Title: TNF-α-induced E3 ligase, TRIM15 inhibits TNF-α-regulated NF-κB pathway by promoting turnover of K63 linked ubiquitination of TAK1

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A B S T R A C T

Ubiquitin E3-ligases are recruited at different steps of TNF-α-induced NF-κB activation; however, their role in temporal regulation of the pathway remains elusive. The study systematically identified TRIMs as potential feedback regulators of the TNF-α-induced NF-κB pathway. We further observed that TRIM15 is "late" response TNF-α-induced gene and inhibits the TNF-α-induced NF-κB pathway in several human cell lines. TRIM15 pro- motes turnover of K63-linked ubiquitin chains in a PRY/SPRY domain-dependent manner. TRIM15 interacts with TAK1 and inhibits its K63-linked ubiquitination, thus NF-κB activity. Further, TRIM15 interacts with TRIM8 and inhibits cytosolic translocation to antagonize TRIM8 modualted NF-κB. TRIM8 and TRIM15 also show func- tionally inverse correlation in psoriasis condition. In conclusion, TRIM15 is TNF-α-induced late response gene and inhibits TNF-α induced NF-κB pathway hence

a feedback modulator to keep the proinflammatory NF-κB pathway under control.

Keywords: TNF-α NF-κB TRIM15 TRIM8 Ubiquitination Functional antagonism

1. Introduction

The NF-κB family of transcription factors are activated by a range of pathophysiological stimuli including viral and bacterial factors (LPS, dsRNA), antigen receptors, DNA damage, reactive oxygen species (ROS), and cytokines (TNF-α, IL-1), [1–3]. Activated NF-κB promotes tran- scription of a several target genes that include growth factors, chemo- kines, cytokines, immune modulators, regulators of apoptosis, acute response genes, cell adhesion molecules [1,3]. NF-κB pathway is tightly regulated by post-transcriptional and post-translational regulatory mechanisms, however, its dysregulation leads to prolonged activation of NF-κB and chronic inflammatory conditions hence critical for organ- ismal survival and fitness [4].

Pleiotropic cytokine TNF-α is an activator of the NF-κB pathway and inflammation [1,5]. The increased level of TNF-α and persistent acti- vation of proinflammatory NF-κB is observed in many pathological conditions including cancer [6–9]. TNF-α activated NF-κB target genes have been classified in 'early' 'mid' and 'late' response genes based on their temporal expression pattern [10]. Some of the early response genes like NFKBIA (IκBα) and TNFIP3 (A20) act as negative feedback regula- tors of the pathway and dynamics of gene expression by controlling NF- κB oscillations [10–12]. The modulation of the NF-κB pathway by late responsive genes and its implication in feedback regulation to restrict inflammation had not yet been explored.

Ubiquitination plays a critical role in the regulation of TNF-α medi- ated NF-κB pathway. cIAP1/2 mediated Lys-63-linked poly- ubiquitination of RIP1, recruits TAK1 [5,13]. After recruiting TAK1 to the complex, TAK1 is K(Lysine)-63-linked polyubiquitinated and acti- vated to recruit IκB kinase (IKK) complex. Dual phosphorylation of IκBα by IKK complex leads its K48-

linked polyubiquitination by $SCF^{\beta TrCP}$ ubiquitin ligase complex [5,13] which is degraded through ubiquitin proteasome system (UPS). Removal of K63-linked ubiquitin chains by deubiquitinase (DUB) like A20 and CYLD negatively regulates NF- κBactivation and plays a pivotal role in modulating NF-κB pathway [4,5,13]. TNF-α stimulation also promotes the expression of several E3 ligases including various TRAFs, cIAPs, and XIAP and most of these modifiers positively regulate the pathway [1]. Surprisingly to date, no TNF-α-induced E3 ligase had been shown regulating the pathway in a negative feedback mechanism.

Ubiquitin E3 ligases identify unique substrates and promote their ubiquitination and topology of the ubiquitin chain assembled on the substrate determines their fate.*Tripartite Motif containing* (TRIM) pro- teins are RING E3 ligases characterized by the signature motif composed of RING, B-Box, and Coiled-coil domain. They are further subclassified based on the presence of the variable C-terminal domain [14,15]. The roles of these proteins are emerging in innate immune regulation, viral restriction, and autophagy [16,17]. The reports from our group and others had shown that TRIM8 and TRIM38 acts as positive and negative (respectively) regulators of the TNF-α and IL1β activated NF-κB pathway [18–20]. Besides, TRIMs have also been identified as regulators of NF-κB in response to diverse stimuli, however, TRIM mediated negative feed- back modulation of TNF-α regulated-κB is not well understood [16,17]. In this study, we systemically identified potential feedback regula- tors of the TNF-α-induced NF-κB pathway and further characterized the role of TRIM15 in negative regulation of TNF-α-induced NF-κB pathway.

2. Materials & methods

2.1. *Cells and reagent*

HEK293, MCF-7 and MDA-MB-231 cells were grown at 37 °C, 5% CO_2 in Dulbecco's Modified Eagle's Medium (DMEM, Cyclone, GE, USA)

and Minimal Essential Media (MEM, HyClone, GE, USA) supplemented with 10% (*v*/v) heat-

inactivated fetal bovine serum (Gibco, Thermo Fisher Scientific, USA) and 1% penicillin, streptomycin, and neomycin (PSN) antibiotic mixture (Gibco, Thermo Fisher Scientific, USA). TRIM15 in pCDNA-3 and all TRIM15-YFP constructs in pZsYellow1-N1 was provided by Dr. Walther H. Mothes and Dr. Pradeep Uchil (Sec- tion of Microbial Pathogenesis, Yale School of Medicine, USA) [26]. HA- TRIM8 in pCGN-HA was gifted by Dr. S. Hatakeyama (Department of Biochemistry, Hokkaido University Graduate School of Medicine) [34]. Flag-TAK1 was provided by Dr. Yan-Yi Wang [49]. Control and TRIM15- siRNA were purchased from Qiagen.

Primary antibodies Anti-HA-HRP, Anti-FLAG-HRP were purchased from Sigma, USA. Mouse polyclonal against ß-Actin and Ubiquitin was from SantaCruz, USA, Rabbit polyclonal against GFP from Abclonal (Woburn, MA, United States) and p-IκBα was from Cell signaling tech- nology, USA. TRIM15 was purchased from St John's Laboratory (United Kingdom). HRP-conjugated secondary antibodies; anti-rabbit and anti- mouse were purchased from Jackson ImmunoResearch, USA. Recom- binant Human TNF-α was purchased from Milteny Biotech, Germany, and PeproTech, USA. MG132, Bafilomycin A1, EZview™ Red Anti-HA Affinity Gel, and M2 FLAG-Affinity Gel were purchased from Sigma- Aldrich, USA. Lipofectamine® 3000 (Invitrogen, USA) was used for siRNA transfection. Dual Glow Luciferase Reporter Kits were purchased from Promega, USA.

2.2. *Gene expression analysis of TRIM family proteins*

HEK293 cells were treated with TNF-α (10 ng/ml) for 10 h and collected in RNA ISO plus reagent (Takara, Japan). Total RNA was iso- lated using RNAiso Plus Reagent and was reverse transcribed to syn- thesize cDNA using PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan) according to the manufacturer's protocol. The expression of TRIMs were analyzed by quantitative Real-Time PCR using TaqMan probes specific for the indicated TRIM gene (Applied Biosystems, Inc., USA). Data were processed using DataAssist v3.01 (Applied Biosystems, Inc., USA). 18S rRNA was used as endogenous control and fold change values ($2^{-\Delta\Delta ct}$) were plotted.

Similarly, mRNA expression of various TRIMs was reconfirmed using Real-time PCR by SYBR Premix Ex Taq II (Tli RNase H Plus) (Takara, Japan) as per the manufacturer's instruction. β-Actin and GAPDH genes were used as multiple endogenous control and expression of indicated genes were calculated using QuantStudio 3 and 5 system's Design and Analysis Software v1.5.1. fold change values ($2^{-\Delta\Delta ct}$) of a minimum of three independent biological replicates were plotted.

Specific primers for the genes are listed in Supplementary Table 1.

2.3. *NF-κB luciferase reporter assay*

Dual-Glo luciferase assay system was used for detecting NF-κB ac- tivity (Promega, USA) as described previously [19]. HEK293 cells were seeded at a density of 2.5×10^5 cells per well in 12 well plates. siRNA
targeting specific TRIMs or indicated vectors were co-transfected with
NF-κB firefly and Renilla luciferase reporter constructs. siRNA and vector co-transfection was done using Lipofectamine 3000 following the manufacturer's protocol. Transfected cells were treated with TNF-α for 10 h or indicated time points and Firefly/Renilla activity was measured by following the manufacturer's protocol using BioTek Synergy HTX multimode plate reader. The Firefly/Renilla ratio was plotted to show the activation of the NF-κB pathway.

2.4. *Fluorescence/confocal microscopy*

HEK293 cells were seeded at a density of 1.5×10^5 cells on coverslips in 24 well plates. Cells were transfected with TRIM15-YFP or TRIM15-
RFP. After 24 h of transfection, cells were treated with indicated chemicals and fixed with 4% Paraformaldehyde (PFA). Coverslips were removed from 24 well plates and slides were prepared using SlowFade™ Gold Antifade Mountant (Thermo, USA). Images were acquired using Nikon Eclipse

Ti2 Inverted Microscope and processes using NIS- Elements Advanced Research software (Nikon, Japan). Similarly, slides were prepared, and images were acquired using Nikon Confocal Microscope A1R HD25 (Nikon, Japan).

Automated image analysis was performed using NIS-Elements Advanced Research software. For measurement of mean fluorescent intensity (MFI) and binary area "field measurement" feature of the "Automated image analysis" tool was used. Acquisition and measure- ment parameters for all YFP images were kept the same for all the im- ages. Similarly, the "object count" feature of the "Automated image analysis" tool was used for counting the YFP puncta of TRIM15.

