Protein Arginine Methyltransferase 5 (PRMT5) Inhibition Induces Lymphoma Cell Death through Reactivation of the Retinoblastoma Tumor Suppressor Pathway and Polycomb Repressor Complex 2 (PRC2) Silencing

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Background: PRMT5, PRC2, and cyclin D1 are overexpressed in non-Hodgkin lymphoma (NHL).

Results: PRMT5 expression inversely correlates with levels of hypophospho-RB1 and RBL2.

Conclusion: PRMT5 inhibition reactivates RB1 and RBL2 and silences PRC2 and cyclin D1.

Significance: PRMT5 inhibition results in NHL growth arrest and cell death.

Epigenetic regulation mediated by lysine- and arginine-specific enzymes plays an essential role in tumorigenesis, and enhanced expression of the type II prmt5 protein arginine methyltransferase PRMT5 as well as the polycomb repressor complex PRC2 has been associated with increased cell proliferation and survival. Here, we show that PRMT5 is overexpressed in three different types of non-Hodgkin lymphoma cell lines and clinical samples as well as in mouse primary lymphoma cells and that it up-regulates PRC2 expression through inactivation of the retinoblastoma proteins RB1 and RBL2. Although PRMT5 epigenetically controls RBL2 expression, it indirectly promotes RB1 phosphorylation through enhanced cyclin D1 expression. Furthermore, we demonstrate that PRMT5 knockdown in non-Hodgkin lymphoma cell lines and mouse primary lymphoma cells leads to RBL2 derepression and RB1 reactivation, which in turn inhibit PRC2 expression and trigger derepression of its CASP10, DAPI, HOXAS, and HRK pro-apoptotic target genes.

We also show that reduced PRMT5 expression leads to cyclin D1 transcriptional repression via loss of TP53K372 methylation, which results in decreased BCL3 expression and enhanced recruitment of NF-κB p52-HDAC1 repressor complexes to the cyclin D1 promoter. These findings indicate that PRMT5 is a master epigenetic regulator that governs expression of its own target genes and those regulated by PRC2 and that its inhibition could offer a promising therapeutic strategy for lymphoma patients.

During B cell development, several DNA recombination and modification events take place so that proper populations of pre-B cells are formed (1, 2). However, as B cells differentiate, aberrant DNA mutations and translocation may occur and lead to B cell transformation (3). Examples of B cell tumors at this stage of B cell development include mantle cell lymphoma (MCL), which represents 3–10% of non-Hodgkin lymphoma (NHL) derived from a subset of naïve pre-germininal center (GC) B cells located in primary lymphoid follicles or in the mantle region of secondary lymphoid follicles. The hallmark of this disease is the translocation (11;14)(q13;q32), which is detected in most if not all of cases and which juxtaposes the cyclin D1 gene at 11q13 to the immunoglobulin heavy chain locus at 14q32 (4). MCL is considered incurable, and affected patients often have a short median survival, highlighting the need for new therapeutic strategies (5, 6).

Upon encountering antigen, naïve B cells are activated and undergo GC reaction before they become mature B cells. During the GC reaction, B cells may transform into either follicular lymphoma or GC B cell-like diffuse large B cell lymphoma (GCB-DLBCL), and even after maturation, B cells remain susceptible to transformation, as shown in the case of activated B cell-like DLBCL (ABC-DLBCL) (2, 3). DLBCL is the most common NHL worldwide and has been divided by global gene expression profiling into three different types: GCB-DLBCL, ABC-DLBCL, and primary mediastinal B cell lymphoma (7, 8). Although most GCB-DLBCL patients can be cured with the anti-CD20 antibody rituximab combined with chemotherapy, >50% of ABC-DLBCL patients do not survive. Despite molecular profiling of MCL, GCB-DLBCL, and ABC-DLBCL, we still do not understand the molecular mechanisms that drive their initiation, maintenance, and progression.

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‡ The abbreviations used are: MCL, mantle cell lymphoma; NHL, non-Hodgkin lymphoma; GC, pre-germinial center; GCB-DLBCL, GC B cell-like diffuse large B cell lymphoma; ABC-DLBCL, activated B cell-like DLBCL; sh-GFP, GFP shRNA; RIPA, radioimmune precipitation assay; TMA, tissue microarray; TRITC, tetramethylrhodamine isothiocyanate.
It is well established that altered expression of oncogenes and/or tumor suppressor genes results in transformation of normal cells into malignant derivatives (9). Both activation and repression of target genes with growth regulatory functions have been reported in every cancer examined, and the mechanisms that lead to their dysregulation almost invariably involve epigenetic modification of chromatin (9–11). Although DNA methylation has clearly been implicated in gene silencing and tumorigenesis, methylation of histones at conserved lysine and arginine residues has also been shown to play an important role in cancer (12). Hypermethylation of histones H3R8 and H4R3 by the protein arginine methyltransferase PRMT5 has been documented in many cancers and shown to be associated with tumor cell growth and survival (13, 14). Similarly, increased levels of EZH2 (enhancer of zeste homolog 2), the catalytic subunit of the polycomb repressor complex PRC2, and its associated H3(Me3)K27 mark have been reported in both solid and lymphoid tumors (15, 16).

PRMT5 is a member of the protein arginine methyltransferase family of enzymes that can symmetrically methylate arginine residues in a variety of cellular proteins, including histones (13). A consequence of PRMT5-induced histones H3R8 and H4R3 is transcriptional repression of target tumor suppressor genes (17–19). PRC2 consists of four core subunits, EZH2, SUZ12 (suppressor of zeste 12 homolog), EED (embryonic ectoderm development), and RBBP4/7 (retinoblastoma binding protein 4/7), and is involved in transcriptional silencing of growth and developmentally regulated genes (20, 21). Previous work has shown that expression of EZH2 and EED is controlled by EZF transcription factors, which are known to be modulated by the retinoblastoma family of tumor suppressors (22, 23). More recently, work by our group has demonstrated that PRMT5 epigenetically silences RBL2, which can in turn potentiate EZF function and promote cell proliferation (18). Given these results and the fact that expression of PRMT5 and PRC2 is enhanced in a variety of cancer cells, we reasoned that through its ability to suppress RBL2 expression, PRMT5 might positively control PRC2 levels.

