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# **TRIM45 negatively regulates NF- $\kappa$ B-mediated transcription and suppresses cell proliferation**

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*Key words:* TRIM45, NF- $\kappa$ B, MAPK, TNF $\alpha$ , cell proliferation

## **Abstract**

The NF- $\kappa$ B signaling pathway plays an important role in cell survival, immunity, inflammation, carcinogenesis, and organogenesis. Activation of NF- $\kappa$ B is regulated by several posttranslational modifications including phosphorylation, neddylation and ubiquitination. The NF- $\kappa$ B 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 TNF $\alpha$ -induced NF- $\kappa$ B-mediated transcription by a luciferase reporter assay and that TRIM45 lacking a RING domain also has an activity to inhibit the NF- $\kappa$ B signal. Moreover, we found that TRIM45 overexpression suppresses cell growth. These findings suggest that TRIM45 acts as a repressor for the NF- $\kappa$ B signal and regulates cell growth.

## 1. Introduction

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

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- $\kappa$ B and suppresses cell proliferation. These findings indicate that TRIM45 regulates cell proliferation via inhibition in the NF- $\kappa$ B 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<sub>2</sub> 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 (Cambrex, 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 BlendTaq (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<sub>3</sub>VO<sub>4</sub>, 400 μmol/l EDTA, 10 mmol/l NaF, and 10 mmol/l sodium pyrophosphate. The cell lysates were centrifuged at 15,000 g 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( $\Delta$ R)) 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  $\mu$ g/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 1:1,000. The cells were further incubated with Hoechst 33258 (1  $\mu$ g/ml) in PBS for 10 min, followed by extensive washing with PBS. The cells were covered with a drop of GEL/MOUNT (Biomedex, 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( $\Delta$ R) 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  $\mu\text{g/ml}$  (Sigma) and the infected clones were expanded in a medium containing puromycin (5  $\mu\text{g/ml}$ , Sigma).

## 2.6. *Dual-luciferase assay*

HEK293T cells were seeded in 24-well plates at  $1 \times 10^5$  cells per well and incubated at 37°C with 5% CO<sub>2</sub> for 24 h. NF- $\kappa$ B luciferase reporter plasmid (NAT-Luc) [25], pRL-TK *Renilla* luciferase plasmid (Promega) and various combinations of FLAG-tagged TRIM45(FL) or FLAG-tagged TRIM45( $\Delta$ R) expression vectors were transfected into HEK293T cells using the Fugene HD reagent (Roche). Twenty-four h after transfection, cells were incubated with TNF $\alpha$  (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( $\Delta$ R), or an empty vector (mock) was stably expressed by using a retroviral expression system were seeded in 24-well plates at  $1 \times 10^5$  cells per well and incubated at 37°C with 5% CO<sub>2</sub> for 24 h. NF- $\kappa$ B luciferase reporter plasmid (NAT-Luc) [24] and pRL-TK *Renilla* luciferase plasmid (Promega) were transfected into each stable cell line using the Fugene HD reagent (Roche). Twenty-four h after transfection, cells were incubated with TNF $\alpha$  (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( $\Delta$ R) and an empty vector (mock) was stably expressed by using a retroviral expression system were seeded in 6-cm dishes ( $1 \times 10^5$  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*( $\Delta$ R)) (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*( $\Delta$ R)) 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( $\Delta$ R) and then stained with an antibody to FLAG with Hoechst33258 to reveal the subcellular distribution of FLAG-tagged TRIM45(FL) or TRIM45( $\Delta$ R) (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( $\Delta$ R) 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.

