Research Synopsis
on

"Investigations on the potential role of Tripartite Motif 34 (TRIM34) expression in Lung Cancer Tumorigenesis"

For the Degree of
Doctor of Philosophy

By

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Table of Contents

INTRODUCTION

Cancer is a multifaceted health condition that arises from the abnormal and uncontrolled growth of cells in the body. This unregulated proliferation can occur in any tissue or organ, leading to the formation of tumors, which can be either benign (non-cancerous) or malignant (cancerous)[1,2]. The complex origins of cancer involve a combination of genetic, environmental, and lifestyle elements, requiring a comprehensive understanding to develop precise approaches for prevention and treatment. Molecular research has played a crucial role in uncovering the fundamental processes, including genetic mutations, disruptions in signaling pathways, and evasion of the immune system, that drive cancer progression [3–5].

The hallmarks of cancer represent a fundamental set of characteristics that collectively influence the initiation, progression, and metastasis of tumors. These traits offer a comprehensive framework for comprehending the biological mechanisms underlying cancer development and behavior. Introduced by Hanahan and Weinberg in 2000, the hallmarks include sustained proliferative signaling, evading growth suppressors, resisting cell death, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis. Understanding these hallmarks provides crucial insights for researchers and clinicians, guiding the identification of potential therapeutic targets in the complex landscape of cancer [6]. Among all, lung cancer persists as a significant worldwide health issue, contributing substantially to cancer-related illness and death.

Lung cancer represents a significant portion of cancer fatalities, comprising the majority of cancer-related deaths (18.0% of all cancer fatalities) [7, 8]. Lung cancer encompasses two major subtypes: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC, representing 80-85% of cases, three subtypes of NSCLC that are characterized histologically are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma [9]. The development of lung cancer is widely acknowledged to result from a complex interplay of genetic and environmental factors, rendering it a multifaceted disease [10, 11]. Understanding its etiology is paramount for formulating effective prevention measures and targeted interventions. Foremost among the causes of lung cancer is tobacco smoke, notably from cigarettes, which stands as the primary culprit. A wealth of epidemiological research consistently underscores the robust correlation between smoking and the risk of lung cancer [12–14]. Cigarette smoke contains carcinogenic substances and harmful compounds, such as polycyclic aromatic hydrocarbons (PAHs), aromatic amines, nitrosamines, and reactive oxygen

species (ROS), capable of initiating DNA damage and genetic modifications within lung cells [10, 11]. These genetic alterations, encompassing mutations in tumor suppressor genes and oncogenes, play a pivotal role in initiating and advancing lung cancer. Additionally, exposure to environmental carcinogens and occupational hazards is associated with susceptibility to lung cancer. Notably, radon, a naturally occurring radioactive gas, stands out as a recognized contributor to lung cancer, particularly in regions characterized by elevated concentrations of radon [15, 16]. Genetic susceptibility, chromosomal aberrations, and epigenetic alterations contribute further complexity to the molecular portrait. More than half of lung adenocarcinomas exhibit somatic mutations in the five primary oncogenes: KRAS, EGFR, ALK, ERBB2, and BRAF [17, 18]. DNA methylation profiling suggests TRIM58 methylation as a promising early diagnostic biomarker for lung cancer, with TRIM58 potentially serving as a tumor suppressor gene [19]. Exploring the genetic landscape of lung cancer reveals its polymorphic state that is resulted failure of treatment strategies. Understanding these multifactorial contributors becomes essential for personalized risk assessment and the formulation of effective prevention strategies.

Altered protein conformation/ amplification is one of the known characteristics of cancer survival along with gene mutation. A disruption in the cellular proteostasis network can arise from various factors, including genetic mutations, environmental stressors, and the natural aging process. This disruption leads to the accumulation of misfolded or dysfunctional proteins within the cells. Consequently, a condition known as proteotoxic stress emerges when there is an imbalance between the production of misfolded or damaged proteins and the cell's capacity to adequately fold, repair, or degrade these proteins through its molecular machinery. This imbalance results in the aggregation of protein and disrupts the normal equilibrium within the cell. Ultimately, this process leads to cellular dysfunction and the onset of cancer [20].

In response to proteotoxic stress, cells have developed sophisticated protein degradation pathways designed to eliminate abnormal proteins and uphold protein quality control. Essential for maintaining cellular proteostasis and preventing the accumulation of harmful protein aggregates, these degradation pathways include the ubiquitin-proteasome system and the autophagy-lysosome pathway [21, 22]. Ubiquitin, a conserved protein found in eukaryotic cells, regulates ubiquitylation, a crucial post-translational modification that directs targeted protein degradation [23]. Aberrations in ubiquitylation pathways are associated with the progression of cancer, significantly influencing tumor biology [24–26]. Irregular ubiquitylation processes result in either the degradation or stabilization of critical regulatory proteins,

impacting cellular functions such as growth, survival, invasion, and metastasis [27, 28]. The ubiquitin-proteasome system (UPS) plays a significant role in breaking down tumor suppressor proteins, thereby promoting unchecked cell growth. Conversely, the abnormal ubiquitylation that stabilizes oncoproteins contributes to tumorigenesis and resistance to therapy. Dysfunctions in ubiquitin ligases and deubiquitylating enzymes present promising targets for therapeutic advancement, with the goal of reinstating regular ubiquitin signaling and impeding oncogenic pathways [29]. In the domain of ubiquitylation, the Tripartite Motif (TRIM) family of proteins, recognized for their E3 ubiquitin ligase functions, stands out as a central player in both cancer and rare genetic disorders [30]. The accurate ubiquitylation and degradation of oncogene proteins, supported by technologies such as PROTAC, offer potential for groundbreaking therapeutic approaches in treating lung cancer [31].

Consequently, TRIM utilizes their 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 [32]. The TRIM protein family is linked to diverse pathophysiological mechanisms, including cell proliferation, DNA repair and signal transduction[33–37]. Along with cellular function, TRIM proteins associates in antiviral activities. Certain TRIMs initiate immune responses and cytokine production, whereas others directly hinder viral replication by targeting viral proteins. Members of the TRIM family participate in essential processes such as innate immunity, autophagy, signaling, and carcinogenesis [38, 39]. In lung cancer, distinct TRIM proteins undergo upregulation, impacting pivotal processes in tumorigenesis and potentially serving as both diagnostic indicators and targets for therapeutic intervention [40]. Wang *et al.* (2015) demonstrated the regulatory roles of TRIMs across various signaling pathways associated with the interferon response and inflammation [41]. It governs diverse cellular processes, encompassing protein quality control, intracellular signaling, innate immunity, inflammation, transcription, autophagy, cell metabolism, developmental processes, chromatin modification, carcinogenesis, and numerous other functions [38, 42–49].

TRIM proteins, integral to innate immunity and antiviral responses, are subject to regulation by Interferons (IFNs) [50]. The expression of numerous TRIM genes is profoundly influenced by both type I and type II interferons (IFNs). This emphasizes the crucial role of IFNs in regulating TRIM proteins that are integral to antiviral immune responses [51]. 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 central position in cancer immunotherapy, showcasing promise in treating cancer, viral infections, and autoimmune disorders [52–54]. 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 [55]. The significance of IFNs is particularly highlighted in lung cancer, known for its heterogeneity, where immunotherapy has emerged as a successful approach in leveraging the body's immune system to target cancer cells [56].

Our research focuses on TRIM34, an antiviral protein that plays a protective role in colon cancer. The primary objective of this investigation is to explore the functions of TRIM34 in lung adenocarcinoma. Also recognized as RING Finger Protein 21 (RNF21), TRIM34 belongs to the ubiquitin E3 ligase family and is induced by interferons. This upregulation by interferons implies its potential role as an effector in the cellular response against viruses and in the realm of cancer therapy. Researchers observed that other TRIM proteins, such as TRIM6, TRIM22, and TRIM5, exhibit functional similarities to TRIM34 [57]. Orozco et al. (2009) noted a strong correlation between TRIM34 expression and copy number variants (CNVs), which impact gene expression and metabolic characteristics in mice [58]. In a recent investigation into the genetic underpinnings of Parkinson's disease, analysis of DNA methylation patterns indicated that TRIM34 was among the genes associated with multiple CpGs linked to epigenetic changes [59].

TRIM34 demonstrates antiviral characteristics and appears to be downregulated in colon cancer, suggesting a potential role as a tumor suppressor gene [60]. We hypothesized that TRIM34 may play dual role in oncogenic crosstalk in combination with interferon as promoter or suppressor. To accomplish this, TRIM34 expression levels were elevated through interferon (IFN) treatment, while CRISPR/Cas9 technology was employed to downregulate TRIM34 in NSCLC.

Further study aimed to explore a possible connection between the regulatory mechanisms of TRIM34 and changes in DNA methylation levels, which can result in either hypo- or hypermethylation. The primary objective was to investigate whether treating non-small cell lung cancer (NSCLC) cell lines with Interferon (IFN) along with an epigenetic modifying agent could enhance the upregulation of TRIM34 expression. The study employed 5-Aza-2'-deoxycytidine (AZA), a well-known inhibitor of DNA methyltransferase, to reduce DNA methylation. This approach aimed to shed light on the potential interplay between TRIM34

regulation, DNA methylation alterations, and the therapeutic implications of combining IFN with an epigenetic modifying agent in the context of NSCLC.

HYPOTHESIS:

Cancer cells convive with the proteotoxic stress created by promoting the UPR and related pathways to induced protein degradation. And literature suggested that Interferon can induce ER stress to induce cell death. Moreover, our preliminary data in which we had seen that TRIM34 expression was under expressed as compared with normal. Hence based on our preliminary data and the relevant literature we hypothesize that *'TRIM34 may regulate lung cancer pathogenesis by modulating interferon and ubiquitin-proteasomal pathways'*. Also, Interferon treatment-induced TRIM34 may induce lung cancer regression.

Specific Objectives:

1. **To characterize the modulatory role of TRIM34 expression in lung cancer cell lines.**
2. **To investigate changes in TRIM34-sensitive ubiquitination pathway genes in lung cancer cell lines.**
3. **To investigate epigenetic regulation of TRIM34 expression in lung cancer cell lines.**

MATERIAL AND METHODS

Cell culture

A549, NCI-H23, and NCI-H522 lung adenocarcinoma cells were obtained from National Centre for cell sciences (NCCS), Pune. Cells were cultured in RPMI-1640 (Himedia, AL162S) supplemented with 10% Fetal Bovine serum (Gibco,10270106) and 1% Penicillin Streptomycin solution (Gibco,15140122). Cells were maintained in a 5% CO_2 incubator at 37℃.

Dose-dependent Study of Interferon Treatment

In this experiment, a total of 0.1 x 10^6 cells were seeded in each well of a 12-well cell culture plate. The cells were allowed to adhere and grow for the required confluence state. Cells were exposed to Interferon Alpha (10,50,100,200, and 500 IU/ml), Interferon Gamma (10,50,100, and 200 ng/ml), and Interferon Lambda (10,50, and 100 ng/ml).

