Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia

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Imatinib-insensitive leukemia stem cells (LSCs) are believed to be responsible for resistance to BCR-ABL tyrosine kinase inhibitors and relapse of chronic myelogenous leukemia (CML). Identifying therapeutic targets to eradicate CML LSCs may be a strategy to cure CML. In the present study, we discovered a positive feedback loop between BCR-ABL and protein arginine methyltransferase 5 (PRMT5) in CML cells. Overexpression of *PRMT5* was observed in human CML LSCs. Silencing *PRMT5* with shRNA or blocking PRMT5 methyltransferase activity with the small-molecule inhibitor PJ-68 reduced survival, serial replating capacity, and long-term culture-initiating cells (LTC-ICs) in LSCs from CML patients. Further, *PRMT5* knockdown or PJ-68 treatment dramatically prolonged survival in a murine model of retroviral BCR-ABL–driven CML and impaired the in vivo self-renewal capacity of transplanted CML LSCs. PJ-68 also inhibited long-term engraftment of human CML CD34+ cells in immunodeficient mice. Moreover, inhibition of PRMT5 abrogated the Wnt/β-catenin pathway in CML CD34+ cells by depleting dishevelled homolog 3 (DVL3). This study suggests that epigenetic methylation modification on histone protein arginine residues is a regulatory mechanism to control self-renewal of LSCs and indicates that PRMT5 may represent a potential therapeutic target against LSCs.

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Targeting methyltransferase PRMT5 eliminates leukemia stem cells in chronic myelogenous leukemia

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Introduction

Chronic myelogenous leukemia (CML) is a disease of hematopoietic stem cells (HSCs) malignantly transformed by the formation of the Philadelphia chromosome (i.e., BCR-ABL fusion gene) due to reciprocal chromosomal translocation t(9,22)(q34;q11) (1). CML is characterized by malignant expansion of myeloid leukemia cells in bone marrow (BM) and peripheral blood circulation (2). Patients with CML usually experience 3 clinical phases: chronic phase (CP), when BCR-ABL is usually the only driver of the disease; accelerated phase (AP); and blast phase/crisis (BP), when additional oncogenic factors are involved and the disease may clinically resemble acute leukemia (1). Consequently, patients with CP-CML respond well to the tyrosine kinase inhibitor (TKI) imatinib mesylate (IM), whereas patients with AP- and BP-CML usually show IM resistance and CML relapse (2, 3).

Acquired resistance to IM accounts for approximately 40%–50% of resistance cases and is mainly due to mutations in the BCR-ABL gene (e.g., T315I, G250E, Q252H, Y253H, and E255K/V) (3, 4). The drug resistance caused by most of the point mutations in BCR-ABL may be conquered with the second generation (e.g., nilotinib and dasatinib) and the third generation (e.g., ponatinib) of TKIs (5, 6). The evolution of BCR-ABL–independent leukemia clones is the second mechanism to render IM resistance (3, 7). Some CML patients show primary resistance to IM. Adult CML patients in AP and BP and 30% of BCR-ABL–positive pediatric patients with acute lymphoblastic leukemia intrinsically fail to respond to the current TKIs, including IM (8). The evolutionary course from CP to BP usually features additional oncogenic hits, which suggests a switch of the disease driver from BCR-ABL to other drivers or formation of a co-driver complex consisting of multiple oncogenic proteins (9). In such settings, the appearance of BCR-ABL–independent clones may confer resistance to IM and other TKIs (10). The evolutionary pressure to form BCR-ABL-independent leukemia clones may become augmented with long-term IM therapy. Identifying and targeting these additional oncogenic proteins may overcome resistance to IM.

Leukemia stem cells (LSCs) are thought to be an important source of IM resistance, including both primary and acquired resistance (11–13). LSCs possess the properties of rarity, quiescence, self-renewal, and reduced differentiation (11, 12, 14, 15). LSCs maintain their pool size via self-renewal but produce a hierarchy consisting of different stages of leukemic blast cells (10). In addition, the BCR-ABL–independent property of LSCs facilitates their insensitivity to IM (16). This ineffectiveness is supported by long-term follow-up clinical trials of IM in CML showing persistence of LSCs even in patients with undetectable levels of BCR-ABL transcripts during IM therapy and nearly inevitable relapse upon withdrawal of IM (14). Obviously, the cure for CML depends on elimination of the LSCs. Unfortunately, a curative approach to eliminate LSCs and then reconstituting the hematopoietic system with normal HSC transplantation can be performed in only a small number of patients and is accompanied by high risks of morbidity...
ity and mortality (10). Therefore, a curative approach for CML should ultimately involve identifying therapeutic targets against LSCs and rationally designing novel small-molecule compounds against specific targets to eradicate LSCs.

LSCs are regulated by multiple mechanisms (17). At the basal level, the fate of LSCs is regulated by survival/apoptosis regulators (e.g., BCL2, BIRC5 [survivin], MCL1) (18). At the second level, the self-renewal capacity of LSCs is regulated by multiple types of proteins: signaling pathways related to HSC development (e.g., Wnt/β-catenin, Hedgehog) (13), metabolism regulators (e.g., ALOX5, SCD) (19), transcription factors (e.g., FOXO3, Hif-1α), and epigenetic regulators (e.g., SIRT1) (15). At the third level, LSCs are tightly regulated by the malignant hematopoietic microenvironment in CML (20). Targeting epigenetic regulators has recently shown promise for eliminating LSCs while sparing normal HSCs (11, 16). Histone deacetylase (HDAC) inhibitors alone or in combination with TKIs can eliminate quiescent LSCs in both CML and AML (11, 16). Besides histone acetylation, methylation of arginine residues of histone proteins is another major mechanism of epigenetic regulation catalyzed by protein arginine methyltransferases and has been linked to leukemogenesis with cancer stem cell–associated (CSC-associated) proteins (21).