### *3.3. TRIM45 negatively regulates TNF $\alpha$ -induced NF- $\kappa$ B-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 ER $\alpha$ -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 ( $\text{I}\kappa\text{B}$ ) kinase (IKK)  $\gamma$  and inhibited the activity of NF- $\kappa$ B-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- $\kappa$ B signal. Therefore, we performed a luciferase reporter assay using an NF- $\kappa$ B promoter-driven luciferase construct (NAT-Luc) in HEK293T cells to examine whether TRIM45 regulates NF- $\kappa$ B-mediated transcription. We transfected expression vectors encoding FLAG-tagged TRIM45(FL) or TRIM45 ( $\Delta$ R) with reporter plasmids into HEK293T cells. Six hours after stimulation with TNF $\alpha$ , luciferase activity was measured. The luciferase assays showed that TRIM45(FL) significantly suppressed NF- $\kappa$ B-mediated transcriptional activity (Fig. 3A). TRIM45( $\Delta$ R) also repressed NF- $\kappa$ B 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- $\kappa$ B-mediated transcription (Fig. 3A). Next, we generated HeLa cells in which FLAG-tagged TRIM45(FL) or TRIM45( $\Delta$ R) 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( $\Delta$ R) inhibited NF- $\kappa$ B activity compared with that of mock, suggesting that TRIM45 suppresses activities of NF- $\kappa$ B-mediated transcription in HeLa cells (Fig. 3C). Interestingly, luciferase assays showed that TRIM45 had a tendency to suppress activities of NF- $\kappa$ B-mediated

transcription without TNF $\alpha$  (or with the level of TNF $\alpha$  included in the culture medium) (Fig. 3C).

#### *3.4. TRIM45 suppresses cell proliferation via downregulation of NF- $\kappa$ B-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- $\kappa$ B-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- $\kappa$ B-mediated transcriptional activity. 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( $\Delta$ R) 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( $\Delta$ R) was slightly repressed compared with that of mock cells. These findings indicate that TRIM45 may affect cell proliferation via downregulation of NF- $\kappa$ B-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- $\kappa$ B 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( $\Delta$ R) (Fig. 3B and C).

NF- $\kappa$ B is one of the transcriptional factors that plays essential roles in immune response, tumorigenesis and cell proliferation. It is well known that ubiquitin-mediated

I $\kappa$ B protein degradation activates canonical and noncanonical NF- $\kappa$ B pathways. Therefore, the role of ubiquitin as an important signaling tag is characteristically illustrated in the NF- $\kappa$ B 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 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- $\kappa$ B transcription and suppresses cancer cell proliferation. Recently, some studies have shown that crosstalk between NF- $\kappa$ B 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- $\kappa$ B 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- $\kappa$ B and/or MAPK signaling pathways.

In conclusion, TRIM45 is likely to be a novel regulator affecting cell growth via TNF $\alpha$ -induced NF- $\kappa$ B-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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## Figure legends

**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 ( $\Delta$ R). Expression vectors encoding FLAG-tagged human TRIM45(FL) and TRIM45( $\Delta$ R) were transfected into HEK293T cells. Lysates were immunoblotted with anti-FLAG antibody. Anti- $\beta$ -actin antibody was used as an internal control.

**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( $\Delta$ R) 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  $\mu$ m.

**Fig. 3.** TRIM45 negatively regulates TNF $\alpha$ -induced NF- $\kappa$ B-mediated transcriptional activity. (A) TRIM45 suppressed TNF $\alpha$ -induced NF- $\kappa$ B activity. HEK293T cells were transfected with an NF- $\kappa$ B luciferase reporter plasmid and an expression plasmid encoding TRIM45(FL). Twenty-four h after transfection, cells were treated with TNF $\alpha$  (20 ng/ml) and cultured for an additional 6 h. Data are means  $\pm$  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( $\Delta$ R). HeLa cells were infected with a retrovirus encoding FLAG-tagged TRIM45 (FL), FLAG-tagged TRIM45 ( $\Delta$ R) 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 TNF $\alpha$ -induced NF- $\kappa$ B activity in HeLa cells. HeLa cell lines stably expressing FLAG-tagged TRIM45 (FL), FLAG-tagged TRIM45 ( $\Delta$ R) and the corresponding empty vector (mock) were transfected with an NF- $\kappa$ B luciferase reporter plasmid. Twenty-four h after transfection, cells were treated with TNF $\alpha$  (20 ng/ml) and cultured for an additional 6 h. Data are means  $\pm$  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- $\beta$ -actin antibodies. Anti- $\beta$ -actin antibody was used as an internal control. (B) TRIM45 affects cell proliferation. NIH3T3 cell lines expressing FLAG-tagged TRIM45 and mock were seeded ( $1 \times 10^5$  cells) in 60-mm dishes and harvested for counting cell numbers at the indicated times. Data are means  $\pm$  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( $\Delta$ R) or mock were seeded at  $1 \times 10^5$  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 means  $\pm$  s.d. of values from three independent experiments.

