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Cancer is a disease characterized by uncontrolled cell growth and is a complex global health challenge [2,395]. Its intricate etiology involves a convergence of genetic, environmental, and lifestyle factors, necessitating a multifaceted understanding to devise targeted strategies for prevention and treatment. Molecular studies have been involved in unraveling the underlying mechanisms, revealing genetic mutations, dysregulation of signaling pathways, and immune evasion contributing to the progression of cancer[396–398]. Lung cancer encompasses two major subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC, representing 80-85% of cases, further stratifies into subtypes like adenocarcinoma and squamous cell carcinoma[34]. The complex interplay of genetic susceptibility and environmental exposures is responsible for DNA damage and mutations in lung cells[39,40]. Lung cancer incidence is increasing due to environmental exposures (radon) and occupational hazards as well as lifestyle (smoking)[43,44,399]. Genetic susceptibility, chromosomal aberrations, and epigenetic alterations contribute further complexity to the molecular portrait. Understanding these multifactorial contributors becomes imperative for personalized risk assessment and the formulation of effective prevention strategies. Exploring the genetic landscape of lung cancer reveals a highly diverse disease influenced by a spectrum of mutations affecting tumor behavior, treatment response, and overall outcomes. Specific genetic alterations, such as mutations in KRAS, EGFR, ALK, ERBB2, and BRAF, are identified in over 50% of lung adenocarcinomas[59,60].

Apart from genetic susceptibility, proteotoxic stress is one of the hallmarks of cancer. It is capable of disrupting cellular protein balance and emerges as a critical player in various cancers[91]. Cells have protein degradation pathways, such as the ubiquitin-proteasome system (UPS) and autophagy-lysosome pathway to manage proteotoxic stress and ensure protein quality control[97,98]. Ubiquitin is a conserved protein in eukaryotic cells governing ubiquitylation, a pivotal post-translational modification leading to targeted protein degradation[115]. Dysregulation in ubiquitylation pathways is implicated in cancer development, exerting profound effects on tumor biology[293,294,400]. Aberrant ubiquitylation events lead to the degradation or stabilization of key regulatory proteins, influencing cell growth, survival, invasion, and metastasis[401,402]. The UPS is a major player in degrading tumor suppressor proteins, facilitating uncontrolled cell proliferation. Conversely, stabilization of oncoproteins through abnormal ubiquitylation drives tumorigenesis and therapy

resistance. Dysregulated ubiquitin ligases and deubiquitylating enzymes offer potential targets for therapeutic development, aiming to restore normal ubiquitin signaling and disrupt oncogenic processes[403]. Within the realm of ubiquitylation, the Tripartite Motif (TRIM) family of proteins known for their E3 ubiquitin ligase activities, emerges as a focal point in cancer and rare genetic disorders[130]. The accurate ubiquitylation and degradation of oncogene proteins, supported by technologies such as PROTAC, offer potential for groundbreaking therapeutic approaches in treating lung cancer[295].

Consequently, TRIM utilizes its CC domain to create homodimers arranged in an anti-parallel manner. This arrangement suggests that the two catalytic RING domains, separated by the extended CC domain, work together in the ubiquitylation process of substrates[404]. The TRIM protein family is linked to diverse pathophysiological mechanisms, including cell proliferation, DNA repair, signal transduction, and transcription[155–159]. TRIM proteins, vital for diverse cellular functions, play a key role in antiviral activities. Some TRIMs trigger immune responses and cytokines, while others directly impede viral replication by targeting viral proteins. Members of the TRIM family are involved in processes like innate immunity, autophagy, signaling, and carcinogenesis[154,298].

In lung cancer, specific TRIM proteins are upregulated, influencing crucial mechanisms in tumorigenesis and serving as potential diagnostic markers and therapeutic targets[405]. Wang *et al.* (2015) have shown the regulatory functions of TRIMs in multiple signaling pathways mediated by interferon response and inflammation [160]. TRIM proteins, integral to innate immunity and antiviral responses, are subject to regulation by IFNs[209]. Both type I and type II IFNs significantly influence the expression of numerous TRIM genes. This underscores their pivotal role in regulating TRIM proteins involved in antiviral immune responses[406]. Understanding these intricate interactions offers insights into the diverse roles of TRIM proteins in the immune system and their modulation by IFNs. Interferons (IFNs) are a diverse group of cytokines that take a central position in cancer immunotherapy, showcasing promise in treating cancer, viral infections, and autoimmune disorders[201–203]. Their dual impact on the cancer immune response, influencing various stages of the cancer immunity cycle, positions them as valuable tools in advancing cancer treatment strategies[204]. The significance of IFNs is particularly highlighted in lung cancer, known for its heterogeneity, where immunotherapy has emerged as a successful approach to leveraging the body's immune system to target cancer cells[205].

