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Reviewer 1: No comments

Reviewer 2:

1. On page 15, the authors state: “The two types of Trim13 siRNA had linear inhibitory effects on cytokine and chemokine production according to the knock-down efficiency”. I think a better way to say this would be “The two types of Trim13 siRNA had inhibitory effects on cytokine and chemokine production proportional to the knock-down efficiency”, since two points cannot be said to be a linear effect.
Answer: As your suggestion, we’ve changed the sentence.

2. At the bottom of page 15, Tab1 is mentioned but not introduced. Since it is included in the Figures, this would be an appropriate place to mention what it is.
Answer: We agree with your comment and mentioned it.

3. In the discussion on Page 23, the authors state that they tried to use transient transfections to study NF-Kb promoter activity, but switched to stable cell lines. I think this would be stronger if they mentioned why (it is hinted at that, but coming out and saying what the problem was would be helpful).
Answer: Thank you very much. We agree with your comment and revised.

Reviewer 3:

1. The authors state they use t tests to compare means for significance. However, they should reanalyze all tests of significance using one- or two-way ANOVA with a post hoc means test correction, such as the Bonferroni.
Answer: Statistical analysis results are expressed as the mean ± standard deviation of a minimum of 3 independent assays. Statistical significance was calculated by analysis of variance using GraphPad Prism software, version 5.01 (GraphPad. Inc., La Jolla, CA, USA). Groups were compared with two-way analysis of variance with a Bonferroni post-test. And we revised about this in Materials and Methods. * $P<0.05$ was considered to indicate a statistically significant difference.

2. The authors should indicate the number of independent replicates that were done for each of their experiments.
Answer: As your suggestion, we’ve described how many did the experiments in each figures.

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Trim13 potentiates TLR2-mediated NF-κB activation *via* K29-linked polyubiquitination of TRAF6

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a) Running Title: Trim13 stimulates NF-κB activity *via* TRAF6 ubiquitination

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d) Abbreviations:

TLR**,** Toll-like receptor; NF-κB, Nuclear factor-κB; TRAF6, TNF receptor-associated factor 6; Trim, Tripartite motif; Ub, ubiquitin

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Abstract

Ubiquitination is a versatile post-translational modification involved in NF-κB activation of TLR signaling. Here, we demonstrated that Trim13, an E3 ubiquitin ligase, is up-regulated in macrophages upon stimulation with TLR2 ligand. Knock-down of Trim13 attenuated TLR2-mediated production of cytokines/chemokines and formation of foam cells, as well as activation of NF-κB. Trim13 interacts with TRAF6 and potentiates NF-κB activity *via* ubiquitination of TRAF6. Overexpression of inactive mutant (C10/13A) or RING deletion mutant of Trim13 did not potentiate ubiquitination of TRAF6 or activation of NF-κB. These results suggest that the effects of Trim13 are dependent on its E3 ligase activity. Trim13 used K29-linked polyubiquitin chains for TRAF6 ubiquitination to promote NF-κB activity and thus potentiated activation of TLR2-mediated immune responses. Our data identify Trim13 as a positive regulator of NF-κB activation and suggest that K29-linked polyubiquitination is a specific ubiquitin-linked pattern involved in the control of TLR2 signaling.

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Introduction

The toll-like receptor (TLR) family plays a pivotal role in inflammation and immunity, including innate and adaptive immune responses (Blasius and Beutler, 2010). These receptors receive and transduce signals from pathogen-associated molecules, thereby activating nuclear factor-κB (NF-κB). Upon ligand stimulation, TLR recruits the adaptor proteins myeloid differentiation primary response 88 (MyD88) and interleukin-1 receptor-associated kinase (IRAK)1/4, which subsequently form a complex, TNF receptor-associated factor 6 (TRAF6), to trigger K63 auto-polyubiquitination. TRAF6 forms a recognition signal for the recruitment of TGF-β-activated kinase 1 (TAK1)-binding protein 1 (TAB1) or TAB2/3 and activation of TAK1. TAK1 subsequently activates IκB kinase (IKK) complex, leading to phosphorylation and subsequent degradation of the IκBα. This cascade of signaling enables the NF-κB complex to translocate to the nucleus and initiate transcriptional responses (Kawai and Akira, 2007).

Ubiquitination along with phosphorylation comprises one of the most common post-transcriptional modifications of NF-κB activation of TLR signaling. Various proteins such as TRAF6, TAB2/3, NF-κB essential modifier (NEMO/IKKγ), and IκBα are ubiquitinated during these processes (Chen, 2005). NF-κB activation by ubiquitination in TLR signaling is usually regulated by the type of E3 ubiquitin ligase, chain specificity, target proteins, and

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deubiquitinases (Park et al., 2014). The tripartite motif (Trim) proteins are really interesting new gene (RING)-type E3 ligases that contain a RING finger, B-boxes, and coiled-coil domain. The 76 members of the Trim family can be classified into eleven subgroups according to the variable C terminus (Li et al., 2014). Despite their common domain structure, Trim proteins have distinct cellular functions, including innate immunity (Rajsbaum et al., 2014), inflammation (Uchil et al., 2013), and cancer (Hatakeyama, 2011). In addition, some Trims regulate NF-κB activity in TLR-driven pathways. NF-κB activation is negatively or positively regulated according to the type of Trim in TLR signaling. Trim21, -27, -30α, and -38 act negatively in TLR-mediated NF-κB activation (McEwan et al., 2013; Shi et al., 2008; Zha et al., 2006; Zhao et al., 2012), but Trim23 and -62 act positively (Poole et al., 2009; Uchil et al., 2013).

Trim13 is an E3 ubiquitin ligase with auto-polyubiquitination properties. Trim13, along with Trim59, comprise a subgroup containing a transmembrane domain in the C-terminal region (Lerner et al., 2007). This E3 ligase may play a role in ER-associated degradation (Lerner et al., 2007). It has been reported that Trim13 ubiquitinates L-type channels and induces proteasomal degradation (Altier et al., 2011). Trim13 enhances apoptosis through Akt (Joo et al., 2011), regulates autophagy caused by ER stress (Tomar et al., 2012), affects tumor cell growth (Gatt et al., 2013), and participates in regulation of melanoma differentiation-

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associated antigen 5 (MDA5)-mediated type I interferon production (Narayan et al., 2014). In addition, Trim13 plays a role in the regulation of NF-κB activity (Gatt et al., 2013; Tomar and Singh, 2014); however, the above reports of NF-κB are controversial. Accordingly, it is necessary to reestablish the exact function of Trim13 in NF-κB activity regulation.

We established the role of Trim13 in TLR2-mediated NF-κB activation, investigated its target protein and studied its physiological function. Here, we report that Trim13 participates in TLR2-induced foam cell formation through various cytokines and chemokines. In this process, Trim13 stimulates NF-κB activity through K29-linked polyubiquitination chain formation of TRAF6.

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Materials and Methods

Animals

A total of 20 male 6-week-old C57BL6 wild-type (WT) mice (average weight) were purchased from Central Lab Animal (South Korea). TLR2 deficient (average weight, C57BL6 background, 6 weeks, male) mice were kindly provided by Dr SJ Lee (Seoul National University, South Korea). For the experiments, a total of 20 mice were used. Each group of mice was housed under standard conditions (temperature at 25±2°C, relative humidity (55 ± 5%), 12h/12h light-dark cycle, and free access to food and water). The mice were sacrificed by cervical dislocation. The study was conducted in strict accordance with the guidelines and protocols approved by the Institutional Animal Care and Use Committee of Yeungnam University College of Medicine (Daegu, South Korea, permit number: YUMC-AEC2011-007).

Antibodies and Reagents

The antibodies used and their sources were as follows: anti-Flag, anti-Flag M2 affinity gel (Sigma-Aldrich, St. Louis, MO, USA); anti-GFP, anti-HA (Cell Signaling Technology, Beverly, MA, USA); anti-TRAF6, anti-IκBα (Santa Cruz Biotechnology, Santa Cruz, CA, USA). The reagents used in the study and their sources were as follows: oil red-O, oxidized LDL (Sigma-Aldrich); protein A/G PLUS Agarose (Santa Cruz Biotechnology); mouse TLR

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agonist kits including Pam_3CSK_4 (Invivogen, San Diego, CA, USA) and Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA); Amaxa reagents (Lonza, Switzerland); RT-PCR kits, restriction enzymes (Takara Bio, Japan); luciferase assay kits (Promega, Madison, WI, USA); enhanced chemiluminescence solution (ECL, GE Healthcare, UK).

