highly expressed in HL60 cells in which FLAG-tagged TRIM32 was expressed (Fig. 2B). Next, to examine whether induction of differentiation with ATRA in HL60 cells affects the expression level of endogenous TRIM32, immunoblot analysis was performed to analyze the expression levels of endogenous TRIM32 after ATRA treatment for the indicated times (Fig. 2C). As previously reported, ATRA caused a decrease in the level of RARα in HL60 cells because of degradation of RARα protein by the ubiquitin–proteasome system [9,13]. Treatment with ATRA in HL60 cells also caused a decrease in endogenous TRIM32 expression. However, TRIM32 was observed until 24 h after ATRA induction. This finding suggests that TRIM32 maintains the activity of RARα-mediated transcription even without ATRA for differentiation of HL60.

3.3. TRIM32 promotes differentiation of HL60 cells without retinoic acid

Since luciferase reporter assays and immunoblot analysis showed that ectopic expression of TRIM32 induces RARα-mediated transcriptional activity and stabilizes RARα even without ATRA, we hypothesized that TRIM32 affects granulocytic differentiation of HL60 cells via RARα-mediated transcription without ATRA. We

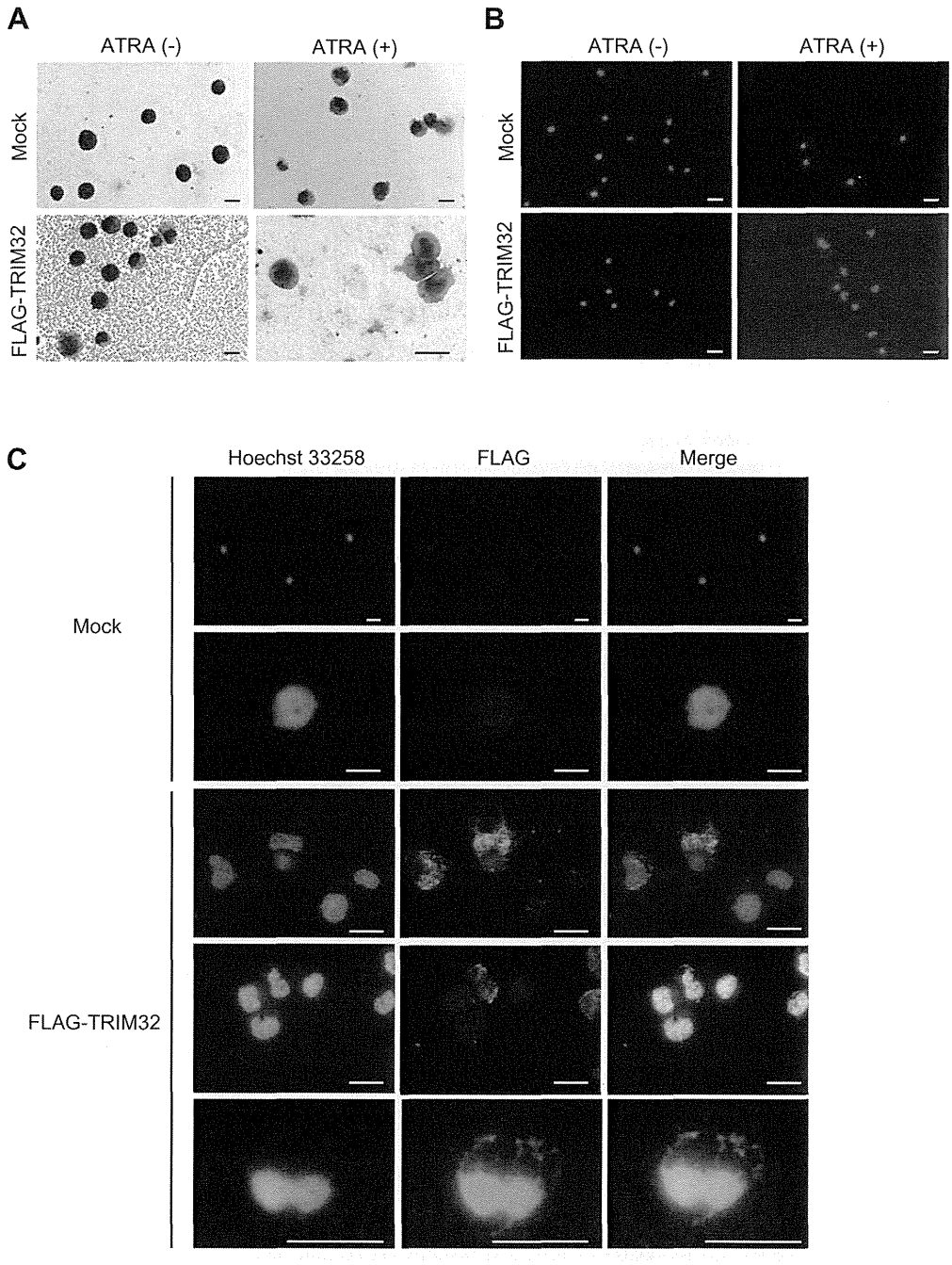

Fig. 3. TRIM32 promotes differentiation of HL60 cells. (A) Morphological changes of HL60 cell lines in which FLAG-tagged TRIM32 had been transfected with or without ATRA. HL60 cells with FLAG-tagged TRIM32 or mock cells were treated with or without ATRA (1 μM) and then stained by May-Grünwald–Giemsa solution. Scale bar, 10 μm. (B) Nuclear morphology of HL60 cell lines in which FLAG-tagged TRIM32 had been transfected. Representative micrographs show Hoechst33258-stained HL60 cells treated with or without ATRA. Scale bar, 20 μm. (C) TRIM32 transfection induces morphological changes of granulocytic differentiation in HL60 cells. HL60 cells were transfected with expression vectors encoding FLAG-tagged TRIM32 or mock by electroporation and then stained with antibodies to FLAG (green) with Hoechst33258 (blue). Scale bar, 10 μm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

analyzed the effects of TRIM32 on morphological changes of the nucleus in the presence or absence of ATRA (Fig. 3A and B). To test cell morphological changes of HL60 cell lines, an expression vector encoding FLAG-tagged TRIM32 was transiently transfected into HL60 cells by electroporation, and the cells were cultured for 72 h with or without ATRA (1 μM) and then stained by the May-Grünwald–Giemsa method. With ATRA treatment, morphological features of granulocytic differentiation such as condensed lobular nuclei and reduced nuclei in size were observed in both mock cells and TRIM32-overexpressing HL60 cells (Fig. 3A). Interestingly, similar morphological features of granulocytic differentiation such as polynucleated cells with granulated cytoplasms and a decreased cytoplasm/nucleus ratio were observed in a part of the population of ATRA-untreated HL60 cells in which Flag-tagged TRIM32 was transiently expressed. A microscope image of nuclei stained by Hoechst 33258 showed similar results (Fig. 3B).

To further confirm that cells in which FLAG-tagged TRIM32 was overexpressed have a tendency to differentiate to granulocytes

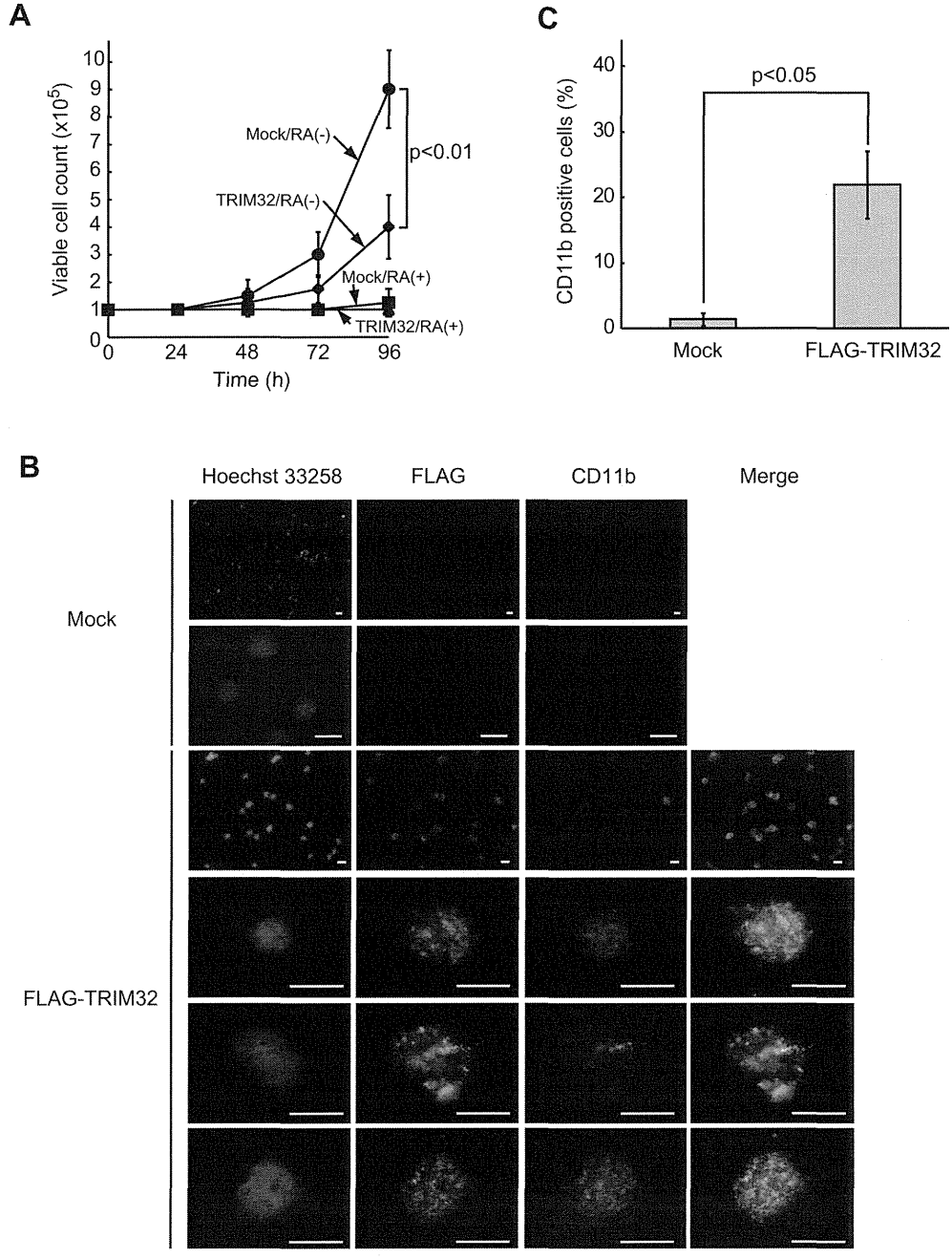

Fig. 4. TRIM32 suppresses proliferation and induces surface expression of CD11b without ATRA in HL60 cells. (A) Overexpression of TRIM32 suppresses HL60 cell proliferation without ATRA. HL60 cells in which FLAG-tagged TRIM32 and mock were transfected by electroporation and seeded at 1×10^5 cells in 60-mm dishes. The cells were cultured with or without ATRA (1 μM) and then counted at the indicated times. Data are means ± SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (B) Expression of CD11b by overexpression of FLAG-tagged TRIM32. HL60 cells were electroporated with an expression vector encoding FLAG-tagged TRIM32 or mock without ATRA and then stained with antibodies to FLAG (green) and CD11b (red) with Hoechst33258 (blue) at 72 h after electroporation. Scale bar, 10 μm. (C) Quantification of CD11b-positive HL60 cells. The cells stained in (B) were counted. Data are means ± SD of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

even in the absence of ATRA, we performed immunofluorescence staining with anti-FLAG antibody. HL60 cells were transfected with an expression vector encoding FLAG-tagged TRIM32 or mock by electroporation and then stained with an antibody to FLAG with Hoechst33258 at 72 h after electroporation. Immunofluorescence analysis showed that granulocytic differentiation with morphological changes was induced in HL60 cells in which Flag-tagged TRIM32 was transiently overexpressed without ATRA (Fig. 3C).

3.4. TRIM32 suppresses HL60 cell proliferation and induces surface expression of CD11b without ATRA

It has been reported that treatment of promyelocytic leukemia cells such as HL60 and NB4 cells with ATRA or As_2O_3 causes differentiation, apoptosis and inhibition of cell proliferation [3,30]. We hypothesized that granulocytic differentiation induced by TRIM32 in HL60 cells is linked to the phenomenon of overexpression of TRIM32 negatively regulating HL60 cell proliferation. To examine whether overexpression of TRIM32 affects cell proliferation, HL60 cells were transfected with a TRIM32 or mock expression vector by electroporation and cultured in the presence or absence of ATRA and then cell numbers were counted at the indicated times. To examine viability of HL60 cells after electroporation, trypan blue staining was performed to count living cells. In the presence of ATRA, HL60 cells in which FLAG-tagged TRIM32 or mock was expressed showed clearly repressed proliferation. Without ATRA, mock cells showed normal rapid proliferation, whereas FLAG-tagged TRIM32 expression delayed cell proliferation (Fig. 4A). These findings suggest that TRIM32 delays cell proliferation and induces granulocytic differentiation via RARα-mediated transcriptional activity even in the absence of ATRA.

Next, we performed immunofluorescence staining to analyze cell surface expression of CD11b antigen as a granulocytic differentiation marker. HL60 cells were transfected with an expression vector encoding FLAG-tagged TRIM32 or mock by electroporation and cultured without ATRA. The cells were stained with antibodies to FLAG and CD11b with Hoechst33258 at 72 h after electroporation to examine whether CD11b expression is higher in TRIM32-transfected cells than in mock cells without ATRA. HL60 cells in which FLAG-tagged TRIM32 (green) was expressed showed high expression level of CD11b (red) in the absence of ATRA (Fig. 4B). We counted the number of cells in which CD11b was expressed. Statistical analysis showed that HL60 cells in which FLAG-tagged TRIM32 was expressed included a significantly higher percentage of CD11b-positive cells than did mock cells (Fig. 4C). These findings suggest that ectopic expression of TRIM32 induces CD11b expression as a granulocyte differentiation marker.

