Pseudokinase Tribbles 1 (TRB1) Negatively Regulates Tumor-Suppressor Activity of p53 through p53 Deacetylation

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TRB1 (TRB1) is one of the mammalian orthologs of Drosophila Tribbles, which regulates development and cell proliferation. TRB1 is suggested to act as a scaffold protein in signaling pathways for important cellular processes. TRB1 has also been identified as a myeloid oncogenic driver and mediates leukemogenesis through the mitogen-activated protein extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway and CCAAT/enhancer binding protein (C/EBP) transcriptional factors. However, the physiological roles of TRB1 in solid tumors have not been clarified. Here, we show that TRB1 interacts with p53 and suppresses its tumor suppressor activity. TRB1 knockdown enhances transcriptional activity of p53 and decreases cell viability. Interestingly, TRB1 enhances histone deacetylase 1 (HDAC1)-mediated p53 deacetylation and decreases DNA binding of p53. These results suggest that TRB1 is involved in the proliferation of tumor cells by inhibiting the activities of tumor suppressor p53 in solid tumors.

Key words  Tribbles 1 (TRB1); p53; histone deacetylase 1 (HDAC1); deacetylation

The pseudokinase Tribbles 1 (TRB1; also termed Trib1, C8FW, SKIP1) is one of the mammalian orthologs of Tribbles, a cell cycle regulator during development in Drosophila.1–2) TRB1 has a conserved motif, which is similar to the catalytic domain of a serine/threonine kinase, but lacks an ATP binding site or one of the conserved catalytic motifs essential for kinase activity.3,4) Therefore, TRB1 is considered as a scaffold protein or an adaptor protein to facilitate the degradation of their target proteins and to regulate several key signaling pathways for important cellular processes.5 In cancer, TRB1 has been identified as a myeloid oncogenic driver and induces acute myeloid leukemia (AML) in mice through the mitogen-activated protein extracellular kinase (MEK)/extracellular signal-regulated kinase (ERK) pathway and CCAAT/enhancer binding protein (C/EBP) transcriptional factors.6,7) Importantly, TRB1 is often overexpressed in AML and myelodysplastic syndrome with gene amplification.8,9) TRB1 is also found to be overexpressed in solid tumors, including prostate cancer,10 thyroid cancer,11 ovarian cancer,12 and colorectal cancer.13 Moreover, Oncomine data analyses show that TRB1 is overexpressed in other types of cancer (e.g. breast, esophageal, head and neck, and melanoma).14,15) However, whether and how TRB1 plays the physiological roles in solid tumors has not been fully investigated.

The tumor suppressor p53 plays an important role in regulating cell proliferation during various stimuli, including genotoxic stress and oncogenic activation, and nearly all cancers show defects in p53 pathway.13–16) p53 primarily functions as a transcriptional factor that activates various genes responsible for cell-cycle arrest, senescence or apoptosis to prevent tumor cell growth.17) The expression of p53 is tightly regulated by a mechanism involving the ubiquitin-proteasome-mediated degradation pathway. When cells experience a variety of stress conditions, p53 becomes stabilized and activated. The mechanisms that activate p53 are generally thought to entail posttranslational modifications of the p53 protein, such as phosphorylation, acetylation and methylation.18,19) In particular, acetylation of p53 enhances its transactivation functions and stability. Moreover, p53 acetylation leads to stimulate its sequence-specific DNA-binding.20) It has been reported that p53 is acetylated by histone acetyltransferases, which include p300, CAMP response element binding protein (CREB)-binding protein (CBP), p300/CBP-associated factor (PCAF), Tip60 and males absent or the first (MOF), and that loss of acetylation blocks p53-dependent growth arrest and apoptosis.21,22)

Conversely, histone deacetylases (HDACs) can deacetyl p53 and repress p53-dependent transcriptional activation, apoptosis and growth arrest. To date, p53 acetylation can be reversed by distinct HDAC complexes containing HDAC1/2 or SIRT1.23) Indeed, p53 hyperacetylation occurs in HDAC1/2- or SIRT1-deficient mice.24) Interestingly, some oncogenes have been shown to stimulate deacetylation of p53, leading to an inhibition of tumor-suppressor activity of p53. For example, oncoprotein Mdm2 can promote p53 deacetylation by recruiting a complex containing HDAC1.25) We previously reported that oncoprotein Ski binds to SIRT1 and stabilizes p53-SIRT1 interaction to promote p53 deacetylation.26) Therefore, it may be that there is a plausible mechanism of p53 inactivation through oncogenes-mediated p53 deacetylation in tumor cells.