Cell culture and transfection

Raw 264.7 and HEK293FT cells were purchased from the American Type Culture Collection (Manassas, VA, USA) and grown in DMEM supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) at 37°C in a humidified atmosphere of 5% CO_2 and 95% O_2. Cells were plated in 35 mm or 100 mm diameter plates at an appropriate density and grown overnight. The NF-κB/293/GFP-*Luc* transcriptional reporter cell line was purchased from System Biosciences (Mountain View, CA, USA). Cells were transduced with a lentivector that co-expresses destabilized GFP and firefly luciferase reporter driven by the minimal cytomegalovirus promoter in conjunction with four copies of the NF-κB consensus transcriptional response element upstream of the minimal cytomegalovirus. Primary bone marrow-derived monocytes were differentiated into bone marrow-derived macrophage (BMDM) over 5-7 days in DMEM supplemented with 10% L929 cell-

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conditioned medium (as a source of M-CSF). siRNA transfection into cells was conducted using Amaxa reagents according to the manufacturer´s instructions. Plasmids were transfected in Raw 264.7 cells employing Amaxa reagents and in HEK293FT cells using Lipofectamine 2000 reagent.

Plasmid constructs and siRNAs

WT Trim13 was kindly provided by Dr JY Kim (Korea Hydro & Nuclear Power Co., South Korea). For Flag-tagged Trim13 and its various deletion mutants, the corresponding DNA fragments were PCR amplified and cloned into the HindIII and XbaI sites of pFlag-CMV2 vector. Trim13 was also cloned downstream from a GFP tag of pEGFP-C1 vector. Site-directed mutagenesis was used to convert WT Trim13 into a catalytic mutant form by mutating cysteine 10 and 13 into alanine (C10/13A). TRAF6 was cloned downstream from a Flag tag at the NotI and BamHI sites of pFlag-CMV2 vector. HA-Ubs (WT, K29R, K48R, K63R, K29, K48, and K63) were kindly provided by Dr. Y. Ye (National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, USA). The plasmid constructs were systematically checked based on their sequence analyses. Stealth nonspecific control and Trim13-specific siRNAs against the following target genes were designed using the Block-IT Stealth RNAi designer (Invitrogen): Trim13 (1), 5´-GAA GCU UGC AGU UAU

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GCA AAC CUA U-3´; Trim13 (2), 5´-TGT ATT CCT GGA ATG GTC TCC ACT T-3´; TLR2, 5´-UAA AAG GGC GGG UCA GAG UUC UCC A-3´.

Foam cell formation assay

Raw 264.7 cells were transfected with individual Trim13 siRNAs using Amaxa reagents. For lipid uptake analysis, macrophages were cultured in 6-well plates, then treated with Pam_3CSK_4 (100 ng/ml) and oxidized LDL (50 μg/ml) for 24 h. Next, cells were washed three times with PBS, fixed with 10% formalin, and stained with oil red-O solution. Intracellular lipid droplets were then detected by light microscopy using a DIAPHOT 300 light microscope (Nikon Corporation, Tokyo, Japan). Images were recorded with an AxioCam ICc I digital camera system (Carl Zeiss, Oberkochen, Germany).

NF-κB promoter activity assay

NF-κB/293/GFP-*Luc* reporter cells were transfected with Trim13 plasmids using Lipofectamine 2000. The cell lysates were then assayed for luciferase activity using the luciferase reporter assay system according to the manufacturer´s protocols. Raw 264.7 cells were transfected using Amaxa regents with siRNA or DNA expressing the Trim13 proteins and plasmid DNA expressing firefly luciferase under control of the NF-κB construct. In

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addition, each transfection mixture contained an internal control reporter plasmid pRL-TK, which expresses *Renilla luciferase* for normalization of transfection efficiencies. After transfection, the cells were incubated with Pam_3CSK_4 for the indicated times. The cell lysates were then assayed for luciferase activity.

***In vivo* ubiquitination assay**

For *in vivo* ubiquitination assay, cells harvested from 100 mm dishes were lysed in buffer (0.5% Nonidet P-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 2 mM $MgCl_2$, 5 mM N-ethylmaleimide and protease inhibitor cocktail). The lysates were then centrifuged at 15,000 rpm for 10 min, after incubated with 1% SDS and 1 mM dithiothreitol at 65°C for 20 min after which they were diluted 10-fold with lysis buffer. The samples were subsequently boiled at 60°C for 20 min to break the protein-protein interactions, after which they were diluted 10-fold with lysis buffer. Next, the diluted samples were incubated with specific antibodies or α-Flag-M2 bead at 4°C for 4 h. Finally, the precipitates were washed five times using RIPA buffer and denatured in SDS loading buffer at 95°C for 10 min.

Western blot analysis

Following SDS-PAGE, the proteins were transferred to nitrocellulose membranes that were

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subsequently blocked with Tris-buffered saline containing 5% skim milk and 0.1% Tween20 for 1 h at room temperature. After blocking, the membranes were incubated with primary antibodies overnight at 4°C. Following washes, the membranes were incubated with HRP-conjugated secondary antibodies (anti-mouse or anti-rabbit 1:10,000) for 1 h at room temperature and visualized using an ECL solution.

Coimmunoprecipitation

Cells were lysed in lysis buffer (20 mM Tris-HCl, pH 8.0, 137 mM NaCl, 5 mM EDTA, 1 mM EGTA, 10 mM NaF, 10% glycerol, and 1% Triton X-100) by incubation on ice for 30 min followed by centrifugation at 15,000 rpm for 10 min. Lysates were incubated with α-Flag-M2 affinity gel at 4°C for overnight. The precipitates were washed five times using lysis buffer and analyzed by immnoblotting.

RT-PCR and quantitative real-time PCR (qPCR)

Total RNA was extracted from cells using TRIzol. First-strand cDNA was then synthesized from 1 μg total RNA by employing random primers, oligo-dT, and reverse transcriptase (Promega). The assays of RT-PCR and qPCR were performed as previously described (Lyu et al., 2015) using the primers listed in Table 1 and 2. Target gene expression was normalized to

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β-actin transcription. qPCR was conducted using a LightCycler 1.5 (Roche Diagnostics) with SYBR-Green I as the florescent dye according to the manufacturer's instructions. For quantification, target genes were normalized against β-actin control and visualized using the Fuji Intelligent Dark Box LAS-3000 Image Reader (FujiFilm, Tokyo, Japan). Densitometric analysis was carried out using LAS-3000 Fujifilm Image Reader and Multi Gauge 3.0 software.

Statistical analysis

Results are expressed as the mean ± standard deviation of a minimum of 3 independent assays. Statistical significance was calculated by analysis of variance using GraphPad Prism software, version 5.01 (GraphPad. Inc., La Jolla, CA, USA). Groups were compared with two-way analysis of variance with a Bonferroni post-test. * $P<0.05$ was considered to indicate a statistically significant difference.

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Results

Trim13 is up-regulated by stimulation of TLRs

A DNA microarray was performed under Pam_3CSK_4 (a TLR2 agonist) treatment in Raw 264.7 cells to identify a new regulator of the TLR2 signaling pathway. Several ubiquitin-related genes (Tnip1, RANK, A20, ABIN3, and Trim13) were included as an upregulated gene by TLR2 activation (Table 3-4). Among them, we focused on the E3 ubiquitin ligase, Trim13. We utilized RT-PCR to verify the expression of Trim13 *via* TLR2 in Raw 264.7 cells. As shown in Fig. 1A, Trim13 gene expression was induced as an early gene by Pam_3CSK_4 in a time-dependent manner. We also confirmed the effects of Pam_3CSK_4 following isolation of BMDMs from the bone marrow of WT or TLR2 knock-out (KO) mice. Increased Trim13 gene expression was observed in WT BMDMs treated with Pam_3CSK_4, whereas Pam_3CSK_4 had no effect on TLR2 KO BMDMs (Fig. 1B). The inducing Trim13 gene is only blocked by TLR2 ligands (Pam3CSK4 and HKLM), not TLR4 ligand (LPS) or TLR9 ligand (CpG), in the TLR2 siRNA cells (Fig. 1C). To test the general effects of TLRs on Trim13 expression, we checked other types of TLR agonists. All tested agonists (TLR1/2, TLR2, TLR3, TLR4, TLR5, TLR6/2, TLR8 and TLR9) induced Trim13 gene expression (Fig. 1D; qPCR & RT-PCR). We next investigate the expression pattern of Trim13 protein. To accomplish this, we used four commercially available Trim13 antibodies to detect Trim13 protein. Even though three of the

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four could detect Trim13 protein in the overexpression system, none of the antibodies detect endogenous or Pam_3CSK_4-induced Trim 13 protein in Raw 264.7 cells. Nevertheless, these data indicate that Trim13 is an important regulator of TLR signaling of macrophages, including TLR2.

