PRMT5 controls the innate immune response



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LIST OF ABBREVIATIONS

ADMA	Asymmetric dimethyl arginine
AML	Acute myeloid leukaemia
ATM	Ataxia telangiectasia mutated
BSA	Bovine serum albumin
CARM	Coactivator-associated arginine methyltransferase
CDK	Cyclin dependent kinase
CHK	Checkpoint kinase
ChIP	Chromatin immunoprecipitation
CRISPR	Clustered regularly interspaced short palindromic repeats
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DP	Dimerisation partner
DSB	Double strand break
E2F	E2 promoter binding factor
EDTA	Ethylenediaminetetraacetic acid
EGFR	epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ERK	Extracellular signal-regulated kinase
FACS	Fluorescence activated cell sorting
FAS	Fas Cell Surface Death Receptor

HR	homologous recombination
IFN	Interferon
IHC	Immunohistochemistry
IL	Interleukin
IRF	interferon regulatory factor
ISG	interferon stimulated genes
IVT	*In vitro* transcription
kDa	kilo Dalton
KMT	lysine methyltransferase
KO	knockout
mTOR	mammalian target of rapamycin
NF-κβ	Nuclear factor kappa-light-chain-enhancer of activated B cells
NHEJ	non-homologous end-joining
PCR	Polymerase chain reaction
PDGFR	platelet derived growth factor receptor
pRB	Retinoblastoma protein
PRMT	Protein Arginine Methyltransferase
PTM	Posttranslational modification
Rad9	radiation-sensitive 9
RAF	Rapidly Accelerated Fibrosarcoma
RI	Retain Intron
RIP	RNA immunoprecipitation
rMATS	replicate multivariate analysis of transcript splicing

RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT	Room temperature
SDMA	Symmetric dimethyl arginine
SDS	Sodium dodecyl sulphate
SHP1	Src homology region 2 domain-containing phosphatase-1
shRNA	Short hairpin Ribonucleic acid
siRNA	Small interfering Ribonucleic acid
SMN	survival of motor neuron
snRNPs	small nuclear ribonucleoprotein particles
SRSF	serine and arginine rich splicing factor
STAT	Signal transducer and activator of transcription
STRAP	stress-responsive activator of p300
TNFα	Tumour necrosis factor alpha
TP53	p53 protein
TRAIL	tumour necrosis factor-related apoptosis-inducing ligand
U2AF	U2 auxiliary factor
UTR	Untranslated region

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ABSTRACT

Protein arginine methyltransferase 5 (PRMT5) is a symmetric di-methyltransferase frequently overexpressed in various cancers, including colorectal cancer. It is known that PRMT5-mediated methylation of E2 promoter binding factor 1 (E2F1) not only supports cancer cell growth but also influences RNA splicing. Previous studies have shown that inhibition of PRMT5 suppresses tumour growth and induces apoptosis in colorectal cancer cells. However, the underlying mechanisms remain insufficiently understood. This study explores a novel pathway involving an innate immune response triggered by PRMT5 and E2F1-mediated RNA splicing. We hypothesised that PRMT5 inhibition could increase retained introns (RIs) in the colorectal cancer cell line HCT116, leading to the production of immunogenic double-stranded RNAs (dsRNAs). Using the PRMT5 inhibitor T1-44, we observed increased intron retention and subsequent accumulation of dsRNAs and interferon-beta (IFN-β) both *in vivo* and *in vitro*, in an E2F1 dependent manner. Previous replicate multivariate analysis of transcript splicing (rMATS) analysis identified a pool of genes undergoing increased intron retention with T1-44 treatment.

Transfection of HCT116 cells with intron-retained genes resulted in elevated dsRNA and IFN-β levels. Validation of the dsRNA structure of RI transcripts was also attempted using dsRNA immunoprecipitation (RIP). Although additional experiments are required to precisely identify the dsRNA-producing intronic regions, our results support the role of the PRMT5-E2F1 axis in modulating immune responses through the regulation of RNA splicing. The findings suggest that inhibiting PRMT5 may enhance the IFN response in cancer cells, potentially improving the efficacy of existing immunotherapies.

1. INTRODUCTION

1.1 PRMT5 and Cancer

1.1.1 Overview of the PRMT family

Posttranslational modifications (PTMs) are crucial to the function of proteins, influencing their localization, stability, activity, and interactions with other proteins, thereby regulating numerous molecular pathways and cellular processes such as cell signalling, gene expression, cell proliferation and apoptosis etc. (Zhong et al., 2023). At a broader scale, the maintenance of PTM homeostasis is an important factor in human health.

There are over 400 different types of PTMs, with the most well studied ones being phosphorylation, acetylation, ubiquitination, succinylation and methylation (Ramazi and Zahiri, 2021). Protein arginine methylation, in particular, plays a significant role in cellular functions including transcription, signal transduction, cell cycle progression, and DNA damage response (DDR). In mammalian cells, between 0.5% and 4% of all arginine residues are methylated, a process catalysed by the family of protein arginine methyltransferases (PRMTs) (Maron et al., 2021). PRMTs are ubiquitously expressed and found in both the nucleus and the cytoplasm, except for PRMT8 which is localised on cell membranes of neurons in the central nervous system (Mark T. Bedford and Steven G. Clarke, 2009). One of the most common motifs PRMTs recognise and methylate in their substrates is the glycine- and arginine-rich (GAR) motif, consisting of repeats of RGG (arginine-glycine-glycine) and RG (arginine-glycine) sequences (Yang, Y. and Bedford, 2013; Wang, Y. and Bedford, 2023). PRMTs transfer the methyl group from S-adenosylmethionine (SAM) to a guanidino nitrogen atom of an arginine residue, creating methylarginines and the by-product S-adenosylhomocysteine (SAH). There are three

types of PRMTs, all capable of monomethylating arginine residues, producing mono-methylarginine (MMA) (Haghandish and Côté, 2016). Type I PRMTs (PRMT1, 2, 3, 4, 6, and 8) subsequently catalyse the addition of a second methyl group to the same guanidino nitrogen atom that was previously methylated, resulting in asymmetric dimethylarginine (ADMA). Type II PRMTs (PRMT5 and 9) add the second methyl group to the previously unmethylated adjacent guanidino nitrogen atom and produce symmetric dimethylarginine (SDMA). Type III PRMTs (PRMT7) are limited to monomethylate arginine residues and produce MMAs (Haghandish and Côté, 2016; Zurita-Lopez et al., 2012) **(Figure 1)**.

Under physiological pH, the guanidino is protonated and contains five potential hydrogen bond donors, which enables the interaction with other proteins through hydrogen bonds. Methylated arginine residues remain positively charged, but each methyl group added removes a potential hydrogen bond donor and possibly affecting the protein-protein interaction (Yang, Y. and Bedford, 2013; Hwang et al., 2021). PRMTs methylate both histone proteins for epigenetic regulation of gene expression, and non-histone proteins, regulating a wide range of cellular processes including RNA processing, translation, signal transduction, DNA damage response, and the cell cycle (Hwang et al., 2021).

The major asymmetric and symmetric arginine methyltransferases are PRMT1 and PRMT5, respectively (Yang, Y. and Bedford, 2013). The global ablation of either of enzyme is embryonically lethal in mice (Yu et al., 2009; Tee et al., 2010)**.** Conditional knockout of *Prmt5* in adult mice resulted in a complete loss of SDMA and severely impacted haematopoiesis (Liu, F. et al., 2015). *Prmt4/Carm1* knockout mice exhibit neonatal lethality, while those lacking

other PRMTs like PRMT2, 3, and 6 are viable (O'Brien et al., 2010; Kim, D. et al., 2010; Yoshimoto et al., 2006; Ganesh et al., 2006; Swiercz et al., 2007; Neault et al., 2012). As the focus of this study, PRMT5 has a housekeeping nature through the variety of biological pathways it regulates.

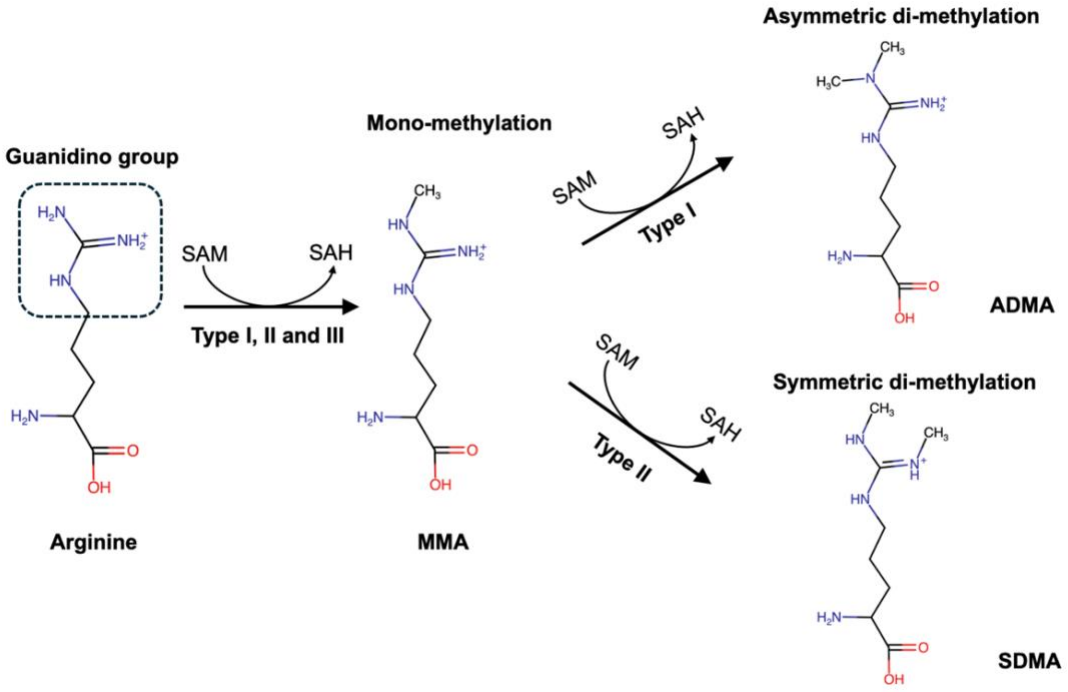


Figure 1: Arginine Methylation.

PRMTs catalyse the methylation of arginine residues on target proteins, using SAM as a methyl donor and producing SAH as a byproduct. All PRMTs are capable of generating mono-methylarginine (MMA). Type I PRMTs further catalyse the formation of asymmetric dimethylarginine (ADMA), while Type II PRMTs, specifically PRMT5 and 9, produce symmetric dimethylarginine (SDMA).

1.1.2 Biological role of PRMT5

Methylation of histone proteins

PRMT5 forms a hetero-octameric complex composed of four PRMT5 proteins and four essential cofactors, MEP50 (methylosome protein 50)/WDR77 (WD repeat domain 77)

(Antonysamy et al., 2012; Ho et al., 2013). Compared to the PRMT5 homodimer, the PRMT5/MEP50 complex has a higher affinity to SAM and to the target substrate (Antonysamy et al., 2012). In association with components of the SWI/SNF chromatin remodelling complex such as BRG1, BRM, and BRD7, the PRMT5/MEP50 complex catalyses dimethylation of four arginine residues within histone tails: H2AR3, H4R3, H3R2 and H3R8 (Kim, H. and Ronai, 2020). H2AR3me2s and H4R3me2s are known to suppress gene transcription, contributing to the developmental role of PRMT5 mentioned earlier (Kim, H. and Ronai, 2020). PRMT5 maintains embryonic stem cell (ESC) pluripotency via H2AR3me2s, and downregulates genes for differentiation such as *GATA4*, GATA*6*, and *HOXD9*. For this reason, *Prmt5*-knockout (KO) prevents the pluripotency of blastocysts and causes embryonic lethality in mice (Tee et al., 2010).

Crosstalk between different types of histone modifications creates a complex regulatory network of transcription. H3R8me2s results in deacetylation of H3K9 as a repressive mark, and H4R3me2s is associated with inhibition of transcriptionally activating lysine acetylation on H4K5 (Pal et al., 2004). By the symmetric demethylation of H4R3, PRMT5 promotes the development and differentiation of oligodendrocyte progenitor cells, which are responsible for myelination in the central nervous system (Scaglione et al., 2018).

In contrast, methylation on H3R2 by PRMT5 is generally associated with transcriptional activation. H3R2 symmetric demethylation recruits a lysine methyltransferase (KMT) complex that catalyses the trimethylation of H3K4, resulting in H3K4me3 which is indicative of an active promoter (Migliori et al., 2012).

Methylation of non-histone proteins

Transcriptional factors

PRMT5 extends its regulatory role beyond histone proteins, influencing gene expression also through direct di-methylation of transcription factors. For example, PRMT5 methylation enhances NF-κB transcriptional activity by enhancing its DNA binding affinity (Wei et al., 2013). This upregulates NF-κB target genes including IL-8 and TNF-α, which are activators of innate immune response (Liu, T. et al., 2017).

p53 is a tumour suppressor and plays a crucial role under cellular stress and DDR. Functioning as a transcription factor, p53 activates genes controlling cell cycle, apoptosis, metabolic pathways and DNA repair (Engeland, 2022). PRMT5 is also recruited to p53 by the stress-responsive activator of p300 (STRAP), where its dimethylation of p53 modulates genes associated with DDR (Jansson et al., 2008). The depletion of PRMT5 in haematopoietic stem cells activates p53 and the mTOR pathway, impairing the splicing of DNA repair genes and reducing cell viability (Tan et al., 2019).

Another key target of PRMT5 is E2F1, a critical transcription factor that controls cell cycle progression, apoptosis, and RNA splicing. Methylation of E2F1 by PRMT5 enhances the expression of genes essential for cell growth and proliferation like p73, Chk1 and Chk2 (Cho et al., 2012). The detailed pathway of PRMT5/E2F1 interaction will be elaborated in later sections.

RNA processing proteins

Besides E2F1, PRMT5 also targets other proteins involved in RNA processing. It methylates SRSF1, a serine/arginine-rich protein necessary for its interaction with target mRNAs and splicing-associated proteins, thus maintaining an intact splicing pattern (Radzisheuskaya et al., 2019). Furthermore, PRMT5 methylation is involved in the assembly of spliceosome. The three Sm proteins, SmD1, SmD3 and SmB, are methylated by PRMT5 to bind to survival of motor neuron (SMN) protein and assembled into small nuclear ribonucleoprotein particles (snRNPs) for pre-mRNA splicing (Meister et al., 2001).

Growth factor signalling

PRMT5 is implicated in the methylation of EGFR at Arg 1175, which enhances EGF-induced EGFR trans-autophosphorylation at Tyr 1173. This modification facilitates the docking of the phosphatase SHP1 and attenuates EGFR-mediated ERK activation (Liu, M. et al., 2011). Additionally, PRMT5 regulates the ERK1/2 signal through the methylation and subsequent degradation of RAF proteins, CRAF and BRAF (Andreu-Pérez et al., 2011).

On the other hand, PRMT5 positively regulates platelet-derived growth factor receptor (PDGFR) signalling in oligodendrocyte progenitor cells by preventing PDGFRα degradation, thereby activating downstream AKT/ERK signalling pathways essential for cell proliferation (Calabretta et al., 2018).

DNA damage response proteins

In addition to p53, other regulators of DDR are also substrates of PRMT5. The methylation of

Rad9 by PRMT5 is necessary for the activation of Chk1 signalling and the maintenance of the S/M and G2/M cell cycle checkpoints (He et al., 2011).

PRMT5 also plays a role in both homologous recombination (HR) and non-homologous end-joining (NHEJ) DNA repair pathways. Its methylation of the TIP60 complex cofactor RUVBL1 facilitates histone H4K16 acetylation by TIP60, enabling the displacement of 53BP1 from double-strand break (DSB) sites, crucial for HR (Clarke et al., 2017).

Moreover, 53BP1, a positive regulator of NHEJ, is competitively methylated by both PRMT1 and PRMT5. While PRMT1 methylation affects its DNA-binding activity, PRMT5 methylation stabilises 53BP1. Loss of PRMT5 results in decreased 53BP1 protein levels, leading to defects in the NHEJ process (Hwang et al., 2020).

Immune function

PRMT5 is essential for the normal functioning of immune cells, playing a pivotal role in T-cell activation and proliferation, as well as in B-cell survival, maturation, proliferation, and antibody production (Litzler et al., 2019; Tanaka et al., 2005).

In regulatory T cells (Tregs), PRMT5 is necessary for suppressing autoimmunity, possibly through the methylation of FOXP3 at R51. Inhibition of PRMT5 in Tregs results in a loss of immunosuppressive function, and mice with PRMT5 deletion develop scurfy-like autoimmunity (Nagai et al., 2019).

Due to its biological significance, deregulation of PRMT5 is implicated in the development of various diseases, including diabetes, Alzheimer's disease, cardiovascular disease, and cancer. The role of PRMT5 in cancer has been a significant focus of research (Motolani et al., 2021).

