

Reversal of promoter hypermethylation with targeted DNA methyltransferase inhibitors in gastric cancer

By

Wenxia Luo

A thesis submitted to the Faculty of Graduate and Postdoctoral Studies, University of Manitoba

In partial fulfillment of the requirements of the degree of:

DOCTOR OF PHILOSOPHY

College of Pharmacy

Rady Faculty of Health Sciences

University of Manitoba

Winnipeg, Manitoba

Abstract

Gastric cancer is one of the leading causes of cancer-related mortality worldwide, and DNA hypermethylation, particularly CDKN2A/p16 promoter hypermethylation, plays a critical role in tumor suppressor gene silencing during gastric carcinogenesis. DNA methyltransferase (DNMT) inhibitors have therefore been explored as epigenetic agents in gastric cancer; however, their biological effects and regulatory consequences remain incompletely understood. This thesis aimed to investigate how DNMT inhibition modulates DNA methylation, gene expression, and regulatory networks in gastric cancer, with a specific focus on CDKN2A/p16 and CDKN2A-associated gene networks under DNMT inhibitor treatment.

To address this objective, multiple gastric cancer cell lines were used to evaluate the effects of three DNMT inhibitors, decitabine, RG108, and GSK3685032, on total DNA methylation, CDKN2A(p16) promoter methylation, gene expression, cell-cycle progression, and histone modification. At low concentrations, DNMT inhibitors reduced total and promoter-specific DNA methylation and increased CDKN2A(p16) expression, resulting in cell-cycle arrest. In contrast, higher concentrations produced a paradoxical increase in DNA methylation at both total and gene-specific levels, accompanied by reduced CDKN2A(p16) expression. These dose-dependent responses were associated with increased expression of DNMT3B and, in some cases, TET enzymes. These findings suggest that, if translatable to gastric cancer treatment, lower doses of decitabine and GSK3685032 may enhance CDKN2A(p16) expression, whereas higher doses may attenuate CDKN2A(p16) expression and potentially reduce therapeutic effectiveness.

To extend these observations beyond individual gene responses, integrative multi-database analysis, bioinformatic analysis, and dynamic DNMT inhibitor dose validation were employed to define CDKN2A-centered regulatory networks in gastric cancer. From large public datasets, a 20-gene CDKN2A-associated panel with clinical relevance and network support was identified and evaluated through network reconstruction and survival analyses. Dynamic dose-response validation of gene expression further supported TRIB3, GET4, and PDE4D as connected components within this network. Among these, TRIB3 emerged as the most consistently supported candidate, based on convergent clinical, network, and DNMT inhibitor-responsive expression evidence, indicating that TRIB3 is a tightly coupled component of the CDKN2A-driven network and a potential co-targeting candidate.

Together, this work demonstrates that DNMT inhibitor responses in gastric cancer are shaped by coordinated, dose-dependent regulation within CDKN2A-centered gene networks rather than simple gene-level effects.

Acknowledgements

Here I would like to thank many people who have helped me in various ways throughout the years of my PhD study. First, I would like to thank my advisor Dr. Ted Lakowski. His guidance, support, encouragement, and patience throughout my research and the writing of this thesis has been invaluable. I would also like to thank Dr. Amir Barzegar Behrooz and Dr. Hamid Latifi Navid for supervising me on bioinformatics parts of my thesis. Their guidance, insights, and expertise has helped me to finish my research and the writing of this thesis. Without the guidance and support from Dr. Ted Lakowski, Dr. Amir Barzegar Behrooz and Dr. Hamid Latifi Navid, this project would not have been continued, and this thesis would not have been finished.

I would also like to thank my committee members Dr. Lucy Marzban, and Dr. Donald Miller for their advice and instruction on the cancer biology of this research. In every committee meeting, the thesis proposal, and the candidacy examination, they asked the insightful questions to get me to think and evaluate the scientific meaning of my research. Their insights and questions have helped steer both my research and my career directions.

I'm grateful to all the members of the Lakowski lab, my lab partner, Oluwafolahanmi Ige, Thordur Hendrick-Rebissant, Dr. Ivan Iurchenko and Dr. Prasanta Paul. Our past students; Ryan Hnatiuk, Phinehas Cheung, Daniel Paetkau for their thought-provoking discussions and comradery. I wish to say particular thanks to my friends Dr. Alekhya Lavu, Dr. Billy Vuong, Dr. Yuhua Fang, Shannon Derie, Nitesh Sanghai, Rushie Tyagi, Danish Malhotra, Laila Aboullata, Jessica Meza Resillas, Walid Shouman, Sherin Paraplammoottil Cheriyan and other Graduate students.

I would like to express my heartfelt gratitude to all my family and friends for their support and encouragement throughout my research journey. To my beloved husband, Jing Zhou, and my beloved daughter, Xinwei Zhou, I offer my deepest thanks for everything they have brought into my life. They have always been by my side, sharing every triumph and sacrifice with me along the way.

Publications and Contributions

Portions of Chapter 1 will be submitted for publication: Wenxia Luo, Ivan Yurchenko, Olufola Ige, Amir Barzegar Behrooz, Thordur Hendrickson-Rebizant, Ted M. Lakowski. Methylation-Driven Insights in Gastric Cancer: Recent Progress and Future Directions in Biomarkers and DNMT Inhibitor Development. I wrote most of the manuscript with guidance from Lakowski, T. M. Co-authors contributed to writing and revision.

Chapter 2 has been published: Wenxia Luo, Olufola Ige, Amir Barzegar Behrooz, Haziqa Kassim, Thordur Hendrickson-Rebizant, Daniel Paetkau, Emmanuel A. Ho, Meaghan J. Jones, James R. Davie, Ted M. Lakowski. Dose Dependent Paradoxical Increases in DNA Methylation, Reductions in p16 Expression, and Changes in Histone Modifications in Gastric Cancer Cells Treated with DNMT Inhibitors. *Biomedicine & Pharmacotherapy*. 2026;198:119221. I conducted the data analysis and wrote the manuscript with guidance from Lakowski, T. M. Co-authors contributed to experimental work, data analysis, and manuscript revision.

Chapter 3 will be submitted for publication: Wenxia Luo, Amir Barzegar Behrooz, Hamid Latifi-Navid, Ivan Yurchenko, Mahsa Koopaei, Thordur Hendrickson-Rebizant, Olufola O. Ige, Ted M. Lakowski. Systematic Identification and Dynamic Validation of CDKN2A-Associated Genes in Gastric Adenocarcinoma Cell Lines under DNMT Inhibitor-Induced Expression Changes. I conducted the data analysis and wrote the manuscript. Co-authors contributed to experimental work, data analysis, and manuscript revision.

Table of Contents

Abstract.....	i
Acknowledgements	iii
Publications and Contributions	v
Table of Contents	vi
List of Tables	xi
List of Figures.....	xiii
List of Abbreviations.....	xv
Chapter 1: Introduction	1
1.1 DNMTs	2
1.1.1 The biological roles of DNMT.....	4
1.2 Hypermethylation biomarker in gastric cancer	7
1.3 Methylomics in identifying abnormal methylation genes for gastric cancer.....	9
1.4 DNMT inhibitors	11
1.4.1 Nucleoside DNMT inhibitors.....	11
1.4.2 Non-nucleoside DNMT inhibitors	13
1.5 Challenges in DNMT inhibitors development in gastric cancer	16
1.6 Hypotheses and objectives	17
Chapter 2: Dose Dependent Paradoxical Increases in DNA Methylation, Reductions in CDKN2A(p16) expression, and Changes in Histone Modifications in Gastric Cancer Cells Treated with DNMT Inhibitors	18
2.1 Introduction	18
2.2 Methods and Materials	21
2.2.1 Cell culture and DNMT inhibitor treatments	21

2.2.2 Cell viability analysis.....	21
2.2.3 qPCR gene expression analysis.....	22
2.2.4 Total DNA methylation assay.....	23
2.2.5 DNA methylation measure at CDKN2A(p16) gene promoter.....	23
2.2.6 Histone modification analysis.....	24
2.2.7 DNMT, TET, p16, p21, ELISA assay.....	24
2.2.8 Flow cytometry analysis	25
2.2.9 Statistical analysis	25
2.3 Results	26
2.3.1 In vitro effects of DNMT inhibitors on gastric cancer cell line growth	26
2.3.2 Total genomic DNA methylation and p16 expression changes in gastric cancer cells treated with DNMT inhibitors.....	28
2.3.3 Total DNA methylation aligned with p16 promoter methylation using decitabine and GSK3685032 in AGS and SNU719 cells	34
2.3.4 Decitabine and GSK3685032 increased DNMT3B and decreased TET in AGS cells and SNU719 cells.....	36
2.3.5 Decitabine and GSK3685032 induce cell cycle arrest in AGS and SNU719 cells....	38
2.3.6 Changes in total histone modifications in AGS and SNU719 cells treated with decitabine and GSK3685032	41
2.4 Discussion.....	43
2.5 Supplementary	53
2.5.1 Total DNA methylation assay validation.....	53
2.5.1.1 LCMS procedure	53

2.5.2 Validation results	56
2.5.3 DNA hydrolysis validation	62
2.5.4 The dynamic changes of total DNA methylation, p16 and Methylation-silenced genes expression.....	63
2.5.5 Decitabine and GSK3685032 increased DNMT3B and decreased TET in AGS cells and SNU719 cells.....	64
2.5.6 The protein level changes in p21 and p16 in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.	65
Chapter 3 : Genes Associated with DNMT Inhibitor-Induced Changes in CDKN2A/p16 expression in Gastric Adenocarcinoma	66
3.1 Introduction	66
3.2 Materials and Methods	68
3.2.1 Integrative Gene and Pathway Dataset Construction for Gastric Cancer Network Analysis.....	68
3.2.1.1 Genes associated with gastric cancer	70
3.2.1.2 Pathways associated with gastric cancer.....	71
3.2.1.3 Cell lines associated with gastric cancer	71
3.2.1.4 Network reconstruction and centrality analysis.....	72
3.2.1.5 CDKN2A-Cell line specific Hub Genes Network reconstruction.....	73
3.2.1.6 Validation of the relationship between the expression of identified genes and CDKN2A gene body/3' UTR CpG -methylation level	74
3.2.2 Systematic validation of the CDKN2A/p16-associated core network gene Set	77
3.2.2.1 Integrated validation of the CDKN2A/p16-associated core network gene set across multi-database platforms	78

3.2.2.2 <i>Validation of correlations between the CDKN2A/p16-associated core network gene set and CDKN2A promoter methylation.....</i>	79
3.2.2.3 <i>Validation of protein level of the CDKN2A/p16-associated core network gene set using the human protein atlas.....</i>	79
3.2.2.4 <i>Dynamic experimental validation using DNMT inhibitors in gastric adenocarcinoma cell lines.....</i>	80
3.2.3 Statistical Analysis.....	85
3.3 Results	87
3.3.1 Integrated validation of the CDKN2A/p16-associated core network gene set across multi-database platforms.....	87
3.3.2 Correlation analysis between CDKN2A/p16-associated core network gene set with CDKN2A/p16 Promoter Methylation.....	92
3.3.3 CDKN2A - related final key axes and enrichment analysis of key genes	93
3.3.4 Validation of CDKN2A/p16-associated core network gene set expression by qPCR and identification of CDKN2A-synchronized response genes under DNMT inhibitor treatment.....	96
3.4 Discussion.....	107
3.5 Supplementary.....	116
Chapter 4 :Conclusions and Future Directions.....	120
4.1 Conclusion.....	120
4.2 Future Directions	122
Reference	123
Appendices.....	151

Table 2A Human CDKN2A/p16 promoter pyrosequencing region and primers	139
Table 3A Genes and qPCR TaqMan gene expression assay for CDKN2A/p16-associated core network gene set (n = 20).....	139
Figure 3A. CDKN2A/p16-associated core network gene set (n = 20) mapped in TaqMan Array (96 well plate).	140

List of Tables

Table 2.1 IC50 values for GSK3685032 and RG108 in gastric cancer cells AGS, SNU216, SNU719, MKN45 and control cells HEK293.....	28
Table 2.2 Summary of changes in histone modifications in AGS and SNU719 cells treated with decitabine and GSK3685032 for 72 h.....	43
Table S2.1 Analytes abbreviation and MRM transitions.....	54
Table S2.3 Enzymatic Hydrolysis Recovery of DNA Modifications (n=3).....	61
Table S2.2-1 Typical calibration curve for analytes.....	55
Table S2.2-2 Precision and accuracy validation for mdC (n=3 with 6 replicates per batch)	56
Table S2.2-3 Precision and accuracy validation for hmdC (n=3 with 6 replicates per batch)	57
Table S2.2-4 Precision and accuracy validation for mdA (n=3 with 6 replicates per batch)	58
Table S2.2-5 Precision and accuracy validation for fdC (n=3 with 6 replicates per batch)	59
Table S2.2-6 Precision and accuracy validation for cadC (n=3 with 6 replicates per batch)	60
Table S2.3 Enzymatic Hydrolysis Recovery of DNA Modifications (n=3).....	61
Table 3.1 List of pathways to build a comprehensive gene database for gastric adenocarcinoma	70
Table 3. 2 Characteristics and rationale for the gastric adenocarcinoma cell lines used in this study.....	83
Table 3.3 Summary of 20 CDKN2A/p16-associated core network gene set across TNM-PLOT, KM-PLOT, OncoDB, cBioPortal methylation correlation analysis and Functional Consistency.....	90

Table 3.4 Summary of Statistically Significant Expression Changes in the CDKN2A/p16-Associated Core Network Gene Set Following DNMT Inhibitor Treatment	101
Table S3.1 The 24 genes identified using the Network-Correlation-Based Approach demonstrated significant correlations with CDKN2A Methylation via considering both the Spearman and Pearson correlation indices.	115
Table S3.2 The 15 genes identified using the Correlation-Based Approach demonstrated significant correlations with CDKN2A Methylation via considering both the Spearman and Pearson correlation indices	116
Table S3.4 The nine genes identified through the TNM-PLOT database exhibited significant correlations in paired tumor and adjacent normal tissues	116
Table S3.5 Eight genes identified using the OncoDB database demonstrated significant associations with various cancer stages, as determined by p-values from Analysis of Variance (ANOVA) tests.	117

List of Figures

Figure 2.1 Dose response curves for DNMT inhibitors in gastric cancer and control cell lines for 72h.....	27
Figure 2.2 The changes in total DNA methylation and p16 expression in gastric cancer and control cell lines treated with DNMT inhibitors for 72h.....	30
Figure 2.3 Correlation of total DNA methylation with CpG methylation-silenced genes in AGS and SNU719 cells treated with decitabine and GSK3685032 for 72h.....	33
Figure 2.4 CpG methylation in the p16 promoter (pyrosequencing) and p16 expression across selected DNMT-inhibitor doses.	36
Figure 2.5 Decitabine and GSK3685032 increased DNMT3B expression and/or decreased TET expression on the mRNA and protein levels in AGS and SNU719 cells after 72 h treatment.	37
Figure 2.6 Induction of cell cycle arrest and the changes in p21 and p16 in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.	40
Figure 2.7 Changes in total histone modifications in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.....	42
Figure S2.1 Pyrosequencing assays for quantifying methylation at CpG sites in the p16 promoter.	62
Figure S2.2 Decitabine and GSK3685032 increased DNMT3B expression and/or decreased TET expression on mRNA level in AGS cells and SNU719 cells after 72 h treatment.....	63
Figure S2.3 The changes in p21 and p16 in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.....	64

Figure 3.1 Integrated workflow for identifying and validating CDKN2A-associated genes in a gastric cancer cell lines.	69
Figure 3.2 Correlation between CDKN2A methylation and gene expression.	75
Figure 3.3 Identification of CDKN2A/p16-synchronized response genes in gastric cancer cell lines treated with DNMT inhibitors.	82
Figure 3.4 Integrated validation of the final 20 CDKN2A/p16-associated core network gene set across multi-database platforms.	88
Figure 3.5 Network reconstruction and pathway enrichment identifying CDKN2A-centered regulatory axes in gastric adenocarcinoma.	95
Figure 3.6 The changes CDKN2A/p16-associated core network gene set gene expression networks.	100
Figure 3.7 The relationship between CDKN2A and a set of 20 database-derived candidate genes.	103
Figure 3.8 The dose-response consistence with CDKN2A genes in gastric cancer cell lines. ...	106
Figure 3.9 Proposed working model of CDKN2A/p16–RB checkpoint control and its disruption in gastric adenocarcinoma.	110

List of Abbreviations

AML	Acute myeloid leukemia
CDK4	Cyclin-dependent kinase 4
CDK6	Cyclin-dependent kinase 6
CDKN2A	Cyclin-dependent kinase inhibitor 2A
CMML	Chronic myelomonocytic leukemia
circRNA	Circular RNA
CTD	Comparative Toxicogenomics Database
DNMT	DNA methyltransferase
EBV	Epstein–Barr virus
ES cells	Embryonic stem cells
EWAS	Epigenome-wide association study
GEO	Gene Expression Omnibus
GETdb	Genetic and Evolutionary features of drug Targets database
GWAS	Genome-wide association study
HDAC	Histone deacetylase
Helicobacter pylori	H.pylori
lncRNA	Long non-coding RNA
MBD	Methyl-CpG-binding domain
MDS	Myelodysplastic syndromes
miRNA	MicroRNA
OncoKB	Oncology Knowledge Base

PDE4D	Phosphodiesterase 4D
PPI	Protein-Protein interaction
SAH	S-adenosylhomocysteine
SAM	S-Adenosylmethionine
TCGA	The Cancer Genome Atlas
TET	Ten–eleven translocations
TRIB3	Tribbles pseudokinase 3
UALCAN	University of Alabama at Birmingham Cancer data analysis portal

Chapter 1: Introduction

Gastric cancer remains a significant global health challenge. In 2022, about 1 million people were diagnosed with gastric cancer and around 660,000 died from it, ranking it the fifth most common cancer in both incidence and deaths[1]. Despite declining incidence and mortality rates in many regions, East Asia, particularly China, Japan, and South Korea, continues to bear a disproportionate burden, contributing significantly to global gastric cancer cases and mortality[2,3]. The etiology of gastric cancer involves modifiable and non-modifiable risk factors, including *Helicobacter pylori* (*H.pylori*) infection, dietary habits, smoking, and genetic predispositions[4]. Recent advancements in early detection techniques and therapeutic strategies, including surgery, chemotherapy, and radiotherapy, have improved survival outcomes. Nevertheless, these benefits are unevenly distributed, and the prognosis remains poor for patients diagnosed at advanced stages. Compounding these challenges, gastric cancer often lacks distinctive early symptoms, resulting in late-stage diagnoses and consequently diminished survival rates. Notably, an alarming trend of rising gastric cancer incidence among populations younger than 45 years has emerged in countries such as the United States and Canada, underscoring the critical need for targeted prevention and intervention efforts[2,3]. Thus, enhancing early detection methods and developing precise interventions during curable stages of the disease are essential to improving patient prognosis and survival.

DNA methylation, which is catalyzed by DNA methyltransferases (DNMTs) is an epigenetic modification that helps to control gene expression. Aberrant DNA methylation, especially hypermethylation, plays a critical role in many cancers, including gastric cancer. DNA methylation is tissue-specific, occurs early in cancer development, and exhibits relative stability, making it especially suitable as a biomarker for early cancer detection[5–7]. Despite this, few promising

biomarkers have transitioned successfully into clinical use, primarily due to challenges in biomarker validation, inter-patient variability, and the stringent requirement for high sensitivity and specificity in detecting early-stage diseases.

DNMT inhibitors have emerged as promising therapeutic candidates, particularly in combination with chemotherapy for gastric cancer treatment[8,9]. DNMT inhibitors can suppress tumor growth by reactivating the hypermethylated tumor suppressor genes[10]. Notably, nucleoside analog DNMT inhibitors, such as azacitidine and decitabine, have achieved clinical success in hematological malignancies like acute myeloid leukemia (AML) and chronic myelomonocytic leukemia (CMML), significantly advancing the field of DNMT-targeted therapeutics. Despite these advances, nucleoside analog inhibitors have notable limitations, including molecular instability, rapid metabolism, low bioavailability, and significant toxicity. Consequently, research has focused on developing new non-nucleoside DNMT inhibitors, can have enhanced specificity and reduced toxicity[11–13]. Although many non-nucleoside DNMT inhibitors lack sufficient selectivity and potency, recent medicinal chemistry and computational advances have provided valuable insights, laying a foundation for the next generation of highly selective inhibitors.

In chapter 1, we comprehensively review the mechanisms and biological functions of DNMT and. We summarize recent advances in the identification of aberrantly methylated genes as diagnostic and prognostic biomarkers in gastric cancer, with particular emphasis on the impact of database-driven methylomics on biomarker discovery. Finally, we discuss the development of DNMT inhibitors and the challenges encountered during their development.

1.1 DNMTs

DNA methylation is a covalent modification of cytosine, where a methyl group is added to the 5th carbon of the pyrimidine ring, forming 5-methylcytosine[14]. In mammals, DNA methylation

predominantly occurs at CpG sites and is catalyzed by DNMT1, DNMT3A, and DNMT3B. DNMT2 and DNMT3L are non-canonical DNMT that lack DNA methylation activity[15]. DNMT1 binds hemi-methylated DNA. It methylates newly synthesized strands of DNA during DNA replication using the parent methylated DNA strand as a template for adding methylation marks in the same CpG sites on the new strand a process called DNA methylation maintenance[14]. Although DNMT1 can also establishing new DNA methylation marks known as *de novo* methylation[16], this process is primarily carried out by DNMT3A and DNMT3B [16,17] and can be facilitated by DNMT3L, a regulatory factor that enhances the catalytic efficiency of DNMT3A and DNMT3B[18].

All DNMT utilize S-Adenosyl methionine (SAM) as a co-substrate methyl source for methyl-transfer reactions. The other substrate is unmethylated or hemi-methylated DNA which combines with the appropriate DNMT to form a complex. The target cytosine flips out and rotates into the catalytic pocket of this enzyme. DNMTs catalyze cytosine methylation via a conserved cysteine in motif IV, which attacks the C6 position of cytosine, aided by a glutamic acid in motif VI. A methyl group from SAM as the methyl donor is then transferred to C5, followed by deprotonation to form 5-methylcytosine. This involves covalent enzyme–DNA intermediate formation and subsequent methyl transfer (**Figure 1.1A**).

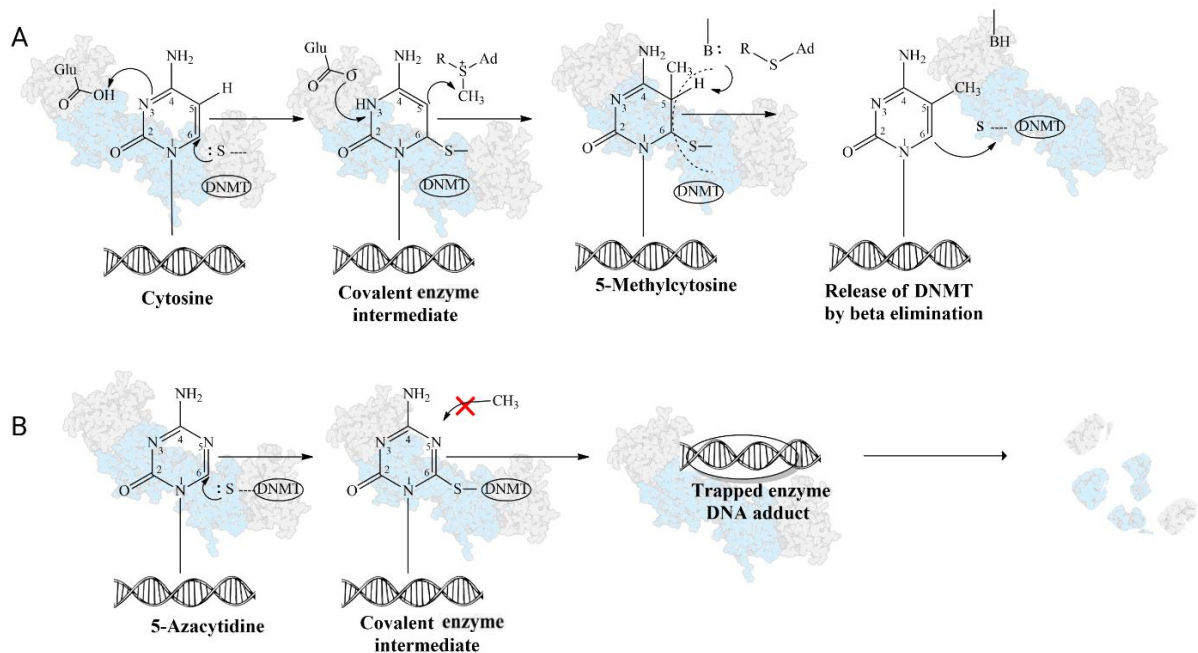


Figure 1.1 Catalytic mechanism of DNMT and the mechanism of nucleoside DNMT inhibitor.

(A) Catalytic mechanism of DNMT; A basic residue, labeled as B in the figure—typically a histidine or lysine—abstracts a proton from the C5 position, enabling cleavage of the covalent bond and release of the enzyme (B) nucleoside DNMT inhibitor mechanisms; Nucleoside analogs such as azacytidine and decitabine mimic cytosine and become incorporated into DNA during replication.

1.1.1 The biological roles of DNMT

The methylation of CpG islands in gene promoters, is an epigenetic mechanism for transcriptional repression by two principal mechanisms[19,20]. First, CpG islands methylation can directly interfere with transcription factor binding by sterically blocking their access to DNA. Second, methylated CpGs can recruit methyl-CpG-binding domain (MBD) proteins, such as MBD2 and MeCP2, which in turn associate with histone deacetylase (HDAC) complexes. The removal of acetyl groups from histones leads to the formation of compact heterochromatin, making the DNA inaccessible to the transcriptional machinery and effectively silencing the gene.

Conversely, methylation in gene body regions is frequently associated with active transcription, although its specific role varies depending on the genomic context[21,22]. In addition to its presence in actively transcribed genes, gene body methylation also helps prevent spurious

transcription by suppressing inappropriate transcription initiation[23]. In repetitive regions, such as retrotransposons, methylation is essential for genomic stability by suppressing their transcription and mobility, thereby preventing genomic instability[14,24].

Through these distinct methylation mechanisms, DNMTs precisely regulate gene expression, thus influencing diverse developmental and cellular processes. Genetic studies using DNMT knockout models across multiple species underscore the critical importance of DNMTs in development, epigenetic inheritance, and cellular function (**Table 1.1**).

Table 1.1 The Roles of DNMTs in Knockout Models Across Different Species

gene	Species	Study subject	Effects of DNMT Deletion or Knockout	Reference
DNMT1	mice	ES cells (Embryonic stem cells)	1) Total DNA methylation reduction; 2) Embryonic mid-gestation lethality; 3) Developmental arrest; 4) Imprinted X-chromosome inactivation defects; 5) Dramatically increased intracisternal A-particle transcription; 6) Loss the capability of repression of long terminal repeats (LTRs).	[25–28]
	mice	Forebrain	1) Anxiolytic and antidepressant-like responses	[29]
	mice	Brain	1) Abnormal long-term hippocampal plasticity; 2) Deficits in learning and memory; 3) Significant decrease in neuronal DNA methylation.	[30]
	mice	smooth muscle cells (SMC)	1) DNA hypomethylation in Embryonic SMC; 2) Massive dysregulation of gene expression; 3) Lethality in gastrointestinal smooth muscle.	[31]
	rats	cardiac-specific DNMT1 knockout rat	1) Restricted reprogramming of gene expression; 2) Total hypomethylation.	[32]
	human	ES cells	1) Rapid reduction in total DNA methylation; 2) Immediate lethality upon DNMT1 loss.	[33]
	human	HCT116 colorectal cancer cell lines	1) Moderate activation of CG-X genes; 2) Moderate hypomethylation.	[34]
DNMT3A	mice	Maternal and zygotic cell	1) Loss of paternal and maternal methylation imprints; 2) Embryonic mid-gestation lethality	[35]
	mice	ES cells	1) Failure in establishing maternal and paternal methylation imprints; 2) Spermatogenesis defects; 3) Gut malfunction.	[17,36]
	human	ES cells	1) Mild reduction in total DNA methylation; 2) Rapid reduction in non-CpG methylation.	[33]
	human	hiPSC-derived cardiomyocytes	1) Mitochondrial damage; 2) Disruption of lipid and glucose metabolism.	[37]
DNMT3B	mice	ES cells	1) Minor satellite DNA hypomethylation; 2) Neural tube defects; 3) Embryonic lethality.	[17]
	human	ES cells	1) Mild reduction in total DNA methylation; 2) Rapid reduction in non-CpG methylation.	[33]
DNMT3B	human	HCT116 colorectal cancer cell lines	1) Strongly induction of gCG-X genes such as MAGE-A1, NY-ESO-1, and XAGE-1.	[34]
DNMT3A, DNMT3B	mice	ES cells	1) Failure in initiating de novo methylation after implantation; 2) Developmental arrest; 3) Dramatic reduction in total DNA methylation.	[17,28]
	human	ES cells	1) Significant reduction in total DNA methylation.	[33]
DNMT1, DNMT3A, DNMT3B	mice	zygotic cell and Embryos	1) Gastrulation arrest; 2) Downregulation of gastrulation pathways in DNMT-null embryos.	[38]

1.2 Hypermethylation biomarker in gastric cancer

In gastric cancer, promoter CpG hypermethylation of tumor suppressor genes silences their expression facilitating tumor progression. Environmental factors, including aging, dietary patterns, smoking, alcohol consumption, chronic inflammation, and infection with pathogens such as *H. pylori* and Epstein-Barr virus (EBV), significantly influence DNA hypermethylation in gastric cancer[39].

H. pylori, one of the most prevalent infectious pathogens, increases the risk of gastric cancer. Hypermethylation of genes including *BARHL2*, *HUS1*, *GSTO2*, *TMEM190*, and *miR-129-2* has been identified as potential biomarkers for *H. pylori*-associated gastric cancer risk and prognosis[40,41]. EBV-associated gastric cancer is characterized by a significantly higher frequency of CpG island methylation. While EBV-associated gastric cancer accounts for an average of only 10% of all gastric cancers globally, more than 95% of adults are infected with EBV, which persists in a lifelong latent state[42]. In 2014, 216 genes were identified as downregulated by EBV-related CpG hypermethylation [43]. The Cancer Genome Atlas (TCGA) classified gastric cancer into four molecular subtypes: EBV-positive, microsatellite instability, genomic stability, and chromosomal instability[44]. EBV-positive gastric cancers are distinct from the other three subtypes in molecular, genetic, and epigenetic features[45]. Analyses of ~300 gastric tumors revealed that EBV-positive tumors exhibit a CpG island methylator phenotype, leading to hypermethylation of ~19% of promoter CpG islands[46]. The EBV-encoded Latent Membrane Protein 1 recruits DNMT1 to *RASSF10* leading to methylation and the suppression of its expression[47]. The scinderin gene (*SCIN*) that inhibits tumorigenesis is characterized by low expression and high methylation comparing EBV-positive to -negative gastric cancer[48]. EBV

also increases *miR-124* promoter methylation and enhances *EZH2* gene expression, further promoting gastric cancer progression[49].

Over 50 hypermethylated genes have been recognized as diagnostic biomarkers, and more than 10 hypermethylated genes have been proposed as potential prognostic biomarkers for gastric cancer (**Figure 1.2**). Over 30 studies have identified *p16* promoter hypermethylation as an epigenetic biomarker for diagnosis, prognosis, and therapeutic targeting in gastric cancer[50,51]. Notably, reducing *p16* hypermethylation restores its expression and inhibits gastric tumor growth[52]. *p16* regulates the cell cycle by inhibiting cyclin-dependent kinases 4 (CDK4) and 6 (CDK6) which prevents phosphorylation of the retinoblastoma protein, thereby slowing the progression from the G1 to the S phase and preventing excessive cell proliferation[53,54]. Decreased *p16* expression, mediated by promoter hypermethylation, is strongly associated with the development of primary gastric cancer[55–57].

In 2024 more than 10 new hypermethylated genes were reported as potential biomarkers for early detection, diagnosis, prognosis, and potential treatment targets in gastric cancer. Among these, a study revealed *PRKCB* hypermethylation inversely correlated with its reduced mRNA expression by using the public database: GWAS (Genome Wide Association Studies) catalog, as verified by TCGA and GEO databases[58].

DNA hypomethylation can also suppress the expression of long non-coding RNAs (lncRNAs), circular RNAs (circRNAs), and microRNAs (miRNAs). For example, the expression of *Linc00472*, *H19*, and *miRNA-770* significantly decrease in gastric cancer due to CpG hypermethylation[59,60].

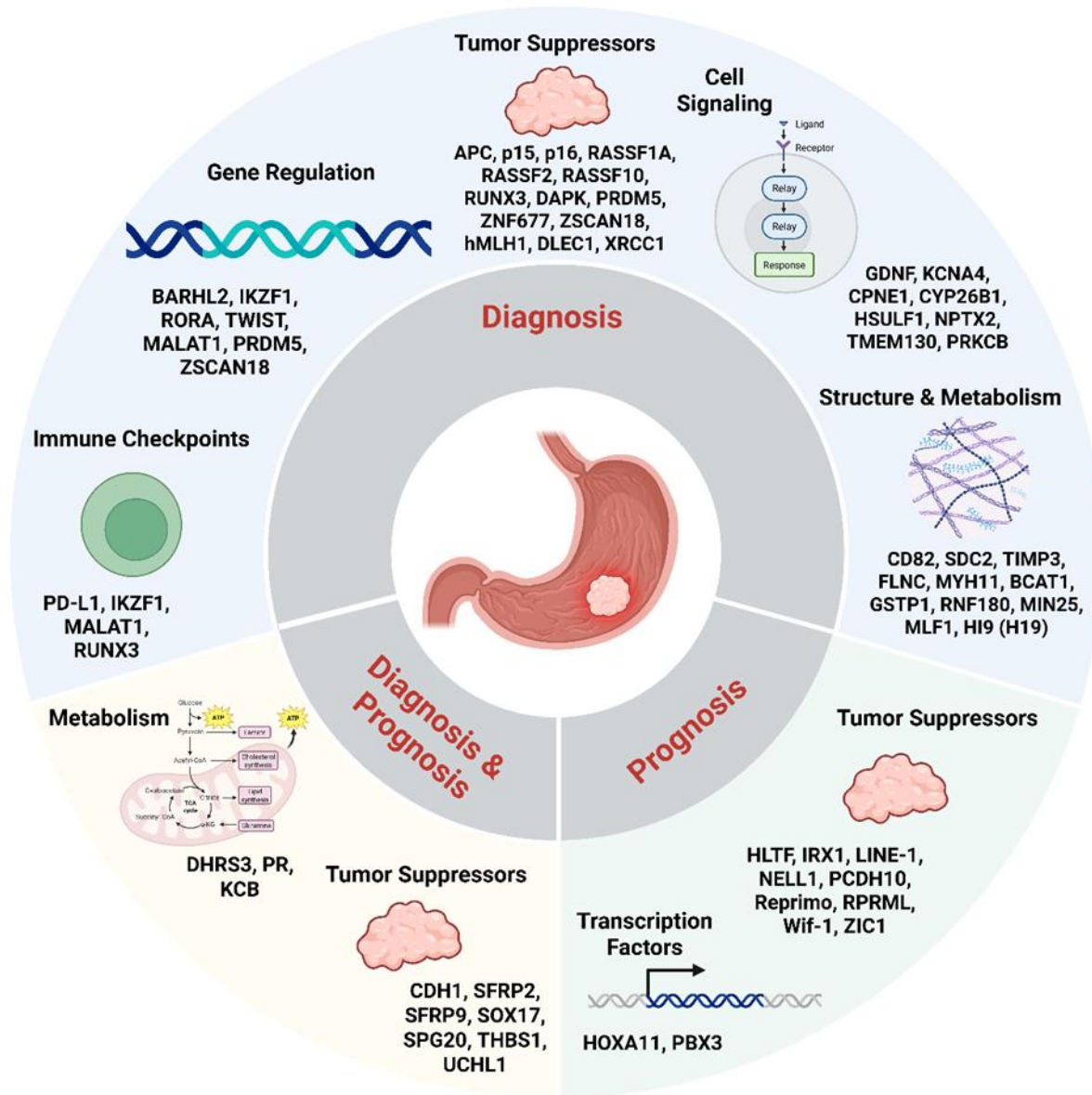


Figure 1.2 The genes hypermethylated in gastric cancer that can be potential biomarkers for diagnosis and prognosis of gastric cancer[51,58,61–117]

1.3 Methyloomics in identifying abnormal methylation genes for gastric cancer

Methyloomics is the comprehensive study of DNA methylation across the genome, aiming to understand how methylation patterns influence biological processes, including cancer development. By analyzing genome-wide methylation changes. For instance, the DNA methylation landscape of a gastric cancer patient exhibited a characteristic bimodal distribution

pattern[58]. Moreover, methylomics studies have identified specific CpG sites as potential biomarkers, moving beyond the traditional focus on hypermethylated genes. For example, cg27284428, cg11798358, cg07880787, and cg00585116 were recognized as biomarker set for gastric cancer, demonstrating the unique advantages of this approach in broadening the scope of biomarker research[118]. Methylomics, leveraging whole-genome methylation sequencing and database analyses (e.g., TCGA, GEO, UALCAN)[44,119,120], has enhanced the efficiency and breadth of biomarker discovery. Incorporating machine learning techniques into methylomics analysis further strengthens its ability to identify specific biomarkers[121]. Incorporating machine learning into methylomics analysis enhances its ability to identify specific biomarkers. For instance, a study on stomach cancer used machine learning to analyze TCGA DNA methylation data, identifying 8 diagnostic probes. This approach demonstrates how machine learning can handle large datasets, select key features, and improve the accuracy of cancer biomarker detection, offering more reliable tools for clinical diagnosis and prognosis[121].

DNA methylation public databases are characterized by continuous data changes and updates so they can become outdated or inaccessible if they are not regularly maintained and updated (**Table 1.2**). Moreover, inconsistent data annotations in public databases, variations in sample processing, and differences in analysis standards across labs uploading data result in a lack of reproducibility[122–124]. Machine learning models, while powerful, rely heavily on accurate high-quality, datasets, and these issues can affect lead to incorrect conclusions[121].

Table 1. 2 The Updated DNA Methylation-Related Databases

Database	Description	Link
GEO (Gene Expression Omnibus)	Public repository for genomics data, including DNA methylation data	https://www.ncbi.nlm.nih.gov/geo/
TCGA (The Cancer Genome Atlas)	Large-scale project providing comprehensive cancer genomics data, including DNA methylation, gene expression, and clinical data.	https://portal.gdc.cancer.gov/
EWAS (Epigenome-wide association studies) Open Platform	Centralized platform for epigenome-wide association studies (EWAS) linking DNA methylation to diseases.	https://ngdc.cncb.ac.cn/ewas
MethBank	Provides comprehensive genome-wide DNA methylation maps across different species and tissues.	https://ngdc.cncb.ac.cn/methbank/
MethMotif	An integrative cell-specific database of transcription factor binding motifs coupled with DNA methylation profiles	https://methmotif.org/
NGSmethDB	a database of methylation data derived from next-generation sequencing data.	https://bioinfo2.ugr.es/NGSmethDB
UALCAN (The University of ALabama at Birmingham CANcer data analysis Portal)	An interactive platform for analyzing cancer omics data, including DNA methylation and gene expression from TCGA.	http://ualcan.path.uab.edu/

1.4 DNMT inhibitors

The role of DNA methylation in cancer has inspired the development of DNA methylation inhibitors as promising novel anticancer agents. Through the understanding DNMT structure and mechanism, a series of DNMT inhibitors have been developed for cancer treatment, which are grouped into two classes the nucleoside analog and non-nucleoside inhibitors.

1.4.1 Nucleoside DNMT inhibitors

The first nucleoside DNMT inhibitor, 5-azacytidine (azacytidine) (discovered in the 70s) is incorporated into RNA and DNA, disrupting normal cellular processes[125,126]. The 2-deoxy analog, 5-aza-2'-deoxycytidine (decitabine) (**Figure 1.3**), was later synthesized to target DNA, resulting in improved DNMT inhibition and reduced off-target effects[127]. Azacytidine and decitabine are triphosphorylated and incorporated into DNA where DNMT bind to them in the

cytidine substrate binding site ultimately covalently trapping the enzyme on DNA and leading to inactivation of DNMT1, DNMT3A and DNMT3B (**Figure 1.1 B**). Over successive replication cycles this leads to passive demethylation of DNA[126]. Azacytidine (Vidaza®) and decitabine (Dacogen™) received FDA approval in 2004 and 2006, respectively, for the treatment of AML and CMML[128,129]. However, their molecular structures are unstable and prone to rapid metabolism by cytidine deaminase, leading to low bioavailability and half-life[130,131]. Their non-selective incorporation into potentially any sequence with a cytosine base and non-selective DNMT inhibition results in toxicity[132]. A series of analogs and prodrugs have been designed to improve stability, enhance efficacy, and achieve better selectivity[133].

Zebularine is more stable decitabine and exhibits minimal cytotoxicity [134]. It selectively inhibits DNMT1 in human cancer cells[135] its clinical application is limited by its rapidly metabolism[136]. 5-fluoro-2'-deoxycytidine, is currently in phase I clinical trials, but is similarly rapidly metabolized[137].

SGI-110, a prodrug of decitabine, offers improved pharmacokinetics and metabolic stability compared to its parent compound due to a phosphodiester bond. SGI-110 effectively inhibits cell growth in hepatocellular carcinoma (HCC) cell lines and delays tumor progression in mouse xenotransplantation models[138]. Currently, SGI-110 is undergoing Phase II clinical trials for the treatment of myelodysplastic syndromes (MDS) and AML[139].

The oral formulations for azacitidine and decitabine has greatly improved patient compliance. Oral azacitidine (CC-486) was approved for AML patients[140]. Oral decitabine ASTX727, combines decitabine with the cytidine deaminase inhibitor cedazuridine, preventing its rapid breakdown and has been approved for the treatment of MDS and AML in multiple countries[141,142].

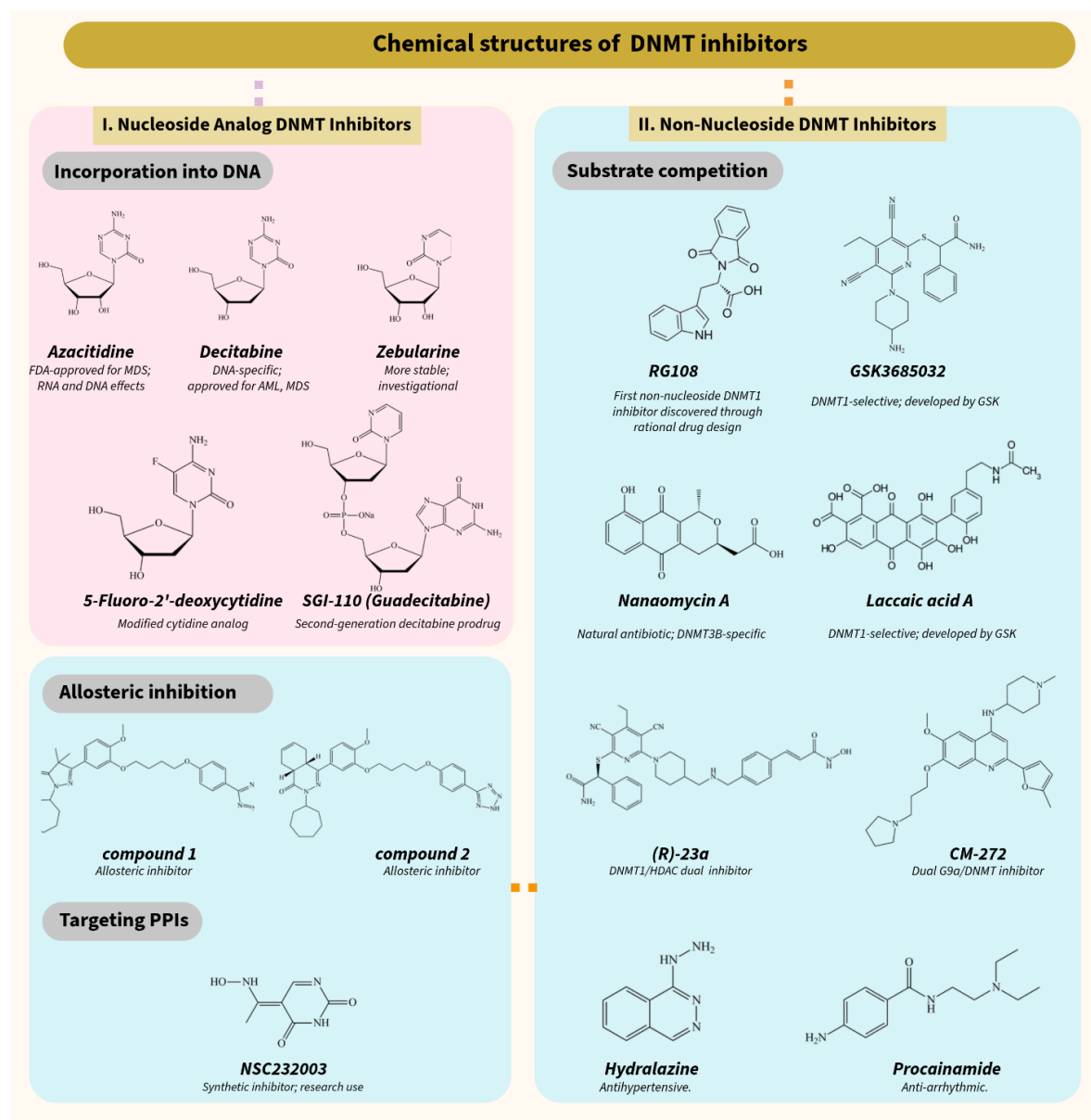


Figure 1. 3 Chemical structures of DNMT inhibitors classified by the inhibition mechanism.

1.4.2 Non-nucleoside DNMT inhibitors

At the same time, extensive efforts have been devoted to identifying non-nucleoside DNMT inhibitors, motivated by the multiple mechanisms through which DNMT activity can be inhibited, including: (1) incorporation into DNA and irreversible enzyme alkylation as described with decitabine and azacytidine (2) competition for the cytidine-DNA or S-adenosylmethionine (SAM)/

S-adenosylhomocysteine (SAH) binding sites (3) targeting protein-protein interactions (**Figure 4**) and (4) allosteric inhibition [9].

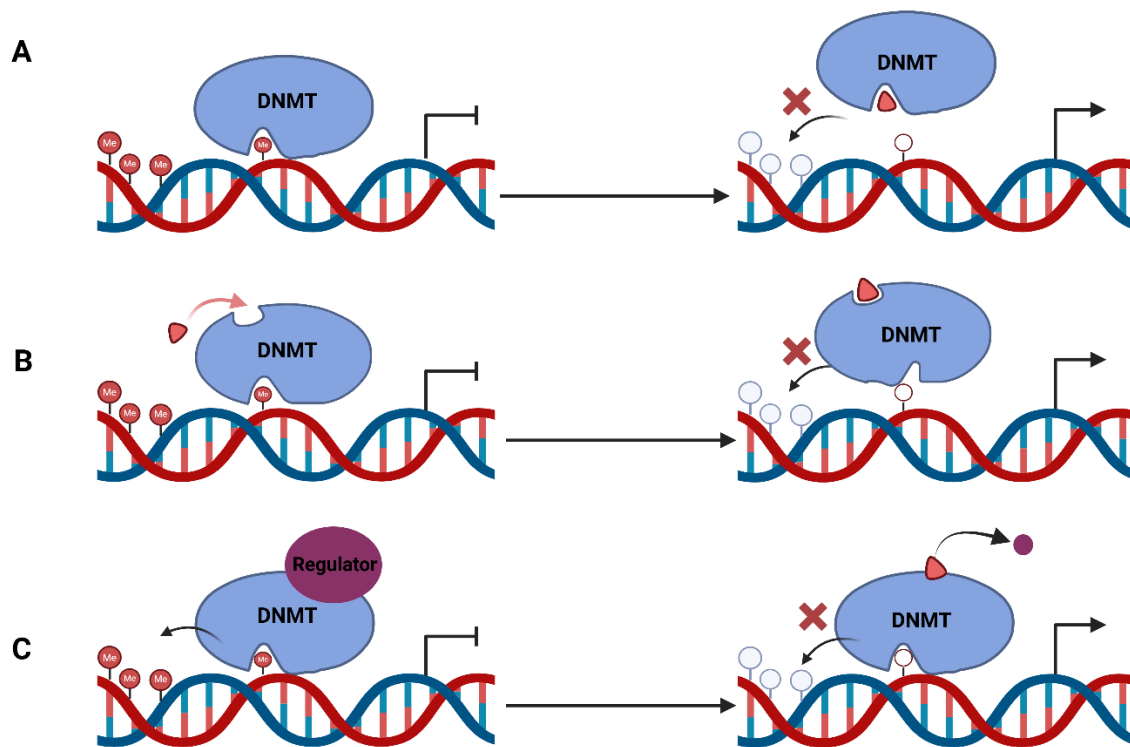


Figure 1. 4 The mechanism of non-nucleoside inhibitors.

