Chapter-3

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INTRODUCTION

Lung cancer stands as the primary contributor to cancer-related deaths in the globe. Extensive research findings have consistently highlighted the significant involvement of epigenetic changes, encompassing DNA methylation, histone modifications, and non-coding RNA expression, in the development of lung cancer[345]. The onset of carcinogenesis arises from the progressive buildup of genetic and epigenetic modifications, leading to the disruption of critical oncogenes, tumor suppressor genes, and DNA repair genes[346]. The likelihood of experiencing these crucial events with pathological implications primarily relies on an individual's exposome, along with variations in their phenotypic traits. Epigenetic diversity, encompassing DNA methylation, histone modifications, and non-coding RNA expression, significantly influences an individual's phenotypic traits, such as xenobiotic metabolism, DNA repair capacity, immunity, and more importantly, susceptibility to developing malignancies[347–349].

Epigenetic alterations play a significant role in carcinogenesis, involving heritable changes in gene expression without altering the DNA sequence[350,351]. Epigenetic alterations have emerged as crucial factors impacting the early diagnosis of cancer and determining patients' outcomes and survival. Although conventional detection methods have limitations, the exploration of epigenetics offers promising avenues for early cancer detection[54,352]. Many specific genes exhibit promoter hypermethylation during the initial stages of mammary gland tumorigenesis, suggesting their potential use in assessing breast cancer risk[353,354]. Notably, in lung cancer, P16INK4a and MGMT gene hypermethylation was found in smokers' sputum years before symptoms of squamous cell carcinoma[352]. Although somatic genetic aberrations, including mutations and copy number alterations, are widely recognized as contributors to oncogenesis, it is important to note that epigenetic alterations are more prevalent than somatic mutations in the context of lung cancer[355]. Epigenetic inactivation of tumor suppressor genes through aberrant promoter methylation is increasingly recognized as a significant mechanism in lung cancer pathogenesis[356,357].

Recent findings reveal that aberrant methylation can be detected in the bronchial epithelium of cancer-free heavy smokers, making it a potential biomarker for lung cancer risk assessment and monitoring chemoprevention trials[358]. Aberrant methylation emerges as the most common mechanism for inactivating cancer-related genes in lung cancer and offers promise for improved diagnostic and therapeutic approaches[358,359]. Epigenetic alterations in peripheral pulmonary adenocarcinoma (ADC) development were investigated in a multistage progression sequence (atypical adenomatous hyperplasia (AAH)-to-adenocarcinoma in situ (AIS)-to-invasive ADC). The methylation status of 18 CpG island loci were analyzed in normal lung tissues, AAH, AIS, and ADC tissues.

Aberrant methylation increased significantly from AAH to AIS and further to invasive ADC. Certain genes (*HOXA1, TMEFF2,* and *RARB*) showed early-stage methylation in preinvasive lesions, while others (*PENK, BCL2, RUNX3, DLEC1, MT1G, GRIN2B, CDH13, CCND2*, and *HOXA10*) exhibited higher methylation in invasive ADC, suggesting their role in tumor invasion during the progression from AAH to AIS to ADC. Epigenetic changes play a critical role in the multistep development of pulmonary ADC[360]. Epigenetic changes were investigated in the histologic progression from normal lung tissue to AAH, with atypia, and finally to adenocarcinoma. The frequency of promoter hypermethylation significantly increased along this progression, with distinct patterns of hypermethylation observed in multifocal AAHs, indicating divergent epigenetic field defects. Promoter hypermethylation of certain genes showed strong associations with advanced histology, with p16 exhibiting the highest odds ratio for hypermethylation in tumor or high-grade AAH compared to low-grade AAH or normal tissue[361].