Protein arginine methyltransferase 5 (PRMT5), a type II arginine methyltransferase, can catalyze the transfer of a methyl group to 2 of 3 guanidino nitrogen atoms within the arginine molecule in the presence of the cofactor molecule S-adenosyl-l-methionine (22). The substrates of PRMT5 include histone and non-histone proteins (e.g., p53). Symmetric dimethylation in histone proteins H4 (S2Me-H4R3) and H3 (S2Me-H3R8) by PRMT5 may remodel the chromatin structure to repress transcription (22). In addition to epigenetic functions, PRMT5 may modify non-histone proteins to regulate various signaling networks (23). PRMT5 is associated with the development of a number of malignancies, such as acute myeloid leukemia, glioblastoma, lymphoma, and lung and ovarian cancer (23, 24). In 5-methylthioadenosine phosphorylase–deficient (MTAP-deficient) cancer cells, PRMT5 expression and activity are preferentially required for cell growth, indicating that PRMT5 is an attractive therapeutic target in cancer with these genetic alterations (25, 26). PRMT5 is essential for maintaining pluripotency in both embryonic and neural stem cells (27, 28). Recently, it has been reported that PRMT5 is critical for sustaining normal hematopoiesis, and its deletion leads to loss of functional hematopoietic stem and progenitor cells (29). However, its role in regulating CSC self-renewal or elimination of CSCs remains unclear.

During a screening for protein targets that regulate LSCs, we discovered a reciprocal positive regulation between PRMT5 and BCR-ABL and the overexpression of PRMT5 in CML LSCs. The objective of this study was to functionally validate PRMT5 as a therapeutic target and to test the effectiveness of specifically targeting PRMT5 by lentiviral shRNA or using a small-molecule inhibitor to eliminate LSCs. Our findings revealed that epigenetic modification by histone arginine methylation is critical for self-renewal of LSCs, and that therapeutic targeting of PRMT5 holds promise to eliminate LSCs for treatment of CML.

Results

**PRMT5 is highly overexpressed in primary CD34+ cells from CML patients.** We first compared PRMT5 expression in CD34+ cells from CML patients and normal BM (NBM) from healthy donors. The intracellular level of PRMT5 protein was significantly higher in CML CD34+ cells than NBM CD34+ cells (Figure 1A). The protein levels of PRMT5 and its histone methylation marks H4R3SDM and H3R8SDM were significantly increased in CML CD34+ cells as determined by Western blot analysis (Figure 1B). The mRNA level of PRMT5 was also greater in CML CD34+ cells than NBM CD34+ cells (Figure 1C). Further, the mRNA level of PRMT5 was markedly elevated in CML CD34+CD38- cells as compared with CD34+CD38+ cells (Figure 1D) and normal counterparts (Supplemental Figure 1A, GSE47927; supplemental material available online with this article; doi:10.1172/JCI8529D). PRMT5 expression is increased by BCR-ABL during malignant transformation of human normal CD34+ cells. Because CML is believed to be derived from malignant transformation of HSCs triggered by the BCR-ABL fusion oncogene (1), we next determined whether PRMT5 was increased during BCR-ABL-mediated malignant transformation of normal CD34+ cells. Purified NBM CD34+ cells were transduced with BCR-ABL retroviral MIGR1 (p210) or empty vector (V). The results demonstrated that the protein level of PRMT5 was upregulated, and the BCR-ABL downstream signaling molecules STAT5, STAT3, and CRKL were activated following the transduction of BCR-ABL in NBM CD34+ cells. The activity of PRMT5, as measured by its specific methylation mark, symmetrical dimethylation of histone H2A arginine 3 (H2AR3SDM), was also increased by BCR-ABL (Figure 1E).

**Activation of PRMT5 transcriptional expression by BCR-ABL fusion gene.** To further confirm the positive regulatory effect of BCR-ABL on PRMT5 expression, we transfected 293T cells with vector or pSG5-BCR-ABL (p210) constructs for 48 hours. We
observed that forced overexpression of BCR-ABL increased the endogenous PRMT5 protein level (Figure 1F). Intriguingly, transfection with pSG5-BCR-ABL (p190) but not HA-tagged BCR or pSG5-ABL upregulated endogenous PRMT5 at both the protein (Figure 1F) and mRNA levels (Supplemental Figure 1B). Thus, it is the BCR-ABL fusion genes, but not BCR or ABL, that upregulate PRMT5 expression.

We next examined whether depletion of BCR-ABL decreased PRMT5 expression. When IM-sensitive CML KBM5 and K562 cells were exposed to escalating concentrations of IM, PRMT5 protein levels were decreased in an IM concentration–dependent manner (Figure 1G and H, left). In contrast, PRMT5 protein levels were not altered by treatment of IM-resistant KBM5-T315I and uveal melanoma Mel270 cells (a control cell line not harboring BCR-ABL) with IM at concentrations that abrogated BCR-ABL tyrosine kinase activity (Figure 1G and H, right). Further, the mRNA levels of PRMT5 were decreased in CD34+CD38+ and CD34+CD38- cell populations after treatment with IM (Figure 1G). Thus, pharmacological inhibition of BCR-ABL activity suppresses PRMT5 expression in CML cells.

