Chapter 7:

Novel DUB-like activity of TRIM15:

RING E3 ligase; regulates TNF-α-

induced NF-κB pathway in a feedback

manner

Ubiquitination plays critical role in regulation of TNF-α mediated NF-κB pathway. cIAP1/2 mediated Lys-63-linked RIP1 polyubiquitination recruits TAK1 kinase complex and IKK complex (Hayden and Ghosh, 2014; Wertz and Dixit, 2010). The activation of IKK complex promotes phosphorylation of IκBα leading to its Lys-48-linked polyubiquitination by SCF^{βTrCP} ubiquitin ligase complex (Hayden and Ghosh, 2014; Wertz and Dixit, 2010). Removal of K63-linked ubiquitin chains by deubiquitinase (DUB) like A20 and CYLD negatively regulates NF-κB activation and play pivotal role in controlling the NF-κB pathway (Afonina et al., 2017; Hayden and Ghosh, 2014; Wertz and Dixit, 2010). TNF-α stimulation also promotes expression of several E3 ligases including various TRAFs, cIAPs and XIAP and most of these modifiers are found to regulate the pathway in a positive manner (Pahl, 1999). Surprisingly till date no TNF-α-induced E3 ligase mediating negative regulation of NF-κB pathway and controlling proinflammatory signaling have yet been described.

Tripartite Motif Containing (TRIM) proteins belong to RING E3 ligases and are characterized by signature motif composed of RING, B-Box, Coiled Coil domain and variable C-terminal domain (Meroni and Diez-Roux, 2005; Reymond et al., 2001). The roles of these proteins are emerging in innate immune regulation, viral restriction and autophagy (Hatakeyama, 2017; Tomar and Singh, 2015). Previous reports had shown that TRIM8 and TRIM38 act as positive and negative (respectively) regulators of TNF-α and IL1β activated NF-κB pathway (Hu et al., 2014; Li et al., 2011; Tomar et al., 2012a). Many TRIMs have been identified as regulators of NF-κB in response to diverse stimuli however systemic TRIMs modulation of TNF-α regulated NF-κB is not well understood (Hatakeyama, 2017; Tomar and Singh, 2015). Our screening had identified TRIM15 as a negative regulator of TNF-α-induced NF-κB pathway therefore we further characterized its role in regulation of the pathway.

7.1 TRIM15 is a late response TNF-α induced gene and inhibits TNF-α-induced NF-κB activation

Previous study exploring DNA methylation patterns associated with gastric cancer had identified TRIM15 as a hyper-methylated gene (Cheng et al., 2014), and the screening showed TRIM15 mRNA expression was enhanced in TNF-α treated cells. Further, TRIM15 inhibited TNF-α-induced NF-κB activity. Therefore, TRIM15 was further characterized for its role in regulation of TNF-α-induced NF-κB pathway.

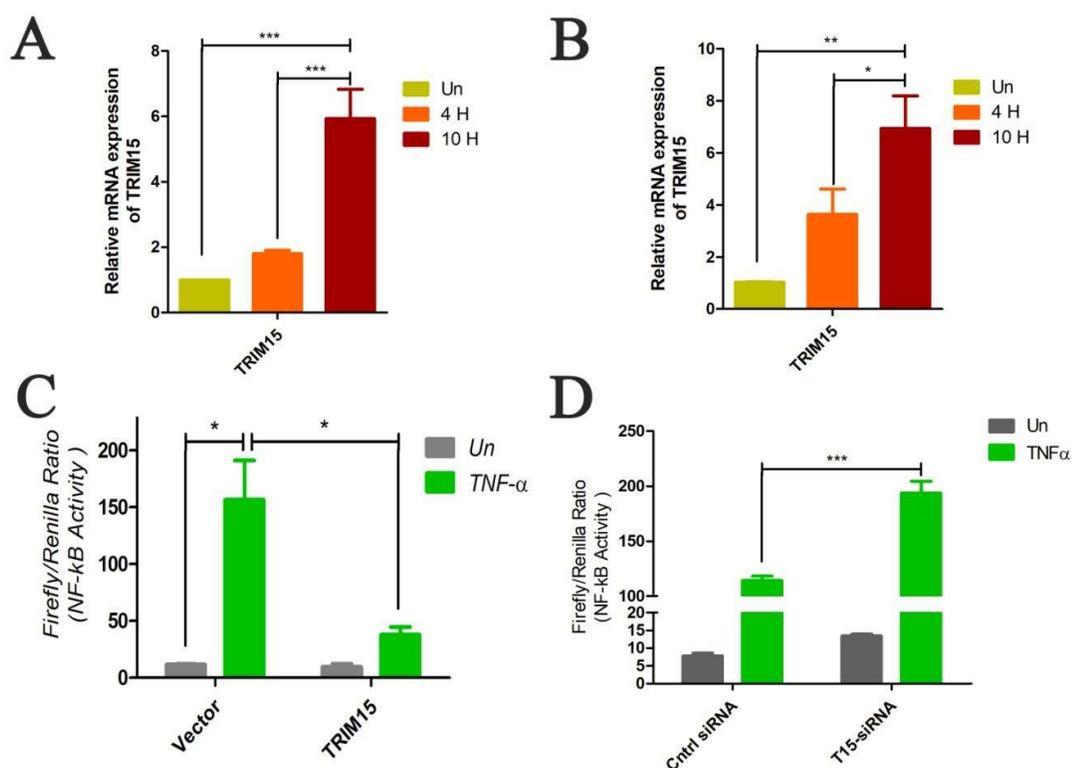***Figure 7.1 (I) : TRIM15 is a late response TNF-α induced gene and inhibits TNF-α-induced NF-κB activation.*** *TNF-α induces expression of TRIM15. (A) HEK293 & (B) MCF-7 cells were treated with TNF-α for indicated time and mRNA expression of TRIM15 was analyzed using qRT-PCR. (C) TRIM15 inhibits TNF-α-induced NF-κB activation. HEK293 cells were co-transfected with control vector or TRIM15 and NF-κB reporter constructs. After 24 hours of transfection, cells were treated with TNF-α for 10 hours and NF-κB activity was*

measured by Dual glow luciferase reporter assay. (D) Control siRNA or TRIM15-sirRNA was co-transfected with NF-κB reporter constructs and 24 hours post transfection, cells were treated with TNF-α for 10 hours and NF-κB activity was measured. 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.

TRIM15 expression was analyzed during "Mid" (4 hours) and "Late" (10 hours) response using qRT-PCR. TRIM15 mRNA transcript levels showed 6 fold increase after 10 hours of TNF-α treatment, whereas no significant change was observed in TRIM15 mRNA levels after 4 hours of treatment (Figure 7.1 (I) A). Expression analysis of TRIM15 in MCF-7 cells treated with TNF-α also showed similar increase in mRNA expression (Figure 7.1 (I) B). It was observed that cultured aortic smooth muscle cells (SMCs) stimulated with TNF-α shows increased expression of TRIM15 at 2 hours and highest expression was observed at 6 hours (Figure 7.1 (II) A) (Lotzer et al., 2010). The data also shows further decrease in TRIM15 transcript levels at 24 hours of TNF-α treatment, suggesting mRNA expression of TRIM15 increases at early time point and comes at the basal within 24 hours (Figure 7.1 (II) A). These results suggest that TRIM15 is primarily a late response TNF-α-induced gene.