The presence of CpG island methylator phenotype (CIMP) was investigated in bladder cancer, head and neck squamous cell carcinomas (HNSCC), non-small-cell lung cancer (NSCLC), and malignant pleural mesotheliomas (MPM). CIMP was found in these solid tumors, suggesting its potential clinical usefulness for disease classification [362]. Liu *et al.* (2008) identified a CIMP in non-small cell lung cancer (NSCLC) samples, showing frequent promoter hypermethylation of tumor suppressor genes on chromosome 3p. CIMP was significantly associated with NSCLC, paired normal tissues, and survival prognosis [363]. In lung adenocarcinoma, CIMP was identified through genome-wide DNA methylation analysis, and a CIMP-high subgroup was associated with wild-type epidermal growth factor receptor (EGFR), males, and heavy smokers. CIMP-H Adenocarcinoma showed a worse prognosis, suggesting personalized treatment strategies using DNA methylation inhibitors for CIMP-positive tumors[364]. The group of enzymes that catalyse the covalent attachment of the methyl group to the cytosine base, known as DNA methyltransferases (DNMT), is increased in NSCLC. Lin *et al.* (2007) demonstrated overexpression of DNA methyltransferases (DNMT1, DNMT3a, and DNMT3b) in lung tumors, particularly in smokers, and their correlation with hypermethylation of tumor suppressor gene promoters, leading to lung tumorigenesis and poor prognosis, especially in squamous carcinoma patients[365].

Covalent modifications of histones and changes in DNA cytosine methylation are common mechanisms. Methylation of CpG islands in gene promoters leads to heritable gene silencing. Unlike genetic alterations, epigenetic modifications are reversible, and treatment with inhibitors of cytosine methylation and histone deacetylation can restore gene transcription by demethylation and chromatin decondensation[366,367]. CpG methylation has the potential to cause point mutations by promoting the deamination of 5-methylcytosine (5mC) or increasing the effect of external carcinogens.

Methylated cytosine can undergo hydrolytic deamination, leading to a C to T transition. Interestingly, over 30% of disease-related germline point mutations are found at CpG dinucleotides[368].

Hypermethylation of promoter region of tumour suppressor genes results in their abnormal silencing[369]. Cytosine methylation is a reversible process susceptible to biochemical and biological manipulation, rendering it an appealing target for therapeutic intervention. Strategies involving demethylation and subsequent reactivation of tumor suppressor genes are rational approaches in cancer treatment[370–372]. The currently available nucleoside-based DNMT inhibitors 5-azacytidine (5-azaC), 5-aza-2′-deoxycytidine (5-azadC), and zebularine (ZEB) are cytosine analogues with similar inhibitory mechanisms[373,374]. Recently, clinical trials combining AZA treatments with additional treatments such as histone deacetylase inhibitors and interleukin-2 [375,376]. Combining epigenetic therapy in which AZA with PD-1/PD-L1 checkpoint blockade could enhance NSCLC treatment response, especially in cases with compromised immune pathways[377].

Sutton *et al.* (2014) observed that TRIM16 transcription increased in melanoma cell lines after 5-Aza treatment, demonstrating direct or indirect de-repression of TRIM16 gene transcription by the demethylating agent[378]. Wang *et al.* (2019) have found that in colorectal cancer TRIM67 downregulation correlated with increased promoter methylation and demethylation treatment restored TRIM67 expression, suggesting DNA methylation-mediated transcriptional silencing[379]. In lung cancer, Shi Y (2021) found that the potential tumor suppressor gene TRIM58 displayed high methylation and low expression[380]. Kaut *et al.* (2017) elucidated the genetics of Parkinson's disease, wherein the signature of DNA methylation suggested that *TRIM34* was one of the genes with multiple CpGs associated with epigenetic variations [217].

Upon thorough investigation of the data presented in Chapter 1 and Chapter 2, we have identified a noteworthy correlation. Specifically, the manipulation of *TRIM34*, involving both its activation and suppression, has demonstrated a significant impact on inducing cancer regression. This observation prompts the consideration of a potential link between the regulatory mechanisms of *TRIM34* and alterations in DNA methylation levels, manifesting as either hypo- or hypermethylation. As our study have revealed that *TRIM34* responds to interferon alpha, gamma, and lambda, our further objective was to ascertain whether the treatment of IFN in combination with an epigenetic modifying agent could maximize the upregulation of *TRIM34* expression in NSCLC cell lines. We have utilized 5-Aza-2'-deoxycytidine (AZA), a known DNA methyltransferase inhibitor, to alleviate methylation, thereby inducing *TRIM34* expression.