1.1.3 PRMT5 in cancer

Hallmarks of Cancer

In order to define the common capabilities of all types of cancer, Weinberg and Hanahan proposed the hallmarks of cancer (Hanahan and Weinberg, 2000). The most recent update in 2022 encompasses eight hallmarks: sustaining proliferative signalling, evading growth suppressors, resisting cell death, enabling replicative immortality, sustaining angiogenesis, activating invasion and metastasis, deregulating cellular energetics, and avoiding immune destruction (Hanahan, 2022). In addition, two emerging hallmarks are proposed: senescent cells and unlocking phenotypic plasticity, which potentially facilitate general oncogenesis (Hanahan, 2022). Four enabling characteristics crucial to acquiring these hallmarks include genome instability and mutation, tumour-promoting inflammation, non-mutational epigenetic reprogramming, and polymorphic microbiomes (Hanahan and Weinberg, 2011; Hanahan, 2022).

Many of the terms were mentioned previously while outlining the biological functions of PRMT5, and sufficient patient data has demonstrated the association of PRMT5's linkage to cancer progression.

PRMT5 overexpression in cancer

PRMT5 is commonly overexpressed in a number of cancer types, including lung, breast, gastric, and colorectal cancers, as well as lymphoma and leukaemia (Abe et al., 2023). The high level of PRMT5 is often associated with larger tumour size, advanced tumour grade, lymph node metastasis and poorer survival across different cancer types (Xiao et al., 2019). PRMT5 expression at both mRNA and protein levels has been identified as a potential biomarker for the diagnosis and prognosis of lung adenocarcinoma, breast cancer, and hepatocellular carcinoma etc., indicating the importance of understanding PRMT5's role in cancer (Wang, J. et al., 2023; Wu, Y. et al., 2017; Sun et al., 2022).

As described previously, PRMT5 is responsible for diverse biological functions, and their deregulation under PRMT5 overexpression could contribute to oncogenesis.

Histone methylation by PRMT5 promotes cancer development

Histone methylation is a key epigenetic modification that regulates gene expression, including the production of microRNAs (miRNAs). This process involves the addition of methyl groups to specific amino acids on histone proteins, which can either activate or repress gene transcription. PRMT5's methylation of histone proteins is crucial in regulating gene expression for tumour growth and progression.

For example, H3R8me2s and H4R3me2s modifications by PRMT5 inhibit tumour suppressor genes like suppressor of tumorigenicity 7 (ST7) and non-metastatic 23 (NM23) transcriptionally (Pal et al., 2004). PRMT5-dependent H3R2me2s modification is also implicated in

transcriptional activation of redox-related genes for re-establishing redox homeostasis under genotoxic stress. This serves as a mechanism of developing chemotherapy resistance in cancer cells (Cao et al., 2019). Furthermore, H3R2me2s on the FOXP1 promoter activates the expression of this transcription factor, contributing to the maintenance of stemness in breast cancer stem cells (Chiang et al., 2017). PRMT5 also promotes epithelial–mesenchymal transition (EMT) through H4R3me2s modification at the E-cadherin promoter, leading to its repression—a hallmark of EMT resulting in the loss of adhesive junctions and the epithelial phenotype (Hou et al., 2008).

Moreover, through epigenetic regulation, PRMT5 affects the expression of cancer specific miRNAs. PRMT5-mediated H4R3me2s modification suppresses miR-29b in acute myeloid leukemia (AML), increasing Sp1 level. This in turn activates *FLT3* expression which encodes for a receptor tyrosine kinase, promoting cancer cell growth (Tarighat et al., 2016). In lung cancer, PRMT5 overexpression adds the repressive H4R3me2s mark to the miR-99 family promoter. The decrease of miR-99 family expression allows for higher levels of fibroblast growth factor receptor 3 (FGFR3), which activates ERK1/2 and AKT signalling pathways, enhancing cell migration and invasion (Jing et al., 2018).

Pro-tumour regulation of transcription factors

Beyond histone methylation, PRMT5's direct modification of transcription factors, particularly those acting as tumour suppressors or oncogenic proteins, is vital in cancer development. Methylation of p53 by PRMT5 controls its promoter specificity associated with apoptosis or cell cycle arrest, with studies showing that knocking down or inhibiting PRMT5 activates p53-

mediated transcription of cell cycle and apoptosis genes such as p21 and MDM4, attenuating growth in haematopoietic and solid tumours with wildtype p53 and sensitising melanoma to CDK4/6 inhibition (AbuHammad et al., 2019; Gerhart et al., 2018).

Kruppel-like factor 4 (KLF4), a crucial transcription factor for DNA damage response and apoptosis, is stabilized through PRMT5-induced methylation, which prevents its ubiquitination (Hu, D. et al., 2015). Stress induced by DNA damage increases PRMT5 protein levels and subsequently facilitates the methylation and accumulation of KLF4, increasing transcription of downstream genes like *p21* and *Bax*, therefore attenuating cell cycle arrest and allows survival of cancer cells (Hu, D. et al., 2015).

Retinoblastoma protein (pRB) is a tumour suppressor protein that binds and inhibits the transcription factor E2F, preventing the transcription of genes needed for the G1 to S phase transition (Engeland, 2022). Under high glucose conditions, PRMT5 directly interacts with CDK4, which activates CDK4-pRb-E2F mediated transcription, advancing cell cycle progression in hepatocarcinoma cells which are highly metabolically active (Yang, H. et al., 2016). Additionally, PRMT5 methylation of E2F1 decreases E2F1 stability, under DNA damage, this methylation is reduced and higher level of E2F1 protein subsequently cause pro-apoptotic transcription (Cho et al., 2012). In cancer, elevated PRMT5 levels suppress E2F1's apoptotic functions, aiding cancer development. It has been shown that the loss of PRMT5 methylation of E2F1 can therefore slow down the growth and proliferation of cancer cells (Yang, H. et al., 2016).

PRMT5 and cancer growth signalling

Growth signalling pathways related with ERK and AKT are regulated by PRMT5 via its methylation of the receptors EGFR and PDGFR. In turn, PRMT5 dampens ERK activation and inhibits cell growth (Sapir et al., 2021). However, many publications indicate that whether PRMT5 activate or deactivate ERK may be cancer type specific, and further research is still required to establish PRMT5's role in ERK signalling. In pancreatic cancer, a study found that PRMT5 promotes EMT via the EGFR/AKT/β-catenin pathway, and combinatorial targeting of PRMT5/ERK has shown potential synergy effect in triple negative breast cancer (TNBC) (Vinet et al., 2019; Ge et al., 2020).

For AKT, whose overexpression leads to cell division dysregulation, there is a positive correlation with PRMT5 expression in various cancer types (Revathidevi and Munirajan, 2019). PRMT5 enhances the proliferative functions of AKT and activates mTOR phosphorylation via the PI3K/AKT pathway, influencing cancer cell survival, invasion, and metastasis (Roudnicky et al., 2017).

PRMT5 and cancer immune evasion

Furthermore, PRMT5 plays a role in cancer immune evasion. While it is involved in T cell maintenance and B cell development, inhibition of PRMT5 does not appear to weaken the immune system. Instead, methylation regulation through FOXP3 allows PRMT5 to impede the anti-tumour immune response. The infiltration of $FOXP3^+$ Tregs into tumour sites is associated with poor prognosis across various cancers (Togashi et al., 2019). Inhibition of PRMT5 sensitizes HER2 antibody-resistant CT26 cells to combined HER2 antibody treatment by

enhancing CD8+ T cells and NK cell infiltration and reducing Treg activity (Smil et al., 2015). Our group has also found that in murine CT26 colorectal cancer cells, PRMT5 affects the lncRNA derived peptide repertoire presented by MHC class I, where its inhibition promotes antigenic lncRNA expression. This is followed by a high influx of cytotoxic CD8+ T cell along with a moderate increase of helper CD4+ T cells in the tumour microenvironment (TME) in the CT26 model. The expression of lncRNA encoding immunogenic peptides regulated by PRMT5 was also validated in human colorectal cancer cell line HCT116 (Barczak et al., 2023).

Furthermore, PRMT5 regulates EMT associated gene expression through pathways like PRMT5/AKT1 axis or PRMT5/SNAIL/NuRD complex (Zheng, Y. et al., 2022; Yin et al., 2021). EMT-associated genes suppress the infiltration of CD8+ T cells and facilitate the infiltration of Tregs, myeloid-derived suppressor cells (MDSC), and tumour-associated macrophages (TAM) into the TME (Taki et al., 2021; Abe et al., 2023). These cancer cells with EMT gene expression also exhibit low MHC class I and high PD-L1 levels, which leads to impaired antigen presentation and prevents T cell killing, contributing to PRMT5's role in immune evasion (Taki et al., 2021).

In summary, PRMT5 plays a multifaceted role in cancer progression by regulating various molecular pathways and gene expressions. Through its mediation of epigenetic modifications and direct interactions with various non-histone proteins, such as transcription factors, PRMT5 influences a range of processes from growth signalling and apoptosis resistance to metastasis initiation and immune evasion. These hallmarks of cancer regulated by PRMT5 make it a promising target for therapeutic intervention. Understanding PRMT5’s mechanisms will offer

potential pathways for developing more effective treatments for cancers with PRMT5 overexpression.

1.2 E2F1 under PRMT5 control

1.2.1 Biological functions of E2F1

E2F1 is a transcription factor within the E2F family, and like PRMT5, it is also commonly overexpressed in cancer. A meta-analysis on E2F1 expression across human cancers has shown that elevated levels of E2F1 correlate with poorer overall survival and disease-free survival in patients, underscoring its prognostic significance (Li et al., 2023). E2F1 is regulated many types of PTMs, such as acetylation and phosphorylation during DDR, sumoylation under oxidative stress, and methylation (Biswas and Johnson, 2012; Graves et al., 2020). E2F1 is a methylation target of PRMT5, and understanding the implications of PRMT5's regulatory effects on E2F1 is crucial for elucidating its role in oncogenesis (Cho et al., 2012).

E2F1 is predominantly involved in cell cycle control through its interaction with the retinoblastoma protein (pRb). During the G1 phase of the cell cycle, elevated levels of cyclin-dependent kinases (CDKs) phosphorylate pRb, leading to the release of E2F1 from the pRb/E2F1 complex (Leone et al., 2001). E2F1 is then able to bind to promoter regions of target genes with dimerization partner (DP) proteins, and drive gene expression for cell cycle progression from G1 to S phase (Leone et al., 2001; Stevens and La Thangue, 2003). The tumour suppressor pRb is often deregulated in cancer cells due to Rb gene mutation, increased cyclin/CDK activity, or as a result of oncogene activation (Stevens and La Thangue, 2003; Leone et al., 2001). The resulting aberrant activation of E2F1 therefore sustains cancer cell

proliferation, which is a classic hallmark of cancer.

E2F1 also plays a pivotal role in apoptosis, particularly in response to DNA damage. Kinases such as CHK1 and CHK2 can phosphorylate pRb, influencing E2F1 activity (Stevens and La Thangue, 2004). Under DNA damage, E2F1-Rb complexes suppresses the expression of cell-cycle genes like *cyclin A2* and activate transcription of pro-apoptotic genes such as *APAF1, TP73, CASP3* and *CASP7* (Biswas and Johnson, 2012).

E2F1 degradation is prevented by the phosphorylation of DNA damage protein kinases ataxia telangiectasia mutated (ATM) /ataxia telangiectasia and Rad3 (ATR), and Chk2 (Stevens and La Thangue, 2004; La Thangue et al., 2003). Under genotoxic stress, E2F1 is also stabilised through acetylated by p300/CBP, P/CAF and TIP60, which enhances E2F1 binding to the promoter and driving expression of the TP73 gene (Van Den Broeck et al., 2012).

E2F1's dual role in cell proliferation and apoptosis, and its regulation through methylation by PRMTs, particularly PRMT5, are crucial for fine-tuning cellular response.

1.2.2 PRMT5 methylation of E2F1

PRMT5 prefers arginine residues sandwiched between two glycine residues, known as the GRG motif (Musiani et al., 2019). E2F1, which contains the PRMT5 methylation motif RGRGR, undergoes symmetric methylation by PRMT5 at R111 and R113 (Cho et al., 2012). Notably, E2F1 is also methylated by PRMT1 at R109. The methylation by these two enzymes is competitive and mutually exclusive, serving as a regulatory switch that modulates E2F1's role

in cellular survival processes (Zheng, S. et al., 2013).

Methylation by PRMT1 is a response to DNA damage and enhances the expression of genes associated with apoptosis. In contrast, methylation by PRMT5 destabilizes E2F1 and suppresses the expression of pro-apoptotic genes, favouring genes that promote proliferation instead. The symmetrical methylation marks on R111 and R113 are recognised by the Tudor domain-containing protein p100-TSN. p100/TSN is an RNA binding protein involved in transcription activation, RNA splicing and miRNA decay. P100/TSN knockout mice is viable with smaller body and organ size, and modest changes of mRNA expression in liver and increase in hypoxia-response transcription (Saarikettu et al., 2023). Interaction between E2F1 and p100-TSN occurs exclusively when E2F1 is methylated by PRMT5. p100-TSN plays a crucial role in translating the SDMA mark on E2F1 into specific biological outcomes, as its binding reduces the half-life of E2F1. Intriguingly, p100-TSN is found at the promoters of many E2F1 target genes that drive proliferation but is absent from those that trigger apoptosis. This suggests that p100-TSN may inhibit E2F1-mediated apoptosis by selectively relocating E2F1 to target genes that promote growth (Zheng, S. et al., 2013).

The PRMT5-E2F1 axis is also implicated in enabling metastasis. Under conditions where PRMT5 is inhibited, the expression of E2F1 target genes associated with focal adhesion and cell migration, such as cortactin (CTTN), is reduced (Barczak et al., 2020). Consequently, PRMT5-inhibited colon carcinoma HCT116 cells exhibit increased adhesion alongside decreased invasion and migration capabilities (Barczak et al., 2020).

Beyond transcriptional suppression of apoptosis and support for cell growth and migration, the PRMT5-E2F1 axis also involves RNA splicing regulation as another mechanism driving oncogenesis. This highlights the complex and multifaceted roles of PRMT5 in cancer, impacting various aspects of cellular function through its regulation of E2F1.

1.2.3 E2F1 mediated RNA splicing

Introduction to mRNA splicing

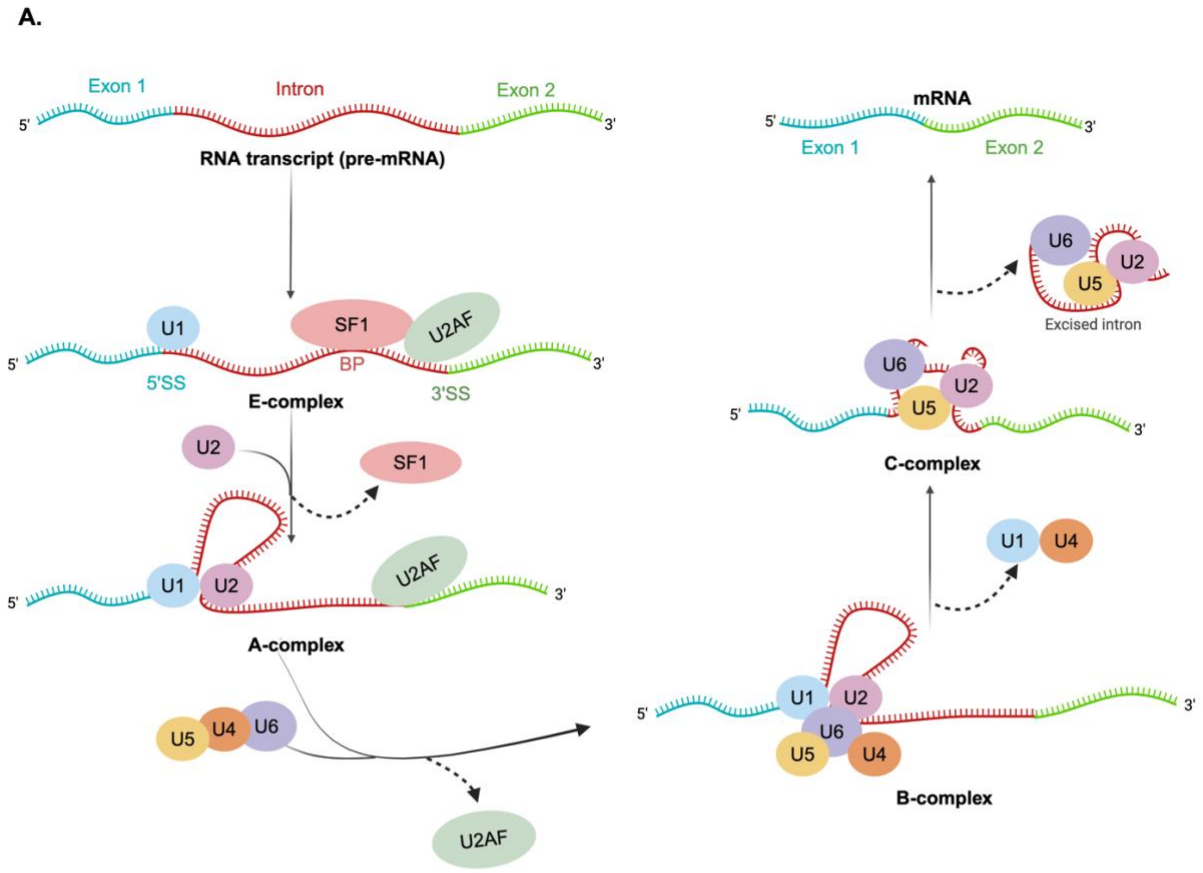
RNA splicing is a type of RNA processing, where introns are removed from pre-mRNAs and the remaining exons are connected to produce mature mRNA. Many genes contain alternative splicing cassettes, so different exons and introns are removed or included to create different combinations from one single gene. This process of alternative splicing can alter the mRNA stability and translation or change protein function as different domains are excluded or retained (Zhang, Y. et al., 2021). Therefore, alternative splicing controls protein expression and function in myriads of cellular processes, and its dysregulation is associated with the development of cancer (Zhang, Y. et al., 2021).