Screening results showing the effect of TRIM15 on NF-κB activation was reconfirmed by Dual Glow Luciferase Reporter assay. The expression of TRIM15 decreased TNF-α-induced NF-κB activation as compared to control (Figure 7.1 (I) C). The knockdown of TRIM15 by siRNA increased TNF-α-induced NF-κB activation (Figure 7.1 (I) D). Temporal analysis of TRIM15's effect on NF-κB inhibition showed that ectopic expression of TRIM15 inhibits NF-κB activity at both 4 hours and 24 hours (Figure 7.1 (II) B).
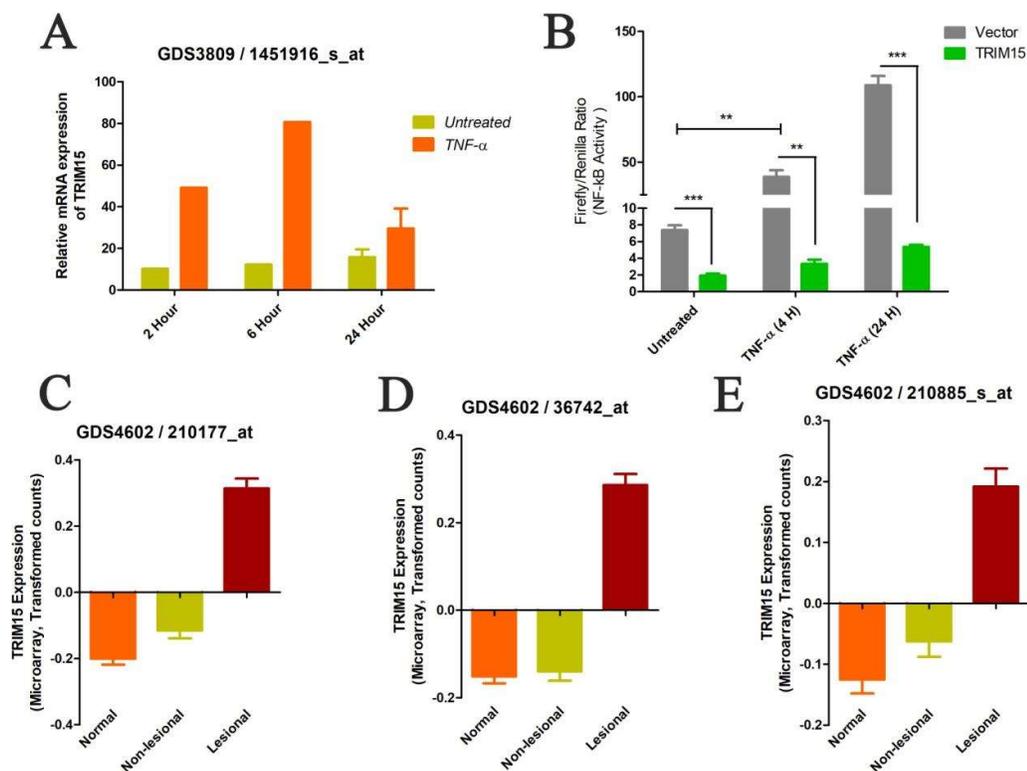
Figure 7.1 (II) : Temporal Expression of TRIM15 and effect on NF-κB activation. (A) Microarray expression values from GEO dataset GDS3809 was plotted for probe set 1451916_s_at (TRIM15). (B) MCF-7 cells were co-transfected with control vector or TRIM15 and NF-κB reporter constructs. After 24 hours of transfection cells were treated with TNF-α for indicated time and NF-κB activity was measured by Dual glow luciferase reporter assay. (C, D & E) Microarray expression values (transformed counts) of GEO dataset GDS4602 was plotted for TRIM15 probe sets as indicated. Asterisk (**) and (***) indicates NF-κB activity statistically significant from control; p value <0.01 and <0.001 (respectively), SEM of minimum three independent experiments.

Further the GEO database for disease progression related to inflammatory conditions was analyzed. Interestingly, gene expression analysis of microarray dataset comparing expression between normal, non-lesional and lesional psoriasis tissue showed marked increase in TRIM15 mRNA expression in lesional psoriasis tissue compared to normal control and non-lesional

(psoriasis) tissue (Figure 7.1 (II) C, D & E). All these evidences strongly suggest that TRIM15 may have critical role in regulation of inflammation, hence may be implicated in chronic inflammatory conditions.

7.2 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 (Hayden and Ghosh, 2014; Liu et al., 2017). To further investigate the mechanism of TRIM15 mediated inhibition of NF-κB activity; the step regulated by TRIM15 was analyzed. After co-transfection of TRAF2 with TRIM15 in HEK293 cells, NF-κB activation in presence/absence of TNF-α was analyzed. The expression of TRAF2 significantly enhanced NF-κB activation whereas TRIM15 significantly reduced the activity in untreated cells (Figure 2A). It was also observed that TNF-α treatment further enhanced TRAF2 activated NF-κB activation whereas TRIM15 co-transfection reduced it (Figure 7.2 A).

Further, the effect of TRIM15 on TAK1 induced NF-κB activation was monitored. TAK1 expression did not affect NF-κB activity in untreated cells but enhanced NF-κB activity in TNF-α treated cells compared to vector (Figure 7.2 B). We also observed that co-transfection of TRIM15 with TAK1 inhibited NF-κB activity (Figure 7.2 B) in presence of TNF-α. The co-expression of TRIM15 with TAK1 reduced the levels of p-IκBα levels in both untreated and TNF-α treated cells compared to vector co-transfected cells (Figure 7.2 F).

Many regulators of TNF-α-induced proteins act on the NF-κB heterodimers to regulate the activation of the pathway (Afonina et al., 2017; Hayden and Ghosh, 2014; Liu et al., 2017). 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

