

Chapter 9:
Summary and Conclusion

9.1 Summary:

Optimal activation of inflammatory response and its temporal resolution is critical for host defense against pathogen and injury. TNF- α -induced NF- κ B pathway is the major proinflammatory pathway required for mounting inflammatory response. Temporal expression induced by TNF- α has been characterized as 'early', 'mid' and 'late' response genes and major emphasis has been given to the early response genes for regulation of the pathway in a feedback manner. Moreover, the role of many late response genes and their cellular functions remain elusive. Investigating the late response genes may provide us novel targetable regulators of TNF- α -induced NF- κ B pathway. PTM by ubiquitination regulates virtually all cellular pathways including TNF- α -induced NF- κ B pathway. Several ubiquitin E3 ligases are recruited at distinct steps of TNF- α -induced NF- κ B pathway; however their role in either modulation had also not been yet studied. Isolated reports have shown the role of TRIMs in regulation of TNF- α -induced NF- κ B pathway but their systematic expression in response of TNF- α and role in feedback regulation is not known. In the present study, TNF- α -induced expression of TRIM E3 ligases and their role in feedback regulation of TNF- α -induced NF- κ B pathway was analyzed. Further, mechanism, cellular localization and dynamics and molecular mechanisms involved in regulation of TNF- α -induced NF- κ B pathway were explored.

9.1.1 TRIM proteins are novel feedback regulators of TNF- α -induced NF- κ B pathway

The evidences here strongly suggest that TRIM E3 ligases are induced during 'late' response of TNF- α treatment to cells, and regulate TNF- α -induced NF- κ B pathway in feedback manner. The major findings of the **chapter 5** are summarized as below:

- The mRNA expression of TRIM genes (TRIM1, 2, 3, 8, 9, 15, 16, 21, 31, 37, 38, 39, 41, 44, 46, 47 and 55) increases greater >2 folds in TNF- α treated cells.
- TNF- α -induced NF- κ B activation is inhibited by TRIM1, 2, 15, 46, 47, 48 and 55.
- TRIM37 promotes activation of TNF- α -induced NF- κ B pathway.

9.1.2 MID2/TRIM1; downregulated in cancers, transcriptionally inhibits TRAF2 oncogene and negatively regulates TNF- α -induced NF- κ B pathway

The major findings of the **chapter 6** are summarized as below:

9.1.2.1 MID2/TRIM1 is a late response TNF- α -induced NF- κ B target gene.

- TNF- α promotes expression of MID2/TRIM1 during late response in HEK293 and MCF-7 cells.
- RelA knockdown inhibits MID2/TRIM1 expression and positive correlation observed between canonical NF- κ B subunits and MID2/TRIM1 expression in various cancer tissues.

9.1.2.2 The turnover of MID2/TRIM1 is high and it is stabilized in presence of TNF- α .

- MID2/TRIM1 level increases in cells treated with MG132 and Bafilomycin-A1 suggesting its turnover through UPS and autophagy pathway.
- MID2/TRIM1 co-localizes with autophagosome marker p62 and lysosomal marker LAMP1.
- TNF- α treatment shows increased MID2/TRIM1 protein levels.

9.1.2.3 RING-B-box domains of MID2/TRIM1 are essential for inhibition of TNF- α -induced NF- κ B pathway.

- Ectopic expression of MID2/TRIM1 inhibits, whereas knockdown of MID2/TRIM1 enhances TNF- α -induced NF- κ B pathway.

- MID2/TRIM1 inhibits I κ B α phosphorylation induced by TNF- α .
- MID2/TRIM1 possesses E3 ligase activity and promotes substrate turnover in TNF- α treated cells.
- E3 ligase activity of MID2/TRIM1 is dependent on its RING-B-box domain.
- RING-B-box domain is sufficient to inhibit TNF- α -induced NF- κ B pathway.

9.1.2.4 MID2/TRIM1 inhibits TRAF2 expression TRAF2 dependant NF- κ B activation.

- Ectopic expression of MID2/TRIM1 inhibits TRAF2 induced NF- κ B activation in TNF- α treated cells.
- Negative correlation between MID2/TRIM1 and TRAF2 is observed in tumor tissue of different origin.

9.1.2.5 MID2/TRIM1 is downregulated in several cancers.

- MID2/TRIM1 expression was downregulated in various cancers
- Lower MID2/TRIM1 expression in tumors of different origin show high of TRAF2 expression.

9.1.3 Novel DUB-like activity of TRIM15: RING E3 ligase; regulates TNF- α -induced NF- κ B pathway in a feedback manner

The major findings of the **chapter 7** are summarized as below:

9.1.3.1 TRIM15 is a late response TNF- α induced gene and inhibits TNF- α -induced NF- κ B activation

- TRIM15 expression is increased during late response (after 10 hours of TNF- α treatment) in HEK293 and MCF-7 cells.
- Ectopic expression of TRIM15 inhibits whereas knockdown enhance TNF- α -induced NF- κ B activation.