MATERIAL AND METHODS

Demethylation Assay

3 x 10^5 Cells were seeded in a 6-well culture plate, and 5 μM 5-Aza-2'-deoxycytidine (AZA) (cat. no. 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 IFN Alpha (100 IU/ml) and AZA + Interferon Alpha (100 IU/ml) and only with for 6 hours in A549 and 8 hours for NCI-H23, and NCI-H522. Cells were harvested for gene expression analysis.

RNA isolation, reverse transcription, and real-time PCR

Detailed methodology is described in the material and methods section.

Genomic DNA Purification

After treatment, genomic DNA purification was carried out using Thermo Scientific GeneJET genomic DNA purification kit (cat. no. K0721). Cells were washed and centrifuged at 250 g for 5 minutes to get cell pellet, that was resuspended in 420 μl of lysis solution containing proteinase K. The sample was incubated at 56 °C for 20 minutes. Following this, 20 μl of RNase A was added and sample was incubated for 15 minutes at room temperature. The prepared lysate was transferred to a GeneJET Genomic DNA purification column inserted into a collection tube, and centrifuged for 1 minute at 6000 g. The flow-through solution was discarded, and purification column was placed into a new 2 ml collection tube. 500 μl of Wash Buffer I was added and centrifuged at 8000 g for 1 minute. Wash Buffer II (with ethanol) was added, followed by centrifugation at 13000 g for 3 minutes. The GeneJET Genomic DNA purification column was then transferred to a sterile 1.5 ml microcentrifuge tube. For elution, 20 μl of elution buffer was added to the column's membrane, followed by a 2-minutes incubation at room temperature and centrifugation at 8000 g for 1 minute. Finally, the purification column was discarded, and the purified DNA was stored at -20 °C until further process.

Quantitative and qualitative analysis of DNA

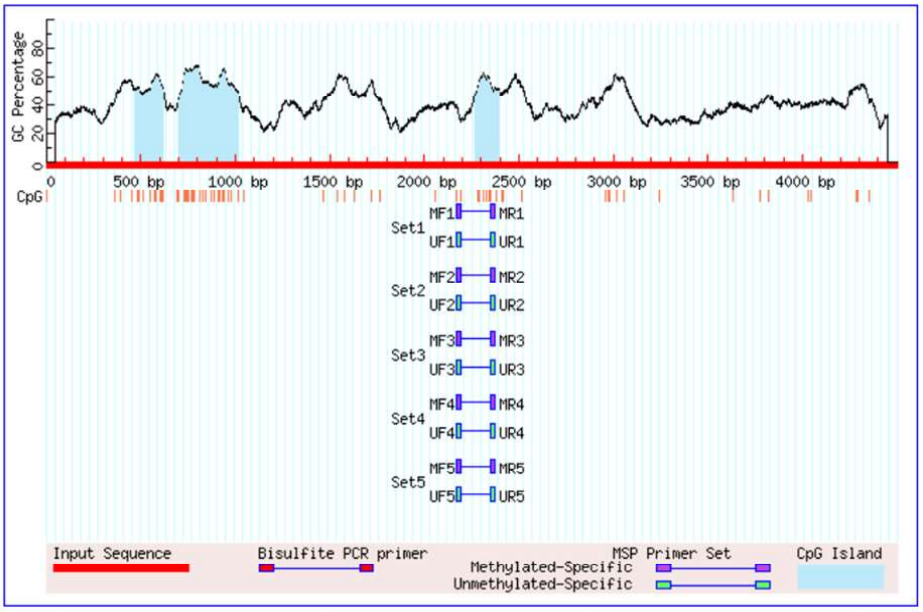
The estimation of DNA concentration was performed using the BioSpec-NanoDrop spectrophotometer by measuring the absorbance ratio at 260/280 nm. A ratio of ~1.8 was considered indicative of DNA purity. The quality of the DNA was assessed through electrophoresis on a 1% agarose gel. Visualization of the DNA band was achieved using an iBright CL1000 Imaging system (Invitrogen, Waltham, MA, USA).