showed no effect on NF-κB activation (Figure 7.2 C). These results confirm that TRIM15 acts upstream of p65 and downstream of TRADD-TRAF complex.
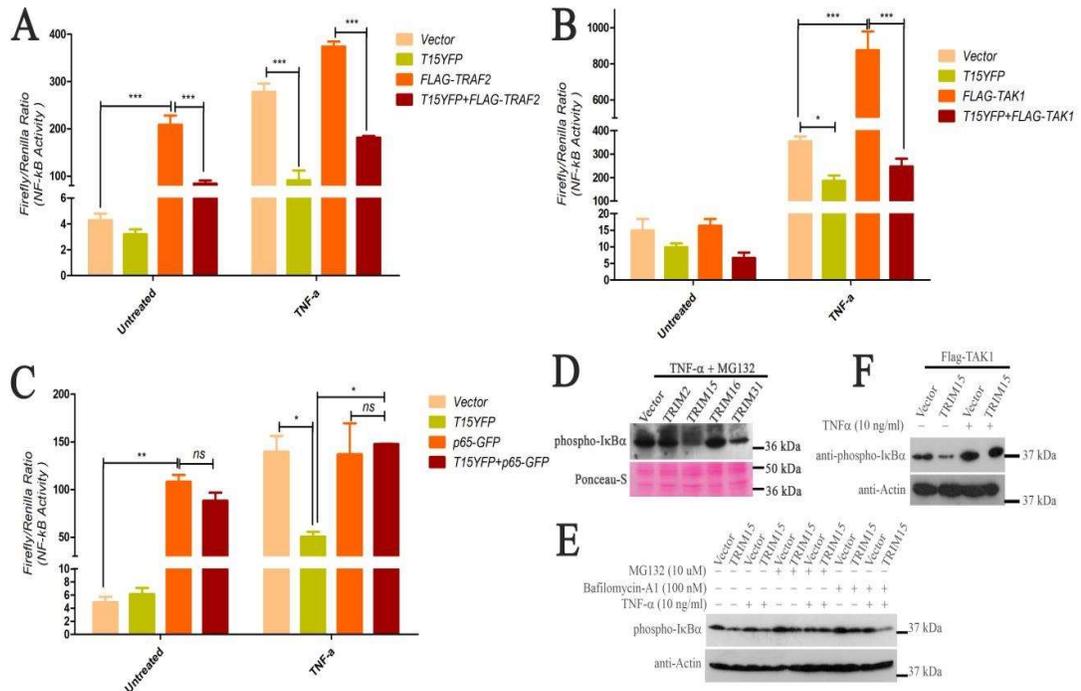

Figure 7.2 : TRIM15 acts upstream of p65 and downstream of TRAF2 in NF-κB pathway. *Vector or TRIM15 was co-transfected as indicated with TRAF2 (A), TAK1 (B) or p65 (C) in HEK293 cells along with NF-κB reporter constructs. 24 hours post transfection cells were treated with TNF-α for 10 hours and NF-κB activity was measured. (D) TRIM15 inhibits IκBα phosphorylation. HEK293 cells were transfected with indicated constructs and treated with TNF-α and MG132 for 10 hours. Western blotting was performed to check the levels of p-IκBα levels using specific antibody. (E) MCF-7 cells were transfected with control vector or TRIM15 and TNF-α, MG132 and Baflilomycin-A1 as indicated, for 10 hours. Western blotting was performed to check the levels of p-IκBα. (F) Flag-TAK1 was co-transfected with either vector or TRIM15 in HEK293 cells and treated with TNF-α for 10 hours. 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.

TRIM15 was able to inhibit TAK1 and TRAF2 activated NF-κB but failed to inhibit p65 induced NF-κB activation, suggesting it acts upstream of p65 heterodimers and possibly regulate IκBα phosphorylation. Phospho-IκBα is ubiquitinated and degraded by ubiquitin proteasome system (UPS) (Hayden and Ghosh, 2014; Liu et al., 2017; Oeckinghaus and Ghosh, 2009). To further confirm the effect of TRIM15 on IκBα phosphorylation, p-IκBα levels were analyzed by western blotting. Cells were transfected with indicated TRIMs and were treated with combination of TNF-α and MG132 (UPS inhibitor). Western blotting showed the 40 kDa band corresponding to p-IκBα was significantly reduced in TRIM15 transfected cells compared to control, suggesting inhibition of IκBα phosphorylation (Figure 7.2 D). Similarly, the p-IκBα levels were also analyzed in MCF-7 cells transfected with control vector or TRIM15 and treated with TNF-α, MG132, Bafilomycin-A1 (Inhibitor of autophagy) or in combination. Western blotting showed significant reduction in levels of 40 kDa band corresponding to p-IκBα in TRIM15 transfected cells compared to control, TNF-α, MG132, Bafilomycin-A1 and TNF-α-Bafilomycin-A1 co-treated cells (Figure 7.2 E). Interestingly, no significant difference was observed 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 NF-κB activation by inhibiting IκBα phosphorylation and does not affect its proteasomal degradation.

7.3 TRIM15: RING E3 Ligase possesses DUB-like activity and inhibits various ubiquitin chain topologies

Recruitment of different ubiquitin ligases brings stringency and specificity to the pathway due to their ability to identify specific substrate and addition of ubiquitin in specific topologies (Kwon and Ciechanover, 2017). Therefore, the effect of TRIM15 on various ubiquitin chain topologies was analyzed to further understand its E3 ligase function. HEK293 cells were co-transfected with HA-Ub-K6, K11, K27, K29, K48, K63 and either vector control or TRIM15 and treated with TNF-α. It was observed that TRIM15 co-transfection significantly reduced K6, K11, K27, K29, K48 and K63 linked polyubiquitination of proteins and reflected most pronounced effect on K6 and K63 linked poly-ubiquitination (Figure 7.3 A), suggesting that TRIM15 may possess strong DUB-like activity.

Therefore, the effect of TRIM15 on cellular ubiquitination was checked by co-transfecting vector or TRIM15 with HA-Ub-K63 and K63 linked ubiquitination was monitored. Interestingly, the higher molecular weight adducts of HA-Ub-K63 linked proteins were increased in presence of TNF-α in vector transfected cells (Figure 7.3 B). Moreover, TRIM15 co-transfection significantly reduced the level of K63 linked ubiquitination in TNF-α treated cells (Figure 7.3 B). Further, marked reduction of HA-Ub-K63 linked proteins was also observed in TRIM15 transfected MG132 and Bafilomycin-A1 treated cells compared to control, suggesting that TRIM15 inhibits K63-linked ubiquitination of proteins and does not promote their turnover through UPS or autophagy (Figure 7.3 B).