- TRIM15 expression is found high in lesional psoriasis tissues suggesting its upregulation during chronic inflammatory conditions.

9.1.3.2 TRIM15 binds and deubiquitinates TAK1 and inhibits I κ B α phosphorylation

- Ectopic expression of TRIM15 inhibits TRAF2 and TAK1 induced NF- κ B activation in untreated and TNF- α treated cells.
- TRIM15 inhibits I κ B α phosphorylation induced by TNF- α and TAK1.

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

- Ectopic expression of TRIM15 significantly reduced K6, K11, K27, K29, K48 and K63 linked polyubiquitination of proteins.
- TRIM15 expression inhibits whereas TRIM15 knockdown enhances TNF- α induced K63 linked polyubiquitination of proteins.
- TRIM15 inhibits total as well as K63 linked polyubiquitination of proteins in both cytosol and nucleus.

9.1.3.4 PRY/SPRY domain mediated DUB-like activity of TRIM15 is essential for inhibition of TNF- α -induced NF- κ B activity

- Deletion of PRY/SPRY domain of TRIM15 rescues K63 linked polyubiquitination of proteins in presence of TNF- α .
- PRY/SPRY domain deletion of TRIM15 completely rescues inhibition of TNF- α -induced NF- κ B activation by full length TRIM15.
- Inhibition of TNF- α -induced NF- κ B activation is independent of TRIM15 E3 ligase activity.
- Functional correlation between cellular K63 linked ubiquitination and NF- κ B activation is observed in HEK293 cells.

9.1.3.5 TRIM15 antagonizes TRIM8 induced NF- κ B activity by inhibiting cytosolic translocation of TRIM8 and TAK1 ubiquitination

- TRIM15 forms distinct punctate structure in cytosol and nucleus and predominantly present in nucleus.
- TRIM15 form higher order structures in presence of TNF- α .
- TRIM8 interacts with TRIM15 only in TNF- α treated cells.
- Ectopic expression of TRIM15 inhibits cytosolic translocation of TRIM8 and TRIM8-induced NF- κ B activity.
- TRIM15 interacts with TAK1 and reduces TAK1 ubiquitination.
- TRIM15 inhibits K63 linked ubiquitination of TAK1.

9.1.4 TRIM8 regulated autophagy modulates the level of cleaved Caspase-3 subunit to inhibit genotoxic stress induced cell death

The major findings of **chapter 8** are summarized below.

9.1.4.1 Genotoxic stress induces expression and stability of TRIM8

- mRNA expression of TRIM8 is induced in etoposide treated cells
- TRIM8 has high turnover and it is stabilized during genotoxic stress.

9.1.4.2 TRIM8 regulates genotoxic stress induced NF- κ B and autophagy

- TRIM8 enhances NF- κ B activation in etoposide treated cells.
- Ectopic expression of TRIM8 induces autophagic flux in a RING domain dependant manner.
- TRIM8 promotes mRNA and protein expression of p62/SQSTM1.
- TRIM8 promotes autophagic flux in etoposide treated cells.

9.1.4.3 TRIM8 promotes lysosomal biogenesis

- Knockdown of TRIM8 reduced the levels of lysosomal markers ATP6V0D1 and LAPT4A.
- Ectopic expression of TRIM8 enhances mRNA expression of LAMP1
- TRIM8 induces LAMP1 protein levels in control and etoposide treated cells.

9.1.4.4 TRIM8 protects cells from genotoxic stress induced cell death

- Ectopic expression of TRIM8 promotes cell viability of etoposide treated cells, whereas its knockdown sensitizes cells from etoposide induced cell death.
- TRIM8 expression inhibits etoposide induced caspase-3 activation, whereas TRIM8 knockdown promotes caspase-3 activation.

9.1.4.5 TRIM8 mediated autophagy is required for its cytoprotective effect during genotoxic stress

- Rescue in cell death observed due to TRIM8 expression is reduced by autophagy inhibition.
- Increased caspase-3 activation is observed in etoposide-wortmannin co-treated cells in presence of TRIM8
- TRIM8 promotes degradation of active caspase-3 subunits by enhanced autophagy.

9.1.4.6 TRIM8 interacts with XIAP and caspase-3 to block caspase-3 activation

- Ectopic expression of TRIM8 promotes XIAP stabilization in etoposide treated cells.
- TRIM8 mediated XIAP stabilization precedes reduction in caspase-3 activation.
- TRIM8 forms a complex with XIAP and caspases-3 in RING independent manner.