Because endogenous PRMT5 proteins are distributed in both the cytoplasmic and nuclear compartments, we further examined the effect of IM on the subcellular distribution of PRMT5 in CML cells. We found that the level of PRMT5 was decreased in both the cytosolic and nuclear fractions following IM treatment (Supplemental Figure 1C). Immunofluorescence microscopy confirmed the decreased expression of PRMT5 in both the cytosolic and nuclear compartments (Supplemental Figure 1D) in IM-treated cells.

To further confirm the dependence of PRMT5 expression on BCR-ABL, we silenced BCR-ABL expression in K562 and in primary CD34+ cells from CML patients by transduction of lentiviral shRNA against BCR-ABL. The results showed that silencing BCR-ABL depleted PRMT5 in K562 cells and in human CML CD34+ cells (Figure 1, J and K). Therefore, BCR-ABL positively regulated PRMT5 expression in these cells.

PRMT5 expression might be regulated at various levels, such as protein phosphorylation, mRNA stability, and transcription (30, 31). We found that blocking BCR-ABL kinase activity by IM had minimal effect on both the protein and mRNA stability of PRMT5 (data not shown) but significantly decreased PRMT5 mRNA levels in K562 cells (Figure 1L). In contrast, forced overexpression of BCR-ABL increased PRMT5 mRNA levels (Supplemental Figure 1B).

BCR-ABL-mediated PRMT5 transcriptional expression is STAT5 dependent. Given that the transcription factor STAT5, a key BCR-ABL downstream signaling molecule, is essential for BCR-ABL transformation (32), we searched for and discovered 2 putative conserved STAT5 binding sites in the PRMT5 gene promoter. Using ChIP assays, we found that STAT5A and STAT5B were recruited to the promoter of PRMT5 (Figure 1M). Further, treating K562 cells with IM abrogated the recruitment of STAT5A and STAT5B (Figure 1M). To further define the role of STAT5 in PRMT5 gene transcription, we knocked down STAT5 using shRNA against human STAT5A and STAT5B in K562 cells and then evaluated the expression of PRMT5. Knockdown of STAT5A and STAT5B expression greatly decreased the protein and mRNA levels of PRMT5 (Figure 1N and data not shown). These data suggest that BCR-ABL mediates PRMT5 transcriptional expression in a STAT5-dependent manner.

BCR-ABL expression depends on PRMT5 in CML cells. To investigate the functional role of PRMT5 in CML, we transfected K562 cells with PRMT5 construct and collected single clones stably overexpressing PRMT5 (#16 and #19). The BCR-ABL protein level was greatly increased in the PRMT5-overexpressing cells (Figure 2A), suggesting that PRMT5 promotes BCR-ABL expression. We also determined whether PRMT5 worked in the setting of T315I BCR-ABL. Ectopic overexpression of PRMT5 in KBM5 cells harboring T315I BCR-ABL elicited an increased level of endogenous T315I BCR-ABL (Supplemental Figure 2, A and B).

Because the conserved arginine residue (R368) is essential for the methyltransferase activity of PRMT5, a catalytically dead PRMT5 was created by mutating the amino acid arginine 368 to alanine (R368A) (24). K562 cells were electrotansfected with pCMV-2B-Flag-PRMT5 or mutant pCMV-2B-Flag-PRMT5 (R368A). PRMT5 upregulated both protein and mRNA levels of BCR-ABL, which was dependent on intact methyltransferase activity of PRMT5 (Figure 2, B and C). To further explore the mechanism of BCR-ABL gene transcription by PRMT5, we transiently cotransfected 293T cells with the BCR-ABL promoter reporter construct and the pCMV-2B-Flag-PRMT5 or mutant pCMV-2B-Flag-PRMT5 (R368A) for 24 hours. PRMT5 increased BCR-ABL promoter reporter activity, which was also dependent on its methyltransferase activity (Figure 2D).

Next, we examined the effect of PRMT5 knockdown on BCR-ABL expression. K562 cells were transduced with pLKO.1-GFP-shRNA or pLKO.1-GFP-shPRMT5 lentivirus (Scramble, shPRMT5 #1 and shPRMT5 #2, respectively). Both the protein and mRNA levels of BCR-ABL were
decreased in the PRMT5 stable knockdown cells (Figure 2, E and F). Similarly, PRMT5 knockdown by shRNA in KBM5-T315I cells decreased both the protein and mRNA levels of BCR-ABL (Supplemental Figure 2, C and D).

To conveniently perform in vitro and in vivo experiments involving the interference of PRMT5 function, we screened and identified a potent small molecule inhibitor of PRMT5 designated PJ-68 (chemical structure shown in Figure 2G). To gain an understanding of binding of PJ-68 to PRMT5, we performed molecular docking of PJ-68 to PRMT5 (Supplemental Figure 2E). The total score was 9.0382 (rank -1.6543). It has been shown that Glu499 and Glu508 located on a hairpin loop connecting β4 and α of (double-E) loop are required for the enzymatic activity of PRMT5 (33). In addition, Phe379 might be important for PRMT’s catalytic activity (33). These residues in the binding pocket were at 3 Å distance from PJ-68, illustrating the electronic/hydrophobic/ aromatic stacking interactions with the compound. Using an in vitro methyltransferase activity assay, we showed that PJ-68 inhibited the methyltransferase activity of PRMT5 with an IC50 of 517 nM (Figure 2H). PJ-68 possessed no inhibitory activity against type I (PRMT1, 3, 4, 6, and 8) PRMT family members, indicating its specificity toward PRMT5 (Figure 2H). PJ-68 –treated K562 (PRMT1, 3, 4, 6, and 8) PRMT family members, indicating its specificity toward PRMT5 (Figure 2H). PJ-68 possessed no inhibitory activity against type I PRMT (33).

