

TRIB1 downregulates hepatic lipogenesis and glycogenesis via multiple molecular interactions

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Abstract

Mammalian tribbles homolog 1 (*TRIB1*) regulates hepatic lipogenesis and is genetically associated with plasma triglyceride (TG) levels and cholesterol, but the molecular mechanisms remain obscure. We explored these mechanisms in mouse livers transfected with a *TRIB1* overexpression, a shRNA template or a control (LacZ) adenovirus vector. The overexpression of *TRIB1* reduced, whereas induction of the shRNA template increased, plasma glucose, TG, and cholesterol and simultaneously hepatic TG and glycogen levels. The involvement of *TRIB1* in hepatic lipid accumulation was supported by the findings of a human SNP association study. A *TRIB1* SNP, rs6982502, was identified in an enhancer sequence, modulated enhancer activity in reporter gene assays, and was significantly ($P=9.39 \times 10^{-7}$) associated with ultrasonographically diagnosed non-alcoholic fatty liver disease in a population of 5570 individuals. Transcriptome analyses of mouse livers revealed significant modulation of the gene sets involved in glycogenolysis and lipogenesis. Enforced *TRIB1* expression abolished CCAAT/enhancer binding protein A (CEBPA), CEBPB, and MLXIPL proteins, whereas knockdown increased the protein level. Levels of *TRIB1* expression simultaneously affected MKK4 (MAP2K4), MEK1 (MAP2K1), and ERK1/2 (MAPK1/3) protein levels and the phosphorylation of JNK, but not of ERK1/2. Pull-down and mammalian two-hybrid analyses revealed novel molecular interaction between *TRIB1* and a hepatic lipogenic master regulator, MLXIPL. Co-expression of *TRIB1* and CEBPA or MLXIPL reduced their protein levels and proteasome inhibitors attenuated the reduction. These data suggested that the modulation of *TRIB1* expression affects hepatic lipogenesis and glycogenesis through multiple molecular interactions.

Key Words

- molecular genetics
- metabolism
- liver
- hyperlipidemia
- fats/fatty acids

*Journal of Molecular
Endocrinology*
(2014) 52, 145–158

Introduction

Plasma concentrations of lipids are a heritable risk factor for the development of atherosclerosis and related cardiovascular diseases (CVDs). High concentrations of LDL-associated cholesterol elevate the risk of ischemic heart disease and high concentrations of HDL-associated cholesterol reduce the risk (Law *et al.* 2003, Lewington *et al.* 2007). An increase in plasma triglyceride (TG) concentrations is also an independent risk factor for CVD (Bansal *et al.* 2007, Nordestgaard *et al.* 2007). The European genome-wide SNP association studies (GWAS) have associated 95 loci with blood lipid levels (Teslovich *et al.* 2010). The downstream linkage disequilibrium (LD) block of mammalian tribbles homolog 1 (*TRIB1*) is one locus that has convincing impact on CVD and levels of TG and LDL across several ethnic groups (Nakayama *et al.* 2009, Teslovich *et al.* 2010). A functional study using knockout mice and overexpression experiment has confirmed *TRIB1* involvement in hepatic lipogenesis, which affects very low density lipoprotein (VLDL) production (Burkhardt *et al.* 2010). However, the molecular mechanisms remain unknown. Therefore, we explored the molecular pathways using a mouse model and functional variations in the LD block that affect levels of *TRIB1* expression.

TRIB1, *TRIB2*, and *TRIB3* share 45% peptide sequence similarity and homology with tribbles (Hegedus *et al.* 2007), a *Drosophila* protein that inhibits mitosis during the early developmental stage by binding to the *CDC25* homolog, string (Grosshans & Wieschaus 2000). Genes in the Tribbles family encode a kinase-like domain but lack catalytic core motifs and kinase activities (Sung *et al.* 2006). A carboxy-terminal hexapeptide motif is shared among the three tribbles proteins and it is the binding site for E3 ubiquitin ligase constitutive photomorphogenic protein 1 (COP1) that participates in the proteasome-mediated degradation of some targets of tribbles (Qi *et al.* 2006, Yokoyama & Nakamura 2011). *TRIB1*–3 also provides a motif for MEK1 binding at near C-termini, which can enhance or inhibit MEK/MAPK signaling dependently on cell type and target molecule (Sung *et al.* 2006). The MEK1 binding domain is also involved in molecular interaction with CCAAT/enhancer binding proteins (CEBPA and CEBPB; Keeshan *et al.* 2006, Yokoyama *et al.* 2010). Furthermore, *TRIB2* and *TRIB3* interact with specific target molecules. Molecular interactions have been identified between *TRIB3* and acetyl-coenzyme A carboxylase (Qi *et al.* 2006), Akt (Du *et al.* 2003), peroxisome proliferator-activated

receptor γ (PPAR γ ; Takahashi *et al.* 2008), CEBP homologous protein (CHOP), and activating transcription factor 4 (ATF4; Ohoka *et al.* 2005). Fewer unique target molecules have been identified for *TRIB1* than for *TRIB3*, among which factors involved in hepatic lipogenesis are likely to be included. Target molecules were searched using mouse models with *TRIB1* overexpression or knock-down predominantly in the liver using an adenoviral gene delivery system.

Experimental procedures

Adenovirus vector constructs

The open reading frame (ORF) of *TRIB1* cDNA under a CMV promoter (NM_025195, pAx-CMV-*TRIB1*) and a mouse *Trib1* shRNA template (pAx-sh*Trib1*; Supplementary Table 1, see section on supplementary data given at the end of this article) were inserted into pAxcwit (Takara Bio, Inc., Otsu, Japan). The control was an adenovirus encoding LacZ, pAxCA-LacZ. Purified adenovirus vectors were titrated and 2.0×10^9 pfu of adenoviruses were injected into the tail veins of mice.

Animal experiments

Male 12-week-old C57Bl6 mice (CLEA Japan, Inc., Tokyo, Japan) were housed in an air-conditioned environment with a 12 h light:12 h darkness cycle. Blood samples were collected from the tail vein during the morning under conditions of food intake *ad libitum* or after 12 h of starvation. Plasma glucose was measured using Accu-Chek (Roche Diagnostics). Plasma TG, total cholesterol, and aspartate aminotransferase (AST) levels were measured using Fuji Dry Chem System (Fujifilm, Kanagawa, Japan). Plasma free fatty acid, β -hydroxybutyrate, and insulin levels were measured by NEFA C Kit (Wako, Osaka, Japan), fluorometric assay kit (Cayman Chemical Company, Ann Arbor, MI, USA), and ELISA system (Morinaga, Yokohama, Japan) respectively. Oxygen consumption (VO_2) and carbon dioxide production (VCO_2) were individually measured indirectly using an Oxyman V5.61 calorimeter (Columbus Instruments, Columbus, OH, USA). Locomotor activity was estimated using an ACTIMO-100 infrared activity monitoring system (Shinfactory, Fukuoka, Japan). The mice were killed at 4 or 8 days post-injection after 12 h of starvation. Liver TGs were

extracted in chloroform:methanol (2:1, v/v) and measured using commercial kits (Wako) (Rahman *et al.* 2007). Glycogens were extracted into 5% trichloroacetic acid and quantified using phenol-sulfuric acid (Matsusue *et al.* 2004). Frozen sections of mouse liver were stained with hematoxylin and eosin (H&E), Oil Red O, or PAS. Hepatocyte apoptosis was assessed using a colorimetric TUNEL System (Promega). Mice that had fasted for 12 h were injected with glucose (1.8 g/kg) or insulin (1 U/kg) i.p. for glucose tolerance test (GTT) and insulin tolerance test (ITT) respectively. The Animal Care and Use Committee of Jichi Medical University approved all procedures involving animals.