RNA splicing can occur in the nucleus through the spliceosome machinery. The spliceosome is a dynamic complex composed of 5 U-rich small nuclear RNAs (snRNAs): U1, U2, U4, U5, and U6, and another over 300 proteins can associate with the spliceosome (Shenasa and Bentley, 2023). The vast majority of mRNA splicing occurs co-transcriptionally, close to the transcription elongation complex (TEC), which comprises RNA polymerase II (Pol II), the nascent mRNA transcript, and the chromatin template. Splicing begins with base-pairing of the 5′ splice site (5’ SS) to the U1 snRNA in U1 snRNP, and recognition of the branch point (BP)

adenosine and the 3′ splice site (3’ SS) by SF1 and U2 auxiliary factor (U2AF), respectively, to generate the E complex. U2 snRNP then binds to the BP in an ATP-dependent step through base-pairing with U2 snRNA and establishes contact with U1 snRNP to form the A complex where 5′ and 3′ SSs are selected. Subsequent recruitment of the U4/6 U5 tri-snRNP and rearrangement ejects the U1 snRNP which is replaced at the 5′ SS by U6 snRNA. Base-pairing between U6 and U2 snRNAs creates an active site which catalyses the first transesterification reaction, which creates two products: exon1 cleaved at the 5′ SS and the intron/exon2 lariat intermediate. Further remodelling converts the active site to the exon ligation conformation that catalyses step 2 transesterification. This reaction joins the exons and releases the intron as a lariat **(Figure 2A)** (Shenasa and Bentley, 2023).

Splicing is modulated by various RNA-binding proteins (RBPs) such as serine and arginine-rich (SR) proteins and heterogeneous nuclear ribonucleoproteins (hnRNPs). SR proteins generally promote splicing by binding to exonic and intronic splicing enhancer elements (ESE and ISE), which facilitates exon inclusion. Conversely, hnRNPs often act as splicing repressors by associating with exonic and intronic splicing silencer elements (ESS and ISS), leading to exon exclusion. These proteins play specific roles that either enhance or inhibit spliceosome function, interacting with regulatory sequences during transcription to influence the outcome of splicing (Shenasa and Bentley, 2023; Smith et al., 2005; Howard and Sanford, 2015). The exact mechanism by which splicing factors interact with the transcription elongation complex (TEC) to regulate spliceosome assembly and its subsequent remodelling are not yet fully understood.
In addition to the core components (snRNPs, SR, and hnRNPs), a vast array of RBPs has been identified genome-wide that are capable of binding to mRNA. At least half of the transcription

factors also function as RBPs, equipped with arginine-rich RNA binding domains (Oksuz et al., 2023). Although E2F1 is not confirmed to be an RBP, it contains arginine-rich motifs and is found to regulate RNA alternative splicing (Roworth et al., 2019).



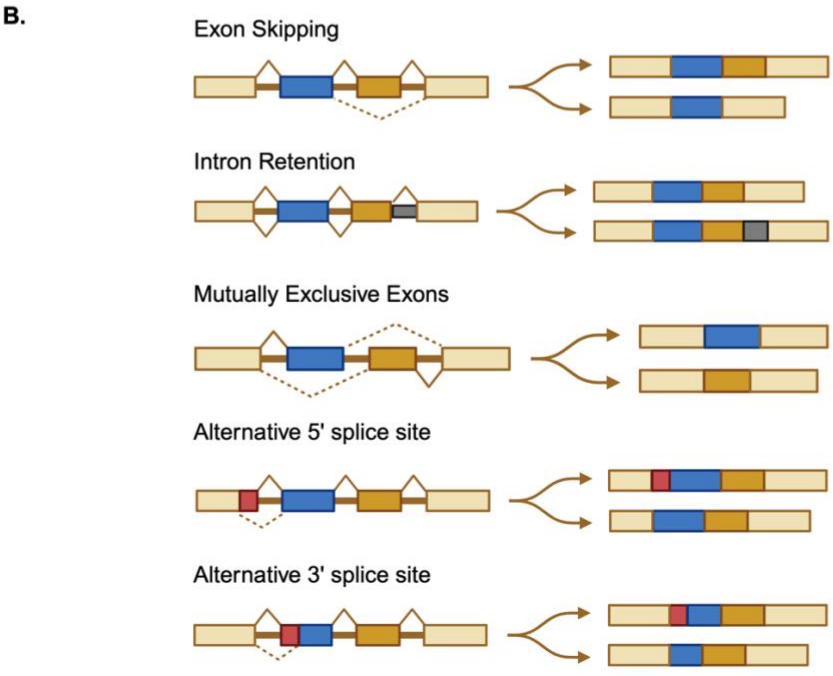


Figure 2: RNA alternative splicing.

A. Mechanism of spliceosome Assembly alternative splicing. The U1 small nuclear ribonucleoprotein (snRNP) binds to the 5′ splice site (SS), while splicing factor 1 (SF1) binds to the branch point adenosine (BP), and U2AF bind to the 3′ SS to form the E complex. Subsequently, U2 snRNP replaces SF1 to form the A complex. The incorporation of U4/U6.U5-tri-snRNP then creates the precatalytic B complex. From here, two key reactions occur: the first reaction forms Complex C, which undergoes rearrangements that drive the second reaction, leading to exon ligation and the formation of mature mRNA. In the final steps, introns are removed and recycled along with snRNPs.
B. Five main types of alternative splicing events. Potential pre-mRNA processing on the left gives rise to different mRNA isoforms on the right.

E2F1 regulates alternative splicing via PRMT5 control

Alternative splicing is a mechanism by which different combinations of exons are included or excluded from the final mRNA, affecting protein function by altering mRNA stability, translation, or protein domains. The primary patterns of alternative splicing include exon skipping, intron retention, mutually exclusive exons, and alternative 5′ and 3′ splice sites **(Figure 2B).** Utilizing the multivariate analysis of transcript splicing (rMATS) algorithm on RNA sequencing data, our group identified that a significant portion of genes (1560 in total) underwent alternative splicing upon E2F1 expression, with 65% being direct E2F1 targets, indicated by the presence of E2F1 binding motifs within their proximal promoter regions (Roworth et al., 2019). Notably, these alternatively spliced genes are typically weak transcriptional targets of E2F1, suggesting that E2F1 extends its regulatory impact through modulating alternative splicing. Among these, exon skipping (SE) and retained introns (RI) emerged as the predominant splicing events influenced by E2F1.

Importantly, the symmetric methylation mark reader p100/TSN is known to participate in spliceosome assembly and enhance splicing activity (Gutierrez-Beltran et al., 2016). With PRMT5 inhibition or mutation of PRMT5 methylation site R111 and R113 on E2F1, the interaction of E2F1 with p100/TSN is lost, and alternative splicing profile of the RNA

populations changes (Roworth et al., 2019; Zheng, S. et al., 2013).

E2F1 was found to associate with spliceosome components U1, U4, U5, and U6 snRNAs as well in various cancer cell types, including U2OS, MCF7 and HCT116 cells. This interaction is also lost with treatment of PRMT5 inhibitor or siRNA targeting p100/TSN. RNA-seq also reported that E2F1 binding to a large set of RNAs (384 in total) was dependent on p100/TSN presence, implying that p100/TSN recognition of PRMT5 methylated E2F1 is crucial to E2F1's association with the splicing machinery and target RNAs. This process is also suggested to take place on chromatin-bound E2F1, as when E2F1 chromatin binding activity is compromised, there is also weaker binding of E2F1 with U6 snRNA (Roworth et al., 2019).

From that study, a model of PRMT5-E2F1 in RNA alternative splicing is proposed **(Figure 3)**. PRMT5 mediates E2F1's binding with p100/TSN, which both permits the transcriptional activation of genes associated with proliferation and allows E2F1 interaction with the spliceosome. Some of these genes can be E2F1 target genes and RNA splicing regulation occurs co-transcriptionally (Roworth et al., 2019).

The treatment of PRMT5 inhibitor T1-44 has anti-tumour effects in terms of growth inhibition and promoting apoptosis (Barczak et al., 2020). This is achieved through the loss of symmetric methylation marks on E2F1, and therefore E2F1 fails to bind with p100/TSN (Zheng, S. et al., 2013). This shifts E2F1 control to both a pro-apoptotic transcriptional profile, and results in altered splicing events that favoured apoptosis (Roworth et al., 2019). A recent study demonstrated that PRMT5-E2F1-regulated exon skipping in crucial apoptotic genes reduces

neuroblastoma cells' sensitivity to apoptosis, underscoring the potential of pharmacological PRMT5 inhibition to enhance apoptotic responses in cancer therapy (Bate-Eya et al., 2024).

In summary, E2F1 mediates cancer cell survival and proliferation both at the levels of DNA transcription and mRNA splicing, controlled by PRMT5 methylation. This methylation facilitates E2F1's binding to p100/TSN, switching its regulatory effects to support cancer cell proliferation by transcriptionally driving the expression of proliferation genes while simultaneously suppressing apoptosis genes through alternative splicing. Thus, targeting the PRMT5-E2F1 axis offers a promising approach for anti-tumour therapy. As the mechanisms behind the effect of PRMT5 inhibition in cancer is still under research, a new aspect of how inhibiting PRMT5-E2F1 axis can potentially trigger the innate immune response through increased intron retention will be discussed in the following section.

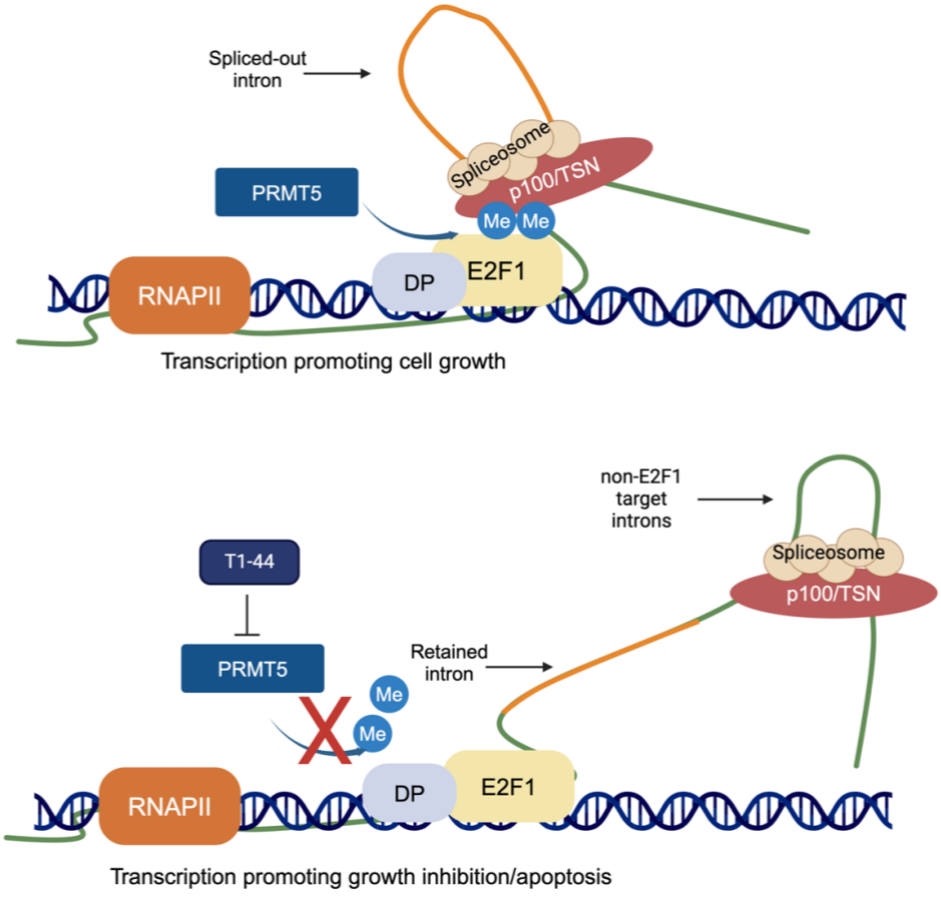


Figure 3: Proposed model of E2F1 regulated RNA splicing.

Top: Under normal conditions, PRMT5 methylates E2F1, enhancing its transcriptional activity that promotes cell proliferation and facilitating its interaction with p100/TSN. This interaction allows E2F1 to complex with p100/TSN and the spliceosome, directing efficient intron splicing. Bottom: In the presence of the PRMT5 inhibitor T1-44, the methylation on E2F1 is depleted, leading to potential intron retention due to the disrupted interaction between E2F1 and p100/TSN. Concurrently, the alteration in E2F1's transcriptional activity shifts towards promoting apoptosis and growth arrest.

1.3 Intron retention leads to dsRNA accumulation

Intron retention is a form of alternative splicing that has significant implications for gene expression and protein function. Our unpublished rMATS analysis of E2F1 WT HCT116 cells treated with and without a PRMT5 inhibitor revealed an increase of over 500 intron retention events. The biological effects of this increased intron retention, particularly following PRMT5 inhibition, are yet to be fully elucidated. Intron retention can influence protein activity by promoting transcript degradation, affecting translation, and producing diverse protein isoforms. Additionally, it contributes to innate immunity by forming double-stranded RNAs (dsRNAs) (Jacob and Smith, 2017; Wu, Q. et al., 2022; Bowling et al., 2021). dsRNA occurrence are rare events in the cell and often associated with viral infection. Therefore, an inflammatory response is activated by dsRNA-binding to its sensors, including TLR3, MDA5, PKR, RIG-I, etc. (de Faria et al., 2013).

However, dsRNA can also result from the intramolecular base-pairing within a single RNA strand, a scenario more likely in the presence of highly repetitive RNA sequences (Sadeq et al., 2021)Endogenous sources of dsRNAs include transposable elements (TEs) like long interspersed nuclear elements (LINEs) and short interspersed nuclear elements (SINEs), which

can be transcribed in bidirectional or inverted repeat manners, forming dsRNA through sense–antisense pairing. The transcription of these elements is typically suppressed epigenetically to prevent immune activation. (Sadeq et al., 2021). Nevertheless, the Alu elements, an abundant family of SINEs, are often embedded in introns and UTRs of the mRNA (Deininger, 2011). Alu elements existing in both sense and antisense orientations in the same transcript and form dsRNA structures recognisable to endogenous dsRNA sensors (Kawahara and Nishikura, 2006).

To mitigate the immunogenic effect of dsRNA, modifications like A-to-I editing by ADAR enzymes and N6-adenosine methylation disrupt dsRNA structures, reducing their recognition by immune sensors (Liu, N. et al., 2015; Wagner et al., 1989). Disruption of these RNA modifications can enhance the levels of endogenous dsRNAs, activating an innate immune response. For instance, knockdown of ADAR1 enhances activation of RIG-I and MDA5 in macrophages, leading to increased production of type I interferon and expression of interferon-stimulated genes (ISGs) (Pujantell et al., 2017).

Another source of dsRNAs is the dysregulation of mitochondrial processes. Depletion of mitochondrial RNA degradosome components, such as polynucleotide phosphorylase (PNPase) or SUV3, increases mitochondrial dsRNA. Specifically, mitochondria deficient in PNPase exhibit leakage of dsRNAs into the cytoplasm, where they can activate cytosolic sensors like PKR, triggering immune responses (Dhir et al., 2018; Kim, Y. et al., 2018).

In general, the presence of endogenous dsRNAs is a sign of cellular stress and dysfunction of

regulatory elements, alerting the innate immune system through dsRNA sensors. Some additional pathways for dsRNA production are still under research, such as how defective RNA splicing, and degradation can elevate dsRNA levels. RNA degradation proteins, such as Dicer, are specialised in cleaving dsRNAs as it contains an RNase III domain (Tarallo et al., 2012). It was found that Dicer-deficient cells have upregulated LINEs and SINEs elements and enhanced inflammation (Tarallo et al., 2012; Heras et al., 2013).

Studies in recent years have illuminated how RNA splicing dysregulation can cause dsRNA formation. Targeting the spliceosome in MYC-driven triple negative breast cancer (TNBC) cells found cytoplasmic accumulation of dsRNAs. Hyperactivation of the transcription factor MYC was known to deregulate alternative RNA splicing, making cancer cells sensitive to spliceosome targeting(Bowling et al., 2021). A global increase of intron retention was observed upon spliceosome targeting. These dsRNAs, enriched by J2-RNA immunoprecipitation and identified through RNA sequencing, trigger apoptosis via the mitochondrial antiviral signalling protein (MAVS) and stimulate both antiviral and adaptive immune responses in murine breast cancer models (Bowling et al., 2021). These findings highlight the promise of targeting RNA splicing in anti-tumour therapies, especially in cancers relying on alternative RNA splicing.