Figure 3. PRMT5 knockdown by lentiviral shRNA reduces growth, survival, and colony formation in human CML CD34+ cells. Human CML CD34+ (A, C, E, and G) versus NBM CD34+ (n = 3 each) (B, D, F, and H) cells were transduced with control shRNA (Scramble), shPRMT5 #1, or shPRMT5 #2 for 48 hours, then treated with IM (2.5 μM) for 24 hours. (A and B) Western blot analysis of PRMT5 and BCR-ABL. (C and D) Cell viability was determined by MTS assay (Promega). (E and F) Apoptosis was detected by flow cytometry after dual staining CML and NBM CD34+ cells with annexin V-FITC and anti-CD38-PE. (G and H) The same number of Scramble, shPRMT5 #1, or shPRMT5 #2 in CML and NBM CD34+ cells (5,000 cells/well) were seeded in methylcellulose medium (H4434). Colonies were counted at 14 days. (I and J) Forty-eight hours after 2 rounds of transduction with control shRNA (Scramble), shPRMT5 #1, or shPRMT5 #2 lentivirus, the human CML CD34+ cells (n = 3) were exposed to PJ-68 at 25 μM for another 24 hours, and cell numbers (I) and CFC formation (J) were determined. *P < 0.05, **P < 0.01, ***P < 0.0001, 1-way ANOVA, post-hoc intergroup comparisons, Tukey’s test.

PRMT5 knockdown alone or in combination with IM significantly inhibited the cell viability of CML but not of NBM CD34+ cells (Figure 3, C and D). PRMT5 knockdown also significantly induced apoptosis in CML CD34+ CD38− cells (Figure 3E) but not in NBM CD34+CD38− cells (Figure 3F). This effect of PRMT5 knockdown on CML CD34+ cells was augmented with combined IM treatment. PRMT5 knockdown alone reduced CML colony-forming cells (CFCs) in the methylcellulose medium, which was further potentiated with IM combination (Figure 3G). In contrast, PRMT5 knockdown inhibited NBM CFC growth to a lesser extent (Figure 3H). Thus, PRMT5 knockdown reduced CML CD34+ cell survival and growth, and these effects were augmented with IM combination. Additionally, combination treatments of the human CML CD34+ cells with PRMT5 shRNA and PJ-68 provoked a further reduction in cell growth colony-forming abilities (Figure 3, I and J).

PRMT5 knockdown inhibits in vivo growth of LSCs and prolongs survival of CML mice. In order to define the in vivo role of PRMT5 in CML LSCs, we used a mouse CML model with BM transduction and transplantation (36). C57BL/6 donor BM cells primed by 5-fluorouracil (5-FU) were transduced with retroviral MSCV-IRES-EGFP carrying p210 BCR-ABL. The transduced cells were transplanted into sublethally irradiated recipient mice, which developed CML in -2 weeks. LSCs in BM and spleen of such CML mice would produce leukemia if they were intravenously transplanted into the secondary irradiated recipient mice (Figure 4A).

We knocked down PRMT5 by lentiviral shRNA in splenic GFP+ cells collected from the first generation of CML mice (Figure 4B) and then transplanted the modified splenic cells into sublethally irradiated (550 cGy) C57BL/6 recipient mice. The mice then received IM or vehicle for 2 weeks. PRMT5 knockdown alone or with IM significantly inhibited the splenomegaly of CML mice (Figure 4C). PRMT5 knockdown alone or with IM significantly prolonged the survival of CML mice (Figure 4D). The populations of GFP− (BCR-ABL− expressing leukemia cells) and myeloid cells (Gr-1−Mac-1+) in BM were significantly reduced in the PRMT5− knockdown group and further reduced with IM treatment (Supplemental Figure 3, A and B). Flow cytometry analysis revealed that PRMT5 knockdown alone or with IM significantly decreased LSK (Lin Sca−1−c−Kit+ cells), LT-HSCs (LSK Flt3−CD150+CD48−), and ST-HSCs (LSK Flt3−CD150−CD48+) in BM of CML mice (Figure 4, E−H). The percentages of granulocyte-macrophage progenitors (GMPs, Lin Sca−1−c−Kit−CD34−FcγRII/III+), and common myeloid progenitors (CMPs, Lin Sca−1−c−Kit−CD34−FcγRII/III+ or Lin Sca−1−c−Kit−CD34−FcγRII/III+) were dramatically decreased in BM of CML mice (Supplemental Figure 3, C−E). Similar results were obtained in the splenic cells of CML mice (Supplemental Figure 3, F−I).

In a separate set of experiments, we examined the in vivo effect of PRMT5 knockdown in the LSK cell populations of CML mice.
ed them into the secondary irradiated recipient C57BL/6 mice. Our results showed that knockdown of \textit{PRMT5} in the sorted LSK cells significantly prolonged the survival of the secondary recipients. The median survival for control versus shPRMT5-transduced mice was 40 days compared to 80 days for control, indicating a prolongation of survival.

