TRIM40 promotes neddylation of IKKγ and is downregulated in gastrointestinal cancers

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Running Title: TRIM40 promotes neddylation of IKKγ

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Abbreviations: TRIM, Tripartite motif; Nedd8, neural precursor cell-expressed developmentary down-regulated gene 8; NF-κB, nuclear factor-kappa B; IKK, IκB kinase.

Key words: TRIM40, Nedd8, IKKγ, NF-κB, gastrointestinal cancer

Abstract

Gastrointestinal neoplasia seems to be a common consequence of chronic inflammation in the gastrointestinal epithelium. NF-κB is an important transcription factor for cancinogenesis in chronic inflammatory diseases and plays a key role in promoting inflammation-associated carcinoma in the gastrointestinal tract. Activation of NF-κB is regulated by several posttranslational modifications including phosphorylation, ubiquitination and neddylation. In this study, we showed that TRIM40 is highly expressed in the gastrointestinal tract and that TRIM40 physically binds to Nedd8, which is conjugated to target proteins by neddylation. We also found that TRIM40 promotes the neddylation of IKKγ, which is a crucial regulator for NF-κB activation, and consequently causes inhibition of NF-κB activity, whereas a dominant-negative mutant of TRIM40 lacking the RING domain does not inhibit NF-κB activity. Knockdown of TRIM40 in the small intestinal epithelial cell line IEC-6 caused NF-κB activation followed by increased cell growth. In addition, we found that TRIM40 is highly expressed in normal gastrointestinal epithelia but that TRIM40 is downregulated in gastrointestinal carcinomas and chronic inflammatory lesions of the gastrointestinal tract. These findings suggest that TRIM40 inhibits NF-κB activity via neddylation of IKKγ and that TRIM40 prevents inflammation-associated carcinogenesis in the gastrointestinal tract.

Introduction

Tripartite motif (TRIM) proteins are characterized by the presence of a RING finger, one or two zinc-binding motifs named B-boxes, and an associated coiled-coil region (1). Most TRIM proteins have been reported to have a role in the ubiquitination process. TRIM25/EFP ubiquitinates 14-3-3σ (2) and retinoic acid–inducible gene I (3). Furthermore, several TRIM family members are involved in various cellular processes, such as transcriptional regulation, cell growth, apoptosis, development, and oncogenesis (4-8).

Neural precursor cell-expressed developmentary down-regulated gene 8 (Nedd8) is a small ubiquitin-like protein with 53% identity to ubiquitin that is conserved from yeast to mammals (9-11). Nedd8 is covalently linked to the ε-amino group of lysine residues on target proteins through its carboxy-terminal group as well as a ubiquitination reaction. Nedd8 is first activated by a heterodimeric E1 enzyme, APPBP1-Uba3, and is then transferred to an E2 enzyme, Ubc12. Members of the cullin family are well-characterized substrates for Nedd8 conjugation (10,12,13). Tumor suppressor protein p53 and its relative p73 are also neddylated via a RING-domain containing protein Mdm2 or F-box domain protein FBXO11, and neddylation of p53 causes inhibition of p53-mediated transcription (14-16). Similarly, c-Cbl mediates EGF receptor modification with Nedd8, which enhances subsequent ubiquitination, followed by degradation (17). A well-characterized component of a Cul2-based ubiquitin E3, pVHL, is also neddylated, and Nedd8 modification of pVHL regulates VHL-associated tumorigenesis. (18). Several ribosomal proteins and breast cancer-associated protein3 (BCA3) are modified and regulated by Nedd8 (19,20). Taken together, although

neddylation does not directly mediate proteasomal degradation of target proteins like ubiquitination, Nedd8 modification likely regulates several cellular functions including transcription, translation and signal transductions via structural change or stabilization of target proteins.

The nuclear factor-κB (NF-κB) signaling pathway plays a key role in many aspects of cancer initiation and progression through transcriptional control of genes involved in growth, angiogenesis, anti-apoptosis, invasiveness, and metastasis (21). Regulation of NF-κB signaling occurs at many levels, one of which is through the regulation of protein turnover by the action of SCF complex ubiquitin ligase. Under a resting condition, NF-κB is maintained in an inactive state by binding to IκB proteins. In canonical NF-κB signaling, IκBα binds to p50-p65 and sequesters transcription factors in the cytoplasm, rendering them inactive. On stimulation of the IKK complex, IκBα is phosphorylated at Serine 32 and Serine 36, resulting in its polyubiquitination by a ubiquitin ligase complex, SCF^{Fbw1} (Skp1-Cul1-F-box complex containing Fbw1), and degradation, thus resulting in nuclear accumulation of the complex and transcription of NF-κB target genes (22-25).

Chronic inflammation in the gastrointestinal tract has been closely associated with carcinogenesis (26). The most extensively studied examples are relationships between chronic gastritis resulting from *Helicobacter pylori* infection and gastric cancer, chronic hepatitis and liver cancer, and chronic inflammatory bowel disease and colorectal cancer. Emerging evidence in the past decade has suggested the NF-κB play a critical role in linking inflammation and cancer. NF-κB regulates major inflammatory factors including TNFα, IL-6, IL-1, IL-8, many of which are also potent activators for NF-κB. It is thus likely that NF-κB and inflammation constitute a positive feedback loop in the

milieu of inflammatory sites to induce cellular and DNA damage, promote cell proliferation and transformation, and eventually cause initiation, promotion and progression of cancer (27-30).

In this study, we showed that the TRIM family protein TRIM40 is highly expressed in the gastrointestinal tract including the stomach, small intestine and large intestine. With the aim of elucidating the molecular function of TRIM40 in the gastrointestinal tract, we identified Nedd8 as a novel TRIM40-binding protein by using yeast two-hybrid screening. TRIM40 enhanced neddylation of IKKγ and inhibited the activity of NF-κB-mediated transcription. We also found that knockdown of TRIM40 causes NF-κB activation and increases cell growth. These results provide evidence for a protective role of TRIM40 in inflammation and carcinogenesis in the gastrointestinal tract.

Materials and methods

Cell culture

HEK293T, HeLa and SW480 cell lines were cultured under an atmosphere of 5% CO_2 at 37°C in DMEM (Sigma Chemical Co, St Louis, MO) supplemented with 10% fetal bovine serum (Invitrogen, Carlsbad, CA). IEC-6 cell line was cultured under the same conditions in DMEM supplemented with 10% fetal bovine serum and 0.1 unit/ml bovine insulin (Sigma).

Cloning of cDNAs and plasmid construction

Human *TRIM40* cDNAs were amplified from human stomach cDNA by PCR with KOD-Plus- (Toyobo, Osaka, Japan) using the following primers: 5′-atgatccctttgcagaaggac-3′ (Hs-TRIM40-sense) and 5′-tcagagcttctgagggggctg-3′ (Hs-TRIM40-antisense). Mouse *TRIM40* cDNA was amplified from mouse small intestine cDNA by PCR with KOD-Plus- (Toyobo) using the following primers: 5′-accatgggctctcttgacaaggac-3′ (Mm-TRIM40-sense) and 5′-agactaactgagctttggaccagc-3′ (Mm-TRIM40-antisense). The cDNA fragment lacking a sequence corresponding to amino acids 1-54 was utilized as TRIM40(ΔRING). The amplified fragments were subcloned into pBluescript II SK+ (Stratagene, La Jolla, CA). *TRIM40* cDNAs were subcloned into pCR-FLAG, pCGN-HA, pcDNA3-Myc (Invitrogen), pET30a (Merck, Frankfurter Str, Germany), pGEX-6P1 (GE Healthcare, Piscataway, NJ) and pBTM116 and pACT2 (Clontech, Mountain View, CA). Deletion mutants of mouse TRIM40 cDNA containing amino acids 55-247 were amplified by

PCR and subcloned. Expression vectors encoding IKKα, IKKβ, IKKγ and Nedd8 cDNA were previously described (25).

Yeast two-hybrid screening

Complementary DNA encoding the full-length of mouse *TRIM40* was fused in-frame to the nucleotide sequence for the LexA domain (BD, Franklin Lakes, NJ) in the yeast two-hybrid vector pBTM116. To screen for proteins that interact with TRIM40, we transfected yeast strain L40 (Invitrogen) stably expressing the corresponding pBTM116 vector with a mouse NIH3T3 cDNA library (Clontech).