Here, we show that TRB1 physically interacts with p53 and suppresses p53-mediated functions. TRB1 knockdown enhances the transcriptional activity of p53 and decreases cell survival. Mechanistically, TRB1 enhances HDAC1-mediated p53 deacetylation and decreases the DNA binding of p53. These results suggest that TRB1 is involved in the proliferation of tumor cells by inhibiting the activities of tumor suppressor p53 in solid tumors.

MATERIALS AND METHODS

Cell Lines, Plasmids and Transfections  H1299 cells were cultured in RPMI1640 medium containing 10% fetal bovine serum (FBS) and penicillin/streptomycin. MCF7 cells and 293 cells were grown in Dulbecco’s modified Eagle’s me-
dium containing 10% FBS and penicillin/streptomycin.

The original constructs encoding human p53, p300, HDAC1 and SIRT1 were described previously.25,26 peDNA3/FLAG-TRB1 was polymerase chain reaction (PCR) amplified using mRNA derived from MCF7 cells. All constructs were verified by sequencing. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, U.S.A.) following the manufacturer's instructions.

**Antibodies and Reagents** Anti-p53 (DO-1) was purchased from Calbiochem (San Diego, CA, U.S.A.). Horseradish peroxidase (HRP)-conjugated anti-p53 antibody (DO-1) was purchased from Santa Cruz (Santa Cruz, CA, U.S.A.).

The anti-FLAG (M2) antibody, anti-β-actin (AC-15) antibody, actinomycin D and Nutilin-3 were obtained from Sigma (St. Louis, MO, U.S.A.), and the anti-acetyl-p53 (K382) antibody and HRP-conjugated secondary antibodies were purchased from Cell Signaling Technology (Danvers, MA, U.S.A.). The anti-Myc (4A6) and anti-TRB1 (9-126) antibodies were purchased from Millipore (Billerica, MA, U.S.A.). Anti-p21 was obtained from BD Biosciences (Franklin Lakes, NJ, U.S.A.).

**Immunoprecipitation and Western Blot Analysis** Cells were lysed in TNE buffer (20 mM Tris–HCl, pH 7.5, 120 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.5% Triton X-100) supplemented with protease inhibitors, phospha- tase inhibitors as described previously.27 The lysates were immunoprecipitated with the appropriate antibodies in the presence of protein G sepharose (GE Healthcare, Buckinghamshire, U.K.). For Strept-Tactin pulldown, Strept-Tactin sepharose (IBA Lifesciences, Gottingen, Germany) was used instead of protein G sepharose with bound antibodies. Immunoprecipitates were washed four times with TNE buffer and then subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis. The gels were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore) and then probed with the indicted antibodies. The Western blot membranes were developed using ECL Western blotting detection reagents (GE Healthcare).

**RNA Extraction and Quantitative Real-Time PCR (qPCR)** Total RNA was extracted as described previously.28 First-strand cDNAs were synthesized with PrimeScript reverse transcriptase (TaKaRa Bio, Shiga, Japan) and oligo(dT) primers. q-PCR was performed using GeneAce SYBR qPCR Mix (Invitrogen) according to the manufacturer's protocol.25 The following primer sequences were used: human MDM2 forward, 5' TGC ATG TCC CCA AAG TC-3'; reverse, 5' TGT TTG TAA ATA GCC-3'; TRB1, TRB2 and TRB3 by siRNA in breast cancer cell line to examine whether TRB family members give the sur-
vival advantages to tumor cells, we specifically knockdowned

**RESULTS**

TRB1 Suppresses the Transactivation Ability of p53

In order to examine whether TRB family members give the survival advantages to tumor cells, we specifically knockdowned TRB1, TRB2 and TRB3 by siRNA in breast cancer cell line MCF7 (Fig. 1A). As we mention later, TRB1 knockdown resulted in markedly reduced proliferation and survival of MCF7 cells (Figs. 1F, G). Knockdown of TRB2 or TRB3 also down-regulate the MCF7 proliferation, however, these effects are relatively low (data not shown). We therefore investigated