Transfection study by CRISPR/Cas9

1.5 x 10^5 NCI-H23 cells were seeded per well of a 6-well plate in an antibiotic-free media. After 24 hours cells were transfected with TRIM34 CRISPR/Cas9 KO Plasmid (h) (Santa Cruz Biotechnology, sc-417920) and Negative Control plasmid (Santa Cruz Biotechnology, sc-418922) using UltraCruz Transfection Reagent (Santa Cruz Biotechnology, sc-395739) according to manufacturer's instructions.

Demethylation Assay

3 x 10^5 Cells were seeded in a 6-well culture plate, and 5 µM 5-Aza-2'-deoxycytidine (AZA) (A3656-10MG, Sigma) treatment was given every 24 hours in 1 % FBS containing media for 48 hours. After 48 hours of AZA treatment, Cells were further treated with AZA with Interferon Alpha (100 IU/ml) and only with IFN Alpha (100 IU/ml) for 10 hours in A549, NCI-H23, and NCI-H522. Total RNA in TRIzol was collected. qRT-PCR was performed to check relative expression by a $2^{\wedge-\Delta\Delta CT}$ method.

Gene expression study by qRT-PCR

Cells were seeded and after treatment, total RNA was extracted by using TRIzol reagent (Thermo Fisher Scientific,15596018) and quantified using Nanodrop. cDNA was prepared using a High-Capacity cDNA Reverse Transcription kit (Applied Biosystems,4374966). Real-time PCR (qRT-PCR) was performed using Powerup SYBR Master Mix (Applied biosystems, A25742). GAPDH

level was used as an internal control and the expression level of the TRIM34 gene was checked by using the $2^{\wedge-\Delta\Delta CT}$ method.

Western Blot Analysis

Cells were treated with Interferon gamma (100 ng/ml) for 10 hours. After treatment protein extraction with Pierce RIPA Buffer (Thermo Fisher Scientific, 89900), After that Protein quantification was done by using Pierce BCA Protein Assay kit (Thermo Fisher Scientific, 23227). PAGE was performed using NuPAGE Bis-Tris precast polyacrylamide gels (Thermo Fisher Scientific, NP0323), transfer protein on PVDF membrane using Power Blotter XL (Invitrogen). Incubated membrane with Primary antibody after blocking. After washing, membranes were incubated with secondary antibody and then wash with TBST. ECL reagent was used to observe the protein bands on the blot. Images were observed under the iBright CL1000 Imaging system (Invitrogen).

Cell Apoptosis Assay

The Apoptotic ratio of NCI-H23 cells was determined using Cell Apoptosis Kit with Annexin V Alexa Fluor 488 & Propidium Iodide (Invitrogen, V13241). After treatment apoptosis assay performed according to manufacture protocol using BD FACSAria Instrument. The apoptosis percentage was calculated using BD FACSDiva 8.0.2 software.

Cell viability Assay

Live dead Assay was performed using a Cytotoxicity kit (Invitrogen, L3224). After treatment cells were stained using 2 µM Calcein AM and 4 µM Ethidium homodimer-1 (EthD-1) as per manufacturer's protocol. Live Cells Imaging was done by using Nikon-Ti2E Fluorescence microscopy. Image analysis was done by using Image J Software.

Immunocytochemistry (ICC)

3 x 10^4 NCI-H23 cells were seeded in each well of the 8-well Lab-Tek II chambered slide (Thermo Fischer Scientific, 154534). Post-treatment cells were fixed with methanol, Cells were incubated overnight with Primary Antibody at 4℃ after blocking. After washing, cells were incubated with Fluorophore-tagged secondary antibodies. After washing, Cell was incubated with DAPI for 2 min, with the help of mounting media mounted the slide then observed the images under LSM 710 Confocal microscopy (Carl Zeiss). The acquired images were analyzed using ZEN 3.6 software, which is commonly used for image analysis in confocal microscopy.

The mean intensity value of the fluorescence signal was measured using the software. Additionally, the fluorescence signal was quantified using the Corrected Total Cell Fluorescence (CTCF) formula, Mean of Corrected total cell fluorescence (CTCF) = Integrated density – (Area of selected cell x Mean fluorescence of Background). This calculation helps to normalize the fluorescence intensity based on the size of the selected cell and the background fluorescence.

Scratch Assay

3 x 10^5 Cells were seeded in a 6-well culture dish until they reached 100% confluence. After that, a pipette tip was used to scrape the confluence cultures. Scratching was followed by two gentle medium washes to remove the detached cells. And cells incubated with and without Interferon-gamma (100 ng/ml). At 0, 24, and 48 hrs, scratches were captured in a microscope. Wound area measured using Image J software and Wound closure % was measured using following formula $Wound\ Closure\ \% = \frac{Area\ on\ day\ 0 - Area\ on\ day\ n}{Area\ on\ day\ 0} \times 100$

RNA-Seq sample preparation and data analysis

NCI-H23 cells were seeded and then transfected with TRIM34 CRISPR/Cas9 KO plasmid and Negative control plasmid in UltraCruz transfection reagent according to the manufacturer's instruction. Cells were extracted using TRIzol Reagent. RNA Isolation was done using Purelink RNA mini kit (Invitrogen, 12183018A), Quantity and quality of RNA were checked by using NanoDrop 2000 C (Thermo Fischer Scientific) and Qubit Fluorometer 4 (Invitrogen). Further, the Quality was checked by using Agilent 2200 TapeStation system. The library was prepared by using KAPA RNA HyperPrep Kit with RiboErase (HMR) Illumina Platforms (Roche, KR1351-v2.17) followed by Sequencing was done using NovaSeq 6000 Platform. Ten significantly expressed genes were selected with log2foldchange > = +/- 2 & Padj <0.05.

Statistical Analysis

The results were presented as the Mean ± SEM. Statistical analysis was performed using GraphPad Prism 9 software (GraphPad Software, Inc). Student's t-test was performed for data analysis. A result was deemed statistically significant if the value was $p < 0.05$ (* = $p \leq 0.05$, ** = $p \leq 0.01$, *** = $p \leq 0.001$ and **** $p \leq 0.0001$).

KEY FINDINGS

Chapter 1 delves into the increase of *TRIM34* expression induced by interferon treatment in a lung cancer cell line. Measured basal mRNA level of *TRIM34* gene in lung adenocarcinoma cell lines and lung tissues (Figure 1.1). The expression of *TRIM34* is significantly reduced in various human lung adenocarcinoma cell lines, namely A549, H292, H1650, and H1975 when compared to normal human alveolar epithelial type II cells. This downregulation suggests a lower level of TRIM34 mRNA production in the cancerous lung cells compared to healthy lung cells. Furthermore, when examining the lungs of a cancer-susceptible mouse model strain (AJ mice) and comparing them to the lungs of a cancer-resistant mouse model strain (C57BL6 mice), it was observed that the expression of TRIM34 mRNA was downregulated in the cancer-susceptible mice. This indicates that the production of TRIM34 mRNA in the lungs of the cancer-susceptible mice was notably lower than that in the lungs of the cancer-resistant mice.

Also, we investigated the impact of three types of interferons, namely Type I (IFN-α), Type II (IFN-γ), and Type III (IFN-λ), on the expression levels of *TRIM34*. Varying doses of each interferon were administered to determine the maximum *TRIM34* expression at different time points. Notably, maximum *TRIM34* expression occurred at 6 hours in A549 cells and 8 hours in NCI-H23 and NCI-H522 cells following exposure to IFN-α (Figure 1.2). Substantial upregulation was observed, with significant fold increases in *TRIM34* expression in all cell lines. Moreover, we investigated the impact of IFN-γ and IFN-λ treatments on *TRIM34* mRNA expression, revealing dose-dependent increases across the cell lines. These results underscore the ability of IFN-γ and IFN-λ to significantly enhance *TRIM34* expression in lung cancer cells (Figure 1.3&1.4), suggesting their potential involvement in modulating *TRIM34* dynamics and emphasizing their relevance in lung cancer pathogenesis or therapeutic responses. Additionally, our findings prompted further investigations specifically focusing on *TRIM34* in NCI-H23 cells stimulated with IFN-γ, aligning with previous research demonstrating the sensitivity of NCI-H23 cells to IFN-γ signaling and its implications in initiating an immune response [61].

TRIM34 protein levels were assessed through Western blot (Figure 1.5A&B) and immunocytochemistry (Figure 1.5 C&D) to validate the mRNA expression. IFN-γ treatment significantly increased TRIM34 protein levels in NCI-H23 cells. The immunocytochemistry results further support higher presence of TRIM34. Cell viability assays (Figure 1.6) were conducted to explore the functional consequences of TRIM34 upregulation induced by IFN-γ. The result indicates increase in apoptotic percentage on upregulation of TRIM34 (Figure 1.8).

These findings were further substantiated by assessing apoptosis-related genes (*BAX, BAD, TNF-α*) following IFN-γ treatment, revealing a significant upregulation and implying that TRIM34 overexpression induced apoptosis in lung cancer cells (Figure 1.7). This finding is consistent with the results reported by Vila-del Sol V *et al.* (2008) in murine macrophage cells [62]. 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 [63]. 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 [64]. Our result of *BAX* and *BAD* upregulation is in accordance with the literature [65, 66]. Further to validate apoptosis, FACS-based Annexin V and propidium iodide assays was employed. Result showed 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 proliferations [65, 67, 68]. In scratch assay (Figure 1.9), the cell migration was inhibited on treatment of IFN-γ (100 ng/ml). IFN-γ contribute to cancer regression, potentially impeding cell migration and reducing metastasis.

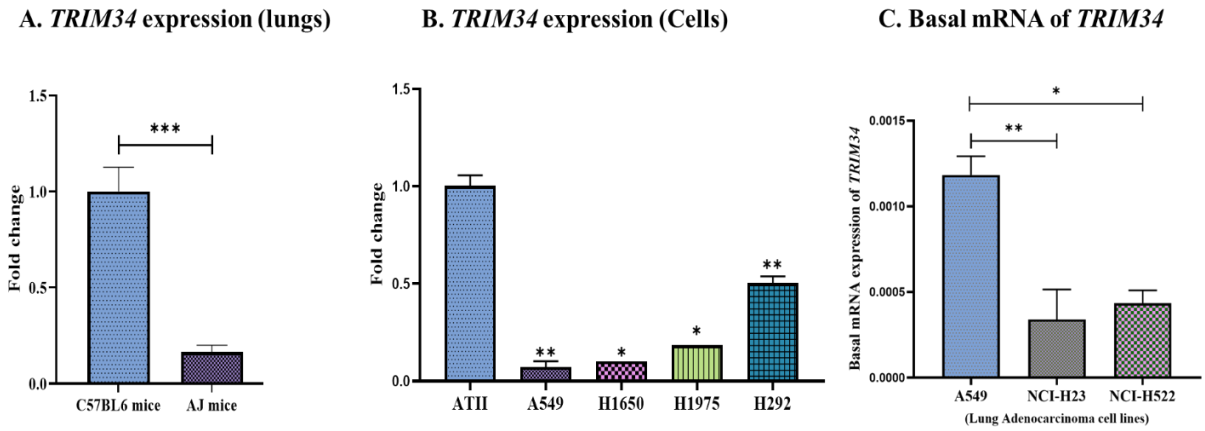


Figure 1.1: Quantification of basal mRNA level of TRIM34 genes in lungs of mice (A) and lung adenocarcinoma cell lines (B-C). Cells were harvested, and Total RNA was isolated and reverse-transcribed. Relative mRNA expression of *TRIM34* was measured by real-time qRT-

PCR and normalized to *GAPDH* gene expression. Graphs are plotted as Mean ± SEM, n=6. Statistical significance is denoted by *, and **, to indicate $p \leq 0.05$, and $p \leq 0.01$ respectively.