TRIM34 is reported to be downregulated in colon cancer, suggesting its potential role as a tumor suppressor gene[218]. The present study aimed to analyze the role of TRIM34 in lung cancer behavior. Upregulation of TRIM34 was done by IFN treatment and CRISPR/Cas9 technology was used to generate TRIM34 knockout cells. Specifically, NSCLC cell lines, namely NCI-H23, A549, and NCI-H522 were utilized for the analysis. These cells were procured from NCCS, Pune.

Chapter 1 focuses on the upregulation of TRIM34 by treatment in lung cancer cell lines. Three types of interferons—Type I (IFN-α), Type II (IFN-γ), and Type III (IFN-λ)—were employed to understand their influence on TRIM34 levels. Various doses of IFN were used to standardize maximum expression of *TRIM34* at different timepoints. Peak *TRIM34* expression occurred at 6 hours in A549 cells and 8 hours in NCI-H23 and NCI-H522 cells post-IFN-α exposure. Significant upregulation ensued in response to IFN-α (Fig. 1.3), with NCI-H23 cells exhibiting an 8.4-fold increase at 200 IU/ml ($p \leq 0.01$), NCI-H522 cells showing a 2.8-fold rise at 500 IU/ml after 8 hours ($p \leq 0.0001$), and A549 cells displaying a 3.1-fold elevation at 100 IU/ml after 6 hours ($p \leq 0.01$). These results underscore substantial *TRIM34* upregulation in lung cancer cells following IFN-α exposure. Additionally, *TRIM34* mRNA expression was analyzed in NCI-H23, NCI-H522, and A549 cells treated with various doses (10, 50, 100, and 200 ng/ml) of IFN-γ for 10 hours, revealing a dose-dependent increase (Fig. 1.4). NCI-H522 cells displayed a 2.9-fold upregulation at 200 ng/ml ($p \leq 0.01$), A549 cells exhibited a significant 14.2-fold increase at 50 ng/ml ($p \leq 0.05$), and NCI-H23 cells demonstrated an 8.9-fold rise at 100 ng/ml. Furthermore, *TRIM34* mRNA expression was evaluated following treatment with various doses (10, 50, 100 ng/ml) of IFN-λ (Fig. 1.5), showing a statistically significant 4.3-fold increase in NCI-H23 cells at 100 ng/ml ($p \leq 0.05$), a substantial 25.7-fold upregulation in A549 cells at 50 ng/ml, and a 4-fold increase in NCI-H522 cells at 100 ng/ml ($p \leq 0.01$). Additionally, we examined the gene expression of *TRIM6-34* (a variant of TRIM34) in A549 cells (Fig. 1.6) treated with IFN-α, with no notable changes observed.

These findings collectively suggest that IFN-γ and IFN-λ treatments also can significantly enhance *TRIM34* expression in lung cancer cells, implicating their potential role in modulating *TRIM34* expression dynamics and emphasizing their relevance in lung cancer pathogenesis or therapeutic responses. Wroblewski *et al.* (2001) demonstrated that NCI-H23 cells exhibit sensitivity to IFN-γ signaling, leading to the upregulation of Major Histocompatibility Complex (MHC) Class I molecules and initiating an immune response[252]. Based on this finding and our results, we conducted additional investigations into TRIM34 specifically in NCI-H23 cells stimulated with IFN-γ.

TRIM34 protein levels were assessed through Western blot (Fig. 1.7A&B) and immunocytochemistry to validate the mRNA expression. IFN-γ treatment significantly increased TRIM34 protein levels in NCI-H23 cells. The immunocytochemistry (Fig. 1.7C&D) results further support higher presence of TRIM34. Cell viability (Fig. 1.8) assays were conducted to explore the functional consequences of TRIM34 upregulation induced by IFN-γ. The result indicates an increase in apoptotic percentage on upregulation of TRIM34. These findings were further substantiated by assessing apoptosis-related genes (*BAX, BAD, TNF-α*) following IFN-γ treatment (Fig. 1.10), revealing a significant upregulation and implying that TRIM34 overexpression induced apoptosis in lung cancer cells. This finding is consistent with the results reported by Vila-del Sol V *et al.* (2008) in murine macrophage cells [266].