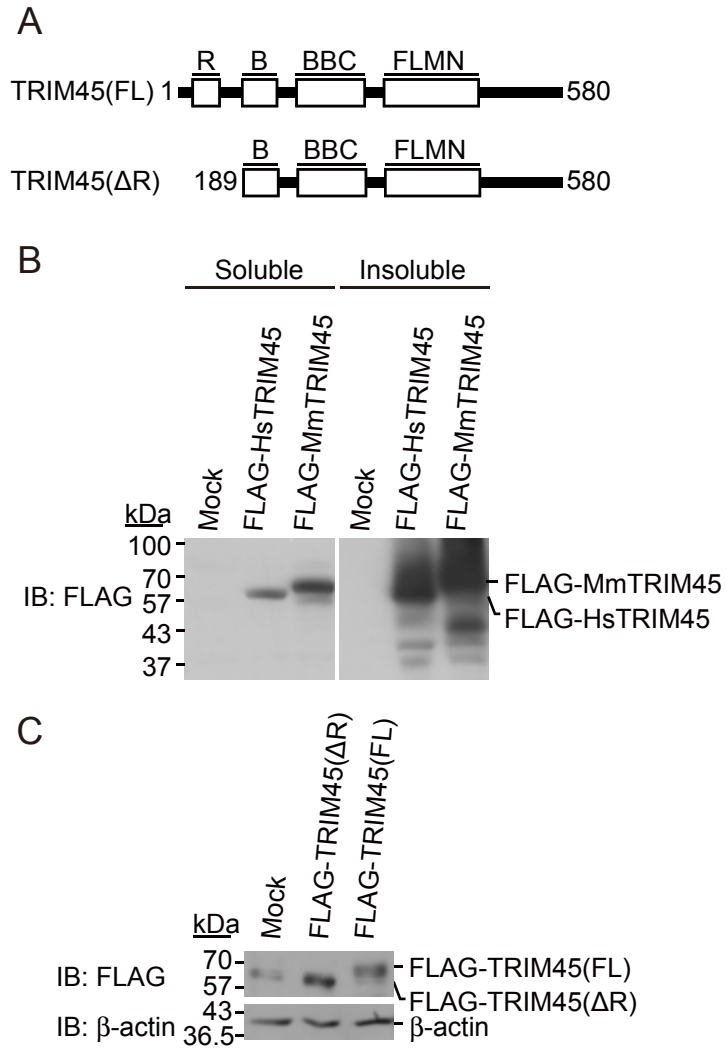


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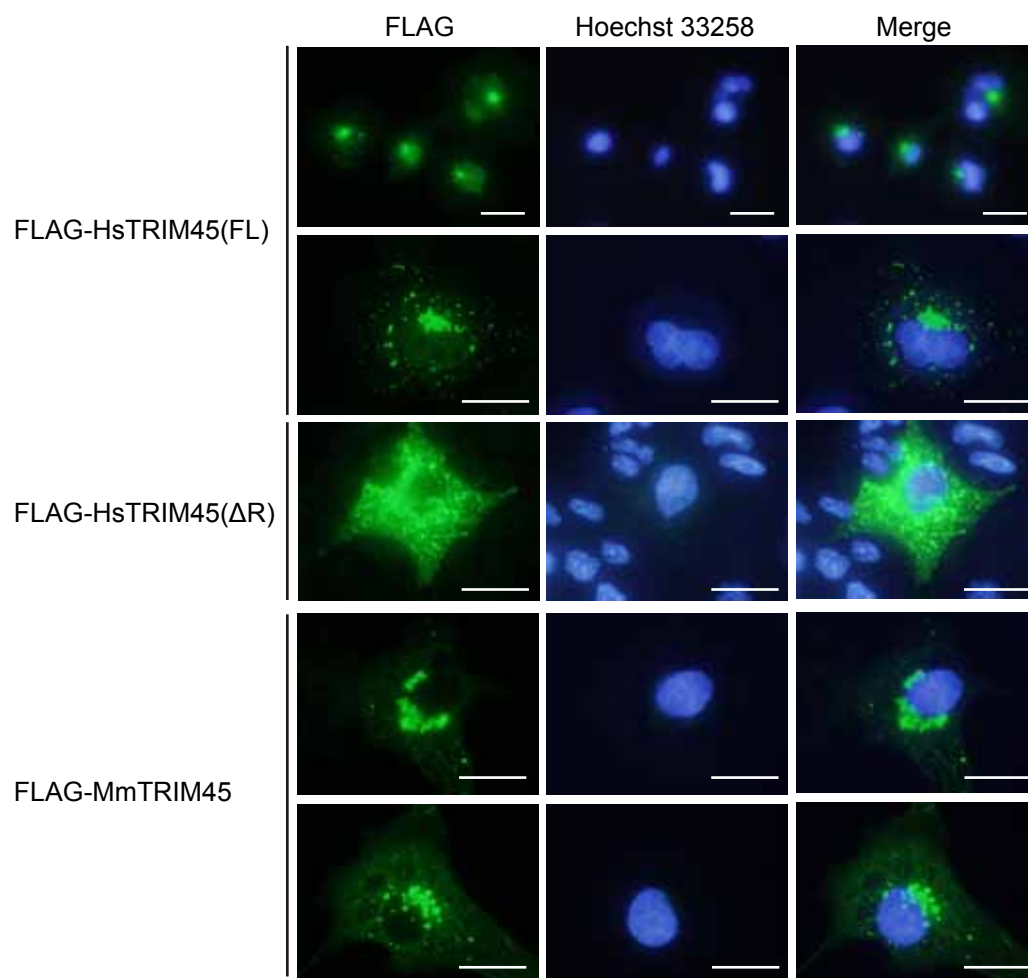