Bisulfite Conversion of DNA

The experiments were conducted using the Thermo Scientific EpiJET Bisulfite Conversion Kit (cat. no. K1461). Modification reagent, wash buffer, and desulfonation buffer were prepared as per manufacturer's protocol. We have used 20 µl of DNA sample, containing 200 ng of purified genomic DNA, into a PCR tube. Following this, 120 µl of prepared Modification reagent solution was added to the 20 µl DNA sample in a PCR tube, and the sample was mixed through pipetting and then centrifuged. The PCR tubes were then placed in a thermal cycler, and the following steps were carried out: 1) Heating to 98°C for 10 minutes, and 2) 60°C for 150 minutes. Subsequently, 400 µl of Binding buffer was added to a DNA Purification micro column within the collection tube. The converted DNA sample was loaded into the column's Binding buffer, mixed thoroughly by pipetting, and the micro column was centrifuged at 12,000 rpm for 30 seconds. The flow-through was discarded, and the micro column was returned to the collection tube. Washing steps were performed by adding 200 µl of wash buffer, followed by centrifugation, discarding the flow-through, and repeating the micro column placement. Desulfonation was achieved by adding 200 µl of desulfonation buffer, allowing the column to stand at room temperature for 20 minutes, and subsequently centrifuging the micro column, discarding the flow-through, and returning the micro column to the collection tube. Further washing steps were executed by adding wash buffer to the micro column, followed by centrifugation and flow-through discarding. Additional washing was repeated once more. The micro column was placed in a sterile 1.5 ml microcentrifuge tube, and 10 µl of Elution buffer were added to the column, followed by centrifugation. The eluted converted DNA was stored at -20 ºC.

Primer Designing for Methylation

The primer designing process was executed as follows: Initially, the genomic information about the *TRIM34* gene's promoter region (5' UTR) was retrieved from the Ensembl genome browser version 110. Subsequently, a segment comprising 4500 base pairs upstream of the promoter region was selected for further analysis and methylation specific PCR (MSP) primer design. The design of primers for the specific purpose of assessing methylation and unmethylation patterns was carried out using the MethPrimer software program[381]. The primers were constructed with a GC concentration of 50% to 60% since this range has been shown to promote stable primer-target interactions. Furthermore, the primers were designed to produce amplicons ranging in length from 100 to 300 bases. The target DNA molecule can be accurately quantified and detected using this length range. To ensure primer specificity, the self-complementarity of both forward and reverse primers was thoroughly examined. During primer design, the identification of three CpG islands within the selected region was accomplished. The designed primers were obtained from Eurofins Genomics India Pvt. Ltd. PCR and Sanger sequencing of Bisulfite converted DNA of untreated and AZA-treated samples were done by SLS private lab, Surat, Gujarat, India.




```
Sequence Name:
Sequence Length: 4500

CpG island prediction results
Criteria used: Island size > 100, GC Percent > 50.0, Obs/Exp > 0.60
 3 CpG island(s) were found in your sequence
              Size         (Start - End)
 Island 1    158 bp        (465 - 622)
 Island 2    316 bp        (700 - 1015)
 Island 3    130 bp        (2270 - 2399)
```

Figure 3.1: Representative image of primer designing in MethPrimer software.

Table 23: List of the primers used for the study. (M: Methylated; UM: Unmethylated)

Promoter	Sequence
TRIM34-F (M)	TTTCGAGTAGTTGGGATTATAGGC
TRIM34-R (M)	ACACTTTAAAAAACCGAAACGAA
TRIM34-F (UM)	TTTTTTGAGTAGTTGGGATTATAGGT
TRIM34-R (UM)	CAACACTTTAAAAAACCAAAACAAA

Table 24: PCR thermal cycling conditions.

Step	Temp. °C	Time	No. of cycles
Initial denaturation	95	5 min	1
Denaturation	95	30 sec	35
Annealing	50	30 sec	
Extension	72	30 sec	
Final Extension	72	5 min	1

RESULTS

Demethylation highly induces TRIM34 expression along with Interferon.