**Figure 4.** \textit{PRMT5} knockdown inhibits LSC growth in the CML mouse model. (A) Schematic strategy of evaluation of the in vivo effect of \textit{PRMT5} knockdown on LSCs in the CML mouse model. (B) The effect of \textit{PRMT5} knockdown in spleen nucleated cells from CML mice was confirmed by Western blot analysis. (C) A representative photograph of spleens from each group. (D) Kaplan-Meier survival curves (n = 8 per group). *P = 0.0142 (Control versus IM), **P = 0.0029 (Control versus shPRMT5), ***P = 0.0259 (Control versus shPRMT5+IM), log-rank test. (E-H) Analysis of LSKs in BM cells of CML mice that received treatment with shPRMT5 ± IM. Control, n = 10; IM, n = 8; shPRMT5, n = 6; shPRMT5+IM, n = 6. (E) Schema for analysis of LSK cells, LT-HSCs, and ST-HSCs. Results for the GFP+ population in BM: LSK cells (F), LT-HSCs (G), and ST-HSCs (H). **P < 0.01, ***P < 0.0001, compared with control; #P < 0.05, ##P < 0.01, shPRMT5 compared with shPRMT5+IM, 1-way ANOVA, post hoc intergroup comparisons, Tukey’s test. (I) A representative photograph of spleens from each group. (J) Kaplan-Meier survival curves (n = 8 per group). *P = 0.0142 (Control versus IM), **P = 0.0029 (Control versus shPRMT5), ***P = 0.0259 (Control versus shPRMT5+IM), log-rank test.

We sorted the murine LSCs (LSK) from the nucleated cells of BM and spleen of the primary-generation CML mice by flow cytometry, and then transduced such LSK cells with lentiviral shRNA against murine \textit{PRMT5} for 48 hours and subsequently transplanted them into the secondary irradiated recipient C57BL/6 mice. Our results showed that knockdown of \textit{PRMT5} in the sorted LSK cells significantly prolonged the survival of the secondary recipients. The median survival for control versus shPRMT5-transduc-
ed cells was 9.5 versus 22 days ($P = 0.0001$, log-rank test) (Figure 4I). These results together suggest that silencing PRMT5 inhibits in vivo growth of CML LSCs and sensitizes them to IM treatment.

**Pharmacological inhibition of PRMT5 reduces survival and self-renewal capacity of CML CD34+ cells.** Next, we determined the effect of PJ-68 on survival of CML CD34+ cells and normal CD34+ cells. CML CD34+ cells or NBM CD34+ cells were incubated with PJ-68 (25.0 μM) in the presence or absence of IM (2.5 μM) for 24 hours. PRMT5 methyltransferase activity was decreased in CML CD34+ cells and NBM CD34+ cells, as indicated by the H2AR3SDM level after PJ-68 treatment alone or with IM (Figure 5A and Supplemental Figure 4A). In parallel, flow cytometry analysis of apoptosis based on dual staining with annexin V-FITC and anti-CD38-phycocerythrin (anti-CD38–PE) antibodies revealed that PJ-68 treatment increased the apoptosis of CML CD34+ cells (Figure 5, B and C) but had minimal effect on NBM CD34+ cells (Supplemental Figure 4, B and C). To further demonstrate the effectiveness of pharmacological inhibition of PRMT5 in quiescent CML LSCs, we labeled CML CD34+CD38– cells or NBM CD34+ cells with CFSE in the presence of PJ-68 (25.0 μM) for 96 hours. With PJ-68 treatment, apoptosis was significantly increased in quiescent CML LSCs (Figure 5D) versus in NBM CD34+ cells (Supplemental Figure 4D).

Given that self-renewal capacity is an important trait of LSCs, we determined whether inhibition of PRMT5 by PJ-68 impaired the self-renewal ability of CML stem cells by CFC/replating and long-term culture-initiating cell (LTC-IC) assay. Human CML CD34+ cells and NBM CD34+ cells were treated with PJ-68 for 24 hours and subjected to 14-day serially plating CFC culture in drug-free methylcellulose medium. With PJ-68 treatment, CFC formation and replating ability was decreased more in CML than in NBM CD34+ cells (Figure 5E and Supplemental Figure 4E). Similarly, the decrease in the number of LTC-IC–derived colony-forming units was greater in CML BM cells (Figure 5F) than in NBM cells (Supplemental Figure 4F). Notably, combination of PJ-68 with IM significantly enhanced CML LTC-IC growth inhibition as compared with either agent alone (Figure 5F).

**PRMT5 modulates the expression of cell cycle regulators in CML CD34+ cells.** Western blot analysis showed that PRMT5 knockdown or pharmacological inhibition of PRMT5 activity with PJ-68 increased p15INK4B and p27KIP1 but not p16INK4A and p57KIP2 expression (Supplemental Figure 5, A and B). Similarly, PRMT5 knockdown or pharmacological inhibition of PRMT5 activity increased p15INK4B mRNA levels in K562 cells (Supplemental Figure 5, C and D) and in CML CD34+ cells (Supplemental Figure 5, E and F). The p15INK4B gene is a negative regulator that prevents cell-cycle progression at the G1/S transition (37). Because PRMT5 specifically catalyzes H4R3SDM, which can recruit DNA methyltransferase DNMT3A to promoter regions of target genes to suppress gene expression (38), we determined whether PRMT5 mediated transcriptional repression of p15INK4B by H4R3SDM and recruitment of DNMT3A at the p15INK4B promoter. ChIP assay showed enrichment of PRMT5 at the p15INK4B promoter, and PRMT5 knockdown by shRNA or inhibition by PJ-68 led to decreased enrichment of PRMT5 at the p15INK4B promoter (Supplemental Figure 5, G and H). Further, PRMT5 knockdown inhibited the enrichment of H4R3SDM (Supplemental Figure 5I) and DNMT3A (Supplemental Figure 5J) at the promoter of p15INK4B in K562 cells. Consistent with this finding, the mRNA level of p15INK4B was dramatically increased following treatment with the DNMT3A inhibitor SGI-1027 in K562 cells and CML CD34+ cells (Supplemental Figure 5, K and L). These results suggest that PRMT5 may modulate the expression of cell cycle regulators including p15INK4B gene by affecting histone arginine methylation.