Antibodies and reagents

The antibodies used in this study were as follows: mouse monoclonal anti-HA (HA.11/16B12, Covance, Princeton, NJ), rabbit polyclonal anti-HA (Y11, Santa Cruz, Santa Cruz, CA), mouse monoclonal anti-FLAG (M2 or M5, Sigma), mouse monoclonal anti-c-Myc (9E10, Covance), mouse monoclonal anti-human IκBα (610690, BD), rabbit polyclonal anti-Nedd8 (PM023, BML, Tokyo, Japan), mouse monoclonal anti-IKKγ (K0159-3, BML), mouse monoclonal anti-Hsp70 (610608, BD), mouse monoclonal anti-p65 (610868, BD), mouse monoclonal anti-GAPDH (6C5, Ambion, TX), mouse monoclonal anti-lamin A/C (612162, BD) and mouse monoclonal anti-β-actin (AC15, Sigma). Tumor necrosis factor α (TNFα), interleukin-1β (IL-1β) and cycloheximide were purchased from Sigma.

Transfection, immunoprecipitation, 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% Triton X-100, 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 15,000 × g for 10 min at 4°C, and the resulting supernatant was incubated with antibodies for 2 h at 4°C. Protein A-sepharose (GE Healthcare) that had equilibrated with the same solution was added to the mixture, which was then rotated for 1 h at 4°C. The resin was separated by centrifugation, washed five times with ice-cold lysis buffer, and then boiled in SDS sample buffer. Immunoblot analysis was performed with primary antibodies, horseradish peroxidase-conjugated antibodies to mouse or rabbit IgG (1:10,000 dilution; Promega, Madison, WI), and an enhanced chemiluminescence system (GE Healthcare). Subcellular fractionation was performed as reported previously (31).

Dual-luciferase assay

HEK293T, HeLa and IEC-6 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 and pRL-TK Renilla luciferase plasmid (Promega) were transfected into HEK293T, HeLa and IEC-6 cells using the Fugene HD reagent (Roche, Basel, Switzerland). Twenty-four h 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).

Immunofluorescence staining

HeLa cells expressing HA-tagged TRIM40 on a glass cover were fixed by phosphate buffer saline (PBS) containing 4% formaldehyde and 0.1% Triton X-100 for 10 min at room temperature, followed by incubation with PBS containing anti-HA antibody (Y-11, 1 μg/ml), anti-FLAG antibody (M5, 1 μg/ml) and anti-p65 antibody (BD, 1 μg/ml) with 0.1% BSA and 0.1% saponin for 1 h at room temperature. Cells were washed three times with PBS, followed by incubation with PBS containing Alexa546-labeled goat anti-mouse IgG antibody, Alexa488-labeled goat anti-rabbit IgG antibody, Alexa546-labeled goat anti-rabbit IgG antibody or Alexa488-labeled goat anti-mouse IgG antibody (Invitrogen) with 0.1% BSA and 0.1% saponin for 1 h at room temperature. The cells were further incubated with Hoechst 33258 (1 μg/ml) in PBS for 10 min, followed by extensive washing with PBS, and then photographed with a CCD camera (DP71, Olympus) attached to an Olympus BX51 microscope.

Recombinant proteins

GST-tagged TRIM40 was expressed in XL-10 cells and then purified by reduced glutathione–sepharose beads (Roche). His_6/FLAG-tagged TRIM40 was expressed in *Escherichia coli* strain BL21 (DE3; Invitrogen) and then purified by using ProBond metal affinity beads (Invitrogen).

Retroviral expression system

Wild-type FLAG-TRIM40 or FLAG-TRIM40(ΔRING) cDNA was subcloned into pMX-puro. Retroviral expression vectors were kindly provided by Dr. Kitamura (University of Tokyo). For retrovirus-mediated gene expression, HeLa cells were

infected with retroviruses produced by Plat-A packaging cells. Cells were then cultured in the presence of puromycin (5 μg/ml).

RNA interference

pSUPER-retro-puro containing a nonfunctional random sequence (5'-cagtcgcgtttgcgactgg-3') or the nucleotides 570 to 588 of rat *TRIM40* cDNA (5'-cttctctgaggcagtaaca-3') was constructed according to the protocol of the manufacturer (OligoEngine, Seattle, WA). For retrovirus-mediated gene expression, IEC-6 cells were infected with retroviruses produced by Plat-E packaging cells. Cells were then cultured in the presence of puromycin (2 μg/ml).

Quantitative PCR analysis

Total RNA was isolated from human samples with the use of an ISOGEN (Nippon Gene, Tokyo, Japan), followed by reverse transcription (RT) by ReverTra Ace (Toyobo, Osaka, Japan). The resulting cDNA was subjected to real-time PCR with a StepOne machine and Power SYBR Green PCR master mix (Applied Biosystems, Foster City, CA). The level of gene expression relative to *GAPDH* was determined. The primer sequences for human *GAPDH* (GenBankTM accession number NM_002046.3) and human *TRIM40* (GenBankTM accession number NM_138700.3) were as follows: human *GAPDH*, 5′-gcaaattccatggcaccgt-3′ and 5′-tcgccccacttgattttgg-3′; and human *TRIM40*, 5′-caacacactgaagaatgctgg-3′ and 5′-cttctgaggggctgaagaag-3′. The primer sequences for rat *GAPDH* (GenBankTM accession number NM_017008.3) and rat *TRIM40* (GenBankTM accession number NM_001009175.1) were as follows: rat

GAPDH, 5′-cacggcaagttcaacggcacagtca-3′ and 5′-gtgaagacgccagtagactccacgac-3′; and rat *TRIM40*, 5′-caccgggcccatactgagctc-3′ and 5′-ttcctgagccttcagccgttg-3′.

Human tissue samples

Tissues from patients who gave informed consent under the guidelines of the Hokkaido University Hospital Ethics Committee were used for this study. Excised samples from lesions and adjacent normal tissues were obtained within 3 h after the operation. All excised tissues were immediately placed in liquid nitrogen and stored at -80ºC until further analysis.

Statistical analysis

The unpaired Student's *t* test and Wilcoxon matched pairs test were used to determine the statistical significance of experimental data.

Results

TRIM40 expression in normal mouse tissues and mammalian cell lines

We and others have shown that some of the TRIM family members are selectively expressed in specific tissues and cell lines (1,6,7). Although several TRIM family members are known to be involved in various cellular processes, the functions of almost all TRIM family proteins have not been elucidated. In this study, we found human and mouse TRIM40 as a new member of the TRIM family and we analyzed the biological function of TRIM40. To examine the expression levels of TRIM40 in several normal tissues at the protein level, we generated a rabbit polyclonal anti-TRIM40 antibody using recombinant mouse TRIM40 protein and performed immunoblot analysis using several mouse normal tissues. Immunoblot analysis showed that TRIM40 is highly expressed in the gastrointestinal tract, heart and cerebellum and faintly in the testis (Figure 1A). To further analyze the expression of TRIM40 in detail, we prepared several gastrointestinal tissues including the esophagus, stomach, small intestine and large intestine. Immunoblot analysis using cell extracts from these tissues showed that TRIM40 is expressed highly in the small intestine, moderately in the stomach, and faintly in the esophagus and large intestine (Figure 1A). Furthermore, immunoblot analysis using several cell lines showed that TRIM40 is highly expressed in the rat small intestinal epithelial cell line IEC-6, the mouse colon adenocarcinoma cell line Colon26 and the human adenoid cystic carcinoma cell line ACC3, which are developmentally derived from parotid glands or gastrointestinal tissues (Figure 1B). These findings suggest that TRIM40 is more highly expressed in normal intestinal cells than in intestinal carcinoma cells.

TRIM40 interacts with Nedd8

To examine the molecular function of TRIM40, we isolated TRIM40-interacting proteins from an NIH 3T3 cDNA library by using a yeast two-hybrid system. We obtained 16 positive clones from 1.2 x 10^6 transformants. Eight of the positive clones had sequence identities with cDNA encoding mouse Nedd8 (NCBI Reference Sequence: NM_008683.3). To examine whether TRIM40 physically interacts with Nedd8 in mammalian cells, we performed an *in vivo* binding assay using cells transfected with expression vectors. We expressed FLAG-tagged TRIM40 together with Myc-tagged Nedd8 in HEK293T cells. Cell lysates were subjected to immunoprecipitation with an antibody to FLAG, and the resulting precipitates were subjected to immunoblot analysis with an antibody to Myc. Myc-tagged Nedd8 was co-precipitated by the antibody to FLAG, indicating that TRIM40 non-covalently interacts specifically with Nedd8 and also is covalently conjugated with Nedd8 (neddylation) (Figure 2A). We also confirmed interaction between FLAG-tagged TRIM40 lacking a RING domain (TRIM40(ΔRING)) and Myc-tagged Nedd8 by immunoprecipitation, suggesting that TRIM40(ΔRING) can be used as a null-functional mutant or a dominant negative mutant (Figure 2A). Furthermore, immunoblot analysis using anti-Nedd8 antibody showed that overexpressed FLAG-tagged TRIM40 is covalently conjugated with Nedd8, but we could not show that overexpressed FLAG-tagged TRIM40 non-covalently interacts with Nedd8 (Figure 2A and B). These findings suggest that TRIM40 is covalently conjugated with Nedd8 and that TRIM40 weakly interacts with monomeric Nedd8.