Another study has explored the effects of inhibiting Type I arginine methyltransferases like PRMT1 in TNBC, observing outcomes similar to those seen with direct spliceosome targeting (Wu, Q. et al., 2022). The use of a PRMT1 inhibitor led to increased intron retention and subsequent dsRNA production. These dsRNAs activated an interferon (IFN) response via sensors such as RIG-I and TLR-3, inhibiting the growth of TNBC cells (Wu, Q. et al., 2022).

This supports the potential of PRMT5 inhibition in activating such viral-mimicry response. However, the specific role of PRMT5 in endogenous dsRNA accumulation remains unclear. It is essential to determine whether the effects of symmetric and asymmetric arginine methylation similarly regulate this innate immune response and to identify which pathways of RNA splicing are disrupted by PRMT5 inhibition. These questions are crucial for understanding whether the pro-apoptotic and immunogenic effect seen after PRMT5 inhibition could be attributed to its role in RNA splicing.

1.4 Type I IFN and their implication in cancer

1.4.1 Pathways activating Type I IFN

Some known sensors of dsRNAs are acting to bridge endogenous dsRNA signals and interferon signalling activation. RIG-I-like receptors (RLRs) such as RIG-I and MDA5 play pivotal roles, each recognizing distinct types of dsRNA molecules (Reikine et al., 2014). RIG-I prefers dsRNAs shorter than 300 bp that possess a 5′ triphosphate group—a feature often absent in viral dsRNAs(Reikine et al., 2014). MDA5 is activated by longer dsRNA molecules, typically ranging from 500 to 1000 bp, without the necessity of a triphosphate group (Peisley et al., 2012). Activation of RIG-I or MDA5 leads to the polymerization of mitochondrial antiviral signalling protein (MAVS) on the mitochondria membrane, which subsequently initiates a cascade of inflammatory responses and IFN signalling (Reikine et al., 2014; Ren et al., 2020). Upon MAVS activation, there is an induction of NF-κB mediated transcription of pro-inflammatory genes and phosphorylation of interferon regulatory factors 3 and 7 (IRF3/7), promoting the transcription of type I IFN genes and the production of IFN-α and β (Ren et al., 2020).

Another key player in the dsRNA-sensing landscape is the interferon-inducible protein kinase R (PKR). PKR binds efficiently to short dsRNAs ranging from 33 bp to 85 bp, with its binding efficiency declining as the length of the RNA duplex increases. Once activated, PKR phosphorylates eukaryotic translation initiation factor 2A (EIF2A), leading to the inhibition of protein synthesis. This phosphorylation event also triggers NF-κB and IFN-β signalling, reinforcing the cell's antiviral defences (McAllister et al., 2012; Chukwurah et al., 2021).

In addition to RIG-I, members of the DExD-box (DDX) and DExH-box (DHX) helicase families also contribute to the antiviral inflammatory responses through dsRNA sensing (Zhang, Z. et al., 2011; Reikine et al., 2014). For instance, DDX1, DDX21, DHX36, and the adaptor molecule TRIF can form complexes that detect dsRNAs ranging from 0.2 to 1 kb in length, thereby activating type I IFN responses (Zhang, Z. et al., 2011). DHX9 acts upstream of MAVS to detect dsRNAs and plays a dual role in maintaining dsRNA homeostasis by unwinding and resolving dsRNA structures, including those from Alu elements (Aktaş et al., 2017; Zhang, Z., Yuan et al., 2011; Zhang, Z. et al., 2011).

In summary, various dsRNA sensors such as RIG-I, MDA5, PKR, and DDX/DHX helicases modulate Type I interferon production. To assess the potential therapeutic benefits of targeting RNA splicing disruptions and dsRNA production, the role of Type I IFN in cancer must be understood.

1.4.2 Type I IFN in cancer cells

Type I IFN produced by cancer cells can act as autocrine signalling molecules or bind to the IFNα and β receptor consisted of subunits IFNAR1 and IFNAR2 (Cheon et al., 2023). Upon ligand binding, the IFNAR activates the Janus kinase 1 (JAK1) and tyrosine kinase 2 (TYK2), which then phosphorylate the signal transducer and activator of transcription proteins, STAT1 and STAT2. The phosphorylated STAT1 and STAT2 form a trimeric complex with interferon regulatory factor 9 (IRF9) known as IFN-stimulated gene factor 3 (ISGF3). This complex translocates to the nucleus where it binds to IFN-stimulated response elements (ISREs) in the promoter regions of target genes, driving their expression and mediating the cellular response to IFN (Cheon et al., 2023; Zhang, X. et al., 2021).

Interestingly, in the steady state, tumours are exposed to chronically low levels of Type I IFN, which was found to have a pro-tumour role by allowing the expression of genes supporting IFN-related DNA damage resistance signature (IRDS), enhancing the cancer cells' ability to resist DNA damage from both endogenous sources and cancer treatments like chemotherapy and radiation (Cheon et al., 2023; Cheon et al., 2021; Weichselbaum et al., 2008). This resistance is also supported by constitutive production of IFN-β in cancer cells with defective p53 or ATM kinase dysfunction (Härtlova et al., 2015; Leonova et al., 2013).

Acute exposure to high levels of type I IFNs, however, is cytotoxic and therefore has an anti-tumour role (Taniguchi et al., 2003; Thapa et al., 2013; Manzella et al., 2016). High concentrations of IFN can induce the expression of p53, leading to anti-proliferative and pro-apoptotic effects. (Kayagaki et al., 1999). They also promote the expression of pro-apoptotic

factors such as TRAIL and FAS, as well as members of the IRF family, which are known to facilitate cell death in various cancer types (Manzella et al., 2016; Hu, G. and Barnes, 2009; Kazaana et al., 2019).

Furthermore, the expression of many ISGs has anti-angiogenic effects, including *GBP1, ISG20, PML etc.* (Raffaella et al., 2004; Weichselbaum et al., 2008; Taylor et al., 2008). Type I IFN also can decrease levels of Vascular endothelial growth factor A (VEGFA) *in vivo* both in mice and patients (Legros et al., 2014; von Marschall et al., 2003; Borden et al., 2011). The production of angiogenesis mediator IL-8 is also inhibited *in vitro* and *in vivo* by IFN-α2 or IFN-β (Singh et al., 1996; von Marschall et al., 2003).

Robust IFN-β signaling can also suppress mesenchymal and cancer stem cell properties in triple-negative breast cancer (TNBC) cells, reducing their migration and restoring an epithelial phenotype (Doherty et al., 2017).

The balance between chronic and acute Type I IFN levels in cancer cells is crucial. Overcoming the chronic, pro-tumoral effects of low-level IFN exposure with a surge of high IFN production can trigger apoptosis, inhibit angiogenesis, and stimulate anti-tumour effects throughout the tumour microenvironment.

1.4.3 Type I IFN in TME

The release of Type I IFN from tumour cells to the TME can give rise to a range of anti-cancer responses of the immune cells. Type I IFNs are essential for the neoantigen response and T-cell

priming by dendritic cells (DCs). Ifnar-/- mice have reduced DC ability to stimulate naive T cells, supporting the role of interferons in neoantigen response (Rouzaut et al., 2010; Diamond et al., 2011a; Mattei et al., 2001). Type I IFN treatment both *in vitro* and in patients enhances the expression of HLA class I and II, as well as the antigen peptide transporter 1 (TAP1) in dendritic cells (DCs), alongside other immunomodulatory ISGs such as CXCL10, CCR7, IL-15, CD80, and CD86 (Diamond et al., 2011b; Heise et al., 2016; Simmons et al., 2012; Mattei et al., 2001).

Plasmacytoid DCs (pDCs), a subset known for their prolific Type I IFN production during antiviral responses, demonstrate enhanced maturation and activation upon IFN exposure(Alculumbre et al., 2019; Montoya et al., 2002). Activated pDCs not only secrete large amounts of Type I IFN but also exhibit lytic activities against cancer cells through the expression of ISG-encoded cytotoxic molecules such as TRAIL, significantly inhibiting breast cancer cell growth (Wu, J. et al., 2017; Kalb et al., 2012).

The trafficking and extravasation of T cells to the tumour site are mediated by chemokines such as CCL2-5 and CXCL9-10 (Kohli et al., 2022). IFN-β has been shown to enhance the expression of CCL5 and the CXCL9 and 10 receptor CXCR3 in melanoma cells, facilitating the recruitment of CD8+ T cells into the tumour (Kohli et al., 2022). Type I IFN also promotes CCL5 synthesis in endothelial cells within the TME, aiding T cell infiltration (Kawai et al., 1999).

Type I IFN signalling increases the expression of MHC class I molecules on cancer cells, improving the presentation and recognition of tumour antigens by T cells (Park et al., 1997;

Propper et al., 2003; Hasegawa et al., 2016; Liu, L. L. et al., 2009; Huang, S. F. et al., 2002). Additionally, it has been shown to enhance the expression of the activation marker CD69, boosting the cytotoxicity of γδ T cells against leukaemia cells (McCarty et al., 2002). Both regulatory T (T_{reg}) cells and myeloid-derived suppressor cells (MDSCs) act to supress cytotoxic T cell activity (Watanabe et al., 2010). Type I IFNs are known to inhibit T_{reg} proliferation and reduce the accumulation and suppressive function of MDSCs (ZOGLMEIER et al., 2011). Furthermore, the maturation, migration, and lytic activities of murine and human NK cells are amplified by Type I interferons, both *in vitro* and *in vivo* (Lena Müller et al., 2017; EDWARDS et al., 1985; Takashima et al., 2016). The expression of ISGs such as CXCL10 and CCL5 in murine melanomas drives NK cell infiltration and activation, which subsequently suppresses tumour growth (Takashima et al., 2016).

Type I IFNs also influence neutrophil differentiation towards an anti-tumour phenotype and suppress the secretion of pro-angiogenic factors like VEGF and angiogenic chemokines by neutrophils in the TME (Pylaeva et al., 2016).

To sum up, type I IFNs exert direct effects on inhibiting proliferation and angiogenesis in cancer cells while simultaneously activating DCs, including pDCs, to enhance T cell priming and cytotoxicity against tumours. They also boost MHC and chemokine expression, enhancing T cell and NK cell activity within the TME. Thus, activating Type I IFN production offers significant potential in cancer therapy for its dual impact on tumour suppression and immune modulation.

1.5 Hypothesis and aims

PRMT5, a protein arginine methyltransferase, is a critical regulator in cancer, influencing numerous cellular processes. The methylation of E2F1 by PRMT5 plays a pro-proliferative role in cancer progression through both transcriptional and RNA-splicing mechanisms. Previous studies by our group have shown that inhibition of PRMT5 using the small molecule active site inhibitor T1-44 suppresses cancer cell growth and induces apoptosis. Additionally, T1-44 treatment in a mouse CT26 tumour model significantly reduced tumour volume and increased T cell infiltration. Intriguingly, treatment with T1-44 also led to an increase in retained intron events in the human colon carcinoma cell line HCT116, suggesting RNA splicing dysregulation. It is known that retained introns due to RNA splicing dysregulation can form dsRNAs in the cytoplasm and trigger an innate immune response via antiviral dsRNA sensors. This causes Type I IFN production by cancer cells and can subsequently drive cancer cell apoptosis and activate anti-tumour immunity in the TME.

Therefore, we hypothesised that PRMT5 inhibition by T1-44 causes accumulation of intron-derived dsRNAs through E2F1-mediated RNA splicing pathway. This in turn activates an innate immune response pathway and lead to Type I IFN production, which has an anti-tumour effect both on cancer cells and in the TME. The hypothesised model is illustrated in **Figure 4**.

This study aims to delineate the potential pathways contributing to the anti-tumour effects of PRMT5 inhibition. The specific objectives are:

1. To confirm whether an increase in cytoplasmic dsRNA can be observed following PRMT5 inhibition and to determine if there is a corresponding increase in Type I IFNs,

specifically IFN-β, both *in vitro* and *in vivo*.

2. To validate specific intron retention events identified during T1-44 treatment. This involved generating plasmids carrying these retained intron (RI) sequences and transfecting them into HCT116 cells to investigate the effects of elevated levels of specific RI transcripts.
3. To establish a direct connection between intron retention and dsRNA levels in cells. This was achieved by synthesizing certain RIs through *in vitro* transcription and employing dsRNA immunoprecipitation techniques to detect their structures.

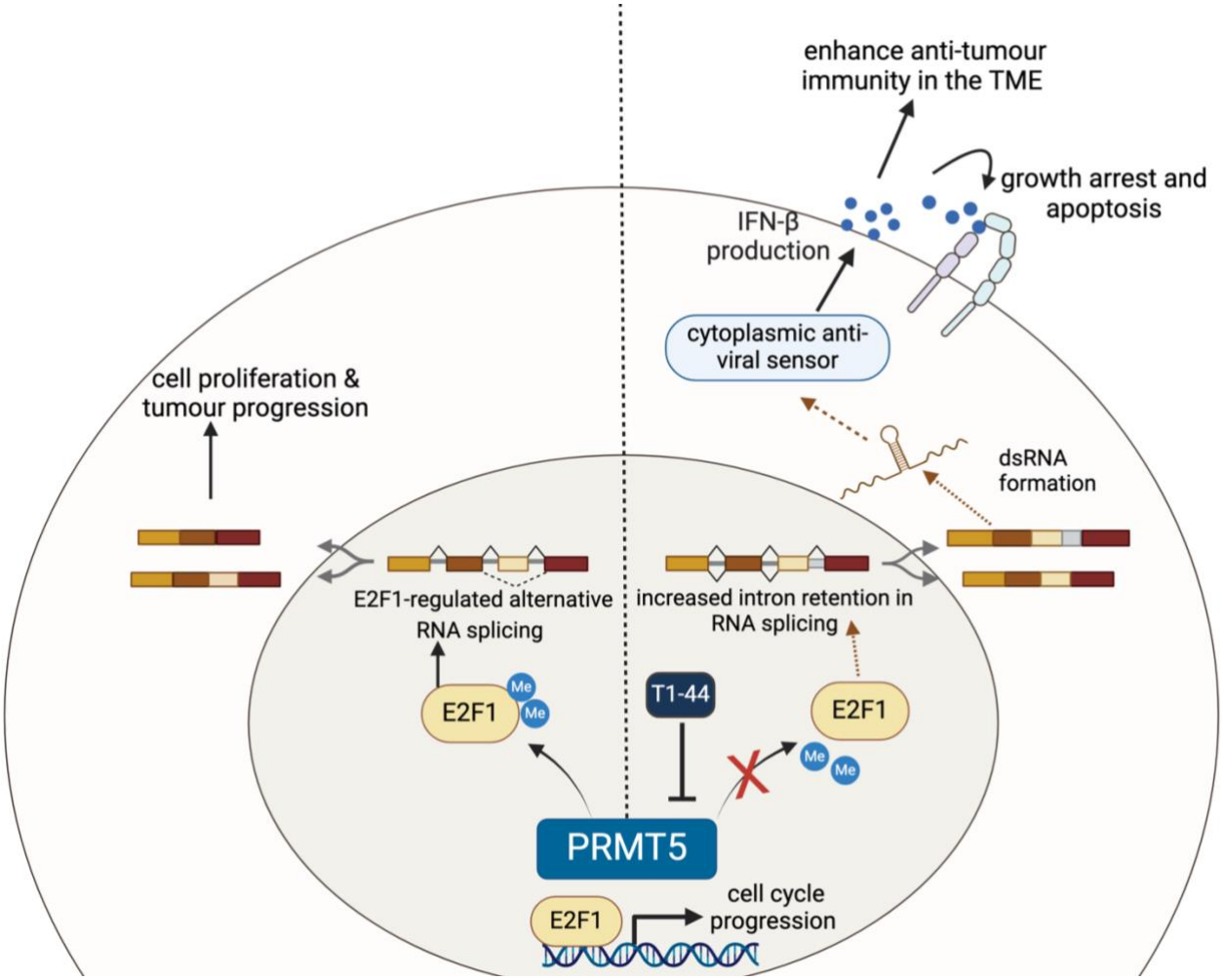


Figure 4: Proposed model of PRMT5 inhibition increasing RI-derived dsRNAs and production of IFN-β.

Under PRMT5 inhibition by T1-44, methylation of E2F1 by PRMT5 is decreased. This will increase intron retention due to altered RNA splicing control. dsRNAs formed from retained introns accumulate in the cytoplasm and can activate anti-viral sensors to trigger the production of IFN-β. Released IFN-β can either bind to IFNAR on the cancer cell to promote growth arrest and apoptosis, or enhance anti-tumour immunity by acting on the immune cells in the TME.

2. MATERIALS AND METHODS

2.1 Cell culture and compound treatments

Human p53-/- HCT116 E2F1 CRISPR and Cas9 control cells were previously generated by members of the group (Barczak et al., 2020). Cells were tested free of mycoplasma contamination with MycoSensor qPCR assay (Agilent, 302107) and cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, D5796) supplemented with 10% foetal bovine serum (Labtech, FCS-SA/500) and 1% penicillin/streptomycin (Gibco, 15140122). Cells were treated with T1-44 (synthesised by Argonaut Therapeutics) at 1μM final concentration or equal volume of DMSO for 48 h.