Figure 2 Shibata et al.

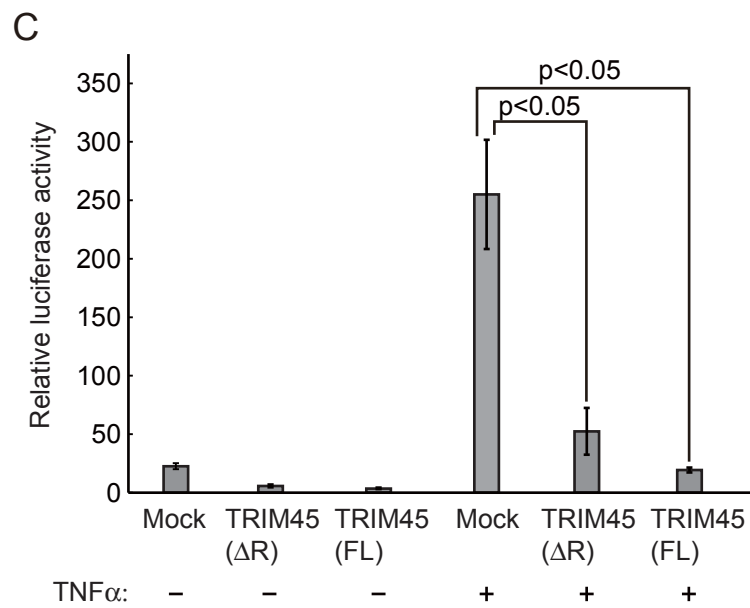
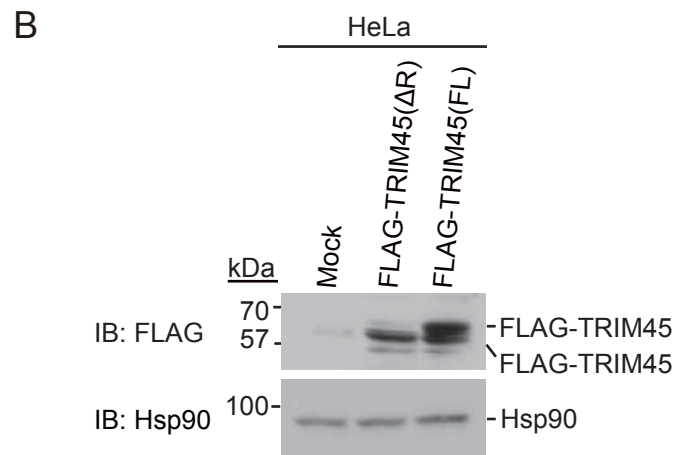
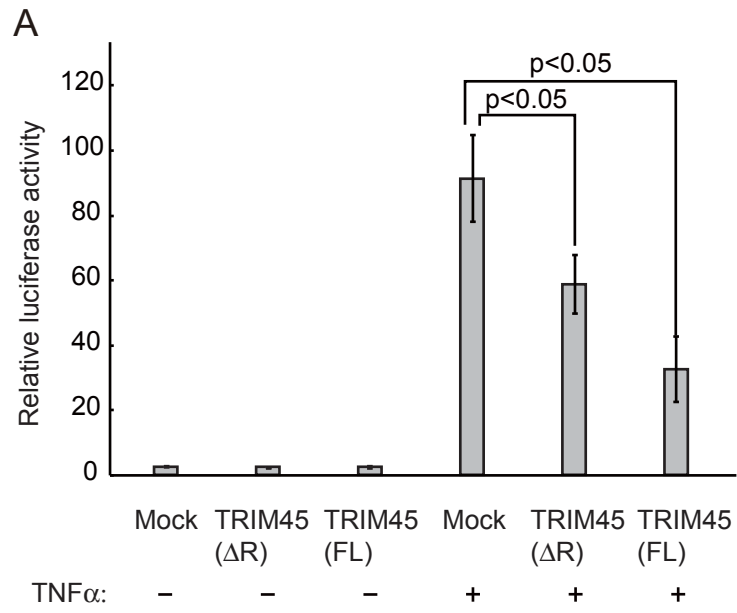