TRIM40 inhibits NF-κB activity

It has been reported that Nedd8 covalently binds to and activates Cul1, which is a component of an SCF complex, and that the SCF complex degrades IκBα phosphorylated by stimulation of TNFα, followed the activation of NF-κB-mediated transcription (12,13). To examine whether TRIM40 affects NF-κB activity, we performed an NF-κB response element luciferase reporter assay. We transfected expression vectors encoding TRIM40 with reporter plasmids into HEK293T cells. Six h after stimulation with TNFα, luciferase activity was measured. The luciferase assays showed that TRIM40 suppressed NF-κB-mediated transcriptional activity in a dose-dependent fashion (Figure 2C). Furthermore, stable HeLa cell lines expressing FLAG-tagged TRIM40(WT) and FLAG-TRIM40(ΔRING) were generated by a retroviral expression system and luciferase assays were performed using these cell lines. Luciferase assays showed that overexpression of TRIM40 inhibits NF-κB activity compared with that of Mock, whereas overexpression of TRIM40(ΔRING) does not affect NF-κB activity, suggesting that a RING domain of TRIM40 is indispensable for inhibition of NF-κB activity (Figure 2D and E). In addition, we showed that TRIM40 also suppressed TNFα-induced NF-κB activity using the human colorectal adenocarcinoma cell line SW480 (Figure 2F). Since a canonical NF-κB pathway can be activated by many stimulations including stimulations with TNFα, IL-1β and LPS, a luciferase reporter assay using IL-1β was performed. The luciferase reporter assay showed that TRIM40 suppressed IL-1β-induced NF-κB activity (Figure 2G). These findings suggest that TRIM40 downregulates NF-κB-mediated transcriptional activity.

TRIM40 inhibits nuclear translocation of NF-κB

It has been reported that the p65 subunit of NF-κB is translocated from the cytoplasm to the nucleus upon stimulation with TNFα (32). To examine whether TRIM40 affects nuclear translocation of p65, immunofluorescent analysis was performed using an anti-p65 antibody. An expression vector encoding HA-tagged TRIM40 was transfected into HeLa cells, and the cells were stimulated with TNFα for 20 or 30 min and then stained with anti-p65 antibody. It was found that p65 was concentrated in the nucleus of mock cells by treatment with TNFα, whereas p65 was weakly concentrated in the nucleus of cells in which TRIM40 was highly expressed (Figure 3A and B). Immunofluorescent staining showed that overexpression of TRIM40 inhibits nuclear localization of endogenous p65 in HeLa cells by stimulation with TNFα, suggesting that TRIM40 inhibits a canonical NF-κB pathway (Figure 3A and B). The effect of TRIM40 on nuclear localization of endogenous p65 by TNFα was further confirmed by biochemical subcellular fractionation of HeLa cells. Immunoblotting with anti-p65 antibody was performed using each subcellular fraction from cells stimulated with TNFα. Endogenous p65 was mainly localized in the nucleus in mock cells by stimulation with TNFα, whereas half of the endogenous p65 remained in the cytosol in TRIM40-expressing cells, suggesting that TRIM40 inhibits nuclear translocation of p65 induced by stimulation with TNFα (Figure 3C and D).

TRIM40 stabilizes IκBα and interacts with IKK complex

Since we found that TRIM40 inhibits nuclear translocation of NF-κB and NF-κB-mediated transcriptional activity, we hypothesized that overexpression of TRIM40 would inhibit degradation of IκBα. To examine whether overexpression of

TRIM40 affects the stability of IκBα, an expression vector encoding TRIM40 was transfected. Immunoblot analysis clarified that overexpression of TRIM40 increases the stability of IκBα. To further confirm that TRIM40 affects the stability of IκBα, a protein stability assay using cycloheximide was performed. HeLa cell lines stably expressing FLAG-tagged TRIM40 were stimulated with TNFα and incubated with cycloheximide for 0 to 60 min. The protein stability assay showed that stimulation with TNFα did not completely degrade IκBα even after 20 minutes of stimulation, suggesting that TRIM40 suppressed TNF-induced IκBα degradation (Figure 4B and C).

Next, we tested whether TRIM40 interacts with IKKα, IKKβ and IKKγ as upstream regulators for IκBα. We transfected expression vectors encoding HA-tagged TRIM40 or HA-tagged TRIM40(ΔRING) and FLAG-tagged IKKα, FLAG-tagged IKKβ or FLAG-tagged IKKγ into HEK293T cells. Cell lysates were subjected to immunoprecipitation with an antibody to HA or FLAG, and the resulting precipitates were subjected to immunoblot analysis with an antibody to FLAG or HA, respectively. Immunoblot analysis showed that HA-tagged TRIM40 was selectively coprecipitated by anti-FLAG antibody and that FLAG-tagged IKKα, FLAG-tagged IKKβ and FLAG-tagged IKKγ were also coprecipitated by anti-HA antibody (Figure 4D, E and F). We also verified interaction between HA-tagged TRIM40(ΔRING) and FLAG-tagged IKKα, FLAG-tagged IKKβ or FLAG-tagged IKKγ by immunoprecipitation (Figure 4D, E and F). TRIM40(ΔR) interacts with IKK subunits much more strongly than TRIM40(WT) does (Figure 4D, E and F), suggesting that the RING domain may inhibit the interaction between TRIM40 and IKK subunits. These findings suggest that TRIM40 is contained in the IKK complex and inhibits the degradation of IκBα in a resting state.

TRIM40 is modified by Nedd8 and promotes neddylation of IKKγ

It has been reported that IKKγ is regulated by several posttranslational modifications including K63-linked or linear polyubiquitination (33,34). Since TRIM40 interacts with Nedd8 and IKKγ, we examined whether Nedd8 affects IKKγ by TRIM40. To examine whether TRIM40 exhibits Nedd8 conjugation on IKKγ, we performed an *in vivo* neddylation assay. Expression vectors encoding FLAG-tagged IKKγ, HA-tagged TRIM40, HA-tagged TRIM40(ΔRING) and Myc-tagged Nedd8 were transfected into HEK293T cells, and cell lysates were subjected to immunoprecipitation with an antibody to Myc and immunoblotted with an antibody to FLAG. Although FLAG-IKKγ was slightly neddylated even without overexpression of TRIM40, overexpression of TRIM40 considerably enhanced neddylation of FLAG-IKKγ (Figure 5A). Next, to elucidate in detail whether TRIM40 neddylates IKKγ, we performed an *in vivo* neddylation assay using several amounts of an expression vector HA-tagged TRIM40. Immunoblot analysis showed that TRIM40 enhances neddylation on IKKγ in a dose-dependent fashion (Figure 5B), suggesting that TRIM40 mediates neddylation on IKKγ and then possibly modulates kinase activity of the IKK complex, followed by stabilization of IκBα. Although immunoblot analysis of IKKα and IKKβ was performed, neddylation of IKKα and IKKβ was not observed (Figure 5C and 5D).

Knockdown of TRIM40 promotes activity of NF-κB and increases cell growth

To evaluate the physiological function of TRIM40, retroviral vectors encoding shRNA specific for rat *TRIM40* (shTRIM40) or non-targeting shRNA as a control (Mock) were infected into IEC-6 cells in which TRIM40 is highly expressed. After puromycin

selection, a stable IEC-6 cell line in which TRIM40 is knocked down was established, and TRIM40 expression at protein and mRNA levels was confirmed by immunoblot analysis and quantitative real-time PCR, respectively (Figure 6A and B). Using these cell lines, we performed an NF-κB response element luciferase reporter assay. Six h after stimulation with TNFα, luciferase activity was measured. The luciferase assays showed that knock-down of TRIM40 enhances NF-κB-mediated transcriptional activity (Figure 6C). Interestingly, knock-down of TRIM40 caused activation of NF-κB-mediated transcription even without TNFα stimulation. In addition, knock-down of TRIM40 increased cell growth (Figure 6D). These findings suggest that TRIM40 downregulates NF-κB-mediated transcriptional activity and that TRIM40 is an important regulator to prevent NF-κB activation in a resting state.