**Pharmacological inhibition of PRMT5 reduces growth of CML LSCs and prolongs survival of CML mice.** To recapitulate the effect of pharmacological inhibition of PRMT5 on CML in vivo, we used the aforementioned BM transduction/transplantation (BMT) CML mouse model (36). The CML mice were then treated with PJ-68 (25 mg/kg/d and 50 mg/kg/d, i.p.), IM (100 mg/kg/d, p.o.), or both (Figure 6A). Vehicle-treated control mice died of CML-like disease approximately 2 weeks after BMT, manifesting splenomegaly (Supplemental Figure 6A), elevated leukocyte counts predominated by myeloid leukemia (GFP+) cells, and lung hemorrhage. We next examined the bulk leukemia cells and stem/progenitor cells in CML mice. Splenomegaly was not observed in the mice that received PJ-68 or both PJ-68 and IM (Supplemental Figure 6A). PJ-68– or IM-treated CML mice showed significantly prolonged survival, and PJ-68 plus IM further extended survival as compared with each agent alone (Figure 6B). The proportion of BCR-ABL–expressing (GFP+) leukemia cells and myeloid cells (Gr-1+Mac-1+) in BM cells was significantly reduced in the PJ-68–treated CML mice; combining PJ-68 with IM further reduced these cell populations (Figure 6, C and D).

PJ-68 alone or in combination with IM, but not IM alone, markedly decreased the proportion of LSK cells, LT-HSCs, and ST-HSCs (Figure 6, E–G), as well as GMP and CMP in BM cells (Figure 6, H and I). Similar results were obtained in spleen cells (Supplemental Figure 6, B–J).

Pharmacokinetic (PK) study showed that PJ-68 $C_{\text{max}}$ values were $3.57 \pm 0.08 \mu$M (1,264.4 ± 28.4 ng/ml) and $7.88 \pm 0.31 \mu$M (2,788.5 ± 109.7 ng/ml), respectively, after a single oral or intravenous administration in SD rats (Supplemental Figure 6K and Supplemental Table 1). Such plasma concentrations of PJ-68 are postulated to be sufficient to block the histone methyltransferase activity of PRMT5 in vitro with an IC$_{50}$ value of 517 nM (Figure 2H).

**Pharmacological inhibition of PRMT5 activity decreases the frequency of in vivo CML LSCs.** To further analyze the frequency of CML LSCs subjected to treatments, we used a limiting dilution assay of BM cells in the secondary transplantation (Figure 7A). Treatment with PJ-68 alone or in combination with IM greatly decreased the engraftment of GFP+ cells and the frequency of CML stem cells in the secondary recipients at 16 weeks after BMT (Figure 7, B and C, and Table 1).

**PRMT5 inhibition reduces long-term multilineage engraftment of human CML CD34+ cells in NSI mice.** We also determined the effect of ex vivo treatment with PJ-68 in human CML CD34+ cells on their ability to be engrafted in NOD-scid IL2rg$^{-/-}$ (NSI) mice (Figure 8A). PJ-68 treatment reduced the engraftment of human CML CD45+ cells in BM (Figure 8, B and C) and spleen (Figure 8D) at 12 weeks after transplantation. The engrafted human CML CD45+ cells sorted by flow cytometry from NSI murine BM cells expressed BCR-ABL, and PJ-68 treatment markedly decreased BCR-ABL mRNA level (Figure 8E). The proportion of engrafted...
Figure 5. Pharmacological inhibition of PRMT5 activity induces apoptosis and suppresses self-renewal of human CML CD34+ cells. (A) Human CML CD34+ and CD14+ myeloid cells in NSI murine BM cells was decreased following PJ-68 treatment, (F and G). Thus, PJ-68 treatment may selectively target human CML CD34+ cells with engraftment capacity in vivo.

PRMT5 activates Wnt/β-catenin signaling in CML CD34+ cells. Canonical Wnt signaling is activated by binding of Wnt proteins to their respective cell surface receptors Frizzled and LRP6 on HSCs, which leads to activation and nuclear translocation of β-catenin, complex formation with TCF4, and transcription of target genes (39). β-Catenin is a critical protein controlling the self-renewal capacity of CML LSCs (13). We wondered whether PRMT5 inhibition blocked the Wnt/β-catenin pathway in CML CD34+ cells. We found that PRMT5 knockdown reduced the protein levels of β-catenin, dishevelled homolog 3 (DVL3, an upstream positive regulator of β-catenin), and target genes (e.g., cyclin D1, MYC, and LEF1) of β-catenin in K562 cells (Figure 9A). Similar results were obtained in PJ-68–treated K562 and primary human CML CD34+ cells (Figure 9, B and C). In contrast, PRMT5 overexpression activated the Wnt/β-catenin pathway in CML cells (Figure 9D). The intracellular activated β-catenin level in the PRMT5 knockdown or PJ-68–treated K562 cells was detected by fluorescence-tagged antibody followed by flow cytometry; loss of PRMT5 function significantly reduced intracellular activated β-catenin level (Figure 9E and Supplemental Figure 7A).