2.2 Poly(I:C) Stimulation

Polyinosinic–polycytidylic acid sodium salt (Poly (I:C)) (Sigma-Aldrich, P1530) was transfected using Lipofectamine 3000 transfection system (Invitrogen, L3000001). Opti-MEM (Gibco, 31985070) was mixed with lipofectamine 3000 and vortexed for 2-3 seconds. In a separate tube, Poly(I:C) was mixed with 3000 reagent and Opti-MEM (Gibco, 31985070). The contents in the two tubes were combined and mixed by flickering and incubated in room temperature for 10-15 minutes before adding dropwise to the cells. The final concentration of Poly (I:C) in the media was 2 μg/mL. Media was changed after 6 hours. Cells were harvested

24 hours after Poly (I:C) stimulation.

2.3 Transient expression of retained-intron sequences

2.3.1 Plasmid Design and Generation

The sequence of four genes: DDX55, CDK10, HDAC6, and A1BG were used for plasmid design. Transcripts containing the retained introns identified in rMATS analysis or a control transcript without the intronic sequences were synthesised and cloned into pcDNA3.1(+) vector by GenScript BioTech (UK) Ltd.

2.3.2 Transformation

For the amplification of the plasmids, the received freeze-dried plasmids were resuspended with DNase/RNase-free water and transformed to DH5α Competent Cells (Invitrogen, 18265-017). 100ng of plasmid DNA was added to DH5α cells and mixed by flickering the tube. The mixture was incubated on ice for 30 minutes and heat shocked at 42°C for 20 s. Then after 2 minutes of incubation on ice, antibiotics-free LB medium was added to the mixtures and incubated for 60 minutes at 37°C, 250 rpm.

Cells were plated onto selection plates made with LB agar with 100 µg/mL ampicillin and incubated at 37°C overnight.

2.3.3 Plasmid extraction

After overnight incubation, a single colony from each plate was inoculated into 5 mL of LB medium containing 100 µg/mL ampicillin and incubated at 37 °C with vigorous shaking (300rpm) for 8 hours. Then the cultures were transferred into 250 mL LB medium with ampicillin and incubated at 37 °C with shaking overnight.

The PureLink™ HiPure Plasmid Filter Maxiprep Kit (Invitrogen, K210016) was used to isolate plasmid DNA from bacteria. The culture was harvested by centrifuging at 1000 xg for 30 minutes and collecting the sediments. Following manufacturer's instructions, cell lysates were passed through filter columns, and DNA was washed and eluted from the filter. Isopropanol was used to precipitate the DNA and the pellet was washed with 70% ethanol before air-drying and resuspending with TE buffer. The isolated plasmids were sequenced at Source BioScience Ltd. to confirm the sequences before being used for transfection and *in vitro* transcription.

2.3.4 Plasmid transfection

Plasmid transfections were performed using the GeneJuice transfection reagent (Novagen, 70967). 1.5 µL of GeneJuice per µg of DNA was added dropwise to Opti-MEM (Gibco, 31985070) and incubated for 5 minutes at room temperature. Then DNA was added to the GeneJuice/Opti-MEM mixture and mixed gently by flickering the tube. Then the mixture was incubated for a further 15 minutes before addition to the dishes with pre-seeded cells. The media was replaced 6 hours after transfection. To ensure high expression of target sequences, cells were re-seeded at around 60% confluency 24 hours after first transfection and plasmid transfection was repeated on the next day following the same procedure. Cells were collected 48-72 hours after second transfection.

2.4 Reverse transcription quantitative real-time PCR (RT-qPCR)

2.4.1 RNA extraction and Reverse Transcription

RNA was isolated from cells using TRIzol (Thermo Fisher Scientific, 15596026) or the Direct-zol RNA MiniPrep kit (Zymo Research, R2050) according to the manufacturer's instructions. 30U DNaseI treatment for 15 mins in room temperature was performed for Direct-zol RNA

extraction. 1 µg of RNA was used for complementary DNA (cDNA) synthesis. Reverse transcription with oligo (dT)20 (Invitrogen, 18418012) or random hexamers for RNA immunoprecipitation experiments (Invitrogen, N8080127) was performed using SuperScript III Reverse Transcriptase (Invitrogen, 18080093) as per manufacturer's instructions.

2.4.2 Quantitative PCR

Quantitative PCR (qPCR) was carried out using cDNA as templates in technical triplicate using the indicated primer pairs and the Brilliant III SYBR Green qPCR Master Mix (Agilent, 600882) with the AriaMx Real-Time PCR System (Agilent, G8830A). Results were expressed as average (mean) fold change compared to control treatments using the ΔΔCt method. The housekeeping gene GAPDH was used as an endogenous reference. See primer sequences in Table 1.

RT-qPCR primer	Forward primer sequence 5'-3'	Reverse primer sequence 5'-3'
DDX55 Intron 4-5	TCAAAGGCTGTTTTCTTGTTGTAG	TCTCAACATCTTCTCCAGGATTC
DDX55 Exon 4-5	CCCCGAGTTCAGCCAGATTC	CCTTGTTGCTTAAACCTCTCAACA
CDK10 Intron 8-9	GGAGAGGAGCCGGCTGTATT	ACATGTCGATGCTGGTGGTCT
CDK10 Exon 8-9	GGTGGTCACTCTCTGGTACCGA	ACATGTCGATGCTGGTGGTCT
HDAC6 Intron 12-13	AGCCTCATGGAGTCTGTTCC	CCTTGCAGGGCATCAAATCC
HDAC6 Exon 12-13	GATGCGGGATGCTGACTACA	GAGGCTGGAACTCGAGGG
A1BG Intron 4-5	GCAGCTACCGGACCGATG	GATGGTTCCCACAGTCACCG
A1BG Exon 4-5	CTACCGGACCGATGGGGAA	TGCAGCGAGCTCCTCAATG
GAPDH	CATCTTCCAGGAGCGAGATCC	ATGACGAACATGGGGGCATC
IVT PCR primer	AGAACCCACTGCTTACTGGCT	CCCTCTAGACTCGAGCGGC

Table 1 List of qPCR or PCR primers used in this study

2.5 Immunoblotting

Cells were harvested by trypsinisation (Sigma-Aldrich, T2601) and pelleted at 300 relative centrifugal force (rcf) for 5 minutes at 4°C. The pellets were washed once in PBS before lysis

in TNN buffer (50 mM Tris-HCl pH7.4, 150 mM NaCl, 1% IGEPAL, 0.1% deoxycholic acid, 1 mM EDTA, 1 mM NaF, 1 mM Na3VO3 and protease inhibitor cocktail [1μg/mL leupeptin, 1 μg/mL aprotinin, 1 μg/mL pepstatin A]) and incubated on ice for 30 minutes. The lysate was then centrifuged at 13,000rpm for 10 minutes at 4°C to remove cellular debris. Protein concentration was determined via Bradford assay following manufacturer's instructions (Sigma-Aldrich, B6916).

Normalised samples were mixed with SDS sample buffer (0.5 M Tris-HCl pH6.8, 0.2M DDT, 2% (w/v) SDS, 20% (v/v) glycerol, 0.1% (w/v) bromophenol blue) and boiled at 95°C for 5 minutes.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by loading samples onto 10% SDS-polyacrylamide gels and running in SDS running buffer (25mM Tris, 192mM glycine, 3.5mM SDS). Color Prestained Protein Standard (New England Biolabs, P7719) was used as a protein ladder to estimate molecular weight.

Proteins were transferred onto nitrocellulose membranes (Pall Corporation, 50852413) using the Trans-Blot Turbo transfer system (Bio-Rad, 1704150) and the Bio-Rad Turbo transfer buffer (Bio-Rad, 10026938) at 1.3 or 2.5 A for 7 minutes.

Following transfer, membranes were blocked in 5% milk in PBS-Tween 0.1% (PBS-T) for 1 hour at room temperature. Primary antibodies were diluted in blocking solution at 1:1000 for E2F1 (Cell Signalling, 3742), or 1:2000 for SDME (Cell Signalling, 13222) and loading control β-actin (Cell Signalling, 3700). Membranes were incubated on a rocker overnight at 4°C, and washed three times in PBS-T. Secondary antibody was performed with HRP-conjugated antibody of 1:10000 dilution for 1 hour at room temperature. Membranes were washed another 3 times with PBS-T before visualisation with Clarity Western ECL Substrate (Bio-Rad,

1705061). Signal was detected by Fuji X-ray films (Fujifilm, 03g010) and developed on the AGFA CP1000 automatic developer.

2.6 Flow cytometry

For intracellular staining of dsRNA and IFN-β, cells were fixed and permeabilised using the Cytofix/Cytoperm kit (BD Biosciences, 554714) after harvesting via trypsinisation, and washing the pellet once in PBS. Cells were incubated with Fixation/Permeabilization solution at 4°C for 15 minutes. 1X BD Perm/Wash buffer was used for washing and antibody dilution. FITC-conjugated IFN-β antibody (Invitrogen, BMS1044F1) was diluted 1:100 and for dsRNA staining, J2 antibody (Nordic Biosite, 246-10010500) was diluted 1:700. Both antibody incubations were for 1 hour incubation at 4°C. For dsRNA staining, anti-mouse Alexa Fluor 488 conjugated secondary antibody (Invitrogen, A-11059) was used in 1:800 dilution as a secondary, and samples were incubated for 1 hour in the dark. Then samples were run and analysed on a flow cytometer (BD Accuri C6 Plus).

2.7 Immunofluorescence

Cells were seeded onto 8-well cell culture chambers on PCA slides (Sarstedt, 94.6140.802) 24 h after second round of plasmid transfection, followed by drug treatment for 48h. Cells were then fixed in 4% paraformaldehyde for 20 minutes at room temperature, and washed before permeabilised with 0.5% Triton X-100 in PBS for another 20 minutes in room temperature. All washes are done three times with 1x PBS.

As a negative control for dsRNA staining, one well was treated with 20U RNase III (Invitrogen, AM2290) for 30 minutes at 37 °C before proceeding to blocking with 1% BSA in PBS-T for 30 minutes at room temperature. The slides were then incubated overnight in humidified chamber

at 4 °C with 1:700 J2 primary antibody (Nordic Biosite, 246-10010500).

Slides were washed before incubation with anti-mouse Alexa Fluor 488 conjugated secondary antibody (Invitrogen, A-11059) at 1:800 dilution for 1 hour in the dark in a humidified chamber. After final wash, slides were fixed using Fluoroshield mounting medium with DAPI (4',6-diamidino-2-phenylindole) (ab104139). Slides were visualised by Dr Wojciech Barczak at Ingenox Therapeutics and analysis were done with ImageJ software.

2.8 Immunohistochemistry (IHC)

In vivo experiment using T1-44 treatment on Colon26 mouse tumour model was performed for a previous publication of the group, where specific conditions were described(Barczak et al., 2023). Daily treatment of 100 mg/kg T1-44 was administrated orally (gavage) using 0.5% Tween/PBS as a vehicle. After 19 days, four formalin-fixed tumour tissue samples from each group were prepared for immunohistochemical staining.

Slides were washed with HistoChoice® Clearing Agent (Merck, H2779) for 5 minutes prior to antigen retrieval with sodium citrate buffer, pH 6 for 20 minutes in 99 °C water bath. Then slides were washed in 6% H202 in Methanol for 15 minutes followed by 20 minutes blocking using blocking solution with goat serum (Genetex, GTX30973). As J2 antibody used for dsRNA detection is a mouse monoclonal Antibody, the Mouse-on-Mouse Polymer IHC Kit (Abcam, ab269452) was used for subsequent blocking of mouse IgG for 1 hour in room temperature. 1:3000 J2 Antibody (Nordic Biosite, 246-10010500) was diluted in PBS and primary Antibody incubation was performed overnight at 4 °C in humidified chamber. A no primary antibody control was used to confirm specificity of the staining. HRP conjugated Polymer supplied by the Mouse-on-Mouse Polymer IHC Kit was used for detection of dsRNAs with 45 minutes

incubation at room temperature.

For IFN-β staining, after blocking with goat serum, slides were incubated in 1:400 Polyclonal IFN-β antibody (Thermo Fisher Scientfic, PA5-20390) at room temperature for 1 hour and detected by VECTASTAIN® Elite® ABC-HRP Kit (Vector Laboratories, PK-6100) following manufacturer's instructions. For amplification and visualization of signal, the DAB substrate Kit (Vector Laboratories, SK-4100) was used following manufacturer's instructions. Finally, slides were counterstained with haematoxylin (Sigma-Aldrich, SLCL4496) for 60 seconds and mounted with VectaMount® AQ Aqueous Mounting Medium (Vector Laboratories, H-5501-60), and pictures of staining were taken by Dr Wojciech Barczak. Analysis of staining intensity was performed by ImageJ using colour deconvolution (H-DAB). Only DAB staining was selected and converted to 8-bit. Then the whole field of view was selected to measure signal intensity. O.D. was calculated by log(255/absorption) were 255 is the maximum intensity.

2.9 *In Vitro* Transcription (IVT) and RNA immunoprecipitation (RIP)

2.9.1 Preparation of PCR templates

The pcDNA3.1(+) plasmids containing either intron-retained or control sequences of DDX55, CDK10, HDAC6 and A1BG were linearised with restriction enzymes. DDX55, HDAC6, and A1BG were linearised with ApaI (Thermo Scientific, ER1411). Because CDK10 sequences contain another ApaI cutting site, the two plasmids for CDK10 were linearised with BclI (Thermo Scientific, ER1411). PCR of the target sequences including the T7 promoter region was performed using Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific, F-530XL). 20ng of linearised plasmids were used to set up 50μL reactions following manufacturer's instructions. The thermocycling conditions were as follows: denaturation at

98°C for 30 seconds, 98°C for 10 seconds, 61°C annealing temperature for 30 seconds and 72°C for 1 minute to a total of 35 cycles, and a final 72°C elongation for 5 minutes. PCR products were visualised on 1% agarose gel with 0.5 μg/mL ethidium bromide.

To ensure purity of the IVT products, PCR products were excised and extracted from agarose gel using QIAquick Gel Extraction Kit (Qiagen, 28704) as per manufacturer's instructions.

2.9.2 *In Vitro* Transcription

IVT was performed using the T7-Scribe™ Standard RNA IVT Kit (Cellscript, C-AS3107). Each reaction contains 1X T7-Scribe Transcription Buffer, 200ng of PCR amplified template DNA, dNTP/dUTP mix each at a final concentration of 7.5 mM, 10 mM DTT, 20 U ScriptGuard™ RNase Inhibitor, 2 μL of T7-Scribe Enzyme Solution, and RNase-Free Water to a final volume of 20 μL. The reactions were incubated at 37°C for 2 hours. Then 1U of RNase-Free DNase I was added to each reaction and incubated for another 15 minutes at 37°C.

RNA was purified by addition of one volume (21 μL) of 5 M ammonium acetate to the reaction. Then the mixture was incubated on ice for 15 minutes and centrifuged at 13,000 x g for 15 minutes at 4°C to pellet the RNA. 70% ice cold ethanol was added to the pellet after discarding the supernatant. Following another centrifugation as before, the ethanol was removed, and pellet was air-dried. The RNA was resuspended in RNase-free water and quantified using NanoDrop (Thermo Scientific, ND2000CLAPTOP).

2.9.3 Cap and Tail

For the purpose of stabilising RNA, 5' cap and Poly A tail were added to the IVT products. The ScriptCap™ m7G Capping System (Cellscript, C-SCCE0625) and the ScriptCap™ 2'-O-

Methyltransferase Kit (Cellscript, C-SCMT0625) were used for capping.

50-60 µg of transcribed RNA was made up to a final volume of 67 µL with RNase-free water and heat denatured for 10 minutes at 65°C. In a separate tube, a cocktail was prepared for the final concentration of 1X ScriptCap Capping Buffer, 1 mM GTP, 500 µM S-adenosyl-methionine (SAM), 100 U of ScriptGuard RNase Inhibitor and 400 U of ScriptCap 2'-O-Methyltransferase after mixing with cooled denatured RNA. Prior to the start of reaction, 40 U of ScriptCap Capping Enzyme was added to each reaction, and the final reaction volume was 100 µL.

The reaction was incubated at 37°C for 1 hour before proceeding to Poly A tailing using the A-Plus Poly(A) polymerase tailing kit (Cellscript, C-PAP5104H).

The 100 µL capping reaction was combined with a final concentration of 1X 10X A-Plus Poly(A) Tailing Buffer, 1mM ATP, 24U of ScriptGuard RNase Inhibitor, and 20 U of A-Plus Poly(A) Polymerase. After mixing, the reaction was incubated at 37°C for 30 minutes before purification with ammonium acetate following the same steps as described in 2.9.2.