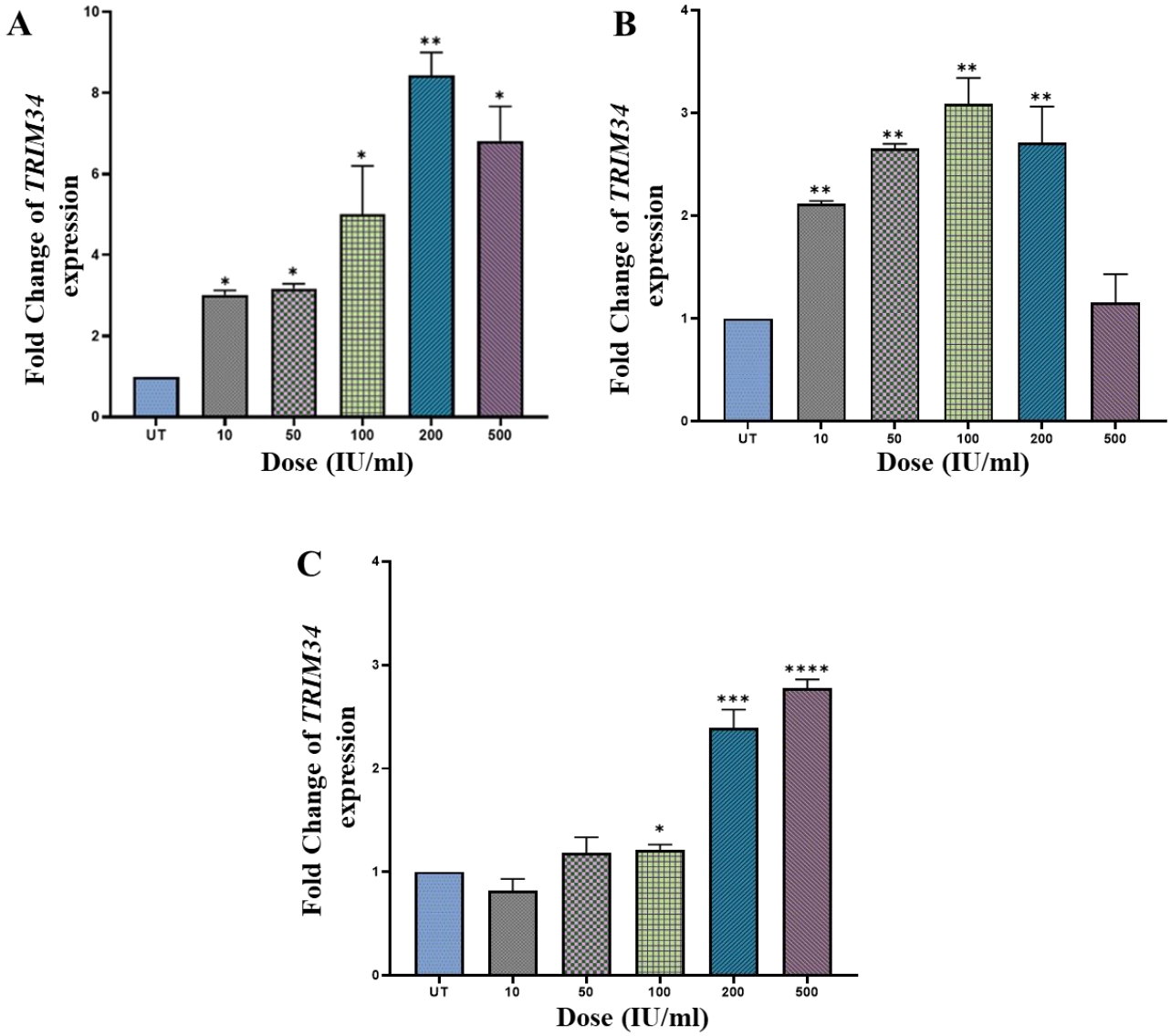


Figure 1.2: Effect of Interferon Alpha on *TRIM34* gene expression in a Dose-dependent manner in different lung adenocarcinoma cells. Cells were stimulated with various doses of Interferon Alpha for 6 hours in A549 and 8 hours in NCI-H23 and NCI-H522. *TRIM34* gene expression level was measured by qRT-PCR and normalized by *GAPDH*. **A.** NCI-H23, **B.** A549, and **C.** NCI-H522 cells. Data was calculated as Mean ± SEM (n=3). Statistical significance is denoted by *, **, ***, and **** to indicate $p \leq 0.05$, $p \leq 0.01$, $p \leq 0.001$, and $p \leq 0.0001$ respectively.

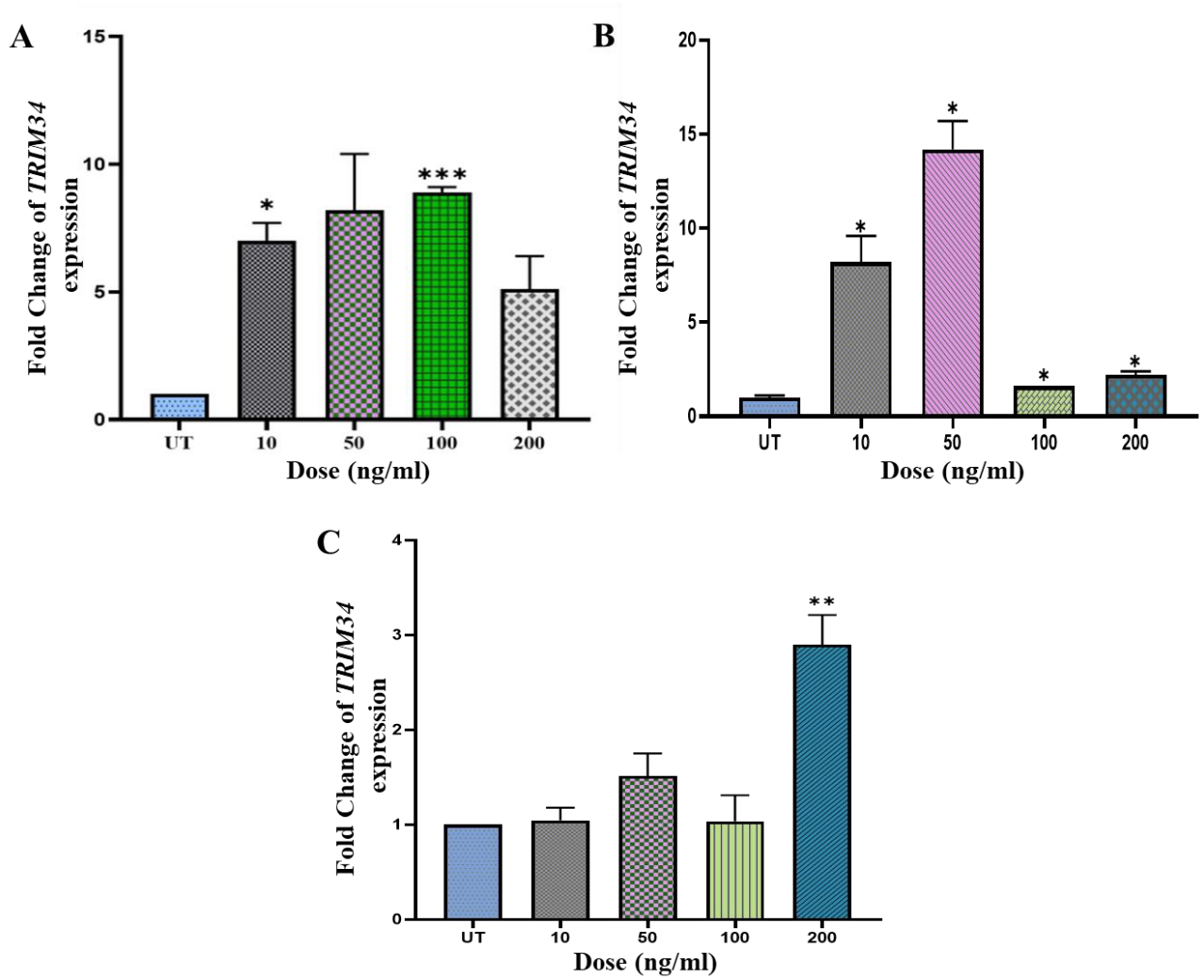


Figure 1.3: Dose-dependent expression of *TRIM34* on exposure of Interferon Gamma in lung adenocarcinoma cells. A549, NCI-H23, and NCI-H522 cells were stimulated by different doses of interferon-gamma for 10 hours. In **A.** NCI-H23, **B.** A549, and **C.** NCI-H522 cells, the expression of the *TRIM34* gene was quantified by qRT-PCR and normalized with *GAPDH* gene. Data was calculated as Mean ± SEM (n=3). Statistical significance is denoted as *, **, and *** to indicate $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$ respectively.