Using patient-derived cell lines from three different NHL cell types, we show that PRMT5 promotes PRC2 expression through transcriptional silencing of RBL2 and hyperphosphorylation of RB1. We also show that inhibition of PRMT5 by shRNA-mediated knockdown reactivates both RB1 and RBL2 tumor suppressors; restores recruitment of repressor complexes to the promoter regions of EZH2, SUZ12, and EED; and re-establishes expression of PRC2 pro-apoptotic target genes CASP10, DAP1 (death-associated protein 1), HOXAS, and HRK (Harakiri). The net outcome of these molecular changes is inhibition of NHL cell growth and induced cell death. Furthermore, we show that the levels of PRMT5, PRC2, and their epigenetic marks are elevated in MCL, GCB-DLBCL, and ABC-DLBCL human clinical samples and that both RB1 and RBL2 are inactivated in NHL patient samples. To substantiate our findings, we show that PRMT5 and PRC2 levels are elevated in mouse primary lymphoma cells and that PRMT5 knockdown re-establishes RB1/RBL2-E2F regulation, which in turn reduces PRC2 levels and derepresses DAP1, HOXAS, and HRK target genes. Taken together, these findings demonstrate the role played by PRMT5 in the control of NHL cell growth and survival.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Cell Infection—PRMT5 knockdown was achieved using lentiviral constructs that express two PRMT5-specific shRNAs (sh-PRMT5-1 and sh-PRMT5-2) as described previously (24). Briefly, plasmids pLenti×2DEST/sh-PRMT5-1, pLenti-CMV-GFP-DEST/sh-PRMT5-1, and pLenti×2DEST/sh-PRMT5-2 were constructed by first cloning double-stranded oligonucleotides that encode PRMT5-specific shRNAs into the entry vector pENTER/pTER+. Plasmids pLenti×2DEST/sh-PRMT5-1 and pLenti-CMV-GFP-DEST/sh-PRMT5-1 were constructed using sense-1 (5′-GATCCCC-CCTAGTGGAGATGCCTTATGTGTGCTGTCATCTACAGGGAATAGGACATCTAAGCTTACCTTGGCTTTTGGAAA-3′) and antisense-1 (5′-AGCCTTTTCCAAGCCCGTGGATGATCCCTATATCTTGGATGCTTGTCTC-3′). sh-PRMT5-1 and sh-PRMT5-2 were individually recombined into either the pLenti×2DEST or pLenti-CMV-GFP-DEST vector as described previously (24), and positive clones were identified by EcoRI digestion and confirmed by DNA sequencing. Next, plasmids pENTER/pTER+/sh-PRMT5-1 and pENTER/pTER+/sh-PRMT5-2 were used to infect NHL or E-DLBCL cell lines and mouse primary E-BRD2 lymphoma cells with lentivirus expressing either control GFP shRNA (sh-GFP) or sh-PRMT5-1 were plated in 6-well plates, and viable cells were counted every 2 days for 1 week using trypan blue.
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dye exclusion. To assess the effect of PRMT5 inhibition on cell death, $8 \times 10^5$ control sh-GFP or test sh-PRMT5-1 cells were seeded into 6-well plates, harvested 72 h later, and stained with anti-annexin V antibody and propidium iodide (BD Biosciences) as specified by the manufacturer before they were analyzed on a Beckman Coulter FC500 flow cytometer. To monitor growth of E\(\mu\)-BRD2 lymphoma cells in vivo, sublethally irradiized 8–9-week-old FVB female mice were injected intravenously with an equal number ($5 \times 10^6$) of GFP cell–sorted primary lymphoma cells infected with lentiviral particles that express GFP along with either control sh-GFP or sh-PRMT5-1. Mice were then monitored every day for the development of lymphoma and were killed at the first appearance of symptoms. All mouse experiments were performed in compliance with Federal and Institutional Animal Care and Use Committee regulations.

Real-time RT-PCR. ChIP, Western Blot, and Immunofluorescence Assays—Total RNA was isolated using TR1zol reagent, and reverse transcription was performed essentially as described previously (19, 24). To measure the mRNA levels of target genes, real-time PCR was carried out using the TaqMan system in a 10-\(\mu\)l reaction as described previously (17). The following primer sets and probes were used in real-time PCR analyses: PRMT5 (forward, 5'-TATGTGTGATCGCTGCACA-3'; reverse, 5'-TGGCTGAGGTTGAACAGG-3'; probe 31), RBL2 (forward, 5'-TTGTTGGTGTCTTCTTTATATAGGTCTG-3'; reverse, 5'-TTCCATATAAAGTCAAGAGC-3'; probe 62), EZH2 (forward, 5'-GAAAACCTGGTAAGGCCTA-3'; reverse, 5'-GGGAGACTATTCTTGATG-3'; probe 45), HOXA5 (forward, 5'-GGAGGACATCTTCTGTAGGGAAG-3'; reverse, 5'-ATCTGCAAGCTGTTCTCCCTG-3'; probe 16), EED (forward, 5'-ATCAATAATTTGCTTATTCTATCCTC-3'; reverse, 5'-ATTGATACCTAATCGCACTTAAAT-3'; probe 21), beta-(forward, 5'-GGTAGACGGCTCTGTGTGG-3'; reverse, 5'-GGGATGAATCAACCTCAAC-3'; probe 2), Casp10 (forward, 5'-GGGAAAAGGGCAGATAAAGCA-3'; reverse, 5'-TCAGAGCTGGTGAGCTCATGAT-3'; probe 18), DAP1 (forward, 5'-ACAGGGATTACAGGAGATTG-3'; reverse, 5'-TGACCCCGAAGATGAACAC-3'; probe 43), SUZ12 (forward, 5'-GGTAGACGGCTCTGTGTGG-3'; reverse, 5'-GCTGTCACCTGAGTGTCTGG-3'; probe 45), HRK (forward, 5'-CCTGAGGACATCGTA-GAAAC-3'; reverse, 5'-TGTGGTTCGAGCTGTTATATTCTT-3'; probe 60), CCND1 (forward, 5'-GAAGATCGTCTGCCAAGCCTG-3'; reverse, 5'-GACCCTCTCCTCCACACCTT-3'; probe 67), BCL3 (forward, 5'-ACTGCCTCTTGACCCACTC-3'; reverse, 5'-GGATGAGGTTGATGACCATGTG-3'; probe 62), TPS3 (forward, 5'-TCCACTCTGTGTGATGCACTC-3'; reverse, 5'-AAGACCCTAAACCCAAAATAGT-3'; probe 75), mouse Prmt5 (forward, 5'-GTCGACCTAGGACATGGTTTTTCTG-3'; reverse, 5'-GATGCTGGAACCTAGGACATGGTTTTTCTG-3'; probe 88), mouse Rbl2 (forward, 5'-TGCTGGTGCTTATATAGCTG-3'; reverse, 5'-GAAATTGACAGATCATCGCTAA-3'; probe 60), mouse Dap1 (forward, 5'-TCCAGCTCTCATGGGACTAC-3'; reverse, 5'-AAATTGGAGGACCATC-3'; probe 64), mouse Hrk (forward, 5'-ATGCTACCTTGTGCTGAGAAGT-3'; reverse, 5'-GGTGACTGCTGCGACCAGACAG-3'; probe 16), mouse Hoxa5 (forward, 5'-ACGGGCCACTACCGCTGACC-3'; reverse, 5'-GTAGGCGGTTGAATGTGAATCTTT-3'; probe 32), mouse Rbl1 (forward, 5'-GGGCGGAATACGCTCTAGA-3'; reverse, 5'-TGCGGCAAATGCAATATAAA-3'; probe 3), mouse Cnd1 (forward, 5'-CTCCTCTCCGACTTCGCT-3'; reverse, 5'-GAAGATTGTCGACCGAGTAGAGATGAAC-3'; probe 74), and mouse Bcl3 (forward, 5'-CTCGACTGCTGCTGACT-3'; reverse, 5'-GAACCAAGAGATCGTTCTTG-3'; probe 94). To normalize mRNA levels, levels of 18 S rRNA were measured in both control and test cell lines using 1X premixed 18 S primer/probe set (Applied Biosystems). To monitor recruitment to target genes, ChIP assays were performed using cross-linked chromatin from either normal or transformed B cells as described previously (19, 24). The following primer sets and probes were used in ChIP assays: RBL2 (forward, 5'-ATTTTTGCGCCCTTTGAAA-3'; reverse, 5'-GCACCCCGATGTTCTGAGCAC-3'; probe 3), EZH2 (forward, 5'-GGGAGAACAGACAAGAGG-3'; reverse, 5'-CGGTCCCTTGTAGTGATAGC-3'; probe 28), SUZ12 (forward, 5'-CGACGTGTTTGGAGGAGACC-3'; reverse, 5'-CGGTTCTTTCCAGTCCTG-3'; probe 19), EED (forward, 5'-GGTACTTTCCATTCGACCA-3'; reverse, 5'-TCCTTGAAGATAGAAATGCAAAAAC-3'; probe 38), CCND1 (forward, 5'-GGGGCTTTGATGAGCCACAGAA-3'; reverse, 5'-GGGCTATGCTGCTGACT-3'; probe 1), and BCL3 (forward, 5'-CTGATACCTAGGATCCAGTG-3'; reverse, 5'-GAGTACCGTCTCATGTTGGA-3'; probe 4).