Since it has been reported that NF-κB activity is upregulated in gastrointestinal carcinomas, we investigated the expression of *TRIM40* mRNA in human cancers and inflammation (29,30). Quantitative real-time PCR was performed and mRNA expression levels of *TRIM40* were compared in gastrointestinal cancers (including Crohn's disease) and normal tissues. Expression levels of *TRIM40* mRNA were lower in samples of gastric cancer (13/13), colon cancer (3/3), rectal cancer (3/4), benign colon tumor (0/1) and Crohn's disease (1/1) than in normal epithelia. Quantitative real-time PCR showed that *TRIM40* mRNA is highly expressed in human normal gastrointestinal tissues and significantly downregulated in gastrointestinal carcinomas and inflammation (Figure 6E and F).

To verify Nedd8-conjugation of IKKγ in human gastrointestinal tissues, immunoprecipitation and immunoblot analysis were performed using normal gastric epithelium and gastric cancer tissue from the same patient. Cell lysates were subjected

to immunoprecipitation with an antibody to anti-IKKγ or anti-Nedd8, and the resulting precipitates were subjected to immunoblot analysis with an antibody to anti-Nedd8 or anti-IKKγ, respectively. Neddylation of IKKγ was selectively detected in normal gastric epithelium (Figure 6F). These findings suggest that endogenous TRIM40 is highly expressed and promotes neddylation of IKKγ, resulting in stabilization of IκBα.

Discussion

In this study, we found that TRIM40 is highly expressed in gastrointestinal tissues and that TRIM40 interacts with the ubiquitin-like protein Nedd8. We focused on the relationship between TRIM40 and Nedd8 because Nedd8 conjugation regulates activity of NF-κB through the SCF complex (12). Although it has been reported that a subunit of SCF complex, Cul1, is positively regulated via Nedd8 conjugation, we did not find interaction of TRIM40 with the SCF complex (data not shown). We further investigated other steps for NF-κB activation and found that TRIM40 interacts with the IKK complex. We showed that overexpression of TRIM40 promotes neddylation of IKKγ and that TRIM40 causes stabilization of IκBα and attenuates NF-κB activity, whereas a mutant of TRIM40 lacking the RING-finger domain does not affect NF-κB activity. Furthermore, knockdown of TRIM40 promoted NF-κB activity and cell growth. Taken together, these results suggest that TRIM40 is a novel negative regulator against inflammation and carcinogenesis in the gastrointestinal tract.

It has been reported that the E3 ubiquitin ligase MDM2 promotes neddylation of p53 and negatively regulates its transcriptional activity (14) and that an F-box protein, FBXO11, which is a component of the SCF type E3 ligase, promotes neddylation of p53 and inhibits its transcriptional activity (16). Therefore, we hypothesized that TRIM40 promotes neddylation of IKKγ via a RING domain and regulates the activity of IKK complex, in which IKKγ is an essential component for activation of the canonical NF-κB pathway. Non-proteolytic Lysine 63 (K63)-linked polyubiquitination has as an important role in IKK activation in the canonical NF-κB pathway (35). IKKγ specifically recognizes K63-linked polyubiquitin chains and is conjugated by

K63-linked polyubiquitin chains, which induces activation of the IKK complex and promotes the NF-κB cascade (33,36,37). In addition, IKKγ is conjugated by amino-terminal-linked linear polyubiquitin chains and linear polyubiquitin of IKKγ is necessary for an NF-κB pathway (34). In this study, we showed that overexpression of TRIM40 results in neddylation of IKKγ and inhibition of NF-κB activity and that knockdown of TRIM40 accelerates NF-κB activity and cell growth. Taken together, these findings indicate that non-proteolytic polyubiquitin chains by K63-linked and linear types on IKKγ positively regulate the IKK complex, whereas Nedd8-conjugation of IKKγ likely functions as a negative regulator for NF-κB activity.

Intestinal epithelial cells provide a primary physical barrier against commensal and pathogenic microorganisms in the gastrointestinal tract, but the influence of intestinal epithelial cells on the development and regulation of immunity to infection is unknown (38). Many kinds of enterobacteria exist in the gastrointestinal tract. Despite the fact that enterobacteria are non-self antigens, the intestinal tract has no immune response for enterobacteria. The normal intestinal tract seems to have an immune suppression system for enterobacteria. In particular, chronic inflammation by pathogenic bacteria such as *Helicobacter pylori* or inflammatory bowel diseases including Crohn's disease and ulcerative colitis are closely associated with cancer (26). Evidence that has accumulated in the past decade has suggested that NF-κB plays a critical role in linking inflammation and cancer (27-30). Tissue reconstruction by chronic inflammation may induce malignant transformation of the gastrointestinal epithelium. Therefore, appropriate regulation of immune responses in the gastrointestinal tract, in which various bacteria cause inflammation, may be required for preventing carcinogenesis. We showed that TRIM40 is highly expressed in normal gastrointestinal epithelia compared with the

expression level in inflammatory gastrointestinal tracts and cancer lesions. TRIM40 may downregulate production of inflammatory cytokines including TNFα, IL-6, IL-1, IL-8 via inhibition of NF-κB and prevent carcinogenesis through inflammation by enteric bacteria. Hence, TRIM40 may functions as an important regulator for maintaining homeostasis of the gastrointestinal tract.

Dysregulation of NF-κB is involved in the etiology of cancer and leukemia. Recently, NF-κB has attracted attention as a target of drugs for cancer and immune regulation. The proteasome inhibitor Bortezomib, one function of which is NF-κB inhibition through reduced IκB degradation, leading to reduced NF-κB-dependent synthesis of anti-apoptotic factors, has been evaluated in a number of published and ongoing trials for solid and hematological malignancies (39). Moreover, it has been reported that the IKKβ inhibitor MLN120B inhibits TNFα-induced NF-κB activation, resulting in inhibition of the growth of multiple myeloma cell lines (40). We showed that a newly developed NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), can be utilized for controlling allograft rejection (41). A recent study has shown that a potent and selective inhibitor of Nedd8-activating enzyme, MLN4924, disrupts cullin-RING ligase-mediated protein turnover, leading to apoptotic death in human tumor cells by dysregulation of S-phase DNA synthesis (42). Furthermore, treatment of diffuse large B-cell lymphoma cells with MLN4924 results in rapid accumulation of phosphorylated IκBα, decrease in nuclear p65 content, reduction of NF-κB transcriptional activity, and G1 arrest, ultimately resulting in apoptosis induction. Therefore, detailed research using MLN4924 may clarify the function of TRIM40 in regulation of the NF-κB pathway. In conclusion, further functional analysis of TRIM40

may provide therapeutic benefits not only for inhibition of the growth of gastrointestinal cancers but also for the prevention of chronic inflammatory bowel diseases.

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Legends to Figures

Fig. 1. Expression levels of TRIM40 in several mouse tissues and cell lines. **(A)** TRIM40 expression in several mouse tissues. The lysates from indicated mouse tissues were subjected to immunoblot (IB) analysis with anti-TRIM40 antibody and anti-Hsp70 antibody as a loading control. **(B)** TRIM40 expression in several cell lines. Immunoblot (IB) analysis with anti-TRIM40 or anti-Hsp70 antibody was performed using cell lysates from human intestine epithelial carcinoma cell lines Caco-2, HCA-7, T84, Lovo, WiDr, DLD-1, SW480, HCT116 and COLO201, rat intestine epithelial cell line IEC-6, mouse colon epithelial carcinoma cell line Colon26, synovial sarcoma cell line SW982, embryonic fibroblast cell line NIH3T3, human pancreatic adenocarcinoma cell line MIApaca 2, human embryonic kidney cell line HEK293T, breast carcinoma cell line MCF7, human hepatocellular carcinoma cell line HepG2 , human lung carcinoma cell line H1299, mouse adenoid cystic carcinoma cell line ACC3 and human cervical carcinoma cell line HeLa. Asterisk, non-specific band.