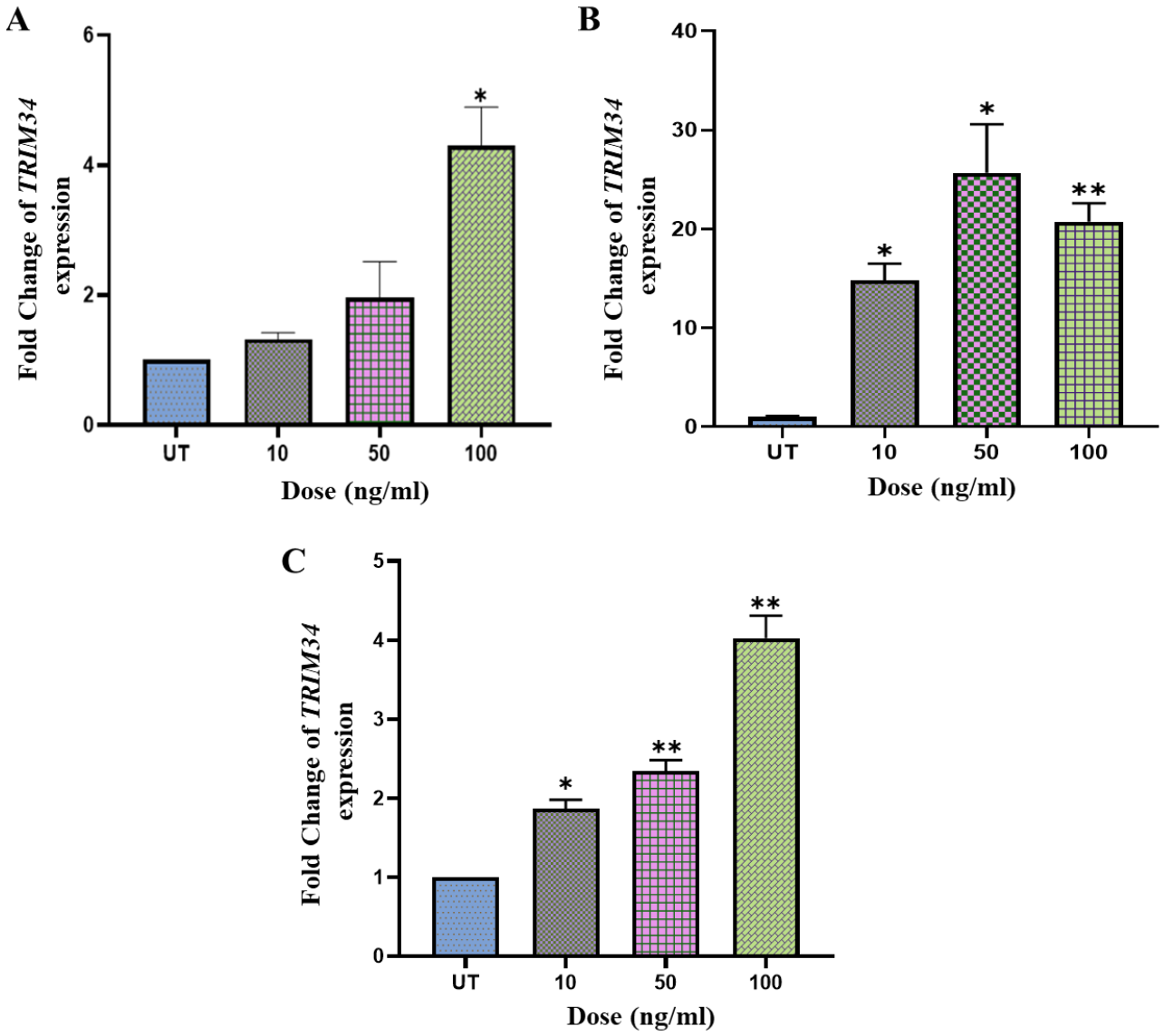
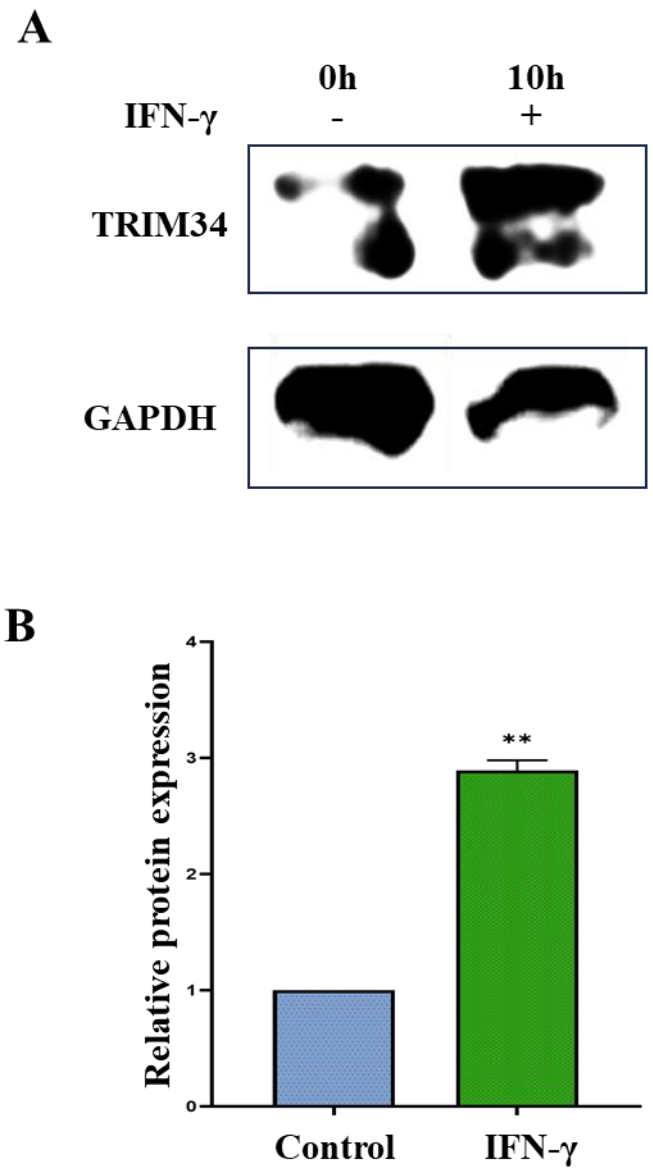


Figure 1.4: Dose-dependent expression of *TRIM34* on exposure of Interferon Lambda in lung adenocarcinoma. The expression of the *TRIM34* gene was measured by qRT-PCR and normalized to *GAPDH* gene. **A**. NCI-H23, **B.** A549, and **C.** NCI-H522 cells. Data was calculated as Mean ± SEM (n=3). *, and ** stands in for $p \leq 0.05$, and $p \leq 0.01$ to indicate statistical significance, respectively.


A
0h
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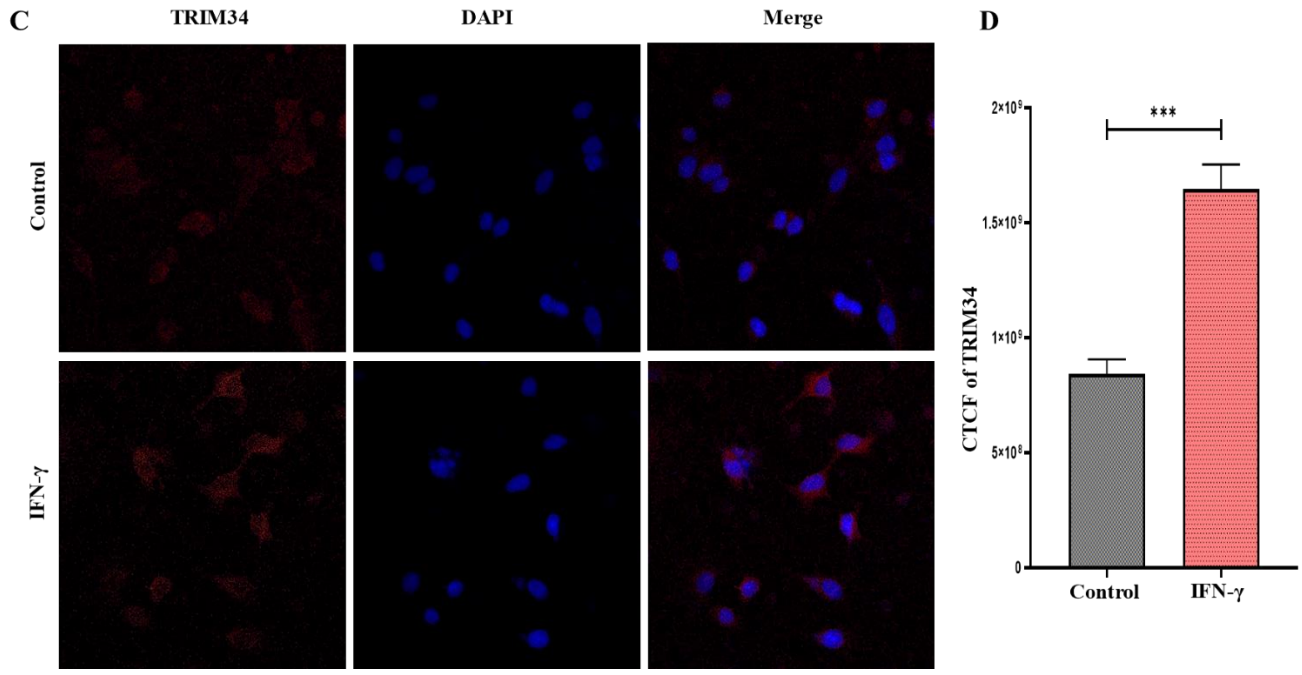

Figure 1.5: Protein level expression of TRIM34 using western blot and Immunocytochemistry techniques after 10 hours of interferon gamma. **A.** Blot of TRIM34 and GAPDH protein expression in NCI-H23 cells. TRIM34 expression was normalized by GAPDH. **B**. Graph represents densitometry analysis of blot using ImageJ software. **C.** TRIM34 localization. TRIM34 protein (red) and Nucleus (Blue). **D**. Graphs represent the corrected total cell fluorescence (CTCF) of TRIM34. 63X magnification. Statistical significance is denoted by **, and *** to indicate $p \leq 0.01$, and $p \leq 0.001$, respectively.

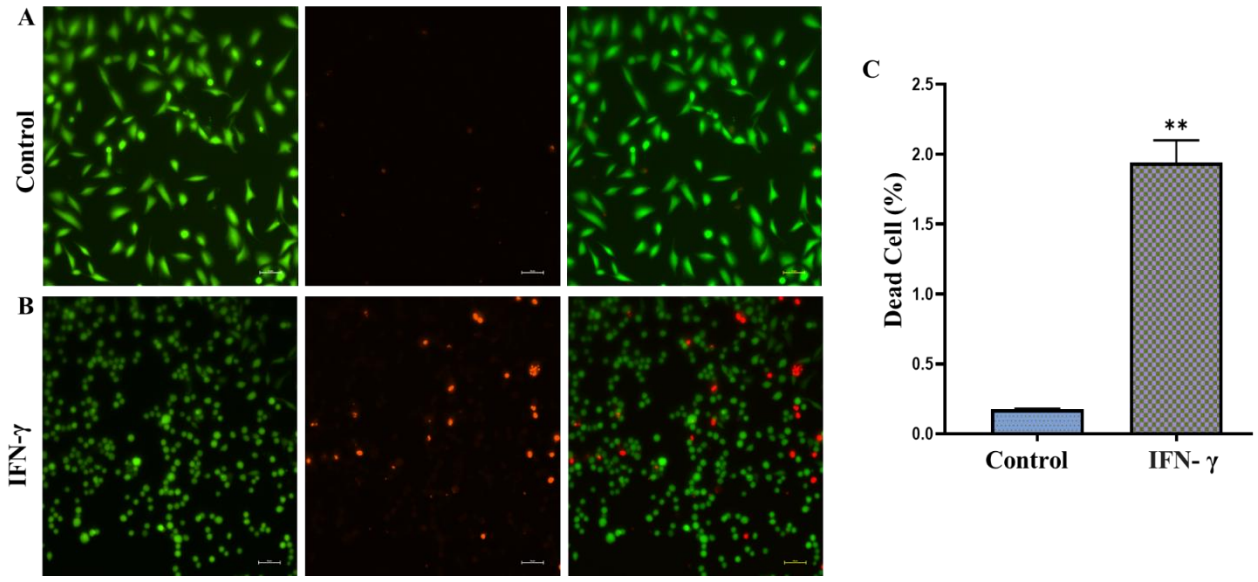

Figure 1.6: Overexpression of TRIM34 induces Cell death in NCI-H23 cells after 10 hours of treatment of IFN-γ. Calcein AM stains for live cells in green, and EthD-1 stains for dead cells in red. Images were seen with a fluorescence microscope at 20X objectives. **A**. Control (Untreated) Cells, **B.** Cells were treated with IFN-γ (100 ng/ml) to observe the cell viability in NCI-H23 cell. **C**. Graph represents % Dead cell. ** Stand for $p \leq 0.01$ to indicate statistical significance.