4. Discussion

Emerging evidence has indicated that E3 ubiquitin ligases do not always participate in the regulation of transcriptional activity in the function of proteolytic activity. Moreover, it has been shown that the activity of RARs is regulated by cofactors that either promote or repress RAR-mediated gene transcription and that RARα interacts with several proteins that belong to the family of E3 ubiquitin ligases. In addition, the HECT domain and ankyrin repeat containing E3 ubiquitin–protein ligase (HACE1) has been shown to repress the transcriptional activity of RARα [31], and another E3 ligase, Rnf41, has also been reported to regulate differentiation of hematopoietic progenitors by modulating the steady state of RARα [32]. In this present study, we demonstrated that TRIM32 stabilizes endogenous RARα and induces granulocytic differentiation in HL60 cells regardless of the presence or absence of ATRA.

There are a few reports that regulation of proteasome activation potentiates the differentiating effect of leukemia cells [33]. It has been shown that proteasome inhibitors have the ability to enhance ATRA-induced cell differentiation of HL60 cell lines, suggesting that stabilization of RARα promotes cellular differentiation of leukemic cells. In our study, in the presence of ATRA, we could not show a significant difference in proliferation of HL60 cells in which TRIM32 is overexpressed and mock cells. However, although the ATRA-induced ubiquitin–proteasome system was activated, expression level of TRIM32 was maintained until 24 h after ATRA induction. These findings suggest that the remaining TRIM32 promotes granulocytic differentiation of HL60 cells via an unknown RARα-independent pathway. A proteasome inhibitor would cause retention of TRIM32, and thereby accumulated TRIM32 may activate RARα transcriptional activity and suppress cellular proliferation followed by induction of granulocytic differentiation. It is important to clarify whether inhibition of the ubiquitin–proteasome pathway promotes differentiation of promyelogenous leukemic cells as well as multiple myeloma and to determine whether a proteasome inhibitor in combination with ATRA is more effective for therapy of APL.

Retinoids have important roles in cancer therapy for their potential effects on cell differentiation and apoptosis. ATRA is now used for initial treatment of APL [34]. However, intractable or relapsed APL would need to be treated by several combination therapies including ATRA. In this study, we showed that TRIM32 is a positive regulator of transcriptional activity of RARα, suggesting that TRIM32 regulates the function of RARα or cofactors involved in differentiation of HL60 cells. It is important to analyze TRIM family proteins that regulate transcriptional activity of nuclear receptors related to cancers or metabolic syndromes. Identification of substrates of TRIM32 and drug discovery for RAR-associated ubiquitin ligases would be helpful for patients suffering from malignant diseases.

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TRIM45 negatively regulates NF-κB-mediated transcription and suppresses cell proliferation

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ABSTRACT

The NF-κB signaling pathway plays an important role in cell survival, immunity, inflammation, carcinogenesis, and organogenesis. Activation of NF-κB is regulated by several posttranslational modifications including phosphorylation, neddylation and ubiquitination. The NF-κB signaling pathway is activated by two distinct signaling mechanisms and is strictly modulated by the ubiquitin–proteasome system. It has been reported that overexpression of TRIM45, one of the TRIM family ubiquitin ligases, suppresses transcriptional activities of Elk-1 and AP-1, which are targets of the MAPK signaling pathway. In this study, we showed that TRIM45 also negatively regulates TNFα-induced NF-κB-mediated transcription by a luciferase reporter assay and that TRIM45 lacking a RING domain also has an activity to inhibit the NF-κB signal. Moreover, we found that TRIM45 overexpression suppresses cell growth. These findings suggest that TRIM45 acts as a repressor for the NF-κB signal and regulates cell growth.

1. Introduction

The nuclear factor-κB (NF-κB) signaling pathway plays an important role in cell survival, immunity, inflammation, carcinogenesis, and organogenesis [1,2]. The NF-κB family has five members: p50, p52, p65 (RelA), c-Rel and RelB. The NF-κB signaling pathway is activated by several distinct signaling mechanisms [2]. Under a resting condition, NF-κB is maintained in an inactive state by binding to IκB proteins. The canonical pathway, which is the most common NF-κB pathway, relies on inhibitor of kappa B (IκB) kinase (IKK) β-mediated phosphorylation of inhibitory IκB proteins. Phosphorylated IκB proteins are degraded by the ubiquitin–proteasome system including Skp1-Cul1-F-box protein complex (SCF)-type ubiquitin ligase, $SCF^{\beta\text{-}TrCP}$ [3]. Ubiquitinated IκB is degraded by the 26S proteasome, allowing NF-κB to enter the nucleus to turn on target genes [4]. Therefore, ubiquitination plays important roles in regulations within NF-κB signaling cascades.

The ubiquitin-mediated proteolytic pathway has very important roles in quality control of proteins and elimination of short-lived regulatory proteins including those that contribute to the cell cycle, cellular signaling, organelle biogenesis, DNA repair and morphogenesis [5,6]. The system responsible for conjugation of ubiquitin to the target protein consists of several components that act in concert [7,8], including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3). The resulting ubiquitin ligation induces formation of polyubiquitinated conjugates that are immediately recognized and degraded by 26S proteasome. E3 is thought to be the scaffold protein that is most directly responsible for substrate recognition [8–10]. E3 ubiquitin ligases have been classified into three families: the HECT (homologous to E6-AP COOH terminus) family [6,10], the RING-finger-containing protein family [11–13] and the U-box family [14–16].

The superfamily of tripartite-motif-containing (TRIM) proteins is characterized by the presence of a RING finger, one or two zinc-binding motifs named B-boxes, a coiled-coil motif and carboxyl-terminal unique domains [17–19]. TRIM family proteins are involved in a broad range of biological processes and their alterations often cause diverse pathological conditions such as developmental disorders, neurodegenerative diseases, viral infection and carcinogenesis [20–22].

TRIM45 is one of the members of the TRIM family and contains a RING finger domain, two B-box domains, a coiled-coil region in its amino-terminal region and a filamin-type immunoglobulin (IG-FLMN) domain in its carboxy-terminal region. The *TRIM45* gene is mapped to chromosome 1q22 and consists of six exons and five introns, and TRIM45 has a predictive 580-amino-acid open reading frame with a calculated molecular mass of 64 kDa. Human TRIM45 shares approximately 88% amino acid sequence identity with mouse TRIM45 protein. It has been reported that TRIM45 has a role as a transcriptional repressor in the mitogen-activated protein kinase (MAPK) signaling pathway [23].

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In this study, with the aim of elucidating the molecular function of TRIM45, which is ubiquitously expressed in human adult tissues, we analyzed cell lines in which TRIM45 was stably expressed by a retroviral expression system. We found that TRIM45 negatively regulates the transcriptional activity of NF-κB and suppresses cell proliferation. These findings indicate that TRIM45 regulates cell proliferation via inhibition in the NF-κB signaling pathway.

2. Materials and methods

2.1. Cell culture

HEK293T, HeLa and COS-7 cells (ATCC, Manassas, VA) were cultured under an atmosphere of 5% CO_2 at 37 °C in DMEM (Sigma–Aldrich Corp., St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). NIH3T3 cells were cultured under the same conditions in DMEM supplemented with 10% calf serum (Camblex, Charles City, IA).

2.2. Cloning of cDNAs and plasmid construction

Human *TRIM45* cDNAs were amplified from a human liver cDNA library and mouse *TRIM45* cDNAs were amplified from a mouse testis cDNA library by polymerase chain reaction (PCR) with Blend-Taq (Takara, Tokyo, Japan) using the following primers: 5′-AGTATGTCAGAAAACAGAAAACCG-3′ (Hs *TRIM45*-sense), 5′-CCATCAGAGAGCCACAGTCCTAAG-3′ (Hs *TRIM45*-antisense), 5′-AAGATGTCAGAAATCAGGAAGCCG-3′ (Mm *TRIM45*-sense) and 5′-GCATCAGAGCGCCACGGTCCTAAG-3′ (Mm *TRIM45*-antisense). The amplified fragments were subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA). The sequences were confirmed by the dideoxy chain termination method with automated sequencing (Applied Biosystems, Foster City, CA). TRIM45 cDNA with a FLAG-tag was then subcloned into pCR (Invitrogen) for expression in eukaryotic cells. A deletion mutant of *Hs TRIM45* lacking a RING-finger domain (*Hs TRIM45(ΔR)*) cDNA was amplified by PCR using the following primers: 5′-CCCTTCTCAGTAGTGGACATC-3′ (Hs *TRIM45 ΔR*-sense) and 5′-CCATCAGAGAGCCACAGTCCTAAG-3′ (Hs *TRIM45 ΔR*-antisense) and sequenced.

2.3. Transfection and immunoblot analysis

HEK293T cells were transfected by the calcium phosphate method and lysed in a solution containing 50 mmol/l Tris–HCl (pH 7.4), 150 mmol/l NaCl, 1% Nonidet P-40, leupeptin (10 μg/ml), 1 mmol/l phenylmethylsulfonyl fluoride, 400 μmol/l Na_3VO_4, 400 μmol/l EDTA, 10 mmol/l NaF, and 10 mmol/l sodium pyrophosphate. The cell lysates were centrifuged at 15,000g for 15 min at 4 °C, and the resulting supernatant was boiled in SDS sample buffer. Immunoblot analysis was performed with the following primary antibodies: anti-FLAG (1 μg/ml; M2, Sigma), anti-Hsp90 (1 mg/ml; 68, BD, Franklin Lakes, NJ) and anti-β-actin (0.2 μg/ml; AC15, Sigma). Immune complexes were detected with horseradish peroxidase-conjugated antibodies to mouse IgG (1:10,000 dilution, Promega, Madison, WI) and an enhanced chemiluminescence system (GE Healthcare). Fugene HD reagent (Roche, Mannheim, Germany) was used according to the manufacturer's protocol.

2.4. Immunofluorescence staining

COS-7 cells expressing FLAG-tagged full-length TRIM45 (TRIM45(FL)) or FLAG-tagged TRIM45 lacking a RING-finger domain (TRIM45(ΔR)) grown on a glass cover were fixed for 10 min at room temperature with 2% formaldehyde in PBS and then incubated for 1 h at room temperature with a primary antibody to FLAG (1 μg/ml, M5, sigma) in PBS containing 0.1% bovine serum albumin and 0.1% saponin. The cells were then incubated with an Alexa488-labeled goat polyclonal antibody to mouse immunoglobulin (Invitrogen) at a dilution of 1:1000. The cells were further incubated with Hoechst 33258 (1 μg/ml) in PBS for 10 min, followed by extensive washing with PBS. The cells were covered with a drop of GEL/MOUNT (Biomeda, Foster City, CA) and then photographed with a CCD camera (DP71, Olympus, Tokyo, Japan) attached to an Olympus BX51 microscope.

2.5. Establishment of stable transfectants by using a retrovirus expression system

Complementary DNAs encoding FLAG-tagged TRIM45(FL), FLAG-tagged TRIM45(ΔR) and the corresponding empty plasmid were subcloned into pMX-puro (kindly provided by Toshio Kitamura, Tokyo University). The resulting vectors were used for transfection into Plat-E or Plat-A cells and then recombinant retroviruses were generated [24]. Forty-eight hours after transfection, culture supernatants were harvested and then used for infection. The infection was carried out in the presence of polybrene at 8 μg/ml (Sigma) and the infected clones were expanded in a medium containing puromycin (5 μg/ml, Sigma).

2.6. Dual-luciferase assay

HEK293T cells were seeded in 24-well plates at 1×10^5 cells per well and incubated at 37 °C with 5% CO_2 for 24 h. NF-κB luciferase reporter plasmid (NAT-Luc) [25], pRL-TK *Renilla* luciferase plasmid (Promega) and various combinations of FLAG-tagged TRIM45(FL) or FLAG-tagged TRIM45(ΔR) expression vectors were transfected into HEK293T cells using the Fugene HD reagent (Roche). Twenty-four hour after transfection, cells were incubated with TNFα (20 ng/ml) for 6 h, harvested, and assayed by the Dual-Luciferase Reporter Assay System (Promega). The luminescence was quantified with a luminometer (Tuner Designs, Sunnyvale, CA).

HeLa cells in which TRIM45(FL), TRIM45(ΔR), or an empty vector (mock) was stably expressed by using a retroviral expression system were seeded in 24-well plates at 1×10^5 cells per well and incubated at 37 °C with 5% CO2 for 24 h. NF-κB luciferase reporter plasmid (NAT-Luc) and pRL-TK Renilla luciferase plasmid (Promega) were transfected into each stable cell line using the Fugene HD reagent (Roche). Twenty-four hour after transfection, cells were incubated with TNFα (20 ng/ml) for 6 h, harvested, and assayed by the Dual-Luciferase Reporter Assay System.

2.7. Cell proliferation assay

NIH3T3 and HeLa cells in which TRIM45(FL), TRIM45(ΔR) and an empty vector (mock) was stably expressed by using a retroviral expression system were seeded in 6-cm dishes (1×10^5 cells) and harvested for determination of cell number at the indicated times.

2.8. Statistical analysis

Student's *t*-test was used to determine the statistical significance of experimental data.

3. Results

3.1. Expression of human and mouse TRIM45 in mammalian cells

It has been reported that *TRIM45* is expressed in various human tissues including the skeletal muscle, brain, heart, and pancreas

[23]. We amplified human *TRIM45* cDNA from HeLa cell, human liver, human prostate and human thymus cDNA libraries by PCR with the forward and reverse primers of human TRIM45 described in Materials and Methods. We also amplified mouse *TRIM45* cDNA from mouse testis and mouse T cell cDNA libraries by PCR with the forward and reverse primers of mouse TRIM45. Amplified human and mouse *TRIM45* cDNAs was isolated and their sequences were verified. To examine the molecular functions and colocalization of TRIM45 in mammalian cells, we generated human and mouse full-length TRIM45 (TRIM45(FL)) and a deletion mutant of human TRIM45 lacking a RING-finger domain (TRIM45(ΔR)) (Fig. 1A). According to immunoblot analysis, transfected TRIM45 was expressed in both soluble and insoluble fractions in HEK293T cells (Fig. 1B). Immunoblot analysis with anti-FLAG antibody showed that a deletion mutant of human TRIM45 lacking a RING-finger domain (TRIM45(ΔR)) protein was also expressed in transfected HEK293T cells (Fig. 1C).