2.5. *Nuclear-cytoplasmic fractionation*

The nuclear and cytosolic fractions were prepared as described previously [19] with minor modifications. HEK293 cells were plated at a density of 1×10^6 in a 60-mm^2 dish and transfected with indicated vectors. After 24 h of transfection, the cells were treated with indicated chemicals. Cells were washed with DPBS (Hyclone, GE, USA) and resuspended in 300 μl of buffer-A (10 mM HEPES buffer, pH 7.9, 0.1 mM EDTA, 10 mM KCl, 0.4% (*v*/v) NP40, 0.5 mM dithiothreitol (DTT), and 1× protease inhibitor cocktail (Roche, Germany) and incubated on ice for 30 min and lysed. Lysates were centrifuged at 15,000*g* for 15 min at4 ˚C and the supernatant was collected as the cytosolic fraction. Pellets were washed three times with buffer-A and resuspended in 70 μl of ice-cold buffer-B (20 mM HEPES buffer, pH 7.9, 400 mM NaCl, 1 mM EDTA, 1 mM DTT, and 1× protease inhibitor cocktail). The resuspended pellet was subjected to a high-speed vortex twice at an interval of 30 min. The nuclear lysate was centrifuged at 15000*g* for 15 min at 4 ˚C and the supernatant was collected as the nuclear fraction. Protein concentrationwas measured by Bradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio-Rad, USA) and an equal amount of proteins (for both cytosol and nuclear fraction) were resolved on 10.5%

SDS-PAGE. Pro- tein expressions in nuclear and cytosolic fractions were analyzed by western blotting using specified antibodies.

2.6. *Western blotting*

To detect the levels of HA/FLAG/GFP tagged proteins, p-IκBα, and ubiquitin western blotting was performed. HEK293 and MCF-7 cell were seeded at a density of 2.5× 10^5 per well in 12 well plates and indicated vectors were transfected using the standard calcium phos- phate transfection method [50]. After 24 h of transfection cells wertreated as indicated and harvested in ice-cold PBS. Cells were lysed in lysis buffer (100 mM NaCl, 50 mM Tris–HCl, 1% Triton-X 100, and 1× protease inhibitor cocktail). Protein concentration was determined byBradford assay (Bio-Rad Protein Assay Dye Reagent Concentrate, Bio- Rad, USA) and an equal amount of proteins were resolved on 10.5% SDS-PAGE. Proteins were electro-blotted on PVDF membrane (Immun- Blot® PVDF Membrane, Bio-Rad, USA) at 110 V for 1 h at 4 ˚C. Themembrane was blocked with 5% blocking buffer (5% non-fat dried milk and 0.1% Tween-20 in TBS) for 1 h at room temperature and incubated with primary antibody overnight at 4 ˚C. After incubation membrane was washed three times with TBS-T (TBcontaining 0.1% Tween-20)and incubated with a secondary antibody at room temperature for 1 h and proteins were detected by using Clarity Western ECL Substrates (Bio-Rad, USA) and exposing to X-ray film or using ChemiDoc MP Im- aging System (Bio-Rad, USA).

2.7. *Co-immunoprecipitation*

To study the protein interaction and ubiquitin conjugation of Flag- tagged proteins, co-immunoprecipitation experiments were performed. HEK293 cells were plated at a density of 2 × 10^6 per 90-mm-diameter

dish and transfected with Flag-TAK1 or HA-TRIM8 in combination with indicated vectors using the calcium phosphate transfection method [50]. After 36 h of transfection, cells were treated with specified chemicals and incubated for 10 h. After treatment, cells were harvested, washed with ice-cold PBS (Hyclone, GE, USA), and lysed in immuno-precipitation buffer (100 mM NaCl, 50 mM Tris–HCl, 1% Triton-X 100, and 1× protease inhibitor cocktail). The cell lysates were incubated with HA or FLAG-Affinity Gel (Sigma, USA) on a roller shaker overnight at 4 °C. The gel beads were washed four times with IP buffer, resuspended in 5× SDS-PAGE sample buffers and separated on 10.5% SDS-PAGE, and analyzed by western blotting using specific antibodies.

2.8. *Gene expression analysis from GEO datasets, CCLE database, and Methylation profile of cancer cell lines*

We explored the Gene Expression Omnibus dataset (GEO) GDS3809 for TRIM15 expression (probe set: 1451916_s_at). The microarray expression values of TRIM15 were plotted as mRNA expression for indicated time points [23]. Similarly, we explored the GDS4062 dataset for expression of TRIM15 (probe set: 36742_at, 210885_at, and 210177_at) and TRIM8 in normal, psoriasis lesional, and non-lesional dataset. Microarray transformed counts were plotted for mRNA expression for each probe set separately. We retrieved mRNA expression data of TRIM15 (both microarray and RNAseq) across various cancer cell lines from the CCLE database [22]. The expression data scatter plots were retrieved for methylation status (X-axis) vs mRNA expression (Y-axis).The methylation status of TRIM15 as bubble plots for breast cancer cell lines was retrieved using the “CpG Methylation Viewer” module of the CCLE database and presented as it is.

2.9. *Statistical analysis*

Data are shown as mean ± SEM for n observations. Comparisons of groups were performed using one-way ANOVA (Newman-Keuls post-test) to determine the levels of significance for each group. The exper- iments have been repeated a minimum of three times independently and probability values of $p < 0.05$ were considered as statistically significant. The data were normalized as the maximum value was considered

as 100% and 0 as 0% for all data set.

1. Results

1.1. *Late response TNF-α induced TRIM15, inhibits TNF-α-induced NF-κB pathway*

TNF-α-induced temporal expression of NF-κB target genes are crucial for optimal inflammatory response and resolution of inflammation. We performed a two-step screening strategy to identify possible feedback regulators of the TNF-α-induced NF-κB pathway. Firstly, we analyzed expression of TRIMs in TNF-α treated HEK293 at 10 h to identify late expressing genes. The mRNA expression of several TRIMs (TRIM1, 2, 3, 8, 9, 15, 16,21, 31, 37, 38, 39, 41, 44, 46, 47 and 55) increased >2 folds

in TNF-α treated cells (Supplementary Fig. 1A). We reconfirmed the

expression of TRIM1, 2, 15, and 16 using a different set of primers in HEK293 cells (Supplementary Fig. 1B).

The early response genes TNFAIP3 and IκBα are the only NF-κB activated genes known to negatively regulate NF-κB [3,4]. We hypoth- esized that TRIMs transcribed during 'Late' response may regulate NF- kBn pathway in feedback manner. Therefore, we analyzed the role of TNF-α-

induced TRIMs in the regulation of NF-κB pathway. In the second step, we knocked down TNF-α upregulated TRIMs using siRNA and monitored NF-κB reporter activity in the presence/absence of TNF-α. The knockdown of TRIM1, 2, 15, 46, 47, 48, and 55 enhanced TNF- α-induced NF-κB activation, whereas TRIM37 knockdown reduced the activity (Supplementary Fig. 1C). These results indicate that TNF- α-induced late response TRIMs act as negative regulators of the TNF-α-induced NF-κB pathway.

A previous study exploring DNA methylation patterns associated with gastric cancer had identified TRIM15 as a hyper-methylated gene [21], hence we analyzed its methylation pattern and its correlation with expression. Firstly, we checked the expression and methylation of TRIM15 in various cancer cell lines using Cancer Cell Line Encyclopedia (CCLE) database [22]. Interestingly, the TRIM15 promoter region was highly methylated in most cancer cell lines except colorectal, pancreatic, and stomach cancer cell lines (Supplementary Fig. 2A, B, C & D). In consonance, TRIM15 mRNA expression was only observed in the colo- rectal, pancreatic, and stomach cancer cell lines (Supplementary Fig. 2A, B & C). Therefore, we reanalyzed the expression of TRIM15 during the "Mid" (4 h) and "Late" (10h) response using qRT-PCR in HEK293 (Fig. 1A) and MCF-7 (Fig. 1C) cells. TRIM15 mRNA transcript levels increased 6-fold after 10 h of TNF-α treatment in both the cell lines (Fig. 1A & C). Besides, while exploring GEO datasets, we also found that cultured aortic smooth muscle cells (SMCs) stimulated with TNF-α show increased expression of TRIM15 at 2 h, and the highest expression was observed at 6 h (Supplementary Fig. 2E) [23]. These results suggest that TRIM15 is primarily a late response TNF-α-induced gene.

Next, we checked protein levels of TRIM15 in TNF-α treated cells. We observed a temporal increase in TRIM15 protein levels in HEK293 (Fig. 1B). In MCF7 cells (Fig. 1D) TNF-α induced TRIM15 at 24 h which reduced at 48 h, whereas MDA-MB-231 (Fig. 1E) cells treated with TNF- α increased TRIM15 protein levels at both 24 and 48 h. Besides TNF-α treatment, we observed increased TRIM15 protein levels in MCF-7 and MDA-MB-231 cells in presence of Interferon-α (IFNα) (Fig. 1D & E).

Further, we validated the role of TRIM15 on NF-κB activation by Dual Glow Luciferase Reporter assay. The expression of TRIM15 decreased TNF-α-induced NF-κB activation as compared to control (Fig. 1F), whereas its knockdown by siRNA increased TNF-α-induced NF-κB activation (Fig. 1G & H). We also analyzed its effect on NF-κB inhibition at different time points and found that ectopic expression of TRIM15 inhibited TNF-α-induced NF-κB activity at both 4 h and 24 h (Supplementary Fig. 2F).

1.2. *TRIM15 form foci in the cytoplasm and nucleus and stabilizes in the presence of TNF-α*

TRIMs are known to dynamically localize in different subcellular compartments [15,19,24]. Therefore, we monitored the subcellular localization of TRIM15. Western blotting showed the presence of TRIM15 in both cytosolic and nuclear fractions Ttreated with TNF-α, MG132 (Proteasome inhibitor), and Bafilomycin-A1 (Baf-A1; Autophagy inhibitor) (Fig. 2A). The level of 50 kDa band corresponding to TRIM15 was higher in nuclear fraction compared to the untreated cells in TNF-α treated condition. Additionally, TRIM15 levels in cyctosolic fraction increased in MG132 (Proteasome inhibitor), Baf-A1 and co-treated cells (Fig. 2A). Further, we monitored subcellular localization using YFP tagged TRIM15. Confocal microscopy showed TRIM15 is present as discrete foci as well as large protein aggregates distributed in the cytosol (Fig. 2B). We also observed the presence of TRIM15 foci in the nucleus (Fig. 2C, Supplementary Fig. 3B).