Expression profiling

Hepatic total RNA was extracted immediately after killing. Expression was profiled by hybridization to an Affimetrix Mouse 430_2 oligonucleotide microarray chip. Pathways that were significantly enhanced or reduced by altered TRIB1 expression were searched using Gene Set Enrichment Analysis (GSEA) Software provided by the Broad Institute (<http://www.broad.mit.edu/gesa/>) and the Kyoto Encyclopedia of Genes and Genomes database. The accuracy of the expression levels of individual genes in the microarray were confirmed by quantitative RT-PCR using the primers listed in Supplementary Table 1. Hepatic protein (20 µg) was separated by electrophoresis in 10% SDS-PAGE gels and candidate TRIB1 target proteins were measured by western blotting using the specific primary antibodies CEBPA, NFκB p65, SREBP1 (against both of SREBP1a and 1c precursor), p-JNK, JNK and β-actin (Santa Cruz), CEBPB and MLXIPL (Abcam, Cambridge, UK), MEK1 (Epitomics, Burlingame, CA, USA), MKK4 and ERK1/ERK2 (R&D Systems, Minneapolis, MN, USA), and p-ERK1/ERK2 (Abnova, Taipei, Taiwan).

Assays of molecular interactions

TRIB1 target protein interaction was assessed using the mammalian two-hybrid and HaloTag mammalian Pull-Down systems (Promega). Full-length *TRIB1* cDNAs or *TRIB1* cDNAs with deleted N-terminal 90 residues (ΔN90) and pseudocatalytic loop (ΔPCL), COP1 (ΔCOP), or MEK (ΔMEK) binding domains were inserted into the pBIND vector to generate GAL4-fusion proteins. The ORFs of *CEBPA* (NM_004364.2), *CEBPB* (NM_005194.2), and *MLX* interacting protein-like (*MLXIPL*, NM_032951.2) were inserted into the pACT vector to generate VP16-fusion proteins. VP16-*MLXIPL* constructs with a deleted nuclear

localization signal (ΔNLS; residues 159–183; Fukasawa *et al.* 2010) and basic helix–loop–helix zipper (ΔbHLH; residue 648–724; Li *et al.* 2006) or leucine-zipper-like (ΔZIP; residue 792–837; de Luis *et al.* 2000) domains were also prepared to search for the interacting domain(s). These constructs were co-transfected using Lipofectamine 2000 (Life Technologies) into COS7 cells harboring the pGL4.31 vector. Empty pBIND and pACT vectors served as negative controls. Firefly reporter expression was measured 48 h later against Renilla luciferase encoded by the pBIND vector. Constructs were co-transfected into COS7 cells and molecular interactions were assessed as relative luciferase activities. The ORFs of *TRIB1*, *CEBPA*, and *MLXIPL* were inserted into the pHTN vector or pCR3 vector with an influenza hemagglutinin (HA)-tag for pull-down assays. Constructs were co-transfected into COS7 cells and then HaloTag fusion and associated proteins were extracted from cell lysates using HaloLink resin. Molecular interactions were compared by western blotting with anti-TRIB1 (Abgent, San Diego, CA, USA) and anti-HA (Sigma–Aldrich) antibodies to the empty pHTN vector as the negative control. Proteasome-mediated degradation of CEBPA or MLXIPL was examined by co-transfection in COS7 cells with TRIB1 in pCR3 vectors with or without an inhibitor of the proteasome system (1 µM epoxomicin or 10 µM MG132 (Sigma)). Cells were harvested 16 h later and the protein levels were measured by western blotting.

Survey of functional SNP in the downstream sequence of *TRIB1*

The upstream sequence of the *TRIB1* gene from –1336 to –1 relative to the ATG start codon was subcloned into the pGL3 basic vector and used as the promoter vector. We selected six conserved sequences in the 25 kb LD block downstream of *TRIB1*, through a database search at ENCODE (ENCODE Project Consortium *et al.* 2011, <http://genome.ucsc.edu/ENCODE/>) and HapMap (<http://hapmap.ncbi.nlm.nih.gov/>) (Supplementary Fig. 4, see section on supplementary data given at the end of this article). Fragments were amplified from several individuals and subcloned into the Sall site of the pGL3-*TRIB1* promoter vector. Constructs encoding SNP variations were verified by sequencing and transfected into HepG2 cells along with the pRL vector (Renilla Luciferase; Promega). Forty-eight hours later, the relative light units (firefly/Renilla light units) were measured using a dual luciferase reporter assay system (Promega) and a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA).

SNP association studies between TRIB1 with non-alcoholic fatty liver disease

The first association study comprised 3013 participants, recruited from outpatients whose general health was assessed at Jichi Medical University Hospital. The second population comprised samples whose hepatic ultrasonographic information was extracted from a database of 21 004 Japanese individuals (Nakayama *et al.* 2009). Experienced clinical technologists performed hepatic ultrasonography under the guidance of clinicians who reviewed photocopies of the scans. We excluded participants with known liver diseases or those who consumed >20 g/day of alcohol. The Ethics Committee at Jichi Medical University approved the study protocol and all recruits provided written informed consent to participate. The genotypes of rs6982502, rs17321515, and rs2980867 were determined using the TaqMan system. The effects of the risk allele on non-alcoholic fatty liver disease (NAFLD) and log-transformed plasma TG were assessed by logistic regression models and multiple regression analysis assuming an additive model of inheritance respectively. Age, sex, BMI, and a clinical history of diabetes were included in both models. Data were statistically analyzed using SPSS 11.0 (SPSS Japan).

Results

Overexpression and knockdown of TRIB1 influenced multiple metabolic parameters in mice

We determined whether variable expression levels of TRIB1 affect hepatic lipid storage using an adenoviral gene delivery system to modulate TRIB1 expression in the mouse liver. X-gal staining of mouse organs injected with pAxCA-LacZ validated nearly exclusive hepatic induction of the adenovirus vector system. The expression of TRIB1 protein that was above physiological levels on day 4 after pAx-CMV-TRIB1 injection (Supplementary Fig. 1A, see section on supplementary data given at the end of this article) became almost equivalent to the level of internal TRIB1 in the control by day 8. However, reduction of the TRIB1 protein level from days 4 to 8 (−98%) was inconsistent with that of the transcript level (−89%) (Supplementary Fig. 1B), suggesting post-translational negative regulation of TRIB1. The knockdown vector, pAx-shTrib1, reduced the protein and mRNA levels to about 60–80% of the control vector without affecting *Trib2* and *Trib3* expression (Supplementary Fig. 1A, C and D). The enforced expression and knockdown of TRIB1 significantly decreased and increased plasma TG levels in mice

fed *ad libitum* respectively (Table 1), while the difference in TG levels between knockdown and control mice at day 4 failed to achieve statistical significance. Plasma TG levels in starved mice did not significantly differ. Total cholesterol levels were also decreased and increased by TRIB1 overexpression and knockdown at day 8 respectively. The overexpression of TRIB1 notably decreased, whereas its knockdown increased plasma glucose levels but did not significantly affect insulin levels. The body weight and epididymal fat pads of mice overexpressing TRIB1 were significantly decreased, although food intake, locomotor activity, and O₂ consumption were equivalent to those of mice harboring the control vector (Table 1, Fig. 1A, and Supplementary Fig. 2, see section on supplementary data given at the end of this article). The respiratory exchange ratio (RER) was significantly repressed by TRIB1 overexpression (Fig. 1A and Supplementary Fig. 2). Increased β-hydroxybutyrate (Table 1) and repressed RER simultaneously suggested relatively decreased carbohydrate consumption and increased lipid β-oxidation in mice overexpressing TRIB1. Knockdown of *Trib1* increased the glucose level at the late phase of GTT (Fig. 1B), while the glucose level in ITT fell essentially to control levels (Fig. 1C), suggesting that hepatic knockdown of *Trib1* impaired glucose tolerance but not systemic insulin sensitivity. Baseline glucose levels of the mice tested GTT and ITT were not equivalent to the data of simple blood collection under fasting, probably due to their diversified nutritional and sampling condition. The knockdown of TRIB1 expression increased hepatic lipid droplets and PAS-stained material at day 8 (Fig. 1D), which reflected the 160 and 166% increases in TG and glycogen levels in the extracts respectively (Fig. 1E and F). In contrast, TRIB1 overexpression decreased TG and glycogen by 29 and 98% respectively. Leukocyte infiltration of the liver under conditions of enforced *Trib1* expression was remarkable at day 8 (Fig. 1D). Leukocytes were barely detectable at day 4, but the number of TUNEL-positive hepatocytes was significantly increased in mice overexpressing TRIB1 (Fig. 1D and G). Hepatocyte destruction might have resulted in the remarkably increased plasma AST level of TRIB1 overexpression (Table 1). The relatively mild AST increase in mice with TRIB1 knockdown and those injected with the LacZ vector might have resulted from inflammation induced by adenovirus.