(A) Substrate competition: Inhibitors block DNMT activity by competing with natural substrates such as DNA, S-adenosylmethionine (SAM), or the catalytic product S-adenosyl-homocysteine (SAH), thereby preventing methyl group transfer. (B) Allosteric inhibition: Small molecules bind to allosteric sites on DNMTs, inducing conformational changes that disrupt catalytic activity and DNA binding without occupying the active site. (C) Targeting protein–protein interactions (PPIs): Inhibitors or regulatory proteins interfere with DNMT interactions with essential cofactors or regulatory partners, destabilizing the complex and impairing DNMT function. The red triangle represents non-nucleoside DNMT inhibitors.

The selective DNMT1 inhibitor, RG108 was discovered by using virtual screening based on a comparative model of hDNMT1[143]. This knowledge helped in the design of inhibitors that target specific DNMT isoforms. GSK3685032 is a highly selective, reversible dicyanopyridine inhibitor of DNMT1. It binds to the active site of DNMT1 intercalating into hemi-methylated DNA at CpG dinucleotide gaps, preventing the DNMT1 active-site loop from inserting into the DNA minor groove blocking methyl-transfer. GSK3685032 demonstrates high selectivity for DNMT1 over DNMT3A and DNMT3B, with significantly reduced toxicity compared to decitabine.

Along with synthetic compounds, several natural products, including alkaloids, carotenoids, flavonoids, lignans, phenolic acids, polysaccharides, quinones, saponins, steroids, terpenoids, chalcones, and fatty acids, have been identified as DNMT inhibitors[144]. Examples include nanaomycin A [145], and laccaic acid A [146]. However, these compounds do not directly target DNMT in cells, and their inhibitory activity is generally weak and non-selective [147]

Dual-targeting DNMT and another epigenetic target can overcome resistance mechanisms, while being more effective at suppressing tumor growth. Current dual target combinations include HDAC/DNMT and G9a/DNMT inhibitors. (R)-23a, a first-in-class dual DNMT1/HDAC inhibitor, that has anti-tumor activity by reactivating silenced tumor suppressor genes It reshapes the tumor immune microenvironment, achieving a tumor growth inhibition of 98% in leukemia models with enhanced safety profiles compared to single-agent treatments[148]. The natural flavonoid dihydromyricetin was identified as a DNMT1 inhibitor that induces total DNA hypomethylation and shows potential for anti-aging applications[149]. CM-272 is a dual G9a/DNMT inhibitors that is being developed for cancers that overexpress both enzymes to drive gene silencing. induce immunogenic cell death and sensitize tumors to immunotherapy and chemotherapy.

Procainamide is an anti-arrhythmic, that inhibits DNMT[150,151]. It is believed that procainamide interferes with the DNA binding of DNMTs[151]. Hydralazine, an antihypertensive competitively binds to the DNMT cytidine substrate binding site and is being investigated as a DNMT inhibitor[150].

Two compounds that are structurally related pyrazolone (compound 1) and pyridazine (compound 2) are allosteric DNMT3A inhibitors [152]. This novel mechanism inhibits DNMT3A activity without competing for the substrate cytosine DNA or SAM/SAH binding sites. Compound 2 shows significant therapeutic potential in AML by inducing myeloid differentiation, promoting apoptosis, and reducing total DNA methylation levels. The compound NSC232003 is incorporated into DNA and covalently binds to and irreversibly inhibits DNMT3A. Moreover, NSC232003 also binds ubiquitin-like with PHD and RING Finger Domains 1 (UHRF1) thereby reducing DNMT1/UHRF1 interactions thereby also targeting PPI[153].

1.5 Challenges in DNMT inhibitors development in gastric cancer

In recent years, significant progress has been made in discovering DNA methylation biomarkers in gastric cancer and DNMT inhibitors, providing a solid theoretical foundation and promising clinical applications for cancer research. Despite these advancements, several challenges remain. For DNMT inhibitors, although many candidates are currently in clinical trials, only two nucleoside analogs (azacitidine and decitabine) have been approved by the FDA for blood-borne cancers. No non-nucleoside inhibitors have been approved for any clinical application. This highlights the urgent need to develop more effective and less toxic DNMT inhibitors suitable for gastric cancer therapy. A deeper understanding of DNMT inhibitor mechanisms and effects in gastric cancer is therefore essential to guide the design of improved therapeutic strategies.

1.6 Hypotheses and objectives

We hypothesize that changes in the concentration of DNMT inhibitors will result in corresponding alterations in total genomic DNA methylation, p16 promoter methylation, and p16 expression in gastric cancer cells, with these changes varying across different gastric cancer cell lines. We focus on the dose-dependent response of (p16) expression to DNMT inhibitors and have designed an approach that utilizes this dynamic CDKN2A expression pattern. This approach aims to discover CDKN2A-associated network in gastric cancer and to identify potential genes that can be inhibited along with DNMT (co-targeting genes).

This thesis consists of two main objectives: the first, addressed in Chapter 2, is to investigate the effects of three DNMT inhibitors, decitabine, RG108, and GSK3685032, on various gastric cancer cell lines, specifically examining changes in genomic DNA methylation, p16 promoter methylation, p16 expression, and cell cycle alterations. The second objective, discussed in Chapter 3, is to integrate multi-database network reconstruction with dynamic experimental validation, aiming to reconstruct p16-anchored gene networks using data from TCGA and protein-protein interaction databases, identify hub genes through network analysis, and validate co-targeting candidates via quantitative PCR in gastric cancer cell lines across dose-response experiments.

Chapter 2: Dose dependent paradoxical increases in DNA methylation, reductions in p16 expression, and changes in histone modifications in gastric cancer cells treated with DNMT inhibitors

2.1 Introduction

Methylation of DNA, especially five-methylcytosine (mdC) is often found in CpG sequences in gene promoters where it is associated with reduced or silenced gene expression, and is involved in embryonic development, and stem cell fate [154]. Abnormal DNA methylation plays a role in various cancers, including gastric cancer, where it can silence tumor suppressor genes via promoter CpG hypermethylation or trigger chromosomal instability through genomic DNA hypomethylation, ultimately promoting tumorigenesis[155–157]. The regulation of DNA methylation is a dynamic process involving DNA methyltransferases (DNMTs), which add methylation marks, ten-eleven translocation (TET) enzymes that remove methylation marks[158], and passive demethylation. Given the involvement of DNA methylation in cancer, DNMTs are promising targets for the development of cancer therapeutics[9,159]. A variety of DNMT inhibitors, including nucleoside and non-nucleoside inhibitors, have been developed. Despite significant differences in structure and mechanism, DNMT inhibitors are thought to exert their anti-cancer activities by preventing CpG promoter methylation on tumor suppressors that would otherwise suppress their expression, thereby restoring the expression of the tumor suppressors. This reactivation of tumor suppressors can lead to cell cycle arrest, differentiation, and/or cell death. The cytidine derivative nucleoside DNMT inhibitor, 5-aza-2'-deoxycytidine (decitabine), is currently approved in the USA for treating blood-borne cancers [9]. It is phosphorylated before being incorporated into DNA where the modified decitabine bases within DNA bind to DNMT1, DNMT3A or DNMT3B and irreversibly trap the enzyme in a covalent decitabine-DNA-DNMT

complex, leading to irreversible inhibition of the DNMT. Non-nucleoside DNMT inhibitors have no obvious structural similarities to nucleoside inhibitors and can inhibit DNMT through various mechanisms that are typically reversible, and they are not incorporated into DNA. RG108 is a non-nucleoside DNMT inhibitor that is not currently under clinical investigation. GSK3685032, first reported in 2021, is a dicyanopyridine that can directly interact with the active site loop of DNMT1 while intercalating into the minor groove of DNA. Consequently, it is a selective DNMT1 inhibitor, and it showed improved pharmacological properties and reduced toxicity compared with decitabine[160]. GSK3685032 and related dicyanopyridine-based non-nucleoside DNMT1 inhibitors are being investigated as novel treatments for acute myeloid leukemia (AML) and other disorders having epigenetic etiologies[161–165].

In 2022, approximately 1 million new cases of gastric cancer were diagnosed, with approximately 660,000 deaths attributed to the disease, ranking it fifth among cancers with respect to incidence and mortality[1]. Several studies have reported that promoter hypermethylation of the tumor suppressor cyclin-dependent kinase inhibitor 2A (*CDKN2A*) (*p16*) and its decreased expression are linked to some gastric cancers[55,166–168]. In fact, *p16* promoter methylation is considered a biomarker for certain gastric cancers, with potential applications in diagnosis as well as being a target for treatment[169–172]. DNMT inhibitors have the potential to restore the expression of tumor suppressor genes like *p16*. Decitabine has been approved for hematological malignancies [173,174]. However, it can also reduce *p16* promoter CpG DNA methylation, upregulating *p16* expression, reducing gastric cancer cell viability [52,168]. Despite this, its efficacy in solid tumors, including gastric cancer, has not yet been clearly demonstrated. GSK3685032 increases *p16* expression in lung cancer and AML [160,165]. However, due to its recent discovery, studies on its effects on *p16* expression in gastric cancer are lacking. Current data suggest that DNMT inhibitors

may be useful in gastric cancer. However, given that DNMT inhibitors may cause genome-wide DNA hypomethylation, it's conceivable that they can alter the expression of many genes, which may complicate their use for gastric cancer. Therefore, examining how DNMT inhibitors affect DNA methylation, p16 and associated gene expression, and downstream regulatory responses is essential for defining their therapeutic relevance in gastric cancer.

Here, we investigated the effects of three DNMT inhibitors, decitabine, RG108, and GSK3685032, on various gastric cancer cell lines. We evaluated alterations in total DNA methylation, *p16* promoter methylation, associated gene expression, cell cycle arrest, and histone modifications in gastric cancer cells treated with these DNMT inhibitors. We showed that at low concentrations, DNMT inhibitors decrease total and p16 promoter CpG DNA methylation to a minimum and increase *p16* mRNA and protein. However, at higher concentrations, there was a paradoxical increase in total and p16 promoter CpG DNA methylation from the minimum experienced at low doses and this was accompanied by a concomitant decrease in *p16* mRNA and protein, sometimes back to pre-treatment levels in the gastric cancer cell lines AGS and SNU719. For this manuscript, the term “paradoxical increase” refers to increased DNA methylation relative to the lowest methylation level induced by the DNMT inhibitors we tested, reflecting reduced efficacy of decreasing DNA methylation at higher DNMT inhibitor doses rather than a net increase in DNA methylation above that measured in the absence of DNMT inhibitors. This paradox of increasing then decreasing expression with increasing dose of DNMT inhibitors was also observed with other genes where their expression is controlled by promoter CpG methylation, showing that DNMT inhibitors work throughout the genome. This paradoxical increase in DNA methylation with high doses of DNMT inhibitors was attributed to increases in *DNMT3B* and in some cases, *TET1* and *TET2* mRNA and protein. Low doses of GSK3685032 and decitabine induced increases in

expression of cell cycle regulators *p21* and *p16*, but to different extents. GSK3685032 increased *p16* expression more than decitabine but decitabine increased *p21* expression more than GSK3685032. As a result, GSK3685032 induces G0/G1 cell cycle arrest, whereas decitabine induces G2/M arrest in gastric cancer cells. Decitabine and GSK3685032 also reduced histone lysine acetylation and monomethyl-lysine in AGS cells. However, decitabine decreased dimethyl-lysine, trimethyl-lysine, and asymmetric dimethyl-arginine. In SNU719 cells, decitabine reduced lysine acetylation and monomethyl-lysine, whereas GSK3685032 had minimal effects on histone modifications. If our results in gastric cancer cells can be extrapolated to the treatment of gastric cancer, they suggest that low doses of decitabine and GSK3685032 may increase *p16* expression, making them potentially effective for treatment, whereas higher doses may decrease *p16* expression, making them potentially less effective. Together, these results highlight the dose-dependent nature of DNMT inhibitor activity, and we emphasize the need to optimize dose to effectively reactivate *p16* expression in gastric cancers caused by low *p16* expression.

2.2 Methods and Materials

2.2.1 Cell culture and DNMT inhibitor treatments

AGS (ATCC) and HEK293 (ATCC), SNU-719 and SNU-216 (Korean Cell Line Bank), and MKN45 (RIKEN BRC) cells were used in this study. The gastric cancer cell lines AGS, SNU-216, and MKN45 were derived from female patients, whereas SNU-719 was derived from a male patient. HEK293 is a human embryonic kidney-derived cell line of female origin and was used as a non-gastric control. SNU-719 is Epstein–Barr virus-positive, while MKN45 is derived from poorly differentiated gastric adenocarcinoma. All cell lines were used within 20 passages after thawing. Although established cell lines may not fully represent the heterogeneity and tumor microenvironment of primary gastric tumors, the selected cell lines exhibit distinct *p16* statuses,

allowing comparison of responses across different p16 backgrounds. AGS and HEK293 cells were cultured in F-12K medium (Gibco BRL, USA) and EMEM medium (ATCC, USA), respectively, with 10% fetal bovine serum (FBS, Corning, USA). SNU719, SNU216, and MKN45 were grown in RPMI1640 (Gibco BRL, USA) and 10% FBS. All cell lines were incubated at 37°C in 5% CO₂. Cells were synchronized by 24-hour serum starvation[175], then treated with a series of concentrations of decitabine, GSK3685032 or RG108 for 72 h. Medium-containing decitabine was changed every 24 hours due to its instability in media[176].

2.2.2 Cell viability analysis

Cell lines were seeded at 1×10⁴ cells/well in a 96-well format and treated with decitabine, GSK3685032 or RG108 for 72 h. Cell viability (expressed as a percentage of the control group) was measured using the CellTiter 96® MTS assay (Promega, UK) according to the manufacturer's instructions and a Synergy H1 microplate reader (BioTek Co., USA) at 490nm.

2.2.3 qPCR gene expression analysis

Total mRNA was isolated from cells using the PureLink RNA isolation kit (Life Technologies, USA), cDNA was synthesized using SuperScript VILO Master mix (Life Technologies, USA), and quantitative PCR (qPCR) performed using the Applied Biosystems QuantStudio 6 Flex Real-Time PCR System with TaqMan assays (Life Technologies, USA). PCR reactions were prepared in TaqMan fast universal master mix (Life Technologies, USA) following the manufacturer's instructions. Relative gene expression was quantified by comparative CT ($\Delta\Delta CT$) to no treatment controls and normalized to glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) and beta-2-microglobulin (*B2M*) (Life Technologies, USA)[177,178].

2.2.4 Total DNA methylation assay

Treated cells were harvested, and DNA was isolated using QIAamp DNA Mini Kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA concentration was measured using a NanoDrop spectrophotometer and reconstituted in water to 400 µg/mL, then stored at -80°C. DNA samples were hydrolyzed with 250 U benzonase (E1014, Sigma-Aldrich, USA), 300 mU phosphodiesterase I (P-3243, Sigma-Aldrich, USA), and 200 U alkaline phosphatase (P-7923, Sigma-Aldrich, USA), 5 mM MgCl₂ in a 100 mM ammonium bicarbonate buffer (pH7.9). 10 µL internal standards 5-methyl-2'-deoxycytidine-d3 (mdC-d3) for 5-methyl-2'-deoxycytidine (mdC) and ¹⁵N₅-2'-deoxyguanosine for 2'-deoxyguanosine (dG) were added to 90 µL of digested samples. A liquid chromatography tandem mass spectrometry (LC-MS/MS) was optimized to quantify mdC and dG. Briefly, the analytes and internal standards were separated using an Acquity BEH C18, 1.7 µm, 2.1 × 100 mm column (Waters, USA) with a pH gradient in organic mobile phases (methanol containing 0.1% formic acid, 2 mM ammonium formate and 0.1 mM malic acid) and aqueous mobile phase (water containing 0.1% formic acid, 2 mM ammonium formate and 0.1 mM malic acid) using a Shimadzu Nexera UHPLC. Each analyte was measured in positive MRM mode using a Shimadzu LCMS 8040 mass spectrometer with DUIS ionization. Concentrations of mdC were normalized to total dG concentrations[179].

2.2.5 DNA methylation measure at *p16* gene promoter

DNA extracted from AGS and SNU719 cells treated with different concentrations of decitabine or GSK3685032, was subjected to sodium bisulfite conversion using the Zymo Research kit according to the manufacturer's instructions. The *p16* promoter region was defined according to previously published literature [168,177], and promoter-specific primers were subsequently designed using the PyroMark Assay Design software, version 2.0 (Qiagen, Germany). Sequences

and positions of the analyzed 8 CpG sites are provided in **Appendices 2A**. The bisulfite-converted DNA (bcDNA) was amplified by PCR [180,181], and DNA methylation levels at *p16* were analyzed using the Qiagen Q48 pyrosequencer. In parallel with experimental samples, we included duplicate DNA standards consisting of mixtures of fully methylated and unmethylated controls (Zymo Research, USA, standard curve of 0%, 25%, 50%, 75%, and 100% methylation). To account for any technical bias in measuring DNA methylation by pyrosequencing, raw measurements of percent DNAm per sample were scaled to the standard curve prior to statistical analysis.

2.2.6 Histone modification analysis

After treatment, cells were harvested, and histones were isolated using the EpiQuik (Epigentek, USA) kit with modifications according to the previously validated method[182,183]. Histone samples (10 µg) were either digested with pronase or hydrolyzed in the vapour phase using 6N HCl, followed by analysis of acetyl-lysine, methyl-lysines, and methyl-arginines via LC-MS/MS. The modified and unmodified amino acids from the histone hydrolysates were separated on a Primesep 200 (Sielc, USA) column using a pH gradient of acetonitrile and water on a Shimadzu Nexera UHPLC (Shimadzu, Japan). Each analyte was detected in positive MRM mode with a Shimadzu LCMS 8040 mass spectrometer using DUIS ionization. Quantification was done using standards for acetyl-lysine, mono-, di-, and trimethyl-lysine, monomethyl-arginine, asymmetric dimethyl-arginine, and symmetric dimethyl-arginine. Concentrations of analytes were normalized to total lysine concentrations to determine the percentage of analyte according to the previously reported method[182,183].

2.2.7 DNMT, TET, p16, p21, ELISA assay

After treatment, cells were harvested, and nuclear proteins were extracted using Nuclear Extraction Kit (Abcam, USA). DNMT, p16, and p21, concentrations were detected using DNMT, p16, and

p21 ELISA kits (Abcam, USA), and TET concentrations were detected by TET ELISA kit (MyBioSource, USA) according to the manufacturer's instructions.

2.2.8 Flow cytometry analysis

Cells harvested following treatments were washed with cold phosphate-buffered saline (PBS), and then fixed in ice-cold 70% ethanol for 10 minutes. The fixed cells were rewashed in PBS and resuspended in 1 ml of propidium iodide staining solution (containing 4 mM sodium citrate, 0.1% Triton X-100, 75 μ M propidium iodide, and 10 μ g/ml RNase A) and incubated at 37°C for 10 minutes. The percentage of cells with the G0/G1, S, and G2/M DNA content was determined by propidium iodide staining using Cytoflex LX flow cytometer (Beckman Coulter, USA). Data were processed and analyzed using the FlowJo software platform (Cell Cycle module, USA). Each stage of the cell cycle was gated and their percentages compared among groups.

2.2.9 Statistical analysis

Statistical analysis was performed using GraphPad Prism10 (GraphPad Software, USA) for most experiments, and RStudio (versions 3.6.1 and 4.2) for DNA methylation measurements at the *p16* gene promoter. A t-test was conducted to compare the potency of GSK3685032 and RG108 within the same cell line. The one-way ANOVA was used to compare the difference between the different concentrations in one cell line treated with the same DNMT inhibitors (P-value <0.05 as a significant). When ANOVA indicated a significant difference, a Tukey's test was performed to determine which specific groups differed. Pearson correlation statistical analysis was used to investigate the relationship between *p16* and other selected methylated-silenced genes as well as DNA methylation. Additionally, the correlation between *p16*, *p21*, and the percentage of DNA content was analyzed.

2.3 Results

2.3.1 In vitro effects of DNMT inhibitors on gastric cancer cell line growth

Decitabine, GSK3685032, and RG108, demonstrated varying capacity to reduce gastric cancer and control cell line viability. Decitabine concentrations of 50-500 μ M for 72 hours could not reduce cell viability below 40% (**Figure 2.1A**), which is consistent with decitabine inducing cell cycle arrest [184,185]. GSK3685032 and RG108 reduced viability in the gastric cancer cell lines AGS, SNU216, SNU719, and MKN45, and the human embryonic kidney-derived HEK293 cell line, which was included as a non-gastric cancer control, with the IC₅₀ values presented in **Table 2.1** and the viability curves in **Figure 2.1B** and **2.1C**. Both GSK3685032 and RG108 were more potent and efficacious than decitabine in reducing cell viability across all tested cell lines. Although RG108 appears more potent than GSK3685032, the difference between the two drugs is only statistically significant when comparing their IC₅₀ values in SNU719 cells.

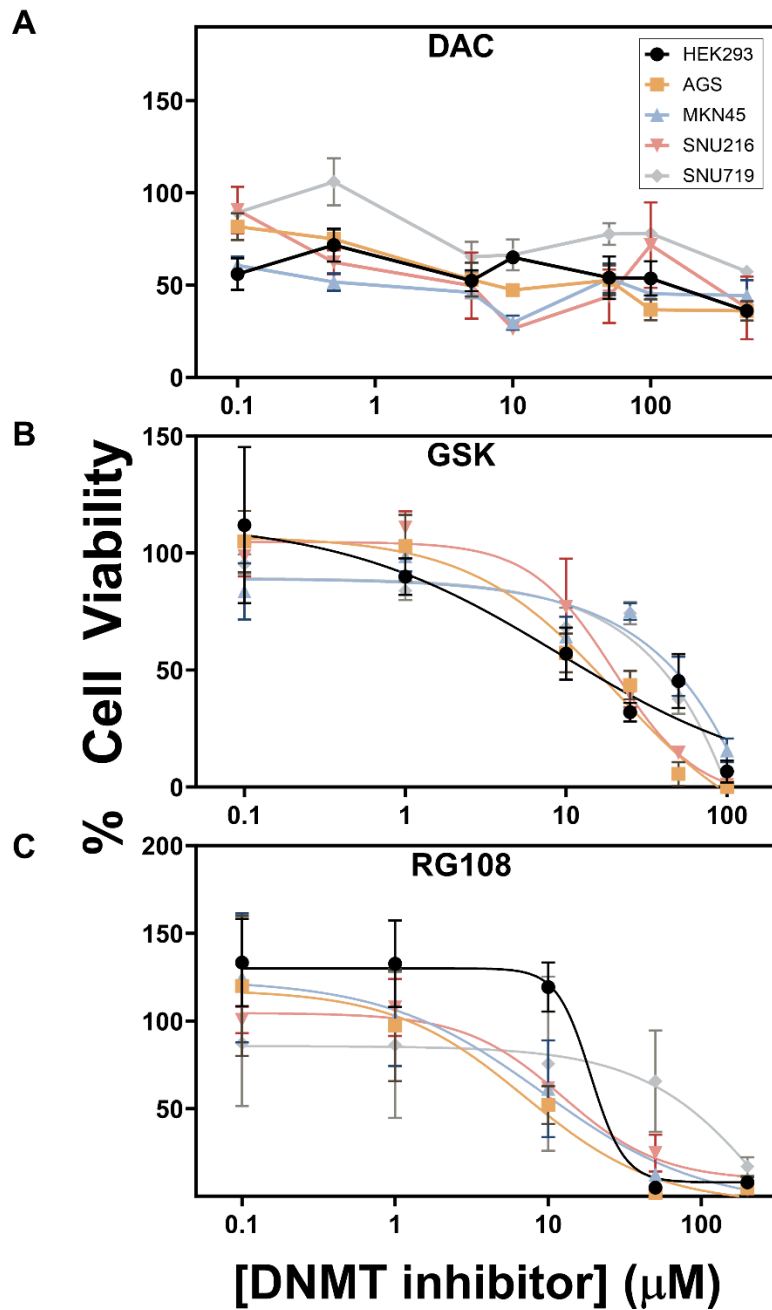


Figure 2.1 Dose response curves for DNMT inhibitors in gastric cancer and control cell lines for 72 h. The percentage of viable cells was measured in control (HEK293) and gastric cancer cells (AGS, MKN45, SNU216, and SNU719) after 72 h of treatment with increasing concentrations of decitabine (A), GSK3685032 (B), and RG108 (C). Viability was assessed using the CellTiter 96® MTS assay. Values represent the mean of three measurements with error bars indicating standard deviation (SD). DAC: decitabine; GSK: GSK3685032.

Table 2.1 IC₅₀ values for GSK3685032 and RG108 in gastric cancer cells AGS, SNU216, SNU719, MKN45 and control cells HEK293

Cell line	IC ₅₀ (μM)				
	AGS	MKN45	SNU216	SNU719	HEK 293
GSK365032	29.00±17.92	303.6±294.81	18.21±5.33	128.3±3.04	8.22±5.18
RG108	10.26±1.25	10.13±2.54	15.14±3.48	2.50±3.49	13.52±1.23
P-value [†]	0.1451	0.1598	0.4505	0.0001	0.1598

[†] Calculated using a t-test

2.3.2 Total genomic DNA methylation and *p16* expression changes in gastric cancer cells treated with DNMT inhibitors

The gastric cancer cell lines AGS, MKN45, SNU216, and SNU719 exhibit low or no *p16* expression, in some cases due to CpG promoter hypermethylation, which in some cases can be reversed by decitabine, resulting in *p16* expression [52,168,186]. To assess the effect of DNMT inhibitors decitabine, GSK3685032, or RG108 on total genomic (total) DNA methylation in these gastric cancer cells, we developed and validated an LC-MS/MS assay (**Table S2.1- S2.3**) to quantify mdC derived from DNA extracted from cells, then correlated this with *p16* expression. HEK293, a non-gastric cancer cell line with relatively high *p16* expression, was used as a control[187].

An initial maximum concentration of 5 μM decitabine was selected because our preliminary data, and previous studies showed that this concentration reduces DNA methylation across many cell lines[168,186] (**Figure 2.2A**). In AGS and SNU719 cells, an increase in decitabine concentration up to 5 μM resulted in a gradual decrease in total DNA methylation as represented by mdC concentration (**Figure 2.2A**), and a progressive increase in *p16* expression (**Figure 2.2B**). Although DNA methylation decreased with increasing decitabine concentrations in MKN45 and

SNU216 cells, there was no change in p16 expression (**Figure 2.2B**). A dose-dependent reduction in total DNA methylation was observed with GSK3685032 in HEK293, SNU216, and MKN45 cells, eventually reaching a minimum (**Figure 2.2C**). Low concentrations of GSK3685032 in AGS and SNU719 cells resulted in a decrease in total DNA methylation reaching a minimum, however, as drug concentrations increased, there was a paradoxical increase in total DNA methylation from the minimum produced by low concentrations, which was statistically significant (**Figure 2.2C**). Accordingly, low concentrations of GSK3685032 initially increased p16 expression in AGS and SNU719 cells; however, at higher concentrations, p16 expression significantly decreased sometimes to pretreatment levels, likely due to a paradoxical increase in total DNA methylation (**Figure 2.2C, 2.2D**). RG108 had no significant impact on total DNA methylation in any of the gastric cancer cell lines we studied, even at ~20-fold above its IC₅₀ (200 μ M) (**Figure 2.2E**). However, at the highest concentrations, RG108 resulted in a statistically significant 12-fold increase in *p16* expression in SNU719 cells (**Figure 2.2F**). There was a strong negative correlation between *p16* gene expression and total DNA methylation (**Figure 2.2G, 2.2H**) following treatment with decitabine and GSK3685032 in AGS and SNU719 cells.

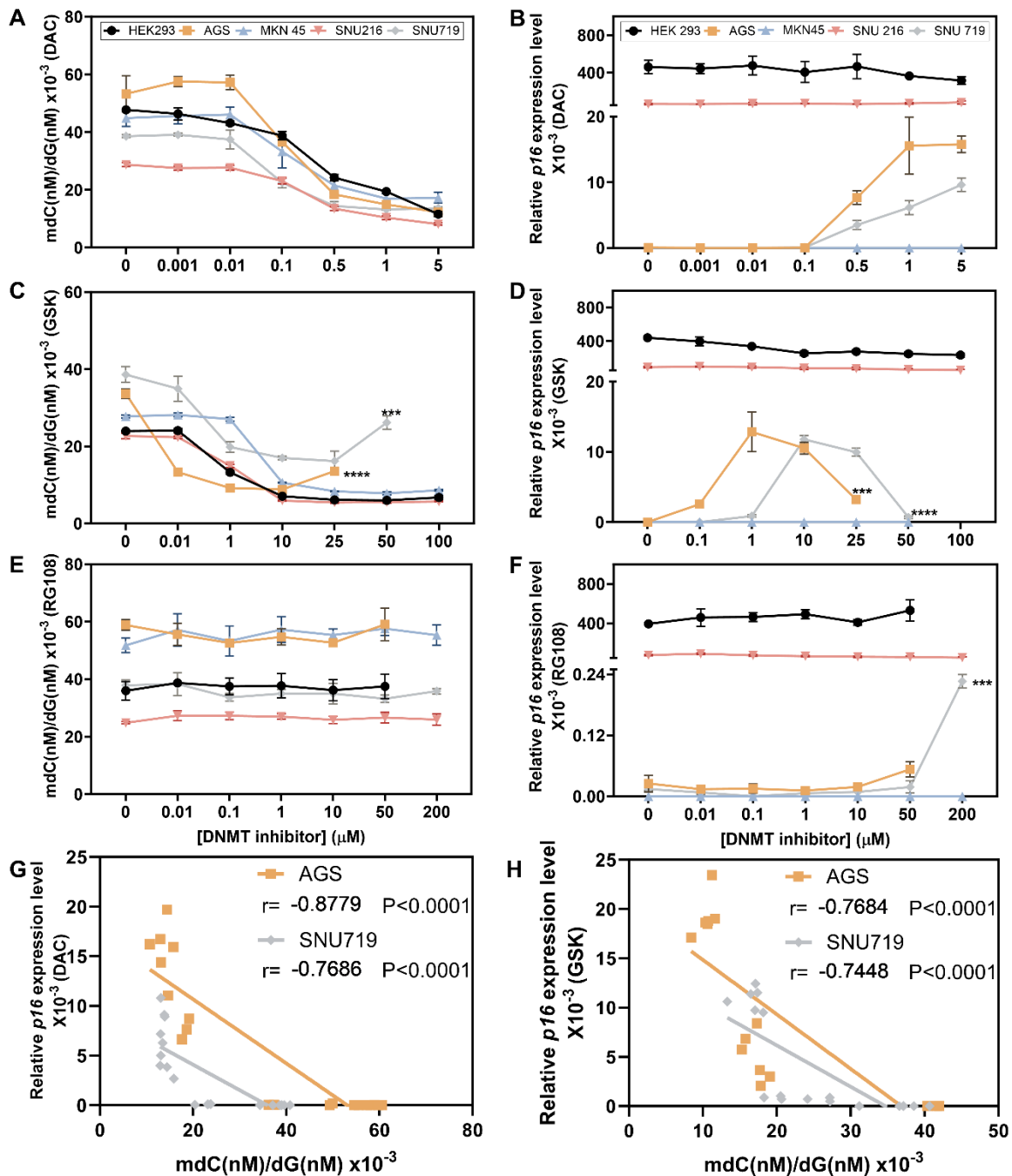


Figure 2.2 The changes in total DNA methylation and p16 expression in gastric cancer and control cell lines treated with DNMT inhibitors for 72 h.

Shown are the changes of total DNA methylation measured by LC-MS/MS in gastric cancer cell lines (AGS, MKN45, SNU216 and SNU719) and control cell lines (HEK 293) and the corresponding changes in p16 gene expression measured by qPCR (normalized using combination of B2M-GAPDH) treated with decitabine (A, B), GSK3685032 (C, D), and RG108 (E, F), respectively. The missing mdC and p16 data are due to insufficient cell collection, resulting from reduced cell viability at the highest doses. In HEK293 and SNU216 cells, p16 expression changes were not significant (P -value > 0.05) with decitabine or RG108 (Supporting information Figure S2). A reduction in p16

expression was observed with 100 μ M GSK3685032. A one-way ANOVA was used to compare the difference between the different concentrations in one cell line treated with the same DNMT inhibitors (P-value <0.05 considered significant). All statistically significant results for total DNA methylation and p16 expression across different cell lines and treatment concentrations of three DNMT inhibitors are provided in the supporting information Figure S1. Asterisk (*) indicates statistical significance (*P<0.05, **P< 0.01, ***P<0.001, ****P<0.0001). Values represent the mean of three measurements with error bars indicating SD. DAC: decitabine; GSK: GSK3685032.

Since GSK3685032 paradoxically increased total DNA methylation and decreased p16 expression at the highest concentrations in AGS and SNU719 cells, we wanted to see if high concentrations of decitabine produce similar effects. AGS cells treated with >5 μ M decitabine appeared to show an increase in total DNA methylation, but this was not statistically significant (**Figure 2.3A**). However, a significant decrease in p16 expression was observed at 10 μ M or higher (**Figure 2.3B**). SNU719 cells treated with decitabine exhibited a significant increase in total DNA methylation beginning at 10 μ M (**Figure 2.3C**), peak *p16* expression at 1 μ M and a reduction nearly back to baseline at 10 μ M, correlating with the increase in total DNA methylation (**Figure 2.3D**).

To determine if the paradoxical pattern of increasing total DNA methylation and decreasing *p16* expression observed with high-dose GSK3685032 and decitabine was particular to *p16*, we measured the effect of the drugs on the expression of tumor necrosis factor superfamily, member 9 (*TNS*), Peptidylprolyl isomerase C (*PPIC*), and secernin 1 (*SCRNI*), which are silenced by promoter CpG methylation [188]. *TNS* encodes a protein that supports T-cell activation and survival, which is crucial in immune response and targeting tumors[189]. *PPIC* catalyzes the isomerization of proline residues, impacting cellular signaling and protein trafficking[190]. *SCRNI* regulates exocytosis and plays a role in secretory processes, influencing immune response and cancer progression[191]. These genes are functionally distinct from p16, with no direct involvement of *p16* in their associated pathways or expression. The mRNA expression of *PPIC* and *SCRNI* mirrored the changes in *p16* expression in AGS cells treated with decitabine, showing an increase followed by a decrease (**Figure 2.3B**). *TNS* followed a similar pattern of increase but no statistically significant decrease, and it required higher doses of decitabine to reach a maximum.

In SNU719 cells, decitabine produced an increase in expression of *TNS*, *PPIC*, and *SCRNI* reaching a maximum at 25 μ M, a 25-fold higher dose than where peak expression is achieved with p16 (**Figure 2.3D**). At 50 μ M and 100 μ M decitabine, the expression of all three genes appear to be slightly reduced but these results are not statistically significant. 25 μ M GSK3685032 yielded significant increases in total DNA methylation after the lows produced by 1-10 μ M in AGS cells (**Figure 2.3E**). Accordingly, the expression of *TNS*, *PPIC*, and *SCRNI* increased at 1-10 μ M but significantly decreased at 25 μ M (**Figure 2.3F**). SNU719 cells treated with GSK3685032 exhibited a significant increase in total DNA methylation after the lows experienced at 10-25 μ M in SNU719 cells (**Figure 2.3G**). Although p16 expression peaked at 10-25 μ M and reduced to nearly zero at 50 μ M (**Figure 2.3H**), the expression of *TNS*, and *PPIC*, progressively increased across all GSK3685032 concentrations with only *SCRNI* appearing to decrease at 50 μ M but this was not statistically significant.

There was a negative correlation between the expression of all selected genes and total DNA methylation (**Figure 2.3I**). Following treatment with GSK3685032, the strongest negative correlation was observed between *p16* expression and total DNA methylation in AGS ($r = -0.768$) and SNU719 cells ($r = -0.745$). In contrast, decitabine treatment yielded the strongest negative correlations between *SCRNI* expression and total DNA methylation in AGS cells ($r = -0.710$) and between *TNS* expression and total DNA methylation in SNU719 cells ($r = -0.582$). Notably, the second strongest negative correlations between p16 expression and total DNA methylation were observed in AGS cells ($r = -0.705$) and SNU719 ($r = -0.548$) cells.

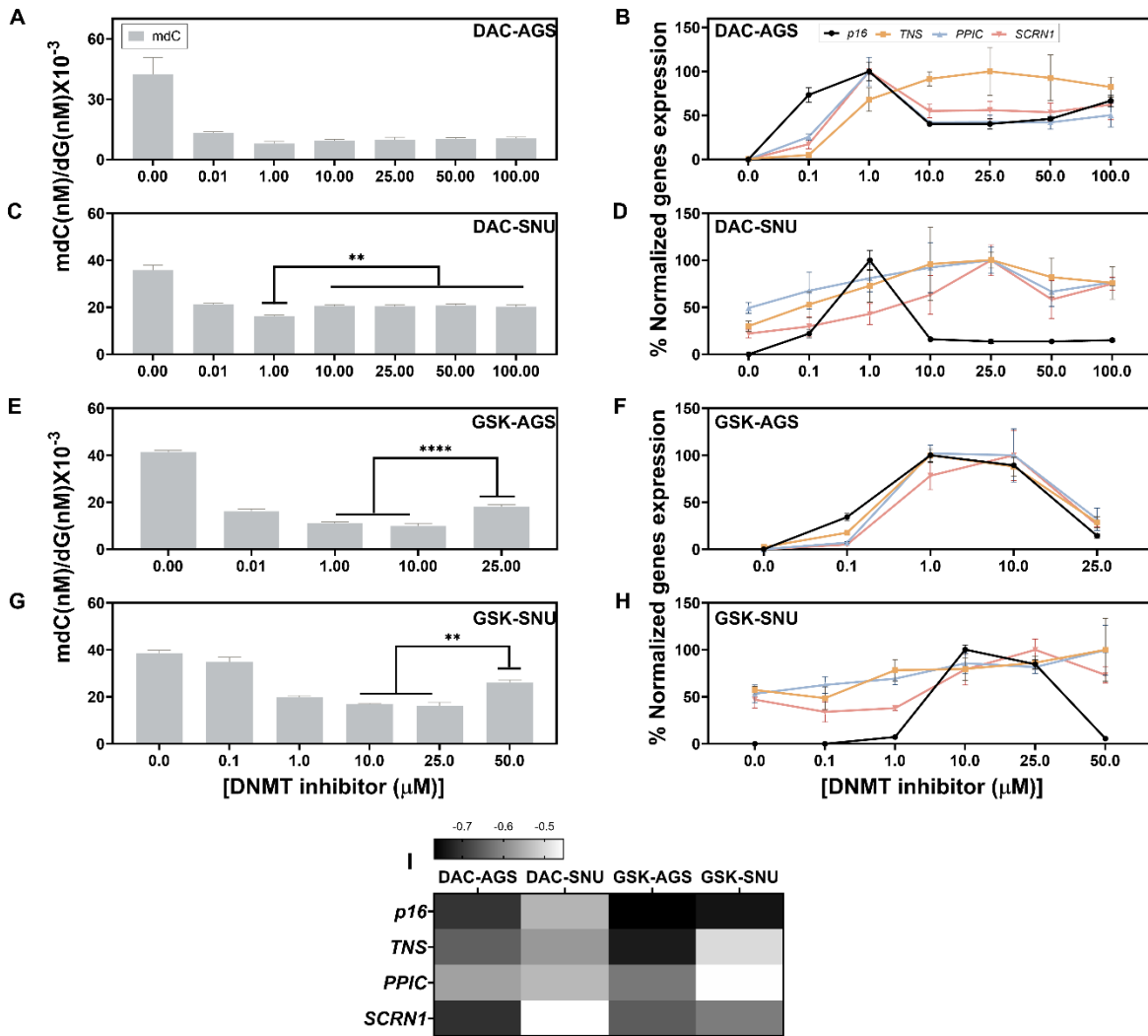


Figure 2.3 Correlation of total DNA methylation with CpG methylation-silenced genes in AGS and SNU719 cells treated with decitabine and GSK3685032 for 72 h.

The mdC levels measured by LC-MS/MS and selected gene expression measured by qPCR (normalized using a combination of B2M-GAPDH) in AGS cells treated with decitabine (A, B), SNU719 cells treated with decitabine (C, D), AGS cells treated with GSK3685032 (E, F), and SNU719 cells treated with GSK3685032 (G, H). The mdC (E, G) and p16 expression (F, H) for these two cell lines treated with GSK3685032 were previously reported in Figure 2C and 2D. The heat map (I) displays the Pearson correlation coefficients (r values) obtained from the statistical analysis, illustrating the relationships between total DNA methylation levels and the expression of CpG methylation-silenced genes. A negative correlation between methylation-silenced genes and total DNA methylation was observed in both AGS and SNU719 cell lines following treatment with decitabine or GSK3685032. A one-way ANOVA was used to compare the difference between the different concentrations in one cell line treated with the same DNMT inhibitors (P-value <0.05 considered significant). The relative gene expression and all statistically significant results for methylation-silenced genes in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72 h are provided in the supporting information Figure S2. Asterisk (*) indicates statistical significance (*P<0.05, **P<0.01, ***P<0.001, ****P<0.0001). The method for normalizing methylation-silenced gene expression is to set the highest expression of each gene expression within the same treatment as 100%. Values represent the mean of three measurements with error bars indicating SD. TNS: Tumor necrosis factor superfamily, member 9; PPIC:

Peptidylprolyl isomerase C; SCRNI1: secernin 1. DAC-AGS: AGS cells with decitabine; DAC-SNU: SNU719 cells with decitabine; GSK-AGS: AGS cells with GSK3685032; GSK-SNU: SNU719 cells with GSK3685032.

2.3.3 Total DNA methylation aligned with p16 promoter methylation using decitabine and GSK3685032 in AGS and SNU719 cells

Pyrosequencing was used to measure *p16* promoter methylation in AGS and SNU719 cells treated with decitabine or GSK3685032, recapitulating the pattern of decreasing then increasing methylation sometimes back to pre-treatment levels with increasing dose. Decitabine at 1 μ M induced a nearly 50% reduction, but 25 μ M restored methylation close to pretreatment levels (**Figure 2.4A, 2.4B and Figure S2.1**). In contrast, 10 μ M GSK3685032 induced a >50% reduction, but a small, statistically significant rebound in promoter CpG methylation at higher concentrations (**Figure 2.4C, 2.4D and Figure S2.1**). These changes in *p16* promoter CpG methylation correlate with the changes in total DNA methylation and in *p16* expression under treatment with GSK3685032 and decitabine (**Figure 2.4E**).

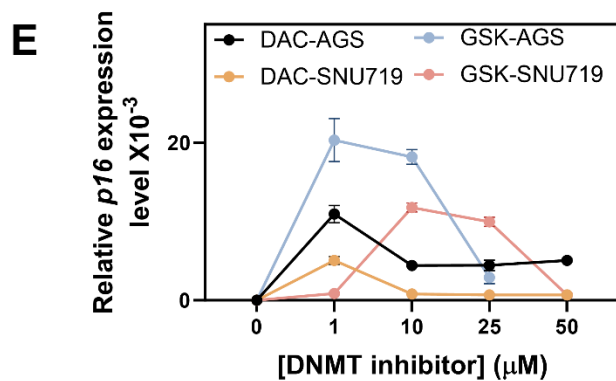
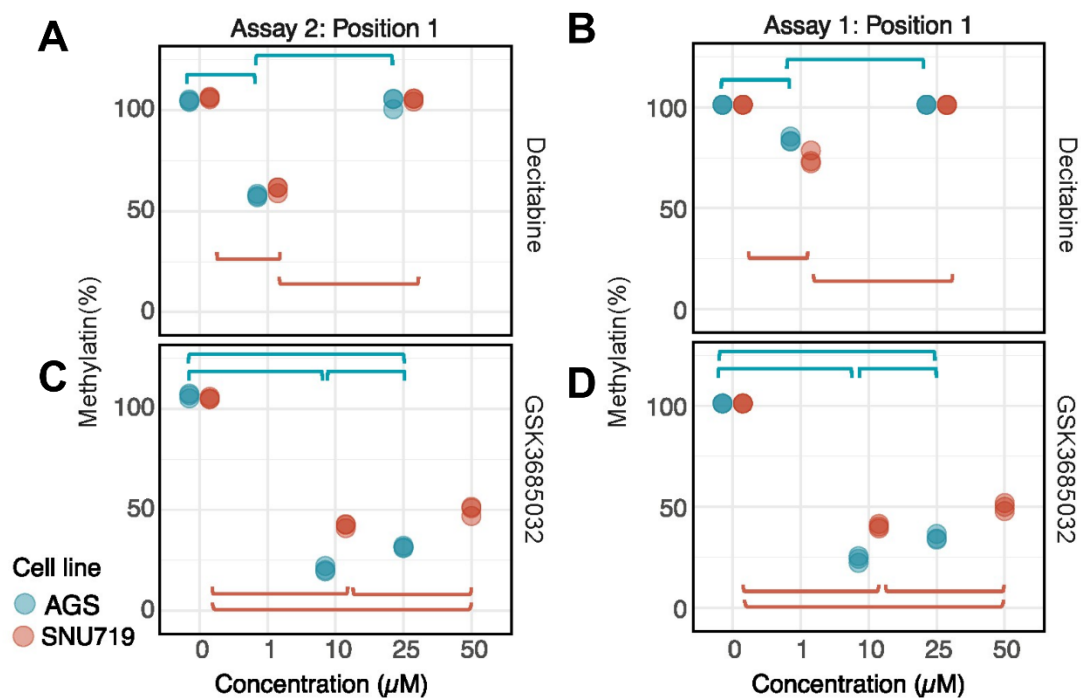
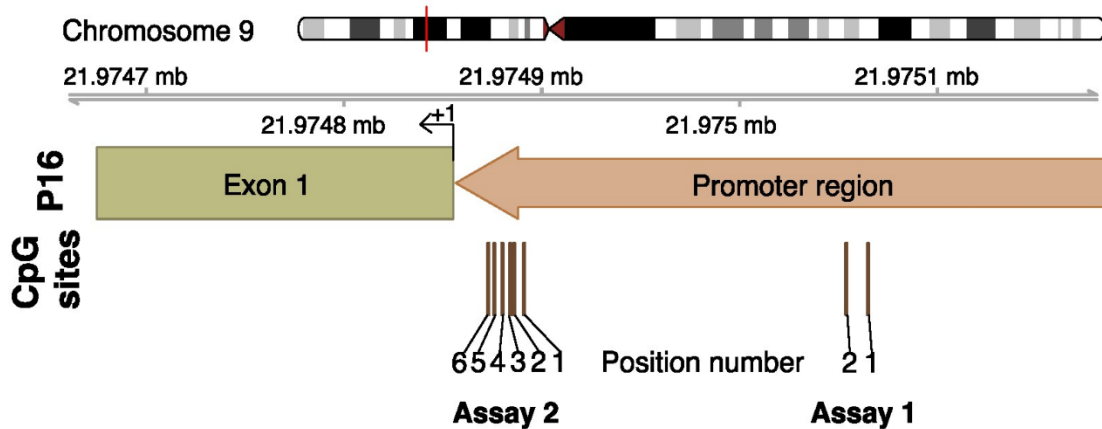


Figure 2.4 CpG methylation in the p16 promoter (pyrosequencing) and p16 expression across selected DNMT-inhibitor doses.