TRIMs are known to dynamically localize to different subcellular compartments under specific stimuli [15,24]. Therefore, we checked the effect of TNF-α on dynamic localization of TRIM15. Interestingly, we observed that both mean fluorescence intensity (MFI) and binary area of TRIM15-YFP increased in TNF-α treated cells compared to control (Fig. 2D, E & F). We further monitored the TRIM15 foci in TNF-α, MG132 and Baf-A1 treated cells. Interestingly, the puncta size of TRIM15-YFP having ≥10 μM increased in TNF-α, MG132 and Baf-A1 treated cells compared to untreated control (Supplementary Fig. 3C).

We also observed a corresponding decrease in puncta size ranging up to ≤2 μM in TNF-α, MG132, and Baf-A1 treated cells compared to control (Supplementary Fig. 3C). The increase in TRIM15 puncta size in presence of MG132 and Baf-A1 suggests its possible turnover through proteasome and autophagy pathway, whereas its increase in TNF-α treatment is possibly due to enhanced transcription of TRIM15 or stabilization at protein levels and formation of higher-order structure.

1.3. *TRIM15 acts downstream of TRAF2 and inhibits IκBα phosphorylation*

TNF-α-induced NF-κB pathway is broadly regulated at three major levels; TRADD-TRAF2 complex at TNF receptor, TAB-TAK complex, and IKK complex respectively [3,5]. To further investigate the mechanism of TRIM15 mediated inhibition of NF-κB activity we analyzed the step regulated by TRIM15. We co-transfected TRAF2 with TRIM15 in HEK293 cells and analyzed NF-κB activation in the presence/absence of TNF-α. The expression of TRAF2 was sufficient to enhance NF-κB activation (Fig. 3A) and TRIM15 co-transfection significantly reduced the activity in untreated cells (Fig. 3A). Similar inhibition of TRAF2 induced NF-κB activation was observed in TRIM15 co-transfected cells treated with TNF-α (Fig. 3A).

Further, we monitored the effect of TRIM15 on the TAK1 induced NF-κB pathway. TAK1 expression did not affect NF-κB activity in untreated cells but enhanced NF-κB activity in TNF-α treated cells compared to vector (Fig. 3B). We also observed that co-transfection of TRIM15 with TAK1 inhibited NF-κB activity (Fig. 3B) in presence of TNF-α. The co-expression of TRIM15 with TAK1 reduced the levels of p-IκBα in both untreated and TNF-α treated cells compared to vector co-transfected cells (data not shown). Many regulators of TNF-α-induced NF-κB pathway act on the NF-κB heterodimers to regulate the activation of the pathway [3–5]. Therefore, we further checked the effect of TRIM15 on p65 induced NF-κB activation. We observed that p65 expression increased NF-κB activity in untreated cells and co-transfection of TRIM15 failed to inhibit NF-κB activation (Fig.

3C). These results confirm that TRIM15 acts upstream of p65 and down- stream of the TRADD-TRAF complex.

Phosphorylated (p) IκBα is ubiquitinated and degraded by the Ubiquitin-Proteasome System (UPS) [2,3,5]. We argued that TRIM15 may affect IκBα phosphorylation, therefore, we analyzed p-IκBα levels by western blotting. Cells were transfected with indicated TRIMs and cells were treated with a combination of TNF-α and MG132. Western blotting showed the 40 kDa band corresponding to p-IκBα was signifi- cantly reduced in TRIM15 transfected cells compared to control, sug- gesting inhibition of IκBα phosphorylation (Fig. 3D). Similarly, we also analyzed the p-IκBα levels in MCF-7 cells transfected with control vector or TRIM15 and treated with TNF-α, MG132, Baf-A1 (Inhibitor of auto- phagy) or in combination. Western blotting showed a significant reduction in the level of 40 kDa band corresponding to p-IκBα in TRIM15 transfected cells compared to control, TNF-α, MG132, Baf-A1, and TNF- α-Baf-A1 co-treated cells (Fig. 3E). Interestingly, we did not observe any significant difference in p-IκBα levels between TRIM15-YFP and control transfected cells co-treated with TNF-α and MG132. These results clearly show that TRIM15 inhibits the NF-κB pathway by inhibiting IκBα phosphorylation.

1.4. TRIM15 enhances turnover of TNF-α-induced K63-linked ubiquitin chains

Recruitment of different ubiquitin ligases brings stringency and specificity to the pathway by identifing specific substrates and the addition of ubiquitin in specific topologies [25]. Therefore, we analyzed the effect of TRIM15 on various ubiquitin chain topologies to further understand its E3 ligase function. We co-transfected HEK293 cells with HA-Ub-K6, K11, K27, K29, K48, K63, and either vector control or TRIM15 and treated them with TNF-α. Interestingly we found that TRIM15 co-transfection significantly reduced K6, K11, K27, K29, K48, and K63 linked polyubiquitination of proteins and reflected the most pronounced effect on K6 and K63 linked polyubiquitination (Fig. 4A).

Therefore, we further checked the effect of TRIM15 on cellular ubiquitination by co-transfecting vector or TRIM15 with HA-Ub-K63 and monitored K63 linked ubiquitination. We observed higher molec- ular weight adducts of HA-Ub-K63 linked proteins were increased in presence of TNF-α in vector-transfected cells (Fig. 4B). TRIM15 co- transfection significantly reduced the level of K63 linked ubiquitina- tion in TNF-α treated cells (Fig. 4B). Interestingly, we found that levels of HA-Ub-K63 linked protein adducts increased in TRIM15 transfected cells in presence of MG132 as compared to untreated and TNF-α (Fig. 4B). Further, we monitored total cellular ubiquitination in the same blot using a ubiquitin-specific antibody, however, no difference was observed in ubiquitination between control or TRIM15 transfected cells in different treatment conditions (Fig. 4B).

To reconfirm, we co-transfected HA-Ub-K63 with control or TRIM15- siRNA and monitored the levels of K63-linked ubiquitination of proteins using HA-specific antibody. We observed a significant increase in levels of higher molecular weight K63-linked protein adducts in TRIM15- siRNA transfected cells compared to control transfected cells both in the absence and presence of TNF-α (Fig. 4C). We also observed an in- crease in total cellular ubiquitination in TRIM15-siRNA transfected cells both in the absence and presence of TNF-α as compared to control (Fig. 4C). Interestingly, a similar increase in K63-linked ubiquitination was also observed in TRIM15 knockdown cells treated with MG132 and in combined treatment of TNF-α and MG132 (Fig. 4C), whereas we observed no difference in total ubiquitination in these conditions, sug- gesting decreased turnover of K63 linked proteins in absence of TRIM15. As observed earlier that TRIM15 is present in both cytosol and nu- cleus (Fig. 2A, B & C), we monitored its effect on nuclear and cytosolic protein ubiquitination levels. We co-transfected HA-Ub-K63 either with control or TRIM15-YFP in HEK293 cells and treated with TNF-α or MG132. Western blotting of cytosolic and nuclear fractions showed that TRIM15 significantly reduced K63-linked ubiquitination in the cytosol and nucleus of both untreated and TNF-α treated cells (Fig. 4D), whereas, its effect on total ubiquitination was less pronounced (Fig. 4D). Consistent with the previous blot (Fig. 4B) we observed increased K63-

linked ubiquitination in TRIM15 transfected cells treated with MG132 as compared to untreated and TNF-α treated cells (Fig. 4D). These results suggest that E3 ligase TRIM15 enhances UPS mediated turnover of K63-linked ubiquitination in both nucleus and cytoplasm.

1.5. *PRY/SPRY domain deletion of TRIM15 rescues inhibiton of TNF-α-induced K63-linked ubiquitination and NF-κB activity*

We further analyzed the effect of TRIM15-mediated inhibition of K63-linked ubiquitination on NF-κB activation. Different domain dele- tion constructs of TRIM15 were co-transfected with HA-Ub-K63 and K63 linked ubiquitination and NF-κB activation was monitored. Surprisingly, transfection of TRIM15 with RING and RING-B-Box deletion showed less rescue of K63-linked ubiquitinated adducts as compared to full-length TRIM15 (Fig. 5A), and strongly inhibited TNF-α-induced NF-κB activity (Fig. 5B). Interestingly, we observed that deletion of SPRY and PRY/SRPY domain of TRIM15 rescued K63-linked ubiquitination and TNF-α-induced NF-κB activity (Fig. 5C). Additionally, transfection of CC domain deleted TRIM15 and CC domain mutant (T15Cm) showed less rescue of K63 linked ubiquitination compared to PRY/SPRY domain deleted TRIM15 (Fig. 5C) and NF-κB activity (Fig. 5D). These results suggest that the PRY/SPRY domain of TRIM15 is essential for the turnover of K63-linked ubiquitination and TNF-α-induced NF-κB activ- ity. Interestingly, these results also confirmed that a significant corre- lation exists between cellular TRIM15-mediated K63-linked ubiquitination and NF-κB activity.

1.6. *TRIM15 inhibits NF-κB activation by turnover of K63-linked ubiquitination of TAK1*

TAK1 is modified by K63-linked ubiquitin chains in presence of TNF- α and IL-1β [20] and we

observed that TRIM15 enhances turnover of K63-linked ubiquitin adducts (Fig. 5C). Therefore, we analyzed whether TRIM15 had any effect on the K63-linked ubiquitination of TAK1. We co-transfected cells with Flag-TAK1 and control vector, TRIM15-YFP or tagless-TRIM15, and treated them with MG132 and TNF-α. TAK1 was pulled down using anti-Flag affinity beads and immunoprecipitation (IP) showed that 75 kDa band corresponding to TRIM15-YFP appeared in the Flag-TAK1 pull-down confirming interaction of TRIM15 and TAK1 (Fig. 6A). Blotting with ubiquitin antibody showed increased higher molecular weight adducts of polyubiquitinated proteins in Flag-TAK1 pull-down, whereas it was reduced in TRIM15 co-transfected cells (Fig. 6A). A similar reduction of TAK1 ubiquitination was observed in Flag-TAK1 co-transfected with tag-less TRIM15 (Fig. 6A).