Next, we determined whether PRMT5 inhibition disrupted the interaction between β-catenin and TCF4. Co-immunoprecipitation (Co-IP) experiments showed that PRMT5 inhibition reduced β-catenin and TCF4 binding in the cells with PRMT5 stable knockdown (Figure 9G). Quantitative real-time PCR (qRT-PCR) analysis showed that PRMT5 knockdown reduced expression of the Wnt target genes AXIN2, MYC, LEF1, and CCND1 (Supplemental Figure 7D). Conversely, PRMT5 overexpression increased expression of these Wnt target genes (Supplemental Figure 7E).

Because the DVL3 but not DVL1 and DVL2 was reduced by PRMT5 inhibition (Figure 9, A–C and data not shown), whereas overexpression of PRMT5 increased the DVL3 level (Figure 9D), we explored the mechanism by which PRMT5 positively affected DVL3 level. K562 cells were pretreated with the proteasome inhibitor MG132 (1.0 μM) for 2 hours, followed by PJ-68 (15.0 μM or 20.0 μM) treatment for 24 hours. MG132 did not prevent PJ-68–mediated reduction of DVL3 protein (Supplemental Figure 7F). By contrast, PRMT5 activated DVL3 gene transcription, and PJ-68 treatment greatly inhibited the DVL3 mRNA level in K562 cells (Figure 9, H and I). Furthermore, we set out to examine whether the promoter of DVL3 was epigenetically regulated by PRMT5. Given that PRMT5 mediates symmetric dimethylation of histone H3R2 (H3R2SDM) and recruits WDR5 to promote H3K4 methylation and gene activation (40, 41), we determined the level of H3R2SDM and found that it was significantly decreased after PRMT5 knockdown or PJ-68 treatment in K562 cells (Figure 9J) or CML CD34+ cells (Supplemental Figure 7G). Further, ChIP experiments with antibodies specific for PRMT5, WDR5, H3R2SDM, and H3K4me3 revealed a significant enrichment of PRMT5 and its epigenetic marks at the DVL3 gene promoter, while knockdown of PRMT5 led to loss of recruitment of PRMT5 and its epigenetic marks on the DVL3 gene promoter in K562 cells (Figure 9K). These data indicate that the DVL3 gene promoter may be epigenetically regulated by PRMT5. Hence, PRMT5 may activate Wnt/β-catenin signaling via transcriptional regulation of DVL3 in CML cells.

**Discussion**

LSCs are insensitive to IM and are seeds of resistance to TKI therapy and relapse in CML. Little is known about the key regulatory proteins and effective therapeutic targets for self-renewal of LSCs. Here, we discovered a reciprocal positive feedback regulation between PRMT5 and BCR-ABL. BCR-ABL activated PRMT5 transcriptional expression via STAT5 during malignant transformation in normal CD34+ cells. PRMT5 upregulated BCR-ABL expression through mIIR-203 in CML cells. PRMT5 was overexpressed in human CML CD34+ cells. Knockdown of PRMT5 by a specific shRNA or pharmacological inhibition of PRMT5 enzyme activity by PJ-68 treatment reduced survival, serially replicating capacity, and LTC-IC in human LSCs. Human CML CD34+ cells were treated with PJ-68, IM, or a combination for 24 hours, and Western blot analysis of protein levels of PRMT5 and its arginine methylation mark H2AR3SDM was performed. Representative flow cytometry plots for apoptosis in human CML CD34+ cells were plated in methylcellulose medium. Colonies were counted on day 14. The cells were harvested and counted, 1,000 cells were replated for the second and third rounds, and colonies were counted on day 14 after each round. Pharmacological inhibition of PRMT5 activity lessened the LTC-IC capacity of CML LSCs. Human CML CD34+ cells (n = 3) were treated with 2 different concentrations of PJ-68 for 24 hours, washed with PBS, and counted; 1,000 cells were plated in methylcellulose medium. Colonies were counted on day 14. The cells were harvested and counted. 1,000 cells were replated for the second and third rounds, and colonies were counted on day 14 after each round. Pharmacological inhibition of PRMT5 activity lessened the LTC-IC capacity of CML LSCs. Two to 10 human CML cells (n = 3) were seeded in LTC-IC medium onto irradiated M2-10B4 cells and treated with PJ-68 (25.0 μM) with or without IM (2.5 μM) for 1 week, and cells were cultured for 5 more weeks in LTC-IC medium with weekly half drug-free medium changes. After 6 weeks, cells were harvested and plated into MethoCult H4435. LTC-IC–derived colonies were counted after 14 days. *P < 0.05, **P < 0.0001, 1-way ANOVA, post hoc intergroup comparisons, Tukey’s test.