Assay 1 measured two CpG sites, and Assay 2 measured six CpG sites. For clarity, one representative CpG site per assay is shown, as results across sites were highly correlated (see supporting information Table S4, Figure S3, S4 for all sites). Decitabine reduced methylation <50% at 1 μ M with frequent rebound to ~baseline at higher doses (A,B), whereas GSK3685032 cut methylation >50% at 10 μ M with only a mild rebound and stronger effects in AGS (C,D). Methylation levels of (A, B, C, D) were calculated using a standard curve generated from methylation standards to correct for assay-specific variation. (E) Replot of p16 data from Figure 3B, 3D, 3F, and 3H, combining both cell lines to compare relative expression across all conditions. Within cell lines, p16 expression rose more with GSK3685032 than with decitabine. Differences in methylation between drug concentrations and cell lines were tested by ANOVA followed by Tukey's post hoc test for pairwise comparisons. Bars indicate significant comparisons ($p < 0.01$). The only non-significant comparison was between 0 μ M and 25 μ M Decitabine in both cell lines and assays.

2.3.4 Decitabine and GSK3685032 increased DNMT3B and decreased TET in AGS cells and SNU719 cells

Since the effects of decitabine and GSK3685032 are not restricted to any particular part of the genome, it is reasonable to expect that they could alter the expression of many genes and we already showed that this was the case with *TNS*, *PPIC*, and *SCRNI*. The most obvious genes that would produce the paradoxical increase in total and *p16* promoter CpG DNA methylation would be the enzymes catalyzing DNA methylation addition and removal: the DNMTs and TETs [158,192]. We therefore measured DNMT1, DNMT3A, DNMT3B, TET1, TET2, and TET3 mRNA and protein using qPCR and quantitative ELISA kits, respectively (**Figure 2.5 and Figure 2.2**).

Increasing decitabine concentrations increased the expression of DNMT3B on the mRNA level by as much as 50% (**Figure 2.5A**) and induced statistically significant increases in DNMT3B protein at 25 and 50 μ M in AGS cells (**Figure 2.5B**). DNMT1 and DNMT3A mRNA also increased, but no corresponding change in protein levels was observed. Decitabine produced statistically significant decreases in TET1 mRNA but no apparent change in protein, and very small decreases in TET2 protein but not mRNA, in AGS cells. In SNU719 cells, decitabine similarly elevated DNMT3B expression on the mRNA and protein levels (**Figure 2.5C, 2.5D**). GSK3685032 increased DNMT3B mRNA, but this was not reflected in a corresponding statistically significant

increase in DNMT3B protein in AGS cells. Reduced TET1 protein was also observed, but there was no corresponding statistically significant change in TET1 mRNA (Figure 2.5E, 2.5F). SNU719 cells treated with GSK3685032 induced significant increases in DNMT3B mRNA and protein (Figure 2.5G, 2.5H).

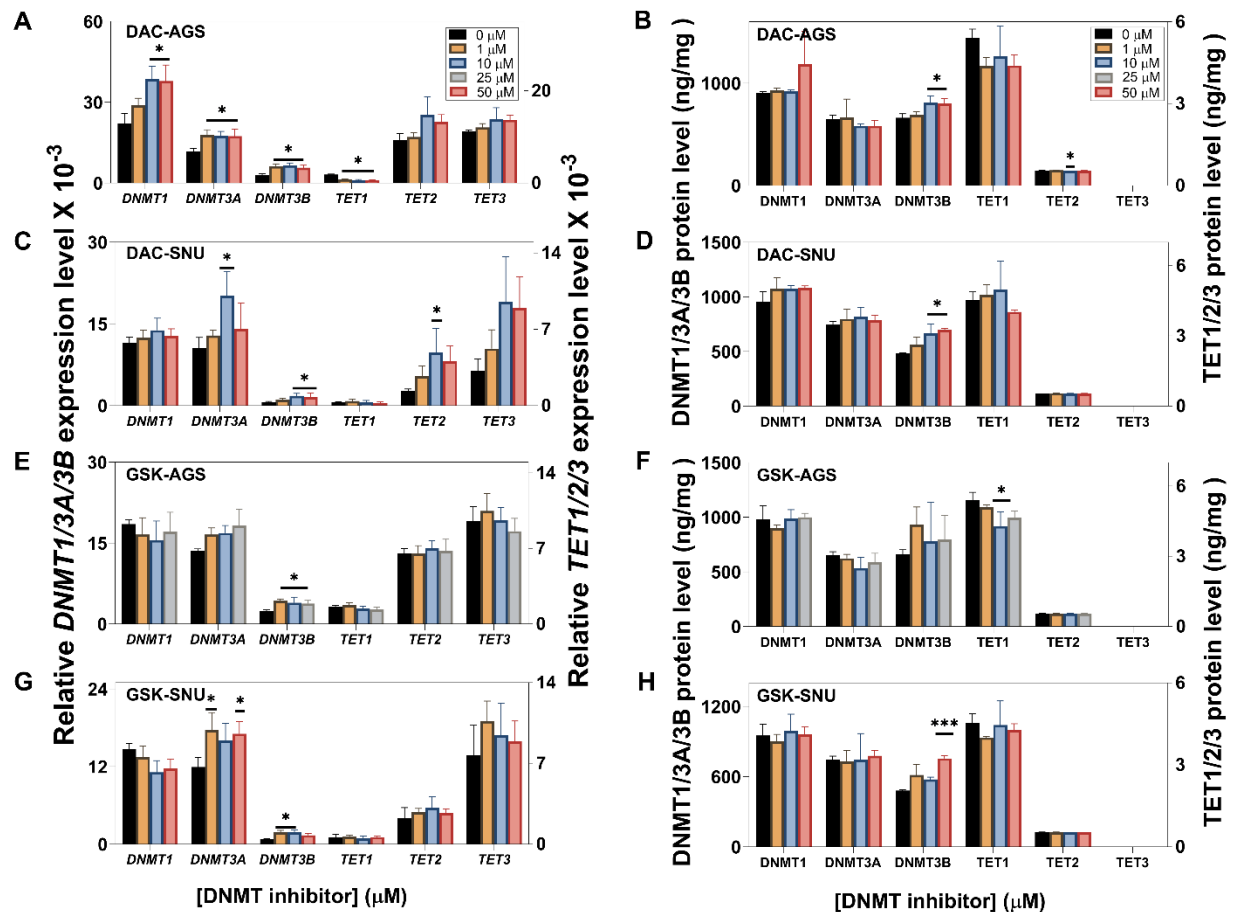


Figure 2.5 Decitabine and GSK3685032 increased DNMT3B expression and/or decreased TET expression on the mRNA and protein levels in AGS and SNU719 cells after 72 h treatment.

Show is the DNMT 1 3A and 3B and TET1, 2, and 3 mRNA and protein concentration in AGS cells treated with decitabine (A, B, respectively), SNU719 cells treated with decitabine (C, D, respectively), AGS cells treated with GSK3685032 (E, F, respectively) and SNU719 cells treated with GSK3685032 (G, H, respectively). The amount of TET3 is below the lower limit of quantitation (0.78 ng/mL). In other figures, gene expression was normalized using a combination of B2M and GAPDH, however, in this figure, normalization was performed using GAPDH alone, as it more closely correlated with significant differences observed at the protein level via ELISA. Gene expression normalized using B2M and GAPDH, or B2M alone, are presented in Figure S5. A one-way ANOVA was used to compare the differences between the different concentrations in one cell line treated with the same DNMT inhibitor (P-value < 0.05 considered significant). Asterisk (*) indicates statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001,

****P<0.0001) compared with the control group (0 mM DNMT inhibitor). Values represent the mean of three measurements with error bars indicating SD. DAC-SNU: SNU719 cells with decitabine; GSK-AGS: AGS cells with GSK3685032; GSK-SNU: SNU719 cells with GSK3685032.

2.3.5 Decitabine and GSK3685032 induce cell cycle arrest in AGS and SNU719 cell lines

Since decitabine and GSK3685032 modulated p16 expression, we investigated if the drugs could influence the cell cycle using flow cytometry. Decitabine induced G2/M phase cell cycle arrest in AGS cells, producing a reduction of cells in the G0/G1 phase, an increase in cells in the G2/M phase (**Figure 2.6A, 2.6B, 2.6I, 2.6K, 2.6M**), but no statistically significant changes in the percentage of cells in the S phase, compared with untreated control. SNU719 cells treated with decitabine show no change in the number of cells in the G0/G1 phase, an accumulation of cells in the G2/M phase, and a decrease in S phase cells (**Figure 2.6C, 2.6D, 2.6I, 2.6K, 2.6M**), compared to untreated controls, suggesting arrest at the G2/M phase. AGS cells treated with GSK3685032 exhibit a significant accumulation of cells in the G0/G1 phase at the highest dose, accompanied by a reduction in cells in the S and G2/M phases (**Figure 2.6E, 2.6F, 2.6J, 2.6L, 2.6N**). SNU719 cells treated with GSK3685032, show an increase in the number of cells in G0/G1 that was statistically significant at the 10 μ M level (**Figure 2.6G, 2.6H**), no apparent change in percentage of the cells in the G2/M and an approximately 56% reduction in the percentage of cells in the S phase at 10 μ M but not at 50 μ M (**Figure 2.6J, 2.6L, 2.6N**). These results suggest that GSK3685032 induces G0/G1 cell cycle arrest.

To better understand the mechanisms underlying cell cycle arrest induced by DNMT inhibitors, we measured mRNA and protein expression of the cell cycle regulators p21 and p16 using qPCR and quantitative ELISA assays (**Figure 2.6 and Figure S2.3**). AGS cells treated with decitabine exhibited a concentration-dependent increase in p21 mRNA expression (**Figure 2.6O**) and protein at 50 μ M (**Figure 2.6S**). In SNU719 cells, p21 mRNA expression exhibited a concentration-dependent increase in response to decitabine (**Figure 2.6Q**) and GSK3685032 (**Figure 2.6R**), with

a maximum 10.0- and 2.4-fold increase, respectively, at 50 μ M. The increase in p21 mRNA induced by decitabine was >4-fold higher than that induced by GSK3685032. Accordingly, p21 protein levels increased significantly with decitabine (**Figure 2.6U**) but not with GSK3685032 (**Figure 2.6V**) in SNU719 cells. Lower concentration of GSK3685032 did not increase p21 protein or mRNA expression in AGS cells (**Figure 2.6P, 2.6T**). GSK3685032 induced greater maximal p16 mRNA expression at concentrations of 1 or 10 μ M than decitabine at 1 μ M in both cell lines (**Figure 2.6P and 2.6R vs 2.6O and 2.6Q**). GSK3685032 and decitabine induced significant increases in p16 protein in AGS cells (**Figure 2.6S, 2.6T, S2.6A and S2.6B**), but unexpectedly, GSK3685032 did not produce a significant increase in p16 protein in SNU719 cells (**Figure 2.6V and S2.6D**), whereas decitabine did (**Figure 2.6U and S2.6C**).

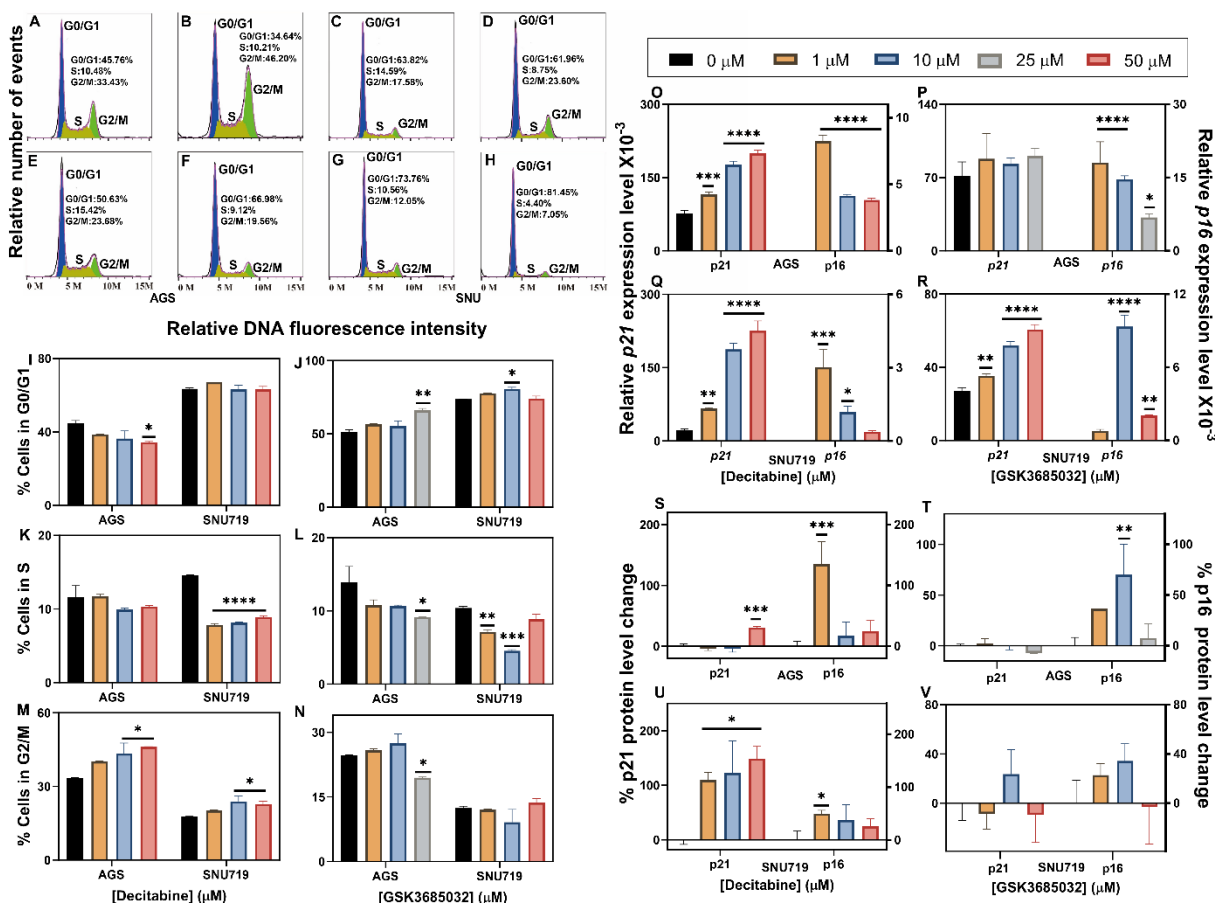


Figure 2.6 Induction of cell cycle arrest and the changes in p21 and p16 in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.

Histograms showing the relative DNA content in G0/G1 (blue), S (brown) and G2/M (green) after treatment with decitabine or GSK3685032 for 72 hours. Shown are, AGS cells treated with 0 μ M decitabine (A); AGS cells treated with 50 μ M decitabine (B); SNU719 cells treated with 0 μ M decitabine (C); SNU719 cells treated with 50 μ M decitabine (D); AGS cells treated with 0 μ M GSK3685032 (E); AGS cells treated with 25 μ M GSK3685032 (F); SNU719 cells treated with 0 μ M GSK3685032 (G); SNU719 cells treated with 10 μ M GSK3685032 (H). The percentage of cells in different phases of cell cycle for AGS and SNU719 cells, which were determined by flow cytometry analysis, after exposure to various concentrations of decitabine (I, K, M) or GSK3685032 (J, L, N) for 72 h. The cell cycle regulators p21 and p16 gene expression measured by qPCR (normalized using combination of B2M-GAPDH) and protein level change (%) measure by Elisa in AGS and SNU719 cells treated with decitabine (O, Q and S, U) or GSK3685032 (P, R and T, V) for 72 h. p21 and p16 protein level measure by Elisa in these two cell lines are presented in Figure S6. A one-way ANOVA was used to compare the difference between the different concentrations in one cell line treated with the same DNMT inhibitors (P-value <0.05 considered significant). Asterisk (*) indicates statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) compared with the control group (0 μ M DNMT inhibitor). Values in flow cytometry analysis represent the mean of two measurements with error bar indicating SD. Values for p21 and p16 expression and protein level changes represent the mean of three measurements with error bars indicating SD.

2.3.6 Changes in total histone modifications in AGS and SNU719 cells treated with decitabine and GSK3685032

We investigated the effects on modifications to histones extracted from AGS and SNU719 cells treated with decitabine and GSK3685032, by quantifying mono-, di-, and tri-methyllysine (Kme1, Kme2, Kme3), acetyllysine (Kac), and mono- and symmetric and asymmetric di-methylarginine (Rme1, Rme2a, Rme2s) derived from hydrolyzed histones (**Figure 2.7, Table 2.2**) using methods we developed previously[193–195]. AGS cells treated decitabine showed a significant reduction in Kac, Kme and Kme2s. Arginine methylation remained relatively stable, except for Rme2a, which showed a small but significant decrease (**Figure 2.7A**). AGS cells treated with GSK3685032 showed reduced Kac and Kme at the highest doses, but no other statistically significant changes in histone modifications (**Figure 2.7B**). Similarly, SNU719 cells treated with decitabine, exhibited significant decreases in Kac and Kme at the highest concentration, but no changes in other histone modifications (**Figure 2.7C**). SNU719 cells treated with GSK3685032 showed no changes in any modifications except a statistically significant increase in Rme2a at the highest concentrations (**Figure 2.7D**). AGS cells treated with decitabine exhibited a decrease in total arginine methylation and total lysine methylation, whereas no significant changes were observed in cells treated with GSK3685032 (**Table 2.2**). GSK3685032 increased total arginine methylation in SNU719 cells, but no other significant changes in histone modifications were observed (**Table 2.2**).

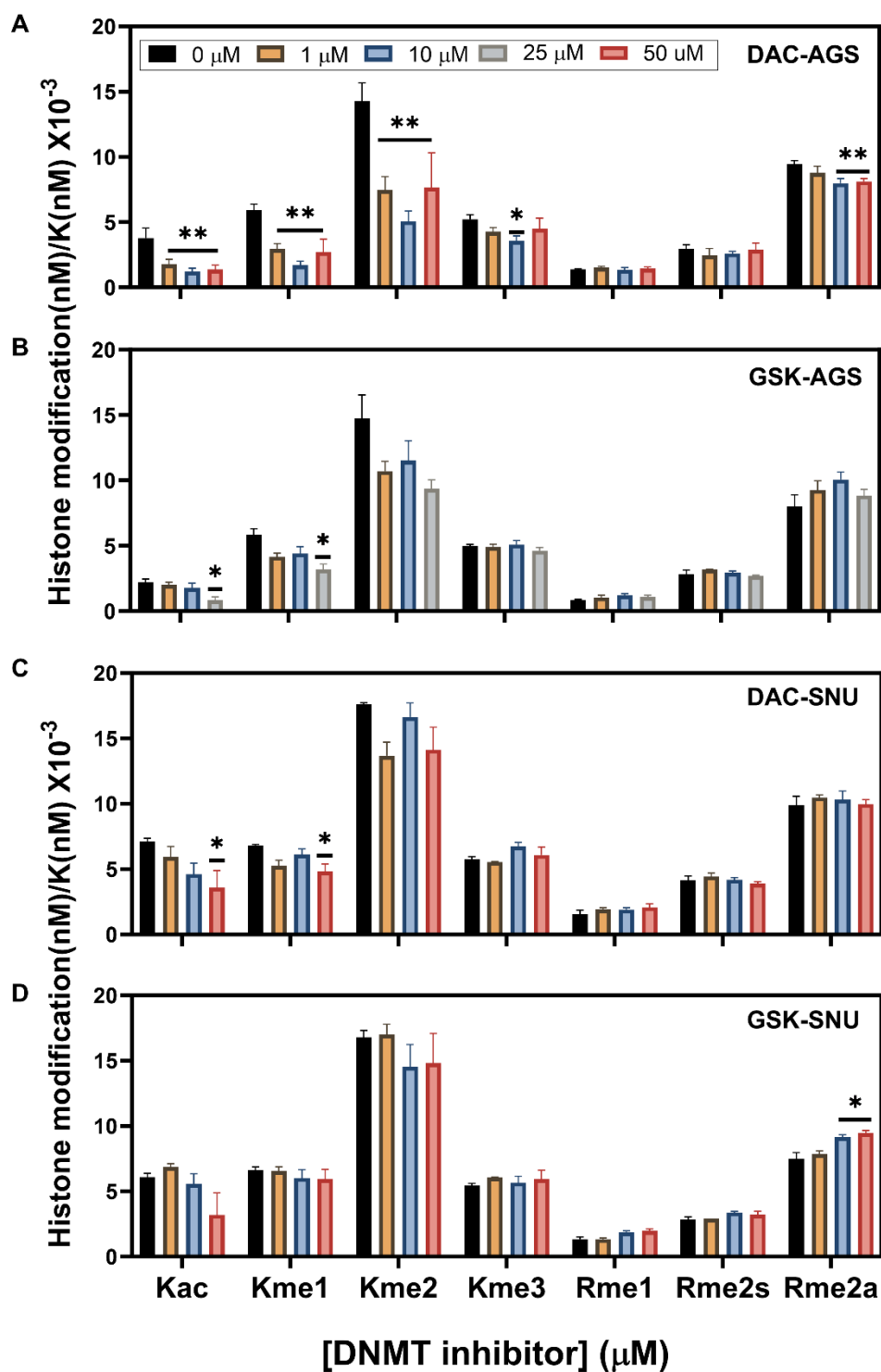


Figure 2.7 Changes in total histone modifications in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72 h.

Histone modification changes measured by LC-MS/MS in AGS cells treated with the indicated concentrations of decitabine (A) or GSK3685032 (B); and in SNU719 treated with the indicated concentrations of decitabine (C) or

GSK3685032 (D). The one-way ANOVA was used to compare the difference between the different concentrations in one cell line treated with the same DNMT inhibitors (P-value <0.05 considered significant). Asterisk (*) indicates statistical significance (*P < 0.05, **P<0.01, ***P<0.001, ****P<0.0001) compared with the control group (0 μ M DNMT inhibitor). Values represent the mean of three measurements with error bars indicating SD. DAC-SNU: SNU719 cells with decitabine; GSK-AGS: AGS cells with GSK3685032; GSK-SNU: SNU719 cells with GSK3685032. Kac: Acetylation lysine; Kme1: Monomethylation lysine; Kme2: Dimethylation lysine; Kme3: Trimethylation lysine; Rme1: Monomethylation arginine; Rme2s: Symmetric dimethylation arginine; Rme2a: Asymmetric dimethylation arginine.

Table 2.2 Summary of changes in histone modifications in AGS and SNU719 cells treated with decitabine and GSK3685032 for 72 h

DNMT inhibitor Cell line	Decitabine		GSK3685032	
	AGS	SNU719	AGS	SNU719
Kac	↓↓	↓	↓	—
Kme1	↓↓	↓	↓	—
Kme2	↓↓	—	—	—
Kme3	↓	—	—	—
Total K Methylated	↓↓	—	—	—
Total methyl group transferred to K	↓↓	—	—	—
Rme1	—	—	—	—
Rme2s	—	—	—	—
Rme2a	↓	—	—	↑
Total R Methylated	↓	—	—	↑
Total methyl group transferred to R	↓	—	—	↑
Total Methyl group transferred to K and R	↓	—	—	—

—: No significant change; ↑: Significant upregulation P<0.05

↓: Significant downregulation P<0.05; ↓↓: Significant downregulation P<0.01.

Kac: Acetylation lysine; Kme1: Monomethylation lysine; Kme2: Dimethylation lysine; Kme3: Trimethylation lysine; Rme1: Monomethylation arginine; Rme2s: Symmetric dimethylation arginine; Rme2a: Asymmetric dimethylation arginine.

2.4 Discussion

This study characterizes dose-dependent responses to DNMT inhibitors within the same gastric cancer cell lines. We show that DNMT inhibitor-induced reductions in DNA methylation do not follow a typical dose response relationship since these reductions in DNA methylation become attenuated at higher doses paradoxically increasing methylation relative to the minimum DNA methylation levels produced by low doses. We combine total and promoter-specific CpG DNA methylation with DNMT/TET/p16 gene regulation on the mRNA and protein levels, histone modifications, and changes in cell-cycle in gastric cancer cell lines. Comparison of mechanistically distinct DNMT inhibitors reveals drug class and dose dependent effects on p16 regulation and cell-

cycle arrest. Together, these findings highlight the potential importance of dose optimization and inhibitor selectivity for effective therapy in gastric cancer. We uncover a dose-dependent switch from *p16* reactivation to re-silencing that may have implications for dosing strategies in gastric cancer.

It was expected that the DNMT inhibitors, decitabine and GSK3685032, would reduce total DNA methylation with increasing dose, and this was observed at low concentrations; however, at high concentrations, there was a paradoxical increase in total DNA methylation relative to the minimum levels of DNA methylation produced by low doses. Decitabine and GSK3685032 increased DNMT3B mRNA levels in both cell lines, corroborated by increases in DNMT3B protein (except for GSK3685032 in AGS cells) at doses consistent with the paradoxical increase in DNA methylation. In addition, TET1 and TET2 protein concentrations decreased in AGS cells treated with GSK3685032 and decitabine, respectively. Thus, the increase in DNMT3B is the most likely cause of the paradoxical increase in DNA methylation at higher doses of decitabine and GSK3685032 in AGS and SNU719 cells (**Figure 2.5**). However, the reductions of TET1 and TET2 may also play a role in AGS cells. A limitation of our study is that we could have used targeted knockdown of DNMT3B or TET1/2 family enzymes to confirm their involvement in the paradoxical increase in DNA methylation with higher doses of DNMT inhibitors. Nevertheless, our findings are supported by previous studies showing that decitabine increased *DNMT3B* expression 4-fold in HL-60, a human leukemia cell line[196] and decitabine reduced *TET1* expression by ~half in the breast cancer cell line JIMT-1[197]. Moreover, the present work is intended to define a pharmacologically relevant, dose and mechanism dependent regulatory framework at the pathway level, rather than to establish genetic cause–effect relationships for individual enzymes.

Previous studies have shown that decitabine can sometimes cause a net increase CpG methylation in selected genes over a no-treatment-control in some cancer cell lines using non-quantitative sequencing methods [198]. It is important to note that in our study, no dose of DNMT inhibitor produced higher total or p16 promoter CpG DNA methylation than the no-treatment controls. Although higher doses of some DNMT inhibitors returned p16 promoter CpG DNA methylation to pre-treatment levels it never exceeded them.

Although DNMT inhibitors have been suggested as potential treatments for gastric and other solid tumor cancers, decitabine is only approved for use in haematological cancers. This and previous studies show that DNMT inhibitors can reduce DNA methylation, inducing expression of silenced tumor suppressors like p16, and could therefore be useful therapeutics for gastric cancers caused by silenced p16 expression. However, if our results with cultured gastric cancer cell lines AGS and SNU719 can be extrapolated generally to gastric cancer tumors, then only particular tissue or tumor concentrations of DNMT inhibitors can possibly increase p16 expression. We observed that concentrations of decitabine below 1 μ M reduced total DNA and p16 promoter CpG DNA methylation and increased p16 mRNA expression and protein, but concentrations higher than 1 μ M led to a paradoxical increase in total DNA and p16 promoter CpG methylation, and a decrease in p16 mRNA expression and protein, sometimes to pre-treatment levels.

Typical plasma decitabine concentrations range from 0.12 to 15 μ M, depending on cancer type and dosing schedule[132,199,200]. For example, administration of 20 mg/m² in AML/MDS patients produced peak plasma concentrations of 0.4–0.9 μ M[201], while higher dose regimens yielded up to 15 μ M[132]. In a phase I trial involving patients with solid tumors, decitabine was administered as a 6-hour IV infusion at doses ranging from 5 to 135 mg/m², and resulting in plasma concentrations of 0.1 to 6 μ M[200]. These decitabine concentration ranges overlap the transition

point between decreased and increased total DNA and *p16* promoter CpG methylation, and increased and decreased *p16* expression that we observed. This suggests that some decitabine dosing schedules may increase, while others decrease *p16* expression depending on the concentration achieved in the tumor. We expect a similar paradigm exists with GSK3685032, but since it has not entered human clinical trials we don't know what its typical plasma concentration range is. Ultimately, selection of the correct doses for decitabine and GSK3685032 may determine their success as potential therapeutics for gastric cancer characterized by *p16* promoter hypermethylation[202] and potentially other solid tumor cancers[203]. Our results also suggest the DNMT inhibitors can cause unanticipated changes in off-target gene expression that may limit their usefulness, an observation we have also made with histone deacetylase inhibitors and histone lysine methyltransferase inhibitors[204,205]. However, it must be noted that the present study is an in vitro proof of concept in gastric cancer cells. Therefore, future translational validation, preferably in humans, will be needed to validate the clinical relevance, of our findings.

We first used LC-MS/MS to quantify total DNA methylation induced by decitabine and GSK3685032 showing a strong negative correlation with *p16* expression, a relationship further supported by *p16* promoter CpG methylation from pyrosequencing. Notably, *p16* expression correlated more strongly with total DNA methylation than with promoter methylation, highlighting the utility of our quantitative total DNA methylation method. These findings suggest that total DNA methylation may serve as a marker for *p16* promoter CpG methylation, especially during treatment with decitabine and GSK3685032. In fact, it may eventually be possible to titrate the dose of decitabine or GSK3685032 to total DNA methylation to ensure optimal induction of *p16* expression.

We confirmed that decitabine increases *p21*[184,185] and *p16* expression in gastric cancer cell lines[168,186] . We showed for the first time that GSK3685032 upregulated *p16* and had a weaker or negligible effect on *p21*, depending on the cell type. Decitabine treatment favored cell cycle arrest in the G2/M phase, whereas GSK3685032 induced G0/G1 arrest. This difference aligned with the roles of *p21* and *p16* in cell cycle regulation, where *p21* regulates both the G1 and G2/M phases[206], while *p16* primarily promotes G0/G1 phase arrest[207]. Therefore, shifts in cell cycle arrest to the G0/G1 or G2/M phases depend on the relative increase in *p16* or *p21* expression, respectively. Decitabine induced a greater maximal increase in *p21* mRNA expression compared to GSK3685032. This was supported by increased *p21* protein levels at 50 μ M in both cell lines (**Figure 2.6S and 2.6U**). Accordingly, decitabine produced statistically significant increases in the percentage of AGS and SNU719 cells in G2/M at 50 μ M where *p16* mRNA and protein was reduced compared to the maximum produced at 1 μ M (**Figure 2.6M, 2.6O, 2.6Q, 2.6S and 2.6U**), and where total DNA methylation increased (**Figure 2.3B and 2.3D**), and where *p16* promoter CpG methylation returned to pretreatment levels (**Figure 2.4A and 2.4B**). In contrast, GSK3685032 at 1-10 μ M in AGS and 10-25 μ M in SNU719 cells induced a greater maximal increase in *p16* mRNA expression compared to decitabine (**Figure 2.3F, 2.3H, 2.6P and 2.6R**), with corresponding significant increases in *p16* protein in AGS but not in SNU719 cells. This aligned with a minimum in total DNA methylation (**Figure 2.3E and 2.3G**) and *p16* promoter CpG methylation (**Figure 2.4C and 2.4D**). Although *p16* expression was most significantly upregulated in AGS cells treated with 1 μ M GSK3685032 (**Figure 2.6P**), the strongest G0/G1 phase arrest was observed at 25 μ M (**Figure 2.6J**). Corroborating our results, previous studies have shown that decitabine increased DNMT3B expression[196]. Our results confirm known effects of decitabine on *p21*, *p16* and G2/M arrest[184,185], but for the first time we demonstrate

that GSK3685032 preferentially enhances p16, has weaker effects on p21, and induces G0/G1 arrest in gastric cancer cells showing the subtle differences in these DNMT inhibitors.

GSK3685032 induced changes in *p16* and *p21* mRNA levels but sometimes failed to show corresponding changes in protein. For example, GSK3685032 increased p21 mRNA in SNU719 cells but not in AGS cells, but there was no significant increase in p21 protein level at any dose or cell line. Similarly, GSK3685032 induced significant increases in *p16* mRNA at 1-10 μ M in AGS and 10-25 μ M in SNU719 cells, but a significant increase in p16 protein was only observed in AGS cells at 10 μ M. Previous reports[160]show that decitabine alters gene expression more rapidly than GSK3685032, with change detectable as early as day 4. This may explain the absence of these expected changes in protein because we measured this after 3 days of GSK3685032 treatment.

The differing effects on *p16* and *p21* expression and cell cycle regulation induced by GSK3685032 and decitabine may be rooted in their differing mechanisms of action. GSK3685032 is a reversible competitive DNMT1 inhibitor, directly interacting with the DNMT1 active site loop and targeting hemi-methylated DNA, without causing DNA damage[164,208]. Indeed, Pappalardi *et al* reported no effects on γ H2AX, a marker of DNA damage, following GSK3685032 treatment[160]. Alternatively, decitabine broadly inhibits DNMT1, DNMT3A and DNMT3B by forming a covalent interaction with the enzyme's active site and exhibiting a non-competitive type of inhibition[209]. This results in replication stress, DNA double-strand breaks, and subsequent DNA damage[210–212]. Decitabine-induced *p21* expression appears to occur through passive demethylation of CpG sites in the *p73* promoter, leading to increased *p73* expression, which subsequently upregulates *p21* [213]. Another mechanism involves the release of HDAC1 from the *p21* promoter without promoter demethylation[214]. Since decitabine induces DNA damage, the

resulting increase in *p21* expression may facilitate DNA repair[215]. The lack of induced DNA damage by GSK3685032 may explain the relatively minor or absent increases in *p21* expression and the difference in cell cycle arrest induced by GSK3685032 compared to decitabine.

The pattern of decreasing then increasing total DNA methylation, increasing then decreasing *p16* and sometimes *TNS*, *PPIC* and *SCRNI* with increasing DNMT inhibitor concentration confirms that the drugs act genome-wide, broadly affecting the expression of many genes. However, over the treatment duration, the effects on *p16* promoter CpG methylation differ between decitabine and GSK3685032. Whereas decitabine reduces *p16* promoter CpG methylation by <50% at 1 μ M with methylation returning to pretreatment levels at 25 μ M, GSK3685032 reduces *p16* promoter CpG methylation by >50% at 10 μ M, and small but significant increases in CpG methylation are observed at 25 or 50 μ M depending on cell line (**Figure 2.4A, 2.4B, 2.4C, and 2.4D**). At these higher concentrations of GSK3685032, *p16* mRNA levels nearly return to pretreatment levels (**Figure 2.4E**) but there is only a significant decrease in *p16* protein levels in AGS and not SNU719 cells (**Figure 2.6 and S2.6**). The significant reduction in *p16* protein observed only in AGS cells may be due to the slower proliferation rate of SNU719 cells (~43 h vs. ~20 h in AGS) [216,217], which may delay the conversion of mRNA changes into detectable protein effects within the 72-hour treatment period. Although there appears to be a decrease in *p16* protein in SNU719 cells, it did not reach statistical significance. In addition, with decitabine, the increases and decreases in *p16* mRNA and protein are significant in both cell lines at 1 μ M. This delay in returning to pretreatment CpG methylation levels with GSK3685032 in comparison to decitabine may be the reason that decitabine induces faster changes in gene expression compared to GSK3685032[160] as we observed above.

GSK3685032 induces fewer changes in histone modifications than decitabine. Previous studies show that decitabine can increase histone H3K4ac and H3K9ac[218], but decrease histone H4K8ac, H4K12ac, and H4K16ac[219]. We find that both GSK3685032 and decitabine decrease total histone lysine acetylation (Kac) in AGS cells. H2AK5ac and H4K16ac are reduced in colorectal cancer cell lines treated with 5-azacitidine. Moreover, the combination of 5-azacitidine and panobinostat, a pan-histone deacetylase (HDAC) inhibitor, demonstrated greater efficacy than either drug alone at inhibiting cell proliferation and reducing tumor growth[220]. This suggests that DNMT inhibitors alone may not fully reactivate gene expression since histone deacetylation (in the absence of CpG methylation) can still maintain chromatin in a closed state, preventing transcription[221]. The loss of histone acetylation that we and others have observed with DNMT inhibitor treatment may represent a compensatory mechanism that counterbalances the reduction in DNA methylation, preserving at least some transcriptional repression for some genes, during treatment. This may also explain why DNMT inhibitors are sometimes used with HDAC inhibitors clinically, aiming to overcome these compensatory mechanisms[221,222].

Histone lysine methylation decreases with decitabine in AGS and SNU719 cells and GSK3685032 in AGS cells, suggesting the DNMT inhibitors may alter the expression of lysine methyltransferases. Indeed, decitabine reduces SUV39H1 expression, decreasing H3K9me3 in diffuse large B-cell lymphoma[223] and H3K9me2 in bladder cancer cells[224], and downregulates EZH2[225], reducing H3K27me3.

Previous studies using Western blots suggested that DNMT1 was degraded during treatment with decitabine and GSK3484862, a compound structurally related to GSK3685032[164,196,226]. Our study failed to show changes in DNMT1 protein using ELISA, which can quantify DNMT concentrations but are not based on size[227]. It is unlikely that even a partially degraded protein

would still bind to both immobilizing and detecting antibodies used in our ELISA kits. Therefore, to understand the difference in results between detection of DNMT with ELISAs or Western blots, it should be noted that decitabine is incorporated into DNA where it forms a stable, covalent, thioether bond with the DNMT, forming a DNMT-decitabine-DNA complex that no part of the Western blot procedure could reverse. The resulting DNMT-decitabine-DNA complex would not have the same mobility as DNMT on SDS-PAGE and would therefore not be detected at the same MW as DNMT using Western blots. In contrast, the DNMT-decitabine-DNA complex was apparently still detected by our ELISA assay kits. In this way, we detected no change in DNMT1 protein by ELISA [228], but Western blots may appear to show degradation because the DNMT-decitabine-DNA complex was not detected by this technique[228]. Alternatively, the difference in results could be because of our extended incubation time of 72h, and any DNMT degradation may be transient, with protein levels recovering by 72 h. A recent study showed that GSK3484862 induced degradation of DNMT1, but it did not show that GSK3685032 had a similar effect [164]. We did not find that GSK3685032 induced degradation of DNMT1, DNMT3A or DNMT3B.

Both control HEK293 cells and gastric cancer SNU216 cells show high *p16* expression with and without DNMT inhibitors, whereas *p16* expression was undetectable in MKN45 cells. However, HEK293, SNU216, and MKN45 cells exhibited sustained reductions in DNA methylation with increasing decitabine and GSK3685032, but not RG108. Promoter hypermethylation of *p16* was reported in SNU719, but not SNU216 cells [229], while MKN45 cells have a homozygous deletion of *p16* [230]. AGS and SNU719 cells are known to exhibit low or no *p16* expression due to CpG promoter hypermethylation[168,229], which we confirmed. Consequently, with DNMT inhibitors, particularly GSK3685032, *p16* expression exhibited the strongest negative correlation with total DNA methylation among all the genes we studied.

RG108 yielded a significant increase in p16 expression at 200 μ M in SNU719 cells (**Figure 2.2F**), despite no significant changes in total DNA methylation. p16 expression is governed by promoter CpG methylation rather than total DNA methylation [52,168], and total DNA methylation change does not necessarily correlate with changes in promoter CpG methylation. Therefore, RG108 may selectively affect *p16* promoter CpG methylation at high concentrations, leading to increased p16 expression without altering total DNA methylation. Unlike GSK3685032 and decitabine, RG108 shows sequence-specific effects on DNA methylation. For example, decitabine decreased methylation at three CpG sites in leukemia cells in the long interspersed nuclear element-1 sequence, which is known to be highly methylated, but even high doses of RG108 induced no significant changes in methylation at these CpG sites[231]. In addition, RG108 did not affect the methylation of centromeric satellite sequences, where decitabine produced significant decreases in methylation [52]. Therefore, RG108 may decrease *p16* promoter methylation without significantly affecting total DNA methylation. However, we did not study RG108 further because it is not used clinically, nor is it in clinical testing, and the doses needed to increase p16 expression were much higher than decitabine or GSK3685032.

2.5 Supplementary

2.5.1 Total DNA methylation assay validation

2.5.1.1 LCMS procedure

The analysis for detecting mdC (5-methyl-2'-deoxycytidine), N6-mdA (N₆-methyl-2'-deoxyadenosine), hmdC (5-hydroxymethyl-2'-deoxycytidine), fdC (5-formyl-2'-deoxycytidine), and cadC (5-carboxyl-2'-deoxycytidine) was conducted using a Shimadzu Nexera UHPLC system coupled to a Shimadzu MS-8040 triple quadrupole mass spectrometer. Detection was performed using electrospray and atmospheric pressure chemical ionization (DUIS) in positive ion mode with

multiple reaction monitoring (MRM) targeting specific m/z transitions for the analytes and their respective internal standards (IS). Detailed m/z values are provided in **Table S2.1**. For mdC, N₆-mdA, fdC, and cadC, separation was performed on an Acquity BEH C18 column (1.7 μ m, 2.1 $\times$ 100 mm, Waters, USA) under a pH gradient with organic mobile phase A (methanol containing 0.1% formic acid, 2 mM ammonium formate, and 0.1 mM malic acid) and aqueous mobile phase B (water containing 0.1% formic acid, 2 mM ammonium formate, and 0.1 mM malic acid) with a flow rate of 400 mL/min. The injection volume was 3 μ L. 5% B is held for 2 min, stepped to 90% B at 4 min and held for 4 min then stepped down to 5% B and held for 5 min.

For hmdC, separation was performed on a Primesep 200 column (2.1 $\times$ 100 mm, 3 μ m) at 40°C. The mobile phase consisted of phase A (water containing 0.05% formic acid, 2 mM ammonium formate, and 0.1 mM malic acid) and phase B (80% acetonitrile and 20% water containing 1% formic acid, 2 mM ammonium formate, and 0.1 mM malic acid) with a flow rate of 500 μ L/min. The gradient was set as follows: 2% phase B held for 1 minute, increased to 90% B and held for 5 minutes, then reduced to 2% B and held for 3 minutes.

Table S2.1 Analytes abbreviation and MRM transitions

Analyte	Abbreviation	Precursor > Product Ion (<i>m/z</i>)	Analyte/Internal Standard (IS)
2'-Deoxyguanosine	dG	267.7 > 152.1	Analyte
5-Methyl-2'-deoxycytidine	mdC	242.1 > 126.3	Analyte
5-(Hydroxymethyl)-2'-deoxycytidine	hmdC	258.1 > 142.1	Analyte
5-Carboxyl-2'-deoxycytidine	cadC	272.1 > 156.1	Analyte
5-Formyl-2'-deoxycytidine	fdC	245.1 > 129.0	Analyte
N6-Methyl-2'-deoxyadenosine	N6-mdA	266.0 > 150.1	Analyte
5-Methyl-2'-deoxycytidine-d3	mdC-d3	245.1 > 129.0	IS for mdC
5-Hydroxymethyl-2'-deoxycytidine-d3	hmdC-d3	261.2 > 145.1	IS for hmdC
15N5-2'-deoxyguanosine	15N5-dG	273.0 > 157.1	IS for dG
N6-Methyl-2'-adenosine-d3	N6-mdA-d3	269.2 > 153.2	IS for N6-mdA
5-Fluorocytidine	Fluo	231.7 > 116.1	IS for cadC and fdC

2.5.2 Validation results

The method was validated based on linearity, accuracy and precision according to the US Food and Drug Administration (FDA) bioanalytical method validation guidelines. The intra- and inter-day precision and accuracy for all analytes in QC and LLOQ samples are given in **Table S2.2**. The intra- and inter-batch bias and CV were less than 15% at LLOQ level. For all the QC samples, the intra- and inter-batch precision was below 15% and the bias was less than 15%. Therefore, the methods presented acceptable accuracy and precision.

Table S2.2-1 Typical calibration curve for analytes

Analyte	Calibration Curve			
	LLOQ(nM)	ULOQ(nM)	typical equation	R ²
mdC	2	800	$y=0.00229252x+0.9967668$	0.9968
hmdC	0.4	160	$y=0.0144525x+0.00132997$	0.9982
mdA	0.2	80	$y=0.0126635x+0.9936312$	0.9936
fdC	0.8	320	$y=0.0113142x+0.997675$	0.9977
cadC	0.8	320	$y=0.00137487x+0.9919478$	0.9919

Table S2.2-2 Precision and accuracy validation for mdC (n=3 with 6 replicates per batch)

Concentration spiked (nM)	Intra-batch			Inter-batch		
	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)
2	1.94 $\pm$ 0.06	2.87	-3.32	2.02 $\pm$ 0.18	0.18	8.82
6	6.51 $\pm$ 0.15	2.26	1.27	6.45 $\pm$ 0.28	4.41	7.57
50	54.59 $\pm$ 0.69	1.27	6.90	55.53 $\pm$ 2.08	3.75	11.06
640	647 $\pm$ 3.19	0.49	0.78	673.83 $\pm$ 25.97	3.85	5.29

Table S2.2-3 Precision and accuracy validation for hmdC (n=3 with 6 replicates per batch)

Concentration spiked (nM)	Intra-day			Inter-day		
	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)
0.4	0.36 $\pm$ 0.03	9.33	0.32	0.40 $\pm$ 0.04	10.61	-0.06
1.2	1.22 $\pm$ 0.03	2.26	2.65	1.24 $\pm$ 0.08	6.19	3.63
10	10.00 $\pm$ 0.23	2.29	-0.62	9.87 $\pm$ 0.22	2.25	-1.26
128	125.67 $\pm$ 1.49	1.19	-1.41	124.94 $\pm$ 1.77	1.42	-2.39

Table S2.2-4 Precision and accuracy validation for mdA (n=3 with 6 replicates per batch)

Concentration spiked (nM)	Intra-batch			Inter-batch		
	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)
0.2	0.21 $\pm$ 0.01	6.57	-5.28	0.20 $\pm$ 0.03	14.84	0.76
0.6	0.64 $\pm$ 0.02	3.44	6.96	0.65 $\pm$ 0.03	4.11	7.73
5	5.10 $\pm$ 0.13	2.49	2.18	5.34 $\pm$ 0.19	3.55	6.81
64	64.65 $\pm$ 1.24	1.93	1.27	68.15 $\pm$ 3.05	4.47	6.48

Table S2.2-5 Precision and accuracy validation for fdC (n=3 with 6 replicates per batch)

Concentration spiked (nM)	Intra-batch			Inter-batch		
	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)
0.8	0.92 $\pm$ 0.04	3.82	12.86	0.86 $\pm$ 0.08	9.08	6.98
2.4	2.63 $\pm$ 0.08	3.06	3.08	2.44 $\pm$ 0.19	7.94	1.70
20	19.13 $\pm$ 0.33	1.73	-4.64	21.0 $\pm$ 1.88	8.93	4.99
256	232.19 $\pm$ 12.95	5.58	-10.46	261.12 $\pm$ 27.52	10.54	2.00

Table S2.2-6 Precision and accuracy validation for cadC (n=3 with 6 replicates per batch)

Concentration spiked (nM)	Intra-batch			Inter-batch		
	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)	Concentration measured (mean $\pm$ SD, nM)	Precision (CV; %)	Accuracy (bias; %)
0.8	0.68 $\pm$ 0.04	5.28	-9.36	0.77 $\pm$ 0.08	10.49	-3.90
2.4	2.26 $\pm$ 0.17	7.56	-7.04	2.30 $\pm$ 0.17	7.44	-3.99
20	20.93 $\pm$ 1.16	5.55	4.07	21.67 $\pm$ 1.23	5.65	8.37
256	125.67 $\pm$ 1.49	1.19	-1.41	279.16 $\pm$ 18.74	6.71	9.05

2.5.3 DNA hydrolysis validation

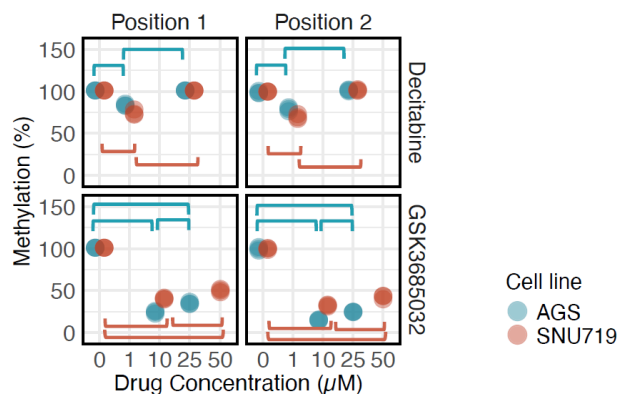
To ensure complete DNA hydrolysis, synthetic double DNA strands containing a single modification (e.g., mdC) were obtained from Integrated DNA Technologies (IDT). These DNA strands were used as standards to evaluate the efficiency of DNA hydrolysis. The digest mix was prepared by adding 250 units of Benzonase, 300 mUnits of phosphodiesterase I (Sigma P-3243), and 200 units of alkaline phosphatase (Sigma P-7923) to a 0.5 mL solution of 100 mM ammonium bicarbonate buffer with 5 mM MgCl₂. The hydrolysis was carried out at 37°C for 96 hours using 20 µg of DNA and 50 µL of the digest mix. The recovery is shown in **Table S2.2**.