Fig. 2. TRIM40 interacts with Nedd8 and downregulates NF-κB activity. **(A)** *In vivo* binding assay between TRIM40 and Nedd8. Expression vectors encoding FLAG-tagged TRIM40, FLAG-tagged TRIM40(ΔR) and Myc-tagged Nedd8 were transfected into HEK293T cells. Cell lysates (WCL) were immunoprecipitated with anti-FLAG or anti-Myc antibody and immunoblotted with anti-Myc and anti-FLAG antibodies. **(B)** *In vivo* binding assay between TRIM40 and Nedd8. Expression vectors encoding FLAG-tagged TRIM40 and FLAG-tagged TRIM40(ΔR) were transfected into HEK293T cells. Cell lysates (WCL) were immunoprecipitated with anti-FLAG

antibody and immunoblotted with anti-FLAG and anti-Nedd8 antibodies. **(C)** TRIM40 reduces TNFα-induced NF-κB activity in a dose-dependent manner. HEK293T cells were transfected with the NF-κB luciferase reporter plasmid and an expression plasmid encoding TRIM40 (100 ng or 300 ng). Twenty-four h after transfection, cells were treated with TNFα (20 ng/μl) and cultured for an additional 6 h. Data are means ± s.d. of values from three independent experiments. The *P* values for the indicated comparisons were determined by Student's t test. **(D)** Luciferase assay for NF-κB activity using HeLa cell lines stably expressing FLAG-tagged TRIM40. Stable cell lines were transfected with the NF-κB luciferase reporter plasmid. Twenty-four h after transfection, cells were treated with TNFα (20 ng/μl) and cultured for an additional 6 h. **(E)** Luciferase assay for NF-κB activity using HeLa cell lines stably expressing FLAG-tagged TRIM40(ΔRING). Stable cell lines were transfected with the NF-κB luciferase reporter plasmid. Twenty-four h after transfection, cells were treated with TNFα (20 ng/μl) and cultured for an additional 6 h, and then luciferase activity was measured. R, RING-domain. **(F)** TRIM40 reduces TNFα-induced NF-κB activity in the human colorectal adenocarcinoma cell line SW480. SW480 cells were transfected with the NF-κB luciferase reporter plasmid and an expression plasmid encoding TRIM40 (300 ng). Twenty-four h after transfection, cells were treated with TNFα (20 ng/μl) and cultured for an additional 6 h and then luciferase activity was measured. **(G)** TRIM40 reduces IL-1β-induced NF-κB activity. HEK293T cells were transfected with the NF-κB luciferase reporter plasmid and an expression plasmid encoding TRIM40 (300 ng). Twenty-four h after transfection, cells were treated with IL-1β (10 ng/μl) and cultured for an additional 6 h and then luciferase activity was measured.

Fig. 3. TRIM40 inhibits translocation of p65 from the cytosol to the nucleus. **(A)** Immunofluorescent staining of p65 in TRIM40-overexpressing cells at low magification. HeLa cells were transfected with an expression plasmid encoding HA-tagged TRIM40. Forty-eight h after transfection, the cells were stimulated with TNFα (20 ng/ml) for 20 min and were stained with anti-HA and anti-p65 antibodies, followed by incubation with Alexa488-labeled anti-rabbit IgG antibody and Alexa546-labeled anti-mouse IgG antibody, respectively. Nuclei were visualized using Hoechst 33258. Scale bars, 10 μm. **(B)** Immunofluorescent staining of p65 in TRIM40-overexpressing cells at high magnification. HeLa cells were transfected with an expression plasmid encoding HA-tagged TRIM40. Forty-eight h after transfection, the cells were stimulated with TNFα (20 ng/ml) for 30 min and were stained with anti-HA and anti-p65 antibodies, followed by incubation with Alexa546-labeled anti-rabbit IgG antibody and Alexa488-labeled anti-mouse IgG antibody, respectively. Nuclei were visualized using Hoechst 33258. Scale bars, 10 μm. **(C)** Subcellular fractionation of p65 from TRIM40-overexpressing cells. HeLa cell lines stably expressing FLAG-tagged TRIM40 were stimulated with TNFα (20 ng/ml). Thirty min after stimulation, biochemically fractionated cytosolic and nuclear extracts were subjected to immunoblot analysis with anti-p65, anti-GAPDH and anti-Lamin A/C antibodies. GAPDH and lamin A/C are used as cytosolic and nuclear markers, respectively. **(D)** Quantification of p65 in cytosol or nuclear fractions. The intensities of p65 bands in (C) were quantified using a densitometer.

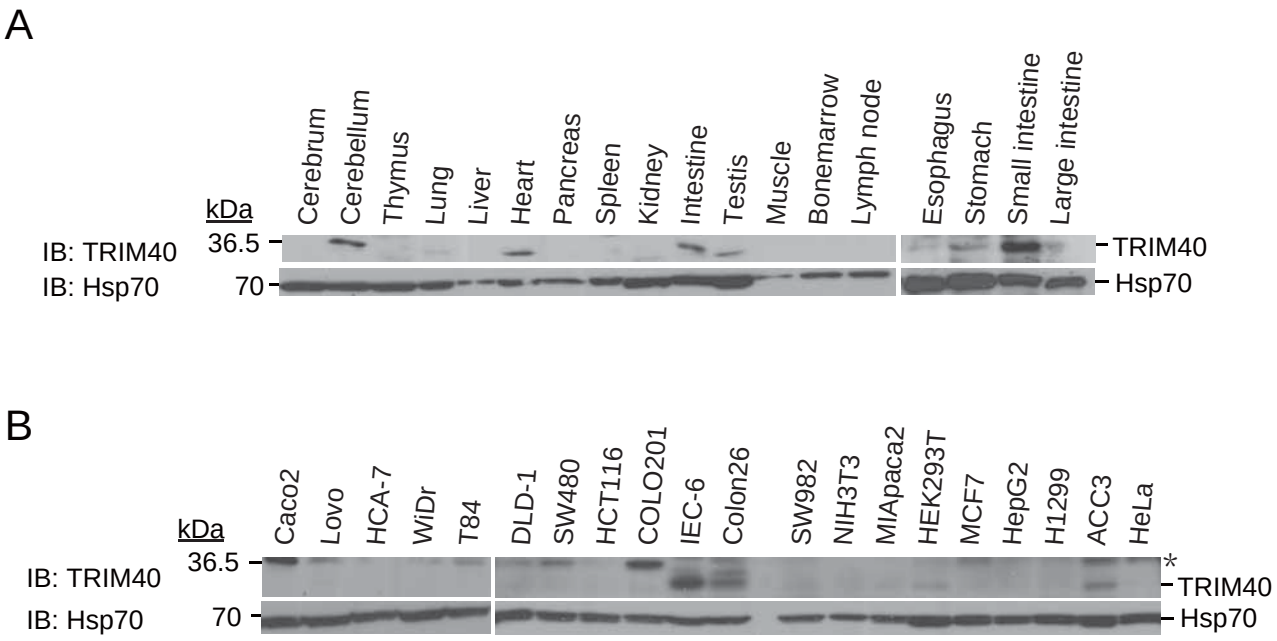
Fig. 4. TRIM40 interacts with IKK complex. **(A)** Upregulation of endogenous IκBα by TRIM40. Immunoblot analysis was performed using HeLa cells stably expressing

FLAG-tagged TRIM40. Cell lysates were subjected to immunoblot (IB) analysis with anti-FLAG, anti-IκBα or anti-Hsp70 antibody. Anti-Hsp70 antibody was used as a loading control. **(B)** TRIM40 affects the stability of IκBα. HeLa cell lines stably expressing FLAG-tagged TRIM40 were stimulated with TNFα (20 ng/ml) and cycloheximide (25 μg/ml) for 0 to 60 min. Cell extracts were analyzed by immunoblotting with anti-IκBα antibody, anti-Hsp70 antibody and anti-FLAG antibody. Anti-Hsp70 antibody was used as a loading control. **(C)** Intensity of the IκBα bands in protein stability analysis in (B) was normalized by that of the corresponding Hsp70 bands and was then expressed as a percentage of the normalized value for time zero. **(D)** Interaction between IKKα and TRIM40. HEK293T cells were transfected with plasmids encoding FLAG-tagged IKKα, HA-tagged TRIM40 and HA-tagged TRIM40(ΔRING), followed by immunoprecipitation (IP) with anti-FLAG antibody or anti-HA antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG or anti-HA antibody. **(E)** Interaction between IKKβ and TRIM40. HEK293T cells were transfected with plasmids encoding FLAG-tagged IKKβ, HA-tagged TRIM40 and HA-tagged TRIM40(ΔRING), followed by immunoprecipitation (IP) with anti-FLAG antibody or anti-HA antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG or anti-HA antibody. **(F)** Interaction between IKKγ and TRIM40. HEK293T cells were transfected with plasmids encoding FLAG-tagged IKKγ, HA-tagged TRIM40 and HA-tagged TRIM40(ΔRING), followed by immunoprecipitation (IP) with anti-FLAG antibody or anti-HA antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG or anti-HA antibody.