3.2. TRIM45 is predominantly localized in the perinuclear region

It has been reported that TRIM45 is expressed in the nucleus and cytoplasm in COS-7 cells [23]. Next, we performed immunofluorescence staining to confirm the subcellular localization of TRIM45 using COS-7 cells. COS-7 cells were transfected with expression vectors encoding FLAG-tagged TRIM45(FL) or TRIM45(ΔR) and then stained with an antibody to FLAG with Hoechst33258 to reveal the subcellular distribution of FLAG-tagged TRIM45(FL) or TRIM45(ΔR) (green) and the nuclei (blue). In COS-7 cells, FLAG-tagged TRIM45(FL) proteins showed a predominantly perinuclear localization pattern (Fig. 2). FLAG-tagged mouse TRIM45 proteins showed a distribution similar to that of FLAG-tagged human TRIM45 (Fig. 2). However, we found that human TRIM45(ΔR) was localized as diffuse dots in the cytoplasm (Fig. 2). These findings suggested that a RING finger domain of TRIM45 is important for localization in the perinuclear region.

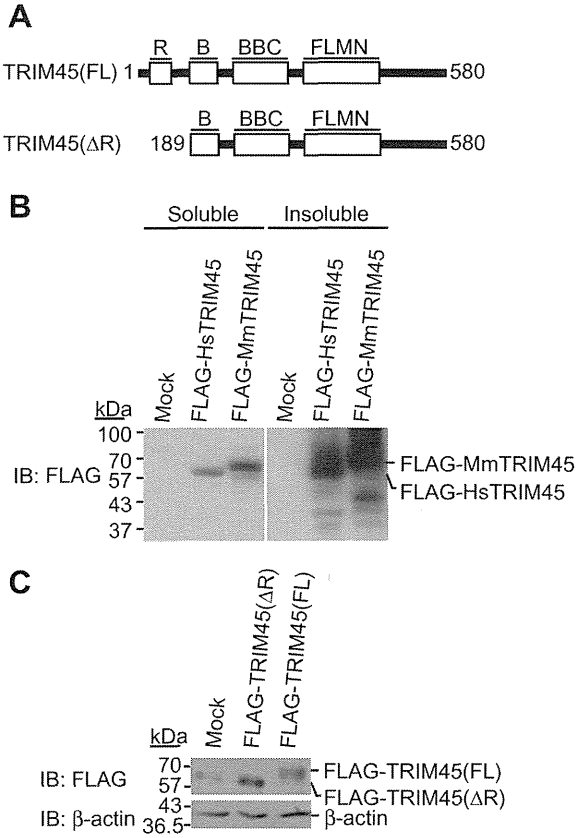

Fig. 1. Overexpression of wild type and deletion mutant of TRIM45 in mammalian cells. (A) Schematic representation of TRIM45 and deletion mutant. FL, full length; R, RING finger domain; B, B-box domain; BBC, B-box C-terminal coiled-coil domain; FLMN, filamin-type immunoglobulin domain. (B) Immunoblot analysis of FLAG-tagged human TRIM45 (Hs TRIM45) and mouse TRIM45 (Mm TRIM45). Expression vectors encoding FLAG-tagged human (Hs) TRIM45 and mouse (Mm) TRIM45 were transfected into HEK293T cells. Lysates were immunoblotted with anti-FLAG antibody. (C) Immunoblot analysis of FLAG-tagged human TRIM45(FL) and TRIM45 (ΔR). Expression vectors encoding FLAG-tagged human TRIM45(FL) and TRIM45(ΔR) were transfected into HEK293T cells. Lysates were immunoblotted with anti-FLAG antibody. Anti-β-actin antibody was used as an internal control.

3.3. TRIM45 negatively regulates TNF-induced NF-κB-mediated transcriptional activity

We previously reported that some members of the TRIM family of ubiquitin ligases regulate activities of several transcriptional factors: TRIM25 and TRIM68 upregulate ERα-mediated transcription and AR-mediated transcription, respectively [21,26,27]. Moreover, we revealed that TRIM40, which is also one of the TRIM family ubiquitin ligases, enhanced neddylation of inhibitor of kappa B (IκB) kinase (IKK) γ and inhibited the activity of NF-κB-mediated transcription [28]. It has been reported that TRIM45 suppresses transcriptional activities of E twenty-six (ETS)-like transcription factor 1 (Elk-1) and activator protein 1 (AP-1), which are targets of the MAPK signaling pathway [23]. Based on these previous reports and our findings of immunofluorescence staining that TRIM45 is localized in the perinuclear region, we hypothesized that TRIM45 acts as a regulator of transcription including the NF-κB signal. Therefore, we performed a luciferase reporter assay using an NF-κB promoter-driven luciferase construct (NAT-Luc) in HEK293T cells to examine whether TRIM45 regulates NF-κB-mediated transcription. We transfected expression vectors encoding FLAG-tagged TRIM45(FL) or TRIM45(ΔR) with reporter plasmids into HEK293T cells. Six hours after stimulation with TNFα, luciferase activity was measured. The luciferase assays showed that TRIM45(FL) significantly suppressed NF-κB-mediated transcriptional activity (Fig. 3A). TRIM45(ΔR) also repressed NF-κB transcriptional activity, but its effect was weaker than that of wild-type TRIM45, suggesting that the RING domain of TRIM45 is partially important for suppression of NF-κB-mediated transcription (Fig. 3A). Next, we generated HeLa cells in which FLAG-tagged TRIM45(FL) or TRIM45(ΔR) was stably expressed by using a retroviral expression system, and we performed luciferase assays using these cell lines (Fig. 3B and C). In agreement with results of the assay using HEK293T cells, the luciferase assays showed that overexpression of TRIM45(FL) and TRIM45(ΔR) inhibited NF-κB activity compared with that of mock, suggesting that TRIM45 suppresses activities of NF-κB-mediated transcription in HeLa cells (Fig. 3C). Interestingly, luciferase assays showed that TRIM45 had a tendency to suppress activities of NF-κB-mediated transcription without TNFα (or with the level of TNFα included in the culture medium) (Fig. 3C).

3.4. TRIM45 suppresses cell proliferation via downregulation of NF-κB-mediated transcriptional activity

Next, we generated NIH3T3 cells in which FLAG-tagged TRIM45 was stably expressed by using a retroviral expression system (Fig. 4A). To clarify whether suppression of NF-κB-mediated transcriptional activity by TRIM45 affects cell proliferation, we performed a cell proliferation assay using these NIH3T3 cell lines. The growth rate of NIH3T3 cells expressing TRIM45 was significantly suppressed compared with that of cells infected with the corresponding empty vector (Fig. 4B). This finding suggests that TRIM45 downregulates NF-κB-mediated transcriptional activity.

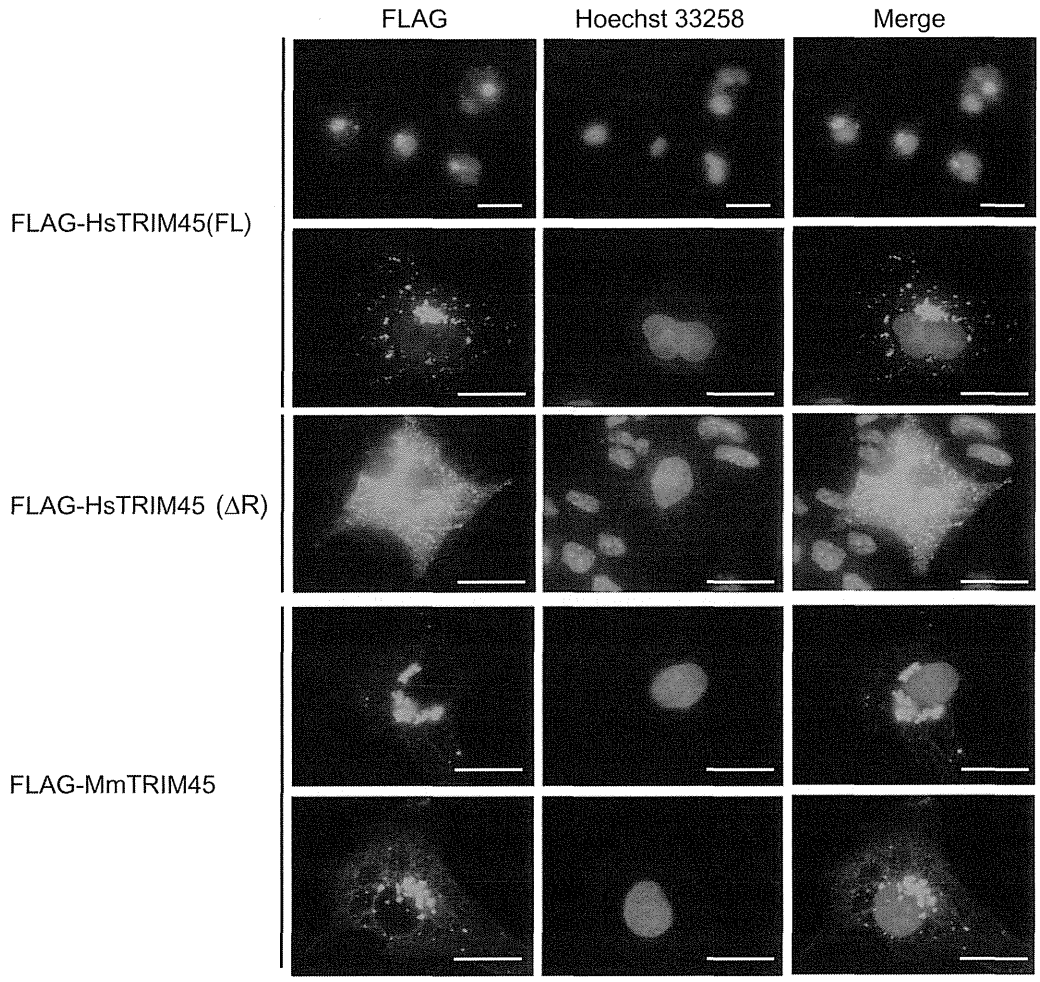

Fig. 2. Subcellular localization of TRIM45 in COS-7 cells. COS-7 cells were transfected with expression plasmids encoding FLAG-tagged human TRIM45(FL), TRIM45(ΔR) or mouse TRIM45(FL). Twenty-four hours after transfection, the cells were stained with anti-FLAG antibody, followed by incubation with Alexa488-labeled anti-mouse IgG antibody. Nuclei were visualized using Hoechst33258. Scale bar, 20 μm.

In addition to the assay using NIH3T3 cells overexpressing FLAG-tagged TRIM45, a cell proliferation assay using stable HeLa cell lines expressing FLAG-tagged TRIM45(FL), TRIM45(ΔR) and the corresponding empty vector was performed (Fig. 4C). The cell proliferation assay using HeLa cell lines showed that the growth rate of HeLa cells expressing TRIM45(FL) was significantly suppressed compared with that of mock cells (Fig. 4C). Moreover, the growth rate of HeLa cells expressing TRIM45(ΔR) was slightly repressed compared with that of mock cells. These findings indicate that TRIM45 may affect cell proliferation via downregulation of NF-κB-mediated transcriptional activity.

4. Discussion

Recent studies have revealed that numerous ubiquitin ligases are involved in regulation of transcriptional factors including the MAPK signaling pathway and NF-κB signaling pathway [29,30]. Moreover, some members of the TRIM family of ubiquitin ligases have been shown to be involved in the regulation of these signaling pathways [23,28,31,32]. Thus, it is important to clarify the correlation between the functions of TRIM family ubiquitin ligases and the effects of these signaling pathways. In this study, we firstly performed immunofluorescence staining to confirm the subcellular localization of FLAG-tagged TRIM45. Immunofluorescence staining showed that human FLAG-tagged TRIM45 proteins had a predominantly perinuclear localization pattern. The characteristic of localization in the perinuclear region of human TRIM45 was also conserved in mouse TRIM45. On the other hand, FLAG-tagged TRIM45 lacking a RING-finger domain is localized as diffuse dots in the cytosol. We hypothesized that FLAG-tagged TRIM45 localizes in the perinuclear region and regulates the function of certain transcription factors. As Reymond et al. demonstrated for other TRIM family proteins [19], we also observed that a deletion of the RING-finger domain induced aberrant cellular localization. This mislocalization may cause attenuation of biological functions as shown by luciferase reporter assays with TRIM45(ΔR) (Fig. 3B and C).