**Chromatin Immunoprecipitation (ChIP) Assay** ChIP assays were performed as previously described.25 Briefly, cells were fixed in 1% formaldehyde with gentle shaking for 10 min at room temperature, and the cross-linking reaction was stopped by adding 2.5 M glycine to a final concentration of 0.125 M glycine. After two washes with cold phosphate-buffered saline, the cells were harvested by scraping, pelleted, and resuspended in SDS lysis buffer (50 mM Tris–HCl, pH 8.0, 1% SDS, 10 mM EDTA, and protease inhibitors). The samples were sonicated three times for 15 s each with 30 s intervals using a UR-20P sonicator (TOMY SEIKO, Tokyo, Japan), and centrifuged at 14000 rpm at 8°C for 10 min. After removing a control aliquot (whole cell extract), the supernatants were diluted 10-fold in ChIP dilution buffer (20 mM Tris–HCl, pH 8.0, 150 mM NaCl, 2 mM EDTA, 1% Triton X-100, and protease inhibitors). Samples were immunoprecipitated with Dynabeads M-280 sheep anti-mouse immunoglobulin G (IgG) (Invitrogen) that had been preincubated with 4 μg of anti-p53 antibody in phosphate-buffered saline, 0.5% bovine serum albumin. After five washes with the ChIP wash buffer (50 mM N-(2-hydroxyethyl)piperazine-N'-2-ethanesulfonic–potassium hydroxide (Hepes–KOH), pH 7.0, 0.5% LiCl, 1 mM EDTA, 0.7% deoxycholate, 1% NP-40) and one wash with TE buffer (pH 8.0), the proteins were eluted from the beads with 0.2 M elution buffer (50 mM Tris–HCl, pH 8.0, 10 mM EDTA, 1% SDS). After reversing the cross-linking, the DNA samples were extracted with a PCR purification kit (FAVORGEN, Pingtung, Taiwan). The purified DNA was analyzed by quantitative real-time PCR. The following specific primers were used, 5'-GGG TGTCCTG ATTGGGTTCCTG-3' and 5'-C TTG AAA CAC GGC ACCTCAAGCA AG-3' for p21 promoter; 5'-GCTG TAC CAC TCTCTG-3' and 5'-G GAATAT GTG TTTA AGA GAC CTCC-3' for Mdm2 promoter; 5'-GTTTGG GCTATTTCACTAGTTG-3' and 5'-ATA AAATGACTGTTAGCCAGAG-3' for HPRT1 first intron were used.

**RNA Interference** Small interfering RNAs (siRNAs) were obtained from Invitrogen. The siRNA duplexes were as follows: TRB1 siRNA sense strand, 5'-CAUCGACUGCA AUAAGGAAACUCA-3'; TR2B siRNA sense strand, 5'-CGC AACCUCUGCAUGUCUUCU-3'; TR3B siRNA sense strand, 5'-UAAGUUGUCAUCCACUCCACCACG-3'; p53 siRNA sense strand, 5'-CAGUUGUUAUCUACUGGGACG GAA-3'. Stealth RNAi29 siRNA Luciferase Reporter Control (Invitrogen) was used as a control. Cells were transfected with the noted siRNAs using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's protocol.25

**Cell Viability Assay** Cells were washed with phosphate buffered saline (PBS). Crystal violet was added to stain cells and the stained cells were lysed in 1% SDS and absorbance was measured at 595 nm.27

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Fig. 1. TRB1 Suppresses the Transactivation Ability of p53