TRIM15 had been identified as an E3 ligase [26] and it showed interaction with TAK1, therefore we analyzed if TRIM15 affects the turnover of TAK1. To address this, we co-transfected Flag-tagged TAK1 with a control vector or TRIM15 and treated the cells with TNF-α, MG132, or in combination. We observed that the 50 kDa band corre- sponding to TAK1 remained unchanged in control and TRIM15 trans- fected cells (Fig. 6B) and no significant difference was observed in the TAK1 levels in control and treated conditions (Fig. 6B).

K63-linked ubiquitination of TAK1 at Lysine 158 is required for TNF- α induced NF-κB activation [2,20,27] and TRIM15 showed reduced levels of K63-linked ubiquitination. Therefore, we checked whether TRIM15 modulates the K63-linked ubiquitination of TAK1. Flag-TAK1 and HA-Ub-K63 were co-transfected with TRIM15 and treated with TNF-α and IP were performed using anti-Flag affinity beads. Western blotting reconfirmed the interaction of TRIM15 and TAK1 and reduction of TAK1 ubiquitination in presence of TRIM15 (Fig. 6C). Detection of K63-linked ubiquitination using HA-specific antibody showed that TNF- α treatment promotes K63-linked ubiquitination of TAK1 whereas TRIM15 co-transfection completely diminished K63-linked ubiquitina- tion of TAK1 (Fig. 6C). Besides, we performed a similar IP and pulled down K63-linked ubiquitin chains using HA-affinity beads. Increased level of ubiquitinated TAK1 was observed in the presence of TNF-α, whereas the transfection of TRIM15 enhanced the turnover of

K63- linked TAK1 in presence of TNF-α (Fig. 6D). Together these results suggest that TRIM15 interacts with ubiquitinated K63-linked TAK1 and reduced its level by enhancing the turnover through UPS.

1.1. *TRIM15 interacts with TRIM8, inhibits its cytoplasmic translocation and TNF-α-induced NF-κB activity*

The previous report from our group had shown that nucleo- cytoplasmic translocation of TRIM8 is required for TNF-α-induced NF-κB activation [19], additionally TRIM8 promotes K63-linked poly- ubiquitination of TAK1 in cytoplasm to induce NF-κB activation [20]. TRIMs are known to homo and heterodimerize for target identification and assembly of signalosomes [28,29]. While exploring interaction partners of TRIM15, the literature survey showed possible interaction between TRIM8 and TRIM15 [30] therefore, we performed co- immunoprecipitation (Co-IP) to confirm their interaction. HA-tagged TRIM8 was transfected with either vector or TRIM15-YFP and treated with TNF-α and MG132 or MG132 alone. TRIM8 was pulled down using anti-HA affinity beads and western blotting showed that the 75 kDa band corresponding to TRIM15-YFP was observed in TRIM15 and HA- TRIM8 co-transfected cells co-treated TNF-α and MG132 (Fig. 7A).

TRIM8's nucleo-cytoplasmic translocation in TNF-α is required for activation of NF-κB [19], therefore we further analyzed the effect of TRIM15 on TRIM8 dynamics. We transfected HA-tagged TRIM8 with TRIM15-YFP or vector control in HEK293 cells and treated with TNF-α and monitored TRIM8 and TRIM15 in nucleo-cytoplasmic fractions. The level of 75 kDa band corresponding to TRIM15-YFP was higher in nu- clear fraction as compared to the cytosol, whereas its cytosolic levels increased in presence of TNF-α (Fig. 7B). The 62 kDa band corre-sponding to HA-TRIM8 was high in nuclear fraction compared to the cytosol (Fig. 7B) and

interestingly, the level of TRIM8 was higher in the nuclear fraction of TRIM8-TRIM15 co-transfected cells (Fig. 7B). We also observed a corresponding decrease in TRIM8 levels in the cytoplasm in TRIM15 co-transfected cells (Fig. 7B).

To further check the functional effect of TRIM15 on TRIM8- enhanced TNF-α-induced NF-κB activity we checked the effect of TRIM15 on TRIM8-regulated TNF-α-induced NF-κB activity . We observed ectopic expression of TRIM8 enhanced NF-κB activity compared to vector-transfected TNF-α treated cells (Fig. 7C) as observed previously [19,20]. Interestingly, we observed that co-expression of TRIM15 with TRIM8 significantly reduced TRIM8-regulated NF-κB activation (Fig. 7C). Since we observed functional antagonism between these proteins in the regulation of the NF-κB pathway, we further explored their physiological significance in inflammatory conditions. We analyzed the GEO databases for disease progression related to in- flammatory conditions, specifically Psoriasis. Interestingly gene expression analysis of microarray dataset comparing expression between normal, non-lesional, and lesional psoriasis tissue showed a marked increase in TRIM15 mRNA expression in lesional psoriasis tissue compared to normal control and non-lesional (psoriasis) tissue (Sup- plementary Fig. 4A, B & C). We also observed that TRIM8 expression was decreased in lesional psoriasis tissue compared to normal control and non-lesional (Supplementary Fig. 4D, & E). Together these results confirm functional and physiological antagonism between TRIM8 and TRIM15 and indicate its implication in the regulation of inflammation and chronic inflammatory condition like psoriasis.

2. Discussion

Increased levels of TNF-α; a prototypic activator of the pro- inflammatory NF-κB pathway, had been observed in several patho- physiological conditions [6–9]. This pathway had been intensively studied, however, the resolution TNF-α-induced inflammatory pathway is still not well understood in pathophysiological conditions. Majority of the studies focused on early response genes however

dysregulation of physiological equilibrium in chronic disease conditions is not well un- derstood. The selective ubiquitination of proteins implicated in presence of TNF-α either leads to its degradation or its recruitment to assemble signaling complexes defining the unique outcome of the TNF-α-patho- physiological responses. In this study, we systematically identified late response TNF-α activated E3 ligases and their role in the regulation of TNF-α-induced NF-κB activation and further characterized TRIM15 in negative feedback regulator of TNF-α-induced NF-κB pathway.

We and others have have previously observed that turnover of TRIMs is high and stabilized during specific pathophysiological conditions [31,32]. Cells keep inflammatory pathways under stringent control and hence it can be inferred that late response genes may have important regulatory functions, hence, we systematically planned to identify late response TNF-α induced TRIMs regulating the NF-κB pathway. In this study we performed screening and identified several TRIM genes (TRIM1, 2, 3, 8, 9, 15, 16,21, 31, 37, 38, 39, 41, 44, 46, 47 and 55) which are late response TNF-α activated genes. Interestingly, most of the late response TRIMs: TRIM1, 2, 15, 46, 47, 48, and 55 inhibit TNF-α-induced NF-κB pathway, suggesting that TRIMs may act as feedback regulator of the TNF-α-induced NF-κB pathway. These results further warrant a more focused investigation of late response genes and their role in feedback regulation of TNF-α induced NF-κB pathway in different pathophysio- logical conditions.

Isolated reports from different groups including our group had shown that TRIMs, a family of RING E3 ligases regulates cellular path- ways by identification and modification of their target substrates [16–18,20,33,34]. Stimuli-specific expression of TRIMs brings precision and specificity to the pathways and determines the physiological outcome [24,31,35,36]. Previously upregualtion of TRIM15 was observed in in THP1-derived macrophages stimulated with ligands of TLR3 and TLR4 [37] but its expression was found unaffected by type-I IFNs in various immune cells [35]. In this study, we suggest that TRIM15 is a TNF-α-induced late response gene and strongly inhibits NF- κB activation in different cell types. TRIM15 has restricted tissue expression and data from

cancer cell line encyclopedia (CCLE) suggest that DNA methylation of TRIM15 promoter regions restricts its mRNA expression. Our observation of TRIM15 gene expression in response to TNF-α is supported by recent reports suggesting that infection [38] and TNF-α promotes [39] DNA demethylation of genes and promote their transcription. This also suggests that hypermethylated genes may also contribute to the regulation of cellular pathways in response to specific stimuli [38,39]. Previous studies from our lab and others have demon- strated that TRIMs have high turnover and stabilizes only specified pathophysiological conditions [16,24,31,32]. Interestingly, unlike other TRIMs, TRIM15 protein is stable and turnover is not high as it had been observed to play an essential role in focal adhesion involved in cell migration, an essential cellular process [26]. TNF-α induced expression of highly methylated gene TRIM15 indicates existence of an additional layer regulation of NF-κB, which is via demethylation of their regulatory genes, however, this concept requires further validation. Ubiquitin E3 ligases are known to modify substrates by adding spe- cific ubiquitin chains and regulate regulate their stability and degrada- tion. Several NF-κB target genes are E3 ligases and found to regulate the pathway positively by modifying specific targets and regulating their turnover [3,5,40–42]. Surprisingly we observed that TRIM15 enhances turnover of K63-linked ubiquitin chains and further characterization revealed the involvement of the PRY/SPRY domain for turnover of K63- linked ubiquitination and inhibition of TNF-α-induced NF-κB activity. K63-linked ubiquitination had been suggested to promote the stability of proteins and function generally independent of UPS, however, emerging evidence suggests that some targets modified by K63- linked ubiquitin chains are degraded by UPS [43]. Further, it had been found that K63- linked chains act as seeds for the formation of K48/K63 branched chains and associates with the proteasome [44]. Furthermore, assembly of K48/K63 branched ubiquitin chains assembled on TRAF6 by E3 ligase HUWE1 protects deubiquitination of K63-linked chains of TRFA6 to positively regulate IL-1β mediated NF-κB pathway [45]. Therefore, it will be interesting to explore if TRIM15 mediated degradation of K63- linked ubiquitination requires the similar deployment of K48/K63- linked branched ubiquitin chains to enhance the turnover of K63- linked substrate to negatively regulate the NF-κB pathway.

Previous studies have shown thatactivation of the TAB-TAK complex is critical for activation of the TNF-α-induced NF-κB pathway and K63 linked ubiquitination of TAK1 is critical for activation [3–5,46]. Our study shows that TRIM15 interacts with TAK1 and strongly inhibits NF- κB activation by enhancing the turonover of K63-linked ubiquitin chain [3–5,20,27]. This is one of the first reports elucidating the role of TNF- α-induced late response genes in negative regulation of the NF-κB pathway.