To examine expression of PRMT5 and its downstream target genes, radioimmuneprecipitation assay (RIPA) extracts were prepared and analyzed by Western blot analysis as described previously (19, 27). When phospho-RB1 levels were measured, RIPA extracts were prepared in the presence of the following inhibitors: 10 \(\mu\)M beta-glycerophosphate, 1 \(\mu\)M NaF, and 50 \(\mu\)M NaF. Antibodies against PRMT5 and its epigenetic marks as well as SUZ12 have been described previously (17, 19, 28). Polyclonal antibodies against RB1, RBL1, RBL2, EZH2, EED, E2F1–4, E2F6, HADC1, HADC2, cyclin D1, CDK4, CDK6, CDKNA2/p16, CDKN1A/p21, HOXA5, HRK, BCL3, p300, and NF-kB p52 were purchased from Santa Cruz Biotechnology. Anti-EZH2, anti-caspase-10, anti-DAP1, anti-caspase-3, and anti-phospho-RB1 (Ser-780, Ser-795, and Ser-807/Ser-811) antibodies were purchased from Cell Signaling Technology, whereas anti-H3(Mej)K27, anti-TP53, and anti-methyl-TP53 antibodies were purchased from Abcam. Both anti-H3K9ac and anti-H3K14ac antibodies were purchased from EMD Milipore, and anti-beta-actin antibody was purchased from Sigma-Aldrich. Immunofluorescence experiments were performed as described previously (17).

Immunohistochemistry—To evaluate PRMT5 expression in NHL patient samples, formalin-fixed primary tumor samples collected from 53 MCL patients (34 common, 14 blastoid, and 5 pleomorphic histologic subtypes) and 62 DLBCL patients (29 GCB and 33 ABC histologic subtypes) were used. Studies that
examined expression of PRMT5, EZH2, and their associated epigenetic marks as well as RB1, phospho-RB1 (Ser-795), and RBL2 levels were performed on a second tissue microarray (TMA) generated from formalin-fixed, paraffin-embedded tissue samples corresponding to 16 MCL and 18 DLBCL cases, which were included in the original TMA studied for PRMT5 expression. The second TMA used in these experiments was constructed as reported previously (29). All cases were retrieved from the archives of the Hematopathology Unit, Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna. MCL cases were classified in the three morphologic variants according to the World Health Organization classification (4): nine common variant, four blastoid variant, and three pleomorphic variant. DLBCL samples were classified as GCB- and ABC-like according to the Hans algorithm (31): 10 GCB-DLBCL and 8 ABC-DLBCL. Paraffin-embedded sections (4-μm thick) were cut from TMA blocks, dewaxed, and submitted to antigen retrieval by heating in Dako PT Link (PT100/PT101, DakoCytomation, Glostrup, Denmark). Slides were placed in EnVision FLEX high pH target retrieval solution (K8004, DakoCytomation) at 92 °C for 5 min and then incubated at room temperature with the following antibodies: anti-PRMT5 (1:120 dilution), anti-H3(Me)R8 (1:180), anti-H4(Me)R3 (1:960), anti-EZH2 (1:120), anti-H3(Me)K27 (1:40), anti-RB1 (1:120), anti-phospho-RB1 (Ser-795; 1:30), and anti-RBL2 (1:120). All antibodies were diluted with Dako REAL antibody diluent (S2022, DakoCytomation). Immunostaining was performed using the Alkaline REAL alkaline phosphatase/RED rabbit/mouse detection system (K5005, DakoCytomation). Sections were counterstained with 2.5–8-fold (p < 10^{-5}) and that RBL2 mRNA levels were suppressed by 2–10-fold (p < 10^{-3}) in NHL cell lines (Fig. 1B).

Using normal B cells as a control and one representative cell line from each NHL cell type, we tested whether PRMT5 and its epigenetic marks can be detected at the RBL2 promoter (−85 to −16) (Fig. 1C). Both PRMT5 and its induced marks were enriched by 2.5–8-fold (p < 10^{-5}) at the RBL2 promoter region in all three NHL cell lines. We also analyzed the global levels of PRMT5, PRC2, and RBL2 by immunofluorescence (Fig. 1D). Consistent with the Western blot results, PRMT5, PRC2, and their associated marks were elevated in NHL cell lines. In contrast, RBL2 protein levels were high in normal B cells and undetectable in NHL cells. These observations suggest that PRMT5, through its ability to suppress RBL2, promotes PRC2 expression.

**RESULTS**

Expression of PRMT5 and PRC2 Inversely Correlates with RBL2 Levels—We previously showed that PRMT5 epigenetically silences RBL2 expression and that its knockdown inhibits lymphoma cell growth (17, 18). However, the molecular mechanisms by which PRMT5 promotes lymphoma cell growth and survival remain unclear. EZH2, the catalytic subunit of PRC2, has been shown to be highly expressed in metastatic prostate and breast cancer as well as in lymphoma (32–34). What is more interesting is that EZH2 is a downstream target gene of E2F transcription factors (23). To investigate the relationship between PRMT5, RBL2, and PRC2, we examined their expression in three different types of NHL cells, including pre-GCB MCL (Mino and JeKo), GCB-DLBCL (Pfeiffer and Toledo), and post-GCB ABC-DLBCL (SUDHL-2 and OCI-Ly3) (Fig. 1A). Both PRMT5 and PRC2 were overexpressed, whereas RBL2 levels were suppressed in NHL cells compared with normal resting or activated CD19+ B cells. In accord with these results, real-time RT-PCR revealed that the mRNA levels of PRC2 components were elevated by 1.6–5-fold (p < 10^{-5}) and that RBL2 mRNA levels were suppressed by 2–10-fold (p < 10^{-3}) in NHL cell lines (Fig. 1B).

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**PRMT5 Knockdown Results in RBL2 Derepression and PRC2 Silencing**—To determine whether PRMT5 can affect RBL2 and PRC2 expression, JeKo, Pfeiffer, and SUDHL-2 cells were infected with lentivirus that expresses either control sh-GFP or two PRMT5-specific shRNAs (sh-PRMT5-1 and sh-PRMT5-2), and total RNA and RIPA extracts were analyzed (Fig. 2). Both RNA and Western blot analyses showed that PRMT5 knockdown triggered RBL2 derepression and PRC2 silencing (Fig. 2, A and B). Because both PRMT5-specific shRNAs gave similar results, we performed further experiments using only sh-PRMT5-1. To further confirm our Western blot results, we measured the global levels of PRMT5, RBL2, and PRC2 by immunofluorescence (Fig. 2C). Our findings show that although PRMT5, PRC2, and their induced epigenetic marks were elevated, RBL2 expression was suppressed in control uninfected and sh-GFP-infected Pfeiffer cells. When PRMT5 was knocked down, RBL2 expression was restored, and PRC2 levels were decreased. Similar results were observed by immunofluorescence in JeKo and SUDHL-2 cells (data not shown). Furthermore, when we measured the proliferation of control sh-GFP- and sh-PRMT5-1-infected NHL cells, there was a clear inhibition of growth in cells in which PRMT5 was knocked down (Fig. 3A), which was accompanied by enhanced cell death as determined by increased annexin V staining (Fig. 3B) and caspase-3 activation (Fig. 3C). In light of these results, we tested whether PRMT5 knockdown could affect expression of PRC2 downstream target genes, which regulate cell death. On the basis of previously published ChIP-Seq data (35, 36), we selected four PRC2 pro-apoptotic target genes, including CASP10, an initiator caspase that activates CASP3 and CASP7 (37); DAP1, which acts as a positive mediator of apoptosis (38); HOXA5, a transcription factor silenced in >60% of breast carcinomas and whose re-expression induces apoptosis (39); and HRK, which activates cell death through inhibition of Bcl-2 and Bcl-xL (40). Real-time RT-PCR analysis showed that despite the fact that the kinetics of reactivation for PRC2 pro-apoptotic target genes varied within and between NHL cell lines, all four target genes were derepressed by 1.5–4-fold (p < 10^{-3}) in PRMT5 knockdown cells (Fig. 3D). Similar results were observed in JeKo and SUDHL-2 cells (data not shown). Importantly, tran-
criptional derepression correlated with increased CASP10, DAP1, HOXA5, and HRK protein expression (Fig. 3E). Collectively, these results indicate that PRMT5 promotes NHL cell growth and survival by indirectly suppressing expression of cell death-inducing genes.