RNA products were visualised by running 100 ng of RNA in a 1% agarose gel prepared using 1X Borax buffer and GelRed Nucleic Acid Stain (Merck, SCT123). An ssRNA Ladder (New England Biolabs, N0362S) was ran alongside for size reference, and RNA was denatured by mixing with 2X RNA Loading Dye and heating at 70 °C for 10 minutes and then placed on ice. Samples were loaded to agarose gel and ran at 150 V for 20 minutes before visualisation using ChemiDoc Imagining System (Bio-Rad).

2.9.4 dsRNA Immunoprecipitation

Protein A/G agarose beads were pre-blocked with 5 mg/mL BSA and 300 µg/mL salmon sperm DNA for 1 hour. Then the beads were washed and resuspended in RIP Lysis buffer (50mM

Tris-HCl pH 7.4, 100 mM NaCl, 1% Igepal, 0.1% SDS, 0.5% Sodium deoxycholate, 1x protease inhibitor cocktail), and kept at 4°C.

For all following procedures, 200 U/mL RNase OUT (Invitrogen, 10777019) were supplemented to the RIP buffer to prevent degradation of RNA.

Equal amounts of transcribed RNA (30-60 µg) were made up to 1mL with RIP buffer, 60 µL of pre-blocked protein A/G agarose beads, and 2 µg of mouse IgG for pre-clearing of samples. The mixtures were rotated at 4°C for 2 hours.

Beads were then spun down at 360 x g at 4°C for 3 minutes, and 1/10 of the supernatant was kept as input and stored at -20°C. The rest of supernatant was split equally to two tubes and topped up to 1 mL with RIP buffer. 2 µg of either J2 dsRNA antibody (Nordic Biosite, 246-10010500) or mouse IgG antibody as a control were added to the samples. The samples were incubated at 4°C on a rotating mixer overnight.

On the next day, 60 µL of A/G beads were added to each tube and samples were incubated for further 2 hours. Then beads were washed 4 times with RIP buffer and resuspended after the fourth wash with TRIzol (Thermo Fisher Scientific, 15596026) to elute RNA from the beads.

Then RNA isolation from beads and input samples was performed using TRIzol according to the manufacturer's instructions. During RNA precipitation, 15 µg of GlycoBlue™ Coprecipitant (Invitrogen, AM9516) was used as a carrier for RNA and increase the visibility of RNA pellets.

RT-qPCR was performed as described in 2.4.2 and 2.4.3, but instead of Oligo(dT)12-18 Primer (Invitrogen, 18418012), Random Hexamers (Invitrogen, N8080127) were used for reverse transcription. The primers used for qPCR are listed in **Table 1**.

2.10 Statistical analysis

All statistical analyses were performed in Graphpad Prism v10. All data has passed the Shapiro-Wilk test for normal distribution. For statistical testing between two groups, two-tailed, unpaired Student's t test was used. For multiple group comparisons, Analysis of variance (ANOVA) was performed.

Splicing analysis from RNA-seq data was performed by Dr Wojciech Barczak using rMATS method. rMATS is a computational tool designed to detect and analyse alternative splicing events from RNA-seq data. Compared to previous version of MATS and methods like SpliceTrap and basic isoform expression analysis from RNA-seq data, rMATs accounts for both the inherent sampling uncertainty in individual RNA-Seq replicates and the biological variability among these replicates. Therefore, it is a more robust and flexible tool for detecting differential splicing events across conditions (Shen et al., 2014).

All Scientific illustrations were created in BioRender.com.

3. RESULTS

3.1 PRMT5 inhibition elevates dsRNA and IFN-β production

PRMT5 is reported to be critical for normal immune system function, such as T-cell maintenance and B-cell development. In an oncogenic setting, however, inhibition of PRMT5 was not previously reported to attenuate anti-tumour immunity (Abe et al., 2023). On the contrary, previous studies have found that PRMT5 inhibition limits Treg function and its migration into tumours in the CT26/HER2 mouse tumour model (Nagai et al., 2019). An earlier study from our group also demonstrated that PRMT5 inhibition leads to an increase in

infiltrating CD8+ and CD4+ T cell populations in the mouse Colon 26 model (Barczak et al., 2023).

In HCT116 human colon carcinoma cell lines, PRMT5 inhibition negatively impacts cell cycle progression, proliferation, and migration (Barczak et al., 2020). To better understand the anti-tumour effects of PRMT5 inhibition, we explored the potential activation of the innate immune response through Type I IFN production. It was established that targeting the spliceosome could result in intron retention during alternative RNA splicing, forming dsRNAs and activating the Type I IFN response (Bowling et al., 2021).

Since PRMT5 is known to regulate RNA splicing, we investigated whether an increase in Type I IFN production under PRMT5 inhibition could coincide with elevated dsRNA levels.

The generation of HCT116 p53-/- cells with E2F1 CRISPR KO or Cas9 control has been described in previous publications by our group(Barczak et al., 2020). E2F1 KO does not significantly affect the growth rate of HCT116 cells, but the effect of PRMT5 inhibition on cell cycle arrest is attenuated in E2F1 KO cells. It is suggested that p53 promoted growth arrest is complementary to the cell cycle regulation by the pRb-E2F1 pathway. Previously studies have revealed a crosstalk between pRb-E2F1 and p53 pathway in cell cycle arrest through arginine methylation, as both E2F1 and p53 are targets of PRMT5 and share a similar sequence motif (Cho et al., 2012; Jansson et al., 2008). The presence of p53 influences the interplay between PRMT5 and E2F1, with a p53-/- background found to increase cell sensitivity to PRMT5 inhibition and showing a greater effect of E2F1 KO (Barczak et al., 2020).

PRMT5 inhibition in this study was achieved using the small molecule active site inhibitor T1-44. The characterization of T1-44, described in previous publications, confirms it as a highly selective and effective inhibitor of PRMT5 with an EC50 of 5.4 nM for PRMT5 enzyme activiy and IC50 of 13.4 nM in HCT116 cells after 6 days of treatment (Barczak et al., 2020). In this study, all T1-44 treatments in HCT116 were for 48 hours at a concentration of 1 μM.

The intracellular levels of dsRNAs and IFN-β were examined in HCT116 p53-/- cells by antibody staining and flow cytometry. Two E2F1 CRISPR KO clones, clone 2 (Cr2) and clone 7 (Cr7), were used alongside E2F1 WT cells to study the effect of E2F1 ablation under PRMT5 inhibition. The dsRNA analogue 2 μg/mL Poly (I:C) was used as a technical positive control for dsRNA-induced IFN-β production. As Poly (I:C) was transfected into the cells with Lipofectamine, a Lipofectamine-only control was tested alongside, and its dsRNA and IFN-β levels were similar to normal WT control cells.

Without PRMT5 inhibition, there was no significant difference in dsRNA and IFN-β levels between E2F1 WT and KO cells. T1-44 treatment increased intracellular dsRNA by an average of 20% and IFN-β by an average of 15% in E2F1 WT cells. In contrast, this effect was not observed in either of the E2F1 KO clones **(Figure 5A)**, indicating a possible dependency of this dsRNA and IFN-β production pathway on E2F1.

The loss of E2F1 in CRISPR KO clones was confirmed by immunoblotting, and the activity of T1-44 was measured by SDME mark, as PRMT5 inhibition would result in decreased symmetric dimethylation **(Figure 5B).** To validate whether the effect of PRMT5 inhibition extends to a physiological setting, tissue samples from the mouse Colon26 tumour model treated

with 100 mg/kg T1-44 or control were used for immunohistochemistry staining of dsRNA and IFN-β (Barczak et al., 2023). The activity of T1-44 is confirmed by staining of the SDME mark. The IHC was performed by Dr Wojciech Barczak previously on the same set of samples **(Figure 5C)** (Barczak et al., 2023).There was a statistically significant increase in dsRNA and IFN-β levels in T1-44 treated samples compared to controls **(Figure 5D, E).**

From the results in HCT116 cell lines and Colon26 tumour models, it can be concluded that PRMT5 inhibition increases dsRNA and IFN-β both *in vivo* and *in vitro* through a possibly E2F1-dependent pathway.

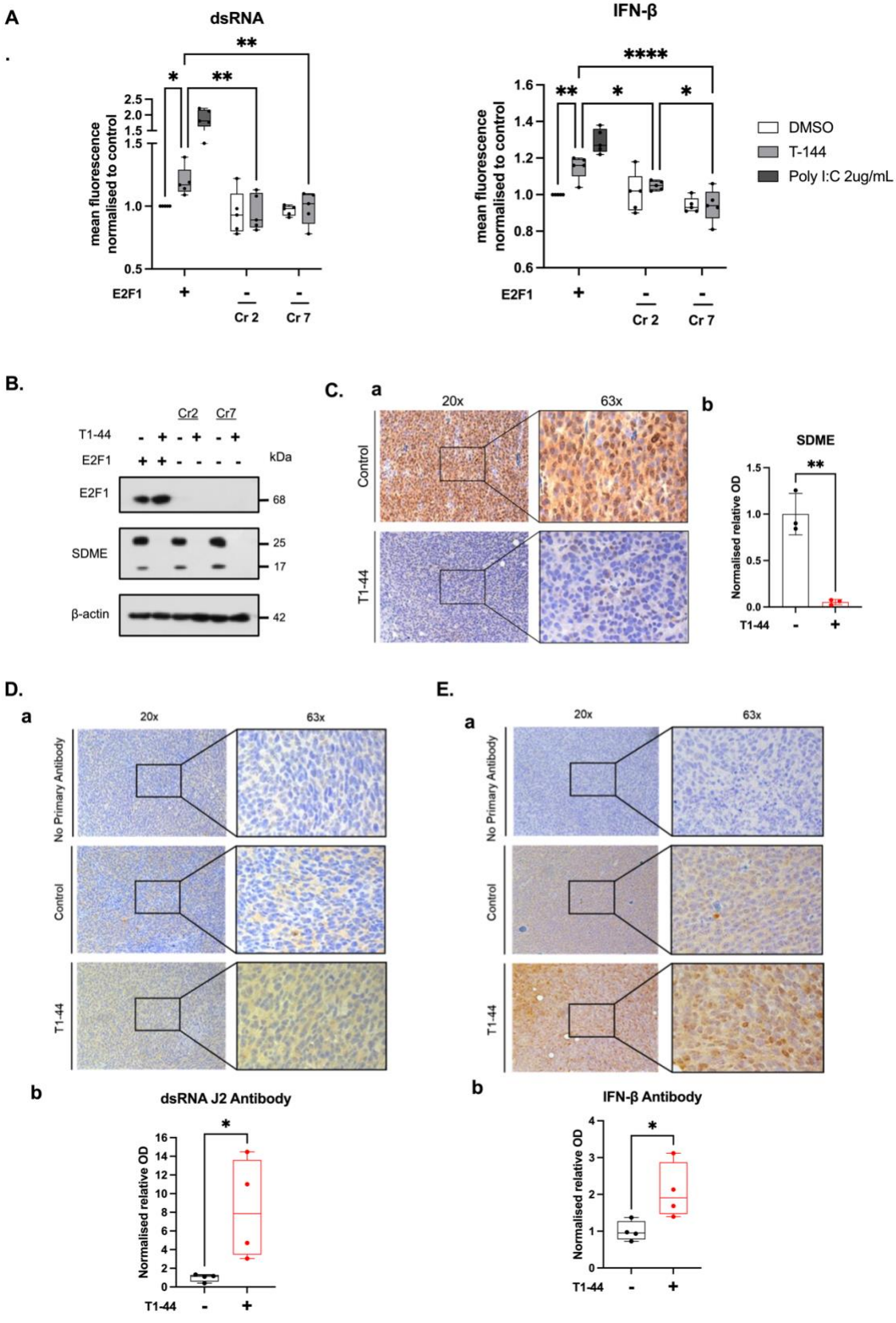

A
dsRNA
IFN-β
mean fluorescence normalised to control
E2F1
Cr 2
Cr 7
DMSO
T-144
Poly I:C 2ug/mL
B.
Cr2
Cr7
T1-44
E2F1
kDa
SDME
β-actin
C.
a
20x
63x
Control
T1-44
b
SDME
Normalised relative OD
D.
No Primary Antibody
dsRNA J2 Antibody
E.
IFN-β Antibody

Figure 5: T1-44 treatment results in dsRNA and IFN beta elevation *in vitro* and *in vivo.*

A) dsRNA (left) and IFN-β (right) intracellular staining of HCT116 E2F1 WT and CRISPR KO clones 2 (Cr2) and 7 (Cr7) treated with DMSO or T1-44. Mean fluorescence was normalised to DMSO treated E2F1-WT cells (control). 2 µg/mL Poly I:C was transfected to WT cells for 24 hours as technical positive control. n=5; * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$ using two-way ANOVA.
B) Representative immunoblot showing E2F1 KO and T1-44 activity by SDME mark.
C) a. Example immunohistochemical staining of SDME on colon26 tissue. **b.** Normalised optical density (O.D.) of each slide. n= 3; * $p < 0.05$ using unpaired Student's t-test.
D) a. Example immunohistochemical staining of dsRNA on colon26 tissue. A slide without primary antibody treatment was used as a negative control for staining. **b.** Normalised optical density (O.D.) of each slide. Each slide analysed was from a different biological replicate. n= 4; * $p < 0.05$ using unpaired Student's t-test.
E) a. Example immunohistochemical staining of IFN-β on colon26 tissue. **b.** Normalised optical density (O.D.) of each slide(top). n= 4; * $p < 0.05$ using unpaired Student's t-test.

3.2 PRMT5 inhibition promotes intron-retention on a population of genes

To explore the mechanism behind the upregulation of dsRNAs in cells treated with the PRMT5 inhibitor T1-44, a replicate multivariate analysis of transcript splicing (rMATS) was conducted on previously obtained RNA-seq data from HCT116 cells. The analysis, provided by Dr Wojciech Barczak, identified an increase in intron-retention events across more than 500 genes, predominantly in T1-44 treated E2F1 WT cells.

Six genes were selected for detailed analysis of intron retention as identified by rMATS (**Figure 6A)**. For each gene, two pairs of primers were designed: one pair spanning the intron-exon junction to detect the retained intron proposed by rMATS (RI primers) and another pair spanning the exon-exon junction to specifically detect spliced transcripts (Exon primers) **(Figure 6B)**. The intron inclusion/exclusion ratio was calculated by dividing the ΔΔCT values obtained from the RI and Exon primers for the same sample.

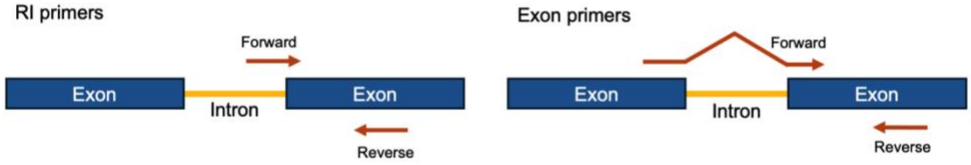
Among the six candidate genes tested—DDX55, CDK10, A1BG, HDAC6, and SPIN2B—five showed an increase in intron retention in T1-44 treated HCT116 E2F1 WT cells **(Figure 6C)**. E2F1 KO and activity of T1-44 were again confirmed by immunoblotting **(Figure 6D).** Interestingly, the effect of PRMT5 inhibition appeared to be attenuated in E2F1 knockout (KO) cells. For one candidate, GALT, T1-44 exhibited an inverse effect where intron retention was reduced, independent of E2F1 presence. This finding indicates that PRMT5 inhibition may have a complex impact on RNA splicing, as some splicing events regulated by PRMT5 are also independent of E2F1.

To determine the impact of these intron-retention events on the production of dsRNA and IFN-β, sequences containing the retained introns (RI) and those without (Exon) were cloned into pcDNA3.1 plasmids for subsequent transfections into HCT116 cells and for *in vitro* transcription. Plasmids were produced for DDX55, CDK10, A1BG, and HDAC6. Example plasmid maps for A1BG are shown in **Figure 6E**.

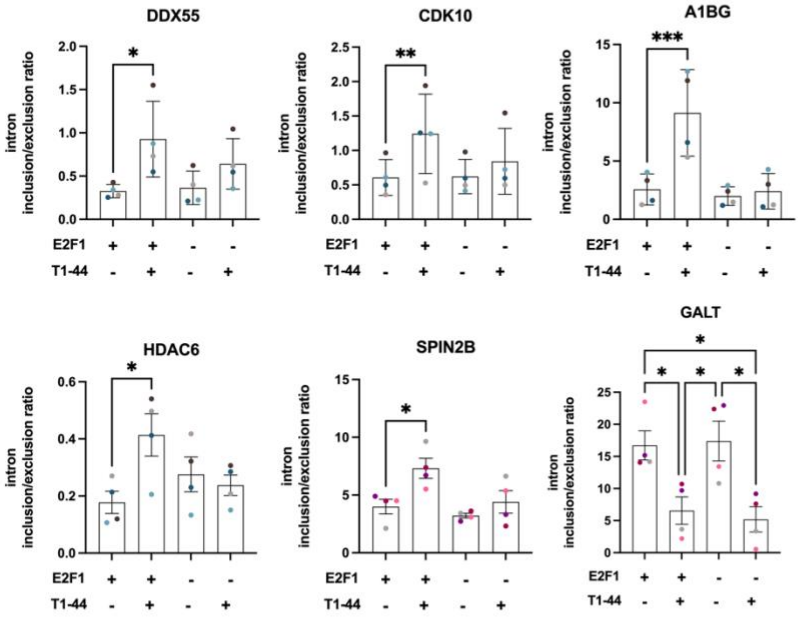
A.