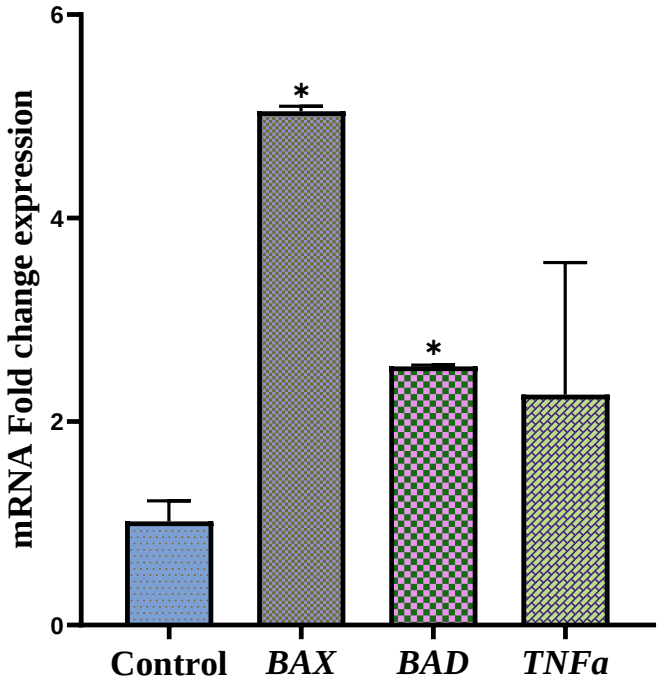

Figure 1.7: Gene expression of cell death markers. NCI-H23 cells were treated with IFN-γ for 10 hours. Evaluation of apoptosis regulating gene expression after IFN-γ exposure. The expression of the *BAX*, *BAD*, and *TNF-α* genes was measured by qRT-PCR and normalized with *GAPDH* gene expression in NCI-H23 cells. Mean ± SEM. (n=3) (*; $p \leq 0.05$).

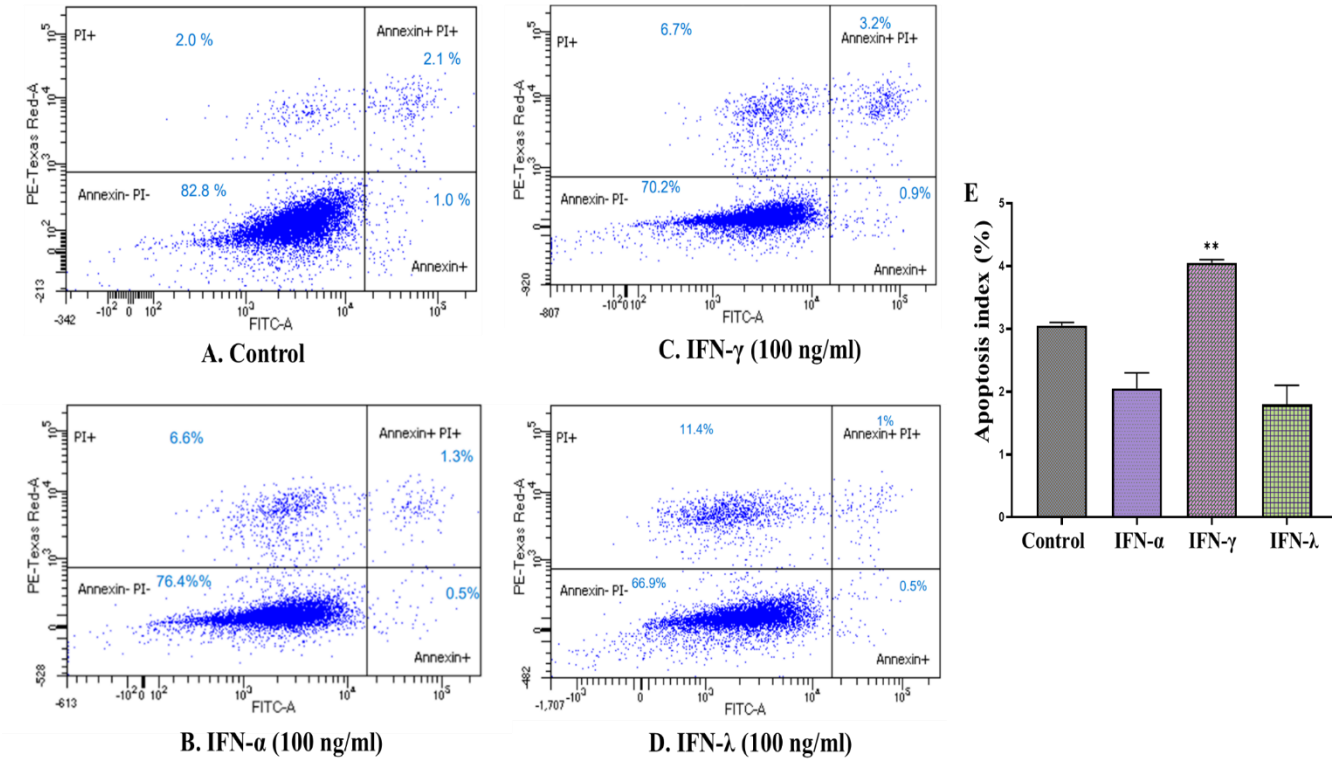

Figure 1.8: Cell death analysis using FACS on IFN treatment for 10 hours to NCI-H23 Cells. **A.** Untreated, **B.** Interferon Alpha (100 IU/ml), **C.** Interferon Gamma (100 ng/ml), and **D.** Interferon Lambda (100 ng/ml) treatment to NCI-H23 Cells. Percent apoptosis measured using E) Apoptosis index percentage. Mean ± SEM. (n=3). * Stand for $p \leq 0.05$ to indicate statistical significance.

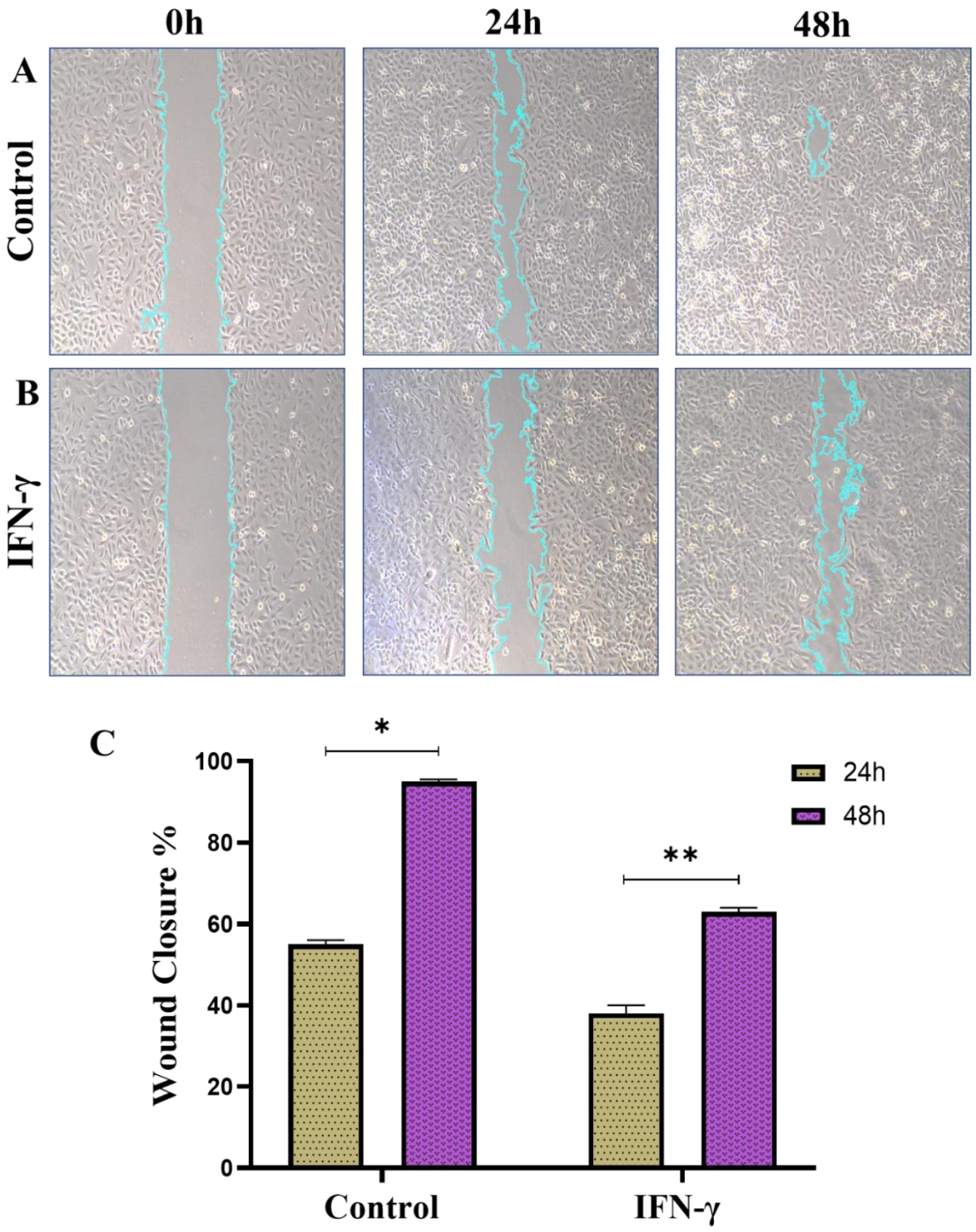

Figure 1.9: Scratch assay to analyze cell migration after Interferon-gamma treatment of 24 and 48 hours. **A.** Untreated, **B**. Interferon Gamma (100 ng/ml) treatment to NCI-H23 Cells. **C.** Percent wound closure measured by ImageJ software. * and ** stands for $p \leq 0.05$ and $p \leq 0.01$ to indicate statistical significance respectively.