Pathway analysis using hepatic transcriptome

We assessed the expression profiles of total RNA in mouse livers at 4 and 8 days after adenovirus injection using

Table 1 Blood parameters and tissue weight of mice injected with AV. Values are mean $\pm$ s.e.m. of seven to eight animals. Blood samples were drawn from tail vein under *ad libitum* feeding (Fed) or 12-h fasting (Fasting) at ZT0 or ZT12 respectively. Killing of the animals was performed after 12-h fasting at the indicated date from the administration of AV vectors

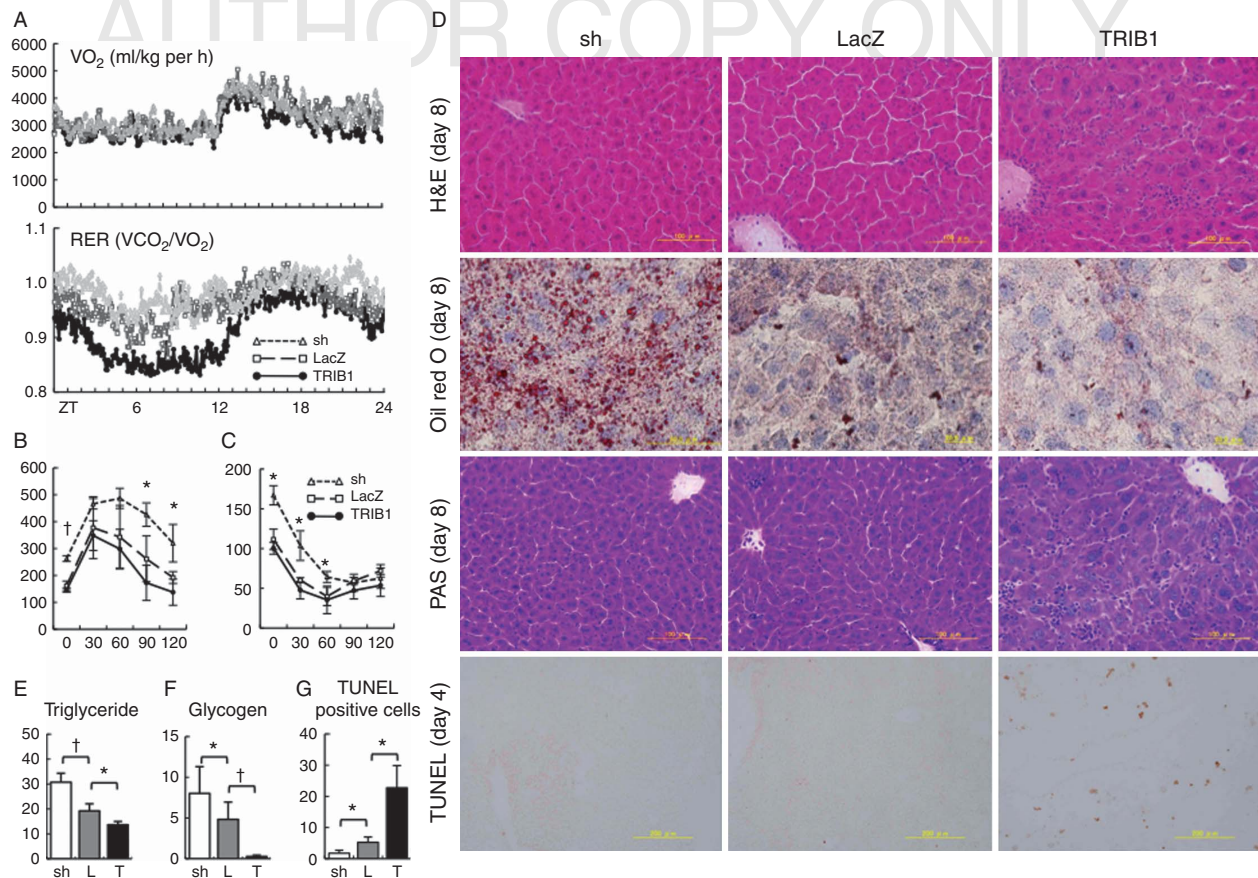
	sh	LacZ	TRIB1	t-test	
				sh vs LacZ	LacZ vs TRIB1
Day 4					
Change of body weight (g)	0.05 $\pm$ 0.32	0.07 $\pm$ 0.37	-1.52 $\pm$ 0.49	NS	<0.01
Food intake (g/day)	2.49 $\pm$ 0.11	2.34 $\pm$ 0.12	2.29 $\pm$ 0.10	NS	NS
Plasma glucose (mg/dl)					
Fed	236.9 $\pm$ 26.9	223.8 $\pm$ 23.9	126.4 $\pm$ 23.9	NS	<0.01
Fasting	196.0 $\pm$ 33.9	172.8 $\pm$ 25.6	95.0 $\pm$ 19.1	NS	<0.01
Plasma triglyceride (mg/dl)					
Fed	95.0 $\pm$ 9.6	93.8 $\pm$ 19.2	22.0 $\pm$ 8.0	NS	<0.01
Fasting	27.8 $\pm$ 2.8	28.0 $\pm$ 5.7	30.8 $\pm$ 3.8	NS	NS
Total cholesterol (mg/dl)					
Fed	70.0 $\pm$ 13.1	58.0 $\pm$ 8.7	67.7 $\pm$ 3.5	NS	NS
Fasting	66.5 $\pm$ 9.8	50.7 $\pm$ 6.8	40.7 $\pm$ 5.9	NS	NS
Free fatty acid (mEq/l)					
Fed	0.47 $\pm$ 0.10	0.58 $\pm$ 0.19	0.54 $\pm$ 0.14	NS	NS
Fasting	0.58 $\pm$ 0.02	0.66 $\pm$ 0.19	0.55 $\pm$ 0.23	NS	NS
β -Hydroxybutyrate (μ mol/l)					
Fasting	84.5 $\pm$ 13.5	79.6 $\pm$ 28.2	124.5 $\pm$ 16.5	NS	<0.05
AST (unit/l)					
Fasting	85.4 $\pm$ 22.7	103.7 $\pm$ 20.6	1007.0 $\pm$ 664.8	NS	<0.01
Epididymal fat pad ^a	1.31 $\pm$ 0.14	1.27 $\pm$ 0.19	0.89 $\pm$ 0.29	NS	<0.01
Insulin (ng/ml)					
Fed	0.19 $\pm$ 0.18	0.21 $\pm$ 0.18	0.16 $\pm$ 0.12	NS	NS
Fasting	0.32 $\pm$ 0.16	0.67 $\pm$ 0.23	0.22 $\pm$ 0.18	NS	NS
Day 8					
Change of body weight (g)	0.46 $\pm$ 0.40	0.175 $\pm$ 0.31	-2.34 $\pm$ 0.52	NS	<0.01
Food intake (g/day)	2.59 $\pm$ 0.15	2.46 $\pm$ 0.28	2.39 $\pm$ 0.12	NS	NS
Plasma glucose (mg/dl)					
Fed	240.8 $\pm$ 4.8	198.8 $\pm$ 29.4	118.0 $\pm$ 4.3	<0.01	<0.01
Fasting	154.8 $\pm$ 29.1	154.3 $\pm$ 26.2	88.3 $\pm$ 10.7	NS	<0.01
Plasma triglyceride (mg/dl)					
Fed	123.3 $\pm$ 22.9	95.3 $\pm$ 21.6	49.3 $\pm$ 14.1	<0.05	<0.01
Fasting	39.5 $\pm$ 7.2	47.0 $\pm$ 6.5	41.8 $\pm$ 9.6	NS	NS
Total cholesterol (mg/dl)					
Fed	91.8 $\pm$ 3.6	82.3 $\pm$ 12.2	48.5 $\pm$ 12.4	<0.05	<0.01
Fasting	54.0 $\pm$ 2.6	51.3 $\pm$ 6.0	37.3 $\pm$ 5.0	NS	<0.01
Free fatty acid (mEq/l)					
Fed	0.53 $\pm$ 0.02	0.49 $\pm$ 0.02	0.487 $\pm$ 0.07	NS	NS
Fasting	0.77 $\pm$ 0.27	0.65 $\pm$ 0.10	0.871 $\pm$ 0.37	NS	NS
β -Hydroxybutyrate (μ mol/l)					
Fasting	99.7 $\pm$ 36.5	123.8 $\pm$ 47.9	257.6 $\pm$ 61.8	NS	<0.05
AST (unit/l)					
Fasting	97.7 $\pm$ 28.1	346.8 $\pm$ 68.0	509.3 $\pm$ 85.4	<0.01	<0.01
Epididymal fat pad ^a	1.11 $\pm$ 0.04	0.95 $\pm$ 0.14	0.66 $\pm$ 0.05	NS	<0.01
Insulin (ng/ml)					
Fed	0.27 $\pm$ 0.35	0.57 $\pm$ 0.66	0.79 $\pm$ 0.28	NS	NS
Fasting	0.50 $\pm$ 0.52	0.51 $\pm$ 0.44	0.16 $\pm$ 0.31	NS	NS