(A) MCF7 cells were transiently transfected with the indicated siRNAs and the knockdown evaluated by quantitative real-time PCR (q-PCR). Error bars represent the S.D. (B) MCF7 cells were transiently transfected with the indicated siRNAs, and treated with 2 nM of Actinomycin D (ActD) for 8 h. mRNA expression levels for p21 (upper panel) and Mdm2 (bottom panel) were determined by q-PCR and normalized to HPRT1 mRNA levels. (C, D) Knockdown of TRB1 augments expression of the p21 mRNA (C) and p21 protein (D) after ActD treatment. MCF7 cells were transfected with the indicated siRNAs, and treated with 2 nM of ActD for 8 h. (C) Quantitative real-time PCR analyses of p21 mRNA levels in MCF7 cells as in (B). (D) Cell lysates were analyzed by immunoblotting with the indicated antibodies. (E) TRB1 suppresses p21 promoter activation by p53. H1299 cells were transfected with p21-promoter Luc and pCMV-βgal in combination with the indicated constructs. After 24 h, the luciferase activity in cell lysates was measured and was normalized with βgal activity. The experiment was run in triplicate, and data are represented as the mean fold activation±S.D. (F, G) TRB1 knockdown decreases cell survival after Nutlin-3 treatment. MCF7 cells were transfected with the indicated siRNAs, and treated with 10 µM of Nutlin-3. After 48 h, the cells were stained with crystal violet (F). Quantitation of data is represented (G).
the expressions of cell cycle regulators and apoptosis regulatory proteins and found the up-regulation of cyclin-dependent kinase inhibitor p21 mRNA in TRB1 knockdown cells (Fig. 1B). As p21 is known to be a well-established p53-target gene, we examined the role of TRB1 in regulating p53 response to cellular stress. Compared to the control siRNA, TRB1 siRNA increased p21 and Mdm2 mRNA levels and p21 protein expression induced by Actinomycin D (ActD) (Figs. 1B, D). The same result was also obtained using HCT116 colon cancer cell line (data not shown). To exclude any off-target effects of the siRNAs, additional TRB1 siRNA was used, and similar results were obtained (data not shown). Simultaneous knockdown of both p53 and TRB1 canceled this ActD-induced increase in p21 (Figs. 1C, D), indicating that the effects mediated by TRB1 are p53-dependent. To next investigate whether TRB1 suppresses the transcriptional activity of p53, a luciferase reporter assay was used. For this purpose, p53-null H1299 cells were co-transfected with p53 and p21-promoter reporter in the absence or presence of TRB1. As shown in Fig. 1E, co-expression of TRB1 inhibited p53 transcriptional activity.

The small molecule Mdm2 antagonist Nutlin-3 exerts anti-tumor effects specifically through direct inhibition of the p53-Mdm2 interaction.29) Nutlin-3 modestly inhibited the cell survival of MCF7 cells, and this effect was diminished upon p53 knockdown (Figs. 1F, G). Importantly, knockdown of TRB1 in MCF7 cells significantly caused additional reduction in survival cells by Nutlin-3 treatment. Taken together, these results suggest that TRB1 suppresses the transactivation ability of p53.

**TRB1 Suppresses the Levels of Acetylated p53 and Reduces the DNA-Binding Activity of p53**

The activity of p53 is largely controlled by p53 protein levels, as well as its DNA-binding activity, subcellular localization, and recruitment of transcriptional coregulators.25) As TRB1 knockdown hardly affected p53 protein levels (Fig. 1D), we next measured the binding activity of p53 to p53-target genes in response to cellular stress by using a chromatin immunoprecipitation (ChIP) assay. We found significantly higher levels of p53 at the p21 promoter and Mdm2 promoter in MCF7 cells than in control cells when TRB1 levels were decreased by siRNA treatment (Fig. 2A). This result indicates that TRB1 represses p53 activity, at least in part by decreasing the DNA binding of p53.

Posttranslational modifications of p53 play critical role for p53 activation in the stress response.18,19) For example, p53 phosphorylation has been shown to regulate its transactivation...
properties. On the other hand, p53 acetylation leads to the activation of sequence-specific DNA binding and stabilization of the p53 protein. As TRB1 knockdown was found to enhance the DNA-binding activity of p53, we next examined the effect of TRB1 on p53 acetylation upon cellular stress. Consistent with previous reports, the levels of K382-acetylated p53 were significantly enhanced after ActD treatment. Importantly, TRB1 knockdown increased the acetylation levels after ActD treatment in MCF7 cells (Fig. 2B). Histone acetyltransferase p300 enhanced p53 acetylation in H1299 cells, while TRB1 inhibited p300-mediated acetylation of p53 (Fig. 2C). These results demonstrate that TRB1 suppressed the levels of acetylated p53 upon cellular stress.