Chapter 2 aimed to elucidate TRIM34's role in ubiquitylation pathways. CRISPR/Cas9 was employed to knockout *TRIM34* in NCI-H23 cells, with three experimental groups: Control, Negative control, and *TRIM34* CRISPR/Cas9 KO Transfection. The study utilized transcriptomics analysis to examine patterns of gene expression (Figure 2.2). Differential analysis was conducted on the knockout (KO) group compared to the control group, aiming to identify genes significantly expressed based on log2 fold changes. The investigation focused on understanding the impact of *TRIM34* knockout on gene expression, revealing substantial changes in 204 upregulated and 69 downregulated genes overall (Figure 2.5). Our aim in this experiment was to identify ubiquitylation genes among the top 10 hub genes. The findings present the top 10 differential genes within the network, ranked by the degree ranking method according to their interactions in the STRING database (Table 1). *DDX58* holds the highest rank, followed by *IFIT3* and *IFIH1*. Notably, *DDX58*, the gene with the highest rank, is associated with ubiquitylation [69, 70]. Literature suggests that DDX58 inhibits the proliferation, migration, and invasion of colon cancer cells [71]. *UBE2L6*, the sixth-ranked gene, is also linked to ubiquitylation [72, 73] and is implicated as a tumor suppressor in melanoma [74].

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were employed to ascertain the participation of differentially expressed genes in cellular compartments and molecular functions. These genes participate in various molecular functions such as binding with ubiquitin-like proteins, ubiquitin protein ligases, thiol-dependent ubiquitin-specific proteases activity, and ubiquitinyl hydrolases. Enrichment analysis revealed that four out of the top ten molecular functions were associated with ubiquitylation among the differentially expressed genes, highlighting the significant impact of *TRIM34* on the ubiquitylation process in non-small cell lung cancer (NSCLC). In the absence of *TRIM34*, a decrease in expression was observed in genes related to ubiquitylation, including *SLC25A5, PRKACB, TANK, ATF6, ZNF746, USP48,* and *RING1* (Table 3). Conversely, there was an increase in the expression of genes associated with ubiquitylation, such as *TXNIP, USP25, DTX3L, BAG5, UBE2L6, USP19, DDX58, CUL4A, POLR2A, USP18, USP3, COPS5, UBA7, FBXW11, HERC5, TRIM5, KIAA1586, RC3H2, UBR4*, and *TRIM38,* under the *TRIM34* knockout condition (Table 2). Cell morphology was observed after cell transfection, more rounded cells were observed in knockout group than control and negative control (Figure 2.1). Assessment of cell viability and apoptosis in *TRIM34* KO NCI-H23 cells showed increased cell death (Figure 2.3) and apoptosis (Figure 2.4) compared to control cells. The involvement

of DDX58 *i*n the STAT3/CSE signaling pathway supports our findings, suggesting enhanced sensitivity to apoptosis with *TRIM34* knockout.

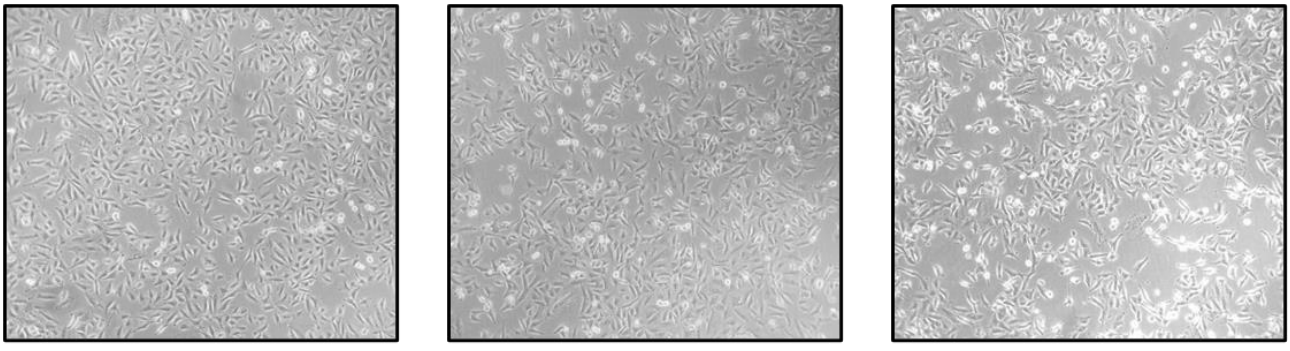

Figure 2.1: Morphological changes of the cells in **A**. Control, **B**. transfection of Negative Control plasmid, and **C.** transfection of TRIM34 CRISPR/Cas9 plasmid.

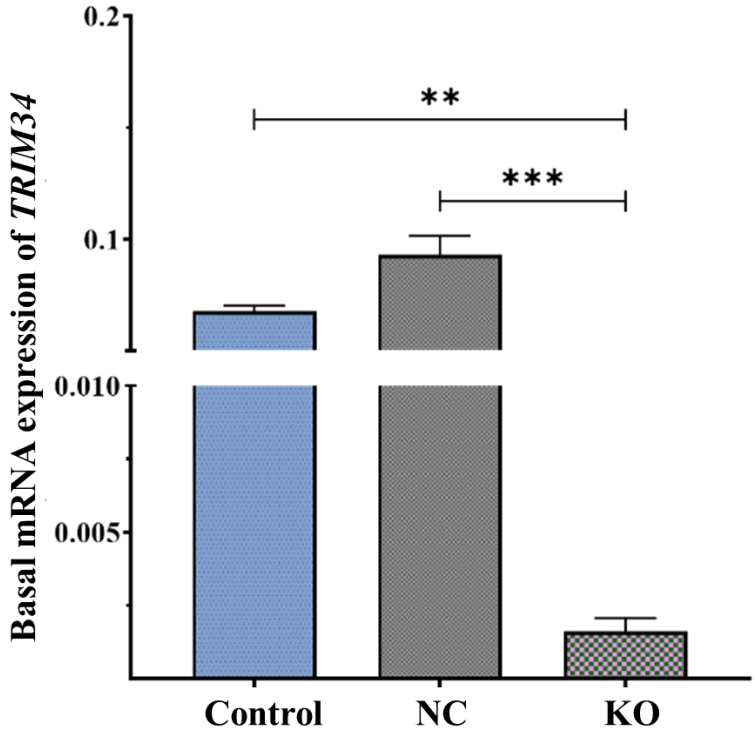

Figure 2.2: Transcript level confirmation of *TRIM34* knockdown in lung adenocarcinoma NCI-H23 cells. mRNA expression of *TRIM34* was measured by real-time qRT-PCR and normalized to *GAPDH* gene expression. Graphs are plotted as Mean ± SEM. (n=3). Statistical significance is denoted by **, and ***, to indicate $p \leq 0.01$, and $p \leq 0.001$, respectively.

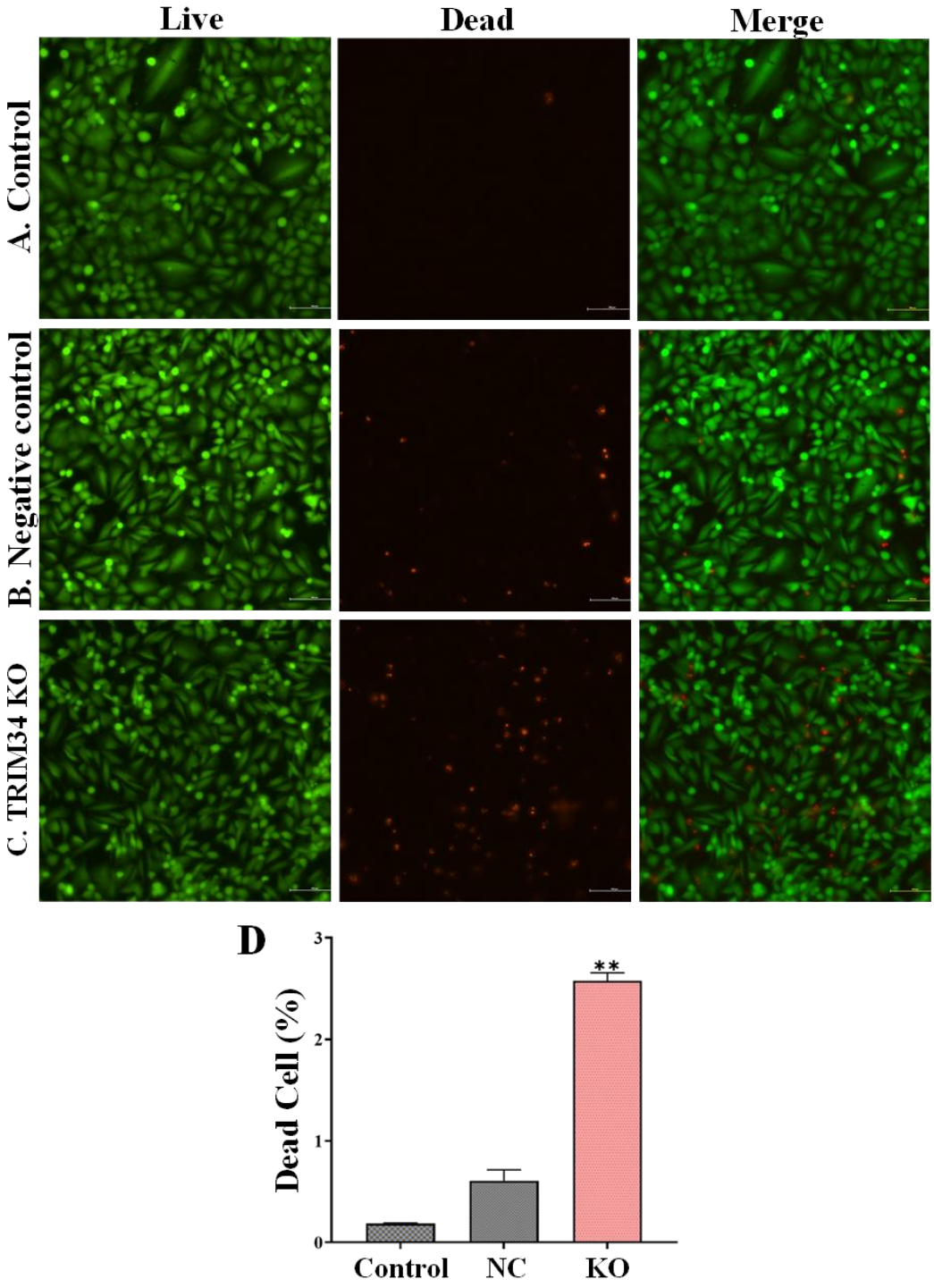

Figure 2.3: Assessment of cell viability using Calcein AM and Ethidium Homodimer-1 (EthD-1) staining in NCI-H23. Calcein AM stains for live cells in green, and EthD-1 stains for dead cells in red. **A**. Control (Untreated) Cells, **B**. Negative Control Group: Cells transfected with CRISPR/Cas9 lacking Guide RNA **C.** *TRIM34* CRISPR/Cas9 KO Transfected cells. **D.** Measurement of the proportion of dead cells in each group using ImageJ software. ** stands for $p \leq 0.01$ to indicate statistical significance. Scale bar: 100 μm.