Trim13 potentiates TLR2-mediated macrophage activation *via* NF-κB

We attempted to identify the role of Trim13 in TLR2 signaling. It is well known that Pam_3CSK_4, a TLR2 ligand, activates NF-κB and then accelerates foam cell formation, eventually leading to atherosclerosis (Mullick et al., 2005). To investigate the effects of Trim13 on NF-κB activation, we constructed two types of Trim13 siRNA and then compared the effects of Pam_3CSK_4 in control siRNA and Trim13 siRNA transfected Raw 264.7 cells. We checked whether two types of Trim13 siRNAs knocked-down gene expression caused by TLR2 stimulation (Fig. 2A, left panel). To confirm the role of Trim13, we measured the change in NF-κB activity in response to Pam_3CSK_4 in Raw 264.7 cells. Pam_3CSK_4 treatment increased NF-κB activity by 64-fold; however, Trim13 knock-down strongly attenuated the NF-κB activity induced by Pam_3CSK_4 (Fig. 2A, right panel). Notably, NF-κB activity was inhibited according to the level of Trim13 expression inhibition. NF-κB is the best known transcription factor regulating cytokines and chemokines. Therefore, we investigated

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expression of cytokines and chemokines using qPCR and conventional RT-PCR. Trim13 knock-down decreased expression of TNF-α, IL-1β, and IL-6, as well as CCL2, CCL3, and CCL4, which are all induced by Pam_3CSK_4. The two types of Trim13 siRNA had inhibitory effects on cytokine and chemokine production proportional to the knock-down efficiency (Fig. 2B and 2C). Because cytokines and chemokines are essential to the formation of foam cells, we tested and compared their formation in control siRNA and Trim13 siRNA transfected cells. As shown in Fig. 2D, foam cell formation by TLR2 stimulation was dramatically decreased in Trim13 knock-down cells. Foam cell formation was also inhibited according to the level of Trim13 gene expression. These results suggest that NF-κB activity is positively regulated by Trim13. Furthermore, these findings indicate that TLR2 stimulation by Pam_3CSK_4 increases Trim13 expression, thereby causing cytokines and chemokines production via NF-κB activation, and eventually promoting foam cell formation.

Trim13 interacts with and ubiquitinates TRAF6

We investigated the mechanism by which Trim13 increases NF-κB activation. TRAF6 is a very important adaptor protein during the initial stages of NF-κB activation induced by TLR2 (Akira and Takeda, 2004). We speculated that TRAF6 was a target of Trim13, and studied

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their interaction. Trim13 and TRAF6 plasmids were co-transfected in HEK293FT cells. We detected clear binding of Trim13 with TRAF6, but not with NEMO and TAB1 (Fig. 3A). Regulation of NF-κB by TRAF6 is dependent on ubiquitination (Zhang et al., 2013; Zhou et al., 2012). In addition, the E3 ligase activity of Trim13 is dependent on the 10^{th} and 13^{th} Cys residues. Therefore, we generated a construct containing a Cys10/13 to Ala10/13 mutation (C10/13A) and then tested for ubiquitination of endogenous TRAF6 by Trim13 in cells. WT Trim13 clearly accelerated TRAF6 ubiquitination, but had no effect on the C10/13A mutant Trim13 (Fig. 3B). We also looked for ubiquitination of exogenous TRAF6 by Trim13 in the above cells. WT Trim13 stimulated exogenous TRAF6 ubiquitination, similar to endogenous TRAF6, but had no effect on C10/13A mutant (Fig. 3C). Trim13 not only plays a role in the ubiquitination of other proteins, but also degrades *via* auto-ubiquitination. Therefore, in the input, WT and the mutant had the level of Trim13 protein to be different. However, not only the WT but also C10/13A mutant Trim13 showed binding with TRAF6 (Fig 3D). These result suggests that Trim13 enzyme activity mediates TRAF6 ubiquitination. A previous study suggested that Trim13 regulates TNFR-mediated NEMO ubiquitination (Tomar and Singh, 2014). Therefore, we investigated and compared TRAF6 and NEMO ubiquitination by Trim13. Interestingly, ubiquitination of NEMO not affected, while ubiquitination of TRAF6 was dramatically increased in HEK293FT cells co-expressing Trim13 (Fig. 3E). We next

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tested NF-κB activation by the combination of Trim13 and TRAF6 using the NF-κB/293/GFP-*Luc* reporter cell line. Before we assayed the NF-κB activity, we checked the expression of TRAF6 and Trim13 in a transfected reporter cell line (Fig. 3F, upper panel). NF-κB activity dramatically increased in response to transfection of TRAF6 alone. NF-κB activity was also much higher in cells co-transfected with TRAF6 and WT Trim13 relative to TRAF6 transfected alone. However, co-transfection of TRAF6 and the C10/13A Trim13 mutant had similar effects as TRAF6 transfection alone (Fig. 3F, lower panel). The endogenous ubiquitination of TRAF6 by TLR2 ligand was investigated in Raw 264.7 cells. Treatment with Pam_3CSK_4 increased TRAF6 ubiquitination of Raw 264.7 cells in a time dependent manner (Fig. 3G). We confirmed that TRAF6 ubiquitination was regulated by Trim13 using siRNA. Trim13 knock-down significantly attenuated the increase in TRAF6 ubiquitination induced by Pam_3CSK_4 (Fig. 3H). Overall, these results suggest that Trim13 stimulates TRAF6 ubiquitination, thereby potentiating NF-κB activity in TLR2 signaling.

Trim13 regulates NF-κB activity *via* its E3 ligase activity

Trim13 is a protein composed of multiple domains, including a RING, B-boxes, coiled coil (CC), and a transmembrane (TM) domain. Each domain has a specific function in the signaling pathway (Li et al., 2014). We constructed deletion mutants to verify the importance

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of each domain in NF-κB activation. The domain composition is shown in Fig. 4A. HEK293FT cells were co-transfected with each Trim13 mutant plus TRAF6 plasmids, after which TRAF6 ubiquitination was investigated. Each Trim13 mutants was successfully expressed. Overexpression of WT Trim13 increased TRAF6 polyubiquitination, but the RING deletion mutant did not have any effect, similar to the C10/13A mutant shown in Fig. 3C. Conversely, deletion of CC or TM domains showed no difference relative to the WT Trim13 effects (Fig. 4B). We next investigated the change in NF-κB activity. The increase in NF-κB activity by WT Trim13 decreased in the RING deletion mutant, but had no effect in the CC and TM deletion mutants (Fig. 4C). These results suggest that TRAF6 polyubiquitination and subsequent NF-κB activation by Trim13 is dependent on the E3 ligase activity.

Trim13 ubiquitinates TRAF6 *via* the K29-linked polyubiquitination chain

TRAF6 ubiquitination was an important mechanism in the increase of NF-κB activity by Trim13. Therefore, we analyzed the type of ubiquitin chain generated on TRAF6 by Trim13. To accomplish this, we first used specific ubiquitin mutants (K29R, K48R, and K63R) to test their effects on TRAF6 ubiquitination in cells overexpressing Trim13. Although K48R and K63R ubiquitin also showed some decrease, but K29R ubiquitin showed the most inhibition of Trim13-mediated TRAF6 polyubiquitination (Fig. 5A). We next used another set of

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ubiquitin mutants in which all except one Lys residue was replaced by Arg. Preliminary experiments showed differences in the transfection efficiency of TRAF6 when various plasmids were co-transfected with different ubiquitin mutants. Nevertheless, K29-linked polyubiquitin chain formation was a strong candidate for TRAF6 (data not shown). Therefore, we confirmed Trim13-induced polyubiquitination of TRAF6 under the same conditions after transfecting each ubiquitin mutant. As shown in Fig. 5B, K29-ubiquitin and K63-ubiquitin promoted chain formation on TRAF6, but the effect was more powerful when K63-ubiquitin was used. Moreover, no chain formation occurred when K48-ubiquitin was used, and Trim13 only potentiated TRAF6 poly-ubiquitination in the K29-ubiquitin assay system. We compared WT or mutant ubiquitin to prove the importance of K29-linked chain formation in regulating NF-κB activity. As shown in Fig. 5C, WT and K29 ubiquitin increased NF-κB activity by Trim13, but K29R ubiquitin could not (Fig. 5C). Thus, our results provide evidence of K29-linked polyubiquitin chain formation on TRAF6 by the Trim13 E3 ligase.

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Discussion

In this study, we conducted screening to identify a new gene regulating the immune signaling pathway using a TLR2 agonist in macrophages. Among the screened genes, we identified Trim13, a RING type ubiquitin E3 ligase. This protein promoted formation of foam cell and production of cytokines/chemokines through NF-κB. Specifically, the effects of Trim13 resulted from the formation of K29-linked polyubiquitination chains of TRAF6, and ultimately increased NF-κB activity.