Table S2.2 Enzymatic Hydrolysis Recovery of DNA Modifications (n=3)

DNA modification	mdC	mdA	hmdC	fdC	CadC
Spiked Amount (nM)	326	32.6	127.4	236.5	288
Measured Amount (nM)	276.457	31.6737	107.275	252.919	215.431
Recovery	84.80%	97.20%	84.20%	106.90%	74.80%
%RSD	2.90%	8.20%	3.80%	18.70%	3.00%

2.5.4 Pyrosequencing assays for quantifying methylation at CpG sites in the p16 promoter

Assay 1



Assay 2

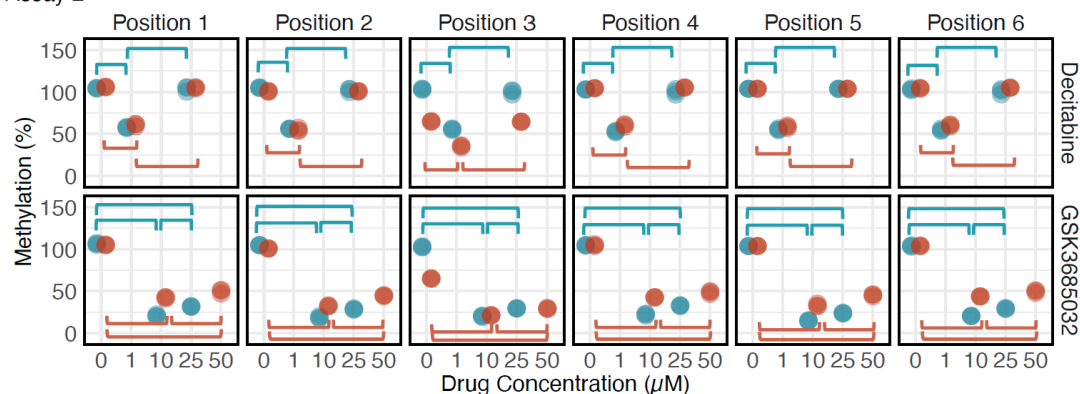


Figure S2.1 Pyrosequencing assays for quantifying methylation at CpG sites in the p16 promoter.

Assay 1 measured two CpG sites, and Assay 2 measured six CpG sites. Methylation levels were calculated using a standard curve generated from methylation standards to correct for assay-specific variation. Differences in methylation between drug concentrations and cell lines were tested by ANOVA followed by Tukey's post hoc test for pairwise comparisons. Bars indicate significant comparisons (p.adj < 0.01). The only non-significant comparison was between 0 μ M and 25 μ M Decitabine in both cell lines and assays.

2.5.5 Decitabine and GSK3685032 increased DNMT3B and decreased TET in AGS cells and SNU719 cells

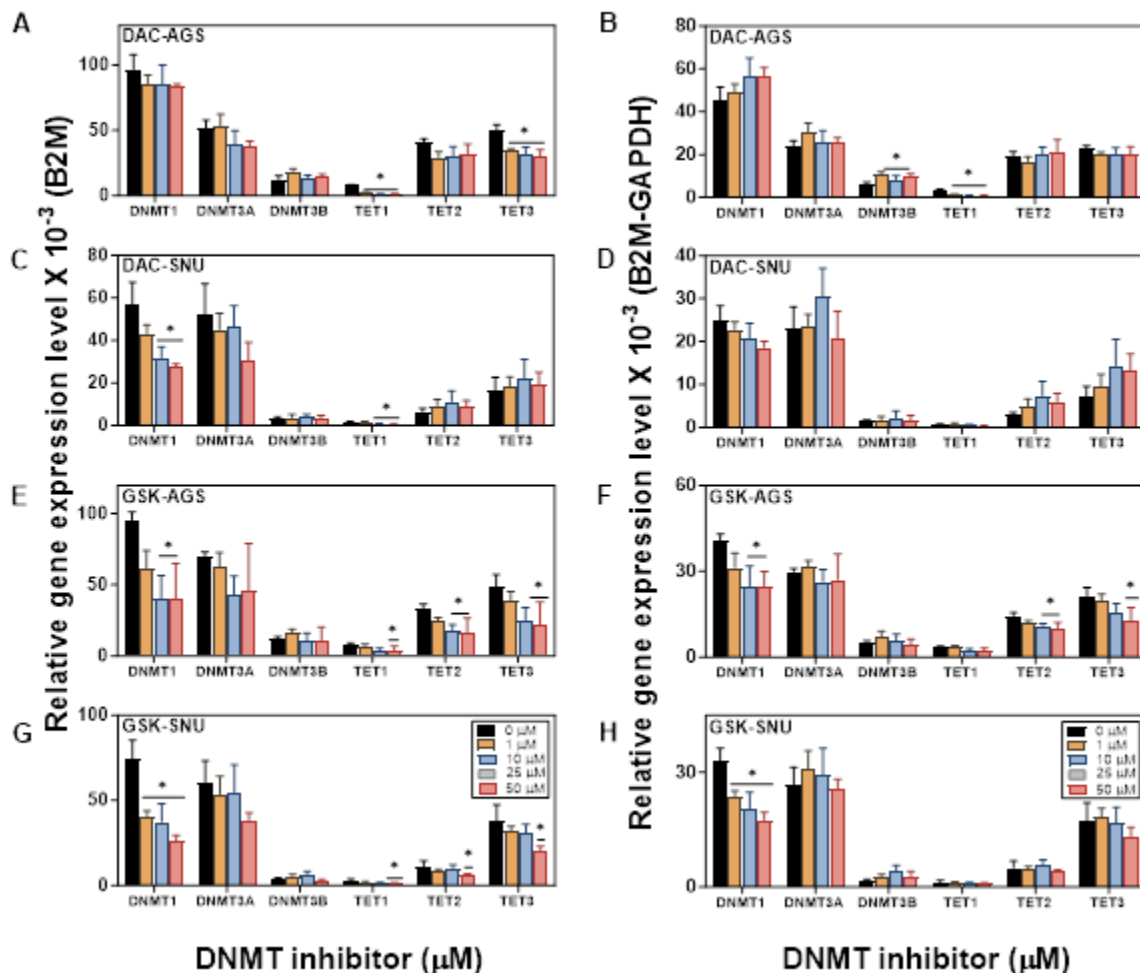


Figure S2.2 Decitabine and GSK3685032 increased DNMT3B expression and/or decreased TET expression on mRNA level in AGS cells and SNU719 cells after 72 h treatment.

The results presented in Figures A, C, E, and G were normalized using *B2M*. In AGS cells, decitabine treatment resulted in a significant reduction in *TET1* and *TET3* expression levels (A), whereas in SNU719 cells, decitabine downregulated *DNMT1* and *TET1* expression (C). Treatment with GSK3685032 led to reductions in *DNMT1*, *TET1*, and *TET3* levels in both AGS and SNU719 cells (E and G). Conversely, Figures B, D, F, and H, normalized using combination of *B2M* and *GAPDH*, demonstrated that in AGS cells, decitabine increased *DNMT3B* expression while decreasing *TET1* levels (B). In SNU719 cells, no significant changes were observed following decitabine treatment (D). For GSK3685032-treated cells, AGS displayed downregulation of *DNMT1*, *TET1*, and *TET2* expression, For GSK3685032-treated cells, AGS cells exhibited downregulation of *DNMT1*, *TET1*, and *TET2* expression (F), whereas SNU719 cells showed a decrease only in *DNMT1* expression (H) Asterisk (*) indicates statistical significance (**P*<0.05, ***P*<0.01, ****P*<0.001, *****P*<0.0001) compared with the control group (0 mM DNMT inhibitor). Values

represent the mean of three measurements with error bars indicating SD. DAC-SNU: SNU719 cells with decitabine; GSK-AGS: AGS cells with GSK3685032; GSK-SNU: SNU719 cells with GSK3685032.

2.5.6 The protein level changes in p21 and p16 in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.

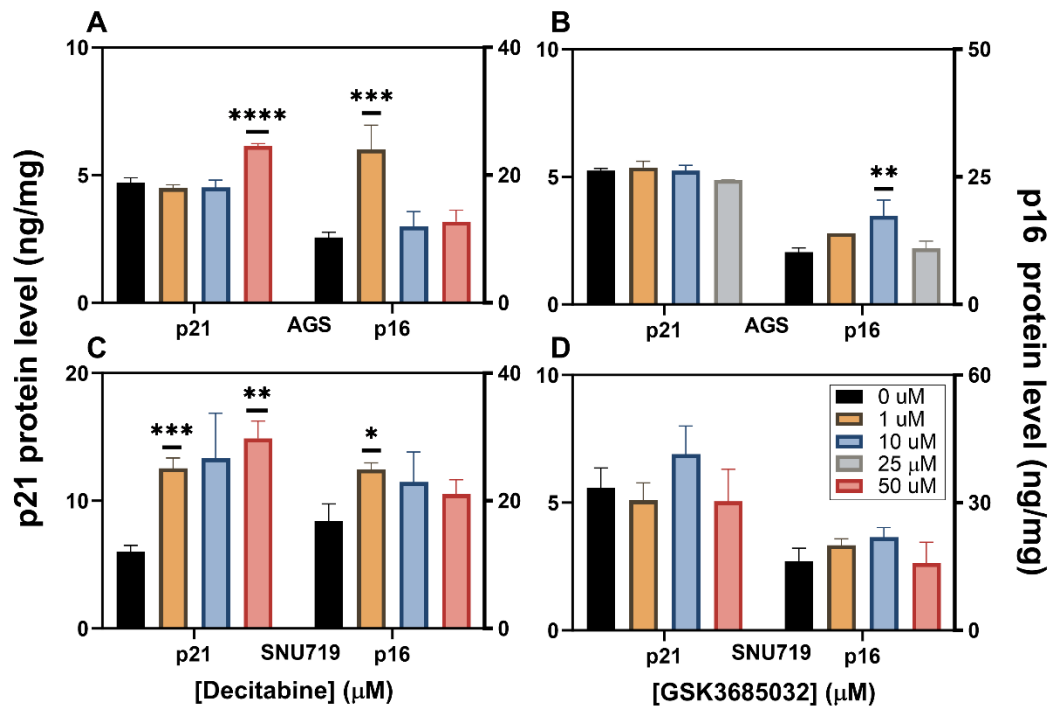


Figure S2.3 The changes in p21 and p16 in AGS and SNU719 cells treated with decitabine or GSK3685032 for 72h.

The cell cycle regulators p21 and p16 protein level measure by Elisa in AGS and SNU719 cells treated with decitabine (A and C) or GSK3685032 (B and D) for 72 h. A one-way ANOVA was used to compare the difference between the different concentrations in one cell line treated with the same DNMT inhibitors (P-value < 0.05 considered significant). Asterisk (*) indicates statistical significance (*P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001) compared with the control group (0 μM DNMT inhibitor). Values for p21 and p16 protein level represent the mean of three measurements with error bars indicating SD.

Chapter 3: Genes Associated with DNMT Inhibitor-Induced Changes in CDKN2A/p16 expression in Gastric Adenocarcinoma

3.1 Introduction

Gastric adenocarcinoma accounts for over 90% of gastric cancer and is sometimes caused by epigenetic dysregulation [232,233], [234]. For example, promoter CpG hypermethylation can silence the tumor suppressor *CDKN2A*, resulting in gastric adenocarcinoma [235]. Understanding how the effects of these epigenetic alterations propagate through gene regulatory networks can identify new therapeutic targets and predict treatment responses.

CDKN2A encodes two distinct tumor suppressor proteins, p16^{INK4a} (p16) and p14^{ARF} (p14) [236,237]. In gastric cancer, *CDKN2A*/p14 expression is generally low compared with *CDKN2A*/p16 [238], and *p16* promoter hypermethylation has been widely recognized as a biomarker of gastric cancer [172,239]. Therefore, because this study focuses on gastric adenocarcinoma, *CDKN2A*/p16 is used when referring collectively to the *CDKN2A* gene and its *p16*-related biological context, including *CDKN2A*/p16-associated networks, *CDKN2A*/p16-synchronized response genes, and *CDKN2A*/p16 expression and reactivation. When a specific molecular level is intended, terminology has been clarified accordingly. *CDKN2A* is used for non-isoform-specific gene-level measurements and database-derived analyses, including gene deletion, mRNA expression, qPCR analysis, and *CDKN2A*-centered network or correlation analyses. *p16* promoter methylation is used when the methylation analysis specifically concerns the *p16*^{INK4a} promoter region. p16 is used when referring to the p16 protein or its established biological functions, including CDK4/6 inhibition and G1/S checkpoint regulation.

DNA methyltransferase (DNMT)-catalyzed *p16* promoter CpG hypermethylation, silences *p16* expression, and occurs in 30-45% of gastric adenocarcinoma cases, correlating with poor prognosis [240,241]. p16 enforces the G1/S checkpoint by inhibiting CDK4/6. Therefore, silencing of p16 permits uncontrolled cell cycle progression in gastric adenocarcinoma [242]. DNMT inhibitors can reverse *p16* promoter CpG hypermethylation thereby reactivating p16 expression, and they have been suggested as potential treatments for gastric adenocarcinoma. However, therapeutic agents aimed at single molecular targets often exhibit limited effectiveness and the

emergence of resistance, primarily as a consequence of inherent features of biological networks, such as robustness, redundancy, pathway crosstalk, and compensatory or neutralizing processes[243,244]. Consequently, increasing attention has been directed toward combination therapies [244]. Moreover, given its role, p16 reactivation is inherently a system-level event rather than an isolated molecular change. Systems-oriented identification of *CDKN2A*/p16-associated genes in gastric adenocarcinoma may facilitate the identification of new genes that can be co-targeted as part of combination therapeutic strategies. Here, co-target genes refer to genes that reside within *CDKN2A*/p16-associated regulatory networks and display expression changes concordant with p16 reactivation under DNMT inhibition.

Decitabine is a DNMT inhibitor used in hematological cancers, but it can also increase *p16* expression in gastric adenocarcinoma cells [245,246]. However, decitabine must be incorporated into DNA where it covalently binds to DNMTs resulting in a complicated mechanism and potential genotoxicity[202]. GSK3685032 is a reversible DNMT1-selective inhibitor that reduces DNA methylation without being incorporated into DNA[160] and it has not been used to dissect *CDKN2A*/p16-associated networks. We distinguish broad from selective DNA methylation-associated effects on p16 using various concentrations of DNMT inhibitors in gastric adenocarcinoma cells.

To identify *CDKN2A*/p16-associated and potential co-targeting genes, we integrated multi-database network reconstruction with experimental validation following treatment of gastric adenocarcinoma cells with DNMT inhibitors. We reconstructed *CDKN2A*/p16-anchored networks using The Cancer Genome Atlas (TCGA) and protein interaction databases; identified highly connected, critical regulators, called hub genes via topology analysis; and validated candidates by quantitative PCR (qPCR) with increasing concentrations of decitabine and GSK3685032 in gastric adenocarcinoma cell lines with differing *CDKN2A* expression status. This approach identifies and validates *CDKN2A*/p16-associated genes during DNMT inhibitor treatment, providing mechanistic insights into gastric adenocarcinoma associated with low *CDKN2A* expression and highlighting potential co-target gene candidates.

3.2 Materials and Methods

3.2.1 Integrative gene and pathway dataset construction for gastric adenocarcinoma network analysis

A multi-step integrative strategy was developed to systematically identify, prioritize, and validate genes associated with gastric adenocarcinoma, with particular emphasis on the CDKN2A/p16 regulatory context across the cell lines AGS, SNU216, SNU719, and MKN45. Candidate genes were first compiled from multiple complementary resources, including GETdb (Genetic and Evolutionary Features of Drug Targets Database), OncoKB (Oncology Knowledge Base), UALCAN (The University of Alabama at Birmingham Cancer Data Analysis Portal), CTD (The Comparative Toxicogenomics Database), KEGG (Kyoto Encyclopedia of Genes and Genomes), NeDRex (Network-based Drug Repurposing and exploration), DisGeNET, and STRING (Search Tool for the Retrieval of Interacting Genes/Proteins), yielding an initial disease-associated gene set. In parallel, genes belonging to seven key signaling pathways implicated in gastric cancer pathogenesis (ErbB, Ras, PI3K-Akt, Wnt/ β -catenin, TGF- β , VEGF, and Hippo) were extracted from KEGG. Cell-line-specific molecular features were additionally incorporated by querying DepMap (CRISPR-Cas9 and RNAi screens) and Cell Model Passports (RNA-seq and proteomics) for the four selected cell lines.

Prior work by our group and others has established that AGS and SNU719 cells exhibit low or undetectable baseline CDKN2A/p16 expression [19,20]. This epigenetic silencing was reversible upon treatment with decitabine or GSK3685032, leading to restoration of CDKN2A/p16 expression. In contrast, SNU216 cells showed relatively high constitutive expression of CDKN2A/p16, whereas CDKN2A/p16 expression was undetectable in MKN45 cells due to a homozygous deletion of CDKN2A [21–23].

Gene lists derived from the disease-module, pathway, and cell-line analyses were independently subjected to network reconstruction in Cytoscape using the GeneMANIA plugin, followed by multi-parameter centrality analysis (degree, betweenness, and additional topological metrics) with the NetworkAnalyzer and CentiScaPe plugins. Hub genes identified across these networks were integrated with CDKN2A to reconstruct cell-line-specific CDKN2A/p16 regulatory networks. Subsequent correlation analyses in the TCGA-STAD cohort (cBioPortal) linked gene expression

to CDKN2A gene-body/3'-UTR and promoter CpG methylation, enabling progressive refinement of a core CDKN2A/p16-associated gene set. This set was further validated through multi-database survival, expression, histological grade, enrichment, and protein-level analyses, culminating in experimental confirmation by TaqMan qPCR following dose-dependent treatment with DNMT inhibitors in the four gastric adenocarcinoma cell lines. **Figure 3.1** summarizes the integrated computational and experimental workflow encompassing multi-database gene and pathway curation, cell-line dependency profiling, network reconstruction and centrality analysis, CDKN2A/p16-centric network refinement, correlation-based prioritization, and final experimental validation.

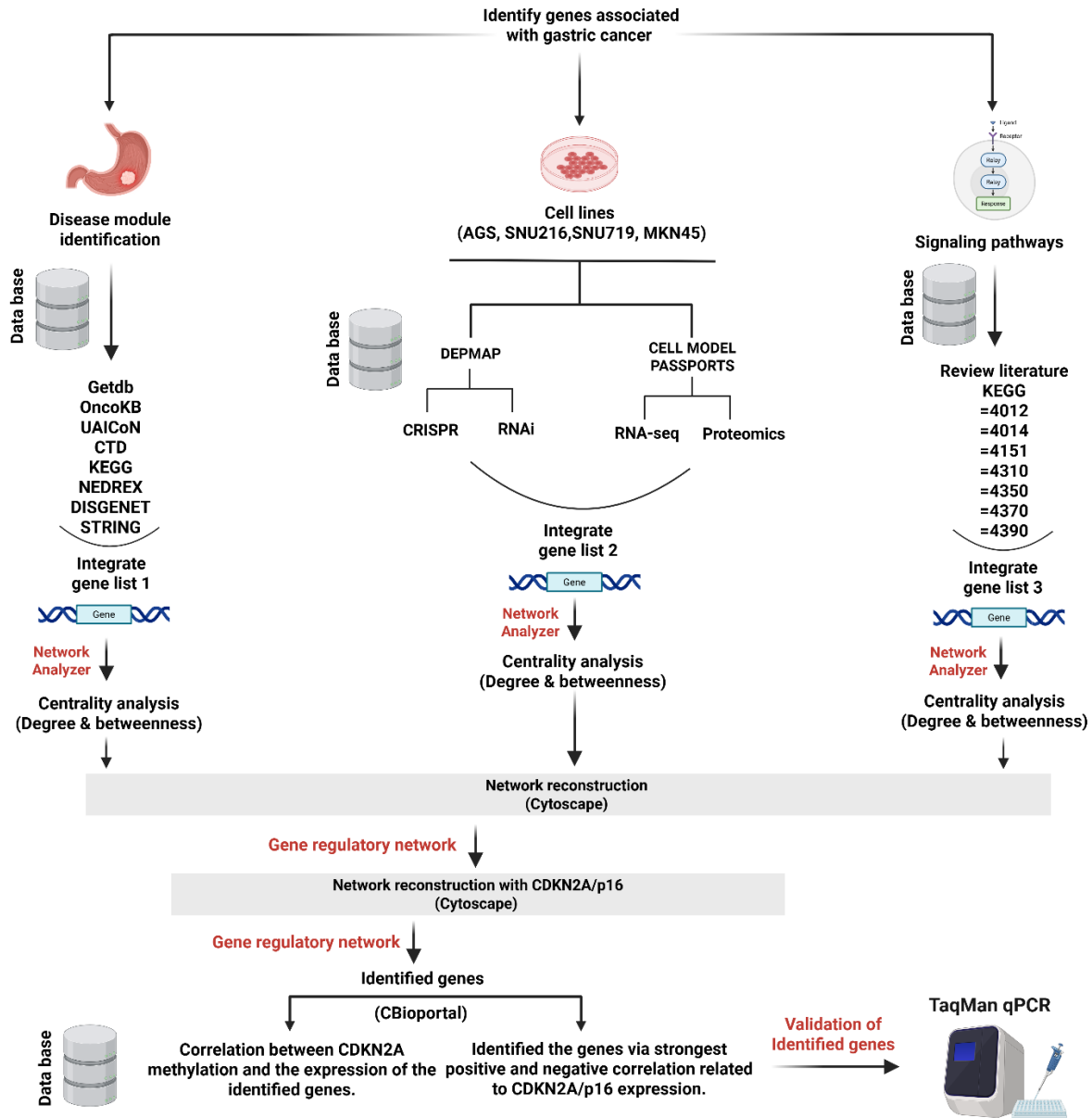


Figure 3.1 Integrated workflow for identifying and validating CDKN2A-associated genes in a gastric cancer cell lines.

Candidate genes were obtained from disease-related databases (Getdb, OncoKB, UALCAN, CTD, KEGG, NeDRex, DisGeNET, STRING), gastric cancer cell line datasets (DEPMAP, Cell Model Passports: CRISPR, RNAi, RNA-seq, proteomics), and signaling pathways (KEGG). Gene lists were integrated and analyzed for centrality (degree and betweenness), followed by Cytoscape network reconstruction, including *CDKN2A*-centered networks. Correlations between *CDKN2A* promoter methylation and gene expression were examined using cBioPortal. The most strongly associated genes were validated experimentally using TaqMan qPCR.

3.2.1.1 Genes associated with gastric adenocarcinoma

To build a detailed gastric adenocarcinoma gene database we extracted data from GETdb (<http://zhanglab.hzau.edu.cn/GETdb/page/index.jsp>)[247], OncoKB (<https://www.oncokb.org/>)[248], UALCAN (<https://ualcan.path.uab.edu/>)[249], CTD (<https://ctdbase.org/>)[250], KEGG (<https://www.genome.jp/kegg/>)[251], NeDRex (<https://nedrex.net/>)[252], and STRING (<https://string-db.org/>)[253]. Through this process, we successfully compiled a collection of 3,839 genes linked to gastric adenocarcinoma.

3.2.1.2 Pathways associated with gastric adenocarcinoma

To ensure that no relevant data were overlooked, we implemented a second complementary strategy for gene selection that differed from the first approach, in which multiple databases were queried collecting 3839 genes. In this second approach, we started with seven key signaling pathways (**Table 3.1**) known to play central roles in gastric cancer pathogenesis. The genes comprising each pathway were then extracted from the KEGG, resulting in 809 unique genes. These two strategies provided a robust and exhaustive list of candidate gastric adenocarcinoma genes.

Table 3.1 List of pathways to build a comprehensive gene database for gastric adenocarcinoma

Targeted Pathways for In-Depth Analysis			
hsa04012	ErbB signaling pathway	hsa04350	TGF-beta signaling pathway
hsa04014	Ras signaling pathway	hsa04370	VEGF signaling pathway
hsa04151	PI3K-Akt signaling pathway	hsa04390	Hippo signaling pathway
hsa04310	Wnt/ β -Catenin signaling pathway	/	/

3.2.1.3 Cell lines associated with gastric adenocarcinoma

We expanded the computational design to incorporate an additional layer of analysis focused on cell line features of the AGS, MKN45, SNU216, and SNU719 cell lines. For this phase, we used The Cancer Dependency Map (DepMap, <https://depmap.org/portal/>)[254] and Cell Model

Passports (<https://cellmodelpassports.sanger.ac.uk/passports/SIDM00850>)[255]. The Cancer Dependency Map (DepMap) uncovers gene dependencies within a wide range of cancer cell lines, by combining >500 RNAi, [256] and >300 CRISPR-Cas9 screens [257] to generate a comprehensive cancer gene dependency map, to identify cancer vulnerabilities and drug targets [254]. The top ten genes and all genes identified through CRISPR-Cas9 and RNAi methodologies were retrieved for analysis across the four cell lines, allowing comparison of genetic dependencies. The Cell Model Passports database combines RNA-Seq and proteomics data to predict genes that drive cancer at the mRNA and protein levels [255]. All genes identified through this dual approach were systematically extracted for our four cell lines, facilitating the analysis of molecular profiles and their implications for cancer research.

3.2.1.4 Network reconstruction and centrality analysis

The network reconstruction was repeated 16 times, incorporating varying gene counts derived from distinct experimental conditions. This process utilized the GeneMANIA plugin [258] within the Cytoscape 3.9.1 platform to build the networks [259]. The input data for the first two networks were derived from genes associated with gastric adenocarcinoma, gathered from multiple databases, and genes linked to signaling pathways that influence gastric adenocarcinoma, respectively. Additionally, 14 networks were independently reconstructed using four distinct data types described in Section 3.2.1.3: DepMap (CRISPR-all genes), DepMap (RNAi-all genes), Cell Model Passports (RNASeq), and Cell Model Passports (proteomics) across the our four different cell lines yielding a total of sixteen network contexts.

Hub genes (also known as highly connected or central regulators in biological networks) are genes (or nodes) that exhibit high connectivity and/or centrality within the reconstructed network,

indicating their potential importance in coordinating biological processes, regulating multiple targets, or maintaining network integrity. We identified hub genes within each reconstructed network by computing network topology parameters: degree, betweenness, closeness, stress, eccentricity, centroid, radiality, eigenvector centrality, bridging centrality, clustering coefficient, neighborhood connectivity, and topological coefficient utilizing the CentiScaPe[260] and NetworkAnalyzer plugins [261] in Cytoscape. The top ten genes with the highest values for each topological parameter were selected and combined for subsequent analysis [262,263].

In the subsequent phase, genes exhibiting the highest frequency across multiple centrality measures were identified using a binary matrix, where one value denoted a gene's presence and zero indicated its absence. A higher frequency for a gene across various centrality metrics indicates its greater importance within the network [264]. By evaluating gene presence across all centrality measures and within both the degree and betweenness groups, we successfully pinpointed the most significant genes.

3.2.1.5 *CDKN2A/p16*-Cell line specific Hub Genes Network reconstruction

After identifying the hub genes associated with each of our four cell lines, a two-step strategy was developed to evaluate the role of *CDKN2A/p16* and elucidate its regulatory network for each cell line. The first step involved integrating data from the DepMap database (CRISPR Top 10, CRISPR hub genes derived from the entire network, RNAi Top 10 genes, and RNAi-hub genes from the whole network), as well as hub genes from RNA-seq and proteomics data obtained from the Cell Model Passports database, and hub genes those associated with disease- and pathway-related networks. In the second step, the *CDKN2A* gene was incorporated into the four refined gene lists, allowing for the reconstruction of a *CDKN2A/p16*-specific regulatory network for each cell line.

At this stage, we identified 35 genes associated with *CDKN2A*/p16 shedding light on its potential regulatory connections.

3.2.1.6 Validation of the relationship between the expression of identified genes and *CDKN2A* gene body/3' UTR CpG -methylation level

3.2.1.6.1 *Network-Correlation-based Approach*

After pinpointing 35 genes associated with *CDKN2A*/p16, we investigated how *CDKN2A* cg12840719 gene body and 3' untranslated region (UTR) CpG methylation levels correlate with the expression of each gene using the cBioPortal database (<https://www.cbioportal.org/>) and Stomach (gastric) Adenocarcinoma cohort (TCGA, PanCancer Atlas)[265]. First, the aim was to identify key genes within the *CDKN2A*/p16 network, focusing on those with significant centrality and strong correlations between their expression and *CDKN2A* gene body / 3'-UTR CpG methylation levels, as measured by Pearson and Spearman correlation methods. Gene body/ 3'-UTR methylation was examined as a complementary database-derived epigenetic feature to identify broader *CDKN2A* methylation-associated transcriptional relationships, recognizing that its association with gene expression may be context dependent. If both correlation analyses were significant, the corresponding genes were selected for further research. The DNA methylation profile was established using a merged dataset from the Infinium HumanMethylation27 (HM27) and HumanMethylation450 (HM450) platforms. The mRNA expression profile was derived from RNA-Seq data processed with the RNA-Seq by Expectation-Maximization (RSEM) algorithm and batch-normalized using the Illumina HiSeq_RNASeqV2 platform. Both datasets were analyzed on a logarithmic scale to facilitate interpretation and comparison. This analysis narrowed the list to 24 genes that met both criteria, significant centrality and strong correlations, as assessed using

spearman rank correlation (**Figure 3.2A**) and Pearson correlation (**Figure 3.2B**) forming the first gene set (Set 1) for further investigation (**Table S3.1**).

3.2.1.6.2 *Correlation-based approach*

To identify genes associated with *CDKN2A* using the TCGA Stomach Adenocarcinoma (TCGA-STAD) PanCancer Atlas dataset, we employed a correlation-based approach. Initially, we selected the top 10 genes exhibiting the highest positive and negative correlations with *CDKN2A* expression, as determined using Spearman and Pearson's correlation coefficient ($P < 0.05$). This resulted in two lists of 10 genes each. These lists were combined, yielding a total of 20 genes. Subsequently, we evaluated the association between the expression of these 20 genes and *CDKN2A* gene body/3'-UTR CpG methylation levels as measured by both Pearson and Spearman correlation methods. If both correlation analyses were significant, the corresponding genes were selected for further analysis. The DNA methylation profile was established using the merged dataset and analysis above (Section 3.2.1.6.1). Following this analysis, 15 genes were retained based on statistically significant associations ($P < 0.05$) with *CDKN2A* gene body/3'-UTR CpG methylation and were assessed using Spearman rank (**Figure 3.2C**) and Pearson correlation (**Figure 3.2D**), forming the second gene set (Set 2) for further investigation (**Table S3.2**).

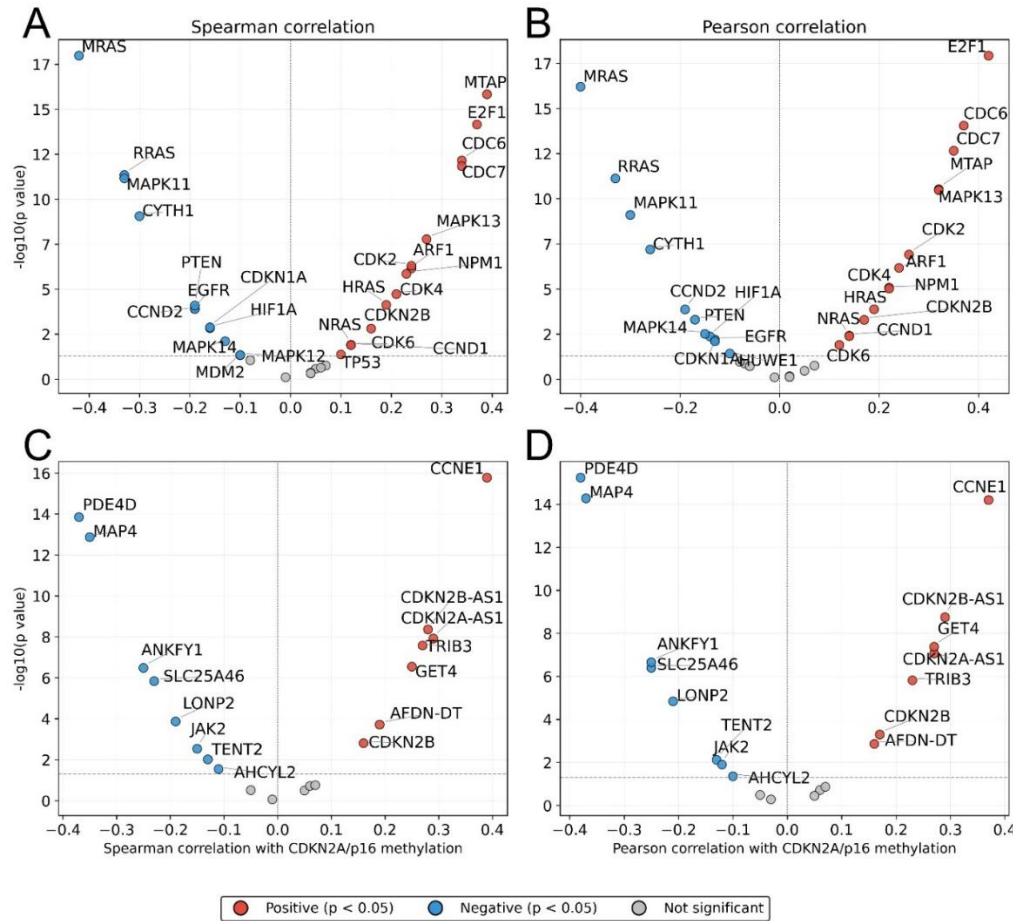


Figure 3.2 Correlation between CDKN2A methylation and gene expression.

Volcano-style correlation plots showing associations between CDKN2A methylation levels and gene expression for 24 genes identified using the network-correlation-based approach (A, B) and 20 genes identified using the correlation-based approach (C, D). Correlations were assessed using Spearman rank correlation (A, C) and Pearson correlation (B, D). The x-axis represents the correlation coefficient (Spearman ρ or Pearson r), and the y-axis represents $-\log_{10}(p\text{-value})$. Genes positively correlated with CDKN2A methylation are shown in red, negatively correlated genes in blue, and non-significant genes ($p \geq 0.05$) in gray. Gene names are labeled for clarity, and the horizontal dashed line indicates the significance threshold ($p < 0.05$).

3.2.1.6.3 Integration and computational selection of the core network gene set

To identify genes for subsequent gene expression analysis, a multi-step computational pipeline was developed. Initially, gene interaction networks were reconstructed independently for two gene sets: a 24-gene set (Set 1) and a 15-gene set (Set 2), using the NetworkAnalyst tool (<https://www.networkanalyst.ca/>)[266]. Next, the six genes with the highest degree centrality and betweenness centrality scores from the 24-gene network (Set 1) were selected, yielding a combined

set of nine genes (due to overlap between the top six genes ranked by degree and betweenness centrality). Then, the seven genes with the highest degree centrality and betweenness centrality scores from the 15-gene network (Set 2) were selected, resulting in a combined set of eight genes (due to overlap between the top seven genes ranked by degree and betweenness centrality). Subsequently, the nine and eight genes from the previous steps were combined, resulting in a consolidated list of 17 genes, after removing two duplicates, 15 unique genes remained, and were defined as set 3. In the fifth step, the association between the expression levels of these 15 genes (Set 3) and the level of *CDKN2A* gene body/3'-UTR CpG methylation levels was assessed using the cBioPortal tool ($p < 0.05$), leading to the exclusion of the MYC and the retention of 14 genes. Finally, six additional genes from the 15-gene set (Set 2), selected for their high correlation with *CDKN2A* methylation levels ($P < 0.05$), were added to the 14 genes, resulting in a final set of 20 genes for gene expression analysis, hereafter referred to as the *CDKN2A*/p16-associated core network gene set.

3.2.2 Systematic validation of the *CDKN2A*/p16-associated core network gene Set

To comprehensively evaluate the robustness and biological relevance of the *CDKN2A*/p16-associated core network gene set, we implemented a validation strategy using independent public databases and complementary analytical approaches. The gene set was validated at the transcriptomic level across tumor and normal tissues, assessed for associations with patient survival and histological grade. In addition, network-based refinement and functional enrichment analyses were performed to elucidate the underlying biological context. Finally, we performed experimental validation by treating gastric cancer cells with increasing concentrations of DNMT inhibitors, followed by qPCR analysis to confirm the gene set and to identify *CDKN2A*/p16-concordant response genes based on dose-dependent expression patterns.

3.2.2.1 Integrated validation of the CDKN2A/p16-associated core network gene set across multi-database platforms

For the *CDKN2A*/p16-associated core network gene set, the Kaplan-Meier Plotter (<https://kmplot.com/analysis/>)[267] was employed to investigate the relationship between genes, derived from both network- and correlation-based approach and the correlation-only approach, and patient survival outcomes.

We validated the findings derived from pan-cancer dot matrix analysis, multi-gene expression studies (including box plots and density plots), and correlation matrix analyses using Pearson and Spearman methods. In addition, gene expression was compared between tumor and normal tissues, including paired tumor and adjacent normal tissues, as well as healthy adult and pediatric tissues. This validation was performed using the TNMplot database (<https://tnmplot.com/analysis/>)[268].

To examine the association between the *CDKN2A*/p16-associated core network gene set and histological grade, we utilized the OncoDB database (https://oncodb.org/clinical_profile_min.html) [269] and selected histological grade as the clinical parameter for comparison. For this clinical parameter, a table of P-values derived from analysis of variance (ANOVA) tests was utilized.

To pinpoint the key hubs in the *CDKN2A*/p16 signaling network, we performed the final network reconstruction step utilizing NetworkAnalyst with the STRING and IMEx databases [270]. Network visualization was performed using through a Steiner forest algorithm.

To elucidate the biological significance of the *CDKN2A*/p16-associated core network gene set, enrichment analysis was conducted using the ExpressAnalyst tool (www.expressanalyst.ca)[271], incorporating gene-set Network and bipartite network approaches. Additionally, Enrichr was

employed utilizing Reactome Pathways 2024 and ShinyGO 0.85 utilizing the KEGG database, and the Enrichr-KG tool [272], utilizing the KEGG, Gene Ontology, and TRRUST databases [273]. The g: Profiler web server [274] was used with the KEGG database, and an enrichment plot was generated using the Cancer Hallmark Genes (CHG) database[275].

3.2.2.2 Validation of correlations between the CDKN2A/p16-associated core network gene set and CDKN2A promoter methylation

Following multi-level validation and prioritization of the *CDKN2A*/p16-associated core network gene set, an additional validation step was performed to specifically assess the relationship between gene expression changes and CpG methylation at the *CDKN2A* promoter site cg03079681. This promoter-focused analysis was performed because p16 promoter methylation is more directly relevant to p16 transcriptional silencing and DNMT inhibitor-induced reactivation. Gene expression and DNA methylation data for gastric adenocarcinoma (TCGA-STAD cohort) were obtained from cBioPortal for Cancer Genomics, originally derived from The Cancer Genome Atlas. Pearson and Spearman analyses were used to calculate correlation coefficients, and statistical significance was defined as $p < 0.05$. Based on the direction of the acceleration across gene body and promoter methylation regions, associations were categorized as concordant or discordant patterns.

3.2.2.3 Validation of protein level of the CDKN2A/p16-associated core network gene set using the human protein atlas

The Human Protein Atlas (<https://www.proteinatlas.org/>) [276] was utilized to confirm the protein-level expression of the *CDKN2A*/p16-associated core network gene set. Immunohistochemistry (IHC) data were obtained, and representative staining images from normal gastric tissues and

gastric cancer tissues were visually assessed. Protein expression patterns were evaluated descriptively by comparing overall staining levels and distribution between normal and tumor samples, with a particular focus on differences in expression consistency and heterogeneity.

3.2.2.4 Dynamic experimental validation using DNMT inhibitors in gastric adenocarcinoma cell lines

3.2.2.4.1 Gastric adenocarcinoma cell Line and treatment with DNMT inhibitors

AGS and HEK293 cells were cultured in F-12K medium (Gibco BRL, USA) and EMEM medium (ATCC, USA), respectively, with 10% fetal bovine serum (FBS, Corning, USA). SNU719, SNU216, and MKN45 cells were grown in RPMI1640 (Gibco BRL, USA) supplement with and 10% FBS. All cell lines were incubated at 37°C in 5% CO₂. Cells were synchronized by 24-hour serum starvation[175] and subsequently treated with zero, low, and high concentrations of decitabine (0, 1 and 25 µM) or GSK3685032 (0, 1 and 25 µM for AGS cells, 0, 1 and 50 µM for SNU216 cells and MKN45 cells, 0, 10 and 50 µM for SNU719 cells) for 72 h. Our previous results showed that *CDKN2A*/p16 expression was low to undetectable at baseline in SNU719 and AGS cells, increased to its maximum at low DNMT inhibitor concentrations, and decreased, sometimes back to baseline levels, at high concentrations [277]. As noted above MKN45 cells are a homozygous deletion of *CDKN2A* do not express *CDKN2A*/p16 whereas SNU216 cells showed relatively high constitutive expression of *CDKN2A*/p16. As decitabine is unstable in culture medium, the medium in treated cells was replaced every 24 hours [278].

3.2.2.4.2 qPCR gene expression analysis

Total RNA was isolated from cells utilizing the PureLink RNA isolation kit (Life Technologies, USA). Complementary DNA (cDNA) was synthesized using SuperScript VILO Master Mix (Life

Technologies, USA). Quantitative PCR (qPCR) was conducted using an Applied Biosystems QuantStudio 6 Flex Real-Time PCR System with TaqMan assays (Life Technologies, USA). PCR reactions were prepared using TaqMan Fast Universal Master Mix (Life Technologies, USA) according to the manufacturer's instructions. A custom TaqMan array system was employed to quantitatively analyze the *CDKN2A*/p16-associated core network gene set (see **Appendices Table 3A, Figure 3A**). In addition to these 20 genes, three reference genes: 18S ribosomal RNA (18S), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and beta-2-microglobulin (B2M) were utilized for normalization purposes (Life Technologies, USA) (see **Appendices Table 3A, Figure 3A**). Relative gene expression was quantified using the comparative Ct ($\Delta\Delta C_t$) method. For each sample, target gene Ct values were first normalized to the corresponding 18S rRNA Ct value measured on the same qPCR plate. The 18S-normalized expression values were then further normalized to the geometric mean of the GAPDH and B2M expression levels from the corresponding sample. Relative expression was calculated against the no-treatment control using the $2^{-\Delta\Delta C_t}$ method, with the control group set to 1. One-way ANOVA was used to compare differences among concentrations within each cell line treated with the same DNMT inhibitor, with $P < 0.05$ considered statistically significant. When ANOVA indicated a significant difference, a Tukey's post hoc test was performed to determine which groups differed.

3.2.2.4.3 *Identification of CDKN2A/p16-synchronized response-Genes under DNMT Inhibitor Treatment*

We defined *CDKN2A*/p16-concordant response genes as genes whose expression trends aligned with DNMT inhibitor-induced changes in *CDKN2A*/p16 from near-zero baseline expression, without implying direct regulation. To identify *CDKN2A*/p16-synchronized response genes, we used the model cell lines AGS and SNU719 which have *p16* promoter CpG hypermethylation and

low or absent *CDKN2A*/p16 expression (**Table 3.3**). Each gene was evaluated under four experimental conditions: AGS-decitabine, AGS-SK3685032, SNU719-decitabine, and SNU719-GSK3685032. **Figure 3.3** illustrates the flowchart for identifying *CDKN2A*/p16-synchronized response genes in gastric cancer cell lines treated with DNMT inhibitors. Step 1) Correlation analysis: Candidate genes were first screened by correlation analysis, requiring strong association with *CDKN2A* expression across dose tiers in both cell lines ($|\rho| \geq 0.70$, FDR $q \leq 0.05$), while genes showing similar correlations in SNU216 exhibiting stable *CDKN2A* expression were excluded; genes were retained if they satisfied the criteria in all four, or at least three of the four, conditions. Step 2) ANOVA with planned contrasts: Genes were required to follow the same dose-response trends. Each gene was further evaluated by one-way ANOVA across dose tiers, followed by two planned contrasts (Low vs Zero; High vs Low). Gene responses were classified relative to *CDKN2A* based on directional consistency, statistical significance after FDR correction, and effect size (Cohen's d). Genes were prioritized if both contrasts were directionally consistent with *CDKN2A*/p16, at least one contrast was statistically significant, and both effect sizes met a minimum magnitude threshold ($|d| \geq 0.50$). Only genes meeting these criteria in all four, or at least three of the four, conditions were retained; Step 3) Joint requirement: To increase robustness, a gene was considered a true *CDKN2A*/p16-synchronized response candidate only if it simultaneously satisfied both the correlation (Step 1) and the ANOVA/contrast (Step 2) criteria. At the gene level, candidates were retained if such joint concordance was observed in all four, or in at least three of the four, conditions. Step 4) Comparison with MKN45: Finally, results were compared with the cell line MKN45 which is a homozygous deletion for *CDKN2A*, and genes were excluded only if they exhibited the same directional changes as MKN45 in at least three of the four conditions; otherwise, they were retained as *CDKN2A*/p16-concordant response. Data

analyses in this section were performed using Python. Raw expression data were processed and statistically analyzed according to predefined directional and statistical criteria described in Steps 1-4 above to identify *CDKN2A*/p16-concordant response genes under DNMT inhibitor treatment.

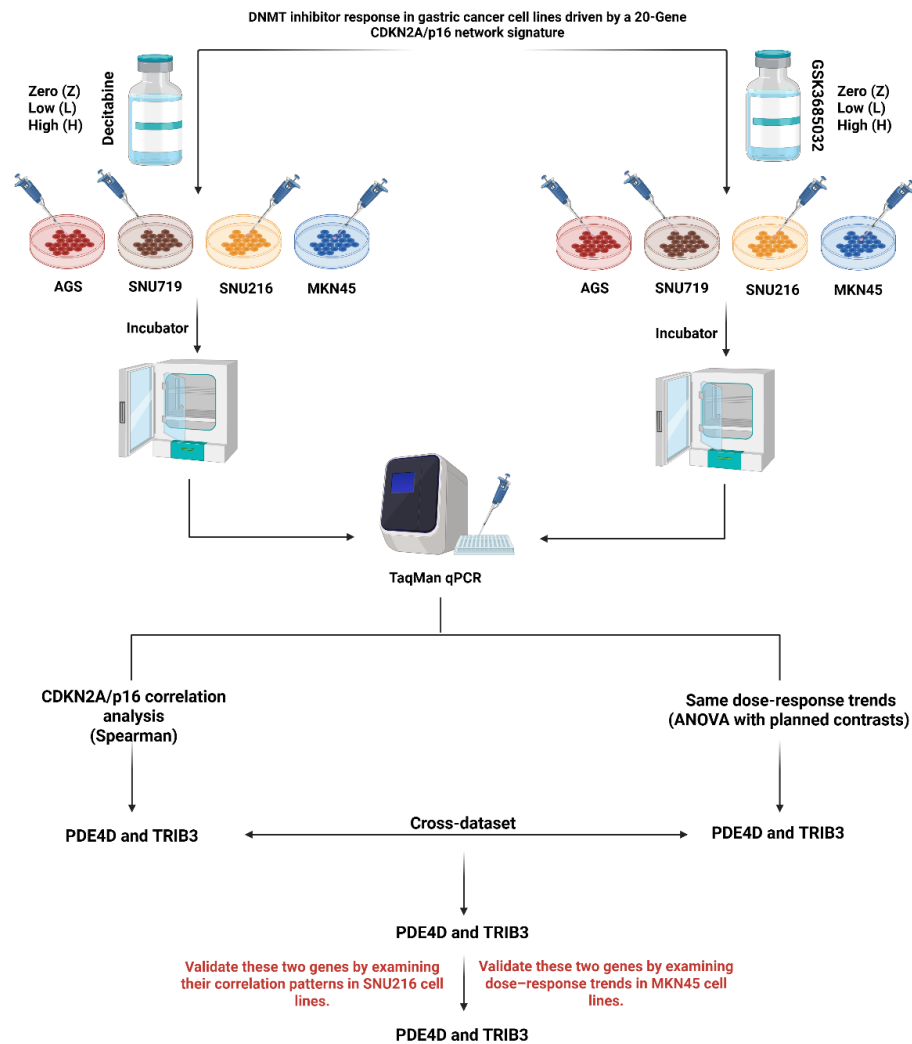


Figure 3.3 Identification of *CDKN2A*/p16-synchronized response genes in gastric cancer cell lines treated with DNMT inhibitors.