Gene	Retained intron location (Chromosome_start_end)	Intron position in protein-coding transcript	Length of Intron
DDX55	chr12_124092070_124092150	Intron 4-5	76
CDK10	chr16_89759875_89760580	Intron 8-9	705
A1BG	chr19_58863053_58863648	Intron 4-5	595
HDAC6	chrX_48673147_48673240	Intron 12-13	93
SPIN2B	chrX_57146534_57146837	Intron 2-3	303
GALT	chr9_34647255_34648111	Intron 2-3	856

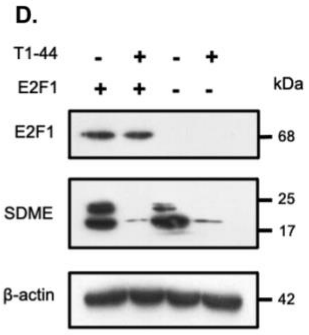
B.



C.



D.



E.

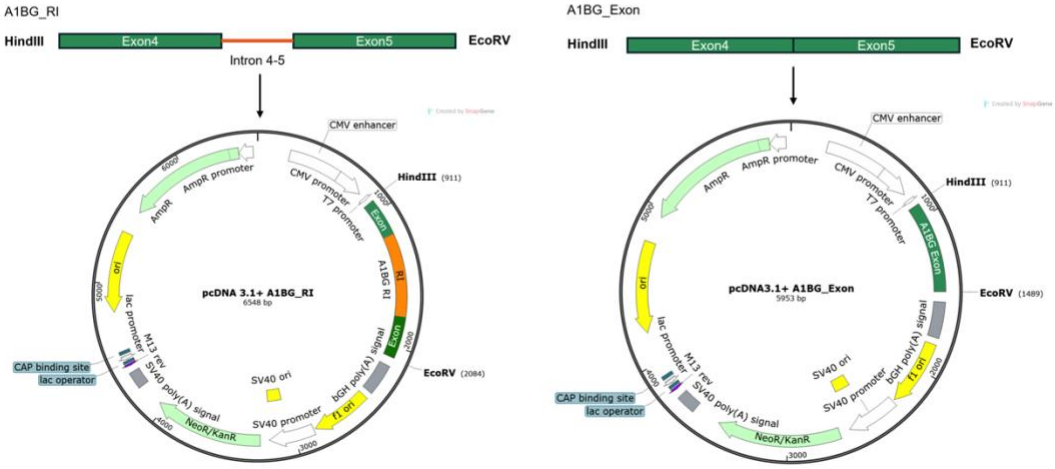


Figure 6: PRMT5 inhibition increases intron retention in a population of genes
A) Table of genes tested for intron retention by RT-qPCR. Intron locations were retrieved from rMATS analysis and positions in transcript were from Ensembl GRCh37 Archive. Genes highlighted in blue were later used in plasmid production.
B) Illustration for qPCR primer design. Primers detecting intron retention (RI) spanned the intron-exon junction and the primers for intron excision (Exon) spanned the exon-exon junction of two adjacent exons.
C) RT-qPCR to determine intron inclusion/exclusion ratio of DDX55, CDK10, A1BG, HDAC6, SPIN2B and GALT in HCT116 E2F1 WT and KO (Cr7) treated with DMSO or T1-44. Colours represent different biological replicates. n=4; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Data are presented as the mean ± SEM.
D) Representative immunoblot showing E2F1 KO and T1-44 activity by SDME mark.
E) Example of plasmid design showing RI and Exon plasmids for A1BG.

3.3 Expression of intron-retained transcripts increases dsRNA and IFN-β production

To investigate the *in vitro* effects of upregulated intron retention, plasmids constructed for two candidate genes—DDX55 and CDK10—were transfected into E2F1 WT HCT116 cells. The cells underwent two rounds of transfection over five days in order to reach a high level of transcript expression, followed by a further two days of incubation. The transfected cells were then prepared for either immunofluorescence analysis to assess dsRNA signal and localisation, or flow cytometry to measure levels of dsRNA and IFN-β **(Figure 7A).**

At the end of the experiment, cells were harvested, and RT-qPCR analysis was conducted to confirm successful transfection by comparing RI and Exon transcription levels to control cells transfected with empty pcDNA3.1(+) plasmid (Ctrl) **(Figure 7B)**. Additionally, the efficacy of T1-44 treatment was verified through immunoblotting **(Figure 7C)**.

Flow cytometry showed that transfection with RI plasmids for DDX55 or CDK10 resulted in significantly elevated levels of intracellular dsRNA and IFN-β compared to cells transfected with Exon plasmids **(Figure 7D)**. This suggests that the specific intron sequences retained under PRMT5 inhibition contribute to the observed effects of T1-44 treatment.

Immunofluorescence staining provided visual evidence of the impact of intron retention. Baseline dsRNA staining was low, indicating minimal accumulation in cells under normal conditions. Both T1-44 treatment and RI plasmid transfection yielded more pronounced dsRNA signals compared to control and Exon transfected cells **(Figure 7E)**. Notably, dsRNAs were primarily located in the cytoplasm, aligning with the proposed model where disruption of splicing activity leads to cytoplasmic dsRNA accumulation.

Quantification of mean fluorescence signals per cell showed no significant dsRNA increase in cells transfected with Exon plasmids compared to controls. However, a trend of increased dsRNA under T1-44 treatment or RI transfection was observed **(Figure 7F)**. This supports the hypothesis that a collection of retained intron events following PRMT5 inhibition contributes to the increase of dsRNA and potentially IFN-β production.

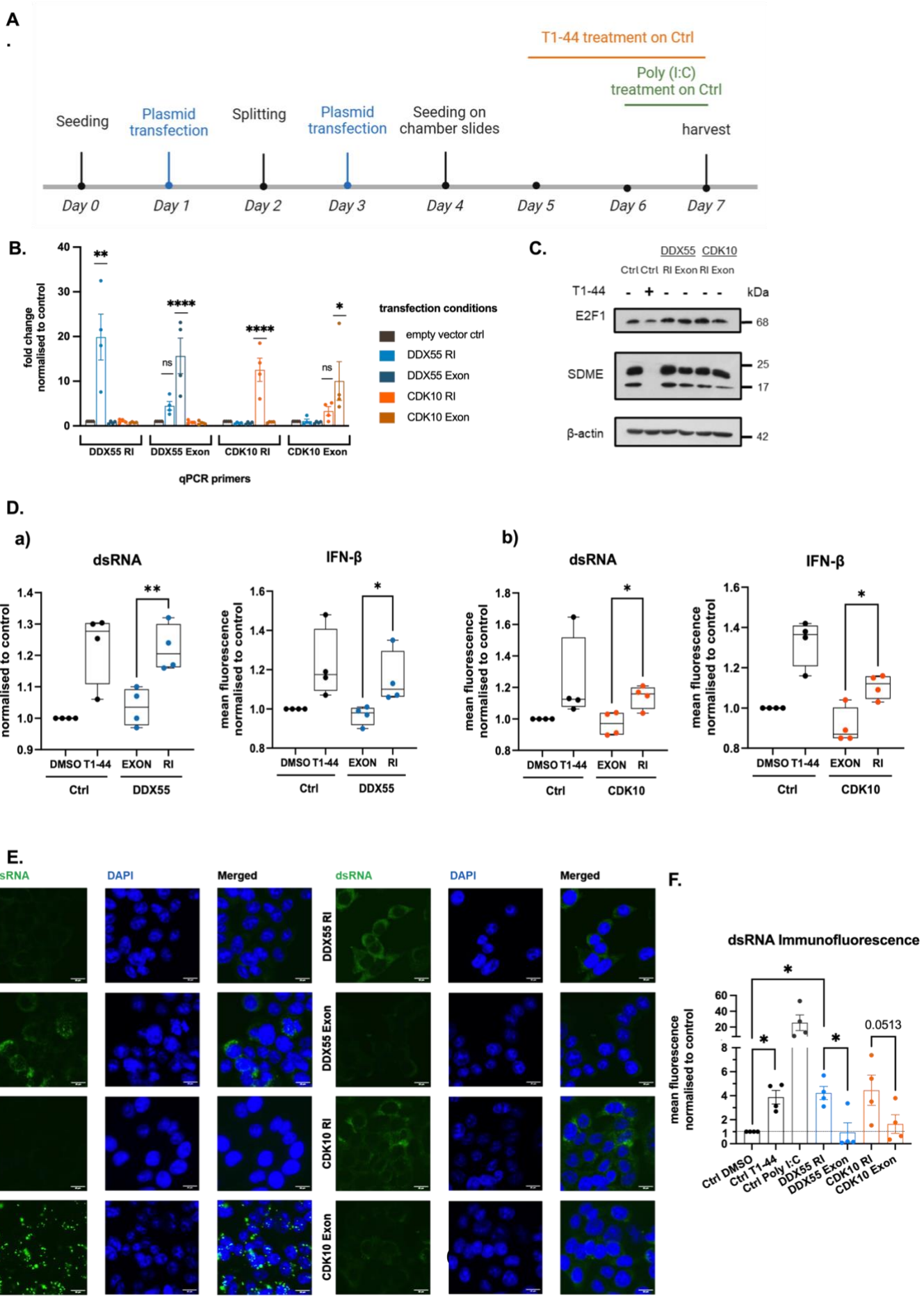

A.
T1-44 treatment on Ctrl
Poly (I:C) treatment on Ctrl
Seeding
Plasmid transfection
Splitting
Plasmid transfection
Seeding on chamber slides
harvest
Day 0
Day 1
Day 2
Day 3
Day 4
Day 5
Day 6
Day 7
B.
fold change normalised to control
transfection conditions
empty vector ctrl
DDX55 RI
DDX55 Exon
CDK10 RI
CDK10 Exon
DDX55 RI
DDX55 Exon
CDK10 RI
CDK10 Exon
qPCR primers
C.
DDX55 CDK10
Ctrl Ctrl RI Exon RI Exon
T1-44
kDa
E2F1
SDME
β-actin
D.
a)
dsRNA
IFN-β
mean fluorescence normalised to control
DMSO T1-44 EXON RI
Ctrl DDX55
b)
dsRNA
IFN-β
DMSO T1-44 EXON RI
Ctrl CDK10
E.
dsRNA
DAPI
Merged
control +DMSO
control +T1-44
RNase treated
Poly I:C
DDX55 RI
DDX55 Exon
CDK10 RI
CDK10 Exon
F.
dsRNA Immunofluorescence
mean fluorescence normalised to control
0.0513
Ctrl DMSO
Ctrl T1-44
Ctrl Poly I:C
DDX55 RI
DDX55 Exon
CDK10 RI
CDK10 Exon

Figure 7: Transfection of intron retained plasmids mimic effect of PRMT5 inhibition

A) Timeline of plasmid transfection and compound treatment. Cells were transfected with pcDNA 3.1(+) plasmids either with retained intron (RI) or without (Exon). Empty pcDNA3.1(+) plasmids were used as control for transfection (empty vector ctrl). After seeding transfected cells to chamber slides for immunofluorescence, the remaining cells were incubated in cell cultures dishes until collection on Day 7 for flow cytometry, immunoblotting and qPCR. 48 hours of 1μM T1-44 treatment on one well of Ctrl cells was represented in orange. Green represents 24 hours of 2 μg/mL Poly (I:C) treatment on one well of Ctrl cells.
B) RT-qPCR relative expression of RI and Exon transcripts confirming transfection of DDX55 and CDK10 plasmids, compared to Ctrl transfected cells. n=4; * $p< 0.05$, ** $p< 0.01$, **** $p<0.0001$ using one-way ANOVA.
C) Representative Immunoblot showing the activity of T1-44 by SDME mark.
D) dsRNA and IFN-β intracellular staining of HCT116 WT cells transfected with either RI or Exon transcripts of DDX55 (a) and CDK10 (b). Signal is relative to control (Exon transfected cells). n=4; * $p< 0.05$, ** $p< 0.01$, *** $p<0.001$ using unpaired Student's t-test.
E) Representative immunofluorescence staining of dsRNAs in HCT116 cells transfected with Ctrl, or RI /Exon plasmids of DDX55 or CDK10. Cells were treated with RNase as negative control or transfected with 2 μg/mL Poly I:C as positive control. Images were cropped from original. Original magnification: 40×, scale bar = 20 μm.
F) Mean fluorescence per cell was calculated using Fiji software. Poly I:C was a technical positive control and not included in statistical analysis. Results were normalised to Ctrl transfected cells treated with DMSO. n=4; * $p< 0.05$ using two-way ANOVA. All data are presented as the mean ± SEM.

3.4 Confirmation of dsRNA structure formed from retained introns

Despite evidence from *in vitro* studies showing that plasmids containing retained introns lead to dsRNA accumulation in the cytoplasm, direct evidence of dsRNA formation from these intronic sequences was lacking.

To address this, RI and Exon plasmids from the four previously tested candidate genes (DDX55, CDK10, A1BG, and HDAC6) were subjected to *in vitro* transcription (IVT) and subsequent dsRNA immunoprecipitation (RIP) **(Figure 8A).** The T7 promoter and RI or Exon sequences were amplified by PCR, and the products were confirmed via gel electrophoresis **(Figure 8B)**.

The PCR products were then gel-purified for the IVT reaction. Stability-enhancing

modifications such as the addition of a 5' Cap and Poly(A) tail were made to the RNA products. An RNA integrity gel showed a single band for most genes, although CDK10 and A1BG RI transcripts appeared less stable **(Figure 8C)**. This could be due to RNase degradation due to inappropriate sample handling that introduced RNase to CDK10 and A1BG RIs. U rich sequences, or RNA secondary structure formation during IVT can also cause early termination of the T7 polymerase and therefore incomplete transcripts (Song and Kang, 2001; Calvopina-Chavez et al., 2022; Artsimovitch and Landick, 1998). It was described that hairpin structure in nascent RNA could cause transcription termination, and given that RIs are predicted to form double-strand structures, this can also be a possible explanation (Artsimovitch and Landick, 1998; Calvopina-Chavez et al., 2022). Equal amounts of RI or Exon RNA were used for RIP, followed by RT-qPCR to compare dsRNA levels.

Normalising to IgG controls, RI transcripts of CDK10 and A1BG showed elevated dsRNA levels, whereas differences for DDX55 and HDAC6 were not significant **(Figure 8D)**. High background levels in IgG-treated samples indicated potential non-specific binding, with significant variability noted particularly in CDK10 replicates, suggesting room for methodological refinement in future experiments. Heat-inactivated J2 antibody might serve as a more suitable negative control. Preliminary data from the dsRNA-RIP experiments, although requiring optimisation, still hold promise for validating dsRNA structures in IVT-synthesised RI transcripts.

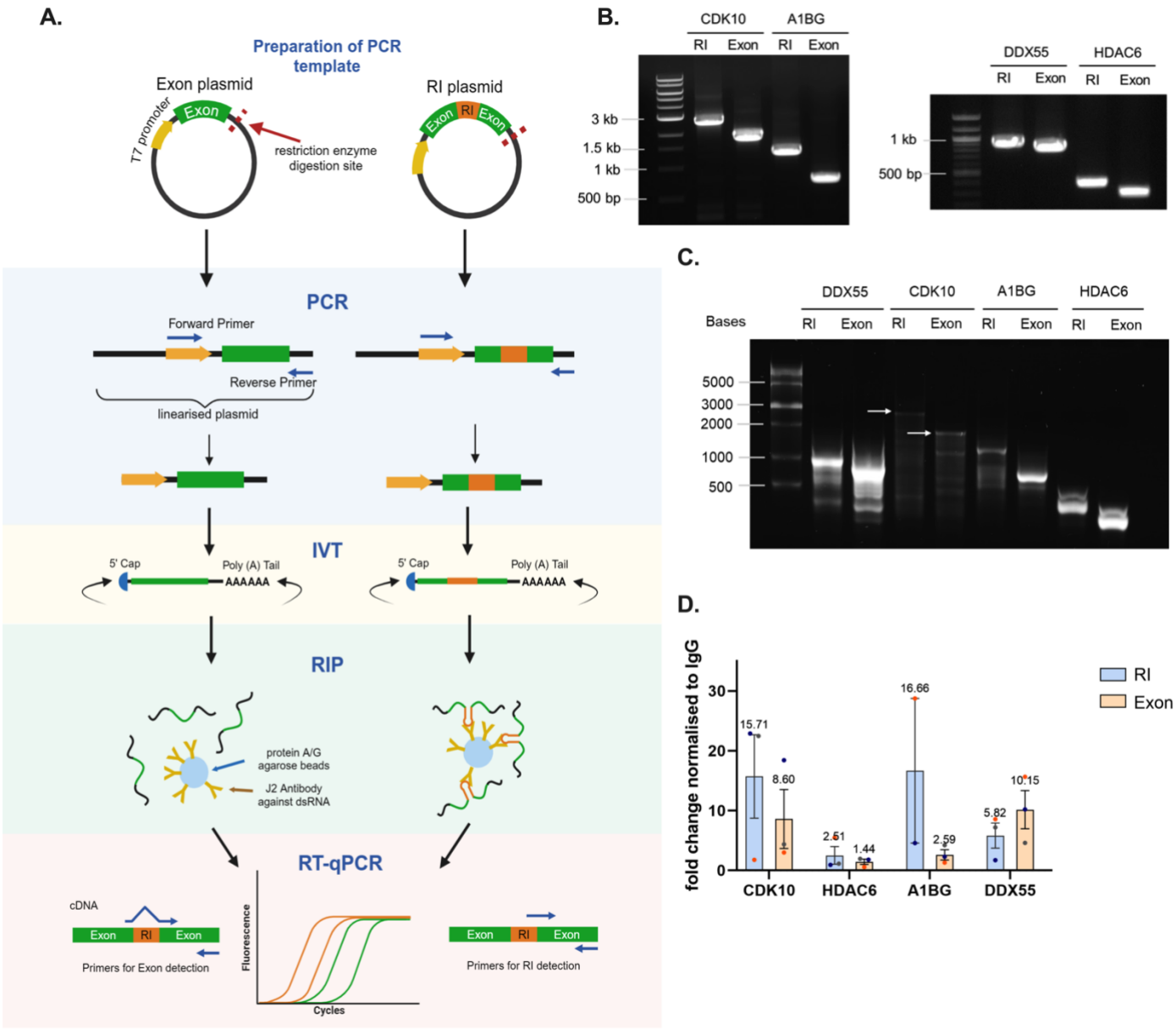


Figure 8: dsRNA RIP from IVT Products to confirm dsRNA formation from retained introns

A) Workflow for *in vitro* transcription (IVT) and dsRNA RIP experiments. Plasmids containing either exonic sequences (Exon) or retained introns (RI) were used to compare dsRNA formation.
B) Representative PCR results showing amplification of Exon and RI sequences for each gene.
C) RNA integrity gel displaying transcripts from IVT for both Exon and RI. Due to the low concentration of transcripts, the arrow indicates the expected positions of CDK10 bands for both RI and Exon.
D) Results of dsRNA RIP for the RI and Exon transcripts. n=3; Due to variability in replication, statistical analysis was not performed.