The Trim family has been shown to play an important role in innate immunity (Rajsbaum et al., 2014). Notably, some Trim family members regulate anti-inflammatory or pro-inflammatory responses through NF-κB activation. Furthermore, there are various target proteins of Trim involved in NF-κB activity regulation. For example, Trim5α and Trim8 target TAK1 (Gong et al., 2011; Li et al., 2011), Trim27 targets IKKβ (Zha et al., 2006), Trim30α targets TAB2/3 (Shi et al., 2008), and Trim40 targets NEMO (Noguchi et al., 2011). TRAF6 is also known as an important target regulating NF-κB activation. TRAF6 is a member of the TRAFs, which mediate TLR intracellular signaling. TRAF6 has been shown to play a critical role in NF-κB activation in that TRAF6 dominant negative cells failed to respond to TLR4 stimulation (Guillot et al., 2004). TRAF6 is also critical for various types of TLRs-mediated NF-κB activation and regulation of the subsequent pro-inflammatory

response. In these TLR pathways, stimulation of the receptors leads to recruitment of MyD88, which further recruits IRAK1 and IRAK4. IRAK1 then binds to TRAF6, which subsequently activates downstream signaling cascades, including those of IKK (Kawai and Akira, 2007). However, Trim38 is the only type of Trim that has been reported to regulate NF-κB activity by targeting TRAF6 in TLR-mediated immune response (Zhao et al., 2012). Therefore, our discovery of Trim13 regulating NF-κB activity by targeting TRAF6 has significant meaning.

The mechanism of Trim13 in regulation of NF-κB activity is currently the subject of debate. Some reports have shown that Trim13 increases NF-κB, while others indicate that it decreases NF-κB activity. For example, Uchil *et al.* reported that screening 43 types of human Trim clearly demonstrated that Trim13 increases NF-κB activity, along with Trim1, -5, -25, and -62 (Uchil et al., 2013). Gatt *et al.* (Gatt et al., 2013) and Versteeg *et al.* (Versteeg et al., 2013) also reported that Trim13 is a NF-κB activator. However, Tomar and Singh reported that Trim13 inhibits NF-κB activity by NEMO ubiquitination in tumor necrosis factor receptor (TNFR) signaling (Tomar and Singh, 2014). Therefore, it is necessary to establish the correct mechanism of Trim13 in NF-κB activity regulation. In the present study, Trim13 was found to increase NF-κB activity, thereby stimulating cytokines and chemokines production. In addition to the increase in NF-κB activity, we believe the acceleration of foam cell formation was also important. We reached these conclusions using a used Trim13 knock-down system

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and confirmed the results with a Trim13 overexpression system to confirm those results. Specifically, the Pam_3CSK_4 effect was compared between WT and C10/13A Trim13 transfected Raw 264.7 cells. NF-κB activity by Pam_3CSK_4 was potentiated in WT Trim13 overexpressed cells when compared to cells transfected with only vector, while C10/13A mutant overexpressed cells had no effect (data not shown). NF-κB activity was also observed in response to the degradation of IκBα protein. We also monitored changes in the IκBα protein stimulated by Pam_3CSK_4 in vector or Trim13-overexpressed cells. Pam_3CSK_4 treatment showed a more rapid degradation of IκBα in Trim13-overexpressed cells (data not shown).

However, our results were contrary to those of Tomar and Singh (Tomar and Singh, 2014), even though the experimental conditions were very similar. Specifically, they reported that Trim13 inhibits TNF receptor (TNFR)-mediated NF-κB activation. There are several possible reasons for this discrepancy, including the difference in TLR and TNFR could be a possible for these differences. Specifically, TLR and TNFR signaling have common signaling pathways for NF-κB activation, but unique processes for each receptor. The pathways through the TAK, IKK, and NF-κB complex are very similar. However, receptor-interacting protein 1 (RIP1) is an important adaptor in early signaling for TNFR, while TRAF6 is associated with TLR. Second, the methods used to measure NF-κB promotor activity could have caused these

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differences. While we used the NF-κB/293/GFP-Luc reporter cell line, Tomar and Singh used transient transfection methods for NF-κB promoter activity. We also attempted transient co-transfection of the NF-κB promoter gene. Even though we compensated for the transfection efficiency by co-transfection of the *Renilla luciferase* gene, we switched over to the cell line system for our experiments. The usage of stable cell line for NF-κB promoter activity ruled out transfection variations in these assays. Third, some Trim mechanisms can have opposite effects according to the target protein. For example, Trim38 acts as a negative regulator when targeting TRAF6 (Zhao et al., 2012), while it acts as a positive regulator when targeting TAB2 (Hu et al., 2014). Trim21 also acts as a negative regulator targeting IKKβ (Niida et al., 2010), while it acts as a positive regulator targeting TAK (McEwan et al., 2013). This possibility cannot be excluded for Trim13 as well.

Protein ubiquitination is a post-translational modification tagging the ubiquitin to the Lys residues of target proteins. Ubiquitin chains linked through different lysine residues have various functions (Chen and Sun, 2009). For example, K48-linked chains act as a signal by targeting substrates for proteasomal degradation. In contrast, K63-linked chains are involved in various signaling pathways. Control of TRAF6 activity also depends largely on K63-linked polyubiquitination. Hence, regulation of TRAF6 polyubiquitination is an important method of controlling NF-κB signaling. Even though the roles of K48- or K63-linked ubiquitin chains

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have been the most widely studied, there is increasing evidence that other types of ubiquitin chains also target substrates for proteasomal degradation (Bedford et al., 2011), protein trafficking (Yuan et al., 2014), and immune signaling (Liu et al., 2014). However, although it has been detected in yeast and mammals, the role of K29-linked ubiquitin chains is poorly understood.

Some reports have suggested the importance of K29-linked polyubiquitination. Specifically, Itch/AIP4 was shown to mediate deltex degradation by K29-linked polyubiquitin chain formation (Chastagner et al., 2006), and Smurf1 regulates Wnt/β-catenin signaling through K29-linked ubiquitin chain formation of axin (Fei et al., 2013). Recently, Kristariyano *et al.* (Kristariyanto et al., 2015) and Michel *et al.* (Michel et al., 2015) suggested the existence of K29-linked polyubiquitin as well. However, no studies have investigated the K29-linked polyubiquitin chain formation of TRAF6 protein and its function. Therefore, we believe that Trim13 accelerating NF-κB activity by K29-linked polyubiquitin chain formation of TRAF6 in TLR2 signaling is an important discovery.

Overall, the results of this study showed that TLR2 stimulus increased Trim13 expression, which regulated foam cell formation following production of various cytokines and chemokines by TRAF6 K29-linked ubiquitin chain formation. This process was followed by NF-κB activation. Thus, Trim13 acts as a positive regulator of TLR2-induced NF-κB

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activation.

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Authorship Contributions

Participated in research design: Suk-Hwan Baek, and Bin Huang

Conducted experiments: Bin Huang

Performed data analysis: Bin Huang, and Suk-Hwan Baek

Wrote or contributed to the writing of the manuscript: Suk-Hwan Baek, and Bin Huang

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Footnotes

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Figure legends

Fig. 1. TLR agonists induce Trim13 gene expression. A, Raw 264.7 cells were treated with Pam_3CSK_4 (100 ng/ml) for the indicated times, after which the Trim13 mRNA levels were analyzed by RT-PCR. B, After monocytes were isolated from WT or TLR2 KO mouse bone marrow and differentiated into BMDMs, they were treated with Pam_3CSK_4 for the indicated times. Trim13 mRNA levels were determined by RT-PCR. C, Raw 264.7 cells were transfected with either control or TLR2 siRNA. The transfected cells were then treated with Pam_3CSK_4, HKLM, LPS, or CpG after which qPCR was performed using Trim13 primers. D, Raw 264.7 cells were treated with various TLR agonists, Pam_3CSK_4, HKLM, Poly I:C, LPS, flagellin, FSL1, ssRNA, and CpG, for 40 min. Trim13 mRNA expression was determined by RT-PCR and qPCR. β-actin was used as a control. The representative experiments were repeated in triplicate with similar results. *P<0.05 vs. the control.