NF-κB is one of the transcriptional factors that plays essential roles in immune response, tumorigenesis and cell proliferation. It is well known that ubiquitin-mediated IκB protein degradation activates canonical and noncanonical NF-κB pathways. Therefore, the role of ubiquitin as an important signaling tag is characteristically illustrated in the NF-κB pathways, which regulate a variety of pathological processes in response to various stimuli [33]. On the other hand, the MAPK signaling pathway, which regulates a broad range of processes including cellular differentiation and proliferation, is also a crucial mediator of signal transduction. In mammalian cells, one of the most extensively studied targets of the MAPK signaling pathway is c-Jun. The gene expression is rapidly modulated by c-Jun by means of conjugating the serum response elements. AP-1 is a transcription factor that is a heterodimeric

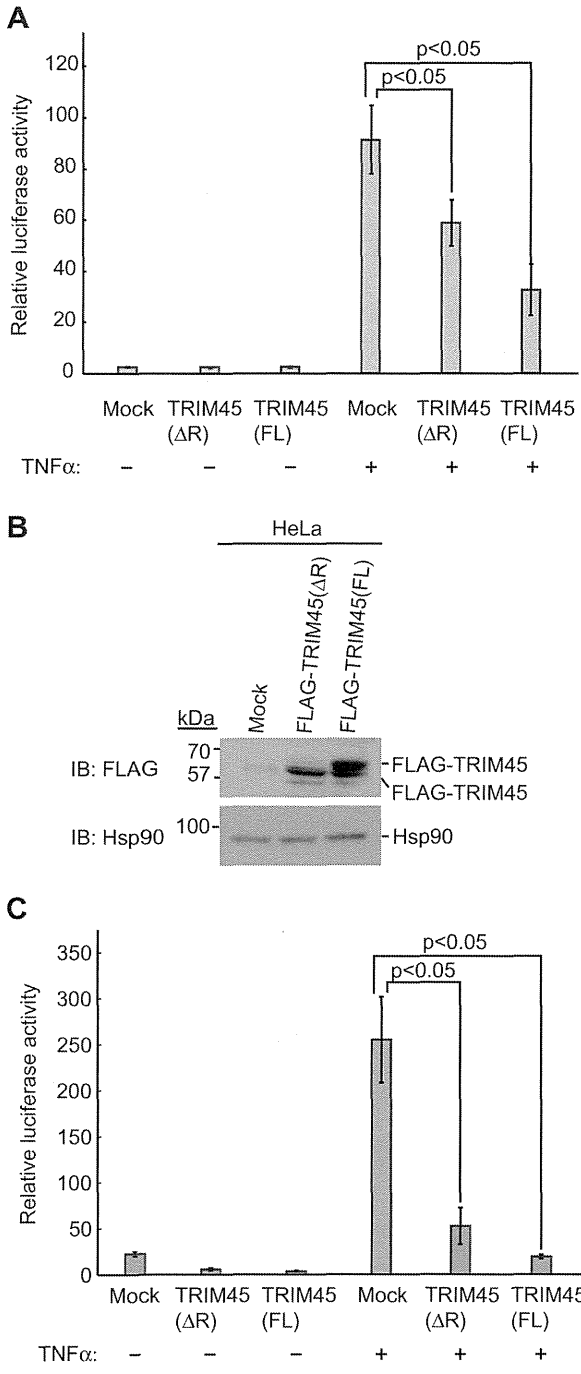

Fig. 3. TRIM45 negatively regulates TNFα-induced NF-κB-mediated transcriptional activity. (A) TRIM45 suppressed TNFα-induced NF-κB activity. HEK293T cells were transfected with an NF-κB luciferase reporter plasmid and an expression plasmid encoding TRIM45(FL). Twenty-four hour after transfection, cells were treated with TNFα (20 ng/ml) and cultured for an additional 6 h. Data are mean ± standard deviation (s.d.) of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test. (B) Establishment of HeLa cell lines stably expressing FLAG-tagged TRIM45(FL) and FLAG-tagged TRIM45(ΔR). HeLa cells were infected with a retrovirus encoding FLAG-tagged TRIM45(FL), FLAG-tagged TRIM45(ΔR) and the corresponding empty vector (mock). Each expression level of TRIM45 protein was checked by immunoblot analysis using anti-FLAG and anti-Hsp90 antibodies. Anti-Hsp90 antibody was used as an internal control. (C) TRIM45 reduces TNFα-induced NF-κB activity in HeLa cells. HeLa cell lines stably expressing FLAG-tagged TRIM45(FL), FLAG-tagged TRIM45(ΔR) and the corresponding empty vector (mock) were transfected with an NF-κB luciferase reporter plasmid. Twenty-four hour after transfection, cells were treated with TNFα (20 ng/ml) and cultured for an additional 6 h. Data are mean ± s.d. of values from three independent experiments. *P* values for the indicated comparisons were determined by Student's *t* test.

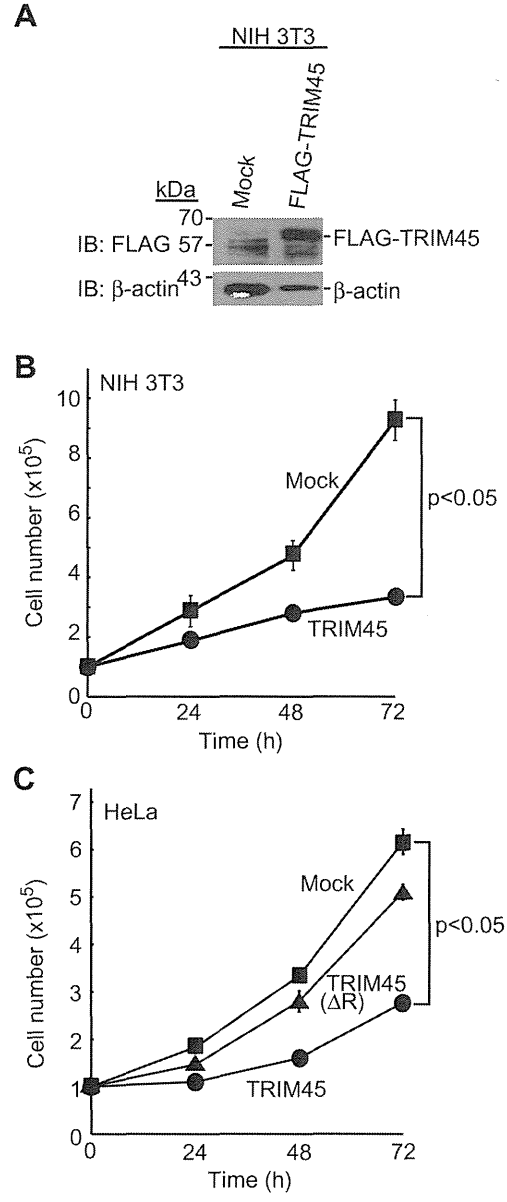

Fig. 4. TRIM45 suppresses cell proliferation. (A) Establishment of NIH3T3 cell lines expressing FLAG-tagged TRIM45. NIH3T3 cells were infected with a retrovirus encoding FLAG-tagged TRIM45 or the corresponding empty vector (mock). Each expression level of TRIM45 protein was checked by immunoblot analysis using anti-FLAG and anti-β-actin antibodies. Anti-β-actin antibody was used as an internal control. (B) TRIM45 affects cell proliferation. NIH3T3 cell lines expressing FLAG-tagged TRIM45 and mock were seeded (1×10^5 cells) in 60-mm dishes and harvested for counting cell numbers at the indicated times. Data are mean ± s.d. of values from three independent experiments. (C) TRIM45 suppresses proliferation of HeLa cells. HeLa cell lines stably expressing FLAG-tagged TRIM45(FL), FLAG-tagged TRIM45(R) or mock were seeded at 1×10^5 cells in 60-mm dishes. These HeLa cells were cultured with a culture medium including 10% fetal bovine serum and harvested for counting cell numbers at the indicated times. Data are mean ± s.d. of values from three independent experiments.

protein composed of proteins belonging to families such as the c-Fos and c-Jun families. In this study, we showed that TRIM45 negatively regulates NF-κB transcription and suppresses cancer cell proliferation. Recently, some studies have shown that crosstalk between NF-κB and MAPK signaling pathways is related to the pathogenesis of various diseases such as cancer and respiratory infectious disease [34,35]. Taken together, TRIM45 may downregulate not only the MAPK signaling pathway through AP-1/Elk-1 transcriptional activity but also the NF-κB signaling pathway.

Otherwise, TRIM45 may act as a repressor between the region downstream of protein kinase C and these signaling pathways. However, further investigations are needed to clarify the proteins that interact with TRIM45 in the NF-κB and/or MAPK signaling pathways.

In conclusion, TRIM45 is likely to be a novel regulator affecting cell growth via TNFα-induced NF-κB-mediated transcriptional activity, and results of further studies on TRIM45 may be useful for revealing the growth activity, cell cycle and immune response. Moreover, analysis by a genetic approach using transgenic or knock-out mice is required to determine whether TRIM45 physiologically functions as a regulator of cell growth.

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TRIM6 interacts with Myc and maintains the pluripotency of mouse embryonic stem cells

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Summary

The proto-oncogene product Myc is a master regulator of cell proliferation through its specific binding to the E-box motif in genomic DNA. It has been reported that Myc has an important role in the proliferation and maintenance of the pluripotency of embryonic stem (ES) cells and that the transcriptional activity of Myc is regulated by several post-translational modifications, including ubiquitination. In this study, we showed that tripartite motif containing 6 (TRIM6), one of the TRIM family ubiquitin ligases, was selectively expressed in ES cells and interacted with Myc followed by attenuation of the transcriptional activity of Myc. Knockdown of TRIM6 in ES cells enhanced the transcriptional activity of Myc and repressed expression of NANOG, resulting in the promotion of ES cell differentiation. These findings indicate that TRIM6 regulates the transcriptional activity of Myc during the maintenance of ES cell pluripotency, suggesting that TRIM6 functions as a novel regulator for Myc-mediated transcription in ES cells.

Key words: ES cell, NANOG, Myc, TRIM6, Ubiquitin

Introduction

The ubiquitin-mediated proteolytic pathway has a crucial role in the elimination of short-lived regulatory proteins (Peters, 1998) and in the quality control of proteins, including those that contribute to cellular signaling, the cell cycle, organelle biogenesis, secretion, DNA repair and morphogenesis (Hershko and Ciechanover, 1998). The system responsible for the conjugation of ubiquitin to the target protein comprises several components that act in concert (Hershko and Ciechanover, 1992; Scheffner et al., 1995), including a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2) and a ubiquitin ligase (E3). The resulting covalent ubiquitin ligation induces the formation of polyubiquitinated conjugates that are immediately detected and degraded by the 26S proteasome. E3 is thought to be the component of the ubiquitin conjugation system that is most directly responsible for substrate recognition (Hershko et al., 1983; Huibregtse et al., 1995; Scheffner et al., 1995). E3 ubiquitin ligases have been classified into three families: the HECT (homologous to E6-AP COOH terminus) family (Hershko and Ciechanover, 1998; Huibregtse et al., 1995), the RING-finger-containing protein family (Freemont, 2000; Joazeiro and Weissman, 2000; Lorick et al., 1999) and the U-box family (Aravind and Koonin, 2000; Cyr et al., 2002; Hatakeyama et al., 2001).

Tripartite motif (TRIM) proteins are characterized by the presence of a RING finger, one or two zinc-binding motifs called B-boxes, an associated coiled-coil region and C-terminal unique domains (Meroni and Diez-Roux, 2005; Nisole et al., 2005; Reymond et al., 2001). TRIM family proteins are involved in a broad range of biological processes and their alterations often cause diverse pathological conditions, such as developmental disorders, neurodegenerative diseases, viral infection and carcinogenesis (Kano et al., 2008; Miyajima et al., 2008; Quaderi et al., 1997).

TRIM containing 6 (TRIM6), a member of the TRIM family (Ozato et al., 2008), also has C-terminal PRY and SPRY (SPla kinase and RYanodine receptor) domains. The *TRIM6* gene is mapped to chromosome 11p15, where it locates within one of the *TRIM* gene clusters. It has also been reported that TRIM6 is localized to cytoplasmic bodies of variable size or nuclear sticks in U2OS and HeLa cells (Reymond et al., 2001). However, the molecular function of TRIM6 has not yet been elucidated.

The proto-oncogene product Myc, a basic helix-loop-helix–leucine zipper (bHLH/LZ)-type transcription factor, is a master regulator of cell proliferation. Myc is transiently expressed and its activity is directly related to the proliferative potential of cells. Wild-type Myc is usually unstable in proliferating cells, whereas mutant Myc, which is often expressed in B cell lymphoma cells, is stable. It has been reported that the ubiquitin–proteasome system controls the abundance of several proteins, especially short-lived regulatory proteins, including Myc. The expression level of the Myc protein must be carefully regulated to avoid carcinogenic transformation.

It has recently been reported that Myc has an important role in the proliferation and maintenance of the pluripotency of embryonic stem (ES) cells. Induced pluripotent stem cells (iPS cells) have been established by expressing four genes, *Oct3/4* [POU class 5 homeobox 1 (*Pou5f1*)], SRY (sex determining region Y)-box 2 (*Sox2*), Kruppel-like factor 4 (*Klf4*) and *Myc*, into fibroblasts of mice and humans (Takahashi and Yamanaka, 2006). However, the potential carcinogenicity of Myc must be suppressed to establish iPS cells for clinical application. Thus, efforts have been made to generate iPS cells without using *Myc* (Nakagawa et al., 2008; Okita et al., 2008). However, if *Myc* is not used, the efficiency of iPS cell establishment is very low. Therefore, appropriate regulation of the expression level of Myc

at a particular stage of stem cell development is thought to be important for maintaining the pluripotency of those cells (Takayama et al., 2010).

In this study, we performed yeast two-hybrid screening using TRIM6 as bait with the aim of elucidating the molecular function of TRIM6, which is selectively expressed in ES cells. We identified Myc as a TRIM6-interacting protein and found that TRIM6 negatively regulated the transcriptional activity of *Myc* and maintained the pluripotency of ES cells. These findings indicate that TRIM6 acts as a corepressor of Myc in mouse ES cells.

Results

TRIM6 interacts with Myc

To examine the molecular function of TRIM6, we isolated TRIM6-interacting proteins from a mouse T cell cDNA library by using a yeast two-hybrid system. We obtained seven positive clones from 3.5×10^5 transformants. One of the positive clones had sequence identities with cDNA encoding mouse Myc (Fig. 1A). To examine whether TRIM6 interacts with Myc in mammalian cells, we performed an in vivo binding assay using cells transfected with expression vectors. We expressed haemagglutinin (HA)-tagged full-length TRIM6 together with FLAG-tagged Myc in HEK293T cells. Cell lysates were subjected to immunoprecipitation with an anti-FLAG antibody, and the resulting precipitates were subjected to immunoblot analysis with an anti-HA antibody. FLAG-tagged Myc was co-precipitated with HA-tagged TRIM6, indicating that Myc specifically binds to TRIM6 in mammalian cells (Fig. 1B). Next, to examine whether the RING-finger domain is required for the interaction between TRIM6 and Myc, we generated a deletion mutant form of TRIM6 lacking the RING-finger domain [TRIM6(ΔR)] (Fig. 1C). An in vivo binding assay showed that TRIM6(ΔR) also interacted with Myc in HEK293T cells, indicating that the RING-finger domain is not required for the interaction between TRIM6 and Myc (Fig. 1D).