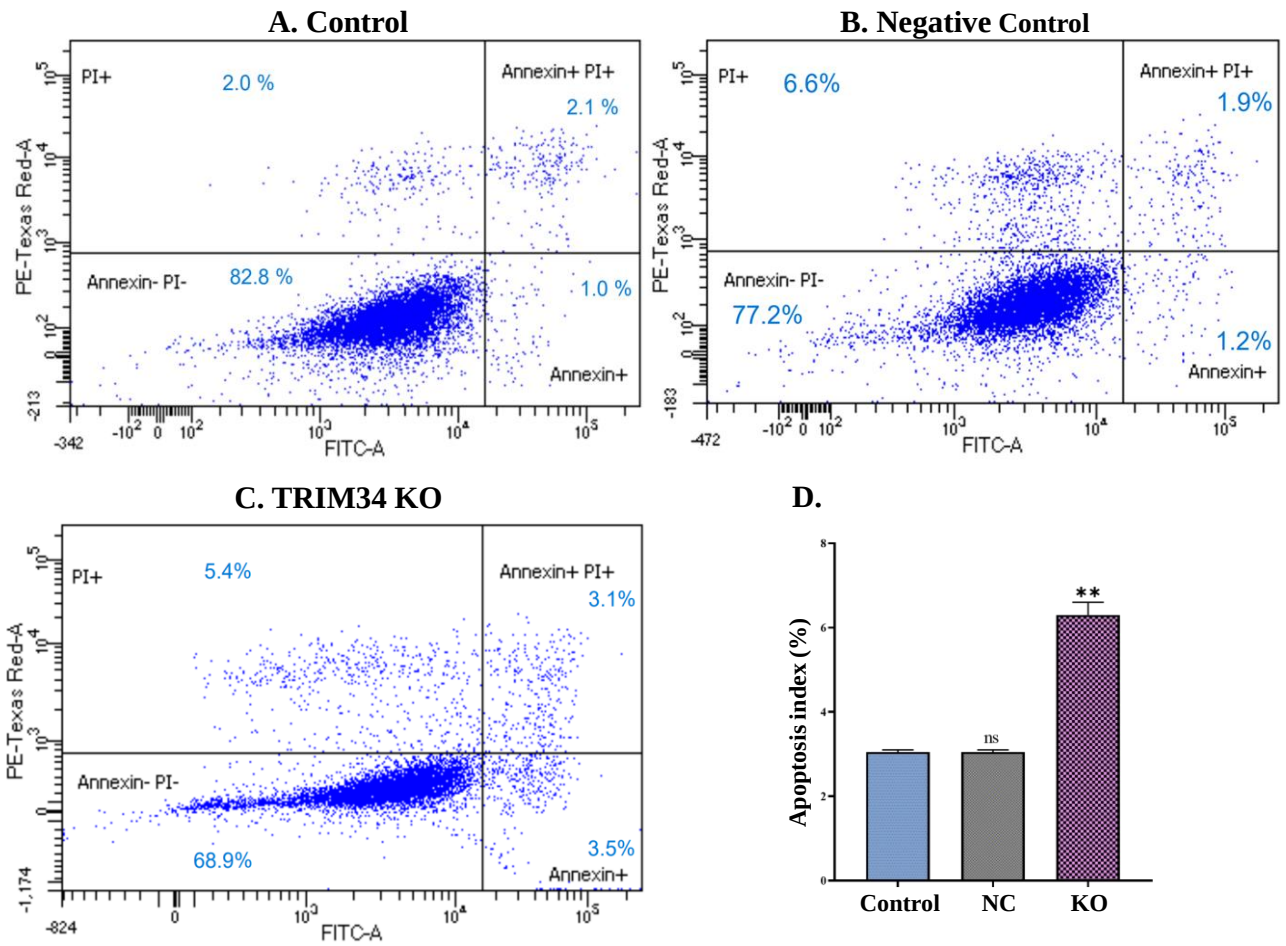

Figure 2.4: Assessment of apoptosis in NCI-H23 cells with *TRIM34* CRISPR/Cas9 Knockout using Annexin V-FITC and PI staining. NCI-H23 cells were stained with Annexin V-FITC and propidium iodide (PI) to assess apoptosis. Representative FACS plot showing the distribution of live cells (Annexin V-FITC negative, PI negative), early apoptotic cells (Annexin V-FITC positive, PI negative), late apoptotic cells (Annexin V-FITC Positive, PI Positive) and necrotic cells (Annexin V-FITC negative, PI Positive). **A.** Control (Untreated) cells, **B.** Negative control group: cells transfected with CRISPR/Cas9 lacking Guide RNA **C.** *TRIM34* CRISPR/Cas9 KO transfected cells. **D.** Apoptosis index (%): Quantification of the percentage of early and late apoptotic in each group, ** stands for $p \leq 0.01$ to indicate statistical significance.

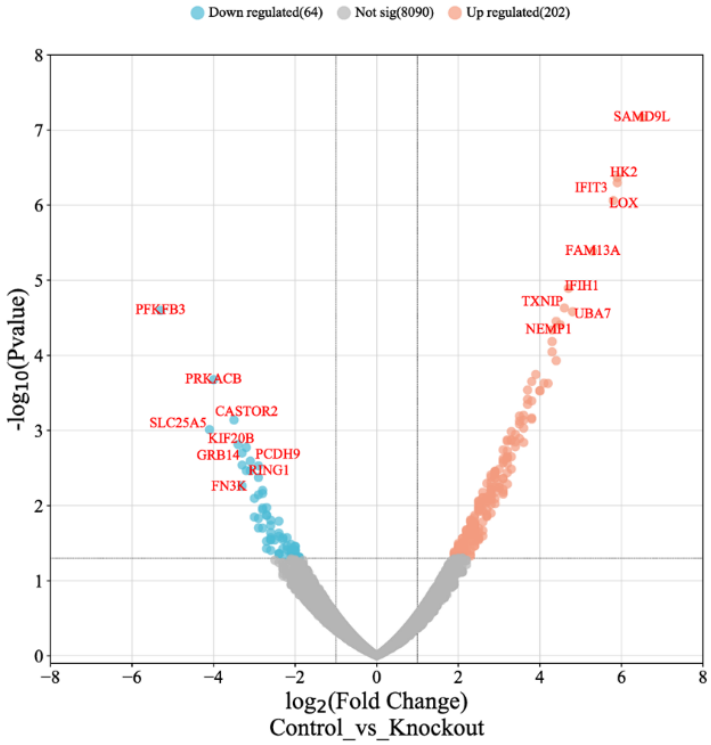

Figure 2.5: Differentially gene expression of *TRIM34* Knockout sample.

Volcano plot depicting differentially expressed genes (DEGs) in *TRIM34* knockout samples compared to control. Genes with a log fold change (log FC) $\geq \pm 2$ and a $p \leq 0.05$ are highlighted, with upregulated genes shown in orange and downregulated genes in blue.

Table 1: Top 10 differential genes in network String interactions ranked by degree ranking method.

Rank	Name	Score
1	*DDX58*	18
2	*IFIT3*	14
3	*IFIH1*	12
4	*IL1B*	11
4	*IFI35*	11
6	*CXCL8*	10
7	*UBE2L6*	9
7	*IRF9*	9
9	*PARP14*	8
9	*H3C14*	8

Table 2: Upregulated ubiquitin pathway genes identified by GO analysis in the *TRIM34* knockout sample.

ID	Description	Genes
GO:0044389	Ubiquitin-like protein ligase binding	*TXNIP, USP25, DTX3L, BAG5, UBE2L6, USP19, DDX58, CUL4A, POLR2A*
GO:0004843	Thiol-dependent ubiquitin-specific protease activity	*USP18, USP3, USP25, USP19, COPS5*
GO:0101005	Ubiquitinyl hydrolase activity	*USP18, USP3, USP25, USP19, COPS5*
GO:0019787	Ubiquitin-like protein transferase activity	*UBA7, DTX3L, FBXW11, UBE2L6, HERC5, TRIM5, KIAA1586, CUL4A, RC3H2, UBR4, TRIM38*
GO:0031625	Ubiquitin protein ligase binding	*TXNIP, USP25, BAG5, UBE2L6, USP19, DDX58, CUL4A, POLR2A*

Table 3: Downregulated ubiquitin pathway genes identified by GO analysis in the *TRIM34* knockout sample.

ID	Description	Genes
GO:0031625	Ubiquitin protein ligase binding	*SLC25A5, PRKACB, TANK, ATF6, ZNF746*
GO:0044389	Ubiquitin-like protein ligase binding	*SLC25A5, PRKACB, TANK, ATF6, ZNF746*
GO:0004843	Thiol-dependent ubiquitin-specific protease activity	*USP48, TANK*
GO:0101005	Ubiquitinyl hydrolase activity	*USP48, TANK*
GO:0055106	Ubiquitin-protein transferase regulator activity	*RING1*

Chapter 3 is aim to unraveling the epigenetic regulation of TRIM34 in non-small cell lung cancer (NSCLC) through methylation analysis. NCI-H23 (Figure 3.1A), NCI-H522 (Figure 3.1C), and A549 (Figure 3.1B) cell lines were examined to observe the promoter methylation pattern of the *TRIM34* gene. The investigation involved the use of 5-Aza-2'-deoxycytidine (AZA), a DNA methylation inhibitor, to demethylate the *TRIM34* promoter in conjunction with IFN.

The combined treatment with AZA and IFN aimed to demethylate the *TRIM34* gene's promoter region, resulting in the restoration of its transcriptional activity. The observed reduction in methylation levels via qRT-PCR and the subsequent increase in *TRIM34* expression following the treatment with AZA and IFN provided additional evidence supporting the role of DNA methylation in suppressing *TRIM34* in lung cancer cells. Elevated *TRIM34* expression in NCI-H23, NCI-H522, and A549 lung cancer cell lines suggests that the altered methylation pattern of *TRIM34* is involved in lung cancer tumorigenesis.

The study implies that TRIM34 upregulation in response to immunotherapy or alteration of the ubiquitin-proteasome system (UPS) by its suppression acts as a double-edged sword in lung cancer treatment.

Demethylation highly induces TRIM34 expression along with Interferon.