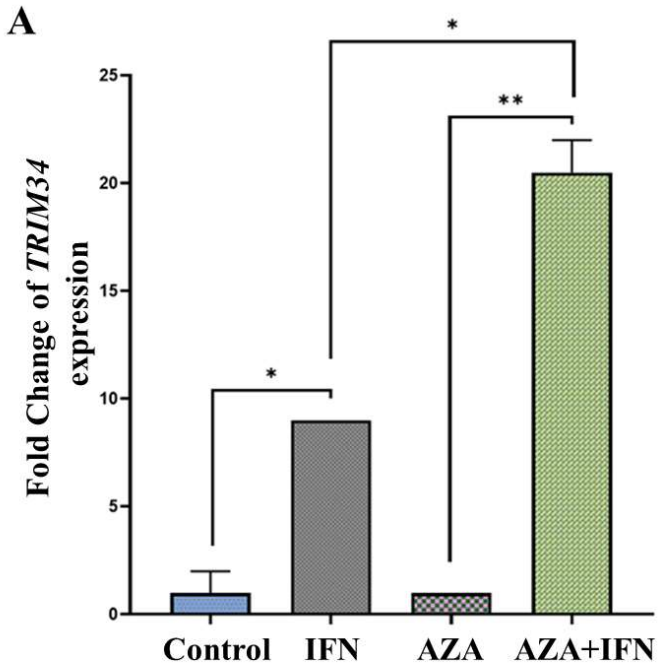


Figure 3.2A: Analysis of methylation pattern of TRIM34 after 48 hours of 5µM 5-Aza-2'-deoxycytidine before being treated with interferon, AZA and AZA+IFN in NCI-H23 cells. GAPDH gene expression was used for normalization. * and ** stands for $p \leq 0.05$ and $p \leq 0.01$ to indicate statistical significance respectively.

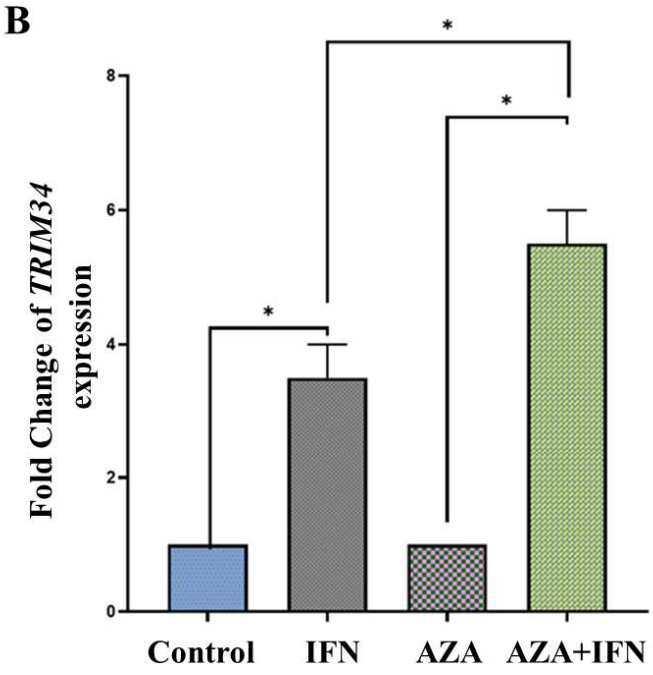


Figure 3.2B: Analysis of methylation pattern of TRIM34 after 48 hours of 5µM 5-Aza-2'-deoxycytidine before being treated with interferon, AZA and AZA+IFN in A549 cells. GAPDH gene expression was used for normalization. * stands for $p \leq 0.05$ to indicate statistical significance.