Phosphorylation of T58 and S62 of Myc does not affect the interaction of TRIM6 with Myc

It has been reported that phosphorylation of threonine-58 (T58) and serine-62 (S62) in Myc box 1 (MB1) is crucial for the stability of Myc and that these two residues are often mutated in various tumor types (Fig. 2A) (Bahram et al., 2000; Lutterbach and Hann, 1994). To determine whether phosphorylation of T58 and S62 in the MB1 of Myc is required for the binding of Myc to TRIM6, we performed an in vivo binding assay using TRIM6 and Myc mutants (T58A, S62A and T58A/S62A) in which either or both T58 and S62 were substituted for alanine to inhibit phosphorylation. We expressed HA-tagged TRIM6 together with FLAG-tagged Myc mutants in HEK293T cells. Cell lysates were subjected to precipitation with an anti-HA antibody and the resulting immunoprecipitates were subjected to immunoblot analysis with an anti-FLAG antibody. FLAG-tagged Myc wild-type and mutants were detected in anti-HA immunoprecipitates, suggesting that phosphorylation within the MB1 of Myc is not required for the interaction between TRIM6 and Myc (Fig. 2B). Next, to determine whether TRIM6 ubiquitinated Myc, we performed an in vivo ubiquitination assay. Expression vectors encoding FLAG-tagged Myc, HA-tagged TRIM6 and His_6-tagged-ubiquitin were transfected into HEK293T cells. Whole-cell lysates were subjected to pull-down with Ni-NTA agarose using denatured conditions with 8 M urea to inhibit noncovalent binding; the resulting precipitates were subjected to immunoblot analysis with anti-FLAG and anti-His_6 antibodies. Immunoblot analysis showed that overexpression of TRIM6 caused extensive ubiquitination of

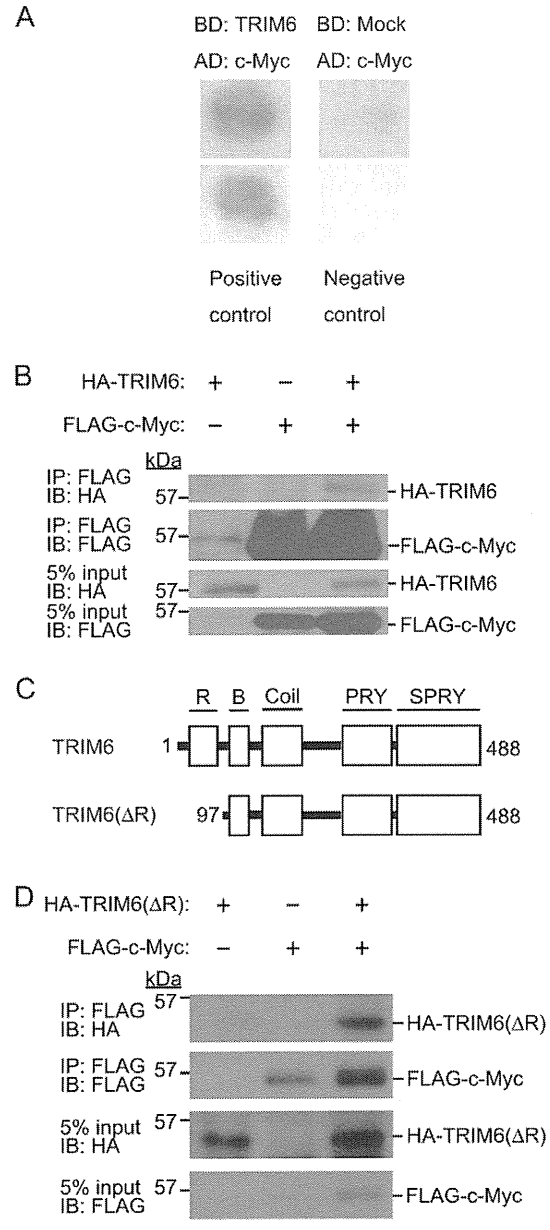

Fig. 1. TRIM6 interacts with Myc. (**A**) Yeast two-hybrid screening for TRIM6-interacting proteins using a mouse T cell cDNA library. pBMT116 and pACT2 vectors were used as negative controls. CHIP and EKN1 cDNAs were used for positive controls. (**B**) In vivo binding assay between TRIM6 and Myc. Expression vectors encoding FLAG-tagged Myc and HA-tagged TRIM6 were transfected into HEK293T cells. Cell lysates were immunoprecipitated with an anti-FLAG antibody and immunoblotted with anti-HA and anti-FLAG antibodies. (**C**) Schematic representation of TRIM6 and a deletion mutant. R, Ring-finger domain; B, B-box domain; Coil, coiled-coil domain; PRY, PRY domain; SPRY, SPRY domain. (**D**) Interaction between TRIM6(ΔR) and Myc in vivo. Expression vectors encoding FLAG-tagged Myc and HA-tagged TRIM6(ΔR) were transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-FLAG antibody and immunoblotted with anti-HA and anti-FLAG antibodies.

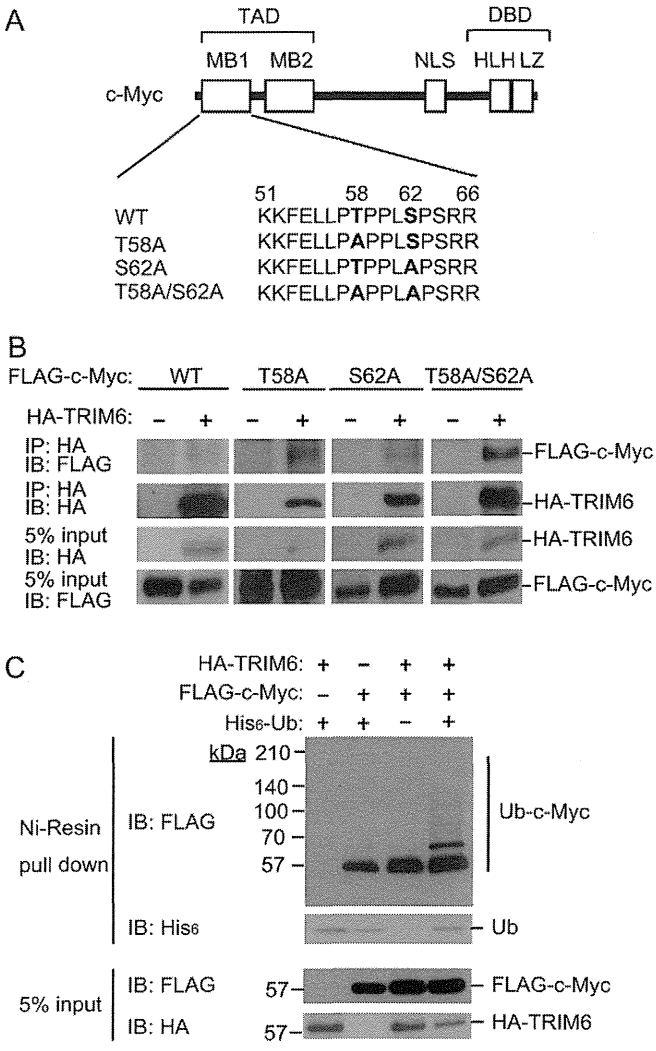

Fig. 2. TRIM6 binds to Myc in a phosphorylation-independent manner at T58 and S62 and mediates Myc ubiquitination. (**A**) Schematic representation of Myc mutants. There are two phosphorylation sites, T58 and S62, in the N-terminus of Myc. DBD, DNA-binding domain; NLS, nuclear localization signal; TAD, transactivation domain. Three Myc mutants in which T58 and/or S62 were substituted for alanine (A) are shown. (**B**) TRIM6 also interacted with Myc mutants that were not phosphorylated at T58 or S62. Expression vectors encoding HA-tagged TRIM6 and FLAG-tagged Myc mutants were transfected into HEK293T cells. Cell lysates were immunoprecipitated with anti-HA antibody and immunoblotted with anti-FLAG and anti-HA antibodies. (**C**) In vivo ubiquitination assay of Myc by TRIM6. Expression vectors encoding FLAG-tagged Myc, HA-tagged TRIM6 and His_6-tagged ubiquitin were transfected into HEK293T cells. Cell lysates were used for a pull-down assay with Nickel-affinity resin and immunoblotted with antibodies against FLAG-tag, His_6-tag or HA-tag.

Myc (Fig. 2C). These findings suggest that TRIM6 interacts with and ubiquitinates Myc without phosphorylation of T58 and S62 on Myc.

TRIM6 represses transcriptional activation of Myc

To determine whether TRIM6 affects Myc-mediated transcription, we performed a luciferase reporter assay using a p4 × E-SVP-Luc reporter plasmid in HEK293T cells (Fig. 3A). The luciferase assay showed that expression of FLAG-tagged Myc induced luciferase activity, whereas overexpression of TRIM6 repressed Myc-mediated transcriptional activity. The luciferase assay also showed that TRIM6 attenuated Myc transcriptional activity in a dose-dependent manner (Fig. 3A). Interestingly, the TRIM6(ΔR) mutant also repressed Myc transcriptional activity in a dose-dependent fashion, but its effect was slightly weaker than that of wild-type TRIM6, suggesting that the RING domain of TRIM6 is important to some degree for suppression of Myc-mediated transcription by TRIM6.

Next, by using a retroviral expression system, we generated Namalwa cells and NIH 3T3 cells in which FLAG-tagged TRIM6 was stably expressed (Fig. 3B). We examined the effect of TRIM6 on the abundance of endogenous Myc using Namalwa cells stably expressing TRIM6. However, the amount of Myc in these cells did not differ from the amount of Myc in Namalwa cells infected with the corresponding empty vector (Fig. 3C). Furthermore, the growth rate of Namalwa cells expressing TRIM6 was similar to that of cells infected with the corresponding empty vector (Fig. 3D). To determine whether TRIM6 affects the stability of Myc in vivo, we performed a protein stability analysis to verify the effect of TRIM6 on the stability of endogenous Myc in NIH 3T3 cells. The protein stability analysis showed that the stability of Myc in cells expressing TRIM6 was similar to that in mock transfectants (Fig. 3E,F). These findings suggest that TRIM6 suppresses Myc-mediated transcription but that its suppression does not affect cell proliferation and that TRIM6 does not change the stability of Myc.

TRIM6 is highly expressed in ES cells and interacts with endogenous Myc

To examine the expression profiles of TRIM6, we compared the protein levels of TRIM6 in various mouse cell lines from different tissues: ES cell line E14, primary embryonic fibroblasts (PEF), embryonic carcinoma cell line P19, myoblast cell line C2C12, fibroblast cell line NIH3T3 and neuroblastoma cell line neuro2a. Immunoblot analysis showed that TRIM6 was highly expressed in mouse embryonic stem cells but not in PEF (Fig. 4A). In addition, TRIM6 was slightly expressed in mouse P19 cells, which have the potential to transform into teratocarcinoma cells. We then verified the interaction between endogenous TRIM6 and Myc using E14 cells by immunoprecipitation with an anti-Myc antibody (Fig. 4B). Immunofluorescence analysis showed that FLAG-tagged TRIM6 was localized predominantly in the cytosol and weakly in the nucleus, whereas endogenous Myc was localized predominantly in the nucleus. Moreover, we found that TRIM6 partially overlapped with Myc in the intranuclear or perinuclear region (Fig. 4C).

TRIM6 represses Myc-mediated transcription in ES cells

To examine whether TRIM6 affects Myc-mediated transcription in not only HEK293T cells, but also ES cells, we performed a luciferase reporter assay using p4 × E-SVP-Luc in ES cells. First, we verified that endogenous Myc was expressed in the mouse ES cell lines E14 and TC-11 (Fig. 5A). We transiently transfected an expression vector encoding FLAG-tagged TRIM6 with the p4 × E-SVP-Luc reporter plasmid. Luciferase assays using ES cells showed that TRIM6 repressed endogenous Myc-mediated transcriptional activity as was observed in the luciferase assay