Reduced PRMT5 Expression Restores Recruitment of RB1/RBL2 Inactivation

PRMT5 Up-regulates PRC2 via RB1/RBL2 Inactivation

**FIGURE 1.** Overexpressed PRMT5 epigenetically silences RBL2 and promotes PRC2 expression in patient-derived NHL cell lines. A, RIPA extracts (20 μg) from normal B (resting or activated), pre-GCB MCL (Mino and JeKo), GCB-DLBCL (Pfeiffer and Toledo), and post-GCB ABC-DLBCL (SUDHL-2 and OCI-Ly3) cells were analyzed by immunoblotting using the indicated antibodies. Anti-β-actin antibody was used as control. B, the levels of RBL2, EZH2, SUZ12, and EED mRNAs were measured in normal and transformed B cells by real-time RT-PCR using gene-specific primers and probe sets. This experiment was conducted three times in triplicate, and 18 S rRNA was used as internal control. C, ChIP experiments were performed on cross-linked chromatin from either normal or transformed B cells using preimmune (PI), anti-PRMT5, anti-H3(Me2)R8, and anti-H4(Me2)R3 antibodies. ChIP assays were carried out twice in triplicate. -Fold enrichment with each antibody was calculated relative to the preimmune sample. D, normal resting and transformed B cells were fixed and incubated with the indicated primary antibodies (Ab), and FITC-labeled goat anti-rabbit antibody was used to detect PRMT5, PRC2, and their marks as well as RBL2. DAPI was used to stain nuclei. Pictures were taken at 100 magnification, and data in each graph are represented as means ± S.D.
sought to assess the role of RB-E2F in PRC2 regulation in NHL cells. First, we examined the promoter regions of EZH2, SUZ12, and EED (−1 to +0.5 kilobase pairs) for the presence of E2F-binding sites using TESS software (41). Our analysis indicated that there are 15 and 17 E2F-binding sites in the EZH2 and SUZ12 promoters, respectively, whereas the EED promoter region contains only one E2F-binding site. Using antibodies that recognize individual E2F transcription factors, we performed ChIP assays using cross-linked chromatin from both normal and transformed B cells (Fig. 4A). Binding of E2F1–4 was enriched by 2–9-fold (p < 10−3) in the promoter region of PRC2 components in normal B cells; however, only E2F1, E2F3, and E2F4 showed increased binding in NHL cells in comparison with the preimmune control. We also examined the recruitment of pocket proteins and discovered that association of RB1 and RBL2 was enhanced by 2.5–6-fold (p < 10−3) in normal B cells but not in NHL cells. Because pocket proteins are known to repress E2F-driven genes through direct interaction with and recruitment of histone deacetylases (22), we also evaluated HDAC2 recruitment and acetylation of H3K9 and H3K14 in the promoter region of PRC2 subunits. Consistent with RB1 and RBL2 binding, recruitment of HDAC2 was increased by 2.5–5.5-fold (p < 10−3) in normal B cells but not in NHL cells, suggesting that deficiency in recruitment of RB1/RBL2-HDAC2 repressor complexes contributes to PRC2 up-regulation in NHL cells.

To evaluate the role of PRMT5 in PRC2 regulation, we knocked down its expression and monitored the recruitment of relevant E2F and RB proteins to the promoter region of PRC2 components in both control sh-GFP and PRMT5 knockdown Pfeiffer cells (Fig. 4B). Our results show that although there was no change in binding of E2Fs, there was a 2–4-fold (p < 10−3) enhancement of RB1 and RBL2 recruitment in PRMT5 knockdown cells compared with control cells. HDAC2 recruitment was also increased and was accompanied by H3K9/H3K14 deacetylation in the promoter region of PRC2 components in PRMT5 knockdown cells, highlighting the important role played by RB1/RBL2-HDAC2 repressor complexes in PRC2 regulation. Similar results were observed in JeKo and SUDHL-2 cells (data not shown). These results show that PRMT5 inhibition reactivates RB1/RBL2 and suppresses PRC2 expression.
PRMT5 Up-regulates Cyclin D1-CDK4/6 Proliferative Signaling in NHL Cells—A major growth regulatory network that is altered in various cancer cells is the cyclin D1-CDK4/6-RB pathway, which controls the E2F-driven transcriptome (42, 43). We have found by ChIP assay that RB1 recruitment is enhanced to the promoter region of PRC2 components in PRMT5 knockdown cells (Fig. 4), suggesting that RB1 has regained its transcriptional inhibitory activity, which may occur only if RB1 becomes hypophosphorylated. To test this hypothesis, we examined the relationship between PRMT5 and phospho-RB1 in NHL cells (Fig. 5A). We discovered that RB1 phosphorylation at multiple sites (Ser-780, Ser-795, and Ser-807/Ser-811) was more abundant in NHL cells than in normal B cells. We also analyzed expression of cyclin D1, CDK4, CDK6, and their inhibitors and found that despite increased expression of CDKN2A/p16 and or CDKN3/p21, RB1 was still hyperphosphorylated, suggesting that cyclin D1-CDK4/6 complexes are functional in NHL cells.

To determine whether PRMT5 is involved in promoting cyclin D1-CDK4/6 signaling, we inhibited its expression by shRNA-mediated knockdown in JeKo, Pfeiffer, and SUDHL-2 cells and monitored cyclin D1-CDK4/6 expression as well as RB1 phosphorylation (Fig. 5B). In all three NHL cell types, PRMT5 inhibition led to decreased cyclin D1 expression. In addition, we noticed a modest decrease in CDK6 levels, whereas CDK4 was unaffected. To measure the activity of cyclin D1-CDK4/6 complexes, we monitored the levels of phospho-RB1 using three distinct antibodies. In agreement with decreased cyclin D1 protein levels, RB1 phosphorylation was inhibited in PRMT5 knockdown NHL cell lines. These results suggest that cyclin D1-CDK4/6-mediated RB1 phosphorylation depends on the presence of PRMT5.
To shed more light on the mechanism by which PRMT5 inhibition triggers cyclin D1 down-regulation, we measured cyclin D1 mRNA and protein expression at different time points after PRMT5 knockdown (Fig. 5, C and D). Cyclin D1 mRNA levels began to drop as early as 36 h, whereas cyclin D1 protein expression decreased 48 h post-infection in knockdown Pfeiffer cells. Similar results were observed in JeKo and SUDHL-2 cells (data not shown). Prior work showed that the NF-κB p52-BCL3 complex drives cyclin D1 expression in H1299 and U2OS cells and that TP53-mediated inhibition of BCL3 results in cyclin D1 transcriptional repression (44). However, the mechanism by which TP53 represses BCL3 remains unknown. The same report also showed that loss of BCL3 expression promotes formation of NF-κB p52-HDAC1 complexes, which actively repress cyclin D1 transcription.