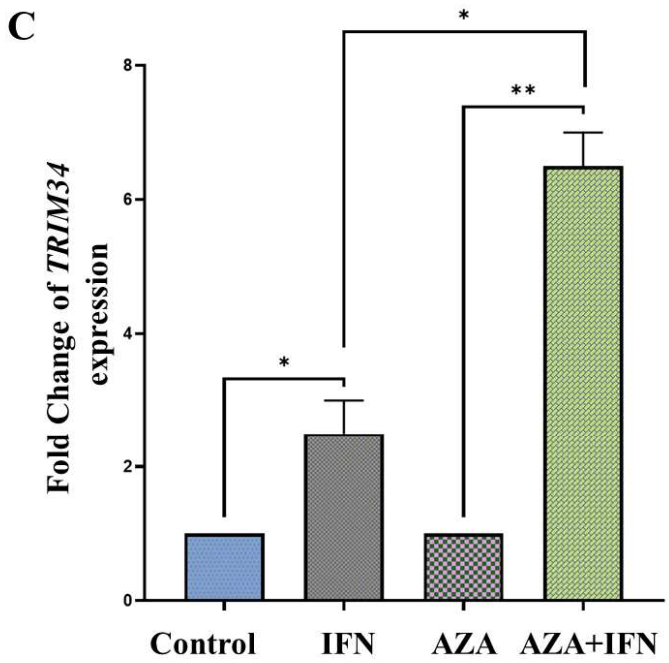


Figure 3.2C: Analysis of methylation pattern of *TRIM34* after 48 hours of 5µM 5-Aza-2'-deoxycytidine before being treated with interferon, AZA and AZA+IFN in NCI-H522 cells. *GAPDH* gene expression was used for normalization. * and ** stands for $p \leq 0.05$ and $p \leq 0.01$ to indicate statistical significance respectively.

qRT-PCR was performed to check the *TRIM34* mRNA expression in lung cancer cells after AZA, IFN and AZA+IFN treatment on non-small cell lung carcinoma (NSCLC) cell lines. To investigate the role of DNA methylation in *TRIM34* regulation, we treated cells with a DNA methylation inhibitor, 5 µM of 5-Aza-2'-deoxycytidine (AZA), in combination with 100 IU/ml of Interferon Alpha. The treatment aimed to demethylate the TRIM34 promoter region. After exposure, we observed a significant reduction in methylation levels, that was indicated by level of mRNA expression. *TRIM34* expression in NCI-H23 cells (Fig. 3.2A) was observed to be significantly upregulated by 20.5-fold ($p \leq 0.01$) in the AZA+IFN group compared to IFN alone (9-fold). Similarly, in NCI-H522 cells, TRIM34 expression showed a significant upregulation of 6.5-fold ($p \leq 0.01$) in the AZA+IFN group compared to IFN alone (2.5-fold). In A549 cells, TRIM34 expression was significantly upregulated by 5.5-fold ($p \leq 0.01$) in the AZA+IFN group compared to IFN alone (3.5-fold). This suggests that AZA combined with IFN treatment can effectively increase the expression of TRIM34 transcripts compared to IFN treatment alone in NCI-H23 (Fig. 3.2A), A549 (Fig. 3.2B), and NCI-H522 cells (Fig. 3.2C). These findings highlight the role of DNA methylation in the suppression of *TRIM34* expression. By inhibiting DNA methylation, the transcriptional activity of *TRIM34* is restored, potentially leading to increased *TRIM34* levels. This implicates DNA methylation as a regulatory mechanism for *TRIM34* downregulation in lung adenocarcinoma and

suggests the potential of combining specific DNA methylation inhibitor with IFN as a strategy to modulate *TRIM34* expression in cancer therapeutics.

The promoter of *TRIM34 is demethylated by AZA treatment*.

Figure 3.3: Demethylation study of *TRIM34* by performing bisulfite conversion and sanger sequencing.

To investigate the impact of an epigenetic modifier (demethylation) on the expression of *TRIM34* in non-small cell lung carcinoma (NSCLC) cell line, a methylation study was performed. A methyl group is transferred from the donor S-adenosyl-L-methionine (SAM) to the cytosine 5′ carbon atom, resulting in 5-methylcytosine (5mC), when DNA is methylated [382]. The most common method for differentiating between methylation and unmethylated cytosine is to treat DNA with sodium bisulfite, which converts unmethylated cytosine to uracil while maintaining 5-methylcytosine (5mC) [383]. After that, the converted DNA was amplified using MSP primers. Sanger sequencing was used to sequence the amplified sequence. Molecular Evolutionary Genetics Analysis (MEGA) software was used for analysis, wherein the ClustalW sequence alignment program was used to displays the multiple alignments between predicted and sequenced amplified sequences.