NS, not significant.

^aPercent of body weight.

microarrays to investigate how TRIB1 modulates hepatic lipid and glycogen storage. The number of genes with over a twofold difference in expression between the overexpression and knockdown groups numbered 2556 and 2158 at days 4 and 8 respectively and 1372 of these genes were shared. We compared pAx-CMV-TRIB1 with

pAxCA-LacZ and pAxCA-LacZ with pAx-shTrib1 at days 4 and 8 using GSEA. The commonly (3/4) enriched gene sets were regarded as significantly affected pathways. Forty-five gene sets were commonly enriched by TRIB1 expression (Supplementary Table 2A, see section on supplementary data given at the end of this article).

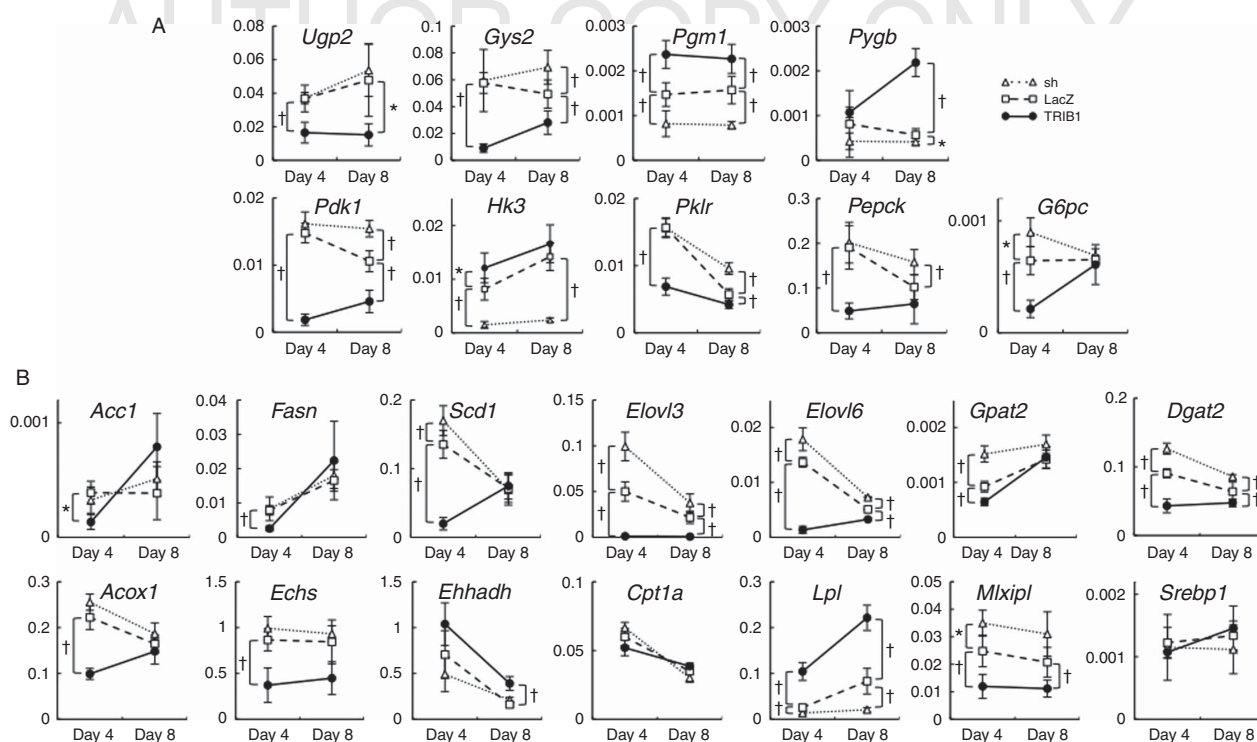
**Figure 1**

Effects of modulating TRIB1 expression on glucose, energy metabolism, liver histology, liver triglyceride (TG), and glycogen storage. (A) Average values of oxygen consumption (VO_2 , ml/kg per h) and respiratory exchange ratios (RER, VCO_2/VO_2) at day 4. Data were collected for 1 min at 5-min intervals. The start of the light period was regarded as Zeitgeber time 0 (ZT0). (B) Glucose tolerance test. Average glucose values (mg/dl) of seven mice per vector (day 8 after vector administration) are shown. (C) Insulin tolerance test. * $P < 0.05$ and $^{\dagger}P < 0.01$ (Student's *t*-test compared with results using pAxCA-LacZ and pAx-shTrib1 vectors). (D) Representative H&E,

Oil Red O, PAS staining (day 8), and TUNEL study (day 4) of mouse liver injected with adenoviral vectors. Scale bars indicate 100 μ m. (E) TG (mg/g) and (F) glycogen (μ g/g) contents in liver at starvation. (G) TUNEL-positive cells in liver section (n/field). Data are presented as means $\pm$ s.d. ($n = 7$ per adenoviral vector). LacZ, control vector encoding LacZ (pAxCA-LacZ); sh, knockdown vector encoding shRNA template (pAx-shTrib1); and TRIB1, overexpression vector (pAx-CMV-TRIB1). * $P < 0.05$ and $^{\dagger}P < 0.01$ (Student's *t*-test; significant change by pAx-shTrib1 vs pAxCA-LacZ or pAxCA-LacZ vs pAx-CMV-TRIB1).