To investigate if cyclin D1 expression is regulated via a similar mechanism in NHL cells, we measured TP53 and BCL3 mRNA and protein expression before and after PRMT5 knockdown. We found that BCL3 transcription was inhibited 36 h post-infection and that this correlated with decreased BCL3 protein expression in knockdown Pfeiffer cells (Fig. 5, C and D). Decreased expression of BCL3 was also observed in knockdown SUDHL-2 cells (data not shown). However, because JeKo cells are TP53-deficient despite detectable TP53 mRNA levels (Ref. 45 and data not shown), we analyzed cyclin D1, TP53, and BCL3 mRNA and protein expression as well as RB1 phosphorylation in Mino cells infected with lentivirus that expresses either sh-GFP or sh-PRMT5-1 (data not shown). Consistent with our findings in Pfeiffer and SUDHL-2 cells, expression of cyclin D1 was inhibited in PRMT5 knockdown Mino cells, as was RB1 phosphorylation (data not shown). Moreover, BCL3 mRNA and protein expression was decreased 48 h post-infection (data not shown). Remarkably, TP53 mRNA and protein expression increased 36–48 h after PRMT5 knockdown in Pfeiffer and SUDHL-2 cells but not in Mino cells (data not shown), which are known to express high levels of TP53 (45), suggesting that TP53 is involved in BCL3 transcriptional repression in NHL cells.

To verify that TP53 is directly involved in BCL3 transcriptional repression, we monitored its recruitment to the BCL3 promoter in Pfeiffer cells (Fig. 5E) and in Mino and SUDHL-2 cells (data not shown). TP53 binding was enhanced by 2–4-fold (p < 10^{-5}) in both control sh-GFP and PRMT5 knockdown NHL cells, suggesting that BCL3 transcriptional repression does not depend on TP53 protein induction. Because TP53 is methylated at Lys-372 by KMT7 (SET7/9), and it is known that this modification enhances TP53 transcriptional activity (46), we measured its levels as well as presence at the BCL3 promoter before and after PRMT5 knockdown. In all three NHL cell lines, TP53K372 methylation was reduced 48–72 h post-infection (Fig. 5D), as was binding of Lys-372-methylated TP53 to the BCL3 promoter, which showed a maximal decline 60–72 h post-infection (Fig. 5E), indicating that loss of TP53K372 methylation correlates with decreased BCL3 promoter activity. We also monitored recruitment of p300 and HDAC2 and found that their binding correlated with TP53-mediated BCL3 transcriptional activation and repression, respectively.

Given that BCL3 down-regulation coincided with cyclin D1 suppression, we examined recruitment of NF-κB p52, BCL3, and HDAC1 to the cyclin D1 promoter in all three NHL cell types (Fig. 5F and data not shown). Our findings indicate that even though NF-κB p52 binding did not change over time,
BCL3 recruitment showed a steady decrease that started at 36 h and became maximal at 72 h post-infection in PRMT5 knockdown NHL cells. Concomitantly, HDAC1 recruitment to the cyclin D1 promoter increased gradually between 36 and 72 h post-infection. Collectively, these results suggest that PRMT5 promotes cyclin D1-CDK4/6 proliferative signaling through modulation of the TP53/NF-κB/p52/BCL3 pathway.

PRMT5 Overexpression Correlates with RB1/RBL2 Inactivation and Enhanced PRC2 Expression in NHL Patient Samples—Having established that PRMT5 is overexpressed in NHL cell lines and that it up-regulates PRC2 expression through inactivation of RB1 and RBL2, we wanted to verify our findings in NHL patient samples. First, we evaluated PRMT5 expression in primary lymphoma samples collected from patients with MCL...
(n = 53), GCB-DLBCL (n = 29), and ABC-DLBCL (n = 33) (Table 1). PRMT5 was abundantly expressed in the majority of MCL, with histologic subtypes showing 85% positivity in common MCL, 93% in blastoid variant tumors, and 100% in pleomorphic variant tumors. When we examined DLBCL samples, 97% of GCB-DLBCL and 100% of ABC-DLBCL showed PRMT5 overexpression.

We next explored the expression profile of PRMT5, EZH2, their associated marks, RB1, and RBL2 in primary tumor samples collected from patients with MCL, GCB-DLBCL, and ABC-DLBCL. However, because of limitation of tissue availability on most TMAs, these studies were performed on a newly constructed TMA as described previously (29). Both PRMT5 and its induced H3(Me2)R8 and H4(Me2)R3 marks were present in all cases of MCL, GCB-DLBCL, and ABC-DLBCL (Fig. 6 and Table 2). EZH2 and H3(Me3)K27 were strongly positive in 100% of MCL and ABC-DLBCL specimens. All GCB-DLBCL samples showed EZH2 expression, with 80% of the clinical samples showing global staining for H3(Me3)K27. Interestingly, all lymphoma histologic subtypes showed a diffuse and strong nuclear staining for RB1 and phospho-RB1 (Ser-795), which were detected in 88% of MCL samples and in 100% of GCB-DLBCL and ABC-DLBCL specimens. Consistent with our findings in patient-derived NHL cell lines, RBL2 was either silenced or weakly expressed in all primary tumor specimens. These results support our cell line data and further demonstrate the positive correlation between PRMT5, phospho-RB1, and PRC2 and the inverse correlation between PRMT5 and RBL2.

**PRMT5 Knockdown Results in Enhanced Mouse Primary Lymphoma Cell Death**—To demonstrate the relevance of PRMT5 in controlling lymphoma cell growth and survival, we examined its expression in mouse primary lymphoma cells derived from the Eμ-BRD2 transgenic mouse, which expresses BRD2 (double bromodomain-containing protein 2) under the control of a murine immunoglobulin heavy chain promoter-enhancer construct (26). A key feature of Eμ-BRD2 transgenic mice is that they develop an aggressive B cell lymphoma similar to human DLBCL as determined by transcriptome analysis (47). In light of our results, we reasoned that PRMT5 expression might be altered in Eμ-BRD2 primary lymphoma cells and that it might up-regulate PRC2 expression through inactivation of the RB-E2F pathway. Therefore, we examined expression of PRMT5 and its target genes in control CD19- B cells and Eμ-BRD2 primary lymphoma cells (Fig. 7, A and B). Real-time RT-PCR and Western blot analyses revealed that PRMT5 and PRC2 expression was elevated in primary lymphoma cells and inversely correlated with RBL2 levels. We also found a positive

### TABLE 1

| Lymphoma histology | Total cases | PRMT5-positive | PRMT5-negative |
|--------------------|-------------|----------------|----------------|
| MCL                |             |                |                |
| Common             | 34          | 29             | 5              |
| Blastoid           | 14          | 13             | 1              |
| Pleomorphic        | 5           | 5              | 0              |
| DLBCL              |             |                |                |
| GCB                | 29          | 28             | 1              |
| ABC                | 33          | 33             | 0              |

**TABLE 2**

| Lymphoma histology | PRMT5 | H3R8/me2 | H4R3/me2 | EZH2 | H3K27/me3 | RB1 | Phospho-RB1 | RBL2 |
|--------------------|-------|----------|----------|-------|-----------|-----|-------------|------|
| MCL                | 9/9   | 9/9      | 9/9      | 9/9   | 9/9       | 9/9 | 8/9         | 2/9  |
| Blastoid           | 4/4   | 4/4      | 4/4      | 4/4   | 4/4       | 4/4 | 3/4         | 0/4  |
| Pleomorphic        | 3/3   | 3/3      | 3/3      | 3/3   | 3/3       | 3/3 | 3/3         | 1/3  |
| DLBCL              |       |          |          |       |           |     |             |      |
| GCB                | 10/10 | 8/10     | 10/10    | 9/10  | 8/10      | 10/10| 10/10       | 5/9  |
| Post-GCB           | 8/8   | 8/8      | 7/8      | 6/8   | 8/8       | 8/8 | 8/8         | 1/8  |

* Faint positivity.
* Faint positivity in three cases.
correlation between enhanced cyclin D1 expression and RB1 hypophosphorylation. We also found that cyclin D1 expression was inhibited at the transcriptional level and correlated with BCL3 repression. More importantly, TP53K372 methylation was also decreased in PRMT5 knockdown primary lymphoma cells, suggesting that the mechanism of cyclin D1 regulation is conserved between human and mouse lymphoma cells. Consistent with decreased PRC2 levels, expression of DAP1, HOXA5, and HRK was increased in PRMT5 knockdown primary lymphoma cells, which is consistent with decreased PRC2 levels.