9.2 Conclusion:

The systematic identification of TNF- α -induced TRIM E3 ligases in the current study suggests several of these modifiers are expressed temporally hence may play a critical role downstream as effectors of inflammatory response or may regulate the pathway in a feedback manner. Interestingly, not only the expression of these proteins but also stability is modulated in the presence of

TNF- α treatment. This finding provides novel insight on TNF- α -induced temporal gene expression specifically late response genes required for controlling the proinflammatory NF- κ B pathway which may have an important role in determining the outcome of chronic inflammatory conditions.

Interestingly most of the identified TRIMs negatively regulated TNF- α -induced NF- κ B suggesting that specific TRIMs are important for the resolution of TNF- α -induced inflammation. Identified TRIMs were further characterized for their role in regulation of TNF- α -induced NF- κ B activation. For example, the characterization of TRIM15's role in negative regulation of TNF- α -induced NF- κ B pathway confirms it acts more like a DUB, suggesting novel NF- κ B regulatory mechanism independent of their E3 ligase activity. The other member of TRIM family proteins containing similar domain architecture may possess similar activity which needs to be further investigated. Functional cooperation and antagonism are required for tight regulation of cellular pathways.

This study showed that TRIM15 antagonizes NF- κ B activating functions of TRIM8 by acting on the same substrate and modulating its dynamics. This evidence further validates the emerging hypothesis that these proteins can heterodimerize and modulate the TNF- α -induced inflammatory pathways. Moreover, TRIM family may have similar functional protein pair essential for tight regulation of associated pathways, this need to be further validated.

Further, we observed that TNF- α -induced TRIMs are recruited at the discreet steps of TNF- α -induced NF- κ B pathway. TRIM1 is recruited at the TRAF2 level and probably interacts with TRAF2; another ligase and prevents the downstream activation of NF- κ B pathway. TRIM1 down regulates the expression of TRAF2; a known NF- κ B target gene. Interestingly, the other late response TNF- α -induced gene TRIM15 is recruited at the TAB-TAK complex and inhibits the downstream pathway by deubiquitinating TAK1. All these

evidences in the study suggest the co-expression of TRIMs and their recruitment at discreet steps NF- κ B may cooperate in inhibition of the proinflammatory NF- κ B pathway. This could also be a strategically important mechanism for controlling chronic inflammation and associated pathological conditions.

This study shows stimuli specific stabilization of TRIM proteins in the case of TRIM8 and TRIM1 and its impact on associated cellular functions. For example, TRIM8 expression and stability are enhanced during genotoxic stress whereas; mRNA expression and stability of TRIM1 are enhanced in TNF- α treated cells. Expression and stability of TRIM8 and TRIM1 have an important role in regulation of genotoxic stress-induced cell death and regulation of TNF- α -induced NF- κ B pathway, respectively. Further characterization of TRIM8 shows its role in regulation of genotoxic stress induced autophagy and cell death. TRIM8 promotes stabilization of XIAP, hence inhibits activation of caspase-3 which enhances survival of cell under genotoxic stress. This indicates toward a novel mechanism by which cancer may acquire chemoresistance.

This study also identifies down regulation of TRIM1 in several cancers. The NF- κ B inhibitory effect of TRIM1 may also control expression of target genes required for proliferation, which needs to be further investigate to confirm the TRIM1-NF- κ B nexus in tumorigenesis. The study also indentifies TRIM1 mediated transcriptional inhibition of TRAF2 which could be strategically important step in controlling over activation of NF- κ B pathway and oncogenic potential of TRAF2. These results also provide preliminary evidences to investigate the role of TRIM8 and MID2/TRIM1 in chemoresistance and oncogenic transformation respectively.