Although 12 of the enriched gene sets were associated with pathways involved in immune responses, nine gene sets were associated with nutrient metabolism. The expression heat map for starch and sucrose metabolism showed that TRIB1 overexpression enriched the genes involved in glycogenolysis and suppressed those involved in glycogen synthesis. The heat map for glycolysis and gluconeogenesis showed that the enrichment and depletion of genes (other than *Pklr*) involved in glycolysis and gluconeogenesis respectively (Supplementary Fig. 3A and B, see section on supplementary data given at the end of this article, and Fig. 2A). The overexpression of TRIB1 enriched the gene set for the insulin signaling pathway whereas knockdown enriched that for maturity onset diabetes (Supplementary

Fig. 3C, D and E, and Supplementary Table 2B). These results are consistent with the low blood glucose levels and reduced glycogen storage identified in mice overexpressing TRIB1. Although GSEA did not appear to alter the pathways of fatty acid biosynthesis and oxidation (possibly due to the small number of genes involved in the pathway), TRIB1 overexpression downregulated, and knockdown increased the expression of genes involved in TG biosynthesis (*Scd1*, *Elovl3*, *Elovl6*, *Gpat2*, and *Dgat2*) and *Acc1* and *Fasn* were downregulated by TRIB1 overexpression at day 4, which was confirmed by real-time PCR (Fig. 2B). However, four (*Acc1*, *Fasn*, *Scd1*, and *Gpat2*) became equivalent at day 8. The expression levels of the genes for fatty acid oxidation (*Acox1*, *Echs*, *Ehhadh*, and *Cpt1a*) were inconsistent with the

**Figure 2**

Expression levels of mRNA involved in glucose–glycogen (A) and lipid (B) metabolism quantified by real-time PCR analysis in the adenovirus-injected liver. Y-axis values show relative expression levels of respective genes

against housekeeping gene, *36b4*. Data represent means $\pm$ s.d. ($n=7$ per adenoviral vector). * $P<0.05$ and † $P<0.01$ (Student's *t*-test).

lipid levels. Among the transcription factors involved in lipogenesis, *Mlxipl* expression was significantly increased by knockdown and decreased by TRIB1 overexpression, but levels of *Srebp1* transcripts did not change (Fig. 2B).

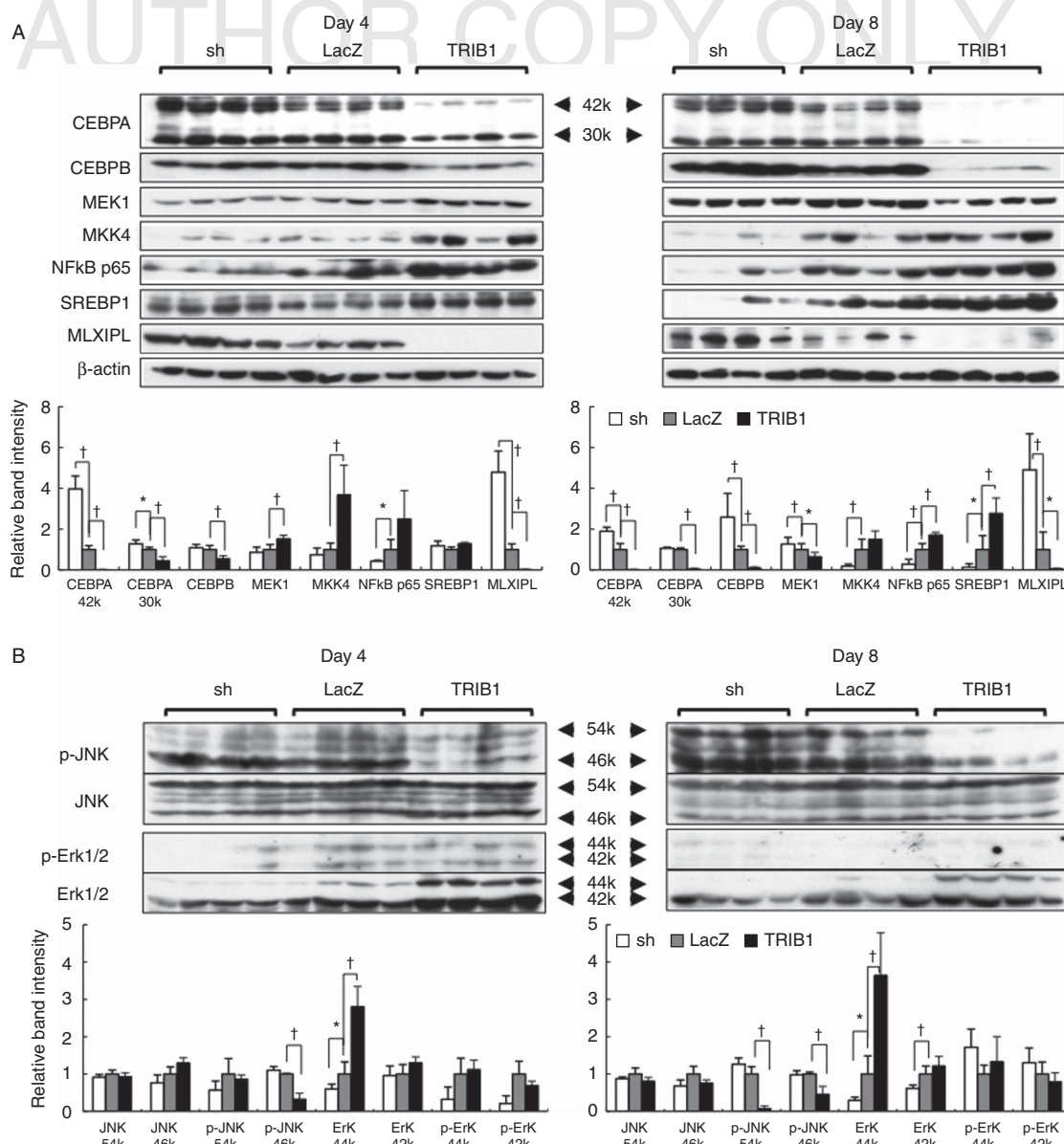
Western blotting of potential targets of TRIB1

TRIB1 interacts with COP1, CEBPA, CEBPB, MEK1, p65/RelA, and MKK4 (Hegedus *et al.* 2007). Levels of CEBPA and CEBPB were significantly decreased in the liver overexpressing TRIB1 and increased by knockdown (Fig. 3A). TRIB1 overexpression increased and decreased MEK1 (MAP2K1) by days 4 and 8 respectively and increased protein levels of MKK4 (MAP2K4) (Fig. 3B). Although MKK4 are scaffold activators of JNK, TRIB1 overexpression, and knockdown did not influence their protein levels, but overexpression downregulated the phosphorylation of 183/185Tyr (Fig. 3B). Erk protein levels were significantly increased by TRIB1 overexpression and decreased by knockdown, while phosphorylation was not influenced (Fig. 3B). Multiple changes in the transcript levels involved in TG synthesis suggested modulation of the hepatic lipogenic master regulators,

SREBP1 and MLXIPL. Enforced TRIB1 expression abolished MLXIPL protein on both days 4 and 8, whereas knockdown increased the protein level to 4.8- and 4.9-fold that of control on days 4 and 8 respectively (Fig. 3A). While TRIB1 expression level did not influence levels of *Srebp1* mRNA, protein levels were increased by TRIB1 overexpression and decreased by knockdown at day 8 (Fig. 3A), indicating that the change in the SREBP1 protein level was a compensatory phenomenon against the exhaustion of hepatic lipid content, which might have resulted in the partial recovery of transcript levels of TG synthetic genes.