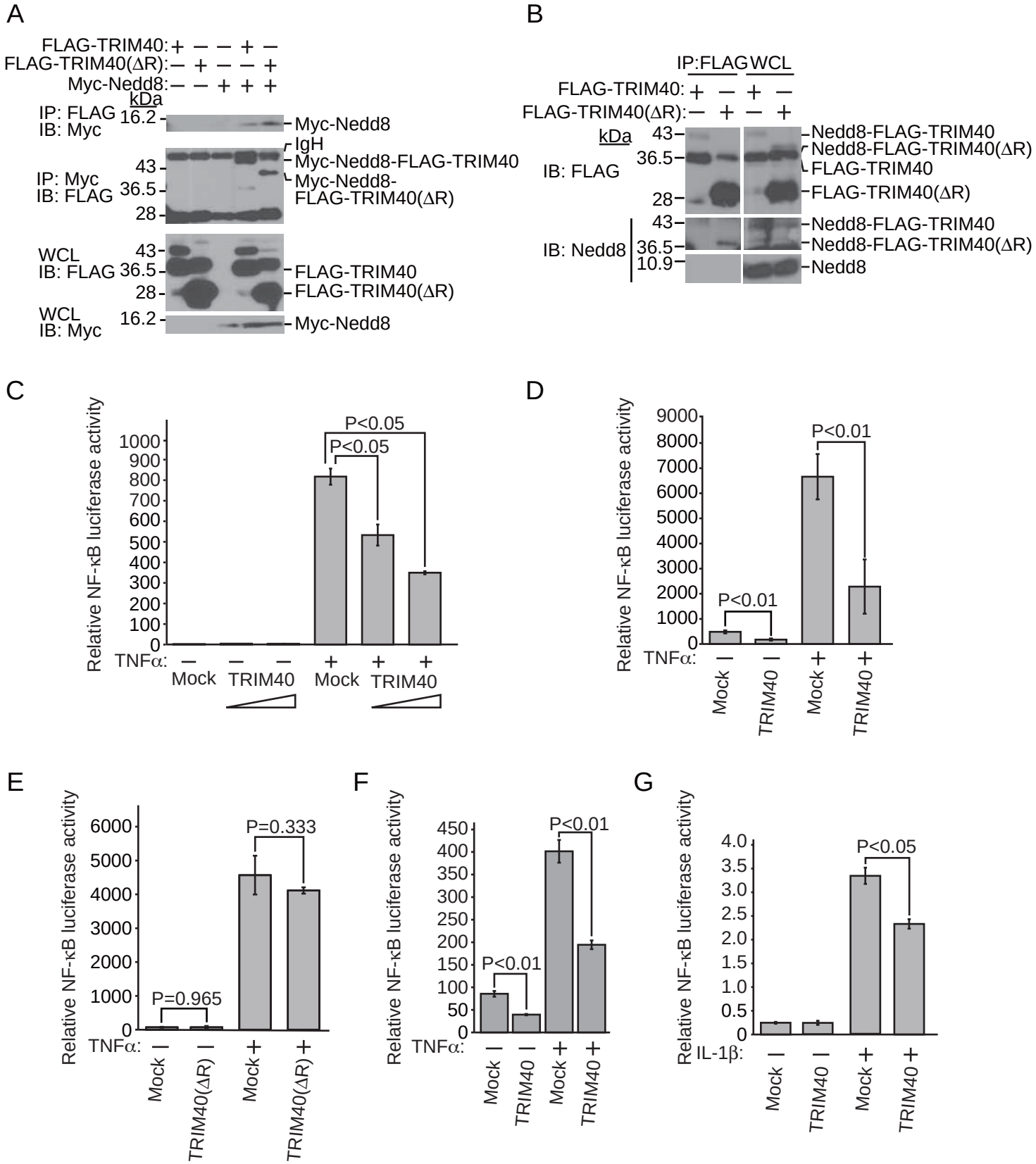
Fig. 5. TRIM40 promotes neddylation of IKKγ. **(A)** *In vivo* neddylation assay of IKKγ by TRIM40. HEK293T cells were transfected with plasmids encoding FLAG-tagged IKKγ, HA-tagged TRIM40, HA-tagged TRIM40(ΔRING) and Myc-tagged Nedd8, followed by immunoprecipitation (IP) with anti-Myc antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG or anti-HA antibody. **(B)** TRIM40 promotes neddylation of IKKγ in a dose-dependent fashion. HEK293T cells were transfected with plasmids encoding FLAG-IKKγ, HA-TRIM40 (1 μg, 3 μg or 6 μg), HA-tagged TRIM40(ΔRING) (3 μg), and Myc-tagged Nedd8, followed by immunoprecipitation (IP) with anti-Myc antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG or anti-HA antibody. **(C and D)** *In vivo* neddylation assay of IKKα or IKKβ by TRIM40. HEK293T cells were transfected with plasmids encoding FLAG-tagged IKKα or IKKβ, HA-tagged TRIM40, HA-tagged TRIM40(ΔRING) and Myc-tagged Nedd8, followed by immunoprecipitation (IP) with anti-Myc antibody. Immunoprecipitates were subjected to immunoblot (IB) analysis with anti-FLAG or anti-HA antibody.

Fig. 6. Knockdown of TRIM40 promotes NF-κB-mediated transcription and accelerates cell growth. **(A)** Establishment of stably knocked-down IEC-6 cell lines with shRNA specific for rat *TRIM40* (shTRIM40) or with non-targeting shRNA as a control (Mock). Immunoblot analysis was performed with anti-TRIM40 or ant-β-actin antibody. **(B)** *TRIM40* mRNA expression in stably knocked-down IEC-6 cell lines with shRNA specific for rat *TRIM40* (shTRIM40) or with non-targeting shRNA as a control (Mock). *TRIM40* mRNA levels in these cell lines were measured by quantitative real-time PCR. **(C)** Knock-down of TRIM40 enhances NF-κB-mediated transcription. Stably

knocked-down cells were transfected with the NF-κB luciferase reporter plasmid. Twenty-four h after transfection, cells were treated with TNFα (20 ng/μl) and cultured for an additional 6 h, and then luciferase activity was measured. Data are means ± S.D. of values from three independent experiments. **(D)** Knock-down of TRIM40 causes growth delay of IEC-6 cells. Cell lines were seeded at 5×10^4 cells in 6-well plates and harvested for determination of cell number at indicated times. Data are means ± S.D. of values from three independent experiments. **(E)** TRIM40 is downregulated in human gastrointestinal carcinomas. *TRIM40* mRNA levels in human gastrointestinal diseases and adjacent normal tissues from 22 cases were compared by quantitative real-time PCR. The expression level of *TRIM40* mRNA was normalized to that of *GAPDH* mRNA and shown as relative expression level. The boxes within the plots represent the 25-75th percentiles. The horizontal line in the boxes indicates median value. The *P* values for the indicated comparisons were determined by Wilcoxon matched pairs test. Samples used in this assay were as follows: gastric cancers, 13; colon cancer, 3; rectal cancer, 4; benign colon tumor, 1; Crohn's disease, 1. **(F)** Neddylation of IKKγ in gastric cancer sample. Cell lysates were subjected to immunoprecipitation with an antibody to IKKγ or anti-Nedd8, and the resulting precipitates were subjected to immunoblot analysis with an antibody to anti-IKKγ, anti-TRIM40 or anti-Nedd8. N, normal tissues; C, cancer tissues.