Also, similar decrease in K63-linked polyubiquitinated proteins was observed in TNF-α-MG132 and TNF-α-Bafilomycin-A1 co-treated cells (Figure 7.3 B). However, there was no difference in total ubiquitination (detected using anti-Ub antibody) between control and TRIM15 transfected cells treated with TNF-α, MG132 and Bafilomycin-A1 (Figure 7.3 B).
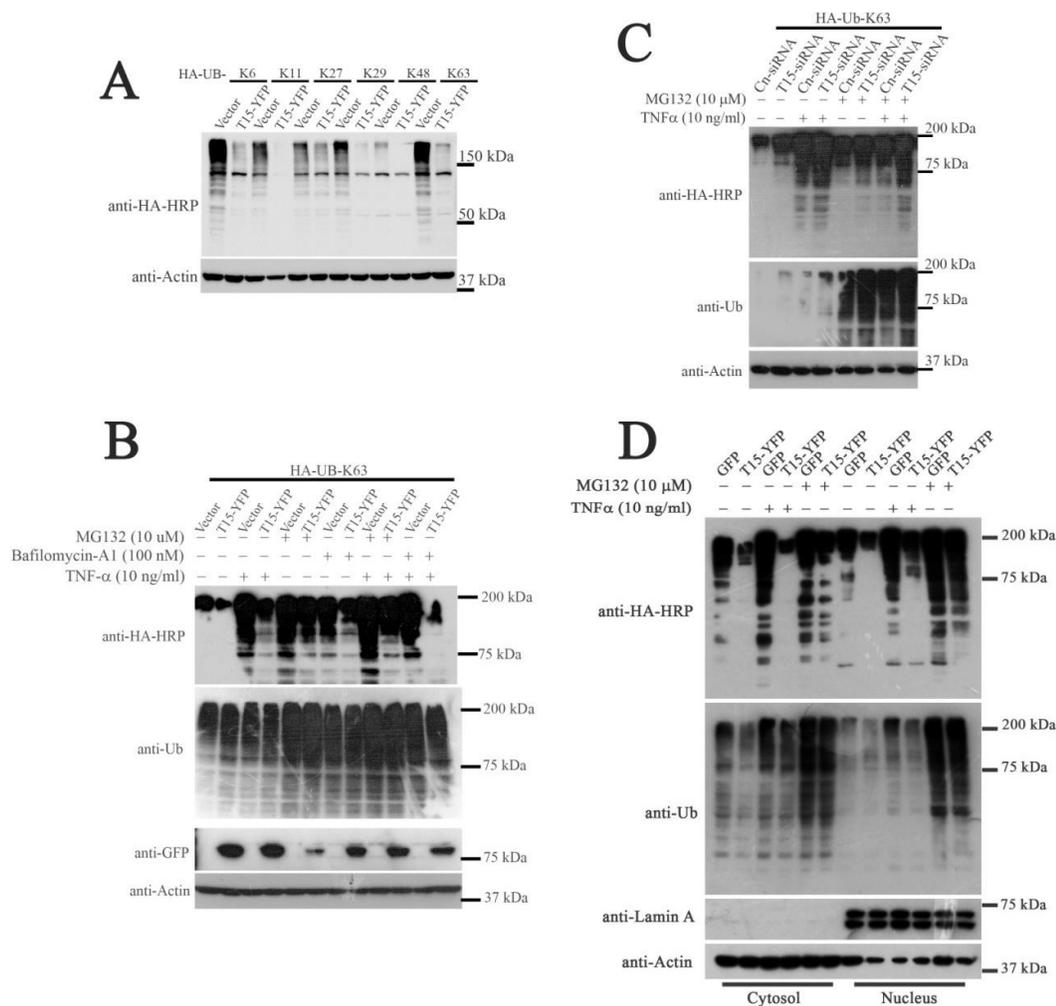
Figure 7.3: TRIM15 possesses DUB-like activity and predominantly inhibits K6 and K63 linked polyubiquitination. *(A) HA tagged Ub-K6, K11, K27, K29, K48 and K63 were co-transfected with either vector or TRIM15-YFP. Cells were treated with TNF-α for 10 hours after transfection and western blotting was performed using HA antibody. (B) TRIM15 inhibits K-63 linked ubiquitination. Vector or TRIM15-YFP constructs were co-transfected with HA-Ub-K63 in HEK293 cells. 24 hours post transfection cells were treated with TNF-α, MG132 and Bafilomycin-A1 as indicated for 10 hours. 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 hours. 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 hours of treatment nuclear-cytosolic fractions were prepared and levels of indicated proteins were detected using specific antibodies.

This was further confirmed by knock down of TRIM15. Control or TRIM15-siRNA were co-transfected with HA-Ub-K63 and the levels of K63-linked ubiquitination of proteins was monitored using HA specific antibody. Significant increase in levels of higher molecular weight K63-linked protein adducts in TRIM15-siRNA transfected cells compared to control transfected cells was observed in both absence and presence of TNF-α (Figure 7.3 C). Interestingly, similar increase in K63-linked ubiquitination was also observed in TRIM15 knockdown cells treated with MG132 and also in combined treatment of TNF-α and MG132 (Figure 7.3 C), whereas slight increase was observed in total ubiquitination in TRIM15-siRNA transfected cells in these conditions (Figure 7.3 C).

TRIM15 is predominantly a nuclear protein; therefore, its effect on nuclear and cytosolic protein ubiquitination was monitored. HEK293 cells were co-transfected with HA-Ub-K63 and control or TRIM15-YFP and later treated with TNF-α or MG132. Cytosolic and nuclear fractions were prepared and monitored by western blotting. It was observed that TRIM15 significantly reduced K63-linked ubiquitination in presence of TNF-α both in cytoplasm and nucleus whereas its effect on total ubiquitination was less pronounced (Figure 7.3 D). Consistent with the previous blot (Figure 7.3 B) K63 ubiquitination was lesser in TRIM15 transfected cells as compared to control when treated with MG132, whereas little or no difference was observed in total ubiquitination (Figure 7.3 D). These results suggest that E3 ligase TRIM15 possesses DUB-like activity, inhibits K-63-linked ubiquitination of proteins in both nucleus and cytoplasm and does not affect their turnover.

7.4 PRY/SPRY domain mediated DUB-like activity of TRIM15 is essential for inhibition of TNF-α-induced NF-κB activity

The domain responsible for the TRIM15 DUB-like activity was further monitored. Different domain deletion constructs of TRIM15 were transfected and K63 linked ubiquitination and NF-κB activation was monitored. Transfection of TRIM15 with RING and B-Box deletion still showed DUB-like activity (Figure 7.4 A) and significantly inhibited TNF-α-induced NF-κB activity (Figure 7.4 B), suggesting that E3 ligase activity of TRIM15 is not required for its DUB-like activity.
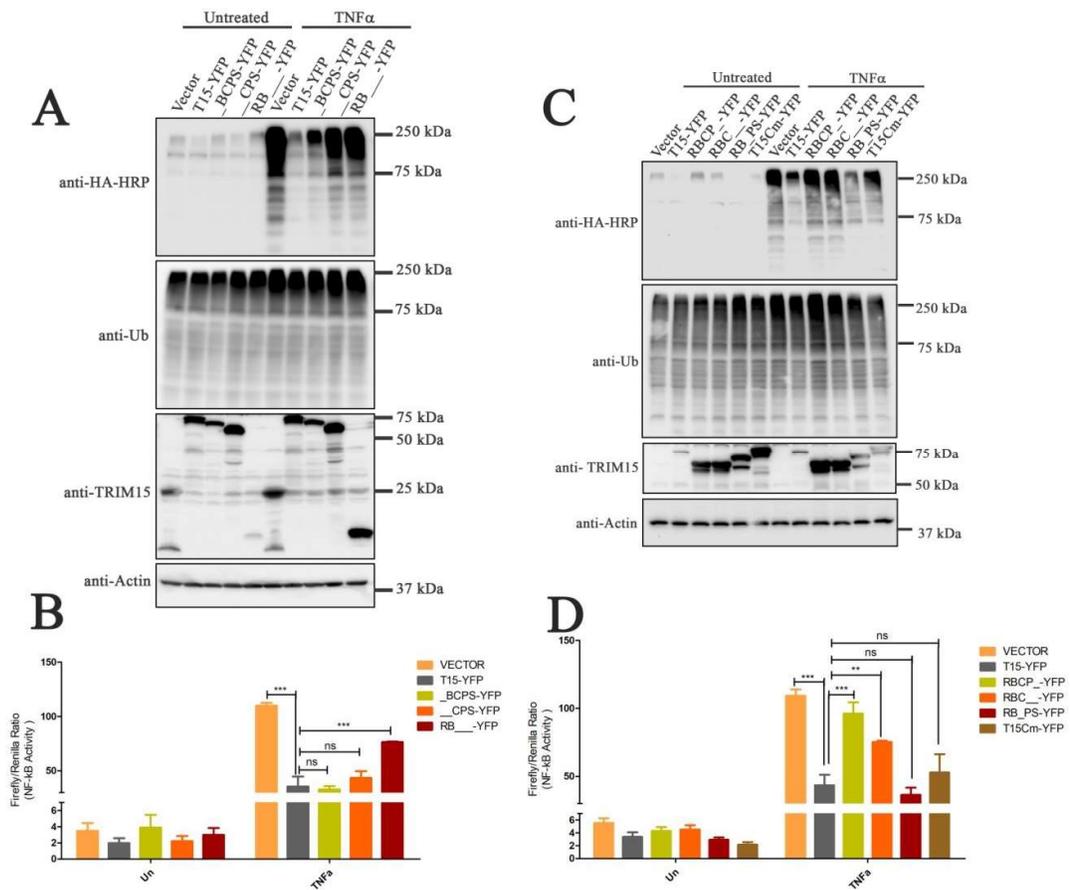