Homo/heteromeric interactions of TRIM proteins had been previ- ously observed [15,30] however their physiological implication had not yet been studied. Cooperation between TRIMs has been observed and reports show antgonostic effect on the same substrate or pathway. Previous reports from our group and others have shown that TRIM8's nucleo-cytoplasmic trafficking is important for NF-κB activation by promoting TAK1 ubiquitination [19,20]. Here we observed that TRIM15 mediated restriction of cytosolic translocation of TRIM8 which may further inhibit TRIM8 mediated K63 linked cytosolic ubiquitination of TAK1 as reported previously. More interestingly the observed functional antagonism between these proteins is also relevant in the chronic inflammatory condition, Psoriasis. Besides its role in regulation of TNF- α-induced NF-κB pathway and virus-induced IFN response [47] TRIM8 is also known to regulate different aspects of cancer signaling including estrogen signaling [48], genotoxic stress induced cell death [32] and chromosome stability [32]. Therefore, it will be interesting to check the relevance of TRIM8-TRIM15 functional antagonism in cancers.

In summary, the current study identified TRIMs as novel regulators of the TNF-α-induced NF-κB pathway and further report TRIM15's role in negatively regulating TNF-α-induced NF-κB activity. Interestingly, TNF-α may induce the expression of several TRIMs which may be selectively stabilized in different subcellular compartments hence different TRIMs may be recruited at the different regulatory steps of TNF-α-induced NF-κB activation. These proteins are developing modu- lators of inflammatory pathways and chronic pathological conditions and further study may provide a novel way of targeting specific TRIMs for therapeutic intervention in given pathophysiological conditions.

Author contribution

MR designed and performed experiments, analyzed/interpreted data, and co-wrote the manuscript. KS and AS provided reagents and provided critical revision of the manuscript. SB and YT provided re- agents and helped with experiments. DG, HV, MM, and FC analyzed data and provided critical revision of the manuscript. RS conceptualized and supervised the research, co-wrote the manuscript, and acquired funding.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Figure legends:

Fig. 1. TRIM15 is a late response TNF-α induced gene and inhibits TNF-α-induced NF-κB activation. TNF-α induces expression of TRIM15. (A) HEK293 &(C) MCF-7 cells were treated with TNF-α for indicated time and mRNA expression of TRIM15 was analyzed using qRT-PCR. (B) HEK293, (D) MCF-7, and (E) MDA-MB-231 cells were treated as indicated and western blotting was performed to check TRIM15 protein levels. (F) TRIM15 inhibits TNF-α-induced NF-κB activation. HEK293 cells were co-transfected with control vector or TRIM15 and NF-κB reporter constructs. After 24 h of transfection, cells were treated with TNF-α for 10 h, and NF-κB activity was measured by Dual glow luciferase reporter assay. (G) Control siRNA or TRIM15-siRNA was co-transfected with NF-κB reporter constructs and 24 h post- transfection, cells were treated with TNF-α for 10 h and NF-κB activity was measured. (H) Control siRNA or TRIM15-siRNA was transfected in HEK293 and mRNA expression of TRIM15 was analyzed using qRT-PCR.Asterisk (*), (**), and (***) indicates fold change or NF-κB activity statistically significant from control; *p*-value
<0.05, <0.01 and < 0.001(respectively), SEM of minimum three independent experiments.

Fig. 2. TRIM15 forms foci in the cytoplasm and nucleus and stabilizes in the presence of TNF-α. (A) HEK293 cells were treated with TNF-α, MG132, and Baf-A1 as indicated for 10 h. Nuclear and cytosolic fractions were prepared and indicated specific antibodies were used to detect protein levels. (B & C) HEK293 cells were transfected with TRIM15-YFP and 24 h post-transfection cells were stained with Hoechst nuclear stain and monitored under a confocal microscope. (D) TRIM15-YFP transfected cells were grown on a coverslip and treated with TNF-α for 10 h. Cells were fixed and stained with DAPI and monitored under a fluorescent microscope for DAPI and YFP fluorescence. (E&F) The binary area and mean fluorescence intensity of TRIM15-YFP was measured from the captured images using the automated measurement feature of Nikon Elements AR software. Asterisk (**) and (***) indicates statistically significant difference in MFI and

binary area from control; p-value

<0.01 and < 0.001 (respectively), SEM of minimum six different fields.

Fig. 3. TRIM15 acts downstream of TRAF2 and inhibits IκBα phosphorylation. (A, B & C) TRIM15 acts upstream of p65 and downstream of TRAF2 in the NF-κB pathway. Vector or TRIM15 was co-transfected as indicated with TRAF2 (A), TAK1 (B), or p65 (C) in HEK293 cells along with NF-κB reporter constructs. 24 h post- transfection cells were treated with TNF-α for 10 h and NF-κB activity was measured. (D) TRIM15 inhibits IκBα phosphorylation. HEK293 cells were transfected with indicated constructs and treated with TNF-α and MG132 for 10 h. Western blotting was performed to check the levels of p-IκBα levels using a specific antibody. (E) MCF-7 cells were transfected with control vector or TRIM15 and TNF-α, MG132, and Baflilomycin-A1 as indicated, for 10 h. Western blotting was performed to check the levels of p-IκBα. Asterisk (*), (**) and (***) indicates NF-κB activity statistically significant from control; *p*-value <0.05, <0.01 and < 0.001 (respectively), SEM of minimum three independent experiments.

Fig. 4. TRIM15 enhances turnover of TNF-α-induced K63-linked ubiquitin chains. (A) HA-tagged Ub-K6, K11, K27, K29, K48, and K63 were co-transfected with either vector or TRIM15-YFP. Cells were treated with TNF-α for 10 h after transfection and western blotting was performed. (B) Vector or TRIM15-YFP constructs were co-transfected with HA-Ub-K63 in HEK293 cells. 24 h post-transfection cells were treated with TNF-α and MG132 as indicated for 10 h. Western blotting was performed to check total ubiquitination and K63 linked polyubiquitinated proteins using indicated antibodies. (C) HA-Ub-K63 was co-transfected with control or TRIM15-siRNA in HEK293 cells and treated with TNF-α and MG132 as indicated for 10 h. Western blotting was performed to check protein levels using indicated antibodies. (D) Vector or TRIM15-YFP constructs were co-transfected with HA-Ub-K63 in HEK293 cells and treated as indicated. After 10 h of treatment nuclear- cytosolic fractions

were prepared and levels of indicated proteins were detected using specific antibodies.

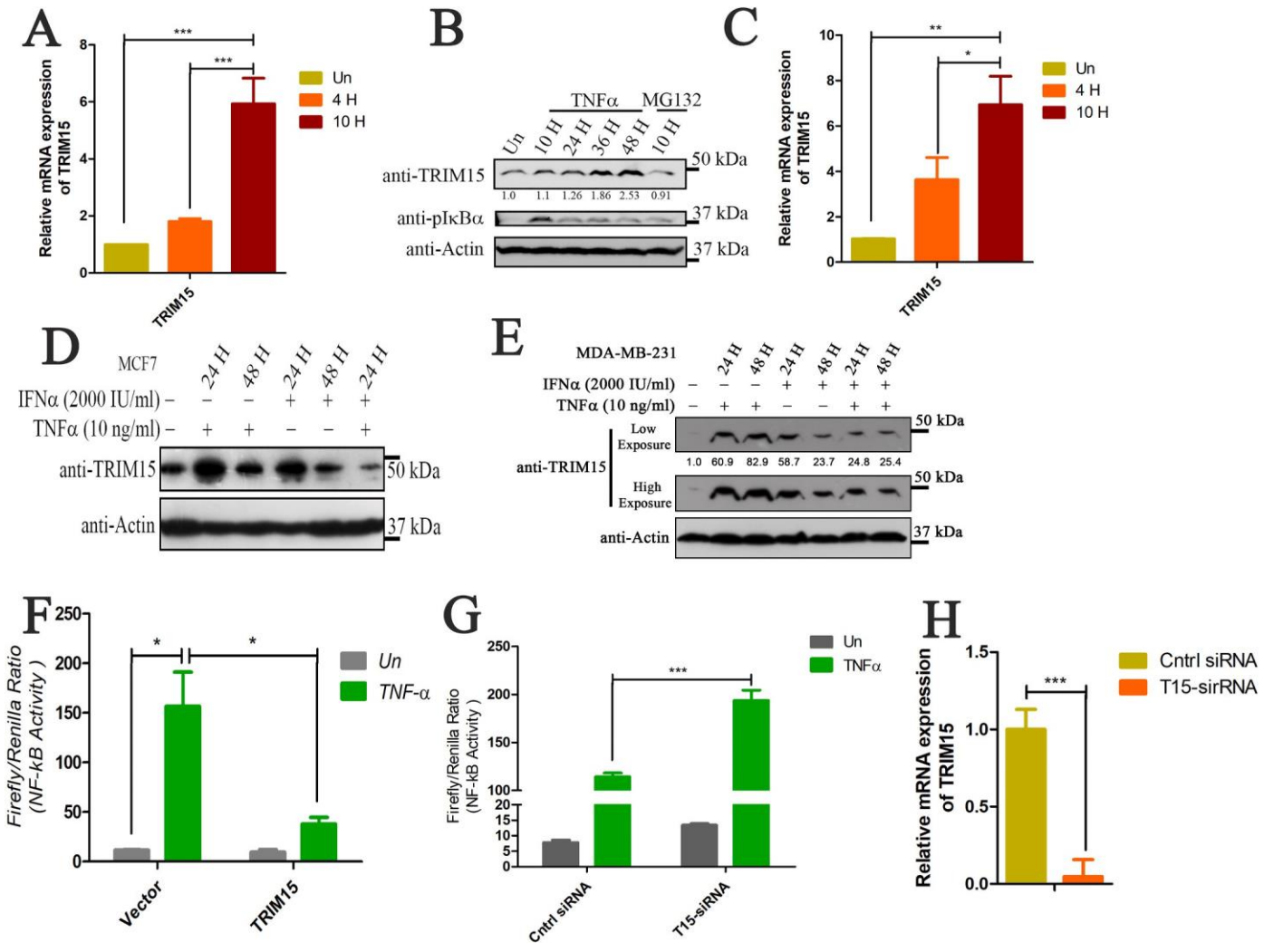
Fig. 5. PRY/SPRY domain deletion of TRIM15 rescues TNF-α-induced K63-linked ubiquitination and NF-κB activity. (A & C) HA-Ub-K63 was co-transfected with either vector or indicated TRIM15 construct in HEK293 and treated with TNF-α for 10 h. Western blotting was performed to check total ubiquitination and K63- linked polyubiquitinated proteins using indicated antibodies. PRY/SPRY domain of TRIM15 is essential for inhibition of TNF-α-induced NF-κB activity. (B & D) Vector or indicated TRIM15 construct were co-transfected with NF-κB reporter constructs and cells were treated with TNF-α for 10 h, later NF-κB activity was measured using dual glow luciferase assay. Asterisk (**) and (***) indicates NF-κB activity statistically significant from control; *p*-value <0.01 and < 0.001 (respectively), SEM of minimum three independent experiments.