Additionally, Boutsikou E *et al.* (2018) demonstrated that the upregulation of TNF-α can enhance immunotherapy outcomes and contribute to the extended survival of NSCLC patients [267].

Yin *et al.* (2019) have shown that IFN-γ induces Bak homodimers and Bax-Bak heterodimers in mitochondria, these events can potentially induce apoptosis by activating caspase-9, caspase-3, and PARP cascades in lung cancer[264]. Our result of *BAX* and *BAD* upregulation by the literature[407,408]. Further to validate apoptosis, FACS-based Annexin V and propidium iodide assays was employed (Fig 1.11). The result showed an increase in apoptotic percentage in response to IFN as compared to untreated cells. FACS and cell viability assay suggest that TRIM34 overexpression dysregulates mitochondrial function, which leads to mitochondrial dependent apoptosis. Mitochondrial morphology is pivotal for cell migration across various tissues. The relocation of mitochondria to the leading edge of migrating cells is integral, as it facilitates the provision of energy and metabolites for cytoskeletal remodeling. This remodeling, in turn, contributes to the dynamic changes in cell shape during movement. Thus, reduced mitochondrial migration is responsible for reduced cell migration and proliferation [407,409,410]. As represented in the scratch assay (Fig. 1.13), the cell migration was inhibited on the treatment of IFN- γ (100 ng/ml).

Literature suggests that elevated doses (≥100 ng/ml) of IFN-γ contribute to cancer regression, whereas lower doses (≤0.2 ng/ml) of IFN-γ enhance the metastatic characteristics of these cancer cells[262]. The protein TRIM34, as mentioned earlier, undergoes increased expression in response to the stimulation of IFN-γ and has been linked to the initiation of apoptosis. This observation suggests that IFN-γ plays a role in impeding cells to migrate/proliferate. This may aid in reducing cancer metastasis. To deepen our understanding, it becomes crucial to delve into the intricate molecular mechanisms that underlie the impact of TRIM34 overexpression on the migration of lung adenocarcinoma cells.

Chapter 2 was designed to understand the role of TRIM34 suppression and induction of apoptosis through ubiquitylation pathways. To suppress TRIM34, CRISPR/Cas9 was used to create TRIM34 KO NCI-H23 cells. The study comprised three experimental groups: the Control group underwent no genetic manipulation, the Negative control group involved cells transfected with a non-targeting sequence to ensure specificity and the *TRIM34* CRISPR/Cas9 KO Transfection group experienced actual *TRIM34* knockout. This examination involved a transcriptomics study to identify differential expression of genes involved in ubiquitylation pathways. Apoptosis and cell cycle analysis was done by FACS.

The study employed Transcriptomics analysis to investigate gene expression patterns. Differential analysis was performed on the KO group in comparison to control group, to identify significantly expressed genes based on log2 fold. The study delved into the impact of *TRIM34* knockout on gene expression, uncovering significant alterations in 204 upregulated and 69 downregulated genes (globally). The top 10 most upregulated (Table 18) and downregulated (Table 19) genes shed light on

potential molecular mechanisms affected by *TRIM34* knockout. The Protein-Protein interaction network and hub gene prediction involved using STRING database to explore interactions among Differentially expressed genes (DEGs). The resulting interactions were visualized in Cytoscape, and hub nodes were identified using the CytoHubba toolkit based on centrality scores in the Protein-Protein Interaction (PPI) network. In this experiment, our goal was to identify ubiquitylation genes among the top 10 hub genes. The presented results (Table 20) outline the top 10 differential genes within a network, ranked by the degree ranking method based on their interactions in the String database. *DDX58* holds the highest rank, followed by *IFIT3* and *IFIH1*. Notably, DDX58, the first-ranked gene, is linked to ubiquitylation [304,305]. Literature suggests that DDX58 inhibits the proliferation, migration and invasion of colon cancer cells[411]. *UBE2L6*, the sixth-ranked gene, is also associated with ubiquitylation [306,307] and is implicated as a tumor suppressor in melanoma[412].