4. DISCUSSION

4.1 Summary of Results

PRMT5 is commonly overexpressed across different cancer types and is considered a crucial contributor to the deregulation of cell proliferation and apoptosis in cancer. The potential of targeting the arginine methyltransferase PRMT5 as an anti-tumour therapy requires an understanding of the pathways controlled by PRMT5 activity. E2F1, as a key PRMT5 target controlling both in transcription and RNA splicing, is also upregulated in a number of cancer cells. Using the PRMT5 inhibitor T1-44, cell cycle arrest and reduced cancer cell proliferation were induced in an E2F1-dependent manner (Barczak et al., 2020). In addition, T1-44 treatment *in vivo* is immunogenic in the TME, although the exact mechanism by which PRMT5 controls the innate immune response is unknown (Barczak et al., 2023).

This study reveals a novel pathway possibly regulated by PRMT5-E2F1. PRMT5 inhibition increases intracellular dsRNA accumulation as well as IFN-β production both *in vitro* and *in vivo*. At the same time, intron-retention was also increased by PRMT5 inhibition, which possibly gave rise to the dsRNAs. Previous rMATS analysis suggested a pool of RI events increased by T1-44 treatment, and several specific RI transcripts (DDX55, CDK10, A1BG, and HDAC6) were tested in qPCR and confirmed this trend. Transfection of RI transcripts of DDX55 and CDK10 produced a similar response to PRMT5 inhibition in terms of dsRNA and IFN-β upregulation. These effects of PRMT5 were attenuated when E2F1 was ablated, supporting the model that E2F1 is a regulator of RNA splicing and its activity depends on PRMT5 methylation.

4.2 Limitations and additional experiments

E2F1's role in PRMT5 pathway

Previously, Bowling *et al.* demonstrated dsRNA to be produced from spliceosome targeted therapy (STT) in TNBC, and an anti-tumour effect could be observed through activating anti-viral dsRNA sensing pathways in the tumour cells (Bowling et al., 2021). Also, it was shown that PRMT1 inhibition in TNBC could also increase dsRNA and trigger an IFN response through RI upregulation (Bowling et al., 2021). However, previous studies did not identify specific RNA splicing components that were responsible for the intron retention. Our study proposes a potential pathway from our previous findings, where PRMT5 acts through E2F1 to maintain normal RNA splicing (Roworth et al., 2019). Additional confirmation of E2F1's role can be accomplished through E2F1 rescue experiments in E2F1 KO cells.

In addition, PRMT5 KD by siRNA in comparison to T1-44 would provide further support to the role of PRMT5 in the PRMT5-E2F1 pathway. siPRMT5 was not performed because it was previously conducted by other members of the group and found to have cellular toxicity when PRMT5 protein is lost. Similar to T1-44 treatment, PRMT5 KD cells have reduced symmetrical methylation on R111/R113 of E2F1, which decreased E2F1 interaction with p100/TSN and caused E2F1-dependent growth inhibition (Zheng, S. et al., 2013).

PRMT1 and PRMT5 sometimes compete for the same substrates, such as E2F1, and produce distinct cellular responses (Zheng, S. et al., 2013). Interestingly, in the study using TNBC cells, PRMT5 expression did not correspond with Type I PRMT inhibitor sensitivity. This indicates that PRMT1 may act through separate pathways in TNBC to regulate RNA splicing.

Validating dsRNA formation from introns

In the study by Bowling *et al.*, retained introns were identified as the source of dsRNA by J2-RIP-seq. Furthermore, the population of introns contained both a well-known source of dsRNAs, the intronic retrotransposable elements (TE), and intronic sequences lacking TE but capable of self-folding into double-stranded structures. However, these analyses were based on RIP-seq results and RNA-fold predictions (Bowling et al., 2021).

Previously, the specific RI transcripts tested—DDX55, CDK10, A1BG, and HDAC6—were predicted to form dsRNA structures and were found to be enriched after T1-44 treatment in rMATS analysis. Observing a similar effect in dsRNA elevation after transfection of DDX55 and CDK10 can partially confirm the bioinformatics prediction. Although in vitro transcription (IVT) followed by J2 antibody RIP still requires optimisation and repetition, it would provide more direct biochemical evidence for the source of dsRNAs found intracellularly after T1-44 treatment. After confirmation of dsRNA structure through IVT, point mutating the intronic sequence will provide the most precise information about which region(s) of the intron are giving rise to the dsRNA structure.

In the future, RIP-seq analysis on HCT116 cells will allow confirmation of whether the increased RI sequences from rMATS analysis will also be found in the dsRNA population after T1-44 treatment. It would also be interesting to confirm whether the dsRNAs in HCT116 originate from intronic TE elements like LINEs and Alu elements.

Elucidating dsRNA sensors

Future experiments also include testing potential sensors of dsRNA during PRMT5 inhibition. In the study by Bowling *et al.*, an shRNA screen indicated that sensors like RIG-I and MDA5 could contribute to the sensitivity of TNBC cells to STT by activating the mitochondrial antiviral-signalling protein (MAVS). Meanwhile, the knockdown of dsRNA sensors that act independently of MAVS also affects the dsRNA-induced apoptosis, suggesting that multiple dsRNA sensors collectively contribute to this anti-tumour effect of STT (Bowling et al., 2021). This complexity of dsRNA sensing would require similar shRNA screening to be conducted in HCT116 cells with T1-44 treatment to see if losing different RNA sensors would affect the sensitivity of these cells to PRMT5 inhibition. It is worth noting that bioinformatics analysis predicted the dsRNA population to be mostly from short intronic sequences, so short dsRNA sensors like PKR may be playing a more important role in PRMT5 inhibition, but other types of dsRNA sensors like MAVS-activating RIG-I and MAVS-independent DDX21 can also be included in the shRNA library for screening (Hur, 2019; Zhang, Z. et al., 2011; Kim, Y. et al., 2018).

Alternative Cell lines

The study was conducted on HCT116 p53-/- background because it was a well-established model in our lab, where RNA-seq of PRMT5 inhibition and E2F1 KO was performed. p53 knockout cells were used as previous studies showed that PRMT5 and/or E2F1 inactivation has a more evident anti-proliferative effect on these cells, e.g., cell cycle arrest. Given that p53 is a target inhibited by PRMT5, and it is frequently inactivated in cancer, using p53 knockout cells excludes the potential transcriptional effect p53 activation might have after PRMT5 is inhibited,

while maintaining a cancer-relevant genetic background to study the PRMT5-E2F1 pathway (Barczak et al., 2020).

However, one limitation of the HCT116 cell line is the low extracellular IFN-β produced. HCT116 cells are not potent secretors of IFN-β, and the level of IFN-β in the cell media was not detectable even after T1-44 treatment. Since interferons are extremely active molecules, they may still have a biological effect on the cells, but later, other cell types, e.g. THP-1, could be used to detect changes in IFN-β in the cell media.

Challenges in delineating the pathway in the landscape of PRMT5 regulation

Overall, PRMT5 inhibition by T1-44 is promising in activating IFN-β response by promoting E2F1-mediated intron-retention and producing dsRNAs. However, one challenge is to validate the physiological effect of dsRNAs induced by PRMT5 inhibition. While the downstream effect of IFN-β production can be tested *in vivo* using an IFNAR1 blocker with T1-44 treatment, and sensors translating dsRNA signal to IFN-β can be identified using RNAi screening, there is no simple method to pinpoint dsRNAs produced from intron retention and show that their production is necessary for the activation of IFN-β response after T1-44 treatment. Because both PRMT5 and E2F1 control a wide range of cellular processes, and RNA splicing would not only affect intron retention but other alternative splicing such as exon skipping, the significance of intron retention to the physiological effect of PRMT5 inhibition remains uncertain. If RIP-seq analysis of dsRNAs can identify some consensus sequences in the retained introns that are prone to form dsRNAs, such as Alu elements, maybe base editing targeting these consensus elements could ablate dsRNA formation in most of the retained introns upregulated by T1-44. Then it would be possible to compare the effect of T1-44 and see whether the loss of RI-derived

dsRNAs would have any physiological effect.

With the current understanding of the PRMT5-regulated dsRNA production, it still remains a challenge to effectively delineate the role of dsRNA production in the overall effect of PRMT5 inhibition.

4.3 Future directions

PRMT5 inhibition may affect RNA surveillance

One emerging question when delineating the dsRNA-IFN-β response, is how the retained introns evade the cellular quality control, such as nuclear retention and non-sense mediated decay. Possible answers include that PRMT5 controls expression of genes for RNA quality control, or they may be in the pool of alternatively spliced genes under PRMT5 inhibition.

The rMATS analysis is a powerful tool to uncover possible RNA splicing targets regulated by the PRMT5-E2F1 axis (Shen et al., 2014). From the rMATS data, several genes encoding proteins for RNA quality control were identified. For example, members of the DDX family were found in the RI upregulated genes when treated with T1-44, such as DDX19A, DDX23, DDX55, and DDX41. The DDX RNA helicase family are responsible for processes like RNA metabolism and remodelling (Cargill et al., 2021).Among the rMATS target genes, DDX19A regulates mRNA nuclear export, and DDX23 is a potential sensor for dsRNAs (Ruan et al., 2019; Folkmann et al., 2011). The perturbation of these pathways due to intron retention of DDX genes could impact subsequent sensing and degradation of alternatively spliced RNAs.

Other genes affected by T1-44 treatment also include NONO and EWSR1. One mechanism of

RNA surveillance in cells is through adenosine to inosine editing of dsRNAs by ADAR, and paraspeckles form within the nucleus to retain these dsRNAs from entering cytoplasm (Fox and Lamond, 2010). NONO and EWSR1 are components of the paraspeckle (Barutcu et al., 2022). If their functions are impaired by PRMT5 inhibition due to intron retention, it would be synergistic with the increase of dsRNA formation to result in elevated dsRNAs in the cytoplasm. Although the increase of intron retention in these genes will need to be validated using qPCR, they may provide explanations to how RI-derived dsRNAs bypass cellular surveillance. Testing the effect of intron retention on these genes may add on to the understanding of PRMT5's realm of control and bring causation when reduced RNA quality control is unwanted in PRMT5 inhibition.

Use of PRMT5 inhibitor in combination with other cancer immunotherapies

Since PRMT5 inhibition could activate the innate immune response pathway by IFN-β production, combining T1-44 with other anti-tumour immunotherapies may provide a more effective treatment strategy. An acute increase in IFN-β is known for its pro-apoptotic and anti-tumour properties (Manzella et al., 2016; Thapa et al., 2013; Taniguchi et al., 2003). Moreover, IFN-β treatments have been shown to enhance the efficacy of anti-PD-1/PD-L1 in melanoma by increasing T-cell infiltration (Uehara et al., 2017). Inhibiting PRMT5 could provide a targeted anti-tumour effect in cells overexpressing PRMT5 and produce IFN-β, which aids the action of immune checkpoint inhibitors. A similar concept was tested using ADAR1-null B16 melanoma model and CT26 colorectal cancer model. The loss of ADAR1, a dsRNA editor that decreases immunogenic dsRNA sensing, results in tumour inflammation and overcomes the resistance of tumours to PD-1 checkpoint blockade in an IFN sensing-dependent pathway (Ishizuka et al., 2019). This further supports the promise of using PRMT5 inhibitor in

combination with immune checkpoint inhibitors in immunologically “cold” tumours.

Additionally, our group has been developing cancer vaccines that enhance the adaptive immune response. One of our projects is developing vaccines from RI-encoded tumour antigens. Combining the vaccine with T1-44 treatment may therefore have a synergistic effect in enhancing both the innate and adaptive arms of the anti-tumour immune response. Previous findings indicate that T1-44 treatment increases T-cell infiltration in CT26 mouse models, but the activation of IFN-β production by T1-44 may affect a broader range of cells within the TME (Barczak et al., 2023). Given IFN-β's impact on various immune cells, including T-cells, NK cells, and neutrophils, future experiments could explore a wider array of TME cells through immunohistochemistry (Zhang, X. et al., 2021). A possible experiment may combine the cancer vaccine or an immune checkpoint inhibitor with T1-44 treatment, and use an IFNAR1 blocker to delineate the role of IFN-β. Such an experiment would reveal the potential benefits of T1-44 when used in combination with other immunotherapies to amplify anti-tumour effects.

Manipulating the dsRNA production in other diseases

It is important to understand the role of PRMT5-E2F1 in controlling dsRNA-activated IFN-β not only in a cancerous setting, but also in normal cells across different cell types. There has been a reported difference in baseline dsRNA levels across cell types, which could also be affecting the response to alterations in RNA splicing. For instance, neuron cells have a significantly higher baseline dsRNA level compared to other human cell types. This was proposed to be a mechanism of defence for neurons against viral infections as neuronal cells have limited regenerative ability (Dorrity et al., 2023). Extending from our current study, in other PRMT5 overexpressed cancer types like neuroblastoma and glioma, it is possible that

these cancer types have higher baseline dsRNA expression due to their close connection with neuronal cells (Han et al., 2014; Huang, L. et al., 2022; Bate-Eya et al., 2024). Therefore, in these tumours, PRMT5 inhibitor T1-44 also has the potential of activating the anti-viral pathway through RI-derived dsRNAs and may even have a better effect than in colorectal cancer. The dsRNA profile in different cancer types has not been systematically studied, and whether they would serve as a prediction for response to PRMT5 inhibition is a question to be answered in future studies.

Type I IFNs, especially IFN-β, have been used therapeutically in diseases beyond cancer. IFN-β treatment has also been tested in neurodegenerative diseases and may be effective in treating Alzheimer's disease (Farhangian et al., 2024).IFN-β is also an FDA-approved treatment against the autoimmune disease multiple sclerosis (MS). It can effectively control inflammatory response and prevent neuronal damage in MS patients (Interferons in the Treatment of Multiple Sclerosis, 2020). However, PRMT5's role in neurodegenerative diseases has not been well-studied (Motolani et al., 2021)Whether it would be possible to exploit the PRMT5-E2F1 pathway in activating IFN-β production while gaining beneficial transcriptional effects through PRMT5 inhibition needs to be researched further.

4.4 Conclusion

In conclusion, this study elucidated how PRMT5 can control the innate immune response, specifically IFN-β production, through an RNA splicing-dependent mechanism.

Corresponding to the aims, main findings include:

1. Treatment with the PRMT5 inhibitor T1-44 resulted in an increase in dsRNA and IFN-β levels in an E2F1 dependent manner both *in vitro* and *in vivo*.

2. PRMT5 inhibition using T1-44 increased intron retention, which is likely under E2F1 control. The expression of specific retained introns could give rise to dsRNAs, in turn activating IFN-β production,
3. Biochemically validate dsRNA-forming intronic sequences through IVT and dsRNA RIP still requires further optimisation.

Future research should clarify the dsRNA sensors and the pathway's physiological roles in non-cancer cells to deepen our understanding of PRMT5's regulatory scope. Consequently, PRMT5 inhibition by T1-44 shows promise as an anti-tumour therapy in combination with existing immunotherapies.

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