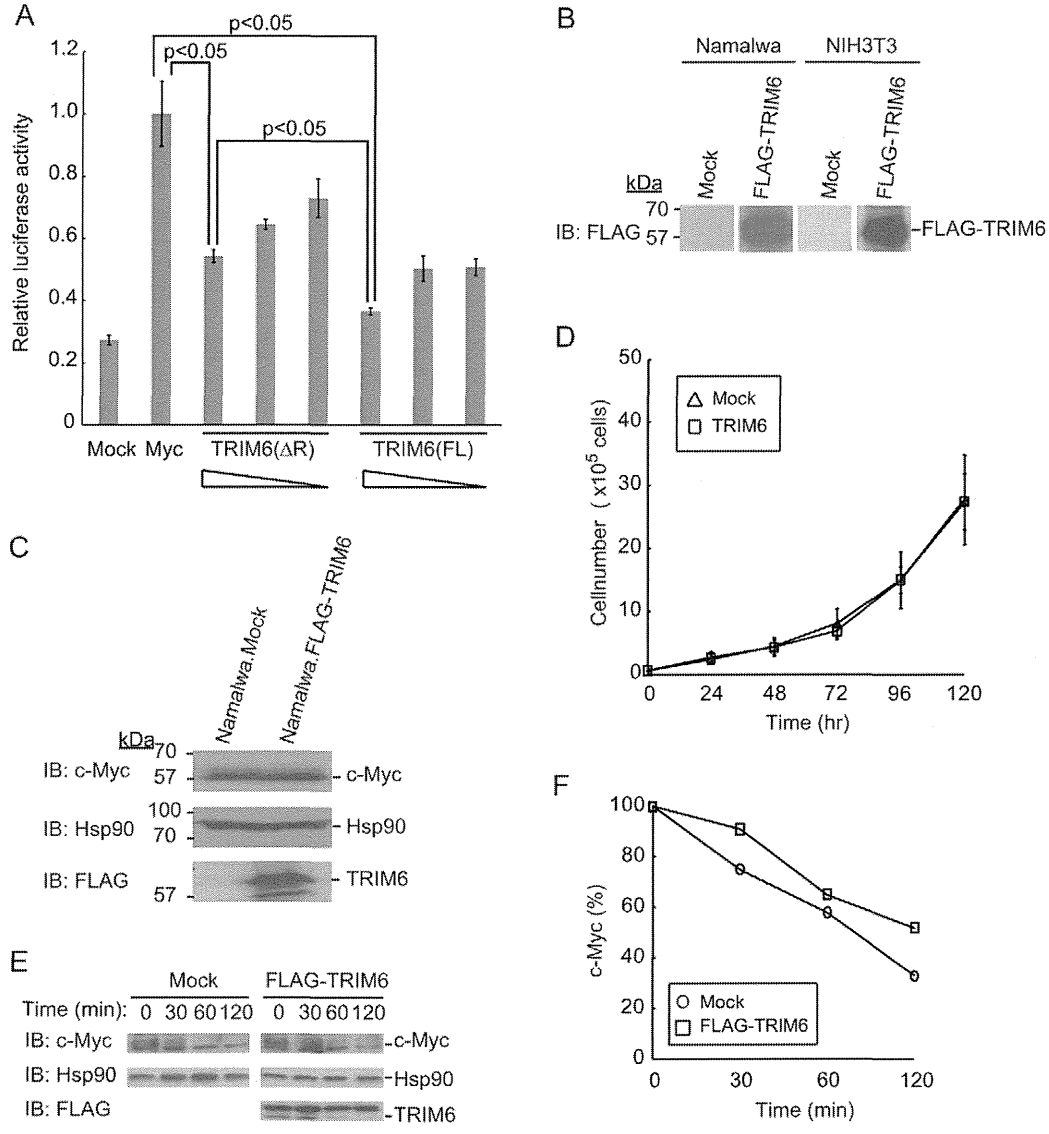

Fig. 3. TRIM6 represses Myc-mediated transcriptional activity. (**A**) TRIM6 represses Myc-mediated transcriptional activity in a dose-dependent manner. The p4 × E-SVP-Luc reporter vector and expression vector encoding various amounts of TRIM6 (FL) or TRIM6(ΔR) were transfected into HEK293T cells. Forty-eight hours after transfection, cells were harvested and assayed for luciferase activity. Data are mean ± standard deviation (SD) of values from three independent experiments. *P* values for indicated comparisons were determined by the Student's *t* test. (**B**) Establishment of Namalwa cell lines and NIH 3T3 cell line stably expressing FLAG-tagged TRIM6 by a retroviral expression system. The cell lines were checked by immunoblot analysis using anti-FLAG antibody. (**C**) TRIM6 does not affect the expression level of endogenous Myc in a Bürkitt lymphoma cell line. Namalwa cells infected with either a retroviral vector encoding FLAG-tagged TRIM6 or the corresponding empty vector (Mock) were lysed and subjected to immunoblot analysis with antibodies to Myc, Hsp90 and FLAG. Anti-Hsp90 antibody was used as an internal control. (**D**) TRIM6 does not affect cell proliferation. Namalwa cells transfected with FLAG-tagged TRIM6 and mock (2×10^4 cells) were seeded in 60-mm dishes and harvested for counting cell numbers at the indicated times. Data are mean ± SD of values from three independent experiments. (**E**) Protein stability analysis of endogenous Myc in cells stably expressing TRIM6. NIH3T3 cells stably expressing FLAG-tagged TRIM6 and Mock were used for a protein stability assay with cycloheximide. Twenty-four hours after seeding on dishes, the cells were cultured in the presence of cycloheximide (50 μg/ml) for the indicated times. Cell lysates were then subjected to immunoblot analysis with anti-Myc, anti-Hsp90 or anti-FLAG antibody. (**F**) Intensity of the endogenous Myc bands in the protein stability analysis in E was normalized with that of the corresponding Hsp90 bands and then expressed as a percentage of the normalized value for time zero.

using HEK293T cells (Fig. 5B). Furthermore, assays using E14 cells showed that TRIM6 attenuated endogenous Myc-mediated transcriptional activity in a dose-dependent manner (Fig. 5B). Next, by using electroporation, we generated three E14 cell lines in which FLAG-tagged TRIM6 was stably expressed (Fig. 5C), and we performed luciferase assays to compare the transcriptional activity of endogenous Myc by using these cell lines. Luciferase assays also showed that TRIM6 repressed endogenous Myc transcriptional activity in E14 cells (E14.TRIM6-1) (Fig. 5D). Moreover, we examined the effect of TRIM6 on the expression level of endogenous Myc. Overexpression of TRIM6 caused no significant change in the

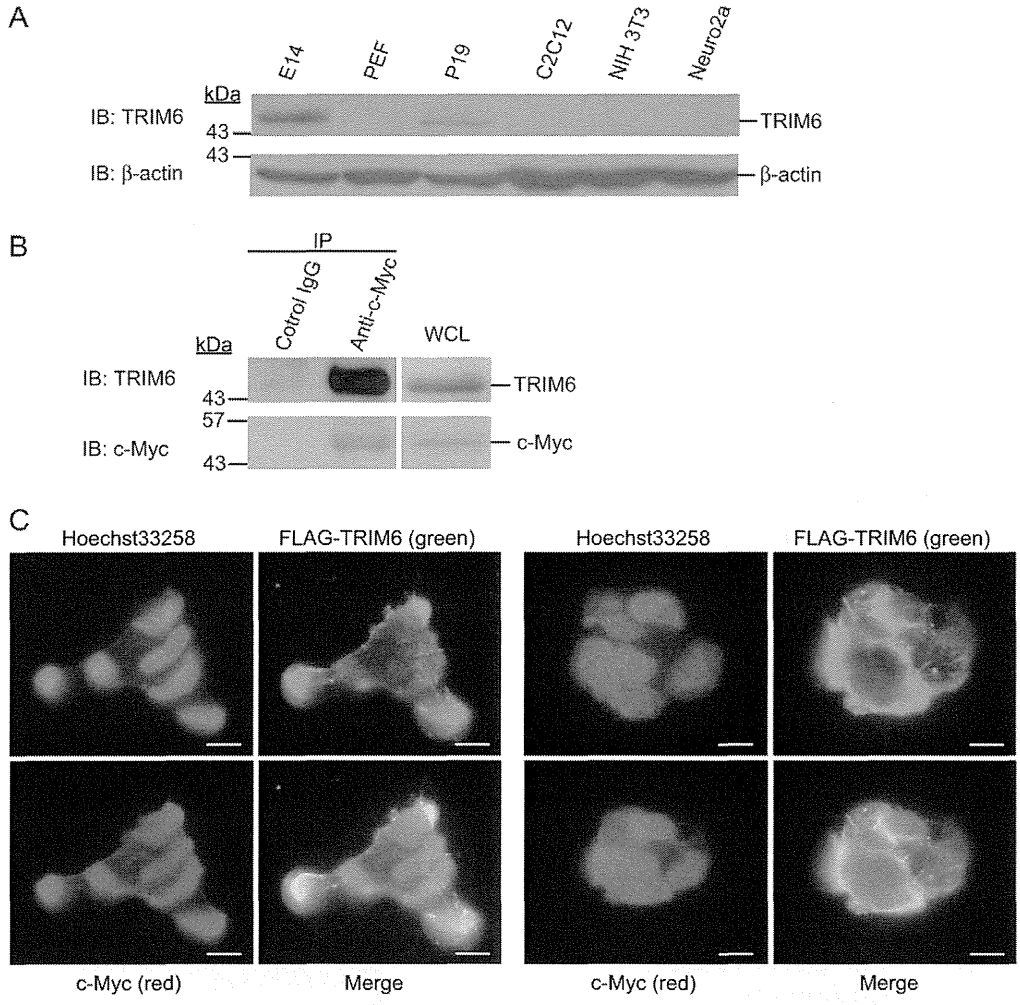

Fig. 4. TRIM6 is selectively expressed in ES cells and interacts with endogenous Myc in ES cells. (**A**) Expression of TRIM6 in various cell lines. Cell lysates from the indicated cell lines were subjected to immunoblot analysis with anti-TRIM6 and anti-β-actin antibodies. (**B**) Interaction between endogenous TRIM6 and Myc in ES cells. Cell lysates from E14 cells were immunoprecipitated with anti-Myc antibody or Mock [control immunoglobulin (IgG)] and immunoblotted with an antibody to TRIM6. (**C**) Colocalization of FLAG-tagged TRIM6 and endogenous Myc by immunofluorescence analysis. E14 cells stably expressing FLAG-tagged TRIM6 were used for the analysis. The cells were fixed and stained with antibodies to FLAG (green), Myc (red) or Hoechst 33258 (blue). Scale bars: 20 μm.

expression level of Myc in E14 cells (Fig. 5E). To verify that TRIM6 affects the stability of endogenous Myc in vivo, we performed a protein stability analysis using cycloheximide and these E14 cell lines. The analysis showed that the degradation rate of endogenous Myc was similar in E14 cells stably expressing TRIM6 to that in mock cells (Fig. 5F). However, overexpression of TRIM6 in E14 cells caused high expression of NANOG, which is an important pluripotent marker of ES cells, suggesting that TRIM6 regulates cell differentiation via Myc-mediated transcription (Fig. 5E). To determine whether forced expression of TRIM6 renders leukemia inhibitory factor (LIF) redundant in ES cells, as in the case of NANOG and KLF4 overexpression (Chan et al., 2009; Darr et al., 2006), we observed E14 cells stably expressing TRIM6 and mock cells without LIF. Although overexpression of TRIM6 in E14 cells caused high expression of NANOG, forced expression of TRIM6 could not maintain the undifferentiation of ES cells without LIF (Fig. 5G). Immunoblot analysis showed that the expression levels of NANOG were decreased in E14 cells stably expressing TRIM6 as well as in mock cells after culture without LIF (Fig. 5H). These findings suggest that forced expression of TRIM6 cannot maintain the pluripotency of ES cells without LIF.

High expression of Myc induced by TRIM6 knockdown in ES cells

To clarify why TRIM6 modulates Myc transcriptional activity in ES cells and does not affect the stability of endogenous Myc by ectopic overexpression of TRIM6, we next generated ES cell lines in which TRIM6 was knocked down. Short hairpin RNAs (shRNAs) targeting TRIM6 were introduced into E14 cells by using a retroviral infection system; silencing of TRIM6 at the protein level in E14 cells was confirmed by immunoblot analysis with an anti-TRIM6 antibody (Fig. 6A). Immunoblot analysis showed that the amount of endogenous Myc in E14 cells in which TRIM6 was knocked down was larger than in mock cells, whereas the expression level of NANOG in TRIM6-knockdown

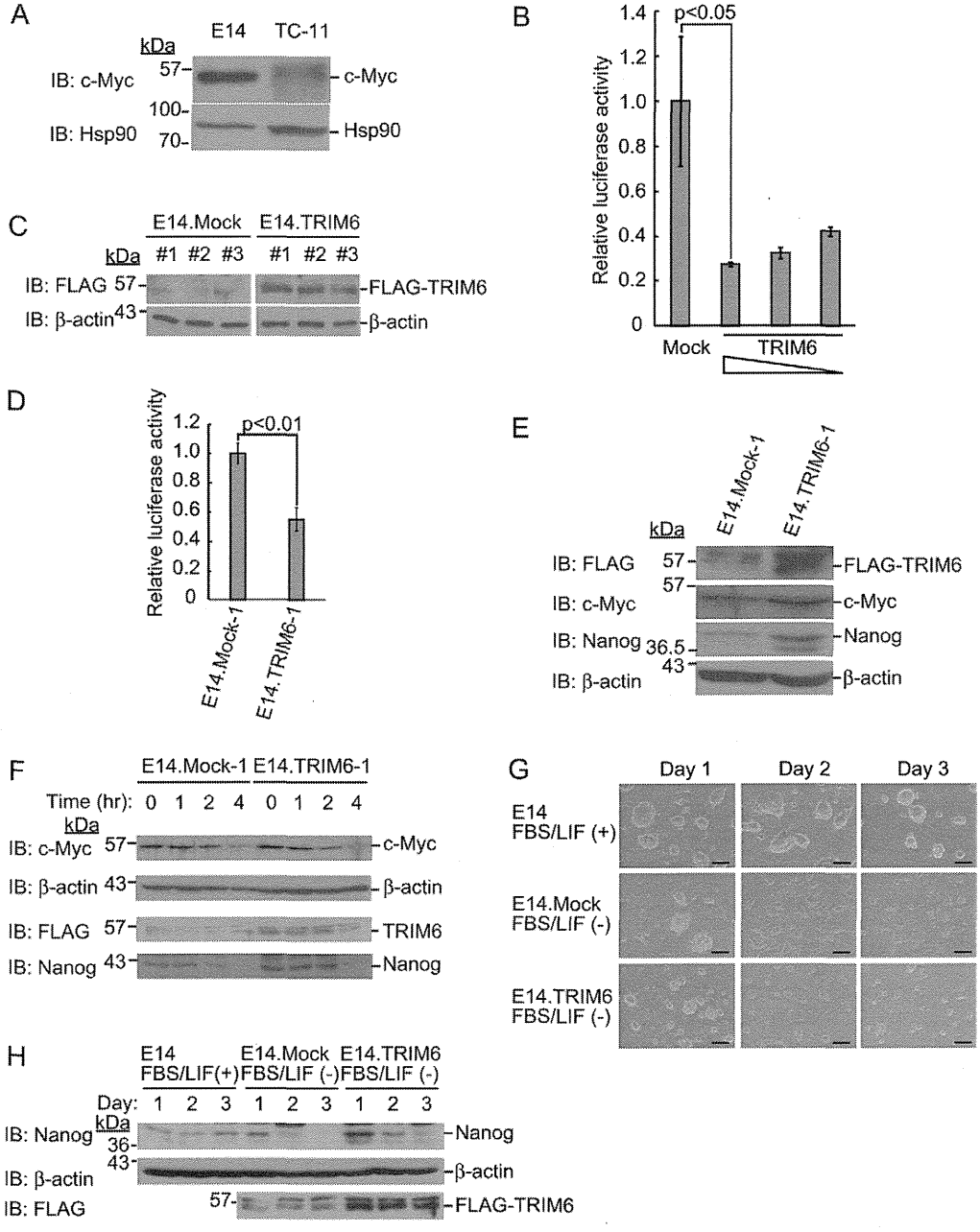

Fig. 5. TRIM6 inhibits transcriptional activation of Myc in ES cells. (**A**) Expression of Myc in mouse ES cell lines. Cell lysates from mouse ES cell lines E14 and TC-11 were subjected to immunoblot analysis with anti-Myc antibody. (**B**) TRIM6 represses Myc-mediated transcriptional activity in a dose-dependent manner. p4 × E-SVP luciferase reporter vector, pRL-TK *Renilla* vector and various amounts of TRIM6 expression vector were transiently transfected into E14 cells. Forty-eight hours after culture with LIF, the cells were harvested and assayed for luciferase activity. (**C**) Establishment of three E14 cell lines stably expressing FLAG-tagged TRIM6 by using electroporation followed by puromycin selection. These cell lines were checked by immunoblot analysis using anti-FLAG antibody. (**D**) TRIM6 represses Myc-mediated transcriptional activity in ES cells stably expressing TRIM6. E14 cell lines stably expressing TRIM6 were assayed for luciferase activity. (**E**) Effect of endogenous Myc and NANOG expression by overexpression of TRIM6 in ES cells. E14 cell lines stably expressing FLAG-tagged TRIM6 and Mock cells were lysed and subjected to immunoblot analysis with antibodies to FLAG, Myc, NANOG and β-actin. (**F**) Protein stability analysis of endogenous Myc in ES cells stably expressing TRIM6. An E14 cell line stably expressing FLAG-tagged TRIM6 and a Mock were used for a protein stability analysis with cycloheximide. The cells were incubated in a conditioned medium including LIF in the presence of cycloheximide (50 μg/ml) for the times indicated. Cell lysates were then subjected to immunoblot analysis with anti-Myc, anti-β-actin, anti-NANOG or anti-FLAG antibody. (**G**) Forced expression of TRIM6 cannot maintain the pluripotency of ES cells without LIF. E14 cell lines with stable overexpression of TRIM6 or Mock cells were seeded at 1×10^6 cells in 60-mm dishes coated with gelatin. These ES cells were cultured with a conditioned medium including 15% FBS. As control, E14 cells were also cultured with the same medium and LIF. Day 1 is the day that each cell line was seeded on dishes. The culture medium with or without LIF was changed daily. Scale bars: 100 μm. (**H**) Immunoblot analysis of NANOG in TRIM6-overexpressing ES cells without LIF. E14 cell lines with stable overexpression of TRIM6 or mock cells were seeded in 60-mm dishes coated with gelatin, and immunoblot analysis with anti-NANOG antibody was performed at the indicated times after seeding. As a control, E14 cells were also analyzed with the same medium and LIF. Two independent experiments were performed. Anti-β-actin antibody was used as an internal control.