Fig. 6. TRIM15 inhibits NF-κB activation by turnover of K63-linked ubiquitination of TAK1. (A) TRIM15 interacts with TAK1 and inhibits its ubiquitination. HEK293 cells were transfected with the control vector, TRIM15-YFP, tagless-TRIM15, and Flag-TAK1 as indicated. Cells were co-treated with TNF-α and MG132 for 10 h and TAK1 was pulled down using Flag affinity beads. Western blotting was performed and indicated antibodies were used to check the interaction. (B) HEK293 cells were co-transfected with Flag-TAK1 and vector control or TRIM15-YFP and treated as indicated. Western blotting was performed to check the levels of indicated proteins using specific antibodies. (C & D) HA-Ub-K63 and Flag-TAK1 were co-transfected with either control vector or TRIM15-YFP and treated with or without TNF-α for 10
h. TAK1 was pulled using Flag affinity beads (C), whereas K63-linked chains were pulled down using HA affinity beads (D), from lysates and western blotting was performed to detect interaction and ubiquitination using indicated antibodies.

Fig. 7. TRIM15 interacts with TRIM8, inhibits its cytoplasmic translocation and TNF-α-induced NF-κB activity. (A) TRIM15 interacts with TRIM8 in TNF-α treated cells. HA-TRIM8 was co-transfected with a control vector or TRIM15-YFP and treated as indicated for 10 h. Cells were collected and TRIM8 was pulled down using HA affinity gel. Western blotting was performed and indicated antibodies were used to check the interaction. (B) TRIM15 inhibits cytosolic translocation of TRIM8. HA-TRIM8 was co-transfected with vector or TRIM15-YFP in HEK293 cells and treated with TNF-α and MG132 as indicated. After 10 h of treatment, nuclear-cytosolic fractions were prepared and levels of indicated proteins were detected using specific antibodies. (C) TRIM15 inhibits TRIM8 enhanced NF-κB activation. HEK293 cells were co-transfected with vector, TRIM15, and TRIM8 as indicated. After 24 h of transfection cells were treated with TNF-α for 10 h and NF-κB activity was

measured. Asterisk (***) indicates NF-κB activity statistically significant from control; p-value <0.001, SEM of minimum three independent experiments.

Fig. 1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Vector
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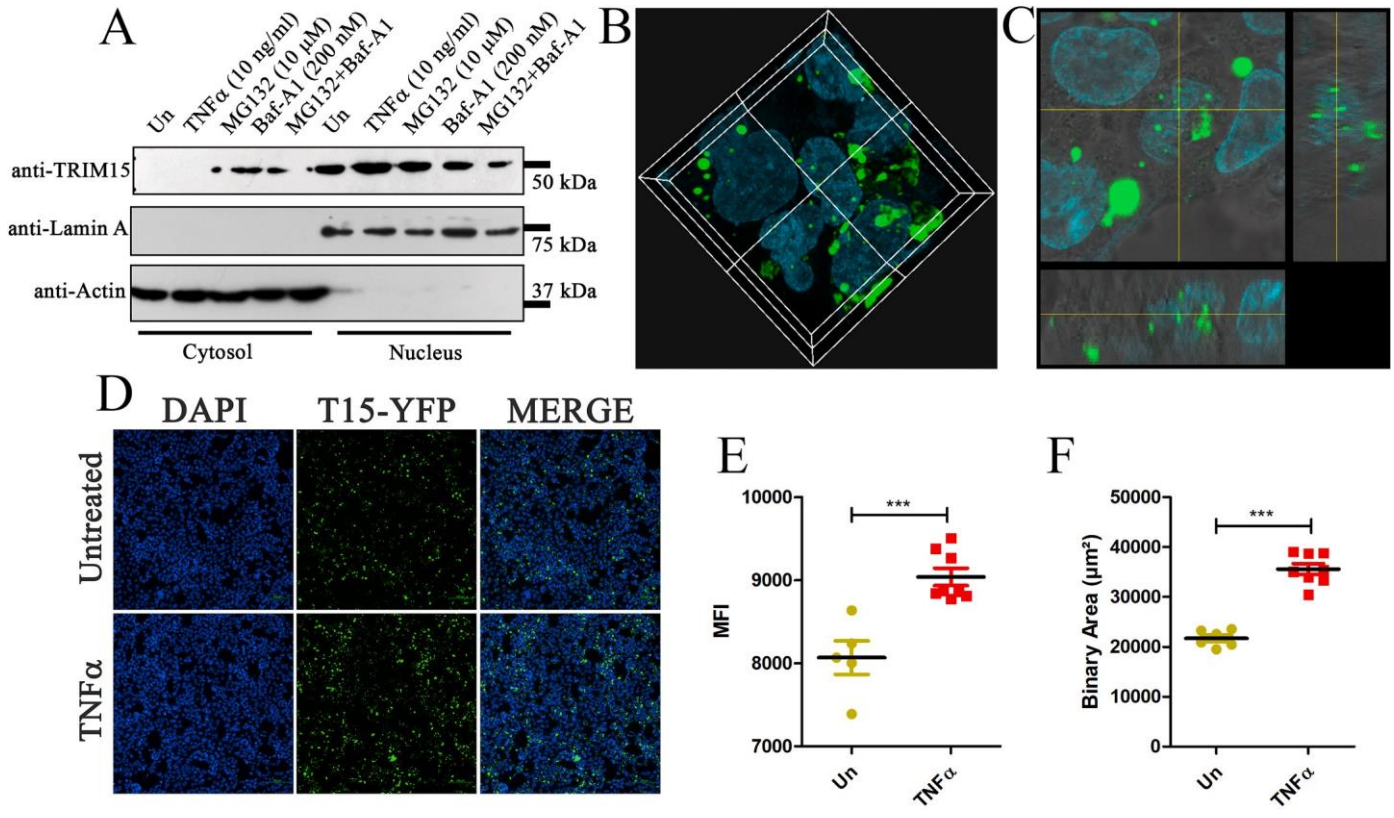
Fig. 2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anti-Actin
50 kDa
75 kDa
37 kDa
Cytosol
Nucleus
B
C
D
DAPI
T15-YFP
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10000
0
***
Un
TNFα

Fig. 3

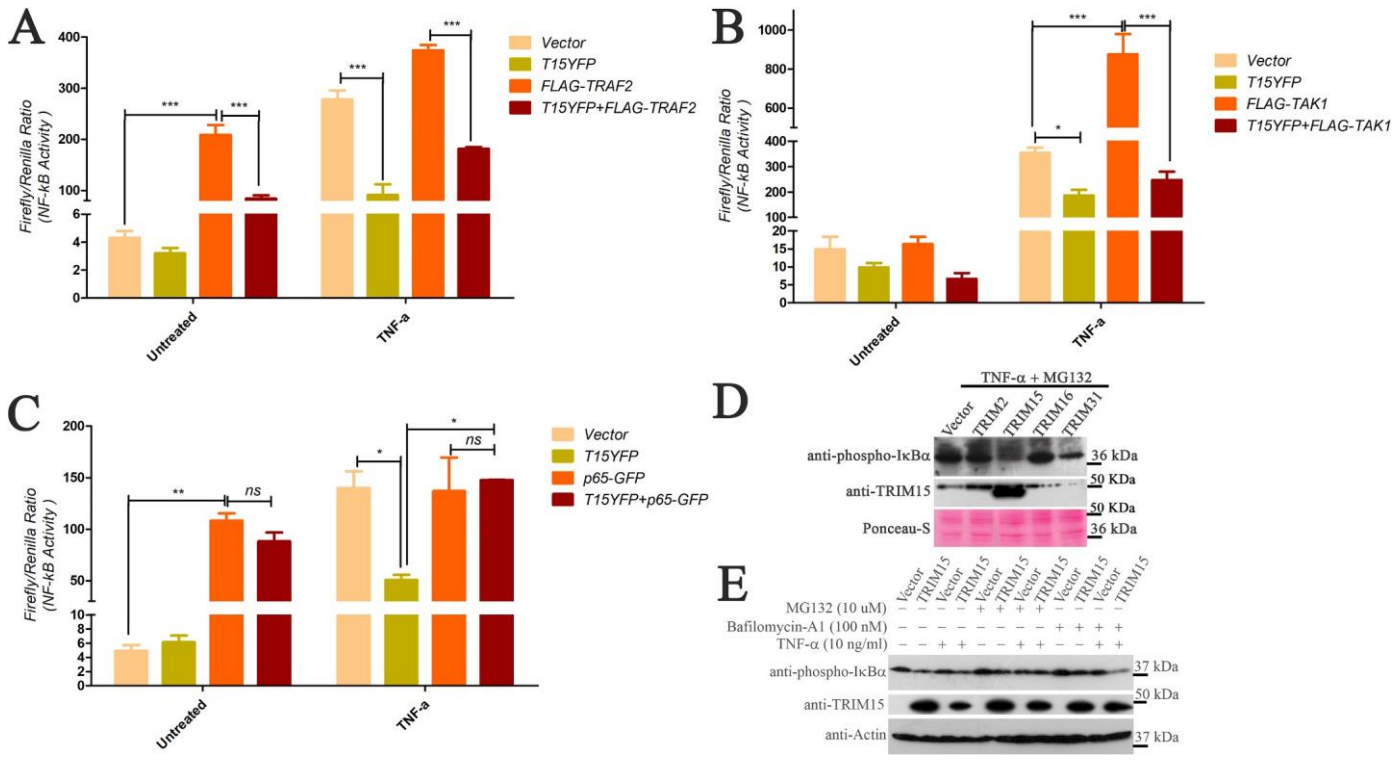

A
Firefly/Renilla Ratio (NF-kB Activity)
Vector
T15YFP
FLAG-TRAF2
T15YFP+FLAG-TRAF2
Untreated
TNF-a
B
Firefly/Renilla Ratio (NF-kB Activity)
Vector
T15YFP
FLAG-TAK1
T15YFP+FLAG-TAK1
Untreated
TNF-a
C
Firefly/Renilla Ratio (NF-kB Activity)
Vector
T15YFP
p65-GFP
T15YFP+p65-GFP
ns
Untreated
TNF-a
D
TNF-α + MG132
Vector
TRIM2
TRIM15
TRIM16
TRIM31
anti-phospho-IκBα
anti-TRIM15
Ponceau-S
36 kDa
50 kDa
50 kDa
36 kDa
E
Vector
TRIM15
MG132 (10 uM)
Bafilomycin-A1 (100 nM)
TNF-α (10 ng/ml)
anti-phospho-IκBα
anti-TRIM15
anti-Actin
37 kDa
50 kDa
37 kDa