Noguchi et al. Figure. 1



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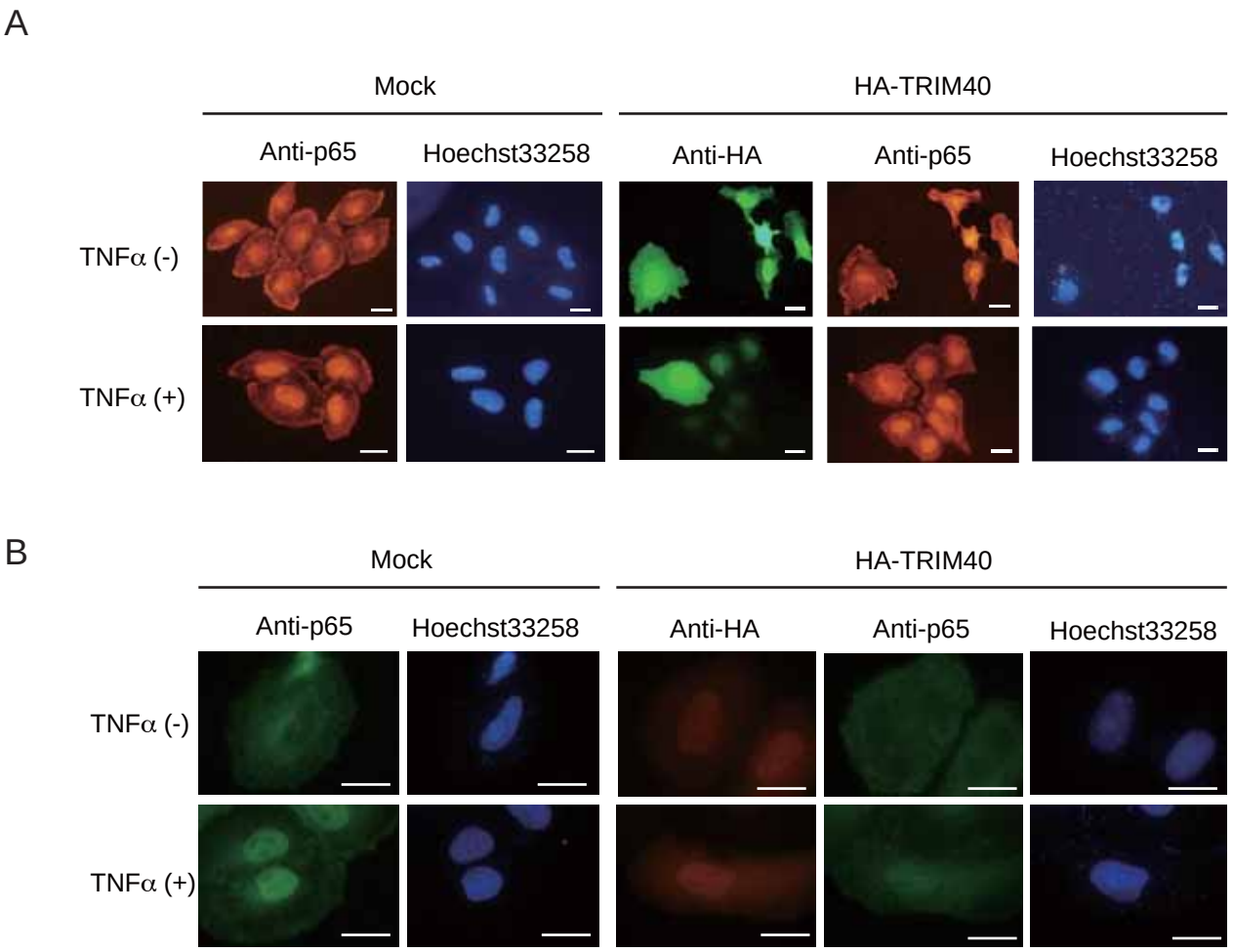
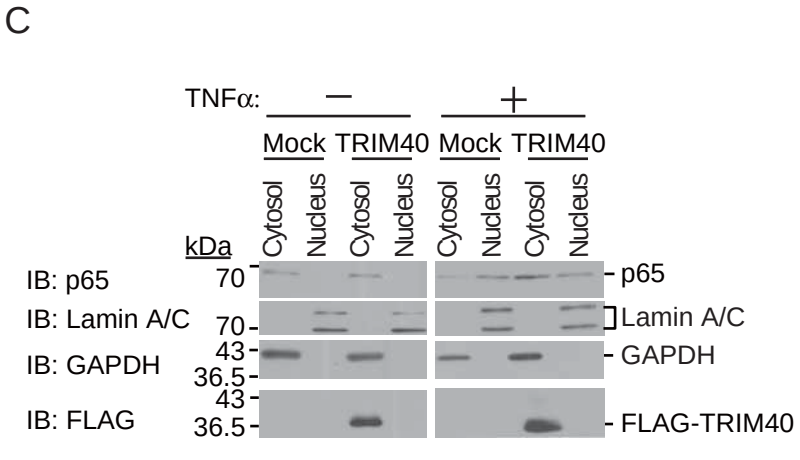
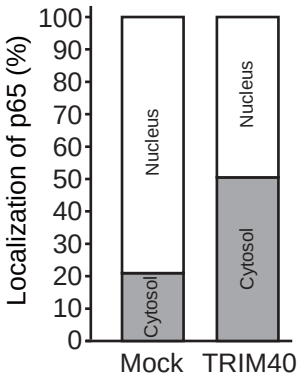
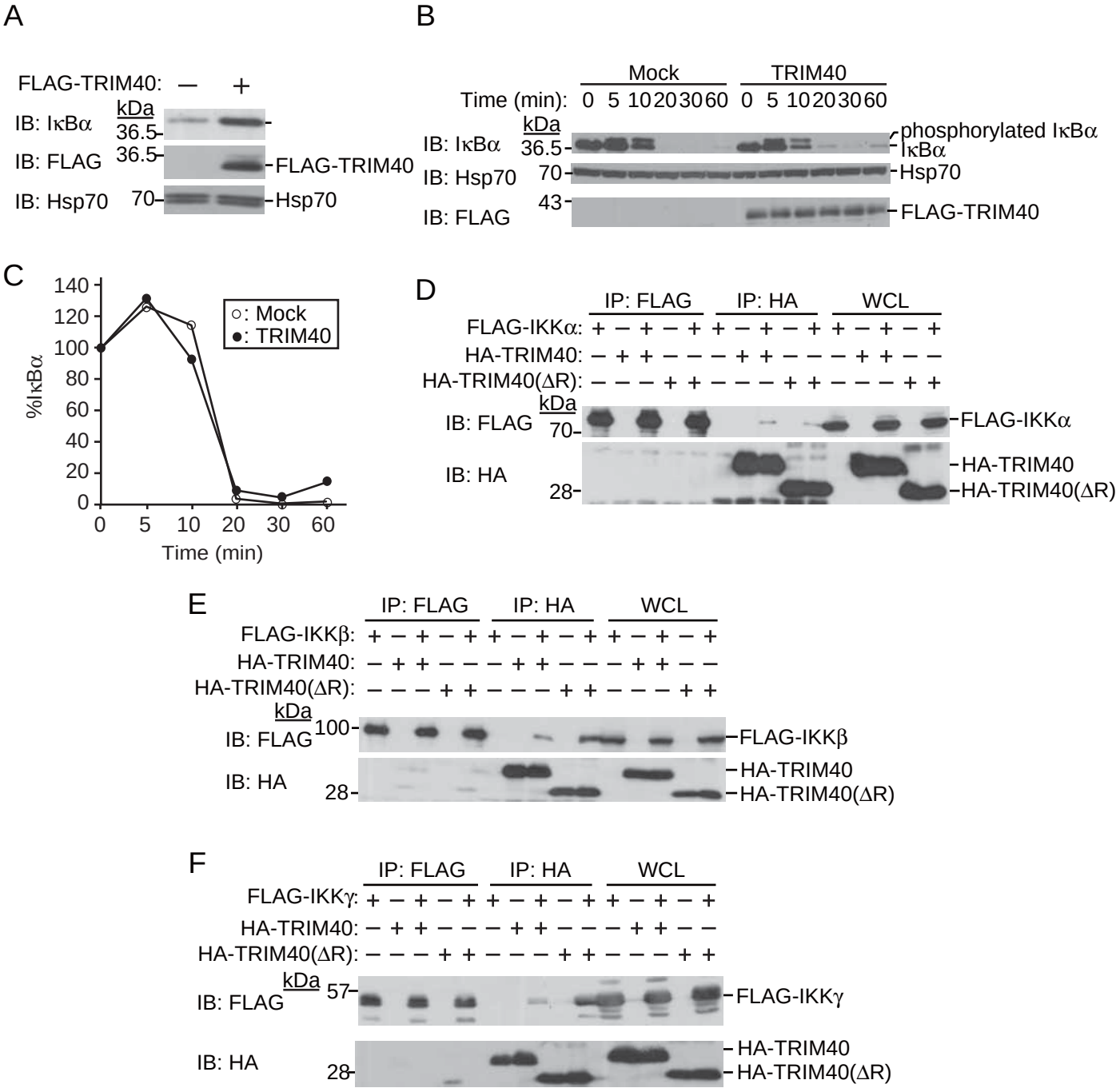