It is evident from Figure 3.3 that a small number of methylated cytosine (mC) may demethylate when treated with AZA. It is well known that when AZA is present, mC demethylates and may convert to thymine or uracil while treated with bisulfite. Among these, mC indicates that red circles could be the demethylated ones. Furthermore, the CpG site is marked with a black square, which may also be shown to be demethylated by AZA.

DISCUSSION

Lung cancer is a leading global cause of cancer deaths where epigenetics changes are playing a key role in its neoplasm[54,345]. DNA methylation is one of the epigenetic markers and it can alter during lung tumorigenesis. It is also linked to histological subtypes, driver gene mutations (e.g., KRAS, EGFR, TP53), and epidemiological factors (e.g., sex, smoking, race)[357]. Elevated expression levels of DNA methyltransferases (DNMTs), notably DNMT1, are commonly identified in lung cancer. Such upregulation is consistently and autonomously correlated with poor prognosis in affected individuals[365,384,385]. Downregulation of DNMT1 and/or DNMT3B led to growth arrest, apoptosis, and the reactivation of tumor suppressor genes (TSGs) in lung cancer cell lines[385]. Ectopic overexpression of MDM2 significantly induces the expression of DNMT3A and DNMT3B, indicating a suppressive influence of MDM2 on RB and FOXO3a in lung cancer [386,387]. Epigenetic regulation of gene expression, like hypermethylation of tumor suppressor genes is known to induce lung cancer [388,389].

Epigenetic alterations have emerged as promising targets in advancing cancer therapies. One of the most common epigenetic changes in mammals is DNA methylation, which occurs at the 5'-position of cytosine and is catalyzed by DNA methyltransferases (DNMTs)[367]. This hypermethylation leads to the silencing of tumor suppressor genes, contributing to tumorigenesis[390,391]. Li *et al.* (2013) found that the stabilization of the DNMT1 protein is governed by suppressing GSK3β-mediated phosphorylation and proteasomal degradation of DNMT1 following the activation of T cell receptor signaling[392].

Karpf *et al.* (1999) found that AZA induces gene expression in colon adenocarcinoma cells, targeting methylation-regulated genes, especially those associated with IFN signaling. This sensitizes cells to IFN-alpha2a, potentially enhancing responsiveness in resistant tumors. The study supports the efficacy of combining immunotherapy with epigenetic approaches in the fight against cancer[393,394]. The combination of IFN with DNA methylation inhibitors could represent a potent strategy to sensitize lung cancer cells to immune-mediated destruction and enhance the efficacy of immunotherapeutic interventions. The combination of AZA, a DNA methylation inhibitor, with IFN was employed to demethylate the promoter region of the *TRIM34* gene and restore its transcriptional activity as observed in Figure 3.2. The decrease in methylation levels and subsequent upregulation of *TRIM34* expression after the AZA and IFN treatment further supports the role of DNA methylation in suppressing *TRIM34* in lung cancer cells. The increase in *TRIM34* expression observed in multiple lung cancer cell lines (NCI-H23, NCI-H522, and A549) highlights the potential relevance of this epigenetic mechanism across different lung cancer subtypes. Modulation of *TRIM34* expression through DNA demethylation has significant implications for lung cancer therapeutics. In chapters 1 and 2, we observed that both, upregulation and downregulation of *TRIM34* contribute to cancer

regression in non-small cell lung cancer (NSCLC). Notably, we found that the *TRIM34* promoter is methylated in lung cancer. However, treatment with AZA and Interferon effectively reduces methylation, leading to increased transcription and subsequent upregulation of *TRIM34*. This suggests the potential therapeutic targeting of *TRIM34* in lung cancer. Interestingly, we recognize its dual role as a double-edged sword in the context of lung cancer.