Figure 7.4: PRY/SPRY domain mediated DUB-like activity of TRIM15 is essential for inhibition of TNF-α-induced NF-κB activity. *PRY/SPRY domain of TRIM15 is responsible for its DUB like activity. (A & C) HA-Ub-K63 was co-*

transfected with either vector or indicated TRIM15 construct in HEK293 and treated with TNF-α for 10 hours. 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 24 hours post transfection cells were treated with TNF-α for 10 hours and 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.

Interestingly, the deletion of SPRY and PRY/SRPY domain of TRIM15 completely reverted the DUB-like activity of full length TRIM15 (Figure 7.4 C) and could not inhibit TNF-α-induced NF-κB activity (Figure 7.4 D). However, transfection with CC domain deleted TRIM15 or CC domain mutant (T15Cm) failed to inhibit K-63 linked ubiquitination (Figure 7.4 C) and inhibited NF-κB activity (Figure 7.4 D).

These results suggest that PRY/SPRY domain of TRIM15 possess the DUB-like activity responsible for inhibition of TNF-α-induced NF-κB activity. Interestingly, these results also confirmed that, significant correlation exist between cellular TRIM15 mediated K63 linked deubiquitination and NF-κB activity.

7.5 Spatiotemporal localization and dynamics of TRIM15

TRIM proteins are dynamic in nature and their spatio-temporal localization may affect their cellular functions. Therefore, cellular localization of TRIM15 using TRIM15-YFP was analyzed in TNF-α, MG132 and Bafilomycin-A1 treated cells. It was observed that TRIM15 is localized in cytosol as well as in nucleus and forms large aggregate in cytosol, whereas appears as punctate in nucleus

(Figure 7.5 (I) A & B). Whereas, no prominent change was observed in cellular localization of TRIM15 in presence of TNF-α, MG132 and Bafilomycin-A1 (Figure 7.5 (I) A & B). Interestingly, the puncta size of TRIM15-YFP having $\geq$ 10 µM increased in TNF-α, MG132 and Bafilomycin-A1 treated cells compared to untreated control (Figure 7.5 (I) C). Also, corresponding decrease in puncta size ranging up to $\leq$ 2 µM was observed in TNF-α, MG132 and Baflilomycin-A1 treated cells compared to control (Figure 7.5 (I) C). Increase in TRIM15 puncta size in MG132 and Bafilomycin-A1 suggest its possible turnover through proteasome and autophagy pathway, whereas its increase in TNF-α treatment is possibly due to enhanced transcription of TRIM15.
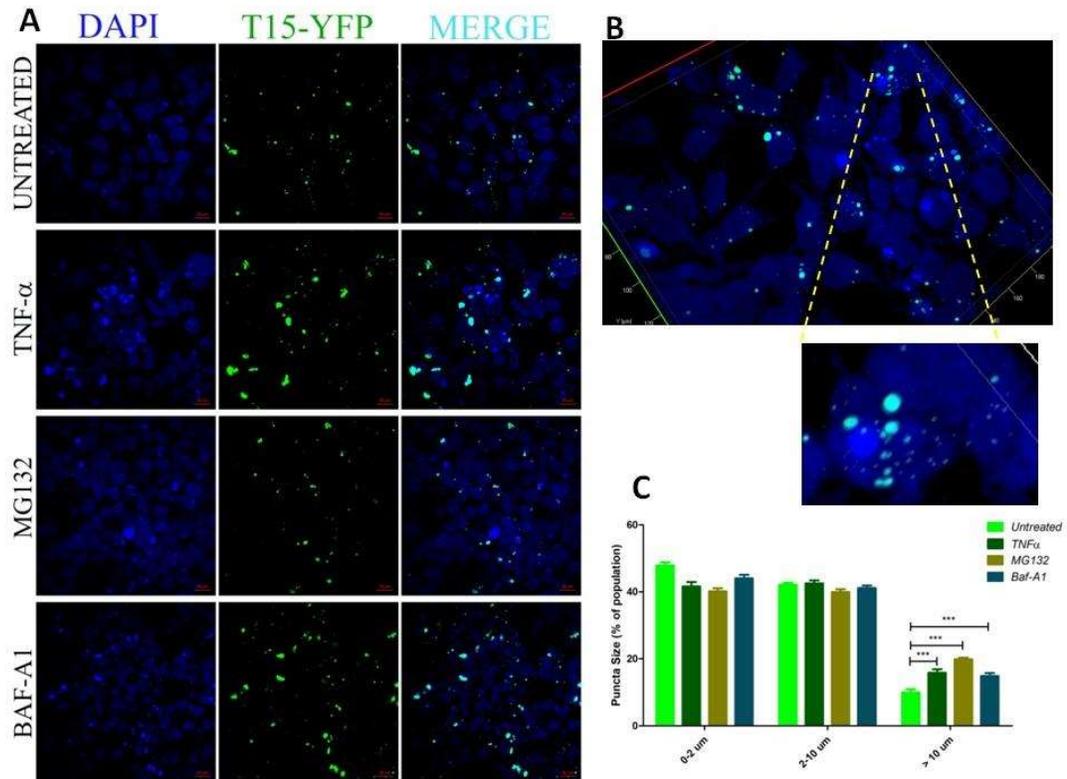

Figure 7.5 (I) : Cellular localization and dynamics of TRIM15. *(A & C) HEK293 cells were transfected with TRIM15-YFP and 24 hours post transfection cells were treated with TNF-α, MG132 or Bafilomycin-A1 for 10 hours. Cells were monitored under fluorescent microscope for YFP*

visualization. (C) From the obtained images YFP puncta were counted using object count feature of Nikon AR image analysis software. Same parameters were applied for all the images and % populations for each size range puncta were plotted. (B) TRIM15-YFP was transfected in HEK293 cells and stained with DAPI. Cells were observed under confocal microscope for observing cellular localization. Asterisk (**) and (***) indicates statistically significant difference in % population from control; p value <0.001, SEM of minimum six different fields.