Molecular interaction between TRIB1 and MLXIPL

We examined whether the disappearance of MLXIPL caused by TRIB1 overexpression resulted from their molecular interaction using mammalian two-hybrid and pull-down assays. Interaction between GAL4-TRIB1 and VP16-MLXIPL fusion proteins significantly enhanced reporter gene expression as did interactions with VP16-CEBPA and VP16-CEBPB (Fig. 4C). The interaction with MLXIPL was attenuated by deletion of the NLS or the

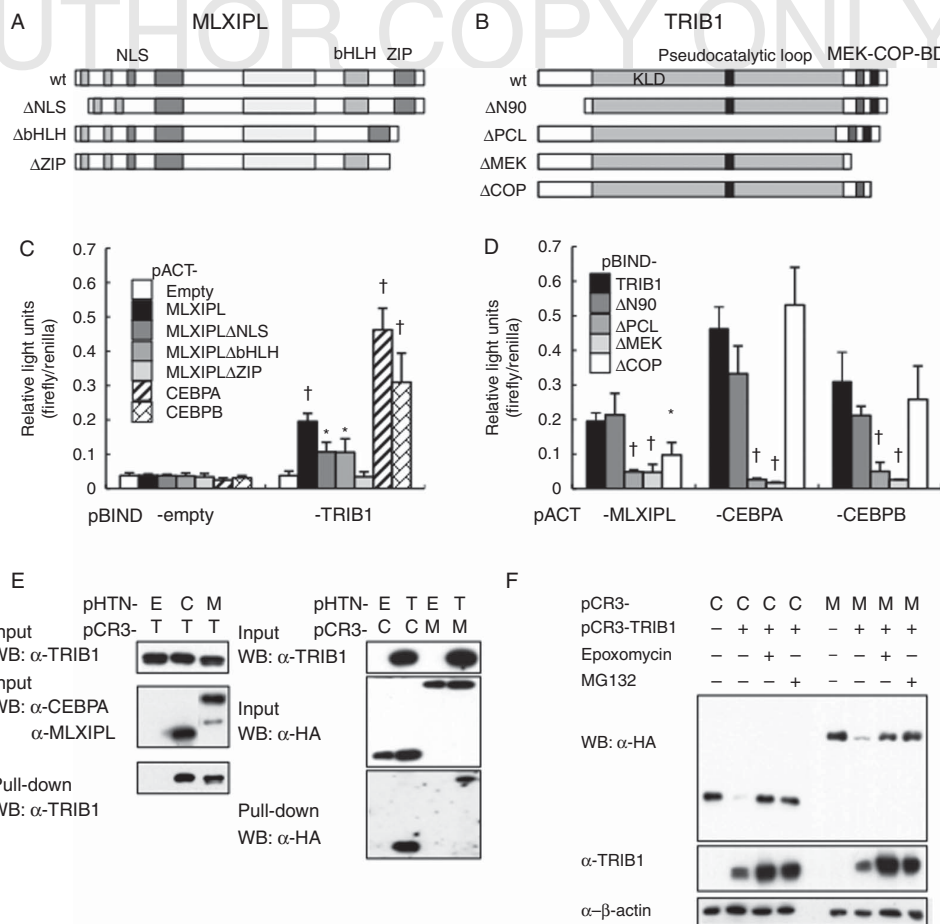
**Figure 3**

Effects of modulation of TRIB1 expression on target proteins. (A) Western blots. Hepatic protein (20 µg) resolved by electrophoresis was transferred onto PVDF membranes and immunoblotted with respective primary antibodies against CEBPA, CEBPB, MEK1, MKK4, NFkB p65, SREBP1, MLXIPL, and β-actin. (B) Effects of modulation of TRIB1 expression on phosphorylation of proteins in MAPK cascade. Western blotting using

antibodies against p-JNK, JNK, p-ERK1/ERK2, and ERK1/ERK2. Signals were quantified by densitometry and normalized to β-actin levels. Bar graphs show relative amounts of respective band intensities (means ± s.d.; n = 7). Control values (pAxCa-LacZ) are defined as 1. * $P < 0.05$ and † $P < 0.01$ (Student's *t*-test).

bHLH domain and disappeared in the absence of a ZIP domain (Fig. 4A and C). The interaction also disappeared when the pseudocatalytic loop or MEK1 binding domain of TRIB1 was removed from the VP16 fusion proteins and became significantly attenuated by deleting the COP1 binding domain (Fig. 4B and D). We tested mutual co-precipitation using HaloTag fusion proteins to verify

interaction between TRIB1 and MLXIPL. Halo-CEBPA and halo-MLXIPL co-precipitated TRIB1 proteins, and, conversely, halo-TRIB1 co-precipitated CEBPA and MLXIPL (Fig. 4E). We then examined the involvement of the proteasome system in the regulation of CEBPA and MLXIPL protein levels through interaction with TRIB1. Co-expression with TRIB1 remarkably reduced CEBPA or

**Figure 4**

Studies of molecular interaction of TRIB1 with CEBPA, CEBPB, and MLXIPL. Constructs of MLXIPL mutants. NLS, nuclear localization signal; bHLH, basic helix–loop–helix zipper domain; and ZIP, leucine-zipper-like domain. (B) Constructs of TRIB1 mutants. BD, binding domain and PCL, pseudo-catalytic loop. (C) Mammalian two-hybrid assay. Vectors pBIND-empty or pBIND-TRIB1 were co-transfected with indicated pACT vectors into COS7 cells harboring pGL4.31 vector. Values are relative light units (firefly/Renilla, means $\pm$ s.d.) of independent experiments in quadruplicate. * $P < 0.05$ and $^{\dagger}P < 0.01$ (Student's *t*-test; pACT vs pACT-empty and pBIND-TRIB1 vectors). (D) Full-length *TRIB1* cDNAs or *TRIB1* cDNAs with deleted N-terminal 90 residues (Δ N90), pseudo-catalytic loop (Δ PCL), COP1 (Δ COP), or MEK (Δ MEK) binding domains were co-transfected with pACT-MLXIPL,

CEBPA, or CEBPB vector. * $P < 0.05$ and $^{\dagger}P < 0.01$; significant reduction compared with pBIND-TRIB1. (E) Pull-down assays. ORFs of *TRIB1* (T), *CEBPA* (C), and *MLXIPL* (M) were inserted into pHTN vector or pCR3 vector with influenza hemagglutinin (HA)-tag. Negative control was empty pHTN (E) vector. Constructs were co-transfected into COS7 cells and then HaloTag fusion and associated proteins were extracted from cell lysates using HaloLink resin. Molecular interactions were assessed by western blotting using anti-TRIB1 or anti-HA antibodies (pull-down). (F) TRIB1 induced degradation of CEBPA and MLXIPL. COS7 cells were transfected with pCR3-HA-CEBPA (A) or -MLXIPL (M) along with pCR3-TRIB1 (+) or pCR3-empty (–) vector with or without proteasome inhibitor (epoxomicin or MG132). Protein levels were assessed by western blotting.

MLXIPL protein levels and proteasome inhibitors rescued CEBPA and MLXIPL from the reduction (Fig. 4F). The proteasome inhibitors simultaneously increased TRIB1 protein levels. These results indicated that molecular interactions between TRIB1 and CEBPA as well as MLXIPL accelerate their decay, and interaction of TRIB1 with the target proteins accelerated the decay of itself. Accelerated decay of TRIB1 may potentially explain the continuously repressed MLXIPL until day 8 of TRIB1 overexpression mice, while TRIB1 protein levels greatly reduced to nearly

physiological levels under still excessive human *TRIB1* mRNA levels (Supplementary Fig. 1A and B).