Genes were selected on the basis of their over and down-expression, and their role was further evaluated by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses to identify their involvement in cellular compartments, and molecular functions. The DEG are listed in Tables 21 and 22. They are involved in molecular functions such as ubiquitin-like protein ligase binding, ubiquitin protein ligase binding, thiol-dependent ubiquitin-specific protease activity and ubiquitinyl hydrolases. Enrichment analysis revealed that four of the top 10 molecular functions were linked to ubiquitylation in differentially expressed genes, indicating a substantial impact of *TRIM34* on the ubiquitylation process in non-small cell lung cancer (NSCLC). In the *TRIM34* knockout condition, a reduction in expression was observed in genes associated with ubiquitylation, including *SLC25A5, PRKACB, TANK, ATF6, ZNF746, USP48,* and *RING1* (Table 22). Conversely, there was an upregulation in the expression of genes linked to ubiquitylation, such as *TXNIP, USP25, DTX3L, BAG5, UBE2L6, USP19, DDX58, CUL4A, POLR2A, USP18, USP3, COPS5, UBA7, FBXW11, HERC5, TRIM5, KIAA1586, RC3H2, UBR4,* and *TRIM38* (Table 21), under the *TRIM34* knockout condition. This implies that *TRIM34* knockout significantly influences genes associated with ubiquitylation pathways, thereby regulating lung cancer pathogenesis.

Exploring the impact of *TRIM34* gene knockout on cell viability (Fig. 2.6) in NCI-H23 cells, the study utilized the Calcein AM and Ethidium homodimer-1 (EthD-1) staining. A significant elevation in cell death in the *TRIM34* CRISPR/Cas9 KO transfection group. Further investigations into the effect of *TRIM34* gene knockout on apoptosis in NCI-H23 cells employed Annexin V-FITC and propidium iodide (PI) staining, followed by FACS analysis. The results indicated a significant increase in apoptotic cell death in the *TRIM34* KO cells compared to the Control and Negative control cells (Fig. 2.7). Cell cycle analysis using PI staining and flow cytometry revealed a significantly higher sub-G1 population in the *TRIM34* KO cells, suggesting increased apoptosis with DNA fragmentation compared to the normal cells (Fig. 2.8D). Deng *et al.* (2022) suggest DDX58 is involved in

STAT3/CSE signaling pathway and consequently affects the proliferation of tumor cells in colon cancer [411]. We found DDX58 as the highest-ranked hub gene, this explains our results as STAT3 silencing enhances sensitivity to apoptosis.

UBE2L6 is associated with EZH2 which regulates the methylation[413]. Methylation is an epigenetic factor and is associated with cancer. So, to understand the methylation role of TRIM34, we have designed ***Chapter 3***, to understand epigenetic regulation of TRIM34 in NSCLC.

NCI-H23, NCI-H522 and A549 cell lines were used to observe the promoter methylation pattern of the TRIM34 gene. The investigation proposes the use of 5-Aza-2'-deoxycytidine (AZA) to alleviate methylation, demethylating the TRIM34 promoter. The utilization of AZA, a DNA methylation inhibitor, in conjunction with IFN, aimed to demethylate the *TRIM34* gene's promoter region, leading to the restoration of its transcriptional activity as depicted in Figure 3.2. The observed reduction in methylation levels via qRT-PCR and the subsequent increase in *TRIM34* expression following the treatment with AZA and IFN provides additional evidence supporting the role of DNA methylation in suppressing *TRIM34* in lung cancer cells. Sanger sequencing of bisulfite converted TRIM34 promoter region revealed a change in methylation pattern (Fig. 3.3). The elevated expression of *TRIM34* in NCI-H23, NCI-H522, and A549 lung cancer cell lines suggests the altered methylation pattern of TRIM34 is involved in Lung cancer tumorigenesis. Our result suggests that TRIM34 upregulation in response to immunotherapy or alteration of UPS by its suppression, acts as a double-edged sword in lung cancer treatment.

The study further explores the impact of DNA demethylation on specific genes, including TRIM16 [378], TRIM67 [379], and TRIM58 [380], in various cancers, underlining the intricate interplay between epigenetic modifications and gene expression. In the context of NSCLC, the downregulation of the *TRIM34* gene is hypothesized to be linked with promoter methylation.