FIGURE 7. Expression of PRMT5, PRC2, and cyclin D1 is elevated in mouse primary lymphoma cells. A, real-time RT-PCR was performed on total RNA from normal B and Eμ-BRD2 cells, and the levels of the indicated target genes were determined as described in the legend to Fig. 1B. β-Actin mRNA levels were shown as a control. B, RIPA extracts (40 μg) were analyzed by immunoblotting using the indicated antibodies. Total RNA (C and E) and RIPA extracts (D and F) were collected 48 h after infection, and the levels of the indicated target genes and proteins were determined. G, Kaplan-Meier curve showing survival of mice injected with activated Eμ-BRD2 cells infected with either control sh-GFP-GFP or sh-PRMT5-1-GFP lentivirus. Eμ-BRD2 cells were activated for 24 h by adding recombinant human interleukin-4 (15 ng/ml) and goat anti-human IgG + IgM (15 μg/ml) before infection with control sh-GFP-GFP or sh-PRMT5-1-GFP lentivirus. GFP-positive control and test cells were purified by FACS analysis and injected into sublethally irradiated FVB mice. H, weight of spleens isolated from mice shown in G. I, activated Eμ-BRD2 lymphoma cells were infected with either control sh-GFP-GFP or sh-PRMT5-1-GFP lentivirus, stained with propidium iodide (PI) and annexin V 72 h later, and analyzed by FACS analysis as described in the legend to Fig. 3B. Activation of Eμ-BRD2 cells was carried out by adding 15 ng/ml recombinant human interleukin-4 and 15 μg/ml goat anti-human IgG + IgM. J, weights of individual mice (n = 11/group) injected with either sh-GFP-GFP- or sh-PRMT5-1-GFP-infected lymphoma cells were monitored for 2 weeks and normalized to one at day 0. The average weight for each group is plotted as means ± S.D.

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knockdown cells and was accompanied by enhanced cell death (Fig. 7, E, F, and J).

Because Ep-βBRD2 primary tumor cells induce an aggressive lymphoma that results in animal death within 2–3 weeks, we assessed the impact of PRMT5 knockdown on their growth and survival in vivo. Mouse primary lymphoma cells were infected with either control sh-GFP-GFP or sh-PRMT5-1-GFP lentivirus, and GFP-positive cells were purified 24–36 h post-infection by FACS analysis and immediately injected into sublethally irradiated FVB mice (Fig. 7G). The Kaplan-Meier survival curve showed that mice (n = 11) injected with control sh-GFP lymphoma cells succumbed to disease within 13–15 days and showed splenomegaly (Fig. 7H) as well as weight loss (Fig. 7J). In addition, necropsy of sick animals showed evidence of lymphoma in peripheral blood, bone marrow, lymph nodes, lungs, liver, kidneys, and pancreas (data not shown). In contrast, mice injected with sh-PRMT5-1-GFP lymphoma cells (n = 11) survived for >24 weeks without any clinical symptoms (p < 10⁻⁴). These results show that PRMT5 inhibition results in primary lymphoma cell growth arrest and death.

**DISCUSSION**

Understanding how cancer cells acquire the ability to survive and proliferate uncontrollably requires the discovery of mechanisms that contribute to aberrant expression of target genes that in turn adversely affect critical regulatory checkpoints. We have determined that PRMT5 is overexpressed in three different types of NHL cell lines and clinical samples and that it up-regulates PRC2 expression through inactivation of RB1 and RBL2. We have demonstrated that PRMT5 epigenetically silences RBL2 and indirectly contributes to RB1 inactivation by phosphorylation. Consequently, these events result in decreased recruitment of RB1/RBL2-HDAC2 repressor complexes to the EZH2, SUZ12, and EED promoters, augmenting PRC2 expression and function in NHL cells. We have shown that PRMT5 knockdown reactivates the RB1/RBL2-E2F tumor suppressor pathway and antagonizes cyclin D1-CDK4/6 signaling in both NHL cell lines and mouse primary lymphoma cells. Further investigation revealed that although the levels of PRMT5, PRC2, and their induced epigenetic marks are enhanced, both RB1 and RBL2 are inactivated in NHL clinical samples. In agreement with our findings in NHL cell lines and patient samples, we have shown that PRMT5 overexpression results in enhanced PRC2 and cyclin D1 levels in mouse primary lymphoma cells and that PRMT5 knockdown restores RB1 and RBL2 tumor suppressor activity, induces expression of pro-apoptotic target genes, and inhibits tumor cell growth and survival in an aggressive DLBCL mouse model.

Our findings show that reactivation of both RB1 and RBL2 restores transcriptional repression of EZH2, SUZ12, and EED promoters, which in turn brings about derepression of CASP10, DAP1, HOXA5, and HRK pro-apoptotic target genes and promotes lymphoma cell death. We have shown that the reason why RB1 becomes dephosphorylated is due primarily to decreased cyclin D1 expression, which depends on loss of TP53K372 methylation. Changes in methylation of TP53K372 probably affect its ability to interact with co-activator proteins and favors its association with co-repressors. In fact, our results support this notion because we have found that recruitment of TP53 to the BCL3 promoter was unaffected, whereas binding of methylated TP53K372 was inhibited after PRMT5 inhibition. Concomitantly, binding of p300 to the BCL3 promoter was reduced, and HDAC2 recruitment was increased. Previous work showed that the consequence of decreased BCL3 expression in H1299 or U2OS cells is formation and recruitment of NF-κB p52-HDAC1 repressor complexes to the cyclin D1 promoter (44). Our results support this model of cyclin D1 transcriptional repression as demonstrated by the gradual loss of BCL3 recruitment, persistent NF-κB p52 binding, and enrichment of HDAC1 at the cyclin D1 promoter in PRMT5 knockdown NHL cells.

One question that arises from these experiments is how PRMT5 inhibition triggers loss of TP53K372 methylation. It is known that TP53 is methylated at different sites by different lysine methyltransferases, including KMT7 and KMT3C (46, 48). In the same way that histones are demethylated by specific demethylases, TP53(Me3)K370 is demethylated by KDM1 (LSD1), an event that renders TP53 less active (49). It is tempting to speculate that PRMT5 controls TP53K372 methylation by suppressing expression of a currently unidentified TP53K372 demethylase. Upon PRMT5 inhibition, derepression of this demethylase would reduce the levels of transcriptionally active, methylated TP53K372 and trigger BCL3 repression. Another possibility is that PRMT5 may play an active role in the maintenance of KMT7 expression. In this case, PRMT5 knockdown would reduce KMT7 expression and de novo TP53K372 methylation. Currently, it is not clear if either or both scenarios are at work, but more investigation is required to determine the contribution of PRMT5 to post-translational regulation of TP53.

We have shown that PRMT5 knockdown results in cyclin D1 suppression and loss of RB1 phosphorylation in JeKo cells despite the fact that they lack TP53 expression. One interpretation of this result is that there are other mechanisms involved in regulating cyclin D1 expression, which are impacted by PRMT5 inhibition. In fact, cyclin D1 is known to be a direct target of the Wnt/β-catenin signaling pathway, which has recently been shown to be constitutively turned on in hepatocellular carcinoma cells through PRC2-mediated suppression of Wnt antagonists AXIN2, NDK1, PPP2R2B, PRICKLE1, and SFRP5 (50). Therefore, it is possible that through its ability to promote PRC2 expression, PRMT5 indirectly contributes to Wnt antagonist suppression and that PRMT5 knockdown could inhibit Wnt/β-catenin signaling, thereby causing cyclin D1 transcriptional repression. Another possible explanation is that PRMT5 epigenetically silences cyclin D1-specific microRNAs, which become derepressed upon PRMT5 knockdown and inhibit cyclin D1 expression. Evidence in support of this mechanism of cyclin D1 regulation comes from recent findings that show that miR-33 inhibits cyclin D1 and CDK6 expression in Huh7 and A549 cell lines (30), and our results showing that, in addition to miR-33, PRMT5 epigenetically silences two other cyclin D1-specific microRNAs, miR-96 and miR-507, in JeKo cells. Furthermore, ongoing studies in our

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laboratory have clearly shown that PRMT5 knockdown results in derepression of miR-33, miR-96, and miR-507. It is worth noting that both transcriptional and translational regulatory mechanisms may be at play in JeKo cells as well as in other NHL cell lines, and it is going to be important to investigate to what extent each regulatory step influences cyclin D1 expression in the context of PRMT5 inhibition.