Identification of regulatory elements and SNP in the downstream of *TRIB1*

To investigate whether the LD block at the downstream of *TRIB1* encodes regulatory elements and whether variations in the sequence affect the regulatory function, we constructed luciferase reporter vectors that individually

encoded six conservative fragments and the variants of high frequent SNPs (Fig. 5A). Whereas none of the constructs exhibited any enhancement activities in 293 and COS7 cells (not shown), fragment 1 (+34 834 to 36 474; relative distance from 1st ATG of *TRIB1*) showed 5.2- and 4.4-fold enhanced activities in HepG2 cells (Fig. 5A). The allelic difference in the activities was statistically significant. Fragments 2, 3, 5, and 6 did not exhibit either enhancing activities or allelic differences in HepG2 cells. Fragment 4 showed weak (1.31 and 1.34) activities but the difference in rs2980867 variation was not statistically significant. Our cloned fragment 1 encoding five SNP variations was further analyzed by constructing deletion derivatives to identify a functional base substitution. The 3' quarter of fragment 1 (+36 183 to 36 474) encoding rs6982502 retained enhancement activities and an allelic difference. Although most of the enhancement activities of the fragment were lost by further 3' deletion, activity differed in the remaining sequence (+36 184 to 36 246) upon nucleotide substitution with rs6982502 (Fig. 5B). Therefore, we speculated that rs6982502 was a principal candidate for functional variation in the 25 kb

LD block, although obvious disruption of binding motifs for transcription factors by the SNP was not predicted through a database search using MatInspector Software (Genomatix; <http://www.genomatix.de/index.html>).

TRIB1 SNP was associated with NAFLD diagnosed by ultrasonography

We assessed the effects of the candidate functional SNPs, rs6982502 and rs2980867, as well as the initially identified tag-SNP, rs17321515, on NAFLD in two populations to determine whether or not *TRIB1* affects hepatic lipid accumulation in humans. The rates of ultrasonographically diagnosed NAFLD in populations 1 and 2 were 34.7 and 28.8% respectively. The genotype frequencies of the three SNPs conformed to Hardy-Weinberg predictions in these populations (Table 2). When age, sex, BMI, and clinical history of diabetes were the covariates, the three SNPs became significantly associated with ultrasonographic NAFLD and plasma TG in both populations (Table 2). The minor allele of rs6982502, C, maximally increased the risk of NAFLD ($P=9.39 \times 10^{-7}$; odds ratio,

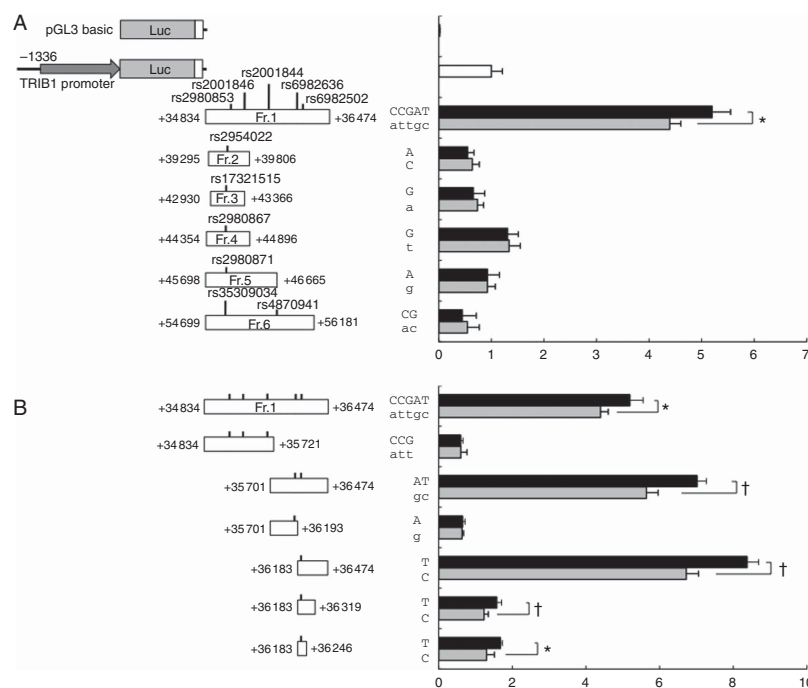


Figure 5

Reporter gene assays of human *TRIB1* downstream sequences. Upstream sequence of *TRIB1* gene from -1336 to -1 relative to ATG start codon was subcloned into pGL3 basic vector and used as parental vector (A). Six conservative fragments (Supplementary Fig. 4) were inserted at 3' position of luciferase in parental vector. Relative positions of fragments are shown at both sides of diagram. Sequence variations in six fragments are indicated

by rs numbers. Major and minor alleles of SNPs are indicated by capital and lowercase letters respectively. Relative activities (firefly/Renilla light units) against parental vector are shown. Relative activities of deletion mutants of Fr.1 (B). * $P<0.05$ and † $P<0.01$ (Student's *t*-test; among major and minor allele constructs).

Table 2 Genetic associations between *TRIB1* SNP with NAFLD and plasma TG. The effects of the risk allele on NAFLD and log-transformed plasma TG were assessed by logistic regression models and multiple regression analysis assuming an additive model of inheritance respectively

	Panel 1			Panel 2			Combined				
	n = 2245, Fem(%) = 58.3			n = 3325, Fem(%) = 56.3			n = 5570, Fem(%) = 56.8				
NAFLD	P	OR (95% CI)	β	P	OR (95% CI)	β	P	OR (95% CI)	Alleles ^a	MAF	P-HWE
rs6982502	7.60 × 10 ⁻³	1.23 (1.06–1.43)		2.19 × 10 ⁻⁴	1.31 (1.16–1.48)		9.39 × 10 ⁻⁷	1.27 (1.17–1.39)	T > C	0.437	0.832
rs17321515	7.82 × 10 ⁻³	1.22 (1.06–1.42)		4.77 × 10 ⁻³	1.19 (1.06–1.35)		6.18 × 10 ⁻⁵	1.22 (1.12–1.34)	G > A	0.489	0.496
rs2980867	0.123	1.13 (0.97–1.33)		0.022	1.16 (1.02–1.32)		5.17 × 10 ⁻⁴	1.18 (1.07–1.29)	G > T	0.337	0.765
TG	P	β	P	P	β	P	P	β			
rs6982502	4.07 × 10 ⁻⁴	0.060		1.57 × 10 ⁻³	0.054		3.58 × 10 ⁻⁶	0.055			
rs17321515	6.41 × 10 ⁻⁴	0.058		3.22 × 10 ⁻²	0.037		1.01 × 10 ⁻⁴	0.046			
rs2980867	5.09 × 10 ⁻³	0.048		6.34 × 10 ⁻³	0.054		1.03 × 10 ⁻⁴	0.052			

Fem(%), female percentage in the studied population; OR, odds ratio, β, β-coefficient of minor allele; MAF, minor allele frequency; P-HWE, probability value in Hardy–Weinberg equilibrium.
^aMinor alleles are shown at right side of greater than.

1.27, 95% CI 1.17–1.39 (combined analysis)) and plasma TG level ($P=3.58 \times 10^{-6}$) among the three SNPs (Table 2).

Discussion

We found that *TRIB1* expression levels in the liver affected plasma and hepatic lipid levels in mouse models. Changes in plasma TG level were consistent with those of previous studies that included systemic knockout mice and hepatic overexpression via adeno-associated virus vector (AAV) (Burkhardt *et al.* 2010), but changes in hepatic lipid and glycogen levels were novel findings of this study. Therefore, we further examined the molecular mechanisms of *TRIB1* in hepatic lipid accumulation using mouse models and genetic associations in a Japanese population that was ultrasonographically diagnosed with NAFLD. Hepatic transcriptome analysis showed that *TRIB1* overexpression downregulated multiple genes that are involved in TG synthesis, gluconeogenesis, and glycogen synthesis. Burkhardt *et al.* (2010) found repressed levels of lipid oxidation transcripts (*Cpt1a*, *Cpt2*, and *Acox1*) in knockout mice, whereas *Acox1* was repressed and *Cpt1a* expression was not affected in mice overexpressing *TRIB1* in this study, suggesting that *TRIB1* does not regulate expression levels of the genes that are involved in lipid oxidation. Modulation of the expression of multiple gene sets involved in nutrition metabolisms suggested that *TRIB1* affects transcription factors or signaling molecules based on its biochemical features.