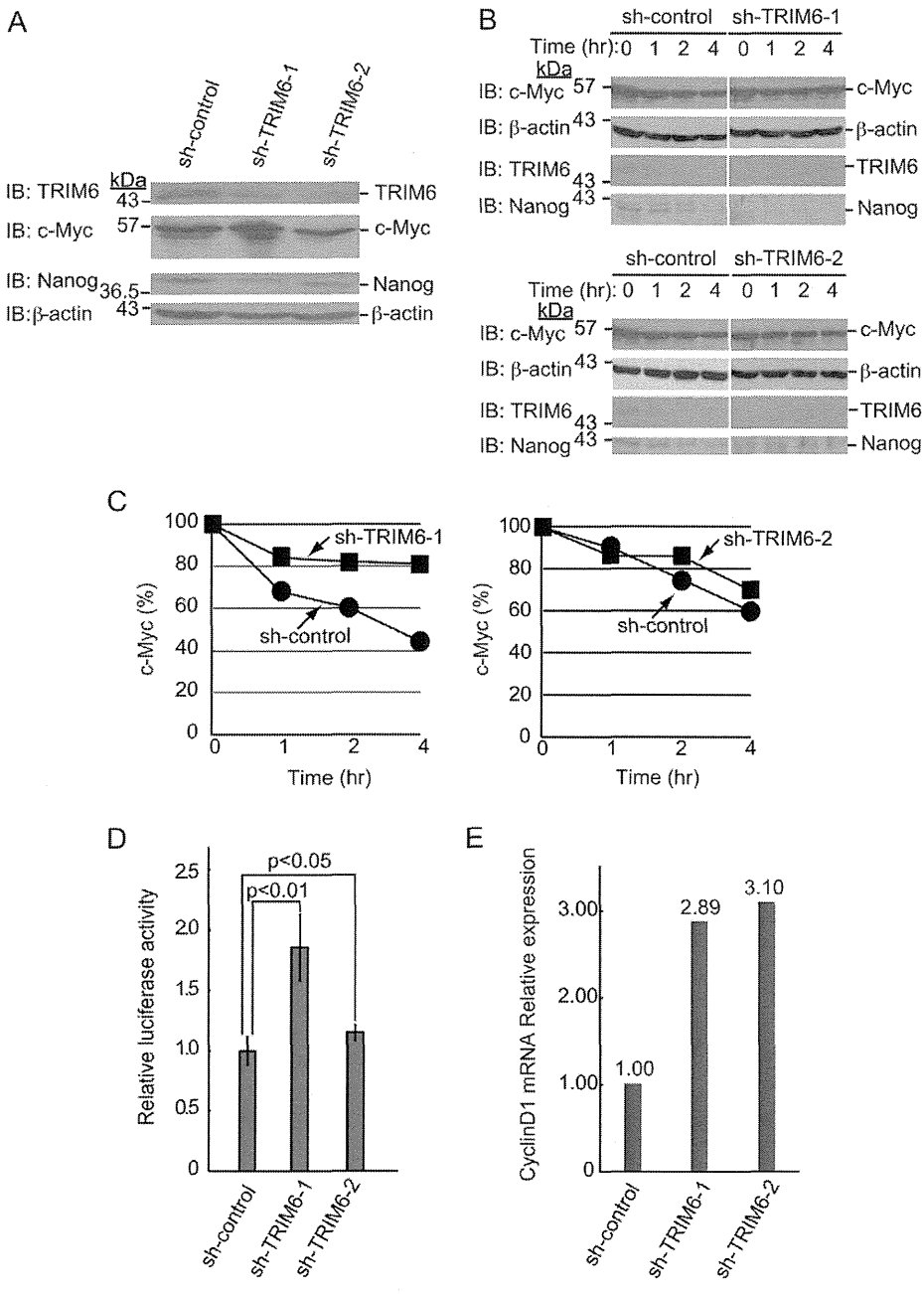

Fig. 6. Effect of silencing TRIM6 on the expression of endogenous Myc and NANOG in ES cells. (**A**) Knockdown of TRIM6 facilitates Myc expression in ES cells. shRNAs targeting TRIM6 (sh-TRIM6-1 and sh-TRIM6-2) or scrambled shRNA as a negative control (sh-control) were introduced into E14 cells using a retroviral expression system. Cells were lysed and subjected to immunoblotting with anti-TRIM6, anti-Myc, anti-Nanog and anti-β-actin antibodies. (**B**) Protein stability analysis of endogenous Myc in knockdown E14 cell lines with sh-TRIM6 or sh-control. E14 cell lines stably knocked-down with sh-TRIM6-1, sh-TRIM6-2 and sh-control were used for a protein stability analysis with cycloheximide. Cell lysates were then subjected to immunoblot analysis with anti-Myc, anti-β-actin, anti-Nanog or anti-FLAG antibody. (**C**) Intensity of the endogenous Myc bands in the protein stability analysis in B was normalized with that of the corresponding β-actin bands and was then expressed as a percentage of the normalized value for time zero. Data are means of values from two independent experiments. (**D**) Knockdown of TRIM6 facilitates Myc-mediated transcriptional activity in ES cells. p4 × E-SVP luciferase reporter vector and pRL-TK *Renilla* vector were transfected into knockdown E14 cell lines silenced with sh-TRIM6. Data are mean ± SD of values from three independent experiments. (**E**) Knockdown of TRIM6 causes upregulation of *Ccdn1* expression in ES cells. mRNAs of *Ccdn1* were measured by real-time quantitative PCR. The relative *Ccdn1* mRNA expression level in cells that had been transfected with sh-control was defined as 1.

E14 cells was less than that in control cells. To confirm that knockdown of TRIM6 affected the stability of endogenous Myc, we performed a protein stability analysis using these knockdown ES cells. The analysis showed that knockdown of TRIM6 caused high stability of Myc (Fig. 6B,C). To further examine the effect of TRIM6 on Myc-mediated transcription, we performed a relative luciferase assay using these knockdown ES cells. The assay showed that Myc-mediated transcriptional activities were increased in TRIM6-knockdown cells compared with those in cells treated with control shRNA (Fig. 6D). In addition, real-time PCR using these cell lines was performed to analyze the mRNA level of the gene encoding cyclin D1 (*Ccdn1*), given that *Ccdn1* is one of the target genes of Myc (Daksis et al., 1994; Dang, 1999; Perez-Roger et al., 1999). Consistent with the results of Myc-dependent transcriptional activity shown by the luciferase assay, the mRNA level of *Ccdn1* was positively regulated in TRIM6-knockdown cells compared with that in control cells (Fig. 6E). These findings suggest that TRIM6 regulates Myc-mediated transcriptional activity and affects the expression levels of target genes of Myc in ES cells.

Knockdown of TRIM6 promotes differentiation of ES cells

Given that our results showed that TRIM6 modulated the expression level of Myc in ES cells and that knockdown of TRIM6 in ES cells caused a decrease in the expression level of NANOG, we hypothesized that the expression of TRIM6 is also changed in several differentiation stages of ES cells. We first performed real-time PCR to quantify the mRNA level of *TRIM6*

in differentiated and undifferentiated ES cells. As expected, the relative mRNA level of *TRIM6* was significantly decreased in differentiated ES cells cultured without LIF compared with the level in undifferentiated ES cells (Fig. 7A,B). We also compared the level of TRIM6 protein in differentiated and undifferentiated ES cells. The expression level of TRIM6 was higher in undifferentiated than in differentiated ES cells (Fig. 7C). Despite the high expression level of TRIM6, the Myc protein expression level was significantly higher in undifferentiated than in differentiated ES cells (Fig. 7C).

Next, to examine whether TRIM6 maintains the undifferentiated state of ES cells, we evaluated the morphological phenotype of ES cells with or without endogenous TRIM6 expression. We seeded each cell line on dishes and cultured the cells with LIF for maintenance of pluripotency. Colonies from control E14 cells grew normally and were enlarged in the presence of LIF for 3 days, whereas TRIM6-knockdown E14 cells (sh-TRIM6-1) differentiated into endoderm-like cells even in the presence of LIF (Fig. 7D). Moreover, we checked the expression levels of NANOG, GATA-binding protein 4 (GATA-4) and alpha-fetoprotein (AFP), which are endodermal differentiation markers in ES cells, and the expression levels of heart and neural crest derivatives expressed 1 (HAND1) and microtubule-associated-protein-2 (MAP2), which are ectodermal differentiation markers in ES cells, by immunoblot analysis at the indicated times (Knofler et al., 2002; Kwon et al., 2006; Soudais et al., 1995; Tropepe et al., 2001). Immunoblot analysis showed that the expression level of NANOG in TRIM6-knockdown E14 cells (sh-TRIM6-1) was lower than in mock cells, whereas the expression levels of GATA-4 and AFP in TRIM6-knockdown E14 cells were higher than in mock cells (Fig. 7E). By contrast, little, if any, HAND1 and MAP2 protein could be detected in either cell line (Fig. 7E). These findings indicated that silencing of TRIM6 in ES cells induced a decrease in NANOG expression level and tended to induce differentiation to endodermal cells rather than ectodermal cells, suggesting that TRIM6 is required for the maintenance of an undifferentiated state of ES cells in the presence of LIF.

To determine whether differentiation induced by TRIM6 knockdown is rescued by transduction of exogenous NANOG, we tried to generate stably NANOG-expressing E14 cell lines in which TRIM6 was knocked down (Fig. 7F). Immunoblot analysis showed that E14 cells in which TRIM6 was knocked down expressed NANOG and OCT3/4, which is encoding by another self-renewal and undifferentiation marker gene in ES cells, at lower levels than in mock cells. However, immunoblot analysis showed that the amount of OCT3/4 was recovered by overexpression of exogenous NANOG, suggesting that differentiation of TRIM6-knockdown cells is rescued by induction of exogenous NANOG and that TRIM6 mainly participates in regulation to maintain the pluripotency of ES cells at a site upstream of NANOG (Fig. 7F).

Furthermore, we checked the morphological phenotype of ES cell lines in which TRIM6 was knocked down and performed immunoblot analysis to confirm the expression levels of NANOG and OCT3/4 in ground-state culture (Ying et al., 2008). For the ground-state culture, pre-formulated NDiff N2B27 base medium was prepared with CHIR99021, which is a specific glycogen synthase kinase 3β (GSK3β) inhibitor, and PD0325901, which is a mitogen-activated protein kinase (MAPK) inhibitor, as two inhibitors (2i). E14 cells as control cells (E14 N2B27+2i), E14 cell lines in which TRIM6 was knocked down (sh-TRIM6 N2B27+2i) and sh-control cells (sh-control N2B27+2i) showed undifferentiated colony formation in the ground-state culture (Fig. 7G). Immunoblot analysis showed that the expression levels of NANOG and OCT3/4 protein were maintained in TRIM6-knockdown E14 cells (sh-TRIM6) as well as in E14 cells and sh-control E14 cells after several passages by means of the ground-state culture (Fig. 7H). The Myc protein levels were downregulated by suppressing the extracellular signal-regulated kinase (ERK) signal in this condition, as previously reported (Fig. 7H) (Ying et al., 2008). These findings suggest that ES cell lines in which TRIM6 has been knocked down differentiate under stimulation of Ras–MAPK kinase (MEK)–ERK signaling cascades.

Discussion

In this study, we identified Myc as a TRIM6-interacting protein in ES cells and revealed molecular functions of TRIM6 in the pluripotency of ES cells. SKP1–CUL1–F-box protein complex (SCF)-type E3 ligases, including SCF^{skp2} and SCF^{Fbw7}, also interact with Myc. Skp2 interacts with Myc via Myc-box 2 (MB2) and the HLH-Zip domain and promotes its ubiquitination and transcriptional activation (Kim et al., 2003; von der Lehr et al., 2003); Fbw7 ubiquitinates and degrades Myc by recognizing phosphorylation on T58 and S62 in MB1 of Myc (Yada et al., 2004). Using yeast two-hybrid screening and immunoprecipitation, we showed that TRIM6 binds to Myc and, in addition, interacts with Myc regardless of its phosphorylation at T58 and S62. We also showed that TRIM6 lacking a RING domain interacts with Myc. These findings suggest that a RING domain of TRIM6 and MB1 domain of Myc are not required for the interaction between them.