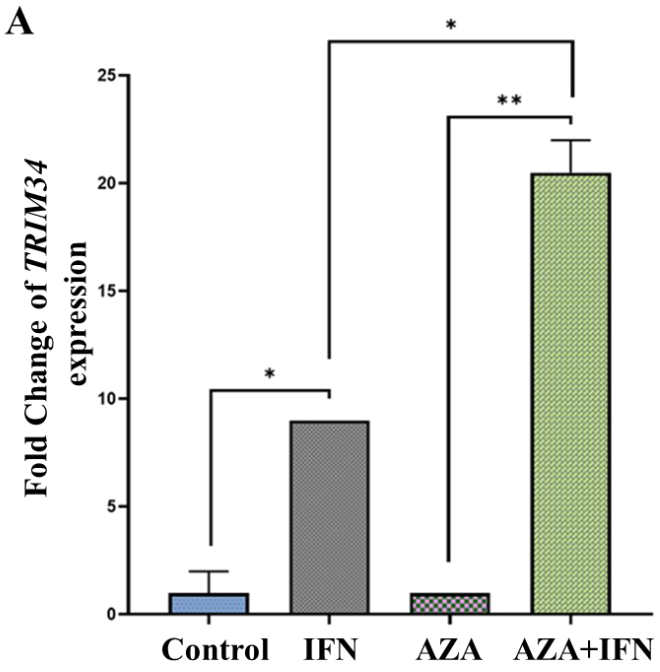

Figure 3.1A: Analysis of methylation pattern of TRIM34 after 48 hours of 5µM 5-Aza-2'-deoxycytidine before being treated with interferon, AZA and AZA+IFN in NCI-H23 cells. GAPDH gene expression was used for normalization. * and ** stands for $p \leq 0.05$ and $p \leq 0.01$ to indicate statistical significance respectively.

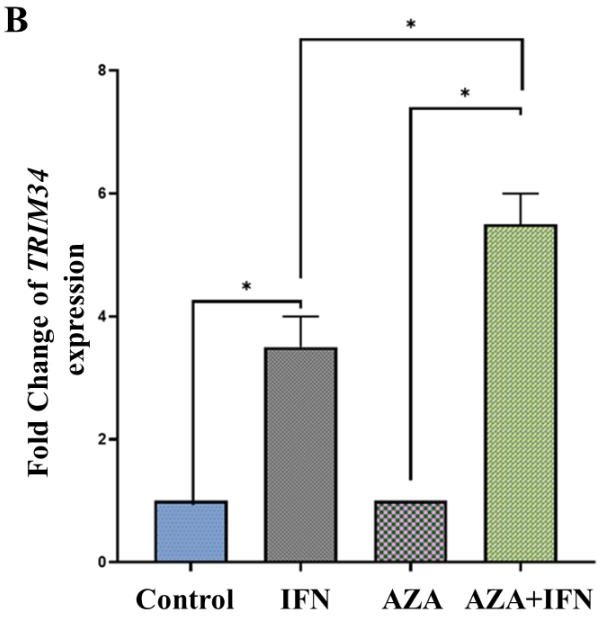

Figure 3.1B: Analysis of methylation pattern of TRIM34 after 48 hours of 5µM 5-Aza-2'-deoxycytidine before being treated with interferon, AZA and AZA+IFN in A549 cells. GAPDH gene expression was used for normalization. * stands for $p \leq 0.05$ to indicate statistical significance.

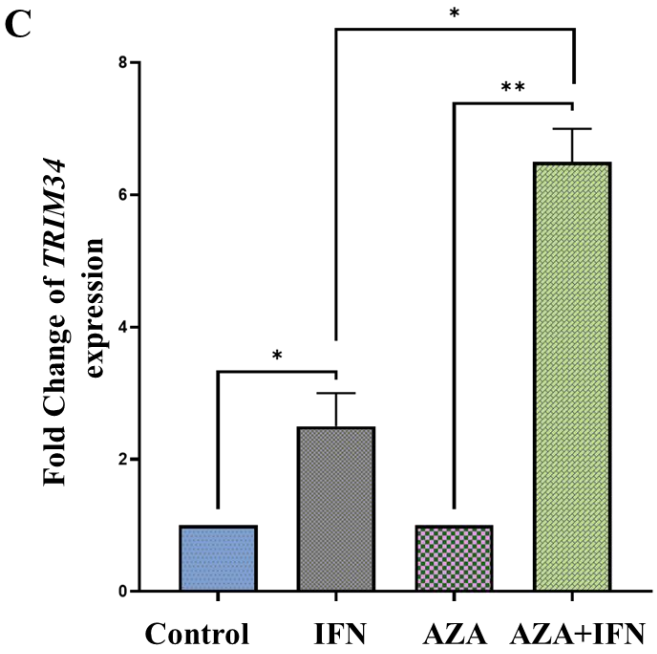

Figure 3.1C: Analysis of methylation pattern of *TRIM34* after 48 hours of 5µM 5-Aza-2'-deoxycytidine before being treated with interferon, AZA and AZA+IFN in NCI-H522 cells. *GAPDH* gene expression was used for normalization. * and ** stands for $p \leq 0.05$ and $p \leq 0.01$ to indicate statistical significance respectively.

CONCLUSION

Our study investigated the role of TRIM34 in non-small cell lung cancer (NSCLC). Expression analyses, particularly in response to various interferons, revealed substantial upregulation, implicating TRIM34 in the intricate network of cellular responses to interferon treatments. The study delved into the impact of IFN-γ on NCI-H23 cells, revealing increased TRIM34 protein levels and providing visual confirmation of its presence post-treatment. Functional consequences, including pro-apoptotic effects, altered expression of apoptosis-related genes, and inhibition of cell migration, suggest a potential therapeutic avenue for IFN-γ induced TRIM34 overexpression in lung adenocarcinoma.

The exploration of *TRIM34*'s role in NSCLC extended to CRISPR/Cas9 gene KO editing, showcasing its impact on cell viability, apoptosis. RNA-seq analysis uncovered significant alterations in expression of genes involved in ubiquitylation pathway upon *TRIM34* knockout. Protein-Protein interaction network analysis shed light on potential molecular mechanisms affected by TRIM34.

Epigenetic studies revealed the impact of DNA methylation on *TRIM34* expression, with 5-Aza-2'-deoxycytidine (AZA) with IFN treatment demonstrating a reduction in methylation levels and a subsequent upregulation of *TRIM34* expression.

The comprehensive exploration of TRIM34 in NSCLC not only advances our understanding of its functional roles but also unveils potential therapeutic avenues, emphasizing the complex interplay between genetics and epigenetics in lung cancer pathogenesis and treatment strategies.

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CONFERENCES/ WORKSHOPS

आज़ादी का अमृत महोत्सव

INCD2022

5th International Conference on Nutraceuticals and Chronic Diseases

"Pharmaceuticals and Nutraceuticals for Cancer and Other Chronic Diseases"

October 7-9, 2022

Certificate of Participation

D 103

This is to certify that Kaushal Chaudhari

has participated as Invited Faculty / Delegate and made an Oral/Poster Presentation entitled Regulation of Tripartite Motif 34 (TRIM34) expression by interferon in lung adenocarcinoma *at the INCD2022 organised by the Department of Zoology, University of Delhi, Delhi, India from October 7-9, 2022.*

Participation Type : OFFLINE/ONLINE

Prof. Alok Chandra Bharti, *Organising Secretary*; Prof. Anju Shrivastava, *Organising Secretary*; Prof. Rina Chakrabarti, *Covenor*; Prof. Bharat Bhushan Aggarwal, *Chairperson*

NATIONAL CONFERENCE

On

CURRENT TRENDS IN BIOLOGICAL SCIENCES - VI

(CTBS-2023)

Saturday, January 07, 2023

Certificate

This is to certify that Mr. Kaushalkumar Chaudhari, had attended and delivered an Oral Presentation on "Type I interferon-dependent TRIM34 expression induces Apoptosis in lung adenocarcinoma", at the conference organized by Post Graduate Department of Biosciences, UGC-Centre of Advanced Study, Sardar Patel University, Satellite Campus, Vadtal Road, Bakrol – 388 315 (Anand), Gujarat.

Dr. Akshaya Gupte
Organizing Secretary

Prof. Ujjval Trivedi
President

1. Attended and presented a poster entitled “Regulation of Tripartite Motif 34 (TRIM34) expression by Interferons in Lung Adenocarcinoma” at the 5th International Conference on Nutraceuticals and Chronic Diseases (INCD 2022) on 7-9 October 2022, at the Department of Zoology, University of Delhi, Delhi, India.

2. Oral presentation entitled “Type I interferon-dependent TRIM34 expression induces Apoptosis in Lung Adenocarcinoma” at the 6th National Conference on Current Trends in Biological Sciences (CTBS 2023) on 7th January 2023, at the Department of Biosciences, Sardar Patel University, Anand, Gujarat, India.

3. The Workshop on Electron Microscopy (FEG-SEM & TEM) took place from October 10, 2019, to October 11, 2019, at the Sophisticated Instrumentation Centre for Applied Research & Testing (SICART) in Vallabh Vidyanagar, Anand.

4. A Short-term Hands-on Training Programme on Application of Real-Time PCR was conducted from November 25, 2019, to November 29, 2019, hosted by Kamdhenu University and Gujarat Biotechnology Research Centre (GBRC) in Gandhinagar.

5. An International Online Conference on COVID-19 was held on April 30, 2020, lasting for one day and organized by the Department of Bioscience at Manipal University Jaipur.

6. Host Immunity and COVID-19 was the focus of an online conference held on May 16, 2020, at Mahamaya Govt. Degree College in Dhanupur, Prayagraj.

7. Development of Viral Vaccine and Other Preventive Measures in Pandemic: A One-Day Online Event Hosted by the Department of Microbiology and Biotechnology at Gujarat University, Ahmedabad on May 17, 2020.

8. An Online Course on DNA Taxonomy and Phylogeny took place from May 21, 2020, to May 23, 2020, hosted by SATHYABAMA Institute of Science and Technology in Chennai.

9. Online Gel-Based Proteomics Workshop by Amazing Biotech Pvt. Ltd. in Chennai from June 21 to June 25, 2020.

10. International Webinar on Modern Trends in Experimental Research: Insights from Regenerative Medicine and Metagenomics was organized by the Quality Assurance Cell, Faculty of Science, and Institute for Interdisciplinary Studies at The M.S. University of Baroda, Vadodara, on May 30, 2020, to May 31, 2020.

11. Ensembl Browser and REST API Course: Hosted by Nextgenhelper in New Delhi from November 17 to November 20, 2020.

12. Online Industrial Training in Bioinformatics, Genomics, and Data Sciences: Offered by Nextgenhelper in New Delhi from February 19 to February 27, 2021.

13. Online Workshop on Flow Cytometry: Organized by the Research and Training Wing at Drawing Pin Publishing in New Delhi from March 8 to March 10, 2021.

14. Online International Bioinformatics Workshop on Genome Informatics: Presented by Decode Life in Haryana from April 5 to April 21, 2021.

15. Statistical Data Analysis Using SPSS: Online Training Program by Science Tech Institute in Lucknow from May 21 to May 27, 2021.

16. Animal Cell Culture & Flow Cytometry Workshop: Hosted by Gujarat Biotechnology Research Centre & Ahmedabad University from September 27 to October 1, 2021.

Signature of Candidate
(Mr. Kaushalkumar D. Chaudhari)

Signature of Ph.D. Guide
(Dr. Hetal Roy)