AGS and SNU719, both characterized by low or absent *CDKN2A*/p16 expression, were designated as the primary models for *CDKN2A*/p16 reactivation, while SNU216 (stable *CDKN2A*/p16) and MKN45 (*CDKN2A*-null) served as control cell lines. Cells were treated with decitabine or GSK3685032 at Zero (Z), Low (L), and high (H) concentrations, and expression of the *CDKN2A*/p16-associated core network gene set was measured by TaqMan qPCR. Candidate

genes were required to correlate strongly with *CDKN2A*/p16 across dose tiers ($|p| \geq 0.70$, FDR $q \leq 0.05$), excluding those with similar associations in SNU216. Dose–response patterns were then evaluated using ANOVA with planned contrasts and corresponding effect sizes (Cohen’s d), with directional consistency with *CDKN2A*/p16 and $|d| \geq 0.50$ as selection criteria. Genes were further compared with those in MKN45, retaining only those with distinct responses, and cross-dataset validation refined the set to *PDE4D* and *TRIB3* as robust *CDKN2A*/p16-concordant response genes.

Table 3. 2 Characteristics and rationale for the gastric adenocarcinoma cell lines used in this study

Cell line	CDKN2A status	p16 promoter methylation status	Baseline CDKN2A/p16 expression	Relevant molecular feature	Rationale for inclusion
AGS	CDKN2A present; low baseline expression	Yes	Low or undetectable at baseline; inducible by DNMT inhibitors	Gastric adenocarcinoma cell line	Primary model for DNMT inhibitor-induced CDKN2A/p16 reactivation and dose-dependent response analysis
SNU 719	CDKN2A present; low baseline expression	Yes	Low or undetectable at baseline; inducible by DNMT inhibitors	EBV-positive gastric adenocarcinoma cell line	Primary model for CDKN2A/p16 reactivation in a biologically distinct gastric cancer context
SNU 216	CDKN2A present	No	Relatively high, stable constitutive CDKN2A expression	Gastric adenocarcinoma cell line	Comparator cell line with stable CDKN2A expression; used to exclude dose-associated patterns independent of CDKN2A/p16 reactivation
MKN 45	Homozygous CDKN2A deletion	Not applicable	CDKN2A expression undetectable	CDKN2A-null gastric adenocarcinoma cell line	Negative-control model to identify and exclude gene-expression changes occurring independently of CDKN2A/p16

3.2.3 Statistical Analysis

Each treatment condition consisted of four parallel culture wells derived from the same cell preparation and treated independently within a single experimental run. RNA extraction, cDNA synthesis, and qPCR measurement were performed separately for each well. One qPCR measurement was obtained for each sample, and no technical qPCR replicates were included. Thus, the four measurements represent within-experiment culture-well replicates rather than fully independent biological replicates generated from separate cell preparations or experimental runs.

Relative gene expression was calculated using the $2^{-\Delta\Delta Ct}$ method, and the resulting relative expression values were used for statistical analysis. For each gene within each cell line and DNMT inhibitor treatment, differences among the zero-, low-, and high-dose groups were evaluated using ANOVA. Benjamini–Hochberg false-discovery-rate correction was applied to the overall ANOVA *P* values across the 20 genes within each cell line–drug combination. When the overall ANOVA indicated a significant concentration effect, Tukey’s honestly significant difference post hoc test was used for pairwise comparisons among the three dose groups. FDR-adjusted $q \leq 0.05$ were considered statistically significant.

For the identification of *CDKN2A*/p16-concordant response genes, the overall concentration effect was additionally summarized using eta-squared (η^2). Two prespecified contrasts, zero versus low dose and low versus high dose, were evaluated using the pooled residual variance from the corresponding one-way ANOVA model. Cohen’s *d* and Hedges’ *g* were calculated to quantify the magnitude of each contrast. Two-sided 95% confidence intervals for the contrast estimates were calculated using the pooled residual variance from the corresponding ANOVA model and the *t* distribution with nine residual degrees of freedom. Confidence intervals were calculated for the

unadjusted mean-difference estimates, whereas statistical significance was determined using Benjamini–Hochberg-adjusted q-values. Benjamini–Hochberg correction was applied separately to the ANOVA *P*-values and to the *P*-values for each of the two prespecified contrasts across the 20 genes within each cell line–drug combination. A gene was considered directionally concordant with *CDKN2A*/p16 when both dose contrasts followed the corresponding directions of *CDKN2A* expression, both contrasts had an absolute Cohen’s *d* of at least 0.50, and at least one contrast had an FDR-adjusted q-value ≤ 0.05 .

Associations between candidate-gene expression and *CDKN2A* expression across the three dose levels and four parallel culture-well replicates were evaluated using two-sided Spearman rank correlation. Benjamini–Hochberg correction was applied across the 19 candidate genes within each cell line–drug combination. Correlations were considered strong and statistically significant when ($|\rho| \geq 0.70$) and FDR-adjusted $q \leq 0.05$. Genes showing corresponding significant correlations in SNU216 cells, which exhibited relatively stable *CDKN2A*/p16 expression, were excluded. Candidates were retained when they met both the correlation and planned-contrast criteria in all four, or at least three of the four, primary AGS and SNU719 cell line–drug combinations. Their directional responses were subsequently compared with those observed in *CDKN2A*-null MKN45 cells.

The assumptions underlying one-way ANOVA were evaluated for each gene within each cell line–drug combination. Normality of the model residuals was assessed using the Shapiro–Wilk test, and homogeneity of variance among the dose groups was assessed using the Brown–Forsythe test. Because each group contained only four parallel culture-well replicates, these diagnostic tests had limited statistical power and were interpreted cautiously rather than as strict decision criteria.

Statistical results were therefore interpreted together with effect sizes, 95% confidence intervals, and FDR-adjusted q-values. For *CDKN2A* and *CDKN2B* in MKN45 cells, expression was uniformly undetectable across all dose groups, consistent with the intrinsic molecular characteristics of this cell line. Because these data exhibited no within-group or between-group variance, ANOVA and the corresponding normality and homogeneity-of-variance assessments were not applicable[279]. All analyses were performed in Python using Jupyter Notebook, and all statistical tests were two-sided unless otherwise specified.

3.3 Results

3.3.1 Integrated validation of the *CDKN2A/p16*-associated core network gene set across multi-database platforms

We evaluated the association between the expression profiles of the *CDKN2A/p16*-associated core network gene set and overall patient survival, as depicted in a forest plot (**Figure 3.4A**). We identified two clear gene groupings: one conferring a protective effect and the other increasing risk, as indicated by hazard ratios.

Comparative analysis of mRNA gene expression in paired tumor and adjacent normal tissues, as well as in normal samples from non-cancerous adult and pediatric patients, using TNMplot revealed that 9 genes in the *CDKN2A/p16*-associated core network gene set, *NRAS*, *CDK2*, *CDKN2A*, *CCNE1*, *CDK4*, *CDK6*, *PDE4D*, *TRIB3*, and *GET4*, exhibited statistically significant upregulation in tumor tissues compared with normal tissues. This was determined using both paired (tumor vs adjacent normal tissues) and unpaired (tumor vs normal samples) comparisons in TNMplot (**Figure 3.4B** and **Table S3.3**).

Using clinical gene expression data from the OncoDB database, we evaluated whether the mRNA expression levels of the *CDKN2A*/p16-associated core network gene set differed across histological grades of gastric adenocarcinoma. Based on an analysis of variance (ANOVA) *PTEN*, *CDKN2A*, *ARF1*, *MAP4*, *PDE4D*, *SLC25A46*, *TRIB3*, and *GET4* were identified as having statistically significant expression differences among grades, indicating that the *CDKN2A*/p16-associated core gene set exhibits grade-dependent expression heterogeneity in gastric adenocarcinoma (**Figure 3.4C**). While the TNMplot results (**Table S3.3**) indicate that many of these genes show increased mRNA expression in tumor tissues relative to normal controls, the OncoDB analysis evaluates differences across tumor stages. Among the eight genes listed above showing significant variation, expression patterns varied across stages and were not uniformly increasing or decreasing. This is expected, as tumor progression does not necessarily follow a monotonic trajectory, and gene-specific regulatory dynamics may vary across disease stages. Tumor–normal differences and stage-dependent dynamics therefore represent distinct biological dimensions and should not be expected to produce identical directional trends. In this context, the use of one-way ANOVA in the OncoDB analysis, which does not assume directionality, enables the identification of grade-associated genes with diverse expression patterns. To further validate the biological relevance of the *CDKN2A*/p16-associated core network gene set, their protein expression profiles of these genes were examined using the Human Protein Atlas (HPA) database, focusing specifically on gastric cancer tissues. The analysis revealed that in normal gastric tissues, these genes exhibited relatively consistent expression patterns, whereas tumor tissues showed marked heterogeneity, with expression ranging from high to undetectable and certain expression states predominating, producing distinct expression patterns between normal gastric mucosa and malignant tissues. The protein levels of *GET4*, *PDE4D*, and *TRIB3* in tumor tissue and normal

tissue are shown in **Figure 3.4D–3.4I**. These three genes are among the top-ranked candidates in **Table 3.3**, exhibiting the highest level of integrated validation, with consistent support across TNMplot, KMplot, OncoDB, and cBioPortal analyses.

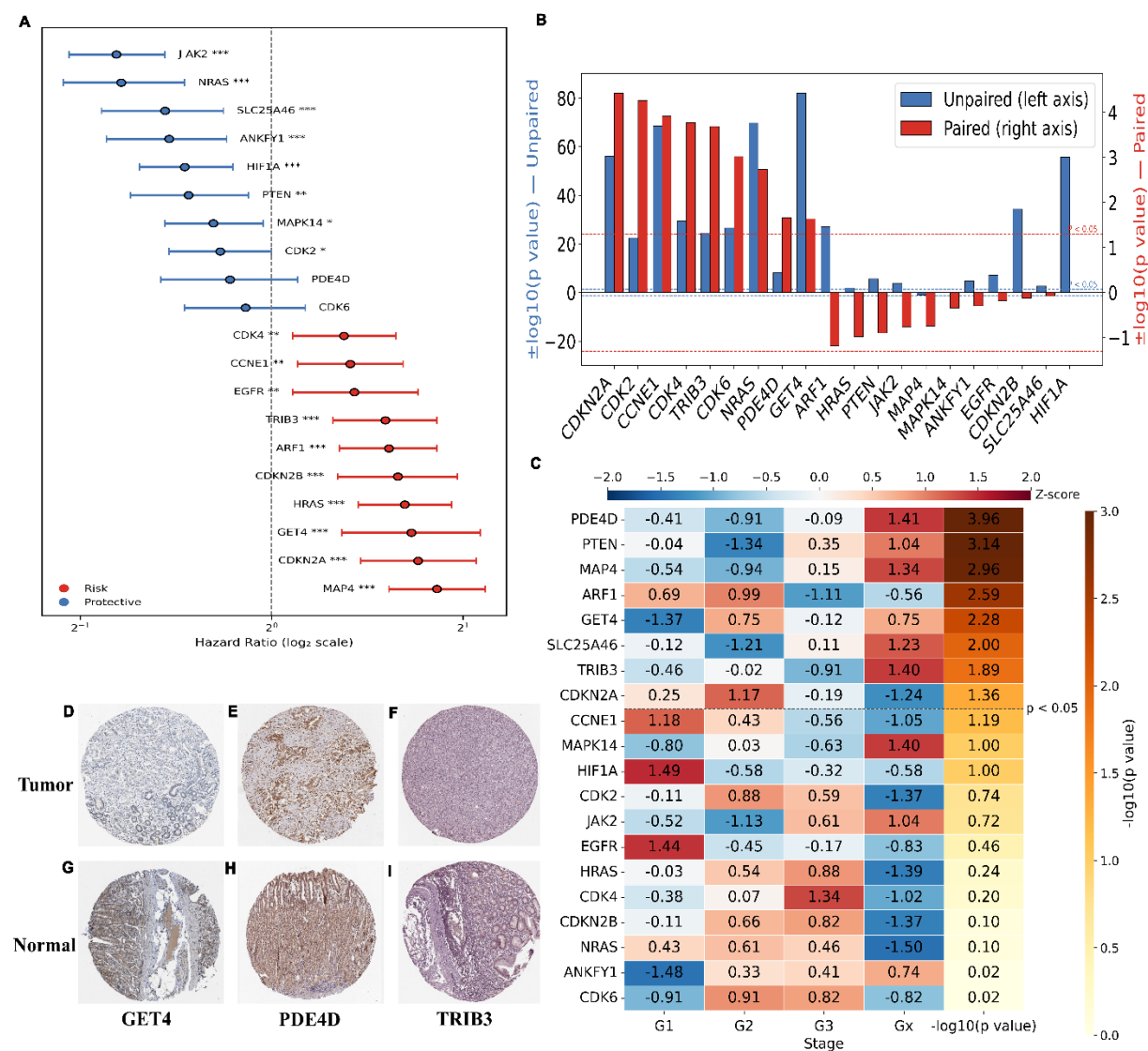


Figure 3.4 Integrated validation of the final 20 CDKN2A/p16-associated core network gene set across multi-database platforms.

(A) Forest plot illustrating the association between the expression of each gene and overall survival in gastric adenocarcinoma patients. Genes with hazard ratios > 1 (red) represent risk factors, whereas those with hazard ratios < 1 (blue) indicate protective factors. Asterisks denote statistical significance levels (*p < 0.05; **p < 0.01; ***p < 0.001). (B) TNM-PLOT expression analysis: Comparison of gene expression between paired tumor versus adjacent normal tissues (red) and unpaired tumor versus normal samples (blue). Nine genes—NRAS, CDK2, CDKN2A, CCNE1, CDK4, CDK6, PDE4D, TRIB3, and GET4—showed significant differential expression in both paired and unpaired datasets. Dashed horizontal lines indicate the statistical significance thresholds at $\pm \log_{10}(p = 0.05)$. (C)

OncoDB stage-specific analysis: Heatmap showing Z-score-normalized expression and corresponding ANOVA p-values ($-\log_{10}$ scale) for the 20 CDKN2A/p16-associated core network genes set across clinical stages G1–Gx. Eight genes (PTEN, CDKN2A, ARF1, MAP4, PDE4D, SLC25A46, TRIB3, and GET4) display significant stage-associated expression changes ($p < 0.05$). (D–I) Human Protein Atlas immunohistochemistry (IHC) validation: Representative immunohistochemical (IHC) staining images from the Human Protein Atlas (HPA) illustrate differential protein expression (both tumor and normal specimens from male donors) for GET4 (Antibody HPA019765), PDE4D (Antibody CAB045895; both tumor and normal specimens from male donors), and TRIB3 (Antibody HPA055442) in gastric cancer tissues (D–F) and normal gastric mucosa (G–I). These genes exhibited distinct expression patterns between normal gastric mucosa and malignant tissues.

Table 3.3 Summary of 20 CDKN2A/p16-associated core network gene set across TNM-PLOT, KM-PLOT, OncoDB, cBioPortal methylation correlation analysis and Functional Consistency

Gene	TNM- PLOT (Tumor vs Normal)	KM- PLOT (Forest plot)	OncoD B (Stage ANOV A)	cBioPortal (<i>CDKN2A/p16</i>)		Functional consistency	Integrated Validation Level
			signific ant	promote	gene body		
				significant			
TRIB3	↑	Risk	YES	YES	YES	YES	Quadruple (TNM+KM+OncoDB+cBioPort al)
GET4	↑	Risk	YES	YES	YES	YES	Quadruple (TNM+KM+OncoDB+cBioPort al)
CDKN2 A/p16	↑	Risk	YES	YES	YES	NO	Quadruple (TNM+KM+OncoDB+cBioPort al)
PDE4D	↑	/	YES	YES	YES	NO	Triple (TNM+OncoDB+cBioPortal)
PTEN	-	Protecti ve	YES	YES	YES	YES	Triple (KM+OncoDB+cBioPortal)
MAP4	-	Risk	YES	YES	YES	YES	Triple (KM+OncoDB+cBioPortal)
SLC25A 46	-	Protecti ve	YES	YES	YES	YES	Triple (KM+OncoDB+cBioPortal)
CCNE1	↑	Risk	NO	YES	YES	YES	Triple (TNM+KM+cBioPortal)
CDK4	↑	Risk	NO	NO	YES	YES	Double (TNM+KM)
NRAS	↑	Protecti ve	NO	NO	YES	NO	Double (TNM+KM)
CDK2	↑	Protecti ve	NO	NO	YES	NO	Double (TNM+KM)
ARF1	-	Risk	YES	NO	YES	YES	Double (KM+OncoDB)
HRAS	-	Risk	NO	YES	YES	YES	Double (KM+cBioPortal)
CDKN2 B	-	Risk	NO	YES	YES	NO	Double (KM+cBioPortal)
MAPK1 4	-	Protecti ve	NO	YES	YES	NO	Double (KM+cBioPortal)
JAK2	-	Protecti ve	NO	YES	YES	NO	Double (KM+cBioPortal)
CDK6	↑	/	NO	NO	YES	NO	Single (TNM only)
EGFR	-	Risk	NO	NO	YES	YES	Single (KM only)
HIF1A	-	Protecti ve	NO	NO	YES	NO	Single (KM only)
ANKFY 1	-	Protecti ve	NO	YES	YES	N.A	Single (KM only)

↑ :significant upregulation in tumor tissues compared with normal tissues (TNMplot).

- : no significant tumor-normal difference.

Risk : higher gene expression is associated with poorer overall survival in KMplot analysis.

Protective indicates that higher gene expression is associated with improved survival.

/ : no significant survival association.

YES : a significant stage-dependent expression pattern based on OncoDB ANOVA analysis.

NO :no significant stage association.

Functional consistency (YES/NO) : whether the prognostic direction (Risk or Protective) is concordant with the known functional role of the gene (oncogenic or tumor-suppressive).

Integrated Validation Level: the concordant identification of a gene across independent clinical databases and methylation correlation in cBioPortal: Genes supported by all four platforms were classified as Quadruple; those supported by three platforms as Triple; those supported by two platforms as Double; and those supported by only one platform as Single.

3.3.2 Correlation analysis between *CDKN2A*/p16-associated core network gene set with *CDKN2A*/p16 Promoter Methylation

Following the identification and multi-level validation of the *CDKN2A*/p16-associated core network gene set prioritized for expression analysis, an additional analytical layer was introduced to refine the mechanistic framework linking gene expression changes with *CDKN2A* methylation dynamics. While previous analyses focused on correlations between these genes and methylation within the *CDKN2A* gene body/3'-UTR, the present step specifically examined whether their expression changes were significantly associated with *CDKN2A* promoter CpG methylation (cg03079681). Unlike the gene body/3'-UTR analysis, which was used to capture broader methylation-associated relationships, the promoter analysis focused on a region with more direct relevance to p16 transcriptional regulation. Two principal correlation patterns were considered. First, concordant patterns, in which gene expression exhibited consistent directional correlations with both gene body and promoter methylation (either uniformly positive or uniformly negative). Second, discordant patterns, in which correlations differed between the two methylation contexts. Concordant and discordant correlations across the *CDKN2A* gene-body/3'UTR and promoter regions were interpreted as distinct methylation-associated relationships and not as evidence of equivalent regulatory effects or direct causality. Regardless of directionality, such associations provide insight into the broader relationships between *CDKN2A* methylation architecture and gene expression in gastric adenocarcinoma. Notably, 13 of the 20 genes in the *CDKN2A*/p16-associated

core network gene set (**Table 3.3**) demonstrated statistically significant correlations between their expression levels and *CDKN2A* promoter CpG methylation, suggesting broader associations with *CDKN2A* methylation.

3.3.3 *CDKN2A* - related final key axes and Enrichment analysis of key genes

To elucidate the interaction context of the *CDKN2A*/p16-associated core network gene set, we reconstructed protein–protein interaction (PPI) networks using both the STRING and IMEx databases. These complementary analyses revealed signaling axes related to *CDKN2A*. In the STRING-derived network (**Figure 3.5A**), *CDKN2A* appeared as a central hub, sitting at the junction emerged: (i) EGFR–JAK2, representing growth factor and cytokine signaling; (ii) CDK4–CCNE1, defining G1/S cell-cycle progression; and (iii) MAPK14–AKT3–TRIB3, associated with stress response and survival adaptation. In contrast, the IMEx network (**Figure 3.5B**) positioned CDK4 as the central hub, from which three functional branches emerge: (i) CCNE1–CDK2–ARF1–PDE4D, linking cell-cycle control to intracellular signaling and trafficking; (ii) *CDKN2A*–HIF1A, connecting *CDKN2A* to hypoxia adaptation; and (iii) PRMT5–JAK2–EGFR, linking *CDKN2A*/p16-associated signaling to histone arginine methylation via PRMT5 and to cytokine and growth-factor signaling via JAK2 and EGFR.

Enrichment analyses using multiple approaches revealed that the genes in the *CDKN2A*/p16-associated core network gene set broadly fall into two major categories: regulators of cell cycle progression and senescence (*CCNE1*, *CDK2*, *CDK4*, *CDK6*, *CDKN2A*, *CDKN2B*, *EGFR*, *HRAS*, *MAPK14*, *NRAS*, *PTEN*), and modulators of cellular signaling and intracellular dynamics (*ANKFY1*, *MAP4*, *PDE4D*, *SLC25A46*, *ARF1*, *HIF1A*, *JAK2*, *TRIB3*, *GET4*). Reactome pathway analysis indicated significant enrichment in G1 phase–related events, including Cyclin D-

associated events in G1, G1 phase, Mitotic G1 phase, and the G1/S transition, as well as cellular senescence and aberrant regulation of mitotic checkpoints. KEGG pathway analysis (**Figure 3.5C**) further highlighted four prominent signaling cascades: PD-L1 expression and the PD-1 checkpoint pathway in cancer, the FoxO signaling pathway, the p53 signaling pathway, and the PI3K–Akt signaling pathway. Network-based enrichment revealed a densely interconnected cluster of cell cycle regulators (CDK2, CDK4, CDK6, CCNE1, CDKN2A/2B) and tumor suppressors (PTEN, TP53, CDKN2A/2B), together with additional links to signaling mediators (JAK2, STAT3, and the MAPK family) (**Figure 3.5D**). Finally, cancer hallmark analysis demonstrated that the *CDKN2A*/p16-associated core network gene set was significantly associated with multiple hallmarks of cancer, including sustaining proliferative signaling, replicative immortality, evading growth suppressors, resisting cell death, tissue invasion and metastasis, and immune evasion (**Figure 3.5E**).

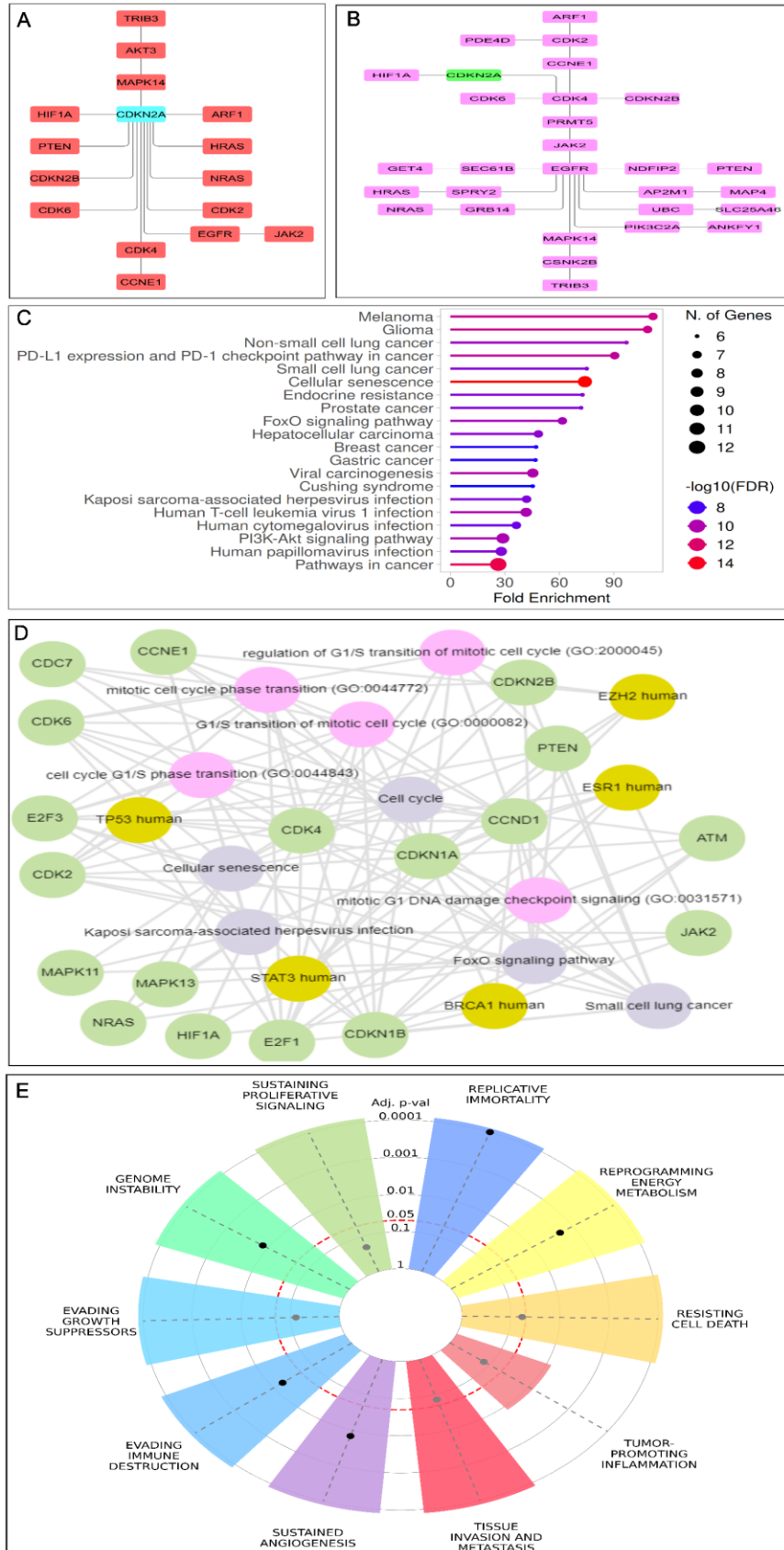


Figure 3.5 Network reconstruction and pathway enrichment identifying CDKN2A-centered regulatory axes in gastric adenocarcinoma.

(A) STRING network: CDKN2A (teal node) is shown at the center of direct interactions with red nodes, representing the final set of key genes (CDK2, CDK4, CDK6, CCNE1, NRAS, HRAS, PTEN, EGFR, MAPK14, JAK2, etc.). These clusters highlight the concentration of CDKN2A-linked regulators in cell cycle and proliferation pathways. (B) IMEx network: Expanded CDKN2A–EGFR connectivity map, with magenta/pink nodes representing additional interactors and green nodes marking secondary hub genes such as EGFR, JAK2, MAPK14, PRMT5, PIK3C2A, and GRB14. (C) Enrichment analysis (Enrichr: ShinyGO 0.85 and Reactome 2024): Purple bars show the top significantly enriched pathways; bubble color gradient (blue → red) represents increasing $-\log_{10}(\text{FDR})$, while bubble size indicates the number of enriched genes. Notable pathways include PD-1/PD-L1 signaling, cellular senescence, and gastric adenocarcinoma. (D) Cancer Hallmark Genes (CHG) network: Green nodes represent genes (e.g., CDKN2A, CDK4, PTEN), purple nodes represent hallmark pathways or terms, and light-yellow nodes denote genes connecting multiple hallmarks. The network emphasizes associations with cell cycle control, senescence, and checkpoint regulation. (E) Cancer hallmark wheel: Each colored sector corresponds to one of the ten cancer hallmarks. Red dashed concentric lines represent adjusted p-value thresholds (0.1, 0.05, 0.01). Significant enrichment is observed for “sustaining proliferative signaling,” “evading growth suppressors,” and “resisting cell death.” In our network analysis, we use the CDKN2A gene symbol, which corresponds to the p16 protein.

3.3.4 Validation of CDKN2A/p16-associated core network gene set expression by qPCR and identification of CDKN2A-synchronized response genes under DNMT inhibitor treatment

Some gastric adenocarcinomas are driven by low or no expression of p16 caused by *p16* promoter CpG hypermethylation and it has been speculated that DNMT inhibitors can reduce this methylation, potentially providing a treatment for this disease. As previously reported by our lab [277], the DNMT inhibitors decitabine and GSK3685032 do indeed reduce global genomic DNA methylation, *p16* promoter CpG methylation and increased p16 expression at low doses but at high doses, the opposite effects are observed[277]. We therefore investigated changes in the expression of the *CDKN2A*/p16-associated core network gene set during treatment of selected gastric adenocarcinoma cell lines with zero, low and high doses of decitabine and GSK3685032. Low doses yielded the greatest induction of CDKN2A expression and high doses reduced *CDKN2A* expression, sometimes back to pretreatment (or zero dose) levels. The *CDKN2A*/p16-associated core network gene set showed significant and coordinated responses depending on DNMT inhibitor dose and *CDKN2A* expression (**Figure 3.6, Table 3.4**). After Benjamini–Hochberg correction, significant overall concentration effects were observed for 16 of 20 genes in decitabine-

treated AGS cells, 15 of 20 genes in GSK3685032-treated AGS cells, 19 of 20 genes in decitabine-treated SNU719 cells, and 15 of 20 genes in GSK3685032-treated SNU719 cells (ANOVA $q < 0.05$; **Figure 3.6 and Table 3.4**). Among cell cycle regulator genes, *CCNE1*, *CDK4* and *CDK6* often showed higher expression at zero or low concentrations, but this expression attenuated at the high DNMT inhibitor concentrations, particularly in AGS and SNU719 cells. In decitabine-treated AGS cells, *CCNE1* increased 1.49-fold at the low dose compared with the zero-dose control ($P < 0.001$) and subsequently decreased to 0.88-fold of the low-dose level at the high dose ($P < 0.005$). In decitabine-treated SNU719 cells, *CDK4* and *CDK6* increased 1.21- and 1.97-fold, respectively, at the low dose ($P = 0.032$ and $P < 0.001$), before decreasing to 0.76- and 0.73-fold of their respective low-dose levels at the high dose ($P = 0.0062$ and $P < 0.001$). However, this low-dose-peak pattern was not observed for all cell-cycle genes. In SNU719 cells, *CCNE1* increased 3.16-fold at the low dose and increased by a further 1.45-fold at the high dose (both $P < 0.001$). *CDK2* also did not attenuate at the high dose, increasing 1.13-fold in SNU719 cells ($P = 0.026$) and 1.81-fold in AGS cells ($P < 0.001$) relative to the corresponding low-dose levels (**Figure 3.6A and 3.6E**). GSK3685032 produced different cell-cycle responses. In AGS cells, *CDK4* and *CDK6* decreased to 0.62- and 0.66-fold of their zero-dose levels at the low dose ($P < 0.001$ and $P = 0.012$, respectively), without significant additional decreases at the high dose. In SNU719 cells, *CDK6* increased 2.57-fold at the low dose ($P < 0.001$) and subsequently decreased to 0.53-fold of the low-dose level at the high dose ($P < 0.005$) (**Figure 3.6C and 3.6G**). Oncogenes such as *EGFR*, *HRAS*, *NRAS*, and *MAPK14* demonstrated variable, cell line-specific responses. In decitabine-treated SNU719 cells, *EGFR* increased 3.28-fold at the low dose and by a further 2.15-fold at the high dose (both $P < 0.001$). *NRAS* similarly increased 1.90-fold at the low dose and by an additional 1.34-fold at the high dose (both $P < 0.001$). *HRAS* increased 1.42-fold between the low and high

doses ($P<0.005$), whereas *MAPK14* increased more modestly by 1.15-fold ($P=0.026$). In contrast, in GSK3685032-treated AGS cells, *NRAS* decreased to 0.82-fold of the zero-dose level at the low dose ($P<0.005$), with no significant additional change at the high dose ($P=0.133$). *HRAS* and *MAPK14* did not change significantly under this condition ($P>0.05$). The oncogene-associated responses in AGS and SNU719 cells are shown in **Figure 3.6A, 3.6C, 3.6E, and 3.6G**. The corresponding responses in SNU216 and MKN45 cells are shown separately in **Figure 3.6I, 3.6K, 3.6M, and 3.6O**.

Among genes involved in signaling and intracellular dynamics, *PDE4D* and *TRIB3* showed the clearest low-dose induction followed by high-dose attenuation. In decitabine-treated AGS cells, *PDE4D* increased 3.64-fold at the low dose and subsequently decreased to 0.65-fold of the low-dose level at the high dose (both $P<0.001$). *TRIB3* increased 18.3-fold at the low dose and decreased to 0.13-fold of the low-dose level at the high dose (both $P<0.001$). In decitabine-treated SNU719 cells, *TRIB3* decreased to 0.23-fold of the low-dose level at the high dose ($P<0.001$); its low-versus-zero fold change could not be calculated because expression was undetectable in the zero-dose group. *PDE4D* increased 1.22-fold at the low dose ($P=0.0071$), but its subsequent decrease to 0.90-fold of the low-dose level was not statistically significant ($P=0.063$) (**Figure 3.6B and 3.6F**). Under GSK3685032 treatment, *PDE4D* increased 2.44-fold in AGS cells and 1.28-fold in SNU719 cells at the low dose ($P<0.001$ and $P=0.024$, respectively). Its expression subsequently decreased to 0.64- and 0.73-fold of the corresponding low-dose levels at the high dose ($P<0.001$ and $P=0.010$, respectively). *TRIB3* decreased to 0.24-fold of the low-dose level in SNU719 cells ($P<0.001$), whereas its decrease to 0.47-fold in AGS cells was not statistically significant ($P=0.133$) (**Figure 3.6D and 3.6H**). By contrast, *ARF1* and *HIF1A* remained stable or were upregulated under certain conditions, suggesting distinct regulatory mechanisms (**Figure 3.6B, 3.6D, 3.6F, 3.6H, 3.6J,**

3.6L, 3.6N, 3.6P). *ARF1* remained unchanged in decitabine-treated AGS cells, showing a 1.00-fold change at the low dose ($P=0.947$) and a 1.07-fold change between the low and high doses ($P=0.068$). By contrast, *HIF1A* increased 1.27-fold at the low dose and by a further 1.44-fold at the high dose in decitabine-treated SNU719 cells ($P=0.042$ and $P=0.0011$, respectively). The signaling and intracellular-dynamics responses in SNU216 and MKN45 cells are shown separately in **Figure 3.6J, 3.6L, 3.6N, and 3.6P**.

Overall, our qPCR gene expression results were consistent with the in silico predictions for the *CDKN2A*/p16-associated core network gene set showing variable but significant changes. Decitabine exerted broader and stronger effects on gene expression than GSK3685032, with the most pronounced changes in AGS and SNU719, while SNU216 and MKN45 showed less pronounced changes.

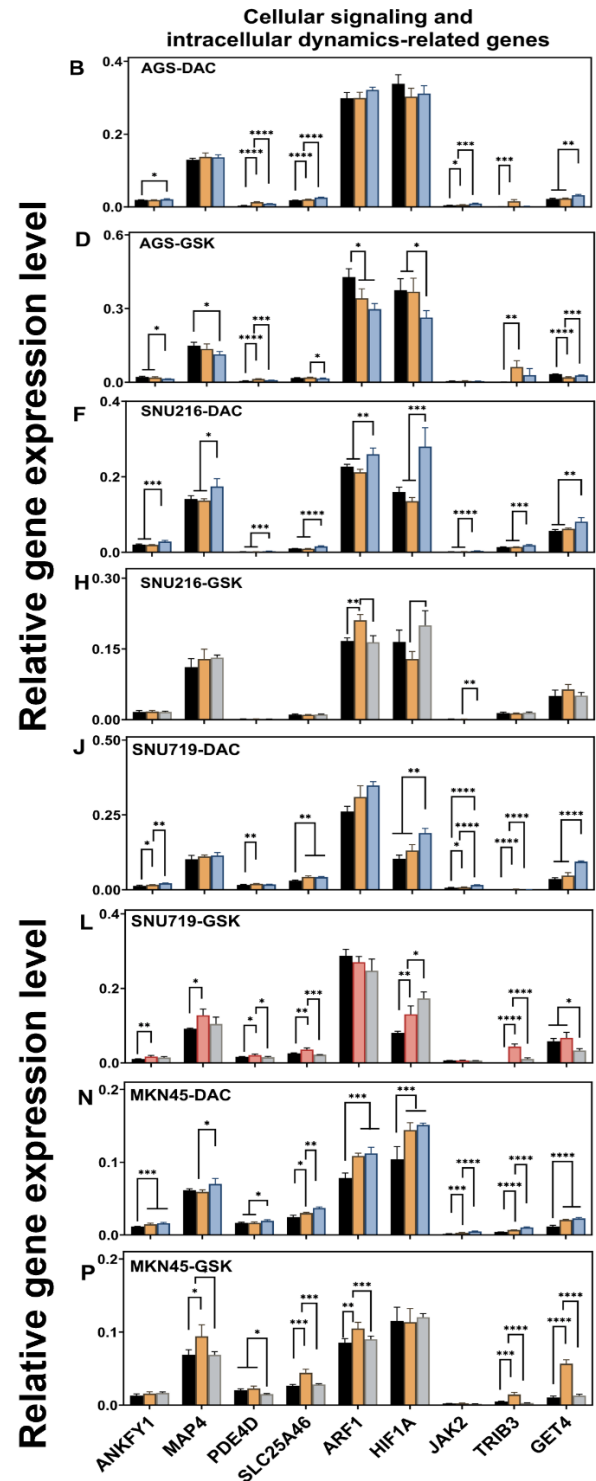
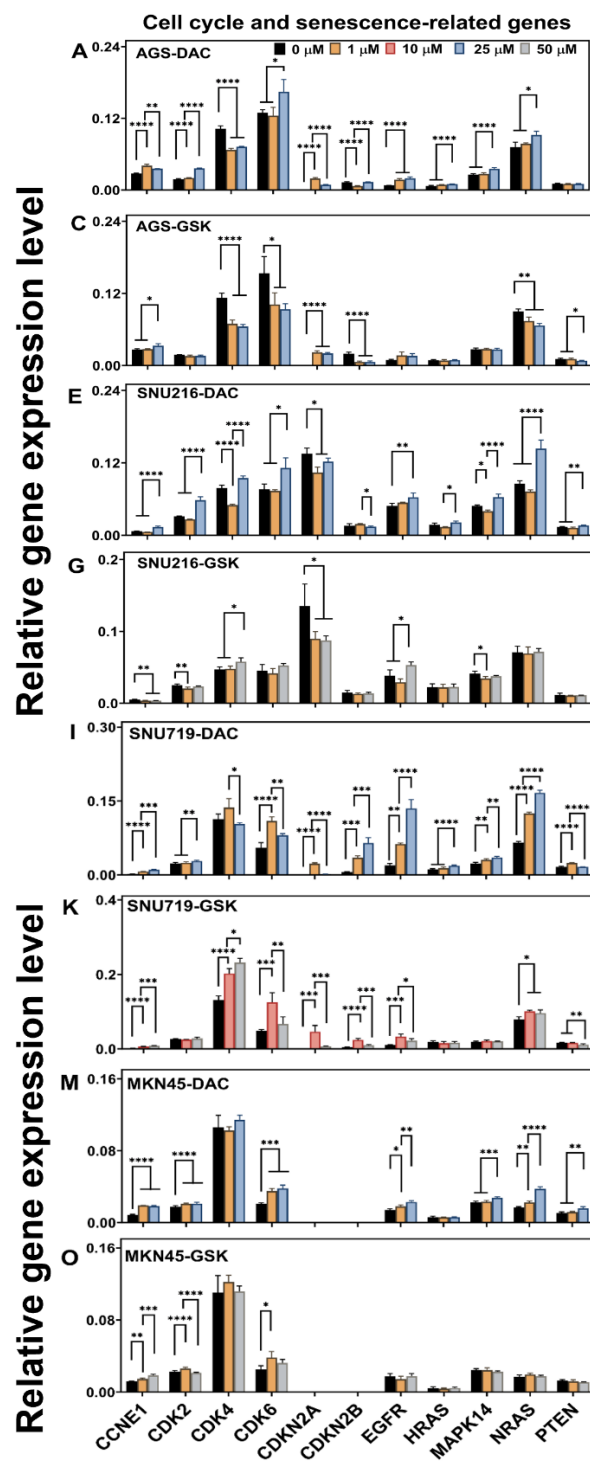


Figure 3.6 The changes CDKN2A/p16-associated core network gene set gene expression networks.

The changes of cell cycle and senescence-related genes, and cellular signaling and intracellular dynamics-related genes measured by qPCR (normalized using a combination of 18S, B2M and GAPDH) in AGS cells treated with decitabine (A, B), AGS cells treated with GSK3685032 (C, D), SNU216 cells treated with decitabine (E, F), SNU216 cells treated with GSK3685032 (G, H), SNU719 cells treated with decitabine (I, J), SNU719 cells treated with GSK3685032 (K, L), MKN45 cells treated with decitabine (M, N), and MKN45 treated with GSK3685032 (O, P). Both DNMT inhibitors induced significant, gene-dependent, and dose-dependent alterations. Decitabine consistently exerted broader and stronger effects than GSK3685032, with the most pronounced responses observed in AGS and SNU719 cells. A one-way ANOVA was used to compare differences across concentrations within a single cell line treated with the same DNMT inhibitors (P-value < 0.05 considered significant). When ANOVA indicated a significant difference, a Tukey test was performed to determine which specific groups differed. Asterisk (*) indicates statistical significance (*P<0.05, **P< 0.01, ***P<0.001, ****P<0.0001). Values represent the mean of four measurements with error bars indicating SD (n=4). DAC: decitabine; GSK: GSK3685032.

Table 3.4 Summary of Statistically Significant Expression Changes in the CDKN2A/p16-Associated Core Network Gene Set Following DNMT Inhibitor Treatment

Cell line	Drug	Gene category	No. significant genes (ANOVA p < 0.05)	Genes with significant change
AGS	DAC	Cell cycle and senescence-related	10	CCNE1, CDK2, CDK4, CDK6, CDKN2A, CDKN2B, EGFR, HRAS, MAPK14, NRAS
		Cellular signaling and intracellular dynamics-related	6	ANKFY1, PDE4D, SLC25A46, JAK2, TRIB3, GET4
	GSK	Cell cycle and senescence-related	7	CCNE1, CDK4, CDK6, CDKN2A, CDKN2B, NRAS, PTEN
		Cellular signaling and intracellular dynamics-related	8	ANKFY1, MAP4, PDE4D, SLC25A46, ARF1, HIF1A, TRIB3, GET4
SNU216	DAC	Cell cycle and senescence-related	11	CCNE1, CDK2, CDK4, CDK6, CDKN2A, CDKN2B, EGFR, HRAS, MAPK14, NRAS, PTEN
		Cellular signaling and intracellular dynamics-related	9	ANKFY1, MAP4, PDE4D, SLC25A46, ARF1, HIF1A, JAK2, TRIB3, GET4
	GSK	Cell cycle and senescence-related	6	CCNE1, CDK2, CDK4, CDKN2A, EGFR, MAPK14
		Cellular signaling and intracellular dynamics-related	3	PDE4D, ARF1, HIF1A
SNU719	DAC	Cell cycle and senescence-related	11	CCNE1, CDK2, CDK4, CDK6, CDKN2A, CDKN2B, EGFR, HRAS, MAPK14, NRAS, PTEN
		Cellular signaling and intracellular dynamics-related	8	ANKFY1, PDE4D, SLC25A46, ARF1, HIF1A, JAK2, TRIB3, GET4
	GSK	Cell cycle and senescence-related	8	CCNE1, CDK4, CDK6, CDKN2A, CDKN2B, EGFR, NRAS, PTEN
		Cellular signaling and intracellular dynamics-related	7	ANKFY1, MAP4, PDE4D, SLC25A46, HIF1A, TRIB3, GET4
MKN45	DAC	Cell cycle and senescence-related	7	CCNE1, CDK2, CDK6, EGFR, MAPK14, NRAS, PTEN
		Cellular signaling and intracellular dynamics-related	9	ANKFY1, MAP4, PDE4D, SLC25A46, ARF1, HIF1A, JAK2, TRIB3, GET4
	GSK	Cell cycle and senescence-related	3	CCNE1, CDK2, CDK6
		Cellular signaling and intracellular dynamics-related	6	MAP4, PDE4D, SLC25A46, ARF1, TRIB3, GET4

To identify *CDKN2A*/p16-concordant response genes (ie., genes whose expression changes were concordant with changes in *CDKN2A* expression across increasing DNMT inhibitor doses) in gastric adenocarcinoma cells, we examined the relationship between *CDKN2A* expression and the

expression of the *CDKN2A*/p16-associated core network gene set at zero, low and high doses of the DNMT inhibitors decitabine and GSK3685032. Spearman correlation analysis revealed that several candidates, including *TRIB3*, *PDE4D*, and *EGFR*, exhibited strong positive correlations with *CDKN2A*/p16 expression in AGS and SNU719 cells but not SNU216 cells ($|\rho| \geq 0.70$, FDR $q \leq 0.05$) (**Figure 3.7A**). The Spearman correlation is also shown for *EGFR*, *PDE4D*, and *TRIB3* versus *CDKN2A* expression (**Figure 3.7B, 3.7C, 3.7D**, respectively) for each DNMT inhibitor dose and zero control with 4 replicates and in AGS and SNU719 cells.