Noguchi et al. Figure. 3



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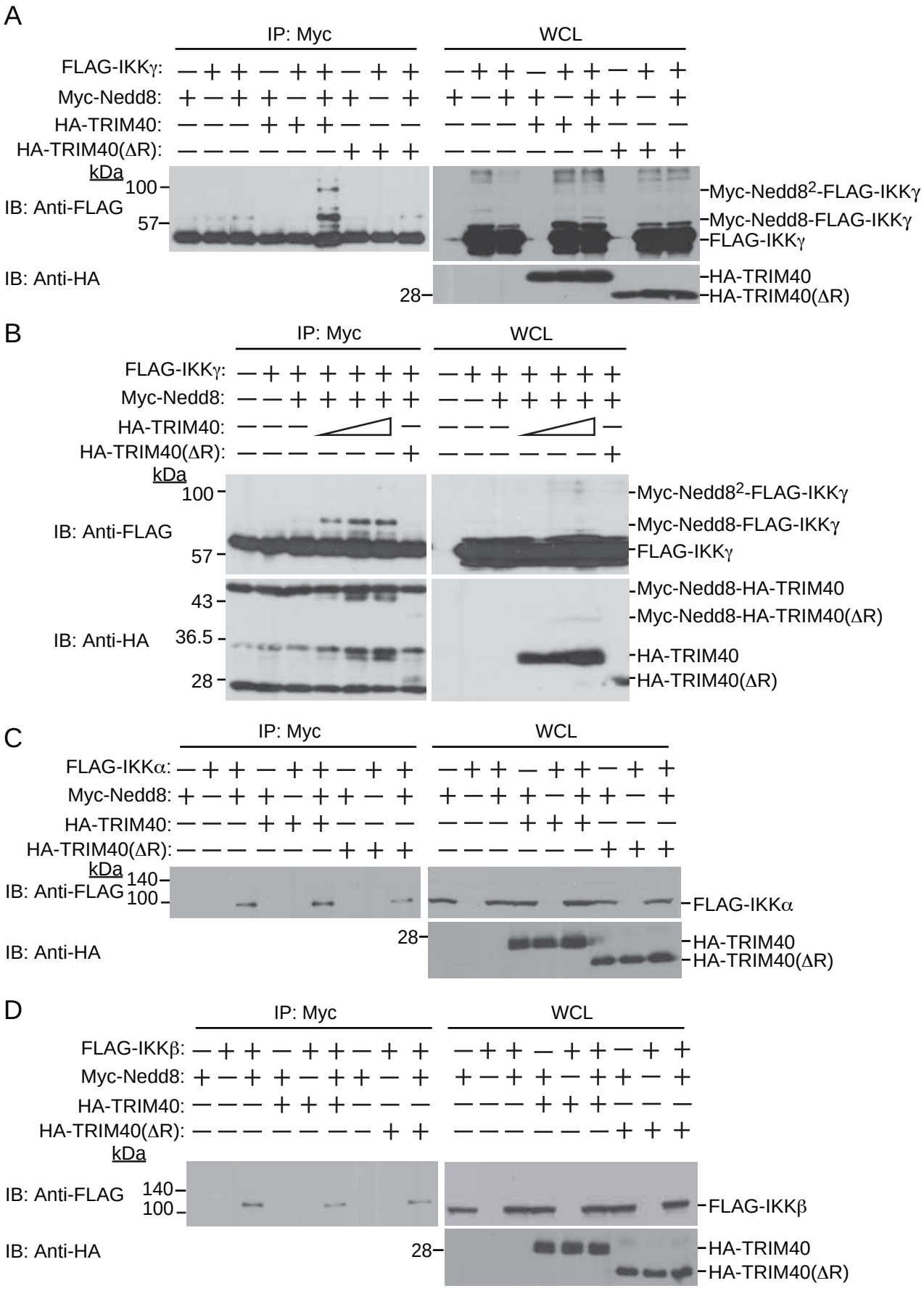

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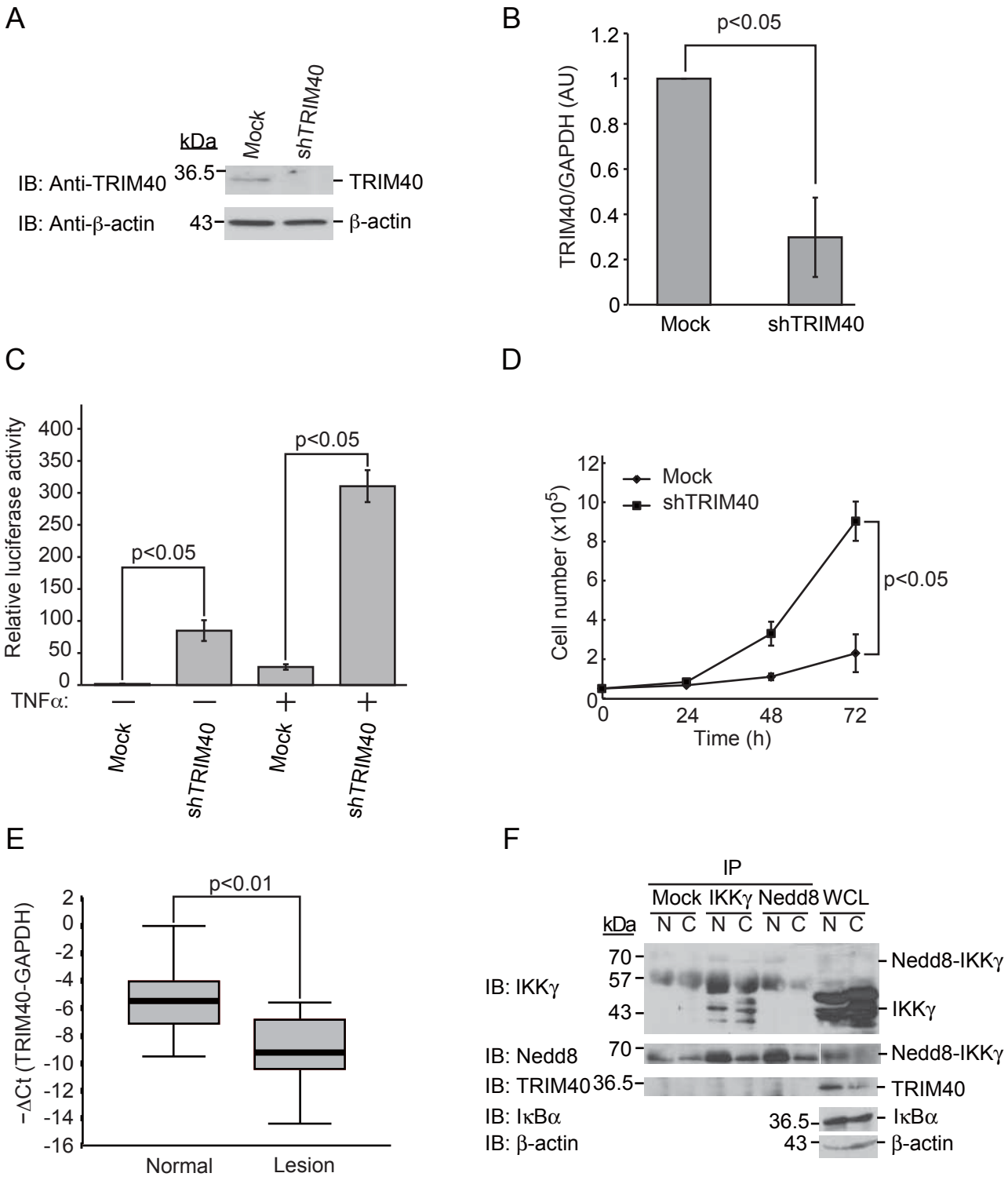

Noguchi at el. Figure. 6

ΔCt values of diseases by real-time PCR

No.	Diseases	Normal tissue ΔCt	Lesion ΔCt
1	Benign colon tumor	8.61	5.94
2	Gastric cancer	0.02	5.57
3	Colon cancer	5.41	6.18
4	Gastric cancer	6.11	10.42
5	Rectal cancer	4.02	9.26
6	Crohn's disease	5.51	6.83
7	Gastric cancer	5.88	6.84
8	Rectal cancer	9.49	9.21
9	Colon cancer	8.4	10.4
10	Rectal cancer	3.38	8.42
11	Rectal cancer	7.39	9.61
12	Colon cancer	8.9	10.35
13	Gastric cancer	7.13	11
14	Gastric cancer	4.81	7.31
15	Gastric cancer	3.88	9.85
16	Gastric cancer	6.89	8.21
17	Gastric cancer	4.6	7.12
18	Gastric cancer	4.96	11.38
19	Gastric cancer	3.73	14.42
20	Gastric cancer	4.19	9.37
21	Gastric cancer	3.59	5.8
22	Gastric cancer	5.69	12.2