Our findings show that PRMT5 inhibition in mouse primary lymphoma cells results in reactivation of the RB1/RBL2-E2F tumor suppressor pathway; suppression of the PRC2 complex; and derepression of DAPI, HOXA5, and HRK pro-apoptotic genes. Furthermore, we have determined that the same regulatory networks restored in patient-derived NHL cell lines are also reactivated in Eμ-BR2 primary lymphoma cells, as evidenced by loss of TP53K372 methylation and decreased expression of BCL3 and cyclin D1. Moreover, when we reduced PRMT5 expression in primary lymphoma cells and examined growth and survival in vivo, we discovered that transplant of PRMT5-specific shRNA-infected primary lymphoma cells into sublethally irradiated FVB mice resulted in significantly improved survival compared with the control group, establishing PRMT5 as an important molecular target and demonstrating that PRMT5 inhibition is a promising epigenetic therapy.

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REFERENCES

1. Küppers, R., Klein, U., Hansmann, M. L., and Rajewsky, K. (1999) Cellular origin of human B-cell lymphoma. Nature. Engl. J. Med. 341, 1520–1529
2. Stevenson, F. K., Sahota, S. S., Oettensmeier, C. H., Zhu, D., Forconi, F., and Hamblin, T. J. (2001) The occurrence and significance of V gene mutations in B cell-derived human malignancies. Adv. Cancer Res. 83, 81–116
3. Nogai, H., Dörken, B., and Lenz, G. (2011) Pathogenesis of non-Hodgkin’s lymphoma. J. Clin. Oncol. 29, 1803–1811
4. Swardlow, S. H., Campo, E., Seto, M., and Müller-Hermelink, H. K. (2008) Mantle cell lymphoma. in WHO Classification of Tumours of Haematopoietic and Lymphoid Tissues (Swardlow, S. H., Campo, E., Harris, N. L., Jaffe, E. S., Pilferi, S. A., Stein, H., Thiele, J., and Vardiman, J. W. eds) pp. 229–232, IARC Publishing Group, Lyon, France
5. Fisher, R. I., Dahlberg, S., and Nathwani, B. (1995) A clinical analysis of two indolent lymphoma entities: mantle cell lymphoma and marginal zone lymphoma (including the mucosa-associated lymphoid tissue and monocyteid B-cell subcategories): a Southwest Oncology Group Study. Blood 85, 1075–1082
6. Teodorovic, I., Pittaluga, S., and Kuin-Nelemans, J. C. (1995) Efficacy of four different regimens in 64 mantle-cell lymphoma cases: clinicopathologic comparison with 498 other non-Hodgkin’s lymphoma subtypes-European Organization for the Research and Treatment of Cancer Lymphoma Cooperative Group. J. Clin. Oncol. 13, 2819–2826
7. Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, Y., Yu, X., Powell, J. I., Wang, Y., Lander, G. E., Myers, G., Moore, T., Hudson, J., Jr., Lu, L., Lewis, D. B., Tibshirani, R., Sherlock, G., Chan, W. C., Greiner, T. C., Weisenburger, D. D., Armitage, J. O., Warnke, R., Levy, R., Wilson, W. H., Jaffe, E. S., Simon, R., Klaussner, R. D., Powell, J., Duffey, P. L., Longo, D. L., Greiner, T. C., Weisenburger, D. D., Sanger, W. G., Dave, B. J., Lynch, J. C., Yose, J. Armitage, J. O., Montserrat, E., López-Guillermo, A., Grogn, T. M., Müller, T. P., LeBlanc, M., Ott, G., Kvaloy, S., Delabie, J., Holte, H., Krajci, P., Stokke, T., and Staudt, L. M. (2002) The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. N. Engl. J. Med. 346, 1937–1947
8. Hanahan, D., and Weinberg, R. (2011) Hallmarks of cancer: the next generation. Cell 144, 646–674
9. Baylin, S. B., and Jones, P. A. (2011) A decade of exploring the cancer epigenome—biological and translational implications. Nat. Rev. Cancer 11, 726–734
10. Bracken, A. P., and Helin, K. (2009) Polycomb group proteins: navigators of lineage pathways led astray in cancer. Nat. Rev. Cancer 9, 773–784
11. Chi, P., Allis, C. D., and Wang, G. G. (2010) Covalent histone modifications—misswritten, misinterpreted and mis-erased in human cancers. Nat. Rev. Cancer 10, 457–469
12. Füllgrabe, J., Kavanagh, E., and Joseph, B. (2011) Histone onco-modifications. Oncogene 30, 3291–3300
13. Karkhanis, V., Hu, Y. J., Baiocchi, R. A., Imbalzano, A. N., and Sif, S. (2011) Versatility of PRMT5-induced methylation in growth control and development. Trends Biochem. Sci. 36, 633–641
14. Yang, Y., and Bedford, M. T. (2013) Protein arginine methyltransferases and cancer. Nat. Rev. Cancer 13, 37–50
15. Chase, A., and Cross, N. C. (2011) Alterations of EZH2 in cancer. Clin. Cancer Res. 17, 2613–2618
16. Pal, S., Baiocchi, R. A., Byrd, J. C., Grefer, M. R., Jacob, S. T., and Sif, S. (2007) Low levels of miR-92b/96 induce PRMT5 translation and H3R8/H4R3 methylation in mantle cell lymphoma. EMBO J. 26, 3585–3569
17. Wang, L., Pal, S., and Sif, S. (2008) Protein arginine methyltransferase 5 suppresses the transcription of the RB family of tumor suppressors in leukemia and lymphoma cells. Mol. Cell. Biol. 28, 6626–6677
18. Tae, S., Karkhanis, V., Velasco, K., Yaneva, M., Erdjument-Bromage, H., Tempst, P., and Sif, S. (2011) Bromodomain protein 7 interacts with PRMT5 and PRC2, and is involved in transcriptional repression of their target genes. Nucleic Acids Res. 39, 5424–5438
19. Simon, J. A., and Kingston, R. E. (2009) Mechanisms of Polycomb gene silencing: knowns and unknowns. Nat. Rev. Mol. Cell Biol. 10, 697–708
20. Margueron, R., and Reinberg, D. (2011) The Polycomb complex PRC2 and its mark in life. Nature 469, 343–349
21. Harbour, J. W., and Dean, D. C. (2000) The Rb/E2F pathway: expanding roles and emerging paradigms. Genes Dev. 14, 2393–2409
22. Bracken, A. P., Pasini, D., Capra, M., Prosperini, E., Colli, E., and Helin, K. (2003) Margueron. EMBO J. 22, 5323–5335
23. Karkhanis, V., Wang, L., Tae, S., Hu, Y. J., Imbalzano, A. N., and Sif, S. (2012) Protein arginine methyltransferase 7 regulates cellular response to DNA damage by methylating promoter histones H2A and H4 of the polymerase δ catalytic subunit gene. POLI1. J. Biol. Chem. 287, 29801–29814
24. Campeau, E., Ruhl, V. E., Rodier, F., Smith, C. L., Rahmberg, B. L., Fuss, J. O., Campisi, J., Yaswen, P., Cooper, P. K., and Kaufman, P. D. (2009) A versatile viral system for expression and depletion of proteins in mammalian cells. PLoS ONE 4, e6529
25. Greenwald, R. J., Tumang, J. R., Sinha, A., Currier, N., Cardiff, R. D., Rothstein, T. L., Faller, D. V., and Denis, G. V. (2004) Eμ-BR2 transgenic mice develop B-cell lymphoma and leukemia. Blood 103, 1475–1484
26. Wang, L., Baiocchi, R. A., Pal, S., Mosialos, G., Caligiuri, M., and Sif, S. (2005) The BRG1- and hBRM-associated factor BAF57 induces apoptosis by stimulating expression of the cylindromatosis tumor suppressor gene. Mol. Cell. Biol. 25, 7953–7965
27. Pal, S., Vishwanath, S. N., Erdjument-Bromage, H., Tempst, P., and Sif, S. (2004) Human SWI/SNF-associated PRMT5 methylates histone H3 arginine 9 and negatively regulates expression of ST7 and NM23 tumor suppressor genes. Mol. Cell Biol. 24, 9630–9645
28. Agostinelli, C., Paterson, J. C., Gupta, R., Righi, S., Sandri, F., Piccaluga, P. P., Bacci, F., Sabattini, E., Pilferi, S. A., and Marafioti T. (2012) Detection of LIM domain only 2 (LMO2) in normal human tissues and haematopoietic and non-haematopoietic tumours using a newly developed rabbit monoclonal antibody. Histopathology 61, 33–46
PRMT5 Up-regulates PRC2 via RB1/RBL2 Inactivation