The expression of *TRIB1* modulated the hepatic protein levels of all potential targets studied. Many studies have implicated CEBPA and CEBPB in hepatic lipid biosynthesis (Wang *et al.* 1995, Lee *et al.* 1997, Matsusue *et al.* 2004, Qiao *et al.* 2006, Rahman *et al.* 2007, Schroeder-Gloeckler *et al.* 2007). Constituents of the MAPK pathway have also been implicated in steatohepatitis (Schattenberg *et al.* 2006). In addition to these, MLXIPL protein levels in the liver injected with adenovirus vectors suggested that MLXIPL regulates the *TRIB1*-induced modulation of hepatic lipid storage. Molecular interaction studies showed that MLXIPL is a novel target protein downregulated by *TRIB1*. MLXIPL is a glucose sensor that is involved in hepatic liponeogenesis (Uyeda & Repa 2006). The SNPs of *MLXIPL* are closely associated with human plasma TG (Teslovich *et al.* 2010) and knockout mice are resistant to steatohepatitis (Dentin *et al.* 2006). Activated and nuclear translocated MLXIPL binds to carbohydrate-responsive element in lipogenic genes and upregulates the transcription of *Pklr*, *Acc1*, *Fasn*, and *Mlxipl* itself (Dentin *et al.* 2006, Denechaud

et al. 2008), which were downregulated by TRIB1 overexpression in this study, supporting the notion that TRIB1 modulates MLXIPL control of lipogenesis.

This study also found that TRIB1 negatively regulates hepatic glycogen storage. Insulin signals are thought to comprise a principal regulator of glycogen synthesis; glycogen synthesis was downregulated while the gene set for the insulin signaling pathway was upregulated. Furthermore, TRIB1 expression downregulated the gene set for gluconeogenesis. These results resembled the changes in glycogen storage caused by the genetic disruption of *Cebpa* in mice, which results in decreased hepatic glycogen and gluconeogenesis (Bezy *et al.* 2007, Wang *et al.* 2009). These facts indicated that enhanced CEBPA decay probably mediated glycogen exhaustion and low plasma glucose in mice overexpressing TRIB1.

Previous studies have identified molecular interaction between TRIB1 and NFκB p65, which enhances inflammation in adipose tissue (Ostertag *et al.* 2010). This study also found that TRIB1 overexpression significantly increased, whereas knockdown decreased levels of p65 protein. The overexpression of TRIB1 also enhanced mRNA levels of the gene set for cytokine–cytokine receptor interaction (Supplementary Table 2A). Leukocyte infiltration on day 8 might have resulted from activation of the NFκB pathway. Hepatic inflammation is thought to be a major contributor to the progression from simple steatosis to non-alcoholic steatohepatitis (NASH; Vanni *et al.* 2010). Insulin-resistant adipose tissue is thought to be the principal pathogenic source of adipokines and cytokines that are involved in hepatic inflammation. Further studies using conditional knockout mice are required to determine whether or not TRIB1 is involved in the pathogenesis of NASH.

Implication of TRIB1 in apoptosis for leukemogenesis is under dispute. Gilby *et al.* (2010) showed that TRIB1 enhanced apoptosis of leukemic cell line through suppression of JNK–Bcl-2 pathway. Yokoyama *et al.* (2010) showed that TRIB1 suppressed apoptosis through enhancement of Erk phosphorylation. TRIB1 overexpression in mouse liver reduced phosphorylation of JNK but did not affect on phosphorylation of Erk and enhanced the apoptosis of hepatocytes, which might have resulted in the massive elevation of plasma AST levels. Molecular mechanisms in cell type-specific regulation of MAPK pathway by TRIB1 should be resolved further.

Through functional screening *in vitro*, we identified a DNA fragment (Fr.1) in the downstream LD block of TRIB1 that had substantial enhancement activities in HepG2 cells but not in COS7 and 293 cells. The presence of an enhancer

element in Fr.1 was supported by the ENCODE database showing methylation of lysine 4 of H3 histone (H3K4Me3), DNaseI hypersensitivity clusters and the ChIP-seq in HepG2 cells (Supplementary Fig. 4), whereas other cell lines show different H3K4Me3 peaks, suggesting cell-type-specific use of enhancer elements. The ChIP fragment generated by anti-p300 of HepG2 cells in the ENCODE database was identical to the minimum enhancer fragment (+36 183 to 36 474). rs6982502 localized in the fragment and modulated the enhancing activity. Base substitution of the minor allele of rs6982502, C, attenuated the enhancement activity to 80.2% of that of the major allele, T. Indeed, –20% is a small effect, but our knockdown experiment (20–40% reduction in production of the transcript) efficiently increased plasma and hepatic lipid, supporting the notion that rs6982502 is a candidate functional SNP in the LD block. Our SNP study of ultrasonographically diagnosed NAFLD showed that a significant association between rs6982502 and the minor allele increased risk for NAFLD 1.27-fold. The minor allele of rs6982502 showed a tight linkage ($D' = 0.985$, $r^2 = 0.713$) with the risk allele of rs17321515 that the GWAS initially identified as a tag-SNP that increased plasma TG and LDL (Kathiresan *et al.* 2008). Relatively lower levels of TRIB1 expression due to the minor allele of rs6982502 might have resulted in increased levels of plasma lipid and progression to hepatic steatosis. While *TRIB1* has not been significantly associated with NAFLD diagnosed by computed tomography (CT) (Romeo *et al.* 2008, Kawaguchi *et al.* 2012), the GWAS of plasma AST levels in Europe found a significant association with the SNP, rs2954021, which localized in the same LD block as rs17321515 (Chambers *et al.* 2011). A weak association between rs2954021 and hepatic steatosis was also identified (Chambers *et al.* 2011). Serum-free fatty acid arising from insulin-resistant adipose tissue and hepatic lipogenesis *de novo* increased by upregulated lipogenic gene expression are regarded as the principal pathogenetic pathways of NAFLD (Vanni *et al.* 2010). TRIB1 is considered a potential genetic factor of NAFLD because of its ability to modulate levels of lipogenic gene expression. Further genetic studies of various ethnic groups and haplotype effects are required to confirm the involvement of TRIB1 in the pathogenesis of NAFLD. The regulatory mechanisms of *TRIB1* expression should also be revealed further to investigate regimen for the reduction of fat accumulation in liver. In our preliminary study, a high-fat diet but not starvation induced the expression of *Trib1* mRNA in mouse liver (Supplementary Fig. 5, see section on supplementary data given at the end of this article).

In summary, chronic (SNP) or acute (adenoviral) modulation of TRIB1 expression affects hepatic lipid storage. Changes in hepatic lipid synthesis caused by TRIB1 were probably mediated, at least in part, by molecular interaction with the novel target, MLXIPL.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-13-0243>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by grants-in-aid from the Japanese Ministry of Education, Culture, Sports, Science, and Technology (numbers 22591001 and 21687021) and by a grant from the Kao Research Council for the Study of Healthcare Science (Tokyo, Japan).

Acknowledgements

The authors are grateful to Takashi Oyamada, Kayo Nagashima and Yukiko Ohashi for excellent technical assistance.

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Received in final form 6 December 2013

Accepted 2 January 2014

Accepted Preprint published online 3 January 2014