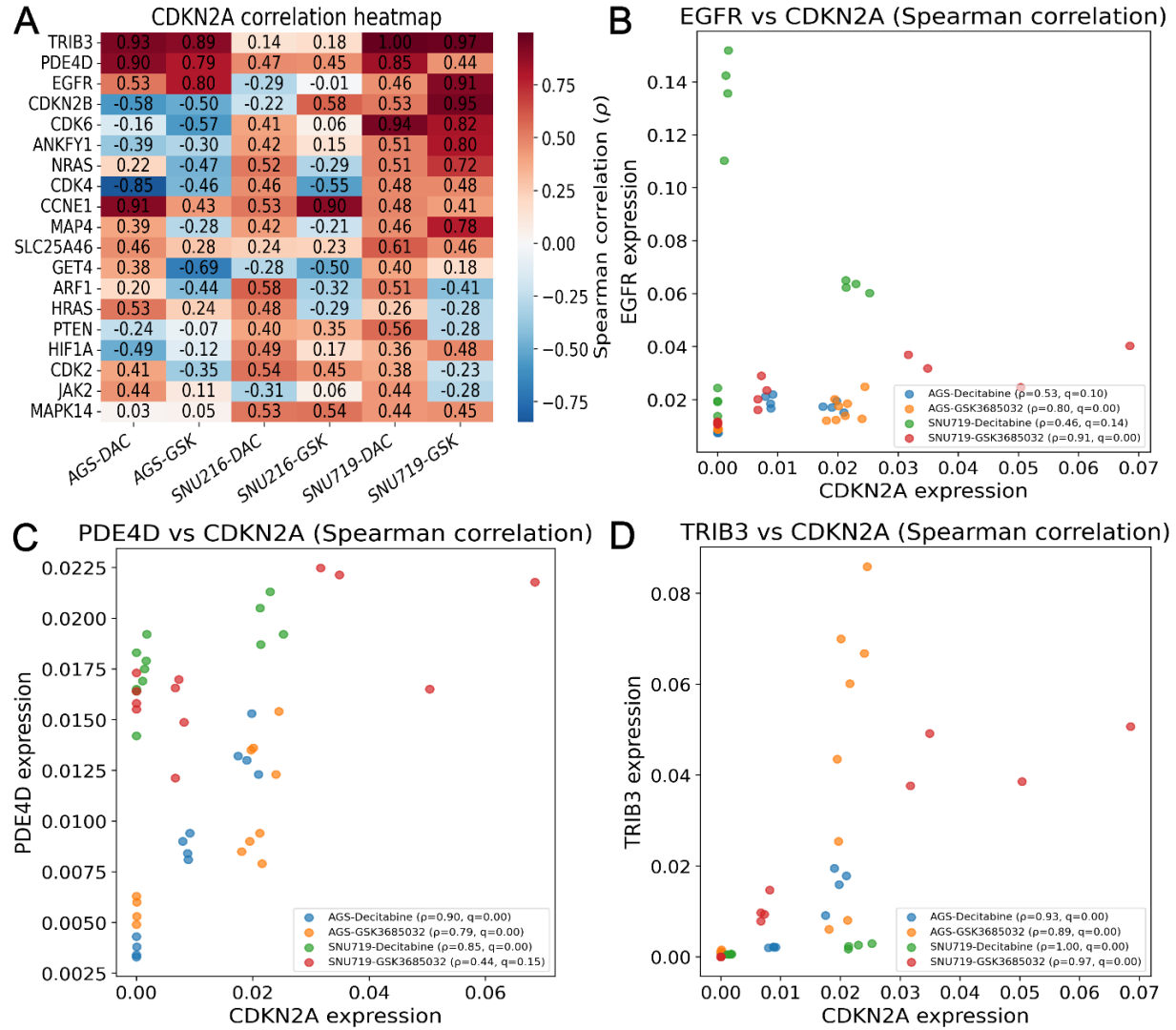


Figure 3.7 The relationship between CDKN2A and a set of 20 database-derived candidate genes.

AGS and SNU719 cells were used as model cell lines for gastric cancer under treatment with decitabine AGS–DAC, SNU719–DAC, and GSK3685032 AGS–GSK3685032 and SNU719–GSK3685032. Candidate genes were prioritized using strong correlation with CDKN2A expression across dose tiers ($|\rho| \geq 0.50$, FDR-adjusted $q \leq 0.05$). (A) Correlation heatmap of CDKN2A with 20 candidate genes across AGS, SNU216, and SNU719 cells treated with DAC or GSK3685032. Positive correlations are shown in red and negative correlations in blue. Notably, EGFR, PDE4D, and TRIB3 consistently exhibited strong positive correlations ($\rho > 0.5$) with CDKN2A expression in at least three experimental conditions. (B–D) Scatterplots showing representative relationships between CDKN2A and EGFR, PDE4D, and TRIB3. Each point represents replicate-level data (3 doses $\times$ 4 replicates), color-coded by condition; Spearman ρ and FDR-adjusted q -values are shown.

To refine these associations, we applied ANOVA with planned contrasts to evaluate directional consistency, statistical significance, and effect size ($|d| \geq 0.50$) of gene responses relative to CDKN2A/p16. This joint strategy yielded a subset of genes that were concordant in at least three

of the four AGS/SNU719 treatment settings (cell line–drug combinations) (**Figure 3.8A**). Notably, *TRIB3* and *PDE4D* demonstrated highly reproducible “low–high–low” expression patterns paralleling changes in the *CDKN2A*/p16 expression changes with increasing dose of DNMT inhibitor doses (Spearman $\rho > 0.7$, FDR $q < 0.05$), meeting all predefined criteria (as defined in section 2.2.4.3) to be designated *CDKN2A*/p16-concordant response genes (**Figure 3.8B–3.8E**). Control comparisons further supported the association of these genes with *p16* expression: SNU216 cells, although *CDKN2A*/p16-positive, exhibited weak, non-dose-dependent responses (**Figure 3.8A**), whereas MKN45 cells, which completely lack p16, showed no comparable induction of *TRIB3* or *PDE4D* (**Figure 3.8F–3.8I**). These indicate that *TRIB3* and *PDE4D* can be robustly defined as *CDKN2A*/p16-synchronized response genes.

A Dose-response consistency with CDKN2A

TRIB3	4.56	4.18	3.34	1.24	6.56	5.01	9.08	6.36
PDE4D	10.07	4.71	7.99	4.93	2.46	1.89	2.15	2.21
CDK6	0.47	2.26	2.18	0.51	5.88	4.60	4.15	2.58
CCNE1	7.47	3.02	0.44	2.54	16.93	3.93	17.03	4.94
CDKN2B	6.72	13.24	6.14	0.07	11.27	3.69	6.47	4.18
EGFR	7.49	1.06	1.80	0.15	12.77	5.69	4.58	1.81
CDK4	8.43	2.74	5.92	0.85	1.55	2.51	5.88	2.27
PTEN	0.66	0.06	0.05	2.23	7.67	14.08	0.91	2.55
SLC25A46	2.56	5.50	1.42	2.71	4.24	0.27	3.16	4.54
NRAS	0.93	3.38	3.00	1.46	23.84	10.13	3.78	0.70
GET4	0.99	5.05	5.74	3.62	1.61	6.51	0.79	2.97
ANKFY1	1.05	2.08	0.64	2.36	2.03	4.82	3.11	0.93
MAP4	0.79	0.16	0.74	1.38	0.98	0.41	3.09	1.34
MAPK14	0.79	3.99	0.28	0.37	3.51	2.19	1.13	0.79
CDK2	2.26	23.65	1.92	0.21	0.86	2.14	0.63	0.83
JAK2	3.16	4.81	0.28	0.80	2.01	11.86	0.06	0.82
HIF1A	1.47	0.38	0.11	2.40	1.75	3.32	3.04	2.10
ARF1	0.04	1.79	2.38	1.40	1.66	1.37	1.09	0.92
HRAS	2.73	1.89	0.38	0.70	0.99	2.77	0.76	0.16

AGS-DAC Z-L(d) AGS-DAC L-H(d) AGS-GSK Z-L(d) AGS-GSK L-H(d) SNU719-DAC Z-L(d) SNU719-DAC L-H(d) SNU719-GSK Z-L(d) SNU719-GSK L-H(d)

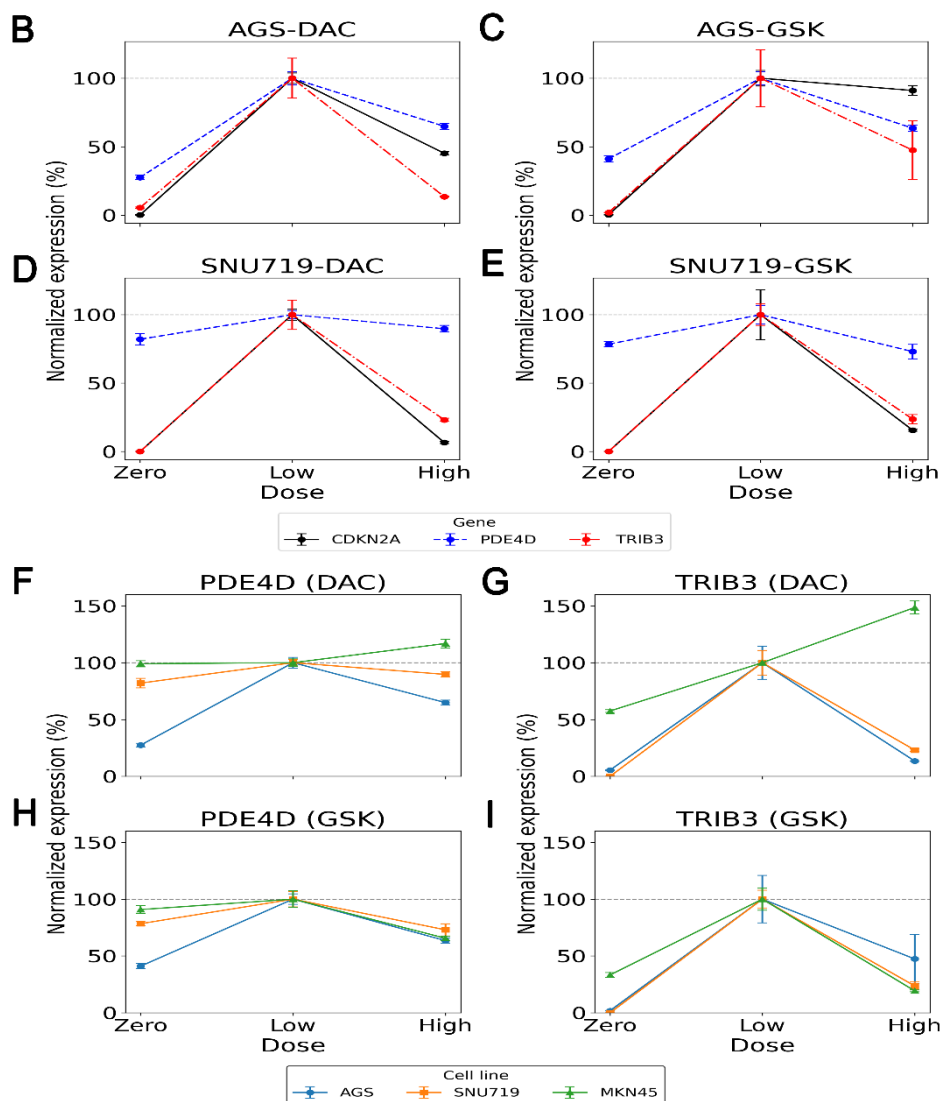
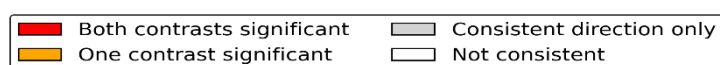


Figure 3.8 The dose-response consistence with CDKN2A genes in gastric cancer cell lines.

AGS and SNU719 cells were used as model cell lines for gastric adenocarcinoma under treatment with decitabine AGS–DAC, SNU719–DAC, and GSK3685032 AGS–GSK3685032 and SNU719–GSK3685032. Candidate genes were prioritized using consistent dose–response concordance with CDKN2A/p16 based on ANOVA with planned contrasts (directional agreement, statistical significance, and effect size $|d| \geq 0.50$). Genes were retained if they showed concordant patterns in at least three of the four primary conditions and were further compared with MKN45 cells (CDKN2A-null) to exclude p16-independent responses. (A) Dose–response consistency matrix (heatmap) summarizing the directional and statistical concordance between CDKN2A and 20 candidate genes across AGS, SNU216, and SNU719 cells treated with decitabine (DAC) or GSK3685032. Each condition is represented by two columns corresponding to Cohen’s d effect sizes $|d|$ for pairwise dose contrasts: Zero–Low (Z–L) and Low–High (L–H). Values indicate the absolute magnitude of standardized expression changes for each contrast, while the overlay colors indicate the level of directional and statistical concordance. Notably, PDE4D and TRIB3 consistently exhibited strong concordance with CDKN2A expression, characterized by an effect size of $|d| \geq 0.50$ and statistical significance across multiple conditions. Red (both contrasts significant): Both contrasts significant and directionally consistent with p16. Genes that show statistical significance in both the Zero–Low (Z–L) and Low–High (L–H) contrasts, and exhibit directional agreement with p16 response (either both upregulated or both downregulated). Orange (both contrasts significant): One contrast significant and directionally consistent with p16. Genes that show statistical significance in either the ML or HM contrast, and exhibit directional agreement with p16 response in at least one contrast. Light Grey (consistent direction only) Directionally consistent with p16 but not significant. Genes that show consistent directional agreement with p16 response across both contrasts, but do not reach statistical significance in either contrast. White (not consistent): Not consistent with p16 response. Genes that either do not show significant changes in expression or do not match the direction of p16 response across contrasts. (B–E) Dose–response trends for CDKN2A and selected genes (PDE4D and TRIB3) in AGS and SNU719 under DAC or GSK3685032 treatment. (F–I) Dose–response trends for selected genes (PDE4D and TRIB3) in AGS, SNU719 and MKN45 under DAC or GSK3685032 treatment. Each point represents replicate-level data (zero, low, and high doses $\times$ 4 replicates).

3.4 Discussion

This study identified a *CDKN2A*/p16-associated gene regulatory network in gastric adenocarcinoma by integrating multi-database bioinformatic analyses with a experimental validation of *CDKN2A*/p16-associated genes in gastric adenocarcinoma cell lines under DNMT inhibitor treatment. Understanding the molecular circuitry of gastric adenocarcinoma requires more than cataloging differentially expressed genes; it necessitates focusing on pivotal regulators that orchestrate disease progression. We therefore designated *CDKN2A* as the anchor gene based on its established role in G1/S cell-cycle regulation, which provides an optimal perspective for exploring regulatory hierarchies. Employing a multi-step strategy, we identified the *CDKN2A*/p16-associated core network gene set from extensive datasets. The majority demonstrated expression patterns, prognostic correlations, and stage-dependent relationships consistent with their established biological functions across TNMplot, KMplot, OncoDB and cBioPortal methylation

analyses (**Table 3.3**), strengthening the credibility of our screening methodology and underscoring their potential as pivotal network components. Among these, *TRIB3* and *GET4* consistently emerged as top-ranked candidates, supported by evidence across expression, prognosis, disease stage analyses and methylation correlation analyses. *PDE4D* also showed consistent tumor–normal upregulation, stage association and methylation correlation analysis but lacked robust prognostic significance. Network reconstruction using STRING and IMEx further supported *TRIB3*, *GET4*, and *PDE4D* as connected components within the *CDKN2A*-associated interaction network. Moreover, *TRIB3* and *PDE4D* showed strong dose-dependent concordance with *CDKN2A*/p16 expression experimental validation of the *CDKN2A*/p16-associated core network gene set under DNMT inhibitor treatment. Across all analyses, *TRIB3* emerged as the most consistent candidate, supported by network reconstruction, DNMT-inhibitor–dose-dependent gene-expression analysis, and quadruple clinical validation, including tumor–normal differential expression, survival association, stage association, and *CDKN2A* methylation correlation. In contrast, *PDE4D* also showed concordant expression in in the DNMT-inhibitor–dose-dependent gene-expression analyses and was supported by network reconstruction. In the clinical datasets, it showed tumor–normal differential expression, stage association, and *CDKN2A* methylation correlation in the clinical datasets, but lacked a significant survival association, and was classified as having triple validation. Taken together, these results indicate that *TRIB3* represents a strongly associated node within the *CDKN2A* methylation-associated regulatory network and a potential cancer therapy co-target candidate. *TRIB3* is a pseudokinase involved in the stress response [280,281]. Accordingly, its expression is induced by hypoxia, endoplasmic reticulum (ER) stress, and nutrient deprivation, and it modulates multiple signaling pathways [282,283]. Activation of these pathways is associated with poor clinical outcomes [281] , and *TRIB3* overexpression has

been linked to tumor angiogenesis and poor prognosis in gastric adenocarcinoma patients [284]. Based on this evidence, TRIB3 may act as a key adaptive resistance node in the stress-response network associated with DNMT inhibitor-mediated p16 reactivation. We and others have noted that DNMT-inhibitor-induced reactivation of p16 causes cell-cycle arrest [184,185,277], thereby disrupting metabolic regulation and protein homeostasis and thereby activating ER stress signaling and metabolic imbalance [285,286]. These stress conditions are known to promote TRIB3 upregulation [283,287]. We observed that TRIB3 and *CDKN2A* expression were congruent at zero, low and high doses of DNMT inhibitor. Importantly, TRIB3 has been reported to promote anticancer drug resistance [288]. In contrast, *TRIB3* siRNA knockdown enhances doxorubicin-induced apoptosis in gastric adenocarcinoma cells [289]. Furthermore, cepharanthine, an autophagy inhibitor, reduces TRIB3 protein levels and improves the antitumor effects of doxorubicin and cisplatin by regulating the FOXO3–FOXM1 axis [290]. Our findings further suggest that inhibiting TRIB3 may enhance the therapeutic effectiveness of DNMT inhibitors when used they could be used in gastric adenocarcinoma, and possibly other cancers.

Despite its role as a tumor suppressor, we found that *CDKN2A* was classified as a risk factor for gastric adenocarcinoma (**Figure 3.4A**) based on Cox regression analysis [291]. This reflects an empirical association between elevated *CDKN2A* expression and with poor clinical outcomes. Mechanistically, p16 inhibits CDK4/6, prevents retinoblastoma (RB) phosphorylation, and blocks the G1–S phase transition, thereby restraining oncogene-driven proliferation and inducing growth arrest and senescence in damaged cells (**Figure 3.9A**) [236,237]. However, in gastric adenocarcinoma this tumor-suppressive function is often impaired but by a variety of mechanisms. Some tumors show complete p16 silencing due to *p16* promoter CpG hypermethylation (e.g., AGS and SNU719 cells) (**Figure 3.9B**), whereas others display paradoxical *p16* overexpression that

does not induce checkpoint activity and is instead associated with poor prognosis [292,293]. Such overexpression has been observed in many cancers and may represent a compensatory feedback response to disruption of the RB pathway, which can include loss of RB activity [294,295], *cyclin D1* overexpression with *CDK4/6* hyperactivation [296,297], and *CDK2* or *CCNE1* overexpression (Figure 3.9C) [298,299]. Although p16 is highly expressed in such gastric tumors, it is ineffective in blocking G1 progression. This proposed feedback mechanism is further supported by the fact that we identified the RB regulators *CDKN2A*, *CDK2*, *CDK4*, *CDK6*, and *CCNE1*, as elevated in TNMplot (**Figure 3.4B, Table 3.3**) and we identified *CDKN2A*, *CDK4*, and *CCNE1* as risk factors for gastric adenocarcinoma (**Figure 3.4A, Table 3.3**). Therefore, elevated expression of *CDKN2A*/p16, as well as *CDK2*, *CDK4*, *CDK6*, and *CCNE1* expression may result from a feedback mechanism caused by defective RB signaling, leading to impaired checkpoint function. These observations further suggest that increasing *CDKN2A* expression alone may only be effective in gastric adenocarcinoma associated with low *CDKN2A* expression caused by promoter CpG hypermethylation.

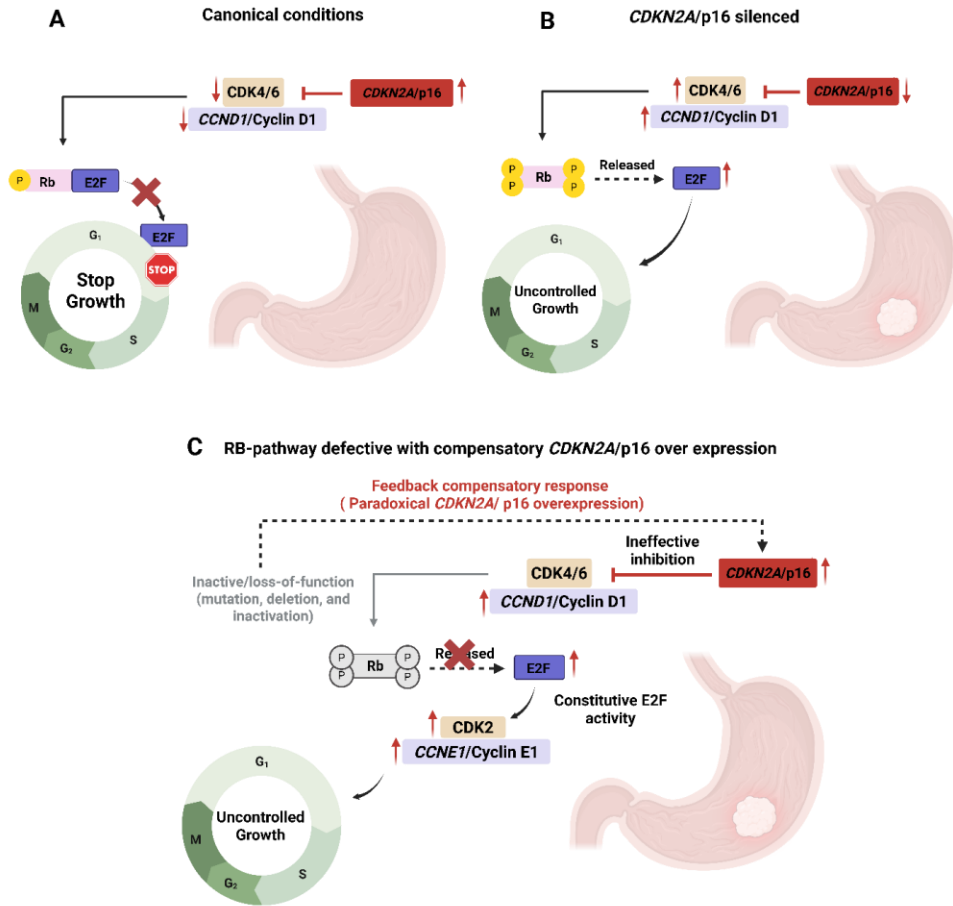


Figure 3.9 Proposed working model of CDKN2A/p16–RB checkpoint control and its disruption in gastric adenocarcinoma.

(A) Under canonical conditions, CDKN2A/p16 inhibits CDK4/6–Cyclin D1 activity, thereby maintaining RB in a hypophosphorylated state, retaining E2F transcription factors, and enforcing G1–S checkpoint arrest. (B) In gastric adenocarcinoma, this tumor-suppressive axis is disrupted by CDKN2A promoter CpG hypermethylation, which silences p16 and permits CDK4/6-mediated RB phosphorylation and E2F release, resulting in uncontrolled proliferation. In this setting, DNMT inhibitor–mediated demethylation may restore p16 expression and potentially recover RB-dependent checkpoint control. (C) In the RB pathway–defective, such as those with RB1 loss or inactivation, p16 overexpression may occur as a compensatory feedback response but remains ineffective in restraining cell-cycle progression. Despite elevated p16, constitutive E2F activity persists, often accompanied by increased CDK2–Cyclin E1 signaling, thereby promoting continued tumor growth. Abbreviations: CDK4/6, cyclin-dependent kinase 4 and 6; CDKN2A/p16, cyclin-dependent inhibitor 2A/p16INK4a; CCND1/Cyclin D1, cyclin D1; CCNE1/Cyclin E1, cyclin E1; CDK2, cyclin-dependent kinase 2; Rb, retinoblastoma protein; E2F, E2F transcription factor; P, phosphorylated state/phosphorylation.

We previously observed that *CDKN2A*/p16 shows a characteristic low-high-low expression pattern with increasing DNMT inhibitor concentration in AGS and SNU719 cells [277]. Building on this, we designed the first approach using this dynamic *CDKN2A*/p16 expression pattern to identify

genes whose transcriptional changes most closely parallel *CDKN2A/p16* in gastric adenocarcinoma cells. Unlike most molecular investigations relying on static tumor versus non-tumor comparisons [300–302] or single-snapshot TCGA datasets [303], our approach adds functional and temporal dimensions, capturing drug-induced transcriptional dynamics essential for understanding treatment adaptation and resistance. In AGS and SNU719 cells, increasing decitabine concent induces the same low-high-low *CDKN2A/p16* expression pattern with *TRIB3* but not in MKN45 cells which do not express *CDKN2A/p16* (**Figure 3.8G**). However, with GSK3685032 treatment the low-high-low expression pattern also seems to be observed with *TRIB3* in MKN45 cells (**Figure 3.8I**) calling into question whether *TRIB3* is truly *CDKN2A/p16*-synchronized. To further clarify the relationship between *TRIB3* and *CDKN2A/p16*, we examined raw expression values (ie not normalized to maximum expression) and dose-dependent responses across cell lines. In AGS cells, *TRIB3* expression closely parallels *CDKN2A/p16* reactivation, showing strong induction at low GSK3685032 doses followed by a coordinated decrease at higher doses. In SNU719 cells, *TRIB3* expression increases from an undetectable baseline to an induced level. In contrast, in MKN45 cells, although *TRIB3* displays a similar directional low–high–low pattern, the magnitude of induction of *TRIB3* expression was approximately 4.4-fold higher in AGS and 3.2-fold higher in SNU719 cells compared to MKN45 cells. This suggests that the pattern of *TRIB3* in GSK3685032 treated MKN45 cells is a different response compared to AGS and SNU719 cells. Another possible explanation is the slightly different mechanism of GSK3685032 [160], which is a DNMT1 selective competitive inhibitor whereas decitabine is a broad inhibitor of DNMT1, DNMT3A and 3B forming permanent covalent interaction with the DNMTs [202,304].

Our findings establish a framework for exploring network-level responses to DNMT inhibitors and led to the discovery of *TRIB3* as a robust *CDKN2A/p16*-synchronized stress effector standing

out across almost all validation results, and positioning TRIB3 as a key adaptive resistance node downstream of the *CDKN2A*/p16-driven stress network, and a co-targeting candidate for DNMT inhibitor response. However, validation was limited to transcript levels using qPCR, without confirming protein activity or functional phenotypes, and drug testing was conducted *in vitro*, without *in vivo* corroboration. Future directions include multi-omics expansion (proteomics and phosphoproteomics), functional perturbation (overexpression/knockout) of TRIB3, and exploring combination strategies co-targeting DNMTs together with the MAPK14–AKT3–TRIB3 stress-adaptation pathway [290]. Together, these findings define a *CDKN2A*/p16-driven stress-adaptive network and identify *TRIB3* as a key effector that may be exploited to overcome adaptive resistance to DNMT inhibitor therapy.

We previously reported that *CDKN2A* shows a characteristic low-high-low expression pattern with increasing DNMT inhibitor concentrations in AGS and SNU719 cells [277]. Building on this, our first approach used this dynamic *CDKN2A* expression pattern to identify genes whose transcriptional changes most closely paralleled those of *CDKN2A* in gastric adenocarcinoma cells. Unlike most molecular investigations relying on static tumor versus non-tumor comparisons [300–302] or single-snapshot TCGA datasets [303], our approach adds functional and temporal dimensions, capturing drug-induced transcriptional dynamics essential for understanding treatment adaptation and resistance. In AGS and SNU719 cells, increasing concentrations of decitabine induces the same low-high-low expression pattern in *CDKN2A*/p16 and TRIB3 but this pattern was not observed in MKN45 cells, which do not express *CDKN2A*/p16 (**Figure 3.8G**). However, with GSK3685032 treatment the low-high-low expression pattern also seems to be observed with TRIB3 in MKN45 cells (**Figure 3.8I**) calling into question whether TRIB3 is truly *CDKN2A*/p16-concordant. To further clarify the relationship between TRIB3 and *CDKN2A*, we

examined raw expression values (ie., values not normalized to maximum expression) and dose-dependent responses across cell lines. In AGS cells, *TRIB3* expression closely paralleled *CDKN2A* reactivation, showing strong induction at low GSK3685032 doses followed by a coordinated decrease at higher doses. In SNU719 cells, *TRIB3* expression increased from an undetectable baseline to an induced level. In contrast, in MKN45 cells, although *TRIB3* displayed a similar directional low–high–low pattern, the magnitude of *TRIB3* induction was approximately 4.4-fold higher in AGS cells and 3.2-fold higher in SNU719 cells compared to MKN45 cells. This suggests that the *TRIB3* response in GSK3685032 treated MKN45 cells differs from that observed in AGS and SNU719 cells. Another possible explanation is the different mechanisms of action of GSK3685032 and decitabine. GSK3685032[18], is a DNMT1 selective competitive inhibitor [160], whereas decitabine is a broad inhibitor of DNMT1, DNMT3A, and DNMT3B that forms covalent interaction with the DNMTs [202,304].

Our findings established a framework for exploring network-level responses to DNMT inhibitors and led to the identification of *TRIB3* as a robust *CDKN2A*/p16-concordant stress response gene. *TRIB3* stood out across nearly all validation results, and positioning it as a key adaptive resistance node in stress-response network associated with DNMT inhibitor-mediated p16 reactivation and as a co-target in DNMT inhibitor therapy. Although the protein expression patterns of selected candidates were descriptively assessed using Human Protein Atlas immunohistochemistry data from normal gastric and gastric cancer tissues, *TRIB3* protein abundance, protein activity, and downstream functional consequences were not directly measured in DNMT inhibitor-treated cell lines. Therefore, validation was limited to transcript levels changes measured by qPCR, without confirming protein activity or functional phenotypes, and drug testing was conducted *in vitro*, without *in vivo* corroboration. Subsequent studies will assess protein-level changes, functional

phenotypes, and in vivo therapeutic responses to determine the translational relevance of TRIB3. Future directions include multi-omics expansion (proteomics and phosphoproteomics), functional perturbation of TRIB3 (overexpression/knockout), and exploring combination strategies co-targeting DNMTs together with the MAPK14–AKT3–TRIB3 stress-adaptation pathway [290]. Together, these findings define a *CDKN2A*/p16-associated stress-adaptive network and identify *TRIB3* as a key effector that may be exploited to overcome adaptive resistance to DNMT inhibitor therapy.

3.5 Supplementary

Table S3.1 The 24 genes identified using the Network-Correlation-Based Approach demonstrated significant correlations with CDKN2A Methylation via considering both the Spearman and Pearson correlation indices.

Expression	CDKN2A Methylation		
Gene Name	Spearman	Pearson	Decision
TP53	Spearman: 0.10 (p = 0.0431)	Pearson: 0.02 (p = 0.736)	--
MYC	Spearman: 0.05 (p = 0.267)	Pearson: 0.05 (p = 0.338)	--
CDK4	Spearman: 0.21 (p = 1.902e-5)	Pearson: 0.22 (p = 8.368e-6)	+
EGFR	Spearman: -0.19 (p = 1.279e-4)	Pearson: -0.13 (p = 6.330e-3)	+
CTNNB1	Spearman: 0.07 (p = 0.177)	Pearson: 0.07 (p = 0.176)	--
HIF1A	Spearman: -0.16 (p = 1.473e-3)	Pearson: -0.14 (p = 4.219e-3)	+
MDM2	Spearman: -0.10 (p = 0.0473)	Pearson: -0.07 (p = 0.134)	--
CCND2	Spearman: -0.19 (p = 1.277e-4)	Pearson: -0.19 (p = 1.359e-4)	+
CDK6	Spearman: 0.12 (p = 0.0127)	Pearson: 0.12 (p = 0.0124)	+
KRAS	Spearman: 0.04 (p = 0.478)	Pearson: 0.02 (p = 0.673)	--
NRAS	Spearman: 0.12 (p = 0.0124)	Pearson: 0.14 (p = 3.818e-3)	+
PTEN	Spearman: -0.19 (p = 8.162e-5)	Pearson: -0.17 (p = 4.982e-4)	+
HRAS	Spearman: 0.19 (p = 7.527e-5)	Pearson: 0.19 (p = 1.332e-4)	+
CCND1	Spearman: 0.12 (p = 0.0129)	Pearson: 0.14 (p = 4.052e-3)	+
HUWE1	Spearman: -0.08 (p = 0.0893)	Pearson: -0.10 (p = 0.0378)	--
RRAS2	Spearman: 0.04 (p = 0.444)	Pearson: -0.08 (p = 0.108)	--
CYTH1	Spearman: -0.30 (p = 8.77e-10)	Pearson: -0.26 (p = 6.50e-8)	+
ARF1	Spearman: 0.24 (p = 6.98e-7)	Pearson: 0.24 (p = 6.75e-7)	+
CDC6	Spearman: 0.34 (p = 7.03e-13)	Pearson: 0.37 (p = 8.96e-15)	+
CDKN2AIP	Spearman: 0.04 (p = 0.475)	Pearson: 0.02 (p = 0.745)	--
CDC7	Spearman: 0.34 (p = 1.46e-12)	Pearson: 0.35 (p = 2.21e-13)	+
MRAS	Spearman: -0.42 (p = 1.05e-18)	Pearson: -0.40 (p = 6.29e-17)	+
RRAS	Spearman: -0.33 (p = 4.53e-12)	Pearson: -0.33 (p = 7.56e-12)	+
MTAP	Spearman: 0.39 (p = 1.49e-16)	Pearson: 0.32 (p = 3.02e-11)	+
CDKN2B	Spearman: 0.16 (p = 1.562e-3)	Pearson: 0.17 (p = 5.104e-4)	+
CDK2	Spearman: 0.24 (p = 5.05e-7)	Pearson: 0.26 (p = 1.24e-7)	+
CDKN1B	Spearman: 0.06 (p = 0.233)	Pearson: 0.02 (p = 0.727)	--
MAPK13	Spearman: 0.27 (p = 1.67e-8)	Pearson: 0.32 (p = 3.39e-11)	+
MAPK12	Spearman: -0.10 (p = 0.0459)	Pearson: -0.06 (p = 0.189)	--
MAPK11	Spearman: -0.33(p = 6.96e-12)	Pearson: -0.30(p = 7.95e-10)	+

Table S3.2 The 15 genes identified using the Correlation-Based Approach demonstrated significant correlations with CDKN2A Methylation via considering both the Spearman and Pearson correlation indices.

Expression Gene Name	CDKN2A Methylation		
	Spearman	Pearson	Decision
TENT2	Spearman: -0.13 (p = 9.722e-3)	Pearson: -0.13 (p = 7.603e-3)	+
JAK2	Spearman: -0.15 (p = 2.924e-3)	Pearson: -0.12 (p = 0.0126)	+
CCNG1	Spearman: -0.05 (p = 0.310)	Pearson: -0.05 (p = 0.327)	--
NSMAF	Spearman: -0.01 (p = 0.873)	Pearson: -0.03 (p = 0.524)	--
AHCYL2	Spearman: -0.11 (p = 0.0288)	Pearson: -0.10 (p = 0.0443)	+
PDE4D	Spearman: -0.37 (p = 1.42e-14)	Pearson: -0.38 (p = 5.85e-16)	+
SLC25A46	Spearman: -0.23 (p = 1.466e-6)	Pearson: -0.25 (p = 4.03e-7)	+
LONP2	Spearman: -0.19 (p = 1.370e-4)	Pearson: -0.21 (p = 1.456e-5)	+
MAP4	Spearman: -0.35 (p = 1.35e-13)	Pearson: -0.37 (p = 5.35e-15)	+
ANKFY1	Spearman: -0.25 (p = 3.30e-7)	Pearson: -0.25 (p = 2.22e-7)	+
CDKN2B-AS1	Spearman: 0.28 (p = 4.39e-9)	Pearson: 0.29 (p = 1.79e-9)	+
CDKN2A-AS1	Spearman: 0.29 (p = 1.22e-8)	Pearson: 0.27 (p = 8.69e-8)	+
CDKN2B	Spearman: 0.16 (p = 1.562e-3)	Pearson: 0.17 (p = 5.104e-4)	+
TRIB3	Spearman: 0.27 (p = 2.66e-8)	Pearson: 0.23 (p = 1.530e-6)	+
GET4	Spearman: 0.25 (p = 2.87e-7)	Pearson: 0.27 (p = 4.24e-8)	+
AFDN-DT	Spearman: 0.19 (p = 1.947e-4)	Pearson: 0.16 (p = 1.377e-3)	+
H4C14	Spearman: 0.05 (p = 0.323)	Pearson: 0.06 (p = 0.193)	--
PROC	Spearman: 0.06 (p = 0.195)	Pearson: 0.05 (p = 0.357)	--
MESP2	Spearman: 0.07 (p = 0.177)	Pearson: 0.07 (p = 0.137)	--
CCNE1	Spearman: 0.39 (p = 1.69e-16)	Pearson: 0.37 (p = 6.36e-15)	+

Table S3.3 The nine genes identified through the TNM-PLOT database exhibited significant correlations in paired tumor and adjacent normal tissues

Gene Expression Comparison between Tumor and Normal	paired tumor and adjacent normal tissues	Mann_Whitney.p.	normal samples from non-cancerous patients and further pediatric tissues	Mann_Whitney.p.
EGFR	--	6.57e-01	+	7.2e-08
HRAS	--	1.05e-01	+	1.16e-02
NRAS	+	1.86e-03	+	1.78e-70
CDK2	+	5.43e-05	+	3.41e-23
PTEN	--	1.27e-01	+	2.08e-06
MAPK14	--	4.56e-01	--	8.49e-01
CDKN2A	+	3.78e-05	+	7.37e-57
ARF1	--	6.61e-02	+	9.57e-28
HIF1A	--	9.9e-01	+	1.92e-56
JAK2	--	1.75e-01	+	2.03e-04
CCNE1	+	1.21e-04	+	2.85e-69
CDKN2B	--	7.46e-01	+	4.41e-35
CDK4	+	1.7e-04	+	4.27e-30
CDK6	+	9.61e-04	+	2.61e-27
ANKFY1	--	5.09e-01	+	1.94e-05
MAP4	--	1.82e-01	--	1.19e-01
PDE4D	+	2.18e-02	+	8.29e-09
SLC25A46	--	8.38e-01	+	2.27e-03
TRIB3	+	2.06e-04	+	2.88e-25
GET4	+	2.32e-02	+	1.15e-82

Table S3.4 Eight genes identified using the OncoDB database demonstrated significant associations with various cancer stages, as determined by p-values from Analysis of Variance (ANOVA) tests.

Gene Name	Stage	Samples	Average	Median	Std	ANOVA-p value
EGFR	G1	12	168.4	44.0	442.4	3.5e-01
	G2	139	65.2	38.2	149.0	
	G3	234	80.3	42.1	211.6	
	Gx	9	44.5	45.8	24.2	
HRAS	G1	12	45.3	42.6	15.9	5.7e-01
	G2	139	47.5	44.4	19.3	
	G3	234	48.8	44.5	20.5	
	Gx	9	40.1	35.2	11.9	
NRAS	G1	12	50.2	47.4	19.0	8.0e-01
	G2	139	50.8	48.3	20.2	
	G3	234	50.3	46.0	22.3	
	Gx	9	43.7	43.2	13.6	
CDK2	G1	12	27.0	23.7	10.7	1.8e-01
	G2	139	31.0	28.6	14.6	
	G3	234	29.8	27.3	13.2	
	Gx	9	21.9	21.9	4.6	
PTEN	G1	12	36.0	34.6	10.0	7.3e-04
	G2	139	31.3	29.3	13.8	
	G3	234	37.4	37.5	13.7	
	Gx	9	39.9	42.3	18.1	
MAPK14	G1	12	45.6	47.9	13.4	1.0e-01
	G2	139	50.4	46.8	22.9	
	G3	234	46.6	44.9	16.5	
	Gx	9	58.4	53.7	23.7	
CDKN2A	G1	12	65.0	11.5	133.8	4.4e-02
	G2	139	86.1	32.1	128.3	
	G3	234	55.0	16.1	96.8	
	Gx	9	30.9	20.8	42.8	
ARF1	G1	12	828.6	819.9	194.7	2.6e-03
	G2	139	841.4	777.8	284.6	
	G3	234	750.5	726.8	186.6	
	Gx	9	774.3	677.7	255.5	
HIF1A	G1	12	200.4	140.2	185.6	1.0e-01
	G2	139	138.0	119.8	70.4	
	G3	234	145.7	120.2	85.2	
	Gx	9	137.9	97.6	79.1	
JAK2	G1	12	14.8	12.7	10.5	1.9e-01
	G2	139	12.9	10.0	9.5	
	G3	234	18.3	13.0	28.6	
	Gx	9	19.6	12.3	18.5	
CCNE1	G1	12	81.6	13.1	232.5	6.5e-02
	G2	139	59.3	18.0	135.3	
CDKN2B	G3	234	29.4	12.0	101.5	7.9e-01
	Gx	9	14.8	14.2	6.9	
	G1	12	15.4	13.4	10.3	
	G2	139	17.4	13.5	17.2	
CDK4	G3	234	17.8	13.7	18.2	6.3e-01
	Gx	9	12.1	10.1	8.4	
	G1	12	66.6	55.3	47.5	
	G2	139	68.5	61.2	36.3	

	G3	234	73.9	63.0	47.7	
	Gx	9	63.9	69.1	20.2	
CDK6	G1	12	26.7	22.3	22.4	9.6e-01
	G2	139	38.7	20.7	98.9	
	G3	234	38.1	19.0	95.3	
	Gx	9	27.3	19.4	17.1	
ANKFY1	G1	12	26.0	25.2	11.4	9.5e-01
	G2	139	28.2	25.5	14.4	
	G3	234	28.3	26.1	12.6	
	Gx	9	28.7	33.0	12.3	
MAP4	G1	12	123.1	116.8	42.1	1.1e-03
	G2	139	115.1	106.7	48.0	
	G3	234	137.0	128.2	59.9	
	Gx	9	161.0	135.3	85.2	
PDE4D	G1	12	11.9	8.8	10.4	1.1e-04
	G2	139	9.5	7.4	7.4	
	G3	234	13.4	11.0	10.4	
	Gx	9	20.5	18.1	15.5	
SLC25A46	G1	12	28.9	31.7	12.7	1.0e-02
	G2	139	25.6	24.0	10.6	
	G3	234	29.6	28.7	11.7	
	Gx	9	33.0	33.1	13.2	
TRIB3	G1	12	43.8	21.9	50.7	1.3e-02
	G2	139	50.9	37.3	47.4	
	G3	234	36.4	21.8	47.0	
	Gx	9	74.1	20.0	112.8	
GET4	G1	12	2.3	1.9	1.3	5.2e-03
	G2	139	4.0	3.4	2.1	
	G3	234	3.3	2.8	2.0	
	Gx	9	4.0	2.9	3.9	

Chapter 4: Conclusions and Future Directions

4.1 Conclusion

Based on the findings of this thesis, a critical need was identified to understand how DNMT inhibitors regulate tumor suppressor genes within adaptive epigenetic and transcriptional networks in gastric cancer. While DNMT inhibitors are commonly viewed as agents to decrease DNA methylation, the results presented here indicate that their biological effects are strongly influenced by dose-dependent and coordinated gene-network responses. These observations underscore the importance of moving beyond single-gene reactivation toward a network-based framework when evaluating epigenetic therapies in gastric cancer. Recognizing this complexity provided the rationale for the mechanistic analyses in Chapter 2 and the CDKN2A-centered network investigations in Chapter 3.

In Chapter 2, we examined the effects of three DNMT inhibitors—decitabine, RG108, and GSK3685032—across multiple gastric cancer cell lines. Changes in total DNA methylation, CDKN2A/p16 promoter methylation, gene expression, and cell-cycle progression were evaluated following DNMT inhibitor treatment. At low concentrations, DNMT inhibitors reduced total DNA methylation and p16 promoter CpG methylation, resulting in increased p16 mRNA and protein expression. In contrast, higher concentrations induced a paradoxical increase in DNA methylation at both total and gene-specific levels, accompanied by decreased p16 mRNA and protein expression in AGS and SNU719 cells sometimes back to zero inhibitor levels. Similar dose-dependent patterns were observed for additional genes regulated by promoter CpG methylation, indicating that DNMT inhibitors exert genome-wide effects. The paradoxical increase in DNA methylation at higher inhibitor concentrations was associated with elevated expression of DNMT3B and, in some cases, TET1 and TET2 at both the mRNA and protein levels. Low doses

of decitabine and GSK3685032 increased the expression of the cell-cycle regulators p16 and p21, although to different extents. GSK3685032 preferentially enhanced p16 expression, whereas decitabine more strongly induced p21 expression. Consistent with these differences, GSK3685032 primarily induced G0/G1 cell-cycle arrest, while decitabine predominantly caused G2/M arrest in gastric cancer cells. Taken together, the findings in Chapter 2 suggest that, if translatable to clinical settings, low doses of decitabine and GSK3685032 may enhance p16 expression and thus hold therapeutic potential for gastric cancer, whereas higher doses may reduce p16 expression back to zero inhibitor levels and reduce efficacy. These results provide mechanistic insight into the dose-dependent and context-specific responses that likely contribute to the inconsistent efficacy of DNMT inhibitors observed in solid tumors.

Building upon the dose-dependent epigenetic responses characterized in Chapter 2, Chapter 3 expanded the scope of investigation from the single tumor suppressor gene CDKN2A to a broader regulatory network centered on CDKN2A. Large-scale database mining, network topology analysis, and dynamic pharmacological validation were integrated to systematically identify CDKN2A-associated adaptive genes. Starting from 3,839 gastric cancer-related genes curated from GETdb, OncoKB, UALCAN, CTD, KEGG, NeDRex, and STRING, pathway-relevant and cell-line-specific networks were refined using DepMap and Cell Model Passports datasets. Network reconstruction and centrality analyses across 16 independent network contexts yielded a CDKN2A-centered hub set, which was further filtered using TCGA-based correlations between CDKN2A methylation and gene expression, resulting in a 20-gene panel with strong clinical and network support. Clinical validation using TNMplot, KM-plotter, and OncoDB demonstrated that multiple genes, including TRIB3 and GET4, were significantly associated with tumor-normal expression differences, disease stage, and patient survival. Protein-protein interaction analyses

using STRING and IMEx revealed CDKN2A-linked signaling axes connecting cell-cycle control (CDK4–CCNE1), growth-factor signaling (EGFR–JAK2), and stress-adaptation pathways (MAPK14–AKT3–TRIB3). Dynamic qPCR profiling in gastric cancer cell lines treated with decitabine and GSK3685032 identified TRIB3 and PDE4D as robust CDKN2A-synchronized response genes, exhibiting dose-dependent concordance with CDKN2A/p16 reactivation and absent responses in CDKN2A-null cells. By combining database-driven discovery with dynamic experimental validation, chapter 3 establishes a framework for identifying drug co-targeting genes based on CDKN2A-synchronized gene networks under DNMT inhibitor treatment. Collectively, this systems-level framework reveals how epigenetic therapy activates a CDKN2A-driven stress network and identifies TRIB3 as a rational drug co-targeting candidate for DNMT-inhibitor-CDKN2A reactivation therapy in gastric cancer.

4.2 Future Directions

The results presented in this thesis suggest several directions for future investigation. First, extending the current in vitro findings to in vivo gastric cancer models will be important to determine whether the observed dose-dependent epigenetic paradox is reproduced within the tumor microenvironment. Second, further investigation of the regulatory mechanisms controlling DNMT3B and TET enzyme induction, together with adaptive stress-response regulators such as TRIB3, may help clarify how compensatory epigenetic responses arise at higher inhibitor doses. Finally, the identification of TRIB3 as a CDKN2A-synchronized response gene suggests that co-targeting adaptive stress-response pathways alongside DNMT inhibition may represent a rational direction for improving the durability of CDKN2A reactivation in gastric cancer.