Figure 3 Shibata et al.

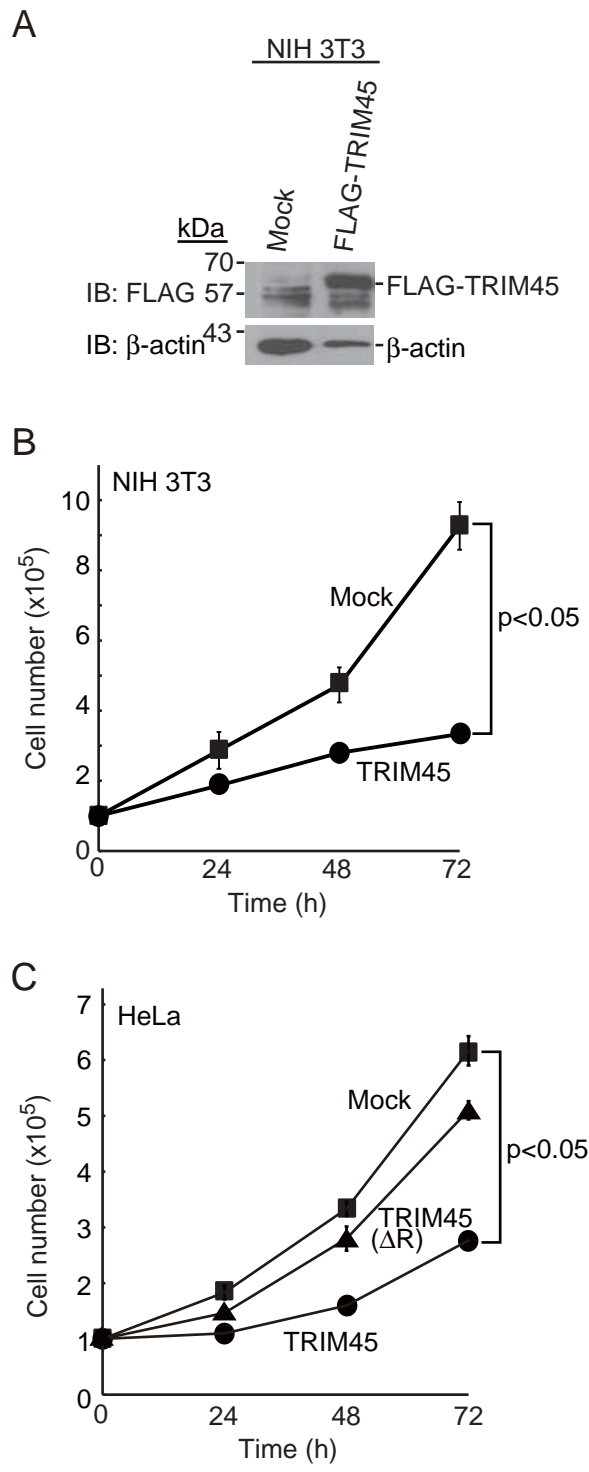


Figure 4 Shibata et al.