**TRB1 Interacts with HDAC1 and Enhances HDAC1-Promoted p53 Deacetylation**

We next examined whether TRB1 interacts with p53. H1299 cells were transiently transfected with FLAG-TRB1 and 6Myc-p53, and co-immunoprecipitation analyses showed that p53 co-precipitated with TRB1 (Fig. 3A). Reciprocal co-precipitation experiments showed that interaction between TRB1 and p53 was also detected (Fig. 3B). As acetylation of p53 can be reversed by distinct HDAC complexes containing HDAC1/2 or SIRT1, we investigated whether TRB1 interacted with HDAC1 or SIRT1. We found that TRB1 preferentially bound HDAC1 but interacted weakly with SIRT1 (Fig. 3C). This result suggested that TRB1 might be able to potentiate HDAC1-mediated p53 deacetylation. We therefore investigated whether TRB1 and HDAC1 cooperate to inhibit the acetylation of p53. HDAC1 expression modestly deacetylated p53, but co-expression of both HDAC1 and TRB1 dramatically reduced the level of acetylated p53 (Fig. 3D). Collectively, these results suggest that TRB1 and HDAC1 cooperate to inhibit the acetylation of p53, leading to the inactivation of p53.

**DISCUSSION**

Results described above show that TRB1 is a negative regulator of p53. Ectopic expression of TRB1 suppressed p53 transactivation activity, and knockdown of endogenous TRB1 augmented the tumor-suppressor activity of p53. TRB1 repressed the activity of p53 by reducing the acetylation of p53 and decreasing the DNA binding of p53 in response to cellular stress. We found that TRB1 interacted with HDAC1, and that TRB1 potentiated HDAC1-mediated p53 deacetylation. These results indicate that TRB1 suppresses p53 activity and triggers uncontrolled cell proliferation, and suggest that TRB1 may be a new molecular target for cancer therapy.

TRB1 has been identified as a myeloid oncogenic driver and induces AML in mice through the MEK/ERK pathway and C/EBP regulation. Importantly, TRB1 is also found to
be overexpressed in solid tumors, including prostate cancer, thyroid cancer, ovarian cancer, and colorectal cancer. In prostate cancer, Mashima et al. have shown that TRB1 is a critical factor for cancer cell stemness and tumor propagating through expression of the endoplasmic reticulum chaperone GRP78. On the other hand, p53 is known to inhibit breast cancer stemness through suppressing CD44 expression. We also observed that TRB1 knockdown led to a reduction of CD44 expression in MCF7 cells (our unpublished data). When considering TRB1 as a negative regulator of p53, TRB1 is likely to mediate the cancer cell stemness through p53 activity regulation. Further studies will be required to clarify this concern.

In many tumors that retain wild-type p53, the function of p53 can be compromised by several mechanisms. Viral oncogenes (e.g., papillomavirus E6 and adenovirus E1B) and a cellular oncogene Mdm2 induce p53 degradation. Interestingly, Mdm2 can promote p53 deacetylation by recruiting a complex containing HDAC1. ETBE1 (also called HSCO), which is frequently overexpressed in hepatocellular carcinomas, forms a complex with HDAC1 and p53, enhances p53 deacetylation. In the present study, TRB1 interacts with p53 and enhances HDAC1-mediated p53 deacetylation. We previously reported that oncoprotein Ski interacts with SIRT1 and stabilizes p53-SIRT1 interaction to promote p53 deacetylation. Therefore, we propose that there is a common mechanism of p53 inactivation through oncogenes-mediated p53 deacetylation in tumor cells. In future, we may be able to design effective chemotherapeutic interventions by inhibiting p53-oncogenes binding and recovering the p53 response in tumor cells.

In conclusion, we have identified a new molecular mechanism by which TRB1 provides its oncogenic potential by inactivating the tumor suppressor p53 in solid tumors. Thus, our study may provide a rationale for using TRB1 as a diagnostic marker and an attractive molecular target for tumor therapy.

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Conflict of Interest The authors declare no conflict of interest.

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