Given that it has been reported that Myc is involved in regulation of cell proliferation and cell differentiation (van Riggelen et al., 2010), we further examined the relationship between TRIM6 and Myc-mediated transcription. By using a luciferase reporter assay, we found that TRIM6 functions as a negative regulator of Myc and that TRIM6 suppressed the transcriptional activity of Myc in HEK293T cells; however, ectopic expression of FLAG-tagged TRIM6 did not change the expression level of Myc and cell proliferation rates in Namalwa cells. Furthermore, ectopically expressed TRIM6 did not cause a change in Myc in NIH3T3 cells. Similar results were obtained in experiments using ES cells. These findings suggest that ectopically expressed TRIM6 suppresses transcriptional activity of Myc but does not have sufficient activity to alter the function of Myc to affect cell proliferation and cell differentiation. Recently, it was reported that Myc has important roles in self-renewal and cell fate determination in ES cells and often causes tumor formation when its activity is dysregulated (Cartwright et al., 2005). Furthermore, there is accumulating evidence that the activity level of Myc is high in undifferentiated ES cells. It has been reported that ectopic Myc expression maintains the pluripotency of mouse ES cells without LIF (Cartwright et al., 2005). However, it has also been reported that Myc promotes progenitor cell differentiation in some contexts (Watt et al., 2008; Wilson et al., 2004). It has been shown that OCT3/4, which has been reported to prevent differentiation directly, is appropriately regulated in undifferentiated mouse ES cells, suggesting that its deregulated expression induces differentiation of mouse ES cells (Niwa et al., 2000). Although the detailed molecular mechanisms are unclear, the expression levels of Myc might also be

appropriately regulated in mouse undifferentiated ES cells. Based on these results, we speculated that TRIM6 strictly modulates the expression level or activity of Myc to prevent differentiation of mouse ES cells. We found increased polyubiquitination of Myc by TRIM6 in the in vivo ubiquitination assay, suggesting that TRIM6 functions as a ubiquitin ligase for Myc in ES cells. However, the reason why the expression level of Myc is maintained in ES cells despite the fact that Myc-mediated transcriptional activation is repressed by TRIM6 remains to be determined. It was recently reported that mouse ES cell culture conditions using MAPK and GSK3β inhibitors render Myc dispensable and that ES cells are not necessarily dependent on Myc to maintain their specific features (Hishida et al., 2011). The same authors also reported that stabilized Myc suppresses MAPK signaling to maintain the pluripotency of ES cells, suggesting that Myc finely modulates the balance of LIF–signal transducer and

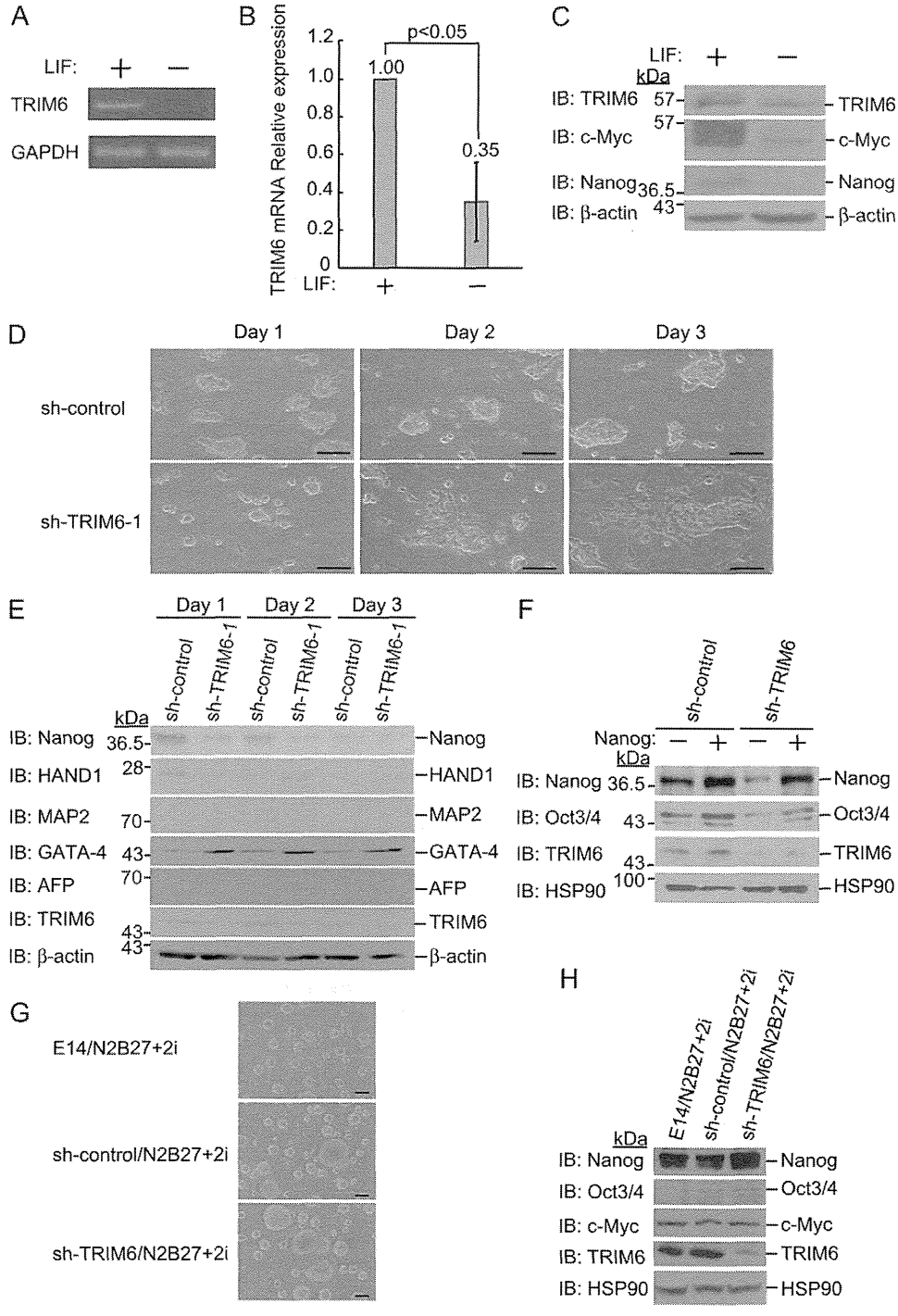

Fig. 7. See next page for legend.

activator of transcription 3 (STAT3) signaling and MAPK signaling activated by LIF and/or fibroblast growth factor (FGF), to prevent differentiation of ES cells. Based on our findings and the results of previous studies, we propose that TRIM6 is one of the interacting proteins that enable Myc to modulate its transcriptional activity appropriately. However, future work is needed to clarify the currently unknown mechanism that induces Myc expression in ES cells.

It has been reported that the pluripotency of ES cells is regulated by three intracellular transduction systems: the Janus kinase (JAK)-STAT3 pathway, phosphatidylinositol 3-kinase (PI3K)–AKT pathway and SHP2 [protein tyrosine phosphatase, non-receptor type 11 (PTPN11)]–RAS–MAPK pathway (Niwa et al., 1998; Watanabe et al., 2006). It has also been demonstrated that JAK–STAT3 and PI3K–AKT pathways are essential and sufficient to mediate LIF signals to maintain the pluripotency of ES cells (Niwa et al., 1998; Watanabe et al., 2006). By contrast, it has been shown that the SHP2–RAS–MAPK pathway, which is related to adjustment of the expression level of Myc, functions as a negative regulator of the maintenance of the pluripotency of ES cells (Kunath et al., 2007) and as a negative regulator of *Nanog* and the gene encoding T-box 3 (*Tbx3*) (Niwa et al., 2009). In our study, TRIM6 expression level was found to be higher in undifferentiated than in differentiated ES cells, and Myc expression level was found to be elevated in TRIM6-knockdown cells. These cells differentiated rapidly even in the presence of LIF. Therefore, TRIM6 could regulate Myc expression level within optimal ranges by repressing the transcription activity of Myc to maintain the pluripotency of undifferentiated ES cells. TRIM6 might also bind to Myc as a negative regulator of Myc in an undifferentiated state with LIF signaling. Upon differentiation of ES cells, TRIM6 is rapidly downregulated and Myc can be transiently activated. However, Myc protein might be degraded by another ubiquitin ligase or another proteolysis system independent of TRIM6 in the differentiated state, although the molecular mechanisms involved have not yet been clarified. Indeed, Myc has been shown to have roles in blocking cell differentiation and in maintaining progenitor cells in vivo (Knoepfler et al., 2002; Satoh et al., 2004). Therefore, TRIM6 probably functions as an important regulator of Myc expression and its downstream molecules to maintain the pluripotency of ES cells.

Recently, it has been reported that tripartite motif family-like 1 (TRIML1), one of the TRIM family proteins, is expressed in the embryo before implantation and that its knockdown causes a reduction in the number of blastocysts and failure to give rise to neonates after embryo transfer (Tian et al., 2009). Given that both TRIML1 and TRIM6 regulate the pluripotency and proliferation of ES cells and blastocysts, it will be important to analyze the functional interaction of TRIML1 and TRIM6 in future studies.

In conclusion, our study clarified that TRIM6 is highly expressed in ES cells and interacts with Myc to inhibit its transcriptional activity, followed by maintenance of pluripotency. Results of future studies aimed at clarifying the relationship between TRIM6 and interacting proteins, including Myc, should help advance understanding of developmental biology and cancer biology. Functional analysis of TRIM6 might provide benefits not only for the establishment of iPS cells, but also for their suppression in lymphomas and leukemias.

Fig. 7. TRIM6 is downregulated in differentiated ES cells and knockdown of TRIM6 induces ES cell differentiation. (**A**) *Trim6* mRNA expression is decreased in differentiated ES cells. The levels of *Trim6* mRNA derived from undifferentiated and differentiated ES cells were measured by real-time quantitative PCR. Differentiated E14 cells were cultured without LIF for 1 week. Amplified *Trim6* and *Gapdh* cDNAs were subjected to agarose gel electrophoresis. (**B**) Quantification of the amount of *Trim6* by real-time quantitative PCR using undifferentiated and differentiated ES cells in A. Data are means ± SD of values from three independent experiments. *P* values for indicated comparisons were determined by the Student's *t* test. (**C**) Immunoblot analysis of TRIM6 in undifferentiated and differentiated ES cells. Immunoblot analysis was performed with anti-TRIM6, anti-Myc, anti-Nanog and anti-β-actin antibodies. (**D**) Knockdown of TRIM6 induces ES cells differentiation. Stably knocked-down E14 cell lines with sh-TRIM6-1 or sh-control were seeded at 1×10^6 cells in 60-mm dishes coated with gelatin. These ES cells were cultured with a conditioned medium including 15% FBS and LIF. Day 0 is the day that each cell line was seeded on dishes. The culture medium with LIF was changed daily. Scale bars: 100 μm. (**E**) Immunoblot analysis of NANOG, HAND1, MAP2, GATA-4 and AFP in TRIM6-knockdown ES cells. Stably knocked-down E14 cell lines with sh-TRIM6-1 or sh-control were seeded at 1×10^6 cells in 60-mm dishes coated with gelatin, and immunoblot analysis with each antibody was performed at the indicated times after seeding. Two independent experiments were performed. Anti-β-actin antibody was used as an internal control. (**F**) Differentiation of TRIM6-knockdown cells is rescued by transduction of exogenous Nanog. By using a retroviral expression system, sh-TRIM6 or sh-control was introduced into E14 cells in which NANOG was stably expressed. Cells were lysed and subjected to immunoblotting with anti-OCT3/4, anti-NANOG, anti-TRIM6, and anti-HSP90 antibodies. Anti-HSP90 antibody was used as an internal control. (**G**) TRIM6-knockdown cells maintain an undifferentiated state in ground culture. Each ES cell line cultured for four passages (total of 14 days) in N2B27 base medium with CHIR99021 and PD0325901 (N2B27+2i) without serum and LIF. Scale bars: 100 μm. (**H**) Immunoblot analysis of NANOG and OCT3/4 in ES cells after 14 days in N2B27+2i culture. Myc protein expression level is suppressed in this condition. Anti-HSP90 antibody was used as an internal control.

Materials and Methods

Cell culture

HEK293T cells and NIH3T3 cells (ATCC, Manassas, VA) were cultured under an atmosphere of 5% CO_2 at 37℃ in DMEM (Sigma-Aldrich, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA) or 10% calf serum (Camblex, Charles City, IA). Namalwa cells (ATCC) were cultured in RPMI-1640 (Sigma-Aldrich) with 10% fetal bovine serum (Invitrogen). Mouse ES E14 cells and TC-11 cells were cultured with or without feeder cells in DMEM (Invitrogen) supplemented with 15% heat-inactivated fetal bovine serum (Invitrogen), 55 μM β-mercaptoethanol (Invitrogen), 2 mM L-glutamine, 0.1 mM MEM non-essential amino acid and 1000 U/ml LIF (Millipore Corporation, Bedford, MA).

Yeast two-hybrid screening

Yeast strain L40 (*MATa LYS2::lexA-HIS3 URA3::lexA-lacZ trp1 leu2 his3*) (Invitrogen) was transformed both with the plasmid pBTM116 encoding the FLAG-tagged mouse TRIM6 and with a mouse T cell cDNA library in the pACT2 vector (Clontech, Mountain View, CA). The cells were then streaked on plates of medium lacking histidine to detect interaction-dependent activation of *HIS3* according to the manufacturer's protocol (Clontech).

Cloning of cDNA and plasmid construction

Mouse *Trim6* cDNA and mouse *Nanog* cDNA were amplified from ES cells by PCR with BlendTaq (Takara, Tokyo, Japan) using the following primers: 5′-ACAATGACTTCAACAGTCTTGGTG-3′ (*Trim6*-sense), 5′-ACCTCAGGAAG-TTGGCCGCCGCAG-3′ (*Trim6*-antisense), 5′-GACATGAGTGTGGGTCCTC-CT-3′ (*Nanog*-sense), and 5′-GTCTCATATTTCACCTGGTGG-3′ (*Nanog*-antisense). The amplified fragments were subcloned into pBluescript II SK^+ (Stratagene, La Jolla, CA), and the sequence was verified. Deletion mutants of *Trim6* cDNA containing amino acids 97–488, which was used as TRIM6 lacking a RING finger domain [TRIM6(ΔR)], were amplified by PCR and subcloned. A deletion mutant of the RING domain of TRIM6 was generated using the following primers: 5′-CGGACCTCCTATCAGCTGGG-3′ [TRIM6(ΔR)-sense] and 5′-AC-CTCAGGAAGTTGGCCGCCGCAG-3′ [TRIM6(ΔR)-antisense]. FLAG-tagged or HA-tagged TRIM6 and TRIM6(ΔR) cDNAs were then subcloned into the