Fig. 2. Trim13 regulates foam cell formation and cytokines/chemokines production *via* NF-κB. A, Raw 264.7 cells were co-transfected with either control siRNA or two types of Trim13 siRNA (#1, #2) with NF-κB-*Luc* promoter for 24 h. The transfected cells were then treated with Pam_3CSK_4 (100 ng/ml), after which qPCR was performed using Trim13 primers or samples were assayed luciferase activity. *P<0.05 vs. the control. B-C, Raw 264.7 cells

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were transfected as described above. The siRNA transfected cells were then treated with Pam_3CSK_4 for 40 min, after which cells were harvested and the mRNA of the indicated genes was measured using qPCR (B) and RT-PCR (C). β-actin was used as a control. *P<0.05 vs. the control. D, The siRNA transfected cells were then treated with Pam_3CSK_4 in the presence of oxLDL for 24 h and stained with oil red-O solution (magnification, x400). The representative experiments were repeated in triplicate with similar results.

Fig. 3. Trim13 binds and ubiquitinates TRAF6 and activates NF-κB. A, HEK293FT cells transfected Flag-tagged TRAF6, NEMO, or TAB1 with GFP-tagged Trim13. Total lysates were isolated from the transfected cells, IP was then conducted using Flag beads, and samples were subjected to electrophoresis and immunoblotting with anti-GFP antibody. B, HEK293FT cells were transfected with either GFP-tagged WT Trim13 or mutant (C10/13A). 24 h later, cells were harvested in RIPA buffer with high SDS concentrations. The samples were then diluted 10-fold with RIPA buffer, after which they were subjected to IP using anti-TRAF6 antibody, followed by electrophoresis and immunoblotting with anti-TRAF6 antibody. The ubiquitinated TRAF6 was detected by anti-HA antibody. C, TRAF6 was transfected with either WT Trim13 or mutant (C10/13A) in HEK293FT cells for 24 h. The cell lysates of transfected cells were subjected to *in vivo* ubiquitination assay by IP using Flag beads, after

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which the ubiquitinated TRAF6 was detected by anti-HA antibody. D, Flag-TRAF6 was transfected with either WT Trim13 or mutant (C10/13A) in HEK293FT cells for 24 h. IP was then conducted using Flag beads, and samples were subjected to electrophoresis and immunoblotting with anti-GFP antibody. E, HEK293FT cells were transfected with either Flag-tagged TRAF6 or NEMO with Trim13 and HA-Ub for 24 h. The cell lysates of transfected cells were then subjected to *in vivo* ubiquitination assay by IP using Flag beads, after which the ubiquitinated TRAF6 or NEMO was detected by anti-HA antibody. F, Flag-TRAF6 was co-expressed with either WT GFP-Trim13 or mutant (C10/13A) in NF-κB/293/GFP-*Luc* reporter cells for 24 h. Luciferase activity was measured at 24 h post-transfection. *$P<0.05$ vs. the control. TRAF6 and Trim13 expression was determined by immunoblotting using GFP or Flag antibody. G, Raw 264.7 cells were treated with Pam_3CSK_4 for the indicated times, after which TRAF6 ubiquitination was analyzed by *in vivo* ubiquitination assay. H, Raw 264.7 cells were transfected with either control siRNA or Trim13 siRNA for 24 h. The siRNA-transfected cells were then treated with Pam_3CSK_4 for 30 min, after which TRAF6 ubiquitination was analyzed by *in vivo* ubiquitination assay. Representative experiments were repeated in triplicate with similar results.

Fig. 4. Trim13 regulates TRAF6 ubiquitination and NF-κB activity *via* E3 ligase activity of

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the RING domain. A, Domain organization of Trim13. B, Flag-tagged TRAF6 was co-expressed with either GFP-tagged WT Trim13 or different deletion mutants in HEK293FT cells for an *in vivo* ubiquitination assay as described in the Fig. 3C legend. The protein expression of different Trim13 mutants was determined by immunoblotting using anti-GFP antibody. The protein expression of TRAF6 was determined by immunoblotting using anti-Flag antibody. C, NF-κB/293/GFP-*Luc* reporter cells were transfected with the indicated plasmids of Trim13 WT or each deletion mutants with Flag-tagged TRAF6. Luciferase activity was measured at 24 h post-transfection. The representative experiments were repeated in triplicate with similar results. *P<0.05 vs. the control.

Fig. 5. Trim13 ubiquitinates TRAF6 *via* K29-linked polyubiquitination chain. A, HEK293FT cells were transfected with a combination of Flag-tagged TRAF6, GFP-tagged Trim13, and various types of HA-Ub (wild-type (WT), or substitution of Lys with Arg (K29R, K48R, and K63R)). An *in vivo* ubiquitination assay was performed after 24 h and the expression of samples was determined by immunoblotting. B, HEK293FT cells were transfected with a combination of specific HA-Ub mutants (wild-type (WT), or Lys-linked (K29, K48, and K63)), Flag-TRAF6, and GFP-Trim13 for 24 h. The cell lysates of transfected cells were subjected to *in vivo* ubiquitination assay by IP using Flag beads, after which the ubiquitinated

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TRAF6 was detected by anti-HA antibody. The representative experiments were repeated in triplicate with similar results. C, NF-κB/293/GFP-*Luc* reporter cells were transfected WT or ubiquitin mutants (K29 or K29R) with Flag-TRAF6 and GFP-Trim13 combination. Luciferase activity was measured at 24 h post-transfection. The representative experiments were repeated in triplicate with similar results. The representative experiments were repeated in triplicate with similar results. *P<0.05 vs. the control.

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Table 1. RT-PCR primers used in the study

Trim13	Forward	AGGAGCAGCGGATGGCCTTCAACAT
	Reverse	AGTGAACACACCTGTGTCTTGCGGC
TNF-α	Forward	TTCTGTCTACTGAACTTCGGGGTGATCGGTCC
	Reverse	GTATGAGATAGCAAATCGGCTGACGGTGTGGG
IL-1β	Forward	GAAGCTGTGGCAGCTACCTGTGTCT
	Reverse	CTCTGCTTGTGAGGTGCTGATGTAC
IL-6	Forward	TTCACAGAGGATACCACTCC
	Reverse	GTTTGGTAGCATCCATCATT
CCL2	Forward	AGAGAGCCAGACGGGAGGAA
	Reverse	GTCACACTGGTCACTCCTAC
CCL3	Forward	ACTGCCCTTGCTGTTCTTCTCT
	Reverse	AGGCATTCAGTTCCAGGTCAGTGA
CCL4	Forward	AAACCTAACCCCGAGCAACA
	Reverse	CCATTGGTGCTGAGAACCCT
TGF-β	Forward	ATTCAGCGCTCACTGCTCTT
	Reverse	TCTCTGTGGAGCTGAAGCAA
CXCL2	Forward	GAACTGCGCTGTCAATGCCT
	Reverse	GTTAGCCTTGCCTTTGTTCA
TF	Forward	GAAGGATGTGACCTGGGCC
	Reverse	GTACCATTCTTTCTGACTAA
LOX-1	Forward	AGGTCCTTGTCCACAAGACTGG
	Reverse	ACGCCCCTGGTCTTAAAGAATTG
β-actin	Forward	TCCTCCGTTGCCGGTCCACA
	Reverse	CGTCTCCGGACTCCATCACA

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Table 2. qPCR primers used in the study

Trim13	Forward	AGGAGCAGCGGATGGCCTTCAACAT
	Reverse	AGTGAACACACCTGTGTCTTGCGGC
TNF-α	Forward	TTCTGTCTACTGAACTTCGGGGTGATCGGTCC
	Reverse	GTATGAGATAGCAAATCGGCTGACGGTGTGGG
IL-1β	Forward	GAAGCTGTGGCAGCTACCTGTGTCT
	Reverse	CTCTGCTTGTGAGGTGCTGATGTAC
IL-6	Forward	TTCACAGAGGATACCACTCC
	Reverse	GTTTGGTAGCATCCATCATT
CCL2	Forward	AGAGAGCCAGACGGGAGGAA
	Reverse	GTCACACTGGTCACTCCTAC
CCL3	Forward	ACTGCCCTTGCTGTTCTTCTCT
	Reverse	AGGCATTCAGTTCCAGGTCAGTGA
CCL4	Forward	AAACCTAACCCCGAGCAACA
	Reverse	CCATTGGTGCTGAGAACCCT
β-actin	Forward	TCCTCCGTTGCCGGTCCACA
	Reverse	CGTCTCCGGACTCCATCACA

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Table 3. List of up-regulated genes in the microarray analysis