To further monitor the effect of TNF-α on TRIM15, cells were transfected with TRIM15-YFP and treated with TNF-α for 10 hours. YFP fluorescence in control and TNF-α treated cells (Figure 7.5 (II) A) were monitored using fluorescent microscope. Mean fluorescence intensity (MFI) and binary area in untreated and TNF-α treated cells were measured. It was observed that MFI and binary area of YPF tagged TRIM15 (Figure 7.5 (II) B & C) significantly increased in presence of TNF-α. Similar increase in MFI and binary area was observed in TRIM15-RFP transfected cells treated with TNF-α (Figure 7.5 (II) D & E). These results further confirmed TRIM15 increased expression and formation of higher order structure in presence of TNF-α.

The endogenous levels of TRIM15 were further monitored using western blotting in nuclear and cytoplasmic fraction of cells treated with or without TNF-α, MG132, Bafilomycin-A1 or in combination. Interestingly, it was observed that the 50 kDa band corresponding to TRIM15 was high in nuclear fraction compared to cytosol (Figure 7.5 (II) F).
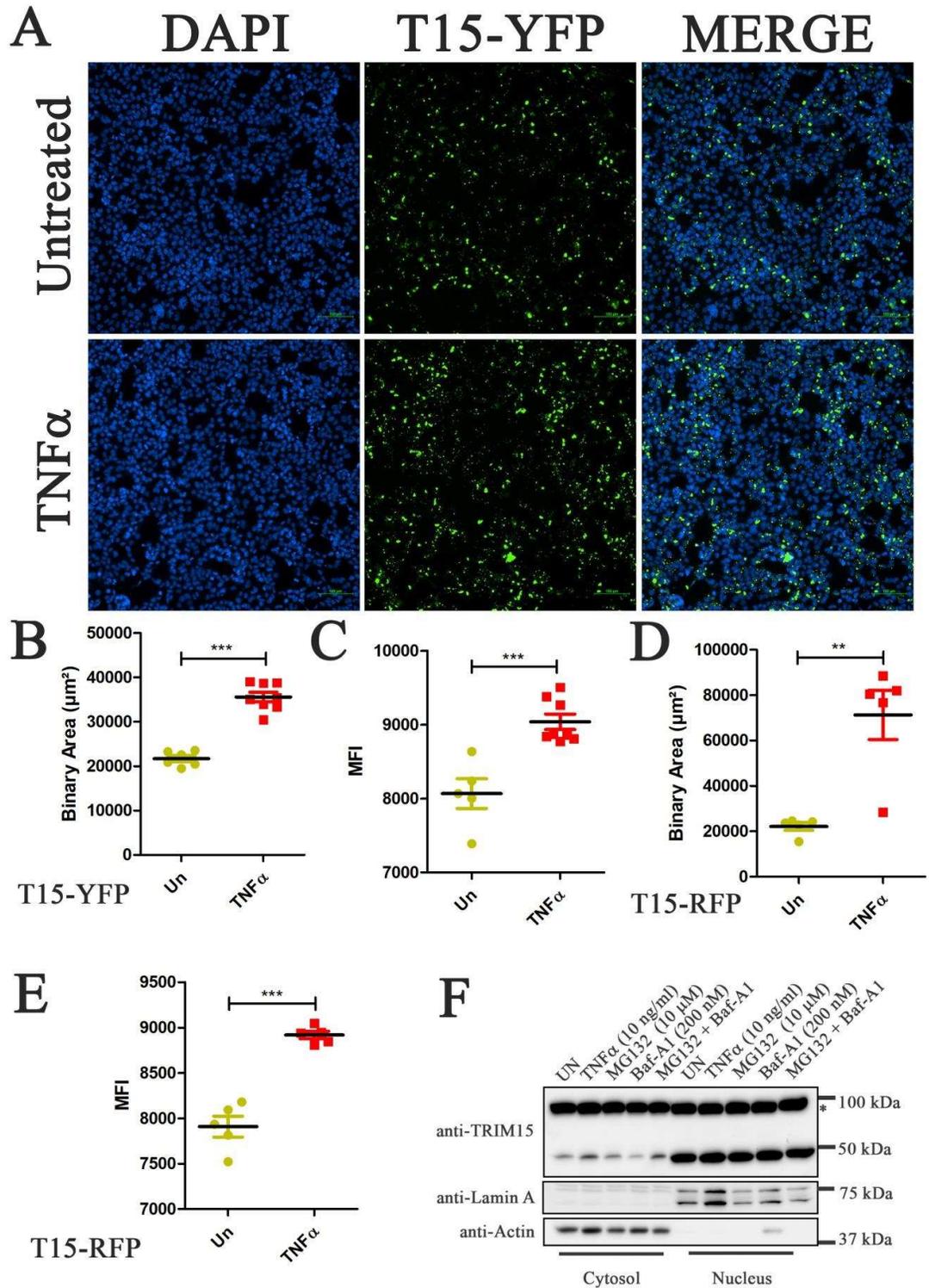
Figure 7.5 (II) : TRIM15 forms higher order structures. *(A) TRIM15-YFP transfected cells were grown on cover slip and treated with TNF-α for 10*

hours. Cells were fixed, stained with DAPI and monitored under fluorescent microscope for DAPI and YFP fluorescence. (B & C) Binary area and mean florescence intensity of TRIM15-YFP and (D & E) TRIM15-RFP were measured from the captured images using automated measurement feature of Nikon Elements AR software. (F) HEK293 cell were treated with TNF-α, MG132 and Bafilomycin-A1 as indicated for 10 hour. Nuclear and cytosolic fractions were prepared and indicated specific antibodies were used to detect protein levels. Asterisk (**) and (***) indicates statistically significant difference in MFI and binary area from control; p value <0.01 and <0.001 (respectively), SEM of

Moreover, another prominent band corresponding to 100 kDa representing the dimeric form of TRIM15 reported previously (Lee et al., 2015) was observed, suggesting that TRIM15 forms a highly stable dimer found in both nucleus and cytoplasm, whereas the monomeric form of TRIM15 was predominantly found in nucleus. Further, significant increase in 50 kDa band corresponding to monomeric TRIM15 increased in the cytosolic fraction compared to untreated cell was observed (Figure 7.5 (II) F). Indicating, it is dynamic in nature and accumulates in cytosol in TNF-α treated cells.