Fig. 4

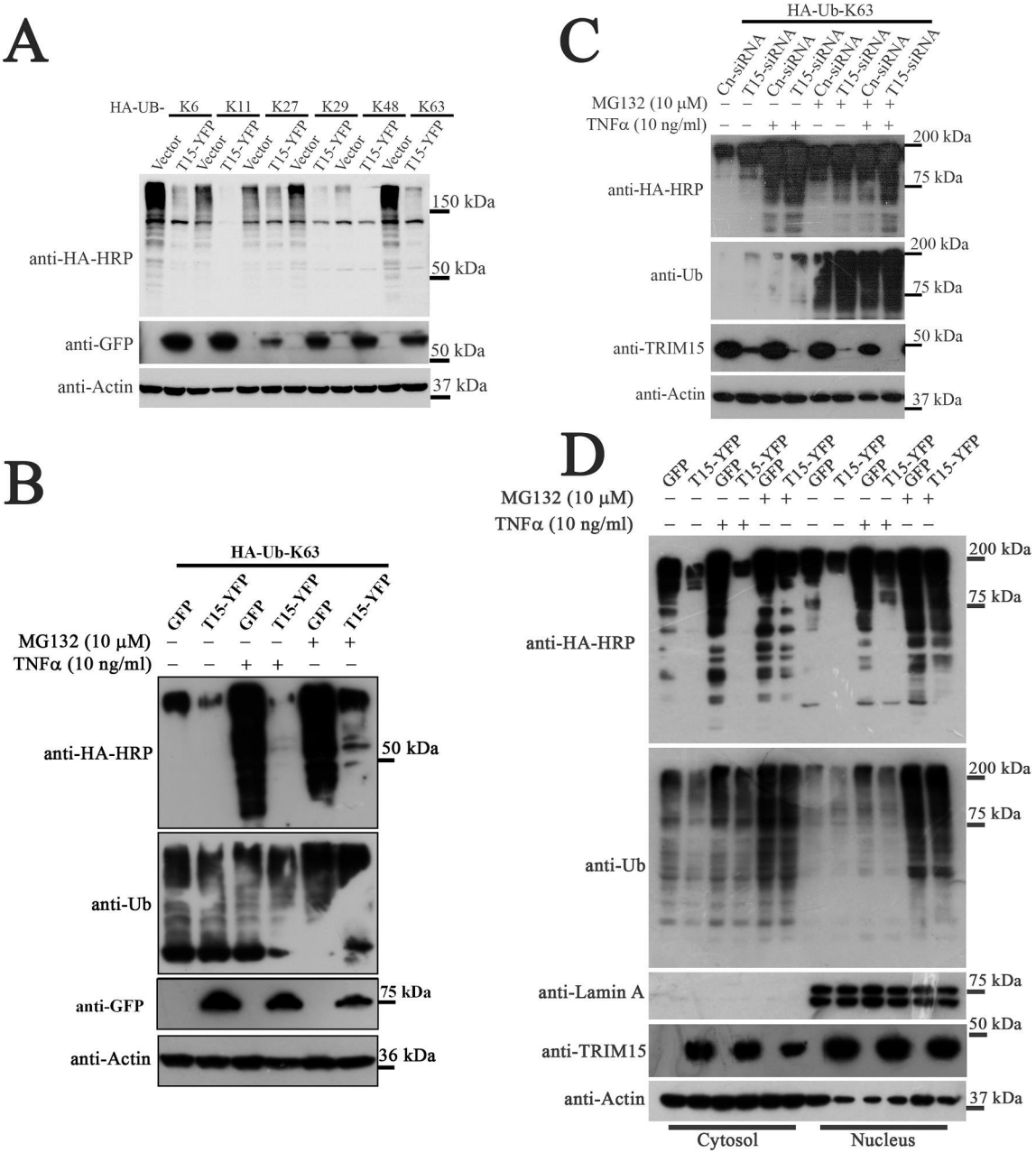

A
HA-UB-
K6
K11
K27
K29
K48
K63
Vector
T15-YFP
anti-HA-HRP
anti-GFP
anti-Actin
150 kDa
50 kDa
50 kDa
37 kDa
B
HA-Ub-K63
GFP
T15-YFP
MG132 (10 μM)
TNFα (10 ng/ml)
anti-HA-HRP
anti-Ub
anti-GFP
anti-Actin
50 kDa
75 kDa
36 kDa
C
HA-Ub-K63
Cn-siRNA
T15-siRNA
MG132 (10 μM)
TNFα (10 ng/ml)
anti-HA-HRP
anti-Ub
anti-TRIM15
anti-Actin
200 kDa
75 kDa
200 kDa
75 kDa
50 kDa
37 kDa
D
GFP
T15-YFP
MG132 (10 μM)
TNFα (10 ng/ml)
anti-HA-HRP
anti-Ub
anti-Lamin A
anti-TRIM15
anti-Actin
200 kDa
75 kDa
200 kDa
75 kDa
75 kDa
50 kDa
37 kDa
Cytosol
Nucleus

Fig. 5

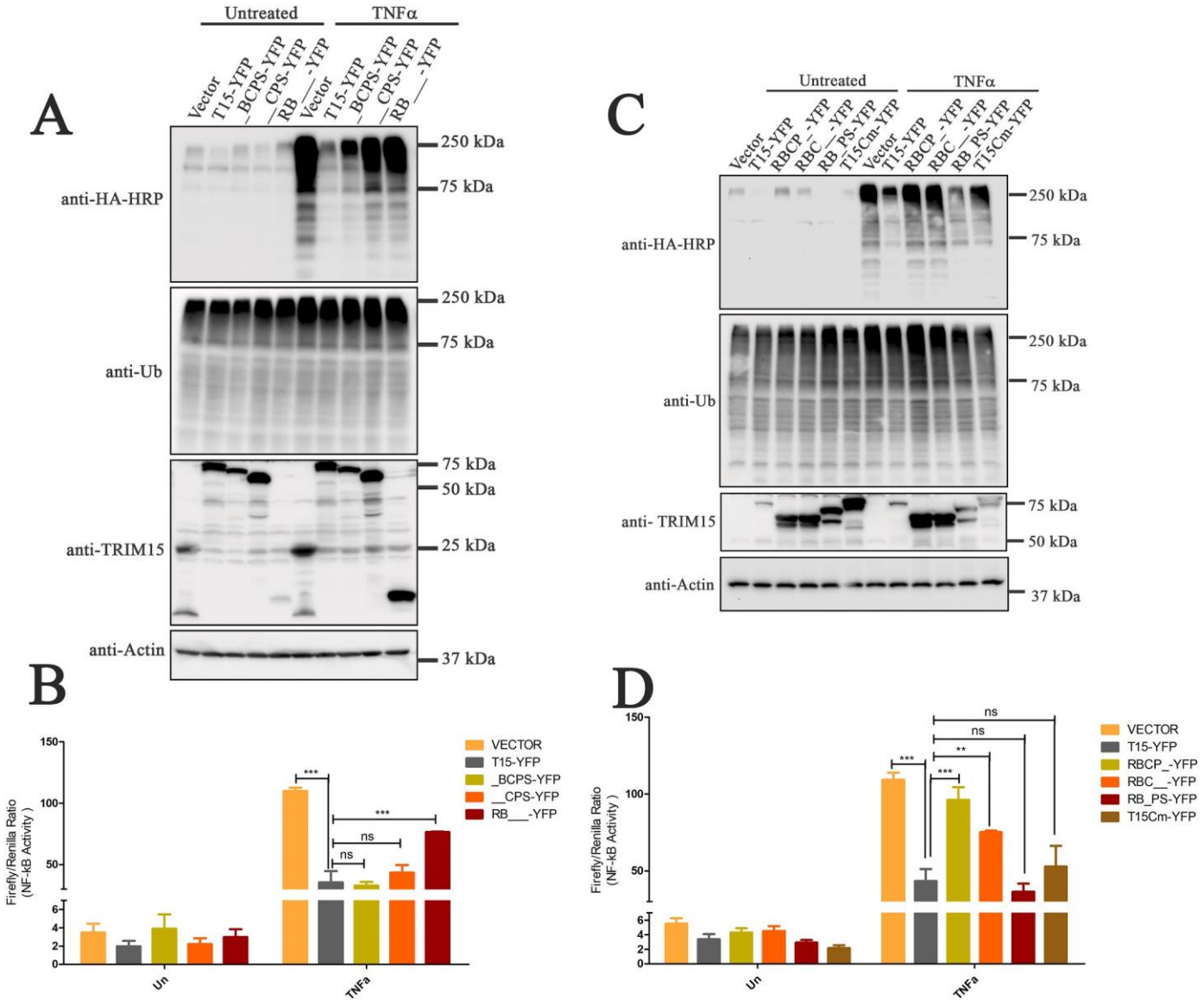

A
Untreated
TNFα
Vector
T15-YFP
_BCPS-YFP
_CPS-YFP
RB___-YFP
anti-HA-HRP
anti-Ub
anti-TRIM15
anti-Actin
250 kDa
75 kDa
50 kDa
25 kDa
37 kDa
B
Firefly/Renilla Ratio
(NF-kB Activity)
VECTOR
T15-YFP
_BCPS-YFP
__CPS-YFP
RB___-YFP
Un
TNFa
C
RBCP_-YFP
RBC__-YFP
RB_PS-YFP
T15Cm-YFP
anti- TRIM15
D
RBCP_-YFP
RBC__-YFP
RB_PS-YFP
T15Cm-YFP
ns