Affy_Probe Set ID	Gene Symbol	Fold change
1417483_at	Nfkbiz	288.52
1418835_at	Phlda1	204.84
1436329_at	Egr3	45.05
1427348_at	Zc3h12a	42.55
1417262_at	Ptgs2	41.95
1450826_a_at	Saa3	35.74
1420331_at	Clec4e	35.67
1437199_at	Dusp5	28.19
1417930_at	Nab2	25.35
1420353_at	Lta	23.85
1419561_at	Ccl3	22.63
1419607_at	Tnf	21.49
1419212_at	Icosl	21.40
1450698_at	Dusp2	19.65
1433699_at	Tnfaip3 (A20)	18.95
1424067_at	Icam1	18.63
1448793_a_at	Sdc4	16.51
1418847_at	Arg2	16.32
1450165_at	Slfn2	15.75
1458299_s_at	Nfkbie	15.02
1452521_a_at	Plaur	14.75
1428834_at	Dusp4	14.38
1419647_a_at	Ier3	12.59
1416010_a_at	Ehd1	12.04
1440346_at	Jmjd3	11.43
1425412_at	Nlrp3	11.00

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Table 4. List of up-regulated genes in the microarray analysis

Affy_Probe Set ID	Gene Symbol	Fold change
1415899_at	Junb	10.71
1417065_at	Egr1	9.74
1456212_x_at	Socs3	8.24
1447432_s_at	Zfp263	8.14
1420591_at	Gpr84	7.45
1434431_x_at	Adora2b	6.93
1448724_at	Cish	6.93
1435626_a_at	Herpud1	6.61
1417406_at	Sertad1	5.94
1418572_x_at	Tnfrsf12a	5.22
1425902_a_at	Nfkb2	5.22
1440104_at	Ranbp2	4.61
1443086_at	Alcam	4.54
1422924_at	Tnfsf9	4.23
1454676_s_at	Ticam1	3.92
1416029_at	Klf10	3.68
1460004_x_at	Stx6	3.55
1418401_a_at	Dusp16	3.52
1456381_x_at	Mcl1	3.39
1427689_a_at	Tnip1	3.32
1417888_at	Trim13	3.25
1459488_at	Zc3h13	3.03
1431218_at	Zdhhc20	2.95
1425837_a_at	Ccrn4l	2.87
1434980_at	Pik3r5	2.86
1420888_at	Bcl2l1	2.85

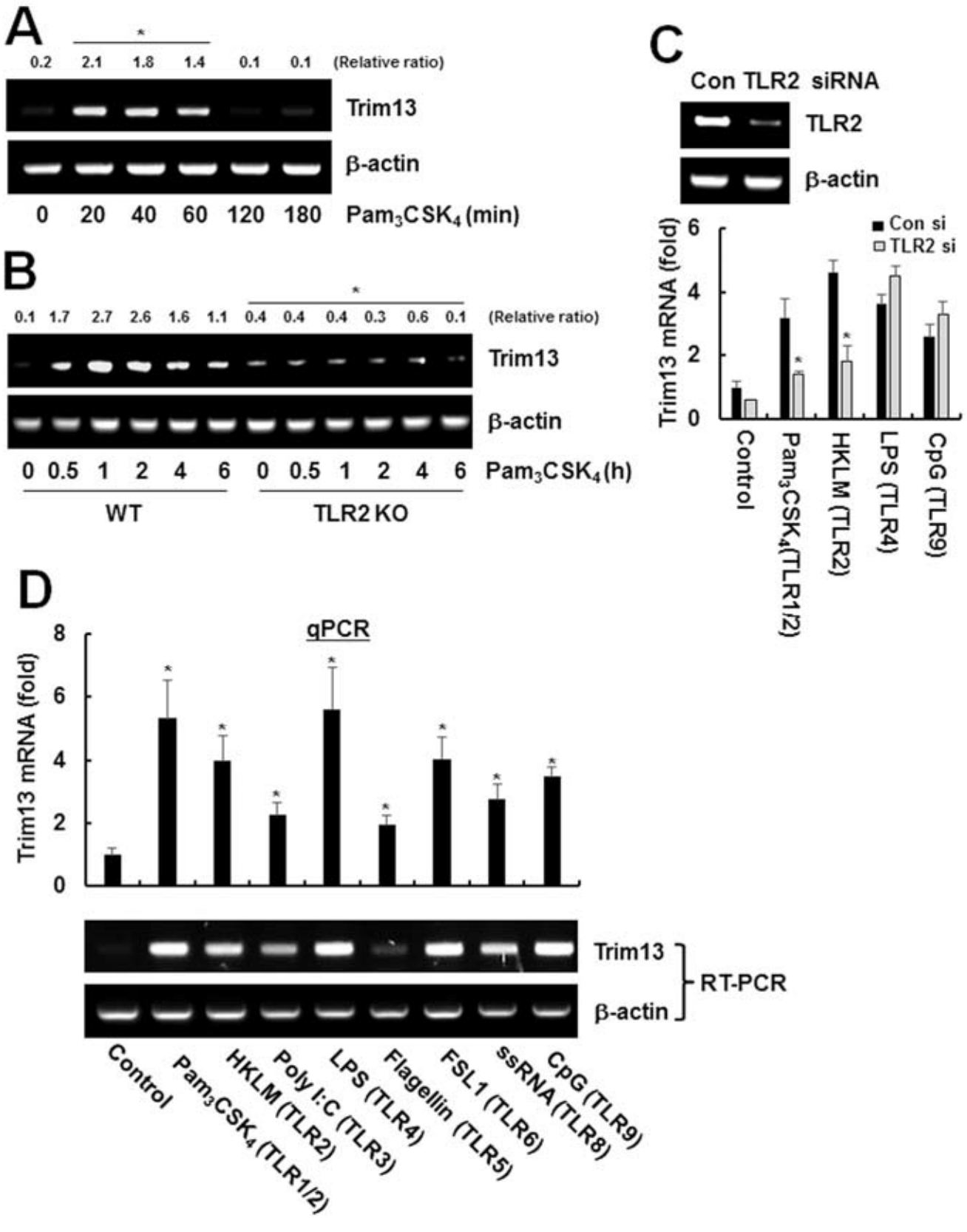
A
0.2 2.1 1.8 1.4 0.1 0.1 (Relative ratio)
Trim13
β-actin
0 20 40 60 120 180 Pam3CSK4 (min)
B
0.1 1.7 2.7 2.6 1.6 1.1 0.4 0.4 0.4 0.3 0.6 0.1 (Relative ratio)
Trim13
β-actin
0 0.5 1 2 4 6 0 0.5 1 2 4 6 Pam3CSK4 (h)
WT
TLR2 KO
C
Con TLR2 siRNA
TLR2
β-actin
Trim13 mRNA (fold)
Con si
TLR2 si
Control
Pam3CSK4 (TLR1/2)
HKLM (TLR2)
LPS (TLR4)
CpG (TLR9)
D
qPCR
Trim13 mRNA (fold)
Trim13
β-actin
RT-PCR
Control
Pam3CSK4 (TLR1/2)
HKLM (TLR2)
Poly I:C (TLR3)
LPS (TLR4)
Flagellin (TLR5)
FSL1 (TLR6)
ssRNA (TLR8)
CpG (TLR9)

Figure 1

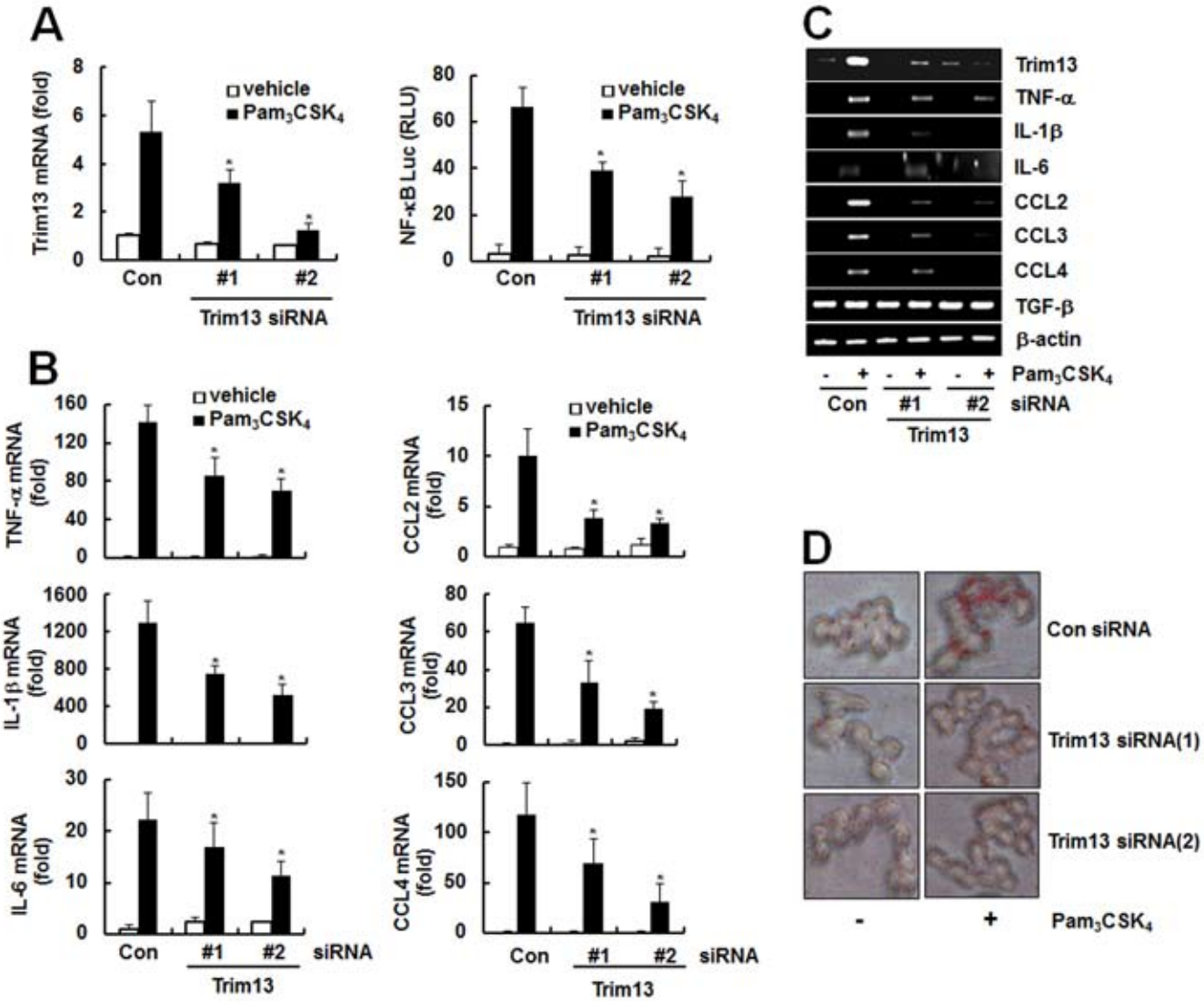
A
Trim13 mRNA (fold)
vehicle
Pam3CSK4
Con
#1
#2
Trim13 siRNA
NF-κB Luc (RLU)
B
TNF-α mRNA (fold)
CCL2 mRNA (fold)
IL-1β mRNA (fold)
CCL3 mRNA (fold)
IL-6 mRNA (fold)
CCL4 mRNA (fold)
siRNA
C
Trim13
TNF-α
IL-1β
IL-6
CCL2
CCL3
CCL4
TGF-β
β-actin
D
Con siRNA
Trim13 siRNA(1)
Trim13 siRNA(2)

Figure 2

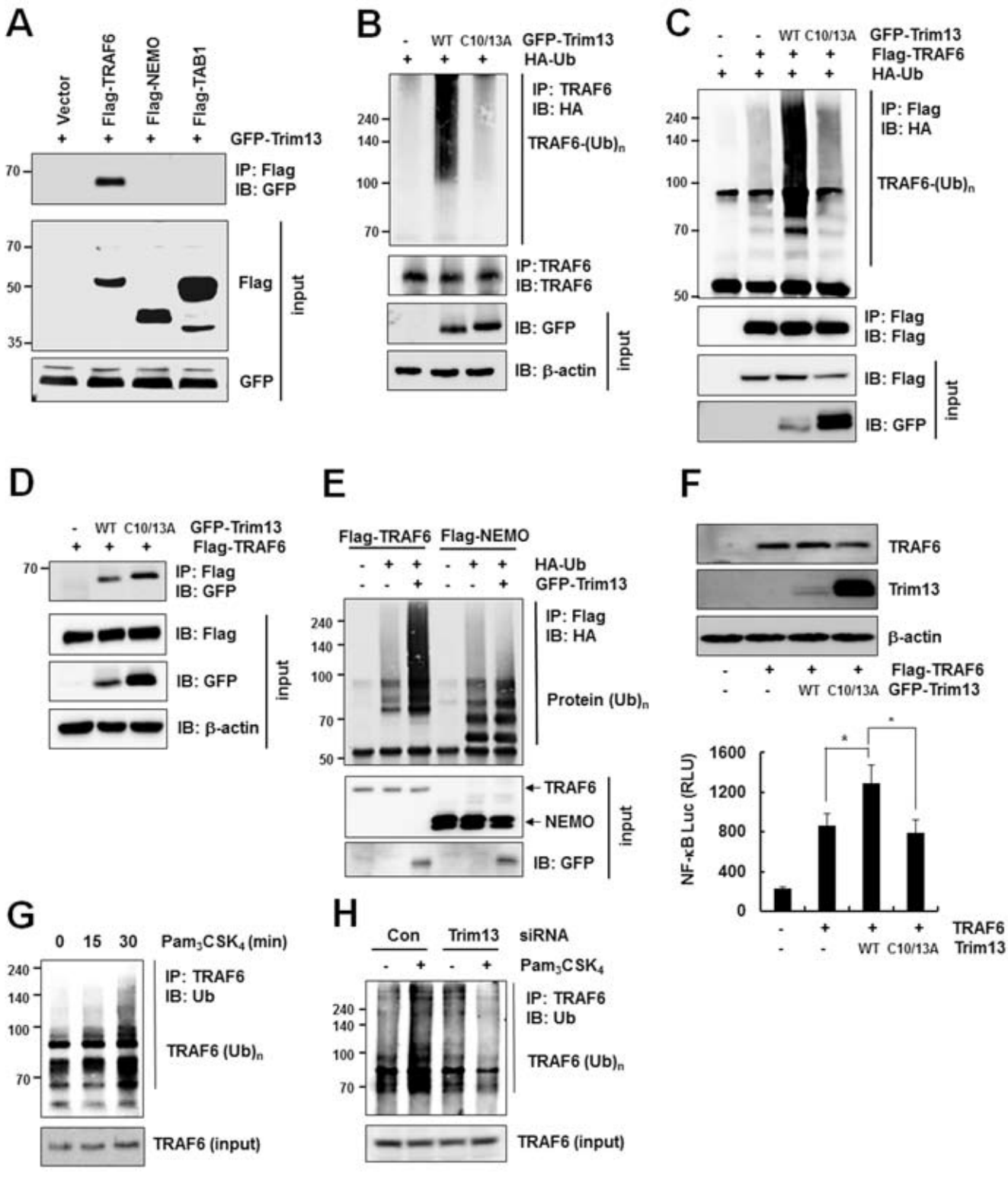
A
+ Vector
+ Flag-TRAF6
+ Flag-NEMO
+ Flag-TAB1
GFP-Trim13
70
IP: Flag
IB: GFP
70
50
35
Flag
GFP
input
B
- WT C10/13A GFP-Trim13
+ + + HA-Ub
240
140
100
70
IP: TRAF6
IB: HA
TRAF6-(Ub)n
IP:TRAF6
IB:TRAF6
IB: GFP
IB: β-actin
input
C
- - WT C10/13A GFP-Trim13
- + + + Flag-TRAF6
+ + + + HA-Ub
240
140
100
70
50
IP: Flag
IB: HA
TRAF6-(Ub)n
IP: Flag
IB: Flag
IB: Flag
IB: GFP
input
D
- WT C10/13A GFP-Trim13
+ + + Flag-TRAF6
70
IP: Flag
IB: GFP
IB: Flag
IB: GFP
IB: β-actin
input
E
Flag-TRAF6 Flag-NEMO
- + + - + + HA-Ub
- - + - - + GFP-Trim13
240
140
100
70
50
IP: Flag
IB: HA
Protein (Ub)n
TRAF6
NEMO
IB: GFP
input
F
TRAF6
Trim13
β-actin
- + + + Flag-TRAF6
- - WT C10/13A GFP-Trim13
NF-κB Luc (RLU)
1600
1200
800
400
0
*
*
- + + + TRAF6
- - WT C10/13A Trim13
G
0 15 30 Pam3CSK4 (min)
240
140
100
70
IP: TRAF6
IB: Ub
TRAF6 (Ub)n
TRAF6 (input)
H
Con Trim13 siRNA
- + - + Pam3CSK4
240
140
100
70
IP: TRAF6
IB: Ub
TRAF6 (Ub)n
TRAF6 (input)