7.6 TRIM15 interacts with TRIM8 inhibits its cytoplasmic translocation and TNF-α-induced NF-κB activity

Previous report had shown that TRIM8's nucleo-cytoplasmic translocation is required for TNF-α-induced NF-κB activation (Tomar et al., 2012a), whereas another report had shown that TRIM8 promotes K63-linked polyubiquitination of TAK1 to induce NF-κB activation (Li et al., 2011). TRIM proteins are known to homo and heterodimerize for target identification and assembly of signalosomes (Koliopoulos et al., 2016; Li et al., 2014). Therefore, the interaction of TRIM15 and TRIM8 was checked by co-immunoprecipitation (Co-IP). HA tagged TRIM8 was co-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 was performed to check the interaction. It was observed that the 75 kDa band corresponding to TRIM15 appeared in TRIM15 and HA-TRIM8 co-transfected cells co-treated TNF-α and MG132 (Figure 7.6 A).
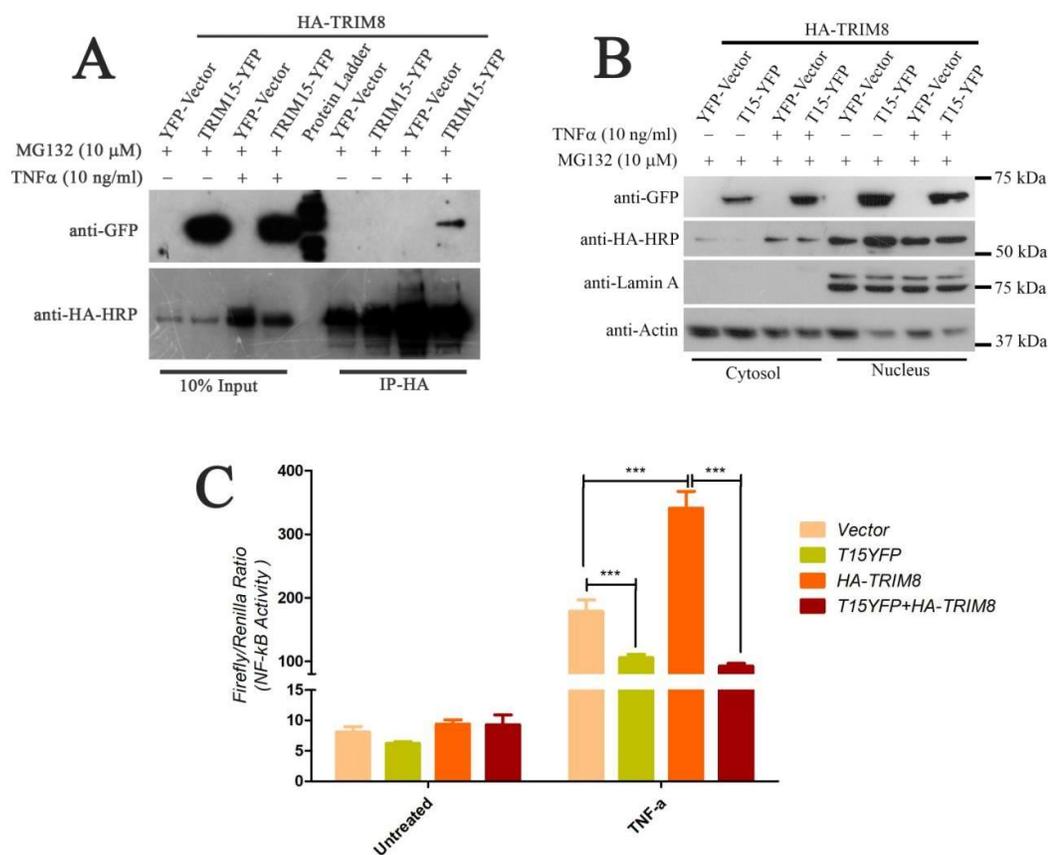

Figure 7.6 : TRIM15 interacts with TRIM8 and inhibits TRIM8-enhanced NF-κB activation. *(A) TRIM15 interacts with TRIM8 in TNF-α treated cells. HA-TRIM8 was co-transfected with control vector or TRIM15-YFP and treated as indicated for 10 hours. Cells were collected and TRIM8 was pulled down using HA 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 hours 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 hours of transfection cells were treated with TNF-α for 10 hours 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.

The effect of TRIM15 on TRIM8 dynamics was further analyzed. HA-tagged TRIM8 was co-transfected with TRIM15-YFP or vector control in HEK293 cells and treated with TNF-α. TRIM8 and TRIM15 levels were monitored in nucleo-cytoplasmic fractions. The level of 75 kDa band corresponding to TRIM15-YFP was higher in nuclear fraction as compared to cytosol, whereas its cytosolic levels increased in presence of TNF-α (Figure 7.6 B). The 62 kDa band corresponding to HA-TRIM8 was high in nuclear fraction compared to cytosol (Figure 7.6 B) and interestingly, the level of TRIM8 was higher in nuclear fraction of TRIM8-TRIM15 co-transfected cells (Figure 7.6 B). Corresponding decrease in TRIM8 levels in cytoplasm in TRIM15 co-transfected cells was also observed (Figure 7.6 B).

To further check the functional effect of TRIM15 on TRIM8-enhanced TNF-α-induced NF-κB activity; the effect of TRIM15 on TRIM8-regulated TNF-α-induced NF-κB activity was checked. Ectopic expression of TRIM8 enhanced NF-κB activity compared to vector transfected TNF-α treated cells (Figure 7.6 C) as observed previously (Li et al., 2011; Tomar et al., 2012a). Interestingly, the co-expression of TRIM15 with TRIM8 significantly reduced the TRIM8 regulated NF-κB activation (Figure 7.6 C). These results clearly suggest that TRIM15 dynamically interacts with TRIM8, inhibit its nucleo-cytoplasmic translocation and inhibits TRIM8-regulated NF-κB activity.

7.7 TRIM15 inhibits NF-κB activation by inhibiting K63-linked ubiquitination of TAK1

TRIM8 is known to promote TNF-α-induced NF-κB activity by promoting TAK1 ubiquitination (Li et al., 2011) and it was observed that TRIM15 enhances nuclear retention of TRIM8 and inhibits TRIM8-regulated NF-κB activity (Figure 7.6 B). Therefore, TRIM15's effect on TAK1 ubiquitination was monitored. We co-transfected cells with Flag-TAK1 and control vector, TRIM15-YFP or tagless-TRIM15, and treated 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 (Figure 7.7 A). 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 (Figure 7.7 A). Similar reduction of TAK1 ubiquitination was observed in Flag-TAK1 co-transfected with tag-less TRIM15 (Figure 7.7 A).
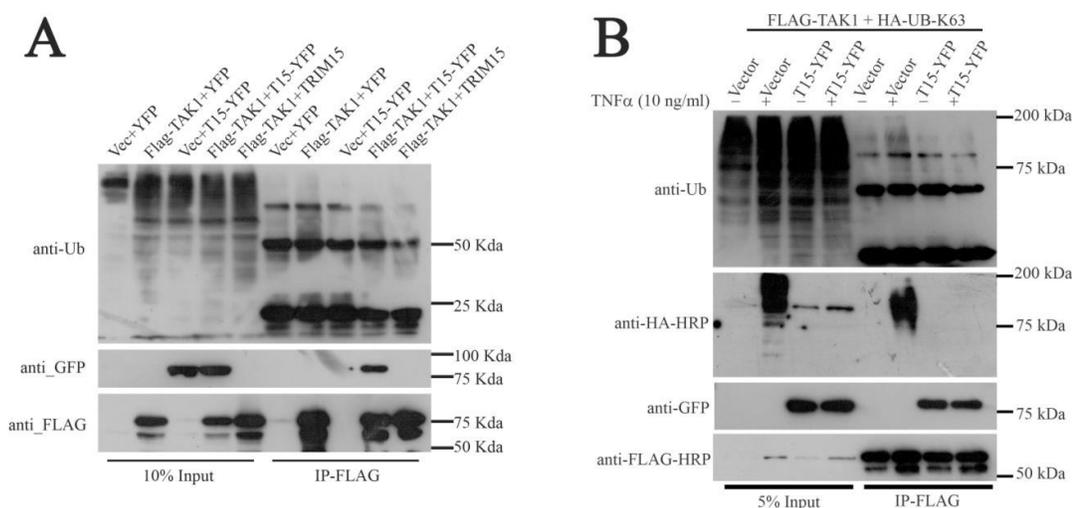