Fig. 6

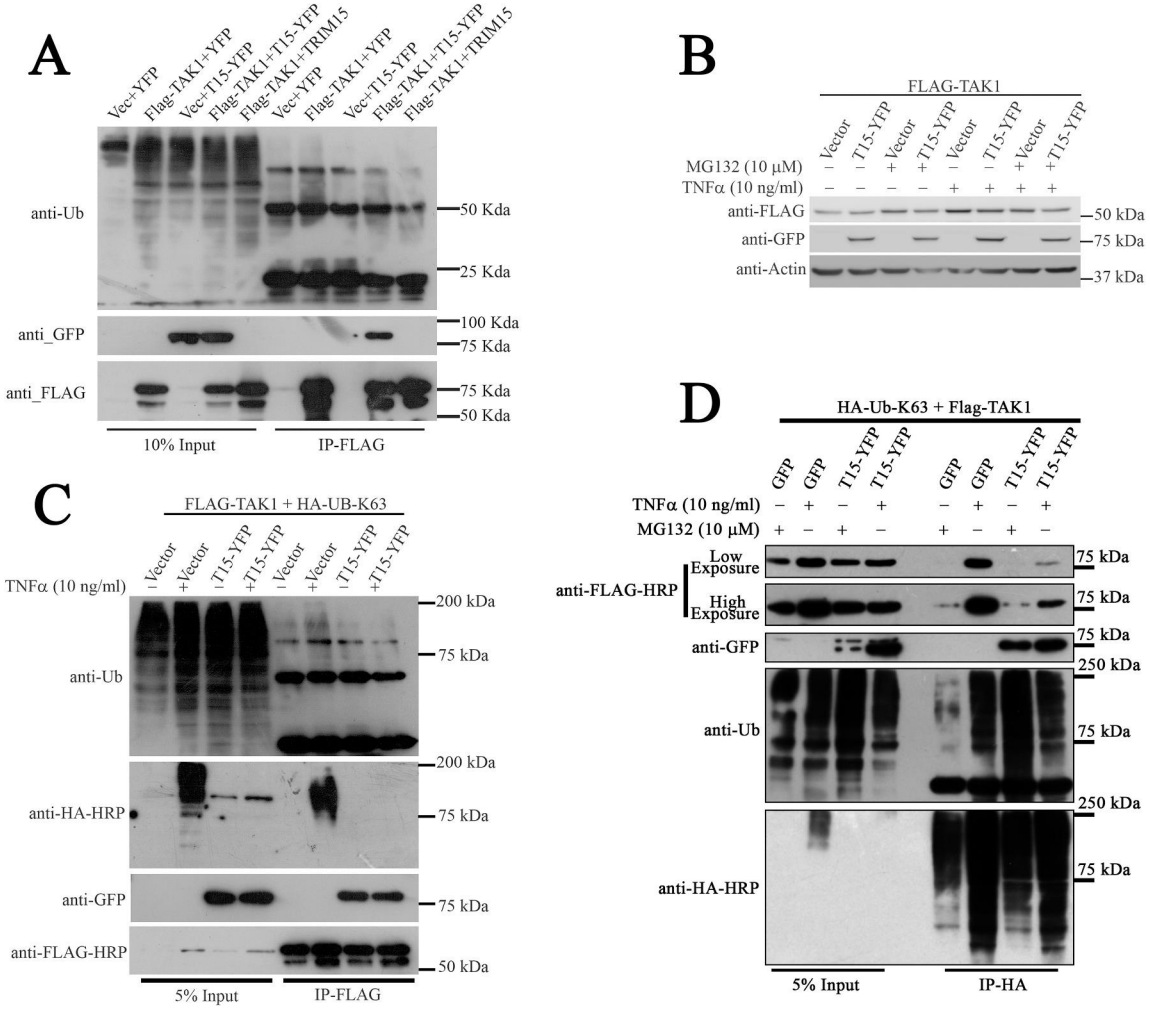
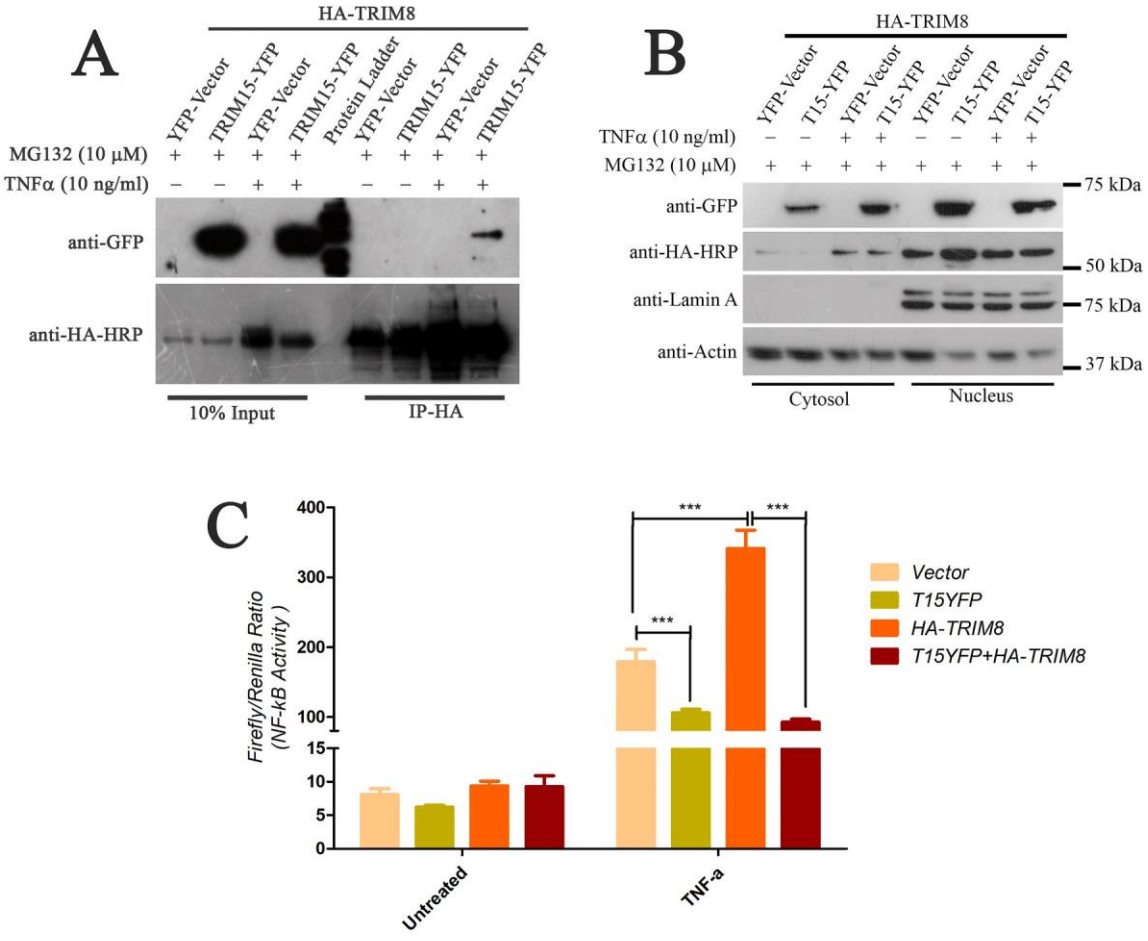

A
Vec+YFP
Flag-TAK1+YFP
Vec+T15-YFP
Flag-TAK1+T15-YFP
Flag-TAK1+TRIM15
Vec+YFP
Flag-TAK1+YFP
Vec+T15-YFP
Flag-TAK1+T15-YFP
Flag-TAK1+TRIM15
anti-Ub
50 Kda
25 Kda
anti_GFP
100 Kda
75 Kda
anti_FLAG
75 Kda
50 Kda
10% Input
IP-FLAG
B
FLAG-TAK1
Vector
T15-YFP
Vector
T15-YFP
Vector
T15-YFP
Vector
+T15-YFP
MG132 (10 μM)
TNFα (10 ng/ml)
anti-FLAG
anti-GFP
anti-Actin
50 kDa
75 kDa
37 kDa
C
FLAG-TAK1 + HA-UB-K63
TNFα (10 ng/ml)
−Vector
+Vector
−T15-YFP
+T15-YFP
−Vector
+Vector
−T15-YFP
+T15-YFP
anti-Ub
anti-HA-HRP
anti-GFP
anti-FLAG-HRP
200 kDa
75 kDa
200 kDa
75 kDa
75 kDa
50 kDa
5% Input
IP-FLAG
D
HA-Ub-K63 + Flag-TAK1
GFP
GFP
T15-YFP
T15-YFP
GFP
GFP
T15-YFP
T15-YFP
TNFα (10 ng/ml)
MG132 (10 μM)
anti-FLAG-HRP
Low Exposure
High Exposure
anti-GFP
anti-Ub
anti-HA-HRP
75 kDa
75 kDa
75 kDa
250 kDa
75 kDa
250 kDa
75 kDa
5% Input
IP-HA

Fig. 7


A
HA-TRIM8
YFP-Vector
TRIM15-YFP
YFP-Vector
TRIM15-YFP
Protein Ladder
YFP-Vector
TRIM15-YFP
YFP-Vector
TRIM15-YFP
MG132 (10 μM)
TNFα (10 ng/ml)
anti-GFP
anti-HA-HRP
10% Input
IP-HA
B
HA-TRIM8
YFP-Vector
T15-YFP
YFP-Vector
T15-YFP
YFP-Vector
T15-YFP
YFP-Vector
T15-YFP
TNFα (10 ng/ml)
MG132 (10 μM)
anti-GFP
anti-HA-HRP
anti-Lamin A
anti-Actin
75 kDa
50 kDa
75 kDa
37 kDa
Cytosol
Nucleus
C
Firefly/Renilla Ratio
(NF-kB Activity)
400
300
200
100
15
10
5
0
***
***
Vector
T15YFP
HA-TRIM8
T15YFP+HA-TRIM8
Untreated
TNF-a

[52]