30. Cirera-Salinas, D., Pauta, M., Allen, R. M., Salerno, A. G., Ramírez, C. M., Chamorro-Jorganes, A., Wanschel, A. C., Lasuncion, M. A., Morales-Ruiz, M., Suarez, Y., Baldan, Á., Espugues, E., and Fernández-Hernando, C. (2012) Mir-33 regulates cell proliferation and cell cycle progression. Cell Cycle 11, 922–933

31. Hans, C. P., Weisenburger, D. D., Greiner, T. C., Gascoyne, R. D., Delabie, J., Ott, G., Müller-Hermelink, H. K., Campo, E., Braziel, R. M., Jaffe, E. S., Pan, Z., Farinha, P., Smith, L. M., Falini, B., Banham, A. H., Rosenwald, A., Staudt, L. M., Connors, J. M., Armitage, J. O., and Chan, W. C. (2004) Confirmation of the molecular classification of diffuse large B-cell lymphoma by immunohistochemistry using tissue microarrays. Blood 103, 275–282

32. Pietersen, A. M., Horlings, H. M., Hauptmann, M., Langerød, A., Ajouaou, A., Cornelissen-Steiger, P., Wessels, L. F., Jonkers, J., van de Vijver, M. J., and van Lohuizen, M. (2008) EZH2 and BMI1 inversely correlate with prognosis and TP53 mutation in breast cancer. Breast Cancer Res. 10, R109

33. Varambally, S., Dhanasekaran, S. M., Zhou, M., Barrette, T. R., Kumar-Sinha, C., Sanga, M. G., Ghosh, D., Pienta, K. J., Sewalt, R. G., Otte, A. P., Rubin, M. A., and Chinnaiyan, A. M. (2002) The polycomb group protein EZH2 is involved in progression of prostate cancer. Nature 419, 624–629

34. Visser, H. P., Gunster, M. J., Kluij-Nelemans, H. C., Manders, E. M., Raaphorst, F. M., Meijer, C. J., Willemze, R., and Otte, A. P. (2001) The Polycomb group protein EZH2 is upregulated in proliferating, cultured human mantle cell lymphoma. Br. J. Haematol. 112, 950–958

35. Kirmizis, A., Bartley, S. M., Kuzmichev, A., Margueron, R., Reinberg, D., Green, R., and Farnham, P. J. (2004) Silencing of human polycomb target genes is associated with methylation of histone H3 Lys 27. Genes Dev. 18, 1592–1605

36. Bracken, A. P., Dietrich, N., Pasini, D., Hans, K. H., and Helin, K. (2006) Genome-wide mapping of Polycomb target genes unravels their roles in cell fate transitions. Genes Dev. 20, 1123–1136

37. Wang, J., Chun, H. J., Wong, W., Spencer, D. M., and Lenardo, M. J. (2001) Caspase-10 is an initiator caspase in death receptor signaling. Proc. Natl. Acad. Sci. U.S.A. 98, 13884–13888

38. Dey, I. P., Reavill, E., Berissi, H., Cohen, O., and Kimchi, A. (1995) Identification of a novel serine/threonine kinase and a novel 15-kD protein as potential mediators of the γ interferon-induced cell death. Gene Dev. 9, 15–30

39. Chen, H., Chung, S., and Sukumar, S. (2004) HOXA5-induced apoptosis in breast cancer cells in mediated by caspases 2 and 8. Mol. Cell. Biol. 24, 924–935

40. Inohara, N., Ding, L., Chen, S., and Núñez, G. (1997) harakiri, a novel regulator of cell death, encodes a protein that activates apoptosis and interacts selectively with survival-promoting proteins Bcl-2 and Bcl-XL. EMBO J. 16, 1686–1694

41. Schug, J. (2008) Using TESS to predict transcription factor binding sites in the DNA sequence. Curr. Protoc. Bioinformatics 21:2.6.1–2.6.15 10.1002/0471250953.bias02621

42. Giacinti, C., and Giordano, A. (2006) RB and cell cycle progression. Oncogene 25, 5220–5227

43. Kang, J. K., and Diehl, J. A. (2009) Nuclear cyclin D1: an oncogenic driver in human cancer. J. Cell. Physiol. 220, 292–296

44. Rocha, S., Martin, A. M., Meek, D. W., and Perkins, N. D. (2003) p53 represses cyclin D1 transcription through down regulation of Bcl-3 and inducing association of the p52 NF-κB subunit with histone deacetylase 1. Mol. Cell. Biol. 23, 4713–4727

45. Amin, H. M., McDonnell, T. J., Medeiros, L. J., Rassidakis, G. Z., Leventaki, V., O’Connor, S. L., Keating, M. J., and Lai, R. (2003) Characterization of 4 mantle cell lymphoma cell lines. Arch. Pathol. Lab. Med. 127, 424–431

46. Chuikov, S., Kurash, J. K., Wilson, J. R., Xiao, B., Justin, N., Ivanov, G. S., McKinney, K., Tempst, P., Prives, C., Gamblin, S. J., Barle, N. A., and Reinberg, D. (2004) Regulation of p53 activity through lysine methylation. Nature 432, 353–360

47. Lenburg, M. E., Sinha, A., Faller, D. V., and Denis, G. V. (2007) Tumor-specific and proliferation-specific gene expression typifies murine transgenic B cell lymphomagenesis. J. Biol. Chem. 282, 4803–4811

48. Huang, J., Perez-Burgos, L., Placek, B. J., Sengupta, R., Richter, M., Dorsey, J. A., Kubicek, S., Opravil, S., Jenuwein, T., and Berger, S. L. (2009) Repression of p53 activity by Smyd2-mediated methylation. Nature 444, 629–632

49. Huang, J., Sengupta, R., Espejo, A. B., Lee, M. S., Feng, H., Ching, A. K., Cheung, K. F., Wong, H. K., Tong, J. H., Jin, H., Choy, K. W., Yu, J., To, K. F., Wong, N., Huang, T. H., and Sung, J. J. (2011) EZH2-mediated concordant repression of Wnt antagonists promotes β-catenin-dependent hepatocarcinogenesis. Cancer Res. 71, 4028–4039