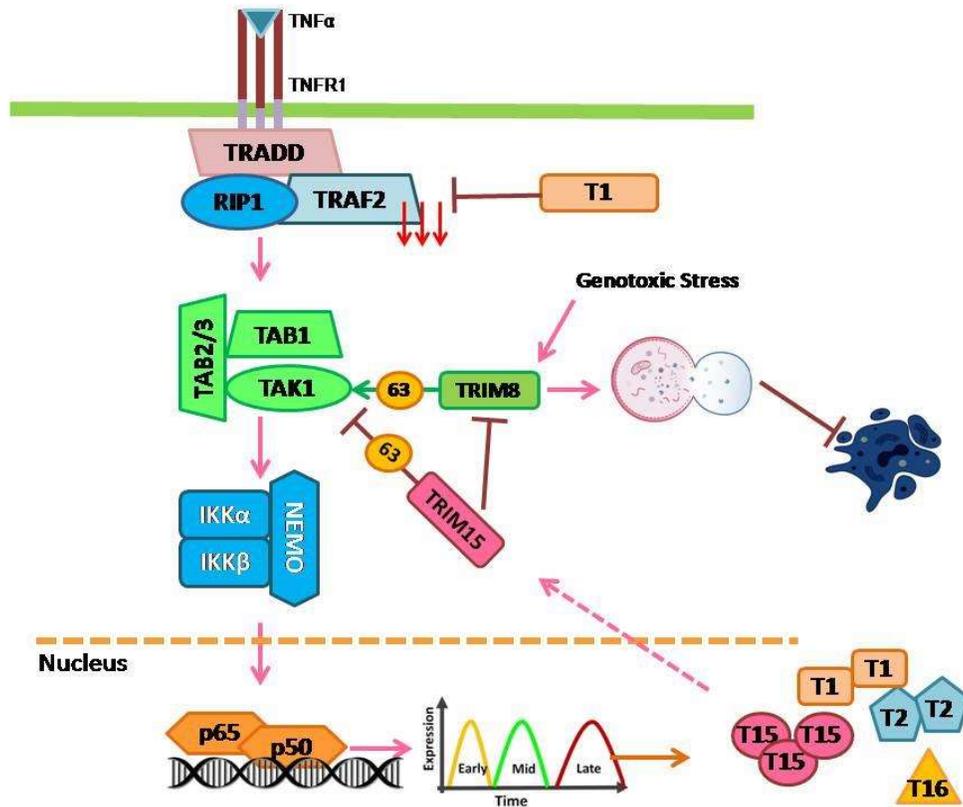


Figure 9.1: Graphical abstract of the current study. *TNF- α induces expression of TRIM proteins. TRIM1 and TRIM15 inhibit NF- κ B activation induced by TNF- α . Whereas TRIM8 regulates genotoxic stress induced NF- κ B, autophagy and cell death.*

Overall the study systematically identifies novel feedback regulators of TNF-induced NF- κ B pathway and mechanism involved in its regulation. The study also provides evidences to support the hypothesis that these proteins may have evolved to control the chronic inflammation by a multilayer feedback mechanism involving discreet steps of NF- κ B pathway. Study further validates that these proteins have stimuli dependant cellular functions essential for regulation of cellular homeostasis. This study will have impact on better understating of chronic inflammatory and associated pathological conditions.

9.3 Limitations of the study:

This study identifies novel regulatory mechanisms of inflammatory NF- κ B pathway and also provides functional relevance to TNF- α -induced late response E3 ligases. The study also highlights their possible association with cancer and chronic inflammatory conditions. However, these are the limitation of the study:

- *Validation of MID2/TRIM1's role in tumorigenesis:* The tumor suppressor role of MID2/TRIM1 needs to be validated in organ specific knockout mice models MID2/TRIM1.
- *Identification of MID2/TRIM1 substrates:* Other than transcriptional downregulation of TRAF2, MID2/TRIM1 can have ubiquitin dependent role in inhibition of TNF- α -induced NF- κ B pathway. This could be further investigated in detail.
- *Physiological relevance of TRIM15:* Analysis of TRIM15 expression in chronic inflammatory conditions can further validate the role of TRIM15 in negative regulation of TNF- α -induced NF- κ B pathway and provide physiological relevance to TNF- α -induced TRIM15 expression.
- *Validation of DUB activity of TRIM15:* Whether TRIM15 itself acts as a DUB or recruits other DUBs to the TAB-TAK complex remains elusive. In-vitro DUB assay with purified TRIM15 protein can validate TRIM15 as a DUB.
- *Validation of chemoresistance induced by TRIM8:* Knockout and overexpressing TRIM8 mice models should confirm the chemoresistance properties of TRIM8.

9.4 Future Perspective:

The current investigation warrants further investigations on MID2/TRIM1, TRIM8 and TRIM15 on chronic inflammatory conditions and cancer. These proteins should be further investigated in the following directions:

- The late response expression of NF- κ B inhibitory TRIMs may have functional synergy essential for controlling chronic inflammatory conditions. Double knock out study of MID2/TRIM1 and TRIM15 may provide evidenced if these proteins act as multilayer feedback regulator of TNF- α -induced NF- κ B pathway.
- MID2/TRIM1's role in NF- κ B inhibition can provide better understanding of NF- κ B's role in tumorigenesis. Hence MID2/TRIM1 knockout mice models should be investigate further for better understanding tumorigenesis. SNPs and mutation in MID2/TRIM1 gene may have association with chronic inflammatory conditions as well as in cancer.
- TNF- α and TLR mediated activation of NF- κ B may have different outcomes in terms of inflammation and immunomodulation. It will be interesting to investigate if TRIMs are involved in regulation of specific arm of immune signaling needs to be investigated for better understanding of chronic inflammatory conditions.
- TNF- α -induced NF- κ B pathway is also critical for immune modulation and bridging between innate and adaptive immune system. Therefore these NF- κ B regulators may have important role to play in physiological regulation of systemic infection, local and chronic inflammation and response to injury. These aspects needs father investigation.