Reference

1. Bray F, Laversanne M, Sung H, Ferlay J, Siegel RL, Soerjomataram I, et al. Global cancer statistics 2022: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. *CA Cancer J Clin* [Internet]. 2024;74:229–63. <https://doi.org/10.3322/caac.21834>
2. Yang W-J, Zhao H-P, Yu Y, Wang J-H, Guo L, Liu J-Y, et al. Updates on global epidemiology, risk and prognostic factors of gastric cancer. *World J Gastroenterol* [Internet]. Baishideng Publishing Group Inc; 2023;29:2452–68. <https://doi.org/10.3748/wjg.v29.i16.2452>
3. Lin JL, Lin JX, Lin GT, Huang CM, Zheng CH, Xie JW, et al. Global incidence and mortality trends of gastric cancer and predicted mortality of gastric cancer by 2035. *BMC Public Health*. BioMed Central Ltd; 2024;24. <https://doi.org/10.1186/s12889-024-19104-6>
4. Machlowska J, Baj J, Sitarz M, Maciejewski R, Sitarz R. Gastric Cancer: Epidemiology, Risk Factors, Classification, Genomic Characteristics and Treatment Strategies. *Int J Mol Sci* [Internet]. MDPI AG; 2020;21:4012. <https://doi.org/10.3390/ijms21114012>
5. Yu X, Zhao H, Wang R, Chen Y, Ouyang X, Li W, et al. Cancer epigenetics: from laboratory studies and clinical trials to precision medicine. *Cell Death Discov* [Internet]. Springer Nature; 2024;10:28. <https://doi.org/10.1038/s41420-024-01803-z>
6. Tahara T, Arisawa T. DNA methylation as a molecular biomarker in gastric cancer. *Epigenomics* [Internet]. 2015;7:475–86. <https://doi.org/10.2217/epi.15.4>
7. Ebrahimi V, Soleimanian A, Ebrahimi T, Azargun R, Yazdani P, Eyvazi S, et al. Epigenetic modifications in gastric cancer: Focus on DNA methylation. *Gene*. 2020;742. <https://doi.org/10.1016/j.gene.2020.144577>
8. Fattahi S, Golpour M, Amjadi-Moheb F, Sharifi-Pasandi M, Khodadadi P, Pilehchian-Langroudi M, et al. DNA methyltransferases and gastric cancer: Insight into targeted therapy. *Epigenomics*. 2018;10:1477–97. <https://doi.org/10.2217/epi-2018-0096>
9. Yu J, Xie T, Wang Z, Wang X, Zeng S, Kang Y, et al. DNA methyltransferases: emerging targets for the discovery of inhibitors as potent anticancer drugs. *Drug Discov Today* [Internet]. Elsevier Ltd; 2019;24:2323–31. <https://doi.org/10.1016/j.drudis.2019.08.006>
10. Morris LGT, Chan TA. Therapeutic targeting of tumor suppressor genes. *Cancer* [Internet]. John Wiley and Sons Inc; 2015;121:1357–68. <https://doi.org/10.1002/cncr.29140>
11. Lopez M, Halby L, Arimondo PB. DNA Methyltransferase Inhibitors: Development and Applications. Jeltsch A, Jurkowska RZ, editors. *Adv Exp Med Biol* [Internet]. Cham: Springer International Publishing; 2016;945:431–73. https://doi.org/10.1007/978-3-319-43624-1_16

12. Xu P, Hu G, Luo C, Liang Z. DNA methyltransferase inhibitors: an updated patent review (2012-2015). *Expert Opin Ther Pat.* 2016;26:1017–30. <https://doi.org/10.1080/13543776.2016.1209488>
13. Mehdipour P, Chen R, De Carvalho DD. The next generation of DNMT inhibitors. *Nat Cancer* [Internet]. Nature Research; 2021;2:1000–1. <https://doi.org/10.1038/s43018-021-00271-z>
14. Moore LD, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* [Internet]. Nature Publishing Group; 2013;38:23–38. <https://doi.org/10.1038/npp.2012.112>
15. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases Non-invasive sexing of preimplantation stage mammalian embryos. *Nature America Inc* [Internet]. 1998;19:219–20. <https://doi.org/https://doi-org.uml.idm.oclc.org/10.1038/890>
16. Haggerty C, Kretzmer H, Riemenschneider C, Kumar AS, Mattei AL, Bailly N, et al. Dnmt1 has de novo activity targeted to transposable elements. *Nat Struct Mol Biol.* Nature Research; 2021;28:594–603. <https://doi.org/10.1038/s41594-021-00603-8>
17. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* [Internet]. 1999;99:247–57. [https://doi.org/10.1016/s0092-8674\(00\)81656-6](https://doi.org/10.1016/s0092-8674(00)81656-6)
18. Jia D, Jurkowska RZ, Zhang X, Jeltsch A, Cheng X. Structure of Dnmt3a bound to Dnmt3L suggests a model for de novo DNA methylation. *Nature.* 2007;449:248–51. <https://doi.org/10.1038/nature06146>
19. Newell-Price J, Clark AJL, King P. DNA methylation and silencing of gene expression. *Trends in Endocrinology and Metabolism.* 2000;11:142–8. [https://doi.org/10.1016/S1043-2760\(00\)00248-4](https://doi.org/10.1016/S1043-2760(00)00248-4)
20. Kass SU, Pruss D, Wolffe AP. How does DNA methylation repress transcription? *Trends in Genetics.* 1997;13:444–9. [https://doi.org/10.1016/S0168-9525\(97\)01268-7](https://doi.org/10.1016/S0168-9525(97)01268-7)
21. Yang X, Han H, DeCarvalho DD, Lay FD, Jones PA, Liang G. Gene body methylation can alter gene expression and is a therapeutic target in cancer. *Cancer Cell.* Cell Press; 2014;26:577–90. <https://doi.org/10.1016/j.ccr.2014.07.028>
22. Jones PA. Functions of DNA methylation: islands, start sites, gene bodies and beyond. *Nat Rev Genet* [Internet]. 2012;13:484–92. <https://doi.org/10.1038/nrg3230>
23. Jjingo D, Conley AB, Yi S V, Lunyak V V, Jordan IK. On the presence and role of human gene-body DNA methylation. *Oncotarget* [Internet]. 2012;3:462–74. <https://doi.org/10.18632/oncotarget.497>
24. Pappalardo XG, Barra V. Losing DNA methylation at repetitive elements and breaking bad. *Epigenetics Chromatin* [Internet]. BioMed Central Ltd; 2021;14:25. <https://doi.org/10.1186/s13072-021-00400-z>

25. Li E, Bestor TH, Jaenisch R. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* [Internet]. 1992;69:915–26. [https://doi.org/10.1016/0092-8674\(92\)90611-f](https://doi.org/10.1016/0092-8674(92)90611-f)
26. Lei H, Oh SP, Okano M, Jüttermann R, Goss KA, Jaenisch R, et al. De novo DNA cytosine methyltransferase activities in mouse embryonic stem cells. *Development* [Internet]. 1996;122:3195–205. <https://doi.org/10.1242/dev.122.10.3195>
27. Sado T, Fenner MH, Tan SS, Tam P, Shioda T, Li E. X inactivation in the mouse embryo deficient for Dnmt1: Distinct effect of hypomethylation on imprinted and random X inactivation. *Dev Biol*. Academic Press Inc.; 2000;225:294–303. <https://doi.org/10.1006/dbio.2000.9823>
28. Li Z, Dai H, Martos SN, Xu B, Gao Y, Li T, et al. Distinct roles of DNMT1-dependent and DNMT1-independent methylation patterns in the genome of mouse embryonic stem cells. *Genome Biol*. BioMed Central Ltd.; 2015;16. <https://doi.org/10.1186/s13059-015-0685-2>
29. Morris MJ, Na ES, Autry AE, Monteggia LM. Impact of DNMT1 and DNMT3a forebrain knockout on depressive- and anxiety like behavior in mice. *Neurobiol Learn Mem*. Academic Press Inc.; 2016;135:139–45. <https://doi.org/10.1016/j.nlm.2016.08.012>
30. Feng J, Zhou Y, Campbell SL, Le T, Li E, Sweatt JD, et al. Dnmt1 and Dnmt3a maintain DNA methylation and regulate synaptic function in adult forebrain neurons. *Nat Neurosci*. 2010;13:423–30. <https://doi.org/10.1038/nn.2514>
31. Jorgensen BG, Berent RM, Ha SE, Horiguchi K, Sasse KC, Becker LS, et al. DNA methylation, through DNMT1, has an essential role in the development of gastrointestinal smooth muscle cells and disease. *Cell Death Dis*. Nature Publishing Group; 2018;9. <https://doi.org/10.1038/s41419-018-0495-z>
32. Wu TT, Ma YW, Zhang X, Dong W, Gao S, Wang JZ, et al. Myocardial tissue-specific Dnmt1 knockout in rats protects against pathological injury induced by Adriamycin. *Laboratory Investigation*. Springer Nature; 2020;100:974–85. <https://doi.org/10.1038/s41374-020-0402-y>
33. Liao J, Karnik R, Gu H, Ziller MJ, Clement K, Tsankov AM, et al. Targeted disruption of DNMT1, DNMT3A and DNMT3B in human embryonic stem cells. *Nat Genet*. Nature Publishing Group; 2015;47:469–78. <https://doi.org/10.1038/ng.3258>
34. James SR, Link PA, Karpf AR. Epigenetic regulation of X-linked cancer/germline antigen genes by DNMT1 and DNMT3b. *Oncogene*. 2006;25:6975–85. <https://doi.org/10.1038/sj.onc.1209678>
35. Hirasawa R, Chiba H, Kaneda M, Tajima S, Li E, Jaenisch R, et al. Maternal and zygotic Dnmt1 are necessary and sufficient for the maintenance of DNA methylation imprints during preimplantation development. *Genes Dev*. 2008;22:1607–16. <https://doi.org/10.1101/gad.1667008>

36. Kaneda M, Okano M, Hata K, Sado T, Tsujimoto N, Li E, et al. Essential role for de novo DNA methyltransferase Dnmt3a in paternal and maternal imprinting. *Nature* [Internet]. 2004;429:900–3. <https://doi.org/10.1038/nature02633>
37. Madsen A, Höppner G, Krause J, Hirt MN, Laufer SD, Schweizer M, et al. An Important Role for DNMT3A-Mediated DNA Methylation in Cardiomyocyte Metabolism and Contractility. *Circulation*. Lippincott Williams and Wilkins; 2020;142:1562–78. <https://doi.org/10.1161/CIRCULATIONAHA.119.044444>
38. Li Q, Lu J, Yin X, Chang Y, Wang C, Yan M, et al. Base editing-mediated one-step inactivation of the Dnmt gene family reveals critical roles of DNA methylation during mouse gastrulation. *Nat Commun*. Nature Research; 2023;14. <https://doi.org/10.1038/s41467-023-38528-z>
39. Li Y, Liang J, Hou P. Hypermethylation in gastric cancer. *Clinica Chimica Acta*. Elsevier; 2015. p. 124–32. <https://doi.org/10.1016/j.cca.2015.07.001>
40. Sogutlu F, Pekerbas M, Biray Avcı Ç. Epigenetic signatures in gastric cancer: current knowledge and future perspectives. *Expert Rev Mol Diagn*. 2022;22. <https://doi.org/10.1080/14737159.2022.2159381>
41. Watari J, Ito C, Shimoda T, Tomita T, Oshima T, Fukui H, et al. DNA methylation silencing of microRNA gene methylator in the precancerous background mucosa with and without gastric cancer: Analysis of the effects of H. pylori eradication and long-term aspirin use. *Sci Rep*. Nature Publishing Group; 2019;9. <https://doi.org/10.1038/s41598-019-49069-1>
42. Pagano JS, Whitehurst CB, Andrei G. Antiviral Drugs for EBV. *Cancers (Basel)* [Internet]. MDPI AG; 2018;10. <https://doi.org/10.3390/cancers10060197>
43. Liang Q, Yao X, Tang S, Zhang J, Yau TO, Li X, et al. Integrative identification of Epstein-Barr virus-associated mutations and epigenetic alterations in gastric cancer. *Gastroenterology*. W.B. Saunders; 2014;147:1350-1362.e4. <https://doi.org/10.1053/j.gastro.2014.08.036>
44. Spainhour JCG, Lim HS, Yi S V., Qiu P. Correlation Patterns Between DNA Methylation and Gene Expression in The Cancer Genome Atlas. *Cancer Inform*. SAGE Publications Ltd; 2019;18. <https://doi.org/10.1177/1176935119828776>
45. Bass AJ, Thorsson V, Shmulevich I, Reynolds SM, Miller M, Bernard B, et al. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature*. Nature Publishing Group; 2014;513:202–9. <https://doi.org/10.1038/nature13480>
46. Stanland LJ, Luftig MA. The role of ebv-induced hypermethylation in gastric cancer tumorigenesis. *Viruses*. MDPI AG; 2020. <https://doi.org/10.3390/v12111222>
47. Gao Y, Fu Y, Wang J, Zheng X, Zhou J, Ma J. EBV as a high infection risk factor promotes RASSF10 methylation and induces cell proliferation in EBV-associated gastric cancer. *Biochem Biophys Res Commun*. Elsevier B.V.; 2021;547:1–8. <https://doi.org/10.1016/j.bbrc.2021.02.014>

48. Gong Z, Bi C, Liu W, Luo B. Comprehensive Analysis Based on the TCGA Database Identified SCIN as a Key DNA Methylation-Driver Gene in Epstein-Barr Virus-Associated Gastric Cancer. *Biochem Genet.* Springer; 2024; <https://doi.org/10.1007/s10528-024-10702-y>
49. Ghoreshi ZAS, Rezaei Zadeh Rukerd M, Askarpour H, Kheirkhah Vakilabad AA, Nakhaie M, Abbaszadeh Afshar MJ, et al. The Role of Epstein-Barr Virus (EBV) Infected Gastric Cancer in Increasing microRNA124 (miR-124) Promoter Methylation and Enhancer of Zeste Homolog 2 (EZH2) Gene Expression. *Medicine (United States).* Lippincott Williams and Wilkins; 2024;103:E36534. <https://doi.org/10.1097/MD.00000000000036534>
50. He D, Zhang Y wang, Zhang N na, Zhou L, Chen J ning, Jiang Y, et al. Aberrant gene promoter methylation of p16, FHIT, CRBP1, WWOX, and DLC-1 in Epstein–Barr virus-associated gastric carcinomas. *Medical Oncology [Internet].* 2015 [cited 2019 Oct 25];32:1–16. <https://doi.org/10.1007/s12032-015-0525-y>
51. Ma C, Wang B, Pu W, Ma H, Song K, Wang N, et al. Association between eight hypermethylation-related genes and gastric cancer: a systematic review and meta-analysis. *Transl Cancer Res [Internet].* AME Publishing Company; 2022;11:2026–39. <https://doi.org/10.21037/tcr-22-372>
52. Kim JK, Noh JH, Eun JW, Jung KH, Bae HJ, Shen Q, et al. Targeted inactivation of HDAC2 restores p16INK4a activity and exerts antitumor effects on human gastric cancer. *Molecular Cancer Research.* 2013;11:62–73. <https://doi.org/10.1158/1541-7786.MCR-12-0332>
53. Lukas J, Parry D, Aagaard L, Mann DJ, Bartkova J, Strauss M, et al. Retinoblastoma-protein-dependent cell-cycle inhibition by the tumour suppressor p16. *Nature [Internet].* 1995 [cited 2019 Oct 23];375:503–6. <https://doi.org/10.1038/375503a0>
54. Weinberg RA. The retinoblastoma protein and cell cycle control. *Cell [Internet].* 1995 [cited 2019 Oct 23];81:323–30. [https://doi.org/10.1016/0092-8674\(95\)90385-2](https://doi.org/10.1016/0092-8674(95)90385-2)
55. Ficorella C, Cannita K, Ricevuto E, Toniato E, Fusco C, Sinopoli NT, et al. p16 hypermethylation contributes to the characterization of gene inactivation profiles in primary gastric cancer. *Oncol Rep.* 2003;10:169–73.
56. Ding Y, Le XP, Zhang QX, Du P. Methylation and mutation analysis of p16 gene in gastric cancer. *World J Gastroenterol.* 2003;9:423–6. <https://doi.org/10.3748/wjg.v9.i3.423>
57. An C, Choi I-S, Yao JC, Worah S, Xie K, Mansfield PF, et al. Prognostic significance of CpG island methylator phenotype and microsatellite instability in gastric carcinoma. *Clin Cancer Res [Internet].* 2005 [cited 2019 Oct 23];11:656–63. <http://www.ncbi.nlm.nih.gov/pubmed/15701853>. Accessed 23 Oct 2019
58. Li L, Fei X, Wang H, Chen S, Xu X, Ke H, et al. Genome-wide DNA methylation profiling reveals a novel hypermethylated biomarker PRKCB in gastric cancer. *Sci Rep [Internet].* 2024;14:26605. <https://doi.org/10.1038/s41598-024-78135-6>

59. Hu D, Lou X, Meng N, Li Z, Teng Y, Zou Y, et al. Peripheral Blood-Based DNA Methylation of Long Non-Coding RNA H19 and Metastasis-Associated Lung Adenocarcinoma Transcript 1 Promoters are Potential Non-Invasive Biomarkers for Gastric Cancer Detection. *Cancer Control*. SAGE Publications Ltd; 2021;28. <https://doi.org/10.1177/10732748211043667>
60. Tsai KW, Tsai CY, Chou NH, Wang KC, Kang CH, Li SC, et al. Aberrant DNA hypermethylation silenced lncRNA expression in gastric cancer. *Anticancer Res. International Institute of Anticancer Research*; 2019;39:5381–91. <https://doi.org/10.21873/anticancerres.13732>
61. Dong M, Wang HY, Zhao XX, Chen JN, Zhang YW, Huang Y, et al. Expression and prognostic roles of PIK3CA, JAK2, PD-L1, and PD-L2 in Epstein-Barr virus-associated gastric carcinoma. *Hum Pathol. W.B. Saunders*; 2016;53:25–34. <https://doi.org/10.1016/j.humpath.2016.02.007>
62. Azarkhazin F, Tehrani GA. Detecting promoter methylation pattern of apoptotic genes Apaf1 and Caspase8 in gastric carcinoma patients undergoing chemotherapy. *J Gastrointest Oncol. AME Publishing Company*; 2018;9:295–302. <https://doi.org/10.21037/jgo.2017.12.05>
63. Li Z, Lei H, Luo M, Wang Y, Dong L, Ma Y, et al. DNA methylation downregulated mir-10b acts as a tumor suppressor in gastric cancer. *Gastric Cancer. Springer*; 2015;18:43–54. <https://doi.org/10.1007/s10120-014-0340-8>
64. Huang Y, Lu H, Li H. DNA methyltransferase 3a-induced hypermethylation of the fructose-1,6-bisphosphatase-2 promoter contributes to gastric carcinogenesis. *Mol Biol Rep. Springer Science and Business Media B.V.*; 2024;51. <https://doi.org/10.1007/s11033-023-08966-5>
65. Chen J, Zhang J, Li X, Zhang C, Zhang H, Jin J, et al. Downregulation of ADAMTS8 by DNA Hypermethylation in Gastric Cancer and Its Clinical Significance. *Biomed Res Int. Hindawi Limited*; 2016;2016. <https://doi.org/10.1155/2016/5083841>
66. Han F, Sun L-P, Liu S, Xu Q, Liang Q-Y, Zhang Z, et al. Promoter methylation of RNF180 is associated with H.pylori infection and serves as a marker for gastric cancer and atrophic gastritis. *Oncotarget [Internet]*. 2016;7:24800–9. <https://doi.org/10.18632/oncotarget.8523>
67. Zhang J kui, Li Y shuang, Zhang C dong, Dai D qiu. Up-regulation of CRKL by microRNA-335 methylation is associated with poor prognosis in gastric cancer. *Cancer Cell Int. BioMed Central Ltd.*; 2017;17. <https://doi.org/10.1186/s12935-017-0387-9>
68. Feng L, Yao C, Li P, Feng Y, Wang F, Liu YF, et al. Low expression of fibulin-1 correlates with unfavorable prognosis in gastric cancer. *Tumor Biology. Springer Science and Business Media B.V.*; 2016;37:9399–410. <https://doi.org/10.1007/s13277-015-4537-1>
69. Xu X, Chang X, Xu Y, Deng P, Wang J, Zhang C, et al. SAMD14 promoter methylation is strongly associated with gene expression and poor prognosis in gastric cancer. *Int J Clin Oncol. Springer*; 2020;25:1105–14. <https://doi.org/10.1007/s10147-020-01647-4>

70. Kojima K, Minatani N, Ushiku H, Ishii S, Tanaka T, Yokoi K, et al. Prediction of onset of remnant gastric cancer by promoter DNA methylation of CDO1/HOPX/Reprimo/E-cadherin [Internet]. 2019. www.oncotarget.com
71. Yin H, Song P, Su R, Yang G, Dong L, Luo M, et al. DNA Methylation mediated down-regulating of MicroRNA-33b and its role in gastric cancer. *Sci Rep*. Nature Publishing Group; 2016;6. <https://doi.org/10.1038/srep18824>
72. Zhang Z, Yu W, Chen S, Chen Y, Chen L, Zhang S. Methylation of the claudin-3 promoter predicts the prognosis of advanced gastric adenocarcinoma. *Oncol Rep*. Spandidos Publications; 2018;40:49–60. <https://doi.org/10.3892/or.2018.6411>
73. Amini M, Hejazi M, Ghorban K, Mokhtarzadeh A, Baradaran B. Identification of functional methylated CpG loci in PD-L1 promoter as the novel epigenetic biomarkers for primary gastric cancer. *Gene*. Elsevier B.V.; 2021;772. <https://doi.org/10.1016/j.gene.2020.145376>
74. Ignatavicius P, Dauksa A, Zilinskas J, Kazokaite M, Riauka R, Barauskas G. DNA Methylation of HOXA11 Gene as Prognostic Molecular Marker in Human Gastric Adenocarcinoma. *Diagnostics*. Multidisciplinary Digital Publishing Institute (MDPI); 2022;12. <https://doi.org/10.3390/diagnostics12071686>
75. Guo W, Dong Z, Liu S, Qiao Y, Kuang G, Guo Y, et al. Promoter hypermethylation-mediated downregulation of miR-770 and its host gene MEG3, a long non-coding RNA, in the development of gastric cardia adenocarcinoma. *Mol Carcinog*. John Wiley and Sons Inc.; 2017;56:1924–34. <https://doi.org/10.1002/mc.22650>
76. Eftang LL, Klajic J, Kristensen VN, Tost J, Esbensen QY, Blom GP, et al. GFRA3 promoter methylation may be associated with decreased postoperative survival in gastric cancer. *BMC Cancer*. BioMed Central Ltd; 2016;16. <https://doi.org/10.1186/s12885-016-2247-8>
77. Saliminejad K, Soleymani Fard S, Khorram Khorshid HR, Yaghmaie M, Mahmoodzadeh H, Mousavi SA, et al. Methylation Analysis of p16, RASSF1A, RPRM, and RUNX3 in Circulating Cell-Free DNA for Detection of Gastric Cancer: A Validation Study. *Avicenna J Med Biotechnol* [Internet]. 2020;12:99–106. <http://www.ncbi.nlm.nih.gov/pubmed/32431794>
78. Li WH, Zhou ZJ, Huang TH, Guo K, Chen W, Wang Y, et al. Detection of OSR2, VAV3, and PPFIA3 Methylation in the Serum of Patients with Gastric Cancer. *Dis Markers*. Hindawi Limited; 2016;2016. <https://doi.org/10.1155/2016/5780538>
79. Sui BQ, Zhang CD, Liu JC, Wang L, Dai DQ. HOXB13 expression and promoter methylation as a candidate biomarker in gastric cancer. *Oncol Lett*. Spandidos Publications; 2018;15:8833–40. <https://doi.org/10.3892/ol.2018.8371>
80. Sun H, Zhou H, Zhang Y, Chen J, Han X, Huang D, et al. Aberrant methylation of FAT4 and SOX11 in peripheral blood leukocytes and their association with gastric cancer risk. *J Cancer*. Ivyspring International Publisher; 2018;9:2275–83. <https://doi.org/10.7150/jca.24797>

81. Zhang Y, Hu S, Li J, Shi D, Luo B. The promoter aberrant methylation status of TMEM130 is associated with gastric cancer. *Digestive and Liver Disease*. Elsevier B.V.; 2022;54:819–25. <https://doi.org/10.1016/j.dld.2021.05.035>
82. Zhao T, Yang T, Zhang J, Hao H, Wang D, Afirifa J, et al. The promoter methylation drives down-regulation mode of HIC1 in gastric cancer, its molecular characteristics and downstream functional pathways. *Gene*. Elsevier B.V.; 2022;824. <https://doi.org/10.1016/j.gene.2022.146380>
83. Amini M, Ghorban K, Mokhtarzadeh A, Dadmanesh M, Baradaran B. CD40 DNA hypermethylation in primary gastric tumors; as a novel diagnostic biomarker. *Life Sci*. Elsevier Inc.; 2020;254. <https://doi.org/10.1016/j.lfs.2020.117774>
84. Ding L, Tian Y, Wang L, Bi M, Teng D, Hong S. Hypermethylated long noncoding RNA MEG3 promotes the progression of gastric cancer. *Aging* [Internet]. 2019;11:8139–55. <https://doi.org/10.18632/aging.102309>
85. Ma J, Xia LL, Yao XQ, Zheng SM, Li S, Xu LS, et al. BARX2 expression is downregulated by CpG island hypermethylation and is associated with suppressed cell proliferation and invasion of gastric cancer cells. *Oncol Rep*. Spandidos Publications; 2020;43:1805–18. <https://doi.org/10.3892/or.2020.7558>
86. Sumei S, Xiangyun K, Fenrong C, Xueguang S, Sijun H, Bin B, et al. Hypermethylation of DHRS3 as a Novel Tumor Suppressor Involved in Tumor Growth and Prognosis in Gastric Cancer. *Front Cell Dev Biol*. Frontiers Media S.A.; 2021;9. <https://doi.org/10.3389/fcell.2021.624871>
87. Amini M, Foroughi K, Talebi F, Aghagolzade Haji H, Kamali F, Jandaghi P, et al. GHSR DNA hypermethylation is a new epigenetic biomarker for gastric adenocarcinoma and beyond. *J Cell Physiol*. Wiley-Liss Inc.; 2019;234:15320–9. <https://doi.org/10.1002/jcp.28179>
88. Wang J, Xu P, Hao Y, Yu T, Liu L, Song Y, et al. Interaction between DNMT3B and MYH11 via hypermethylation regulates gastric cancer progression. *BMC Cancer*. BioMed Central Ltd; 2021;21. <https://doi.org/10.1186/s12885-021-08653-3>
89. Nissar B, Kadla SA, Wani KA, Shah IA, Ganai BA. Promoter CpG island hypermethylation and down regulation of XRCC1 gene can augment in the gastric carcinogenesis events. *Mol Biol Rep*. Springer Science and Business Media B.V.; 2021;48:405–12. <https://doi.org/10.1007/s11033-020-06064-4>
90. Wang H, Duan XL, Qi XL, Meng L, Xu YS, Wu T, et al. Concurrent hypermethylation of SFRP2 and DKK2 activates the Wnt/ β -catenin pathway and is associated with poor prognosis in patients with gastric cancer. *Mol Cells*. Korean Society for Molecular and Cellular Biology; 2017;40:45–53. <https://doi.org/10.14348/molcells.2017.2245>
91. Lin Z, Luo M, Chen X, He X, Qian Y, Lai S, et al. Combined detection of plasma ZIC1, HOXD10 and RUNX3 methylation is a promising strategy for early detection of gastric cancer

and precancerous lesions. *J Cancer*. Ivyspring International Publisher; 2017;8:1038–44. <https://doi.org/10.7150/jca.18169>

92. Xue WJ, Feng Y, Wang F, Li P, Liu YF, Guo YB, et al. The value of serum RASSF10 hypermethylation as a diagnostic and prognostic tool for gastric cancer. *Tumor Biology*. Springer Science and Business Media B.V.; 2016;37:11249–57. <https://doi.org/10.1007/s13277-016-5001-6>

93. Ushiku H, Yamashita K, Ema A, Minatani N, Kikuchi M, Kojo K, et al. DNA diagnosis of peritoneal fluid cytology test by CDO1 promoter DNA hypermethylation in gastric cancer. *Gastric Cancer*. Springer Tokyo; 2017;20:784–92. <https://doi.org/10.1007/s10120-017-0697-6>

94. Xu G, Fan L, Zhao S, OuYang C. Neuronal pentraxin II (NPTX2) hypermethylation promotes cell proliferation but inhibits cell cycle arrest and apoptosis in gastric cancer cells by suppressing the p53 signaling pathway. *Bioengineered*. Bellwether Publishing, Ltd.; 2021;12:1311–23. <https://doi.org/10.1080/21655979.2021.1915658>

95. Bidar N, Rezaei T, Amini M, Jebelli A, Mokhtarzadeh A, Baradaran B. ZNF677 downregulation by promoter hypermethylation as a driver event through gastric tumorigenesis. *Exp Mol Pathol*. Academic Press Inc.; 2021;121. <https://doi.org/10.1016/j.yexmp.2021.104663>

96. Wang H, Xu M, Cui X, Liu Y, Zhang Y, Sui Y, et al. Aberrant expression of the candidate tumor suppressor gene DAL-1 due to hypermethylation in gastric cancer. *Sci Rep*. Nature Publishing Group; 2016;6. <https://doi.org/10.1038/srep21755>

97. Tahara S, Tahara T, Horiguchi N, Okubo M, Terada T, Yoshida D, et al. Lower LINE-1 methylation is associated with promoter hypermethylation and distinct molecular features in gastric cancer. *Epigenomics*. Future Medicine Ltd.; 2019;11:1651–9. <https://doi.org/10.2217/epi-2019-0091>

98. Deng P, Chang XJ, Gao ZM, Xu XY, Sun AQ, Li K, et al. Downregulation and DNA methylation of ECRG4 in gastric cancer. *Onco Targets Ther*. Dove Medical Press Ltd.; 2018;11:4019–28. <https://doi.org/10.2147/OTT.S161200>

99. Ge Y, Wu Q, Ma G, Shao W, Liu H, Zhang Q, et al. Hypermethylation of EIF4E promoter is associated with early onset of gastric cancer. *Carcinogenesis*. Oxford University Press; 2018;39:66–71. <https://doi.org/10.1093/carcin/bgx110>

100. Chen Z, Hu T, Zhu S, Mukaisho K, El-Rifai W, Peng D-F. Glutathione peroxidase 7 suppresses cancer cell growth and is hypermethylated in gastric cancer. *Oncotarget* [Internet]. 2017;8:54345–56. <https://doi.org/10.18632/oncotarget.17527>

101. Kim MJ, Kim HN, Jacobs JP, Yang HJ. Combined DNA Methylation and Gastric Microbiome Marker Predicts Helicobacter pylori-Negative Gastric Cancer. *Gut Liver*. Editorial Office of Gut and Liver; 2024;18. <https://doi.org/10.5009/gnl230348>

102. Park JW, Kim W, Kang HG, Kim WJ, Lee H, Choi D, et al. DNA Hypermethylation Inhibits the CD82 Metastasis Suppressor Gene in Gastric Cancer. *Anticancer Res. International Institute of Anticancer Research*; 2024;44:2459–70. <https://doi.org/10.21873/anticancer.17053>
103. Chen X, Yang Y, Liu J, Li B, Xu Y, Li C, et al. NDRG4 hypermethylation is a potential biomarker for diagnosis and prognosis of gastric cancer in Chinese population. *Oncotarget* [Internet]. 2017;8:8105–19. <https://doi.org/10.18632/oncotarget.14099>
104. Gao C, Liu H, Zhao Y, Miao X, Zheng H. Is there a relationship between neural EGFL like 1 (NELL1) promoter hypermethylation and prognosis of gastric cancer? *Med Hypotheses. Churchill Livingstone*; 2022;158. <https://doi.org/10.1016/j.mehy.2021.110723>
105. Hu H, Chen X, Wang C, Jiang Y, Li J, Ying X, et al. The role of TFPI2 hypermethylation in the detection of gastric and colorectal cancer. *Oncotarget* [Internet]. 2017;8:84054–65. <https://doi.org/10.18632/oncotarget.21097>
106. Li B, Ren B, Ma G, Cai F, Wang P, Zeng Y, et al. Inactivation of ZSCAN18 by promoter hypermethylation drives the proliferation via attenuating TP53INP2-mediated autophagy in gastric cancer cells. *Clin Epigenetics. BioMed Central Ltd*; 2023;15. <https://doi.org/10.1186/s13148-023-01425-9>
107. Cai M, Guo H, Wang D, Zhao T, Liang X, Li J, et al. Expression, DNA methylation pattern and transcription factor EPB41L3 in gastric cancer: a study of 262 cases. *Cell Commun Signal*. 2024;22:470. <https://doi.org/10.1186/s12964-024-01849-7>
108. Mo W, Deng L, Cheng Y, Ge S, Wang J. IGFBP7 regulates cell proliferation and migration through JAK/STAT pathway in gastric cancer and is regulated by DNA and RNA methylation. *J Cell Mol Med*. 2024;28:e70080. <https://doi.org/10.1111/jcmm.70080>
109. Bayat M, Shirgir A, Kazemi Veisari A, Najjar Sadeghi R. Detection of CDH1 gene promoter hypermethylation in gastric cancer and chronic gastritis. *Pract Lab Med. Elsevier B.V.*; 2024;40. <https://doi.org/10.1016/j.plabm.2024.e00406>
110. Zhang C, Zou Y, Dai DQ. Downregulation of microRNA-27b-3p via aberrant DNA methylation contributes to malignant behavior of gastric cancer cells by targeting GSPT1. *Biomedicine and Pharmacotherapy. Elsevier Masson SAS*; 2019;119. <https://doi.org/10.1016/j.biopha.2019.109417>
111. Kanda M, Shimizu D, Tanaka H, Shibata M, Iwata N, Hayashi M, et al. Metastatic pathway-specific transcriptome analysis identifies MFSD4 as a putative tumor suppressor and biomarker for hepatic metastasis in patients with gastric cancer. *Oncotarget* [Internet]. 2016;7:13667–79. <https://doi.org/10.18632/oncotarget.7269>
112. Han F, Liu S, Jing J, Li H, Yuan Y, Sun LP. Identification of high-frequency methylation sites in rnf180 promoter region affecting expression and their relationship with prognosis of gastric

cancer. *Cancer Manag Res.* Dove Medical Press Ltd; 2020;12:3389–99. <https://doi.org/10.2147/CMAR.S246995>

113. Laven-Law G, Kichenadasse G, Young GP, Symonds EL, Winter JM. BCAT1, IKZF1 and SEPT9: methylated DNA biomarkers for detection of pan-gastrointestinal adenocarcinomas. *Biomarkers.* Taylor and Francis Ltd.; 2024;29:194–204. <https://doi.org/10.1080/1354750X.2024.2340663>

114. Lopes C, Almeida TC, Macedo-Silva C, Costa J, Paulino S, Jerónimo C, et al. MIR124-3 and NKX6-1 hypermethylation profiles accurately predict metachronous gastric lesions in a Caucasian population. *Clin Epigenetics.* BioMed Central Ltd; 2024;16. <https://doi.org/10.1186/s13148-024-01712-z>

115. Ghadami E, Nikbakhsh N, Fattahi S, Kosari-Monfared M, Ranaee M, Taheri H, et al. Epigenetic alterations of CYLD promoter modulate its expression in gastric adenocarcinoma: A footprint of infections. *J Cell Physiol.* Wiley-Liss Inc.; 2019;234:4115–24. <https://doi.org/10.1002/jcp.27220>

116. Zhou C, Pan R, Li B, Huang T, Zhao J, Ying J, et al. GPX3 hypermethylation in gastric cancer and its prognostic value in patients aged over 60. *Future Oncol* [Internet]. 2019;15:1279–89. <https://doi.org/10.2217/fon-2018-0674>

117. Li G. Comprehensive analysis of CPNE1 predicts prognosis and drug resistance in gastric adenocarcinoma. *Am J Transl Res.* e-Century Publishing Corporation; 2024;16:2233–47. <https://doi.org/10.62347/niyr2094>

118. Fan Z, Hao J, He F, Jiang H, Wang J, Li M, et al. Novel DNA methylation markers for early detection of gastric cardia adenocarcinoma and esophageal squamous cell carcinoma. *Sci China Life Sci.* Science China Press; 2024; <https://doi.org/10.1007/s11427-024-2642-8>

119. Barrett T, Wilhite SE, Ledoux P, Evangelista C, Kim IF, Tomashevsky M, et al. NCBI GEO: Archive for functional genomics data sets - Update. *Nucleic Acids Res.* 2013;41. <https://doi.org/10.1093/nar/gks1193>

120. Edgar R, Domrachev M, Lash AE. Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. *Nucleic Acids Res* [Internet]. 2002;30:207–10. <https://doi.org/10.1093/nar/30.1.207>

121. Hosseini M, Lotfi-Shahreza M, Nikpour P. Integrative analysis of DNA methylation and gene expression through machine learning identifies stomach cancer diagnostic and prognostic biomarkers. *J Cell Mol Med.* John Wiley and Sons Inc; 2023;27:714–26. <https://doi.org/10.1111/jcmm.17693>

122. Liu C, Tang H, Hu N, Li T. Methylomics and cancer: the current state of methylation profiling and marker development for clinical care. *Cancer Cell Int* [Internet]. BioMed Central Ltd; 2023;23:242. <https://doi.org/10.1186/s12935-023-03074-7>

123. Li S, Tollefsbol TO. DNA methylation methods: Global DNA methylation and methylomic analyses. *Methods* [Internet]. Academic Press Inc.; 2021;187:28–43. <https://doi.org/10.1016/j.ymeth.2020.10.002>
124. Sugden K, Hannon EJ, Arseneault L, Belsky DW, Corcoran DL, Fisher HL, et al. Patterns of Reliability: Assessing the Reproducibility and Integrity of DNA Methylation Measurement. *Patterns*. Cell Press; 2020;1. <https://doi.org/10.1016/j.patter.2020.100014>
125. Issa J-PJ, Kantarjian HM. Targeting DNA methylation. *Clin Cancer Res* [Internet]. 2009;15:3938–46. <https://doi.org/10.1158/1078-0432.CCR-08-2783>
126. Stomper J, Rotondo JC, Greve G, Lübbert M. Hypomethylating agents (HMA) for the treatment of acute myeloid leukemia and myelodysplastic syndromes: mechanisms of resistance and novel HMA-based therapies. *Leukemia* [Internet]. Springer Nature; 2021;35:1873–89. <https://doi.org/10.1038/s41375-021-01218-0>
127. Briski R, Garcia-Manero G, Kantarjian H, Ravandi F. The history of oral decitabine/cedazuridine and its potential role in acute myeloid leukemia. *Ther Adv Hematol* [Internet]. SAGE Publications Ltd; 2023;14:20406207231205428. <https://doi.org/10.1177/20406207231205429>
128. Issa J-PJ, Kantarjian HM, Kirkpatrick P. Azacitidine. *Nat Rev Drug Discov* [Internet]. 2005;4:275–6. <https://doi.org/10.1038/nrd1698>
129. Gore SD, Jones C, Kirkpatrick P. Decitabine. *Nat Rev Drug Discov*. 2006;5:891–2. <https://doi.org/10.1038/nrd2180>
130. Notari RE, Deyoung JL. Kinetics and mechanisms of degradation of the antileukemic agent 5-azacytidine in aqueous solutions. *J Pharm Sci*. 1975;64:1148–57. <https://doi.org/10.1002/jps.2600640704>
131. Chan KK, Giannini DD, Staroscik JA, Sadee W. 5-azacytidine hydrolysis kinetics measured by high-pressure liquid chromatography and ¹³C-NMR spectroscopy. *J Pharm Sci*. 1979;68:807–12. <https://doi.org/10.1002/jps.2600680705>
132. Karahoca M, Momparler RL. Pharmacokinetic and pharmacodynamic analysis of 5-aza-2'-deoxycytidine (decitabine) in the design of its dose-schedule for cancer therapy. *Clin Epigenetics* [Internet]. 2013;5:1–16. <https://doi.org/10.1186/1868-7083-5-3>
133. Foulks JM, Parnell KM, Nix RN, Chau S, Swierczek K, Saunders M, et al. Epigenetic Drug Discovery: Targeting DNA Methyltransferases. *J Biomol Screen*. 2012;17:2–17. <https://doi.org/10.1177/1087057111421212>
134. Nakamura K, Aizawa K, Nakabayashi K, Kato N, Yamauchi J, Hata K, et al. DNA Methyltransferase Inhibitor Zebularine Inhibits Human Hepatic Carcinoma Cells Proliferation and Induces Apoptosis. *PLoS One*. 2013;8. <https://doi.org/10.1371/journal.pone.0054036>

135. Champion C, Guianvarc'h D, Sénamaud-Beaufort C, Jurkowska RZ, Jeltsch A, Ponger L, et al. Mechanistic insights on the inhibition of C5 DNA methyltransferases by zebularine. *PLoS One*. 2010;5. <https://doi.org/10.1371/journal.pone.0012388>
136. Klecker RW, Cysyk RL, Collins JM. Zebularine metabolism by aldehyde oxidase in hepatic cytosol from humans, monkeys, dogs, rats, and mice: Influence of sex and inhibitors. *Bioorg Med Chem*. 2006;14:62–6. <https://doi.org/10.1016/j.bmc.2005.07.053>
137. Martinet N, Michel BY, Bertrand P, Benhida R. Small molecules DNA methyltransferases inhibitors. *Medchemcomm*. 2012;3:263–73. <https://doi.org/10.1039/c1md00194a>
138. Jueliger S, Lyons J, Cannito S, Pata I, Pata P, Shkolnaya M, et al. Efficacy and epigenetic interactions of novel DNA hypomethylating agent guadecitabine (SGI-110) in preclinical models of hepatocellular carcinoma. *Epigenetics*. Taylor and Francis Inc.; 2016;11:709–20. <https://doi.org/10.1080/15592294.2016.1214781>
139. Liu M, Zhang L, Li H, Hinoue T, Zhou W, Ohtani H, et al. Integrative Epigenetic Analysis Reveals Therapeutic Targets to the DNA Methyltransferase Inhibitor Guadecitabine (SGI-110) in Hepatocellular Carcinoma. *Hepatology* [Internet]. 2018;68:1412–28. <https://doi.org/10.1002/hep.30091/supinfo>
140. Wei AH, Döhner H, Pocock C, Montesinos P, Afanasyev B, Dombret H, et al. Oral Azacitidine Maintenance Therapy for Acute Myeloid Leukemia in First Remission. *New England Journal of Medicine*. Massachusetts Medical Society; 2020;383:2526–37. <https://doi.org/10.1056/nejmoa2004444>
141. Dhillon S. Decitabine/Cedazuridine: First Approval. *Drugs*. Adis; 2020;80:1373–8. <https://doi.org/10.1007/s40265-020-01389-7>
142. Candoni A. Fully oral regimen with decitabine and cedazuridine plus venetoclax: a new step forward for older or unfit patients with acute myeloid leukaemia. *Lancet Haematol* [Internet]. Elsevier Ltd; 2024;11:e245–6. [https://doi.org/10.1016/S2352-3026\(24\)00060-7](https://doi.org/10.1016/S2352-3026(24)00060-7)
143. Siedlecki P, Boy RG, Musch T, Brueckner B, Suhai S, Lyko F, et al. Discovery of two novel, small-molecule inhibitors of DNA methylation. *J Med Chem*. 2006;49:678–83. <https://doi.org/10.1021/jm050844z>
144. Aanniz T, Bouyahya A, Balahbib A, El Kadri K, Khalid A, Makeen HA, et al. Natural bioactive compounds targeting DNA methyltransferase enzymes in cancer: Mechanisms insights and efficiencies. *Chem Biol Interact* [Internet]. Elsevier Ireland Ltd; 2024;392:110907. <https://doi.org/10.1016/j.cbi.2024.110907>
145. Kuck D, Caulfield T, Lyko F, Medina-Franco JL. Nanaomycin A selectively inhibits DNMT3B and reactivates silenced tumor suppressor genes in human cancer cells. *Mol Cancer Ther*. 2010;9:3015–23. <https://doi.org/10.1158/1535-7163.MCT-10-0609>

146. Fagan RL, Cryderman DE, Kopelovich L, Wallrath LL, Brenner C. Laccadic acid A is a direct, DNA-competitive inhibitor of DNA methyltransferase 1. *Journal of Biological Chemistry*. 2013;288:23858–67. <https://doi.org/10.1074/jbc.M113.480517>
147. Jurkowska RZ, Jeltsch A. Mechanisms and Biological Roles of DNA Methyltransferases and DNA Methylation: From Past Achievements to Future Challenges. Jeltsch A, Jurkowska RZ, editors. *Adv Exp Med Biol* [Internet]. Cham: Springer International Publishing; 2022;1389:1–19. https://doi.org/10.1007/978-3-031-11454-0_1
148. Chang Y, Guo H, Li X, Zong L, Wei J, Li Z, et al. Development of a First-in-Class DNMT1/HDAC Inhibitor with Improved Therapeutic Potential and Potentiated Antitumor Immunity. *J Med Chem*. American Chemical Society; 2024; <https://doi.org/10.1021/acs.jmedchem.4c01310>
149. Falckenhayn C, Bienkowska A, Söhle J, Wegner K, Raddatz G, Kristof B, et al. Identification of dihydromyricetin as a natural DNA methylation inhibitor with rejuvenating activity in human skin. *Frontiers in Aging*. Frontiers Media SA; 2023;4. <https://doi.org/10.3389/fragi.2023.1258184>
150. Cornacchia E, Golbus J, Maybaum J, Strahler J, Hanash S, Richardson B. Hydralazine and procainamide inhibit T cell DNA methylation and induce autoreactivity. *J Immunol* [Internet]. 1988;140:2197–200. <http://www.ncbi.nlm.nih.gov/pubmed/3258330>
151. Lee BH, Yegnasubramanian S, Lin X, Nelson WG. Procainamide is a specific inhibitor of DNA methyltransferase 1. *Journal of Biological Chemistry*. 2005;280:40749–56. <https://doi.org/10.1074/jbc.M505593200>
152. Sandoval JE, Ramabadran R, Stillson N, Sarah L, Fujimori DG, Goodell MA, et al. First-in-Class Allosteric Inhibitors of DNMT3A Disrupt Protein-Protein Interactions and Induce Acute Myeloid Leukemia Cell Differentiation. *J Med Chem*. American Chemical Society; 2022;65:10554–66. <https://doi.org/10.1021/acs.jmedchem.2c00725>
153. Myrianthopoulos V, Cartron PF, Liutkevičiute- Z, Klimašauskas S, Matulis D, Bronner C, et al. Tandem virtual screening targeting the SRA domain of UHRF1 identifies a novel chemical tool modulating DNA methylation. *Eur J Med Chem*. 2016;114:390–6. <https://doi.org/10.1016/j.ejmech.2016.02.043>
154. Morgan AE, Davies TJ, Mc Auley MT. The role of DNA methylation in ageing and cancer. *Proc Nutr Soc* [Internet]. 2018;77:412–22. <https://doi.org/10.1017/S0029665118000150>
155. Hudler P. Genetic aspects of gastric cancer instability. *ScientificWorldJournal* [Internet]. 2012;2012:761909. <https://doi.org/10.1100/2012/761909>
156. Qu Y, Dang S, Hou P. Gene methylation in gastric cancer. *Clinica Chimica Acta* [Internet]. The Authors; 2013;424:53–65. <https://doi.org/10.1016/j.cca.2013.05.002>
157. Das PM, Singal R. DNA methylation and cancer. *J Clin Oncol* [Internet]. 2004;22:4632–42. <https://doi.org/10.1200/JCO.2004.07.151>

158. Wu X, Zhang Y. TET-mediated active DNA demethylation: mechanism, function and beyond. *Nat Rev Genet* [Internet]. Nature Publishing Group; 2017;18:517–34. <https://doi.org/10.1038/nrg.2017.33>
159. Da Costa EM, McInnes G, Beaudry A, Raynal NJ. DNA Methylation-Targeted Drugs. *Cancer J*. 2017;23:270–6. <https://doi.org/10.1097/ppo.0000000000000278>
160. Pappalardi MB, Keenan K, Cockerill M, Kellner WA, Stowell A, Sherk C, et al. Discovery of a first-in-class reversible DNMT1-selective inhibitor with improved tolerability and efficacy in acute myeloid leukemia. *Nat Cancer*. 2021;2:1002–17.
161. Wiseman AK, Tiedemann RL, Fan H, Shen H, Madaj Z, McCabe MT, et al. Chromosome-specific retention of cancer-associated DNA hypermethylation following pharmacological inhibition of DNMT1. *Commun Biol* [Internet]. Nature Research; 2022;5:528. <https://doi.org/10.1038/s42003-022-03509-3>
162. Horton JR, Pathuri S, Wong K, Ren R, Rueda L, Fosbenner DT, et al. Structural characterization of dicyanopyridine containing DNMT1-selective, non-nucleoside inhibitors. *Structure*. Cell Press; 2022;30:793–802.e5. <https://doi.org/10.1016/j.str.2022.03.009>
163. Ala C, Joshi RP, Gupta P, Ramalingam S, Sankaranarayanan M. Discovery of potent DNMT1 inhibitors against sickle cell disease using structural-based virtual screening, MM-GBSA and molecular dynamics simulation-based approaches. *J Biomol Struct Dyn*. Taylor and Francis Ltd.; 2024;42:261–73. <https://doi.org/10.1080/07391102.2023.2199081>
164. Chen Q, Liu B, Zeng Y, Hwang JW, Dai N, Corrêa IR, et al. GSK-3484862 targets DNMT1 for degradation in cells. *NAR Cancer* [Internet]. Oxford University Press; 2023;5:zcad022. <https://doi.org/10.1093/narcan/zcad022>
165. Chen X, Lawrence EB, Farmer L, Yang L, Perry RC, Sequeira DJ, et al. Targeting p16 epimutation during lung cancer progression for anticancer therapy. *Cancer Res*. AACR; 2023;83:6010.
166. Wu YC, Lv P, Han J, Yu JL, Zhu X, Hong LL, et al. Enhanced serum methylated p16 DNAs is associated with the progression of gastric cancer. *Int J Clin Exp Pathol*. 2014;7:1553–62.
167. Guo L, Huang C, Ji QJ. Aberrant promoter hypermethylation of p16, survivin, and retinoblastoma in gastric cancer. *Bratisl Lek Listy*. 2017;118:164–8. https://doi.org/10.4149/bl_2017_033
168. He D, Zhang Y, Wang Z, Zhang N, Zhou L, Chen J, Jiang Y, et al. Aberrant gene promoter methylation of p16, FHIT, CRBP1, WWOX, and DLC-1 in Epstein–Barr virus-associated gastric carcinomas. *Medical Oncology*. Humana Press Inc.; 2015;32:1–16. <https://doi.org/10.1007/s12032-015-0525-y>