Figure 3

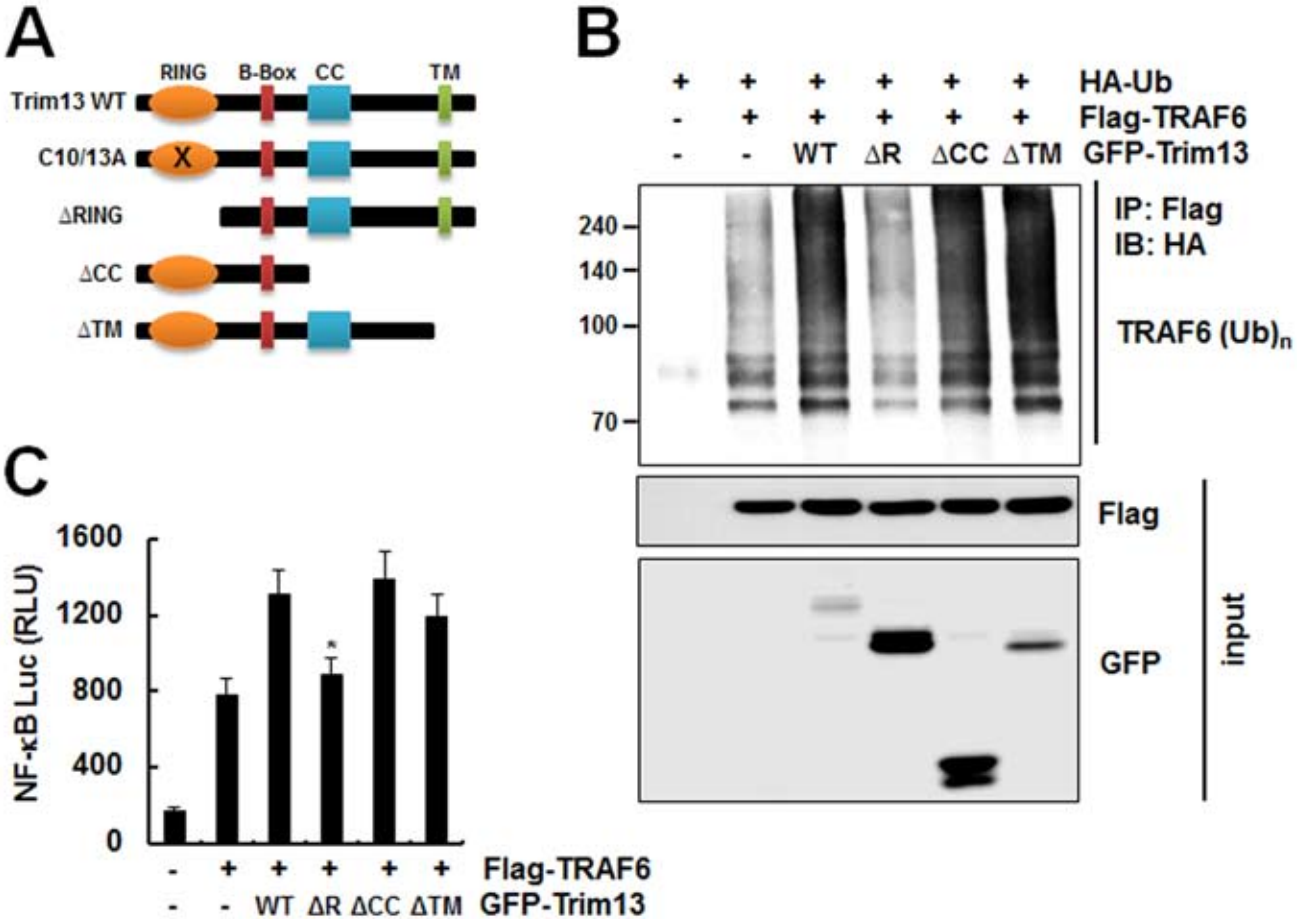
A
RING
B-Box
CC
TM
Trim13 WT
C10/13A
X
ΔRING
ΔCC
ΔTM
B
HA-Ub
Flag-TRAF6
GFP-Trim13
WT
ΔR
ΔCC
ΔTM
240
140
100
70
IP: Flag
IB: HA
TRAF6 (Ub)n
Flag
GFP
input
C
NF-κB Luc (RLU)
1600
1200
800
400
0
Flag-TRAF6
GFP-Trim13
WT
ΔR
ΔCC
ΔTM

Figure 4

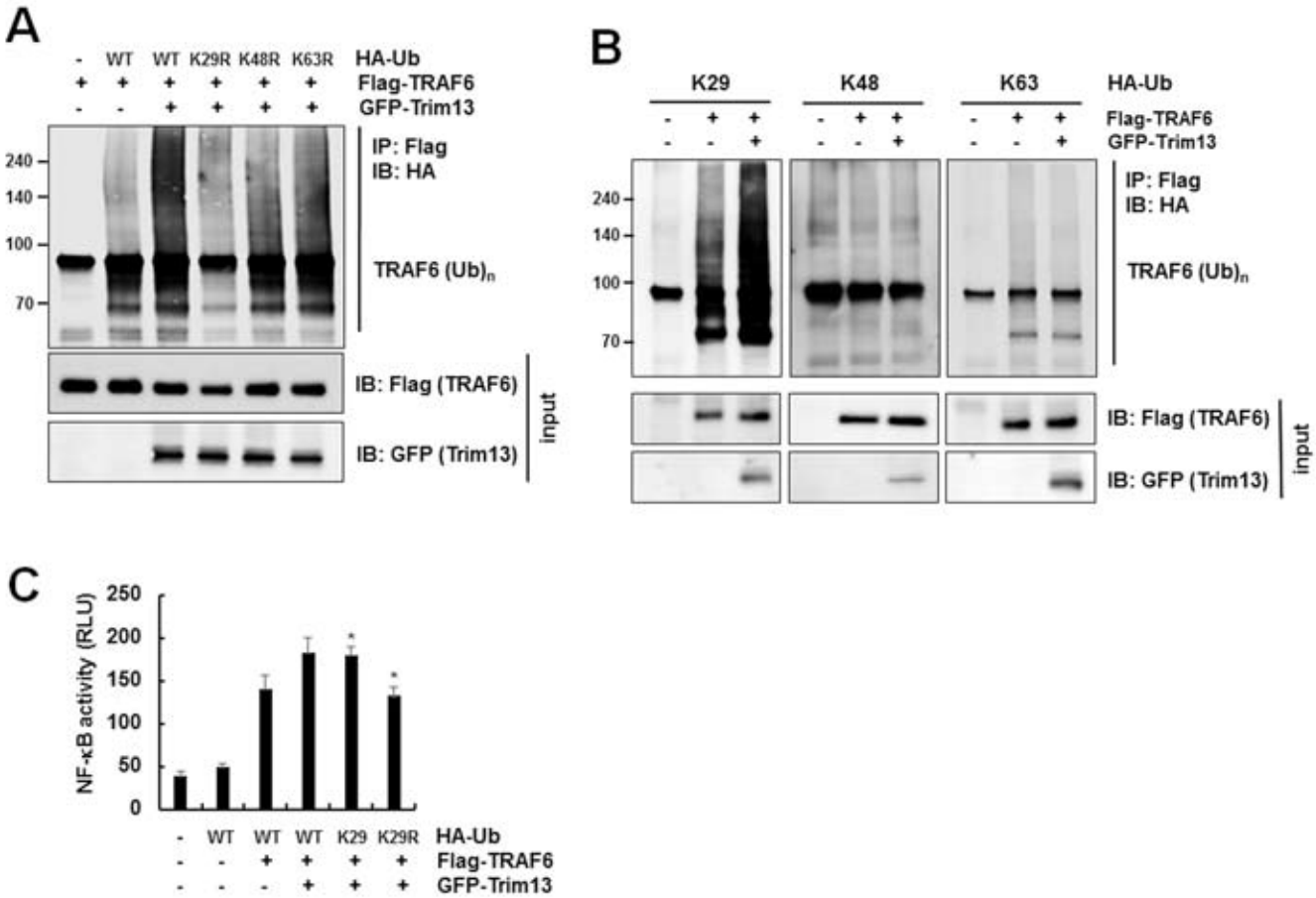
A
- WT WT K29R K48R K63R HA-Ub
+ + + + + + Flag-TRAF6
- - + + + + GFP-Trim13
240
140
100
70
IP: Flag
IB: HA
TRAF6 (Ub)n
IB: Flag (TRAF6)
IB: GFP (Trim13)
input
B
K29
K48
K63
HA-Ub
Flag-TRAF6
GFP-Trim13
IP: Flag
IB: HA
TRAF6 (Ub)n
IB: Flag (TRAF6)
IB: GFP (Trim13)
input
C
NF-κB activity (RLU)
250
200
150
100
50
0
- WT WT WT K29 K29R HA-Ub
- - + + + + Flag-TRAF6
- - - + + + GFP-Trim13

Figure 5