**Figure 5.** PRMT5 activates Wnt/β-catenin signaling in CML CD34+ cells. (A) Human CML CD34+ and CD14+ myeloid cells in NSI murine BM cells was decreased following PJ-68 treatment, (F and G). Thus, PJ-68 treatment may selectively target human CML CD34+ cells with engraftment capacity in vivo. (B) Sort human CML CD34+CD38– cells (n = 3) labeled with CFSE, then cultured with PJ-68 ± IM for 96 hours, and apoptotic cells were analyzed by flow cytometry after labeling with annexin V–PE. (C) Pharmacological inhibition of PRMT5 activity lessened the serial replating capacity of CML LSCs. Human CML CD34+ cells (n = 3) were treated with 2 different concentrations of PJ-68 for 24 hours, washed with PBS, and counted; 1,000 cells were plated in methylcellulose medium. Colonies were counted on day 14. The cells were harvested and counted, 1,000 cells were replated for the second and third rounds, and colonies were counted on day 14 after each round. (D) Pharmacological inhibition of PRMT5 activity lessened the LTC-IC capacity of CML LSCs. 2 × 106 human CML cells (n = 3) were seeded in LTC-IC medium onto irradiated M2-10B4 cells and treated with PJ-68 (25.0 μM) with or without IM (2.5 μM) for 1 week, and cells were cultured for 5 more weeks in LTC-IC medium with weekly half drug-free medium changes. After 6 weeks, cells were harvested and plated into MethoCult H4435. LTC-IC–derived colonies were counted after 14 days. *P < 0.05, **P < 0.0001, 1-way ANOVA, post hoc intergroup comparisons, Tukey’s test.
Figure 6. Pharmacological inhibition of PRMT5 activity reduces growth of CML stem cells in mice. (A) A schematic strategy for testing the efficacy of PJ-68 in combination with IM in a CML mouse model. (B) Kaplan-Meier survival curves of CML mice treated with IM, PJ-68, or their combination. Control (n = 10) versus IM (n = 16), ***P = 0.0003; control versus PJ-68 (25 mg/kg) (n = 8), ***P = 0.0010; control versus PJ-68 (50 mg/kg) (n = 8), ***P = 0.0002; control versus PJ-68 (50 mg/kg)+IM (n = 8), ***P < 0.0001, log-rank test. (C-G) Bulk leukemia cells and stem/progenitor cells in BM were analyzed by flow cytometry. Control (n = 9), IM (n = 8), PJ-68 (25 mg/kg) (n = 8), PJ-68 (50 mg/kg)+IM (n = 8). Results for the GFP+ population in the BM are shown: LSK cells (E), LT-HSCs (F), and ST-HSCs (G). (H) GMPs and (I) CMPs in the BM. *P < 0.05, **P < 0.01, ***P < 0.0001, compared with control; *P < 0.05, **P < 0.01, PJ-68 compared with PJ-68+IM, 1-way ANOVA, post hoc intergroup comparisons, Tukey’s test.
CML stem cells. Similarly, inhibition of PRMT5 prolonged the survival of CML mice and reduced the growth of CML LSCs in mice. In addition, pharmacological inhibition of PRMT5 reduced long-term engraftment of human CML CD34+ cells in NSI mice. Inhibition of PRMT5 abrogated Wnt/β-catenin signaling in CML CD34+ cells. Histone protein arginine methylation may be a novel regulation mechanism to control self-renewal of LSCs, and targeting PRMT5 helped effectively eradicate CML LSCs. Additionally, we identified a potential lead compound against PRMT5 activity and characterized its in vitro and in vivo activity against CML LSCs (Figure 10).

Positive feedback loop between BCR-ABL and PRMT5. A wide spectrum of substrates of BCR-ABL tyrosine kinase has been demonstrated (42, 43). The half-life of BCR-ABL transcripts seems to be a predictor of prognosis in patients with CML in response to TKIs (44). However, little is known about the mechanism regulating BCR-ABL expression. Our results revealed that PRMT5 is a positive regulator of BCR-ABL expression in various settings, such as WT BCR-ABL, T315I mutation, and primary AP-CML CD34+ cells. Conversely, knockdown of PRMT5 by shRNA or blocking its arginine methyltransferase activity by the small-molecule inhibitor PJ-68 greatly reduced BCR-ABL gene transcription. Previous study has shown that miR-203, a potent negative regulator of BCR-ABL oncogene transcription, is transcriptionally modulated by hypermethylation of the Mir203 promoter (35). Given that methylation on histone arginine residues governs the recruitment of histone protein on promoters of miRNAs, which subsequently determine switch-on or -off of their transcription (24, 45), it was postulated that PRMT5 might be recruited to regulate miR-203 through histone arginine methylation. Indeed, our results showed that silencing PRMT5 by shRNA or pharmacological inhibition led to loss of enrichment of PRMT5 and its epigenetic marks H3R8SDM and H4R3SDM on the promoter, and restoration of miR-203 expression. Our study revealed one potential upstream regulator of BCR-ABL transcription by the Argonaute family, only PRMT5 has a solved crystal structure (33). We identified a potent PRMT5 small-molecule inhibitor, PJ-68, that was effective in the LSCs and impaired their self-renewal in human CML LSCs (49). Monitoring the evolution of xenografted human BCR-ABL lymphoblastic LSCs in SCID mice indicates that loss of CDKN2A/B is associated with increased LSC proportion and shortened survival (50). PRMT5 knockdown or pharmacological inhibition of PRMT5 activity increased p15^INK4B mRNA levels, in association with a mechanism by which enrichment of H4R3SDM and DNMT3A is reduced at the promoter of p15^INK4B in CML cells. p27^KIP1 has been demonstrated to be an endogenous CDK inhibitor that negatively regulates the cell cycle and the self-renewal and survival of CML LSCs (12, 51). PRMT5 inhