently shown that TRB3 expression is repressed by nutritional supplies and is increased in a long-term culture of human PC-3 cells by a PI-3K-dependent mechanism (38). In agreement with this report, we also observed that TRB3 expression is oscillated by medium change during adipocyte differentiation. It is possible that the adipogenic program is regulated by nutritional status. Interestingly, mouse TRB3 is a key regulator of glucose homeostasis in hepatocytes and its expression is down-regulated by feeding (22). TRB3 is expressed in mature 3T3-L1 adipocytes and adipose tissues *in vivo* (data not shown) and Qi *et al.* (33) recently reported that TRB3 was increased during fasting and stimulated lipolysis in an adipose tissue. Thus, TRB3 may play an important role for both differentiation and function of adipocytes.

In summary, we show that TRB2 and TRB3 have anti-adipogenic effects. While TRB3 suppresses adipogenesis by strong inhibition of Akt activation, TRB2 blocks adipogenesis through the inhibition of Akt activation and degradation of C/EBPβ and C/EBPδ. TRB3 is increased in IRS-deficient BAT, in which adipocyte differentiation is impaired (39). TRB2 targets C/EBPβ and C/EBPδ in 3T3-L1 cells and mice deficient for both C/EBPβ and C/EBPδ display severe defects in development of brown adipocytes (6). Thus, it will be interesting to investigate whether TRB2 is involved in the development of brown adipocytes *in vivo*.

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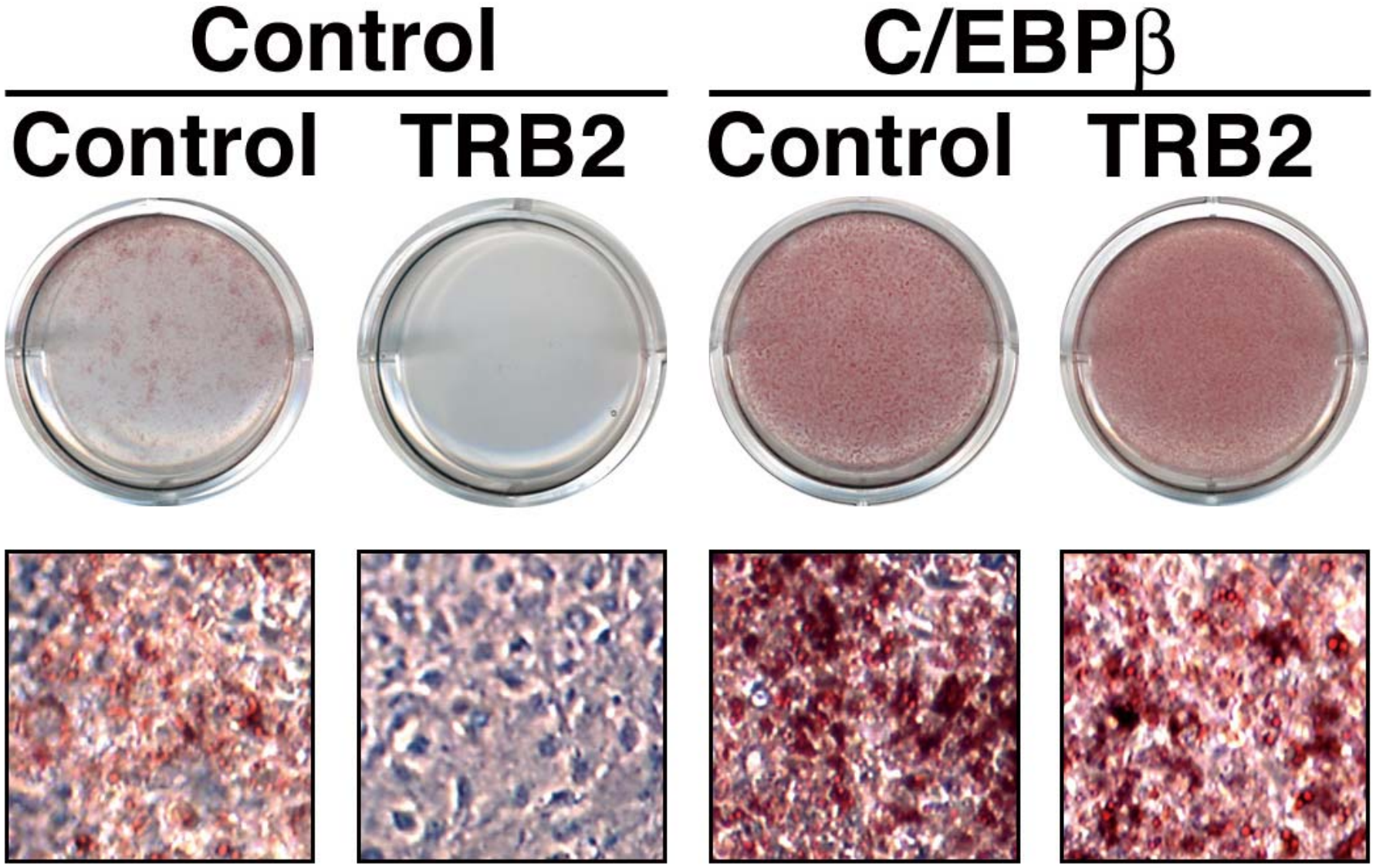
[4] A. Miyajima, unpublished data.

Supplemental Fig. 1. Effect of C/EBPβ expression on adipogenesis in TRB2-expressing cells

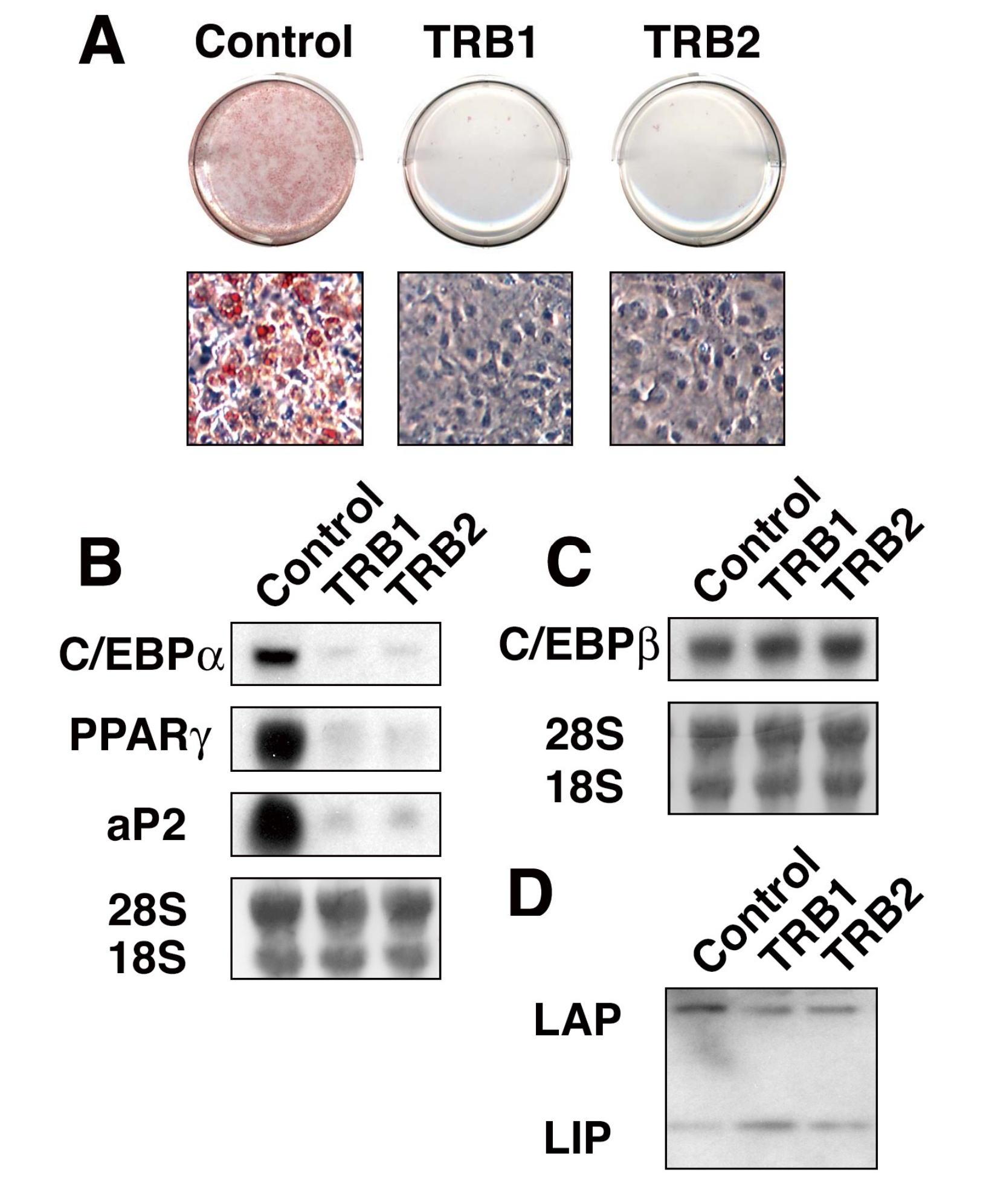
Control or TRB2-expressing cells were infected with virus containing a cDNA for C/EBPβ. Cells were induced to differentiate with MDI. Eight days after induction, lipids were stained by Oil-Red-O. Stained plates (upper panel) and microscopic images (lower panel) are shown.

Supplemental Fig. 2. Effect of TRB1 expression on adipogenesis

(A) 3T3-L1 preadipocytes were infected with control virus or virus containing a cDNA for TRB1 or TRB2. Eight days after induction, cells were stained with Oil-Red-O to visualize the lipid droplets. Stained plates (upper panel) and microscopic images (lower panel) are shown. (B) Five days following induction, total RNA was extracted from cells and then subjected to Northern blot analysis with probes as indicated. (C) Total RNA was extracted from cells 24 hours after induction and the expression of C/EBPβ was examined by Northern blotting. (D) Total protein was extracted from cells 24 hours after induction and the protein level of C/EBPβ isforms were examined by Western blotting.



Naiki et al. supplemental Fig. 1



Naiki et al. supplemental Fig. 2

TRB2, a Mouse Tribbles Ortholog, Suppresses Adipocyte Differentiation by Inhibiting AKT and C/EBP β

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