***Figure 7.7 : TRIM15 interacts with TAK1 and inhibits its K63-linked ubiquitination.** (A) HEK293 cells were transfected with control vector,*

TRIM15-YFP, tagless-TRIM15 and Flag-TAK1 as indicated. Cells were co-treated with TNF-α and MG132 for 10 hours and TAK1 was pulled down using Flag affinity gel. Western blotting was performed and indicated antibodies were used to check the interaction. (B) HA-Ub-K63 and Flag-TAK1 was co transfected with either control vector or TRIM15-YFP and treated with or without TNF-α for 10 hours. TAK1 was pulled using Flag affinity beads from lysates and western blotting was performed to detect interaction and ubiquitination using indicated antibodies.

K63-linked ubiquitination of TAK1 at Lysine 158 is required for TNF-α induced NF-κB activation (Fan et al., 2010; Li et al., 2011; Oeckinghaus and Ghosh, 2009) and TRIM15 showed strong DUB-like activity. Therefore, TRIM15's effect on K63-linked ubiquitination of TAK1 was checked. Flag-TAK1 and HA-Ub-K63 were co-transfected with TRIM15 and treated with TNF-α and IP was performed using anti-Flag beads. Western blotting reconfirmed the interaction of TRIM15 and TAK1 and reduction of TAK1 ubiquitination in presence of TRIM15 (Figure 7.7 B). K63-linked ubiquitination of TAK1 was detected using HA specific antibody and it was found that TNF-α treatment promoted K63-linked ubiquitination of TAK1, whereas, TRIM15 co-transfection completely diminished K63-linked ubiquitination of TAK1 (Figure 7.7 B), suggesting TRIM15 interacts with TAK1 and inhibits its K63 ubiquitination, hence, NF-κB activity.

7.8 Discussion:

Isolated reports from different groups had shown that TRIMs, family of RING E3 ligases regulates cellular pathways by identification and modification of their target substrates (Hatakeyama, 2017; Hu and Shu, 2017; Hu et al., 2014; Li et al., 2011; Okumura et al., 2010; Tomar and Singh, 2015). Stimuli specific expression of these proteins brings precision and specificity to the pathways they regulate (Rajsbaum et al., 2008; Tomar et al., 2012a; Versteeg et al., 2013;

Zhao et al., 2012b). Previously it has been observed that the expression of TRIM15 was up-regulated by TLR3 and TLR4 ligands in THP1-derived macrophages (Jiang et al., 2017) but its expression was found unaffected by type-I IFNs in various immune cells (Rajsbaum et al., 2008). This study suggests that TRIM15 is a TNF-α-induced late response gene and strongly inhibits NF-κB activation in different cell types. TRIM15 is a highly methylated gene but its expression increases in response to TNF-α. This is supported by recent reports suggesting that infection (Pacis et al., 2019) and TNF-α promotes (Jia and Zhao, 2019) DNA demethylation of genes and promote their transcription. This also suggests that hypermethylated genes may also contribute to regulation of cellular pathways in response to specific stimuli (Jia and Zhao, 2019; Pacis et al., 2019). Previous studies had demonstrated that TRIMs have high turnover and stabilizes only specified pathophysiological conditions (Roy et al., 2018; Tomar and Singh, 2015; Tomar et al., 2012a; Versteeg et al., 2013). Interestingly, unlike other TRIMs, TRIM15 protein is stable and turnover is not high as it had been observed to play essential role in focal adhesion involved in cell migration, an essential cellular process (Uchil et al., 2014). TNF-α induced mRNA expression of TRIM15 suggests towards an additional layer of NF-κB regulation that is through methylation of their feedback regulatory genes.

Ubiquitin E3 ligases are known to modify substrates by adding specific ubiquitin chains on them and control their stability and degradation. Several NF-κB target genes are E3 ligases and found to regulate the pathway in a positive manner by modifying specific targets and regulating their turnover (Hayden and Ghosh, 2014; Liu et al., 2017; Lu et al., 2007; Schwenzer et al., 1999; Stehlik et al., 1998). Surprisingly it was observed that TRIM15 has inhibitory effect on cellular ubiquitination and it strongly inhibits K6 and K63 linked ubiquitin chains. Further characterization revealed that PRY/SPRY

domain mediated DUB-like activity of TRIM15 is responsible for its inhibitory effect on TNF-α-induced NF-κB activity. For the first time we show DUB-like activity of a member of E3 ligases family (TRIM15), which is attributed to its PRY/SPRY domain and its implication in regulation of the pro-inflammatory NF-κB pathway. This report also warrants further investigation of TRIM proteins for their possible role in A20-like ubiquitin editing activity and their implication in health and diseases.

Previous studies have found that activation of TAB-TAK complex is critical for activation of TNF-α-induced NF-κB pathway and TAK1 ubiquitination is critical for its activation (Afonina et al., 2017; Hayden and Ghosh, 2014; Liu et al., 2017; Reiley et al., 2007). Current study shows that TRIM15 interacts with TAK1 and inhibits its ubiquitination, consequently, inhibits NF-κB activation. K63 linked ubiquitination of TAK1 is critical for NF-κB activation and we observe that TRIM15 strongly inhibits it (Afonina et al., 2017; Fan et al., 2010; Hayden and Ghosh, 2014; Li et al., 2011; Liu et al., 2017). This is one of the first evidence elucidating the role of TNF-α-induced late response genes in feedback negative regulation of the NF-κB pathway.

Homo/heteromeric interactions of TRIM proteins had been previously observed (Reymond et al., 2001; Woodsmith et al., 2012) however their physiological implication had not yet been studied. Cooperation between TRIM proteins has been observed and reports also shows they might have opposite effect on the same substrate or pathway. The results here show interaction and functional antagonism of TRIMs in regulation of TNF-α-induced NF-κB pathway. Previously it had been shown that TRIM8's nucleo-cytoplasmic trafficking is important for NF-κB activation by promoting TAK1 ubiquitination (Li et al., 2011; Tomar et al., 2012a). This report shows that TRIM15 interacts with TRIM8, restricts its cytosolic translocation and inhibits TNF-α-induced NF-κB activity enhanced by TRIM8. Consequently, inhibits K63-

linked ubiquitination of TAK1 and activation of downstream signaling pathway.

In summary, the current study identifies TRIM15 as a novel feedback regulator of TNF-α-induced NF-κB pathway and further report novel DUB like activity of TRIM15 responsible for negative regulation of TNF-α-induced NF-κB activity. The study also reveals functional antagonism between TRIM8 and TRIM15 and its impact on TNF-α-induced NF-κB activity. TRIM15 could be critical modulator of inflammatory pathways and chronic pathological conditions and further study may provide novel way of therapeutic intervention in given pathophysiological conditions.