169. Abbaszadegan MR, Moaven O, Sima HR, Ghafarzadegan K, A'Rabi A, Forghani MN, et al. p16 promoter hypermethylation: a useful serum marker for early detection of gastric cancer. *World J Gastroenterol*. 2008;14:2055–60. <https://doi.org/10.3748/wjg.14.2055>
170. Ichikawa D, Koike H, Ikoma H, Ikoma D, Tani N, Otsuji E, et al. Detection of aberrant methylation as a tumor marker in serum of patients with gastric cancer. *Anticancer Res*. 2004;24:2477–81.
171. Mitsuno M, Kitajima Y, Ide T, Ohtaka K, Tanaka M, Satoh S, et al. Aberrant methylation of p16 predicts candidates for 5-fluorouracil-based adjuvant therapy in gastric cancer patients. *J Gastroenterol*. 2007;42:866–73. <https://doi.org/10.1007/s00535-007-2113-1>
172. Matsuoka T, Yashiro M. Biomarkers of gastric cancer: Current topics and future perspective. *World J Gastroenterol*. 2018;24:2818–32. <https://doi.org/10.3748/wjg.v24.i26.2818>
173. Yang AS, Doshi KD, Choi SW, Mason JB, Mannari RK, Gharybian V, et al. DNA methylation changes after 5-aza-2'-deoxycytidine therapy in patients with leukemia. *Cancer Res*. 2006;66:5495–503. <https://doi.org/10.1158/0008-5472.CAN-05-2385>
174. Mund C, Hackanson B, Stresemann C, Lübbert M, Lyko F. Characterization of DNA demethylation effects induced by 5-aza-2'-deoxycytidine in patients with myelodysplastic syndrome. *Cancer Res*. 2005;65:7086–90. <https://doi.org/10.1158/0008-5472.CAN-05-0695>
175. Li J, Hao D, Wang L, Wang H, Wang Y, Zhao Z, et al. Epigenetic targeting drugs potentiate chemotherapeutic effects in solid tumor therapy. *Sci Rep*. 2017;7:4035. <https://doi.org/10.1038/s41598-017-04406-0>
176. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res* [Internet]. 2005 [cited 2019 Oct 25];65:6305–11. <https://doi.org/10.1158/0008-5472.CAN-04-2957>
177. Bae HJ, Kang SK, Kwon WS, Jeong I, Park S, Kim TS, et al. p16 methylation is a potential predictive marker for abemaciclib sensitivity in gastric cancer. *Biochem Pharmacol*. 2020;110:6th ed. 2021;183:114320. <https://doi.org/10.1016/j.bcp.2020.114320>
178. Rho HW, Lee BC, Choi ES, Choi IJ, Lee YS, Goh SH. Identification of valid reference genes for gene expression studies of human stomach cancer by reverse transcription-qPCR. *BMC Cancer*. 2010;10:240. <https://doi.org/10.1186/1471-2407-10-240>
179. Li X, Franke AA. High-throughput and cost-effective global DNA methylation assay by liquid chromatography-mass spectrometry. *Anal Chim Acta* [Internet]. Elsevier B.V.; 2011;703:58–63. <https://doi.org/10.1016/j.aca.2011.07.014>
180. Onuzulu CD, Lee S, Basu S, Comte J, Hai Y, Hizon N, et al. Novel DNA methylation changes in mouse lungs associated with chronic smoking. *Epigenetics* [Internet]. Taylor and Francis Ltd.; 2024;19:2322386. <https://doi.org/10.1080/15592294.2024.2322386>

181. Onuzulu CD, Lee S, Basu S, Comte J, Hai Y, Hizon N, et al. Early-life exposure to cigarette smoke primes lung function and DNA methylation changes at Cyp1a1 upon exposure later in life. *Am J Physiol Lung Cell Mol Physiol*. American Physiological Society; 2023;325:L552–67. <https://doi.org/10.1152/ajplung.00192.2023>
182. Lillico R, Sobral MG, Stesco N, Lakowski TM. HDAC inhibitors induce global changes in histone lysine and arginine methylation and alter expression of lysine demethylases. *J Proteomics* [Internet]. 2016 [cited 2019 Oct 25];133:125–33. <https://doi.org/10.1016/j.jprot.2015.12.018>
183. Lillico R, Lawrence CK, Lakowski TM. Selective DOT1L, LSD1, and HDAC Class I Inhibitors Reduce HOXA9 Expression in MLL-AF9 Rearranged Leukemia Cells, But Dysregulate the Expression of Many Histone-Modifying Enzymes. *J Proteome Res*. 20180717th ed. 2018;17:2657–67. <https://doi.org/10.1021/acs.jproteome.8b00118>
184. Shin DY, Sung Kang H, Kim GY, Kim WJ, Yoo YH, Choi YH. Decitabine, a DNA methyltransferases inhibitor, induces cell cycle arrest at G2/M phase through p53-independent pathway in human cancer cells. *Biomedicine and Pharmacotherapy*. 2013;67:305–11. <https://doi.org/10.1016/j.biopha.2013.01.004>
185. Lavelle D, DeSimone J, Hankewych M, Kousnetzova T, Chen YH. Decitabine induces cell cycle arrest at the G1 phase via p21 WAF1 and the G2/M phase via the p38 MAP kinase pathway. *Leuk Res*. Elsevier Ltd; 2003;27:999–1007. [https://doi.org/10.1016/S0145-2126\(03\)00068-7](https://doi.org/10.1016/S0145-2126(03)00068-7)
186. Meng CF, Zhu XJ, Peng G, Dai DQ. Promoter histone H3 lysine 9 di-methylation is associated with DNA methylation and aberrant expression of p16 in gastric cancer cells. *Oncol Rep*. 2009;22:1221–7. https://doi.org/10.3892/or_00000558
187. Sawicka M, Pawlikowski J, Wilson S, Ferdinando D, Wu H, Adams PD, et al. The specificity and patterns of staining in human cells and tissues of p16INK4a antibodies demonstrate variant antigen binding. *PLoS One* [Internet]. 2013 [cited 2019 Oct 25];8:e53313. <https://doi.org/10.1371/journal.pone.0053313>
188. Yamashita S, Tsujino Y, Moriguchi K, Tatematsu M, Ushijima T. Chemical genomic screening for methylation-silenced genes in gastric cancer cell lines using 5-aza-2'-deoxycytidine treatment and oligonucleotide microarray. *Cancer Sci* [Internet]. 2006;97:64–71. <https://doi.org/10.1111/j.1349-7006.2006.00136.x>
189. Croft M. The role of TNF superfamily members in T-cell function and diseases. *Nat. Rev. Immunol*. 2009. p. 271–85. <https://doi.org/10.1038/nri2526>
190. Kumawat M, Singh R, Karuna I, Ahlawat N, Ahlawat S. Salmonella Typhimurium peptidyl-prolyl cis-trans isomerase C (PPIase C) plays a substantial role in protein folding to maintain the protein structure. *World J Microbiol Biotechnol* [Internet]. Springer Science and Business Media B.V.; 2020;36:168. <https://doi.org/10.1007/s11274-020-02943-x>

191. Way G, Morrice N, Smythe C, O'sullivan AJ. Purification and Identification of Secernin, a Novel Cytosolic Protein that Regulates Exocytosis in Mast Cells. *Mol Biol Cell*. 2002;13:3344–54. <https://doi.org/10.1091/mbc.E01-10>
192. Moore LD, Le T, Fan G. DNA methylation and its basic function. *Neuropsychopharmacology* [Internet]. 20120711th ed. 2013;38:23–38. <https://doi.org/10.1038/npp.2012.112>
193. Ige OO, Hendrickson-Rebizant T, Luo W, Cheung P, Lao Y, Zahedi RP, et al. SET7/9 exhibits sigmoidal kinetics on nucleosomes, hyperbolic kinetics on histones by an ordered sequential mechanism, and methylates lysine and arginine. *J Biol Chem* [Internet]. 2025;301:110639. <https://doi.org/10.1016/j.jbc.2025.110639>
194. Lillico R, Sobral MG, Stesco N, Lakowski TM. HDAC inhibitors induce global changes in histone lysine and arginine methylation and alter expression of lysine demethylases. *J Proteomics* [Internet]. 2016 [cited 2019 Oct 25];133:125–33. <https://doi.org/10.1016/j.jprot.2015.12.018>
195. Lillico R, Lawrence CK, Lakowski TM. Selective DOT1L, LSD1, and HDAC Class I Inhibitors Reduce HOXA9 Expression in MLL-AF9 Rearranged Leukemia Cells, But Dysregulate the Expression of Many Histone-Modifying Enzymes. *J Proteome Res*. 20180717th ed. 2018;17:2657–67. <https://doi.org/10.1021/acs.jproteome.8b00118>
196. Chowdhury B, McGovern A, Cui Y, Choudhury SR, Cho I-H, Cooper B, et al. The hypomethylating agent Decitabine causes a paradoxical increase in 5-hydroxymethylcytosine in human leukemia cells. *Sci Rep* [Internet]. Nature Publishing Group; 2015;5:9281. <https://doi.org/10.1038/srep09281>
197. Buocikova V, Tyciakova S, Pilalis E, Mastrokalou C, Urbanova M, Matuskova M, et al. Decitabine-induced DNA methylation-mediated transcriptomic reprogramming in human breast cancer cell lines; the impact of DCK overexpression. *Front Pharmacol* [Internet]. Frontiers Media S.A.; 2022;13:991751. <https://doi.org/10.3389/fphar.2022.991751>
198. Giri AK, Aittokallio T. DNMT inhibitors increase methylation in the cancer genome. *Front Pharmacol*. Frontiers Media S.A.; 2019;10. <https://doi.org/10.3389/fphar.2019.00385>
199. Cashen AF, Shah AK, Todt L, Fisher N, DiPersio J. Pharmacokinetics of decitabine administered as a 3-h infusion to patients with acute myeloid leukemia (AML) or myelodysplastic syndrome (MDS). *Cancer Chemother Pharmacol*. 2008;61:759–66. <https://doi.org/10.1007/s00280-007-0531-7>
200. Ye C, Jiang N, Zheng J, Zhang S, Zhang J, Zhou J. Epigenetic therapy: Research progress of decitabine in the treatment of solid tumors. *Biochim. Biophys. Acta Rev. Cancer*. Elsevier B.V.; 2024. <https://doi.org/10.1016/j.bbcan.2023.189066>
201. Garcia-Manero G, Griffiths EA, Steensma DP, Roboz GJ, Wells R, McCloskey J, et al. Oral cedazuridine/decitabine for MDS and CMML: a phase 2 pharmacokinetic/pharmacodynamic

randomized crossover study [Internet]. 2020. <http://ashpublications.org/blood/article-pdf/136/6/674/1750722/bloodbld2019004143.pdf>

202. Nie J, Liu L, Li X, Han W. Decitabine, a new star in epigenetic therapy: The clinical application and biological mechanism in solid tumors. *Cancer Lett.* Elsevier Ireland Ltd; 2014. p. 12–20. <https://doi.org/10.1016/j.canlet.2014.08.010>

203. Fang JY, Chen YX, Lu J, Lu R, Yang L, Zhu HY, et al. Epigenetic modification regulates both expression of tumor-associated genes and cell cycle progressing in human colon cancer cell lines: Colo-320 and SW1116. *Cell Res.* 2004;14:217–26. <https://doi.org/10.1038/sj.cr.7290222>

204. Lillico R, Lawrence CK, Lakowski TM. Selective DOT1L, LSD1, and HDAC Class I Inhibitors Reduce HOXA9 Expression in MLL-AF9 Rearranged Leukemia Cells, But Dysregulate the Expression of Many Histone-Modifying Enzymes. *J Proteome Res* [Internet]. 2018;17:2657–67. <https://doi.org/10.1021/acs.jproteome.8b00118>

205. Lillico R, Sobral MG, Stesco N, Lakowski TM. HDAC inhibitors induce global changes in histone lysine and arginine methylation and alter expression of lysine demethylases. *J Proteomics* [Internet]. 2016;133:125–33. <https://doi.org/10.1016/j.jprot.2015.12.018>

206. Karimian A, Ahmadi Y, Yousefi B. Multiple functions of p21 in cell cycle, apoptosis and transcriptional regulation after DNA damage. *DNA Repair (Amst).* 2016;42:63–71. <https://doi.org/10.1016/j.dnarep.2016.04.008>

207. Serrano M, Hannon GJ, Beach D. A new regulatory motif in cell-cycle control causing specific inhibition of cyclin D/CDK4. *Nature.* 1993;366:704–7. <https://doi.org/10.1038/366704a0>

208. San José-Enériz E, Agirre X, Rabal O, Vilas-Zornoza A, Sanchez-Arias JA, Miranda E, et al. Discovery of first-in-class reversible dual small molecule inhibitors against G9a and DNMTs in hematological malignancies. *Nat Commun* [Internet]. 2017;8:15424. <https://doi.org/10.1038/ncomms15424>

209. Mehdipour P, Chen R, De Carvalho DD. The next generation of DNMT inhibitors. *Nat. Cancer.* Nature Research; 2021. p. 1000–1. <https://doi.org/10.1038/s43018-021-00271-z>

210. Ibanez V, Banzon V, Kousnetzova T, Vaitkus K, Peterson K, DeSimone J, et al. The Role of DNA Damage/Stress Response Pathways in the Mechanism of Action of Decitabine. *Blood.* American Society of Hematology; 2008;112:490–490. <https://doi.org/10.1182/blood.v112.11.490.490>

211. Pali SS, Van Emburgh BO, Sankpal UT, Brown KD, Robertson KD. DNA Methylation Inhibitor 5-Aza-2'-Deoxycytidine Induces Reversible Genome-Wide DNA Damage That Is Distinctly Influenced by DNA Methyltransferases 1 and 3B. *Mol Cell Biol.* Informa UK Limited; 2008;28:752–71. <https://doi.org/10.1128/mcb.01799-07>

212. Öz S, Raddatz G, Rius M, Blagitko-Dorfs N, Lübbert M, Maercker C, et al. Quantitative determination of decitabine incorporation into DNA and its effect on mutation rates in human

cancer cells. *Nucleic Acids Res* [Internet]. Oxford University Press; 2014;42:e152. <https://doi.org/10.1093/nar/gku775>

213. Schmelz K, Wagner M, Dörken B, Tamm I. 5-Aza-2'-deoxycytidine induces p21WAF expression by demethylation of p73 leading to p53-independent apoptosis in myeloid leukemia. *Int J Cancer*. Wiley-Liss Inc.; 2005;114:683–95. <https://doi.org/10.1002/ijc.20797>

214. Scott SA, Dong WF, Ichinohasama R, Hirsch C, Sheridan D, Sanche SE, et al. 5-Aza-2'-deoxycytidine (decitabine) can relieve p21WAF1 repression in human acute myeloid leukemia by a mechanism involving release of histone deacetylase 1 (HDAC1) without requiring p21WAF1 promoter demethylation. *Leuk Res*. 2006;30:69–76. <https://doi.org/10.1016/j.leukres.2005.05.010>

215. Tieli G, Cazzalini O, Stivala LA, Prosperi E. Revisiting the Function of p21CDKN1A in DNA Repair: The Influence of Protein Interactions and Stability. *Int J Mol Sci* [Internet]. MDPI; 2022;23:7058. <https://doi.org/10.3390/ijms23137058>

216. Barranco SC, Townsend CM, Gasarteli C, Macik BG, Burger NL, Boerwinkle WR, et al. Establishment and Characterization of an in Vitro Model System for Human Adenocarcinoma of the Stomach1 [Internet]. *Cancer Res*. 1983. <http://aacrjournals.org/cancerres/article-pdf/43/4/1703/2415330/cr0430041703.pdf>

217. Kim HJ, Kang SK, Kwon WS, Kim TS, Jeong I, Jeung HC, et al. Forty-nine gastric cancer cell lines with integrative genomic profiling for development of c-MET inhibitor. *Int J Cancer*. Wiley-Liss Inc.; 2018;143:151–9. <https://doi.org/10.1002/ijc.31304>

218. Seelan RS, Mukhopadhyay P, Pisano MM, Greene RM. Effects of 5-Aza-2'-deoxycytidine (decitabine) on gene expression. *Drug Metab. Rev*. Taylor and Francis Ltd; 2018. p. 193–207. <https://doi.org/10.1080/03602532.2018.1437446>

219. Zhang C, Suo J, Katayama H, Wei Y, Garcia-Manero G, Hanash S. Quantitative proteomic analysis of histone modifications in decitabine sensitive and resistant leukemia cell lines. *Clin Proteomics* [Internet]. BioMed Central Ltd.; 2016;13:14. <https://doi.org/10.1186/s12014-016-9115-z>

220. Lee HM, Saw AK, Morris VK, Napolitano S, Bristow C, Srinivasan S, et al. Epigenome Reprogramming Through H3K27 and H3K4 Trimethylation as a Resistance Mechanism to DNA Methylation Inhibition in BRAFV600E-Mutated Colorectal Cancer. *Clin Cancer Res*. 2024;30:5166–79. <https://doi.org/10.1158/1078-0432.CCR-24-1166>

221. Cameron EE, Bachman KE, Myöhänen S, Herman JG, Baylin SB. Synergy of demethylation and histone deacetylase inhibition in the re-expression of genes silenced in cancer. *Nat Genet* [Internet]. 1999;21:103–7. <https://doi.org/10.1038/5047>

222. Huang W, Zhu Q, Shi Z, Tu Y, Li Q, Zheng W, et al. Dual inhibitors of DNMT and HDAC induce viral mimicry to induce antitumour immunity in breast cancer. *Cell Death Discov* [Internet]. Springer Nature; 2024;10:143. <https://doi.org/10.1038/s41420-024-01895-7>

223. Fang Y, Zhang M-C, He Y, Li C, Fang H, Xu P-P, et al. Human endogenous retroviruses as epigenetic therapeutic targets in TP53-mutated diffuse large B-cell lymphoma. *Signal Transduct Target Ther* [Internet]. Springer Nature; 2023;8:381. <https://doi.org/10.1038/s41392-023-01626-x>
224. Nguyen CT, Weisenberger DJ, Velicescu M, Gonzales FA, Lin JCY, Liang G, et al. Histone H3-lysine 9 methylation is associated with aberrant gene silencing in cancer cells and is rapidly reversed by 5-aza-2'-deoxycytidine. *Cancer Res* [Internet]. 2002;62:6456–61. <http://www.ncbi.nlm.nih.gov/pubmed/12438235>
225. Li Z, Li B, Yu H, Wang P, Wang W, Hou P, et al. DNMT1-mediated epigenetic silencing of TRAF6 promotes prostate cancer tumorigenesis and metastasis by enhancing EZH2 stability. *Oncogene*. Springer Nature; 2022;41:3991–4002. <https://doi.org/10.1038/s41388-022-02404-9>
226. Patel K, Dickson J, Din S, Macleod K, Jodrell D, Ramsahoye B. Targeting of 5-aza-2'-deoxycytidine residues by chromatin-associated DNMT1 induces proteasomal degradation of the free enzyme. *Nucleic Acids Res*. 2010;38:4313–24. <https://doi.org/10.1093/nar/gkq187>
227. Tabatabaei MS, Ahmed M. Enzyme-Linked Immunosorbent Assay (ELISA). Christian SL, editor. *Methods Mol Biol* [Internet]. New York, NY: Springer US; 2022;2508:115–34. https://doi.org/10.1007/978-1-0716-2376-3_10
228. Mahmood T, Yang PC. Western blot: Technique, theory, and trouble shooting. *N Am J Med Sci*. 2012;4:429–34. <https://doi.org/10.4103/1947-2714.100998>
229. Ku J-L, Park J-G. Biology of SNU Cell Lines. *Cancer Res Treat*. 2005;37:1. <https://doi.org/10.4143/crt.2005.37.1.1>
230. Murai Y, Hayashi S, Takahashi H, Tsuneyama K, Takano Y. Correlation between DNA alterations and p53 and p16 protein expression in cancer cell lines. *Pathol Res Pract*. Elsevier GmbH; 2005;201:109–15. <https://doi.org/10.1016/j.prp.2005.01.002>
231. Carraway HE, Malkaram SA, Cen Y, Shatnawi A, Fan J, Ali HEA, et al. Activation of SIRT6 by DNA hypomethylating agents and clinical consequences on combination therapy in leukemia. *Sci Rep* [Internet]. Nature Research; 2020;10:10325. <https://doi.org/10.1038/s41598-020-67170-8>
232. Zeng Y, Rong H, Xu J, Cao R, Li S, Gao Y, et al. DNA Methylation: An Important Biomarker and Therapeutic Target for Gastric Cancer. *Front. Genet. Frontiers Media S.A.*; 2022. <https://doi.org/10.3389/fgene.2022.823905>
233. Wang X, Dong Y, Zhang H, Zhao Y, Miao T, Mohseni G, et al. DNA methylation drives a new path in gastric cancer early detection: Current impact and prospects. *Genes Dis. KeAi Communications Co.*; 2024. p. 847–60. <https://doi.org/10.1016/j.gendis.2023.02.038>
234. Karimi P, Islami F, Anandasabapathy S, Freedman ND, Kamangar F. Gastric cancer: Descriptive epidemiology, risk factors, screening, and prevention. *Cancer Epidemiology*

Biomarkers and Prevention. American Association for Cancer Research Inc.; 2014. p. 700–13. <https://doi.org/10.1158/1055-9965.EPI-13-1057>

235. Kalra A, Meltzer SJ. The Role of DNA Methylation in Gastrointestinal Disease: An Expanded Review of Malignant and Nonmalignant Gastrointestinal Diseases. *Gastroenterology*. W.B. Saunders; 2025. p. 245–66. <https://doi.org/10.1053/j.gastro.2024.07.001>

236. Liggett WH, Sidransky D. Role of the p16 tumor suppressor gene in cancer. *J Clin Oncol* [Internet]. 1998;16:1197–206. <https://doi.org/10.1200/JCO.1998.16.3.1197>

237. Romagosa C, Simonetti S, López-Vicente L, Mazo A, Lleonart ME, Castellvi J, et al. p16Ink4a overexpression in cancer: A tumor suppressor gene associated with senescence and high-grade tumors. *Oncogene*. 2011. p. 2087–97. <https://doi.org/10.1038/onc.2010.614>

238. Gil J, Peters G. Regulation of the INK4b-ARF-INK4a tumour suppressor locus: All for one or one for all. *Nat. Rev. Mol. Cell Biol.* 2006. p. 667–77. <https://doi.org/10.1038/nrm1987>

239. Abbaszadegan MR, Moaven O, Sima HR, Ghafarzadegan K, A'rabi A, Forghani MN, et al. p16 promoter hypermethylation: A useful serum marker for early detection of gastric cancer. *World J Gastroenterol*. 2008;14:2055–60. <https://doi.org/10.3748/wjg.14.2055>

240. Spagnol LW, Polettini J, Silveira DA, Wegner GRM, Paiva DFF. p16 gene promoter methylation is associated with oncogenesis and progression of gastric carcinomas: A systematic review and meta-analysis. *Crit. Rev. Oncol. Hematol.* Elsevier Ireland Ltd; 2022. <https://doi.org/10.1016/j.critrevonc.2022.103843>

241. Nakamura J, Tanaka T, Kitajima Y, Noshiro H, Miyazaki K. Methylation-mediated gene silencing as biomarkers of gastric cancer: A review. *World J. Gastroenterol.* WJG Press; 2014. p. 11991–2006. <https://doi.org/10.3748/wjg.v20.i34.11991>

242. Fu DG. Epigenetic alterations in gastric cancer (review). *Mol. Med. Rep.* Spandidos Publications; 2015. p. 3223–30. <https://doi.org/10.3892/mmr.2015.3816>

243. Jia J, Zhu F, Ma X, Cao ZW, Li YX, Chen YZ. Mechanisms of drug combinations: Interaction and network perspectives. *Nat Rev Drug Discov.* Nature Publishing Group; 2009;8:111–28. <https://doi.org/10.1038/nrd2683>

244. Yavuz BR, Jang H, Nussinov R. Discovering anticancer drug target combinations via network-informed signaling-based approach. *Communications Medicine.* Springer Nature; 2025;5. <https://doi.org/10.1038/s43856-025-01150-9>

245. Yu X, Zhao H, Wang R, Chen Y, Ouyang X, Li W, et al. Cancer epigenetics: from laboratory studies and clinical trials to precision medicine. *Cell Death Discov.* Springer Nature; 2024. <https://doi.org/10.1038/s41420-024-01803-z>

246. Nakamura M, Nishikawa J, Saito M, Sakai K, Sasaki S, Hashimoto S, et al. Decitabine inhibits tumor cell proliferation and up-regulates e-cadherin expression in Epstein–Barr virus-associated

- gastric cancer. *J Med Virol.* John Wiley and Sons Inc.; 2017;89:508–17. <https://doi.org/10.1002/jmv.24634>
247. Zhang Q, He Y, Lu YP, Wei QH, Zhang HY, Quan Y. GETdb: A comprehensive database for genetic and evolutionary features of drug targets. *Comput Struct Biotechnol J.* Elsevier B.V.; 2024;23:1429–38. <https://doi.org/10.1016/j.csbj.2024.04.006>
248. Chakravarty D, Gao J, Phillips SM, Kundra R, Zhang H, Wang J, et al. OncoKB: A Precision Oncology Knowledge Base. *JCO Precis Oncol* [Internet]. 2017;2017. <https://doi.org/10.1200/PO.17.00011>
249. Chandrashekar DS, Karthikeyan SK, Korla PK, Patel H, Shovon AR, Athar M, et al. UALCAN: An update to the integrated cancer data analysis platform. *Neoplasia (United States).* Elsevier Inc.; 2022;25:18–27. <https://doi.org/10.1016/j.neo.2022.01.001>
250. Davis AP, Grondin CJ, Johnson RJ, Sciaky D, Wieggers J, Wieggers TC, et al. Comparative Toxicogenomics Database (CTD): Update 2021. *Nucleic Acids Res.* Oxford University Press; 2021;49:D1138–43. <https://doi.org/10.1093/nar/gkaa891>
251. Kanehisa M. The KEGG database. *Novartis Found Symp* [Internet]. 2002;247:91–101; discussion 101–3, 119–28, 244–52. <http://www.ncbi.nlm.nih.gov/pubmed/12539951>
252. Sadegh S, Skelton J, Anastasi E, Bernett J, Blumenthal DB, Galindez G, et al. Network medicine for disease module identification and drug repurposing with the NeDRex platform. *Nat Commun.* Nature Research; 2021;12. <https://doi.org/10.1038/s41467-021-27138-2>
253. von Mering C, Huynen M, Jaeggi D, Schmidt S, Bork P, Snel B. STRING: A database of predicted functional associations between proteins. *Nucleic Acids Res.* 2003. p. 258–61. <https://doi.org/10.1093/nar/gkg034>
254. Pacini C, Duncan E, Gonçalves E, Gilbert J, Bhosle S, Horswell S, et al. A comprehensive clinically informed map of dependencies in cancer cells and framework for target prioritization. *Cancer Cell.* Cell Press; 2024;42:301–316.e9. <https://doi.org/10.1016/j.ccell.2023.12.016>
255. Van Der Meer D, Barthorpe S, Yang W, Lightfoot H, Hall C, Gilbert J, et al. Cell Model Passports - a hub for clinical, genetic and functional datasets of preclinical cancer models. *Nucleic Acids Res.* Oxford University Press; 2019;47:D923–9. <https://doi.org/10.1093/nar/gky872>
256. Tsherniak A, Vazquez F, Montgomery PG, Weir BA, Kryukov G, Cowley GS, et al. Defining a Cancer Dependency Map. *Cell.* Cell Press; 2017;170:564–576.e16. <https://doi.org/10.1016/j.cell.2017.06.010>
257. Behan FM, Iorio F, Picco G, Gonçalves E, Beaver CM, Migliardi G, et al. Prioritization of cancer therapeutic targets using CRISPR–Cas9 screens. *Nature.* Nature Publishing Group; 2019;568:511–6. <https://doi.org/10.1038/s41586-019-1103-9>

258. Mostafavi S, Ray D, Warde-Farley D, Grouios C, Morris Q. GeneMANIA: a real-time multiple association network integration algorithm for predicting gene function [Internet]. 2008. <http://genomebiology.com/2008/9/S1/S4>
259. Shannon P, Markiel A, Ozier O, Baliga NS, Wang JT, Ramage D, et al. Cytoscape: A software Environment for integrated models of biomolecular interaction networks. *Genome Res.* 2003;13:2498–504. <https://doi.org/10.1101/gr.1239303>
260. Scardoni G, Tosadori G, Faizan M, Spoto F, Fabbri F, Laudanna C. Biological network analysis with CentiScaPe: centralities and experimental dataset integration. *F1000Res.* F1000 Research, Ltd.; 2014;3:139. <https://doi.org/10.12688/f1000research.4477.1>
261. Doncheva NT, Assenov Y, Domingues FS, Albrecht M. Topological analysis and interactive visualization of biological networks and protein structures. *Nat Protoc.* 2012;7:670–85. <https://doi.org/10.1038/nprot.2012.004>
262. Latifi-Navid H, Barzegar Behrooz A, Jamehdor S, Davari M, Latifinaid M, Zolfaghari N, et al. Construction of an Exudative Age-Related Macular Degeneration Diagnostic and Therapeutic Molecular Network Using Multi-Layer Network Analysis, a Fuzzy Logic Model, and Deep Learning Techniques: Are Retinal and Brain Neurodegenerative Disorders Related? *Pharmaceuticals. Multidisciplinary Digital Publishing Institute (MDPI);* 2023;16. <https://doi.org/10.3390/ph16111555>
263. Barzegar Behrooz A, Latifi-Navid H, da Silva Rosa SC, Swiat M, Wiechec E, Vitorino C, et al. Integrating Multi-Omics Analysis for Enhanced Diagnosis and Treatment of Glioblastoma: A Comprehensive Data-Driven Approach. *Cancers (Basel).* Multidisciplinary Digital Publishing Institute (MDPI); 2023;15. <https://doi.org/10.3390/cancers15123158>
264. Latifi-Navid H, Soheili ZS, Samiei S, Sadeghi M, Taghizadeh S, Pirmardan ER, et al. Network analysis and the impact of Aflibercept on specific mediators of angiogenesis in HUVEC cells. *J Cell Mol Med. John Wiley and Sons Inc;* 2021;25:8285–99. <https://doi.org/10.1111/jcmm.16778>
265. Gao J, Aksoy BA, Dogrusoz U, Dresdner G, Gross B, Sumer SO, et al. Integrative analysis of complex cancer genomics and clinical profiles using the cBioPortal. *Sci Signal.* 2013;6. <https://doi.org/10.1126/scisignal.2004088>
266. Zhou G, Soufan O, Ewald J, Hancock REW, Basu N, Xia J. NetworkAnalyst 3.0: A visual analytics platform for comprehensive gene expression profiling and meta-analysis. *Nucleic Acids Res.* Oxford University Press; 2019;47:W234–41. <https://doi.org/10.1093/nar/gkz240>
267. Lánckzy A, Györffy B. Web-based survival analysis tool tailored for medical research (KMplot): Development and implementation. *J Med Internet Res.* JMIR Publications Inc.; 2021;23. <https://doi.org/10.2196/27633>

268. Bartha Á, Györffy B. Tnmplot.Com: A web tool for the comparison of gene expression in normal, tumor and metastatic tissues. *Int J Mol Sci. MDPI AG*; 2021;22:1–12. <https://doi.org/10.3390/ijms22052622>
269. Tang G, Cho M, Wang X. OncoDB: An interactive online database for analysis of gene expression and viral infection in cancer. *Nucleic Acids Res. Oxford University Press*; 2022;50:D1334–9. <https://doi.org/10.1093/nar/gkab970>
270. Porras P, Licata L. IMEx Databases: Displaying Molecular Interactions into a Single, Standards-Compliant Dataset. *Methods Mol Biol.* 2022. p. 27–42. https://doi.org/10.1007/978-1-0716-2095-3_2
271. Liu P, Ewald J, Pang Z, Legrand E, Jeon YS, Sangiovanni J, et al. ExpressAnalyst: A unified platform for RNA-sequencing analysis in non-model species. *Nat Commun. Nature Research*; 2023;14. <https://doi.org/10.1038/s41467-023-38785-y>
272. Evangelista JE, Xie Z, Marino GB, Nguyen N, Clarke DJB, Ma'Ayan A. Enrichr-KG: Bridging enrichment analysis across multiple libraries. *Nucleic Acids Res. Oxford University Press*; 2023;51:W168–79. <https://doi.org/10.1093/nar/gkad393>
273. Han H, Cho JW, Lee S, Yun A, Kim H, Bae D, et al. TRRUST v2: An expanded reference database of human and mouse transcriptional regulatory interactions. *Nucleic Acids Res. Oxford University Press*; 2018;46:D380–6. <https://doi.org/10.1093/nar/gkx1013>
274. Raudvere U, Kolberg L, Kuzmin I, Arak T, Adler P, Peterson H, et al. G:Profiler: A web server for functional enrichment analysis and conversions of gene lists (2019 update). *Nucleic Acids Res. Oxford University Press*; 2019;47:W191–8. <https://doi.org/10.1093/nar/gkz369>
275. Zhang D, Huo D, Xie H, Wu L, Zhang J, Liu L, et al. CHG: A Systematically Integrated Database of Cancer Hallmark Genes. *Front Genet. Frontiers Media S.A.*; 2020;11. <https://doi.org/10.3389/fgene.2020.00029>
276. Thul PJ, Lindskog C. The human protein atlas: A spatial map of the human proteome. *Protein Science. Blackwell Publishing Ltd*; 2018;27:233–44. <https://doi.org/10.1002/pro.3307>
277. Luo W, Ige OO, Barzegar Behrooz A, Kassim HB, Hendrickson-Rebizant T, Paetkau D, et al. Dose dependent paradoxical increases in DNA methylation, reductions in p16 expression, and changes in histone modifications in gastric cancer cells treated with DNMT inhibitors. *Biomedicine & Pharmacotherapy [Internet]*. 2026;198:119221. <https://doi.org/10.1016/j.biopha.2026.119221>
278. Brueckner B, Garcia Boy R, Siedlecki P, Musch T, Kliem HC, Zielenkiewicz P, et al. Epigenetic reactivation of tumor suppressor genes by a novel small-molecule inhibitor of human DNA methyltransferases. *Cancer Res [Internet]*. 2005;65:6305–11. <https://doi.org/10.1158/0008-5472.CAN-04-2957>

279. Akama Y, Yasui W, Kuniyasu H, Yokozaki H, Akagi M, Tahara H, et al. Genetic status and expression of the cyclin-dependent kinase inhibitors in human gastric carcinoma cell lines. *Jpn J Cancer Res.* 1996;87:824–30. <https://doi.org/10.1111/j.1349-7006.1996.tb02106.x>
280. Arif A, Alameri AA, Tariq U Bin, Ansari SA, Sakr HI, Qasim MT, et al. The functions and molecular mechanisms of Tribbles homolog 3 (TRIB3) implicated in the pathophysiology of cancer. *Int. Immunopharmacol.* Elsevier B.V.; 2023. <https://doi.org/10.1016/j.intimp.2022.109581>
281. Lei S, Sun J, Xie Y, Xiao X, He X, Lin S, et al. Diverse functions of Tribbles homolog 3 in cancers and its potential as a therapeutic target. *Carcinogenesis.* Oxford University Press; 2024. p. 527–42. <https://doi.org/10.1093/carcin/bgae042>
282. Eyers PA, Keeshan K, Kannan N. Tribbles in the 21st Century: The Evolving Roles of Tribbles Pseudokinases in Biology and Disease. *Trends Cell Biol.* Elsevier Ltd; 2017. p. 284–98. <https://doi.org/10.1016/j.tcb.2016.11.002>
283. Jenkins NC, Liu T, Cassidy P, Leachman SA, Boucher KM, Goodson AG, et al. The p16 INK4A tumor suppressor regulates cellular oxidative stress. *Oncogene.* 2011;30:265–74. <https://doi.org/10.1038/onc.2010.419>
284. Dong S, Xia J, Wang H, Sun L, Wu Z, Bin J, et al. Overexpression of TRIB3 promotes angiogenesis in human gastric cancer. *Oncol Rep.* Spandidos Publications; 2016;36:2339–48. <https://doi.org/10.3892/or.2016.5017>
285. Brewer JW, Diehl JA. PERK mediates cell-cycle exit during the mammalian unfolded protein response. *Proc Natl Acad Sci U S A* [Internet]. 2000;97:12625–30. <https://doi.org/10.1073/pnas.220247197>
286. Ito K, Ito K. Metabolism and the Control of Cell Fate Decisions and Stem Cell Renewal. *Annu Rev Cell Dev Biol.* Annual Reviews Inc.; 2016;32:399–409. <https://doi.org/10.1146/annurev-cellbio-111315-125134>
287. Franco J, Balaji U, Freinkman E, Witkiewicz AK, Knudsen ES. Metabolic Reprogramming of Pancreatic Cancer Mediated by CDK4/6 Inhibition Elicits Unique Vulnerabilities. *Cell Rep.* Elsevier B.V.; 2016;14:979–90. <https://doi.org/10.1016/j.celrep.2015.12.094>
288. Yuan TM, Liu BH, Huang CJ, Huang YC, Chuang SM. TRIB3 as a biomarker of gastric cancer cell sensitivity to chemotherapeutic agents running title: A protective role of TRIB3 on chemotherapy. *SAGE Open Med.* SAGE Publications Ltd; 2024;12. <https://doi.org/10.1177/20503121241292673>
289. Wu IJ, Lin RJ, Wang HC, Yuan TM, Chuang SM. TRIB3 downregulation enhances doxorubicin-induced cytotoxicity in gastric cancer cells. *Arch Biochem Biophys.* Academic Press Inc.; 2017;622:26–35. <https://doi.org/10.1016/j.abb.2017.04.008>
290. Lu Y yang, Fang Y yuan, Wang S sha, Guo J, Song J lin, Zhu L, et al. Cepharanthine sensitizes gastric cancer cells to chemotherapy by targeting TRIB3-FOXO3-FOXM1 axis to inhibit

autophagy. Phytomedicine. Elsevier GmbH; 2024;135.
<https://doi.org/10.1016/j.phymed.2024.156161>

291. Posta M, Györfy B. Pathway-level mutational signatures predict breast cancer outcomes and reveal therapeutic targets. *Br J Pharmacol*. John Wiley and Sons Inc; 2025;182:5734–47. <https://doi.org/10.1111/bph.70215>

292. Tsujie M, Yamamoto H, Tomita N, Sugita Y, Ohue M, Sakita I, et al. Expression of Tumor Suppressor Gene p16 INK4 Products in Primary Gastric Cancer [Internet]. *Laboratory/Clinical Translational Research Oncology*. 2000. www.karger.comwww.karger.com/journals/ocl

293. Kumari S, Kumar P, Kumar M, Singh S, Narayan G. Expression of p27 and p16 and their clinical significance in gastric cancer. *Clinical and Translational Oncology*. Springer Science and Business Media Deutschland GmbH; 2021;23:856–65. <https://doi.org/10.1007/s12094-020-02479-4>

294. Yasutake N, Yamamoto H, Kuga R, Jiromaru R, Hongo T, Katayama Y, et al. Immunohistochemical p16 overexpression and Rb loss correlate with high-risk human papillomavirus infection in endocervical adenocarcinomas. *Histopathology*. John Wiley and Sons Inc; 2024;84:1178–91. <https://doi.org/10.1111/his.15169>

295. Jiromaru R, Yamamoto H, Yasumatsu R, Hongo T, Nozaki Y, Nakano T, et al. p16 overexpression and Rb loss correlate with high-risk HPV infection in oropharyngeal squamous cell carcinoma. *Histopathology*. John Wiley and Sons Inc; 2021;79:358–69. <https://doi.org/10.1111/his.14337>

296. Javed A, Yarmohammadi M, Korkmaz KS, Rubio-Tomás T. The Regulation of Cyclins and Cyclin-Dependent Kinases in the Development of Gastric Cancer. *Int. J. Mol. Sci*. MDPI; 2023. <https://doi.org/10.3390/ijms24032848>

297. He J, Zhao X, Huang C, Zhou X, You Y, Zhang L, et al. Double amplifications of CDK4 and MDM2 in a gastric inflammatory myofibroblastic tumor mimicking cancer with local invasion of the spleen and diaphragm. *Cancer Biol Ther*. Bellwether Publishing, Ltd.; 2018;19:967–72. <https://doi.org/10.1080/15384047.2018.1480290>

298. Sine CC, Watts LP, Fernandez B, Marikar N, Wang J, Knudsen ES, et al. sensitivity to CDK2 inhibitors in cyclin E1-driven ovarian cancers [Internet]. *Sci. Signal*. 2025. <https://www.science.org>

299. Calbó J, Serna C, Garriga J, Graña X, Mazo A. The fate of pancreatic tumor cell lines following p16 overexpression depends on the modulation of CDK2 activity. *Cell Death Differ*. 2004;11:1055–65. <https://doi.org/10.1038/sj.cdd.4401481>

300. Chen XH, Wang A, Chu AN, Gong YH, Yuan Y. Mucosa-associated microbiota in gastric cancer tissues compared with non-cancer tissues. *Front Microbiol*. Frontiers Media S.A.; 2019;10. <https://doi.org/10.3389/fmicb.2019.01261>

301. Yang S, Shin J, Park KH, Jeung HC, Rha SY, Noh SH, et al. Molecular basis of the differences between normal and tumor tissues of gastric cancer. *Biochim Biophys Acta Mol Basis Dis.* 2007;1772:1033–40. <https://doi.org/10.1016/j.bbadis.2007.05.005>
302. Hye SL, Cho SB, Hee EL, Kim MA, Ji HK, Do JP, et al. Protein expression profiling and molecular classification of gastric cancer by the tissue array method. *Clinical Cancer Research.* 2007;13:4154–63. <https://doi.org/10.1158/1078-0432.CCR-07-0173>
303. Bass AJ, Thorsson V, Shmulevich I, Reynolds SM, Miller M, Bernard B, et al. Comprehensive molecular characterization of gastric adenocarcinoma. *Nature.* Nature Publishing Group; 2014;513:202–9. <https://doi.org/10.1038/nature13480>
304. &NA; Decitabine. *Drugs in R and D* [Internet]. 2003 [cited 2019 Oct 24];4:179–84. <https://doi.org/10.2165/00126839-200304030-00007>

Appendices

Table 2A Human p16 promoter pyrosequencing region and primers

CpG sites within the <i>p16</i> promoter region (in red)	GGGGCAGGTGGGGAGGAGCCAGTCCTCCTTCCTTGCCAACGCTGGCTCTGGCGAGGGCTGCTTCCGGCT GGTGCCCCCGGGGAGACCCAACCTGGGGCGACTTCAGGGGTGCCACATT CG CTAAGTGCT CG GAGTTAA TAGCACCTCCTCCGAGCACTCGCTCACGGCGTCCCCTTGCTGGAAAGATAACCGCGGTCCCTCCAGAGGA TTTGAGGGACAGGGTCGGAGGGGGCTCTCCGCCAGCACCGGAGGAAGAAAGAGGAGGGGCTGGCTGGTC ACCAGAGGGTGGGG CGGACCGCGTGCGCTCGGCGGCTG CGGAGAGGGGGAGAGCAGGCAGCGGGCGGCGG GGAGCAGCATGGAGCCGCGGCGGGGAGCAGCATGGAGCCTTCGGCTGACTGGCTGGCCACGGCCGCGGC CCGGGGTCGGGTAGAGGAGGTGCGGGCGCTGCTGGAGGCGGGGGCGCTGCCCAACGCACCGAATAGTTAC GGTCGGAGGCCGATCCAGGTGGGTAGAGGTCTGCAG
Primer sequence 1	PCR Forward: 5'-GTTGGTGTTTTAGGGGAGATTTAATT-3' PCR Reverse: 5'-Biotin-AACCCCTCCTCTTCTTCCT-3' Sequencing Primer: 5'-GGGAGATTTAGGGGTGTTATAT-3'
Primer sequence 2	PCR Forward: 5'-TTTTAGAGGATTGAGGGATAGGG-3' PCR Reverse: 5'-Biotin-CTACCTACTCTCCCCCTCTC-3' Sequencing Primer: 5'-TATTAGAGGGTGGGG-3'

Table 3A Genes and qPCR TaqMan gene expression assay for CDKN2A/p16-associated core network gene set (n = 20)

Gene Name	TaqMan Assay ID
ANKFY1	Hs01019630_m1
ARF1	Hs00796826_s1
B2M	Hs00187842_m1
CCNE1	Hs01026536_m1
CDK2	Hs01548894_m1
CDK4	Hs00364847_m1
CDK6	Hs01026371_m1
CDKN2A	Hs00923894_m1
CDKN2B	Hs00793225_m1
EGFR	Hs01076090_m1
GAPDH	Hs02786624_g1
GET4	Hs00944514_m1
HIF1A	Hs00153153_m1
HRAS	Hs00978050_g1
JAK2	Hs01078136_m1
MAP4	Hs01104794_m1
MAPK14	Hs01051152_m1
NRAS	Hs00180035_m1
PDE4D	Hs01579625_m1
PTEN	Hs02621230_s1
RNA 18S	Hs99999901_s1
SLC25A46	Hs00373382_m1
TRIB3	Hs01082394_m1

	1	2	3	4	5	6	7	8	9	10	11	12
A	RNA 18S	GAPDH	B2M	RNA 18S	GAPDH	B2M	RNA 18S	GAPDH	B2M	RNA 18S	GAPDH	B2M
B	EGFR	HRAS	NRAS	EGFR	HRAS	NRAS	EGFR	HRAS	NRAS	EGFR	HRAS	NRAS
C	CDK2	PTEN	MAPK14	CDK2	PTEN	MAPK14	CDK2	PTEN	MAPK14	CDK2	PTEN	MAPK14
D	ARF1	HIF1A	JAK2	ARF1	HIF1A	JAK2	ARF1	HIF1A	JAK2	ARF1	HIF1A	JAK2
E	CCNE1	CDKN2B	CDK4	CCNE1	CDKN2B	CDK4	CCNE1	CDKN2B	CDK4	CCNE1	CDKN2B	CDK4
F	CDK6	ANKFY1	MAP4	CDK6	ANKFY1	MAP4	CDK6	ANKFY1	MAP4	CDK6	ANKFY1	MAP4
G	PDE4D	SLC25A46	TRIB3	PDE4D	SLC25A46	TRIB3	PDE4D	SLC25A46	TRIB3	PDE4D	SLC25A46	TRIB3
H	GET4	CDKN2A	RNA 18S	GET4	CDKN2A	RNA 18S	GET4	CDKN2A	RNA 18S	GET4	CDKN2A	RNA 18S

Figure 3A. CDKN2A/p16-associated core network gene set (n = 20) mapped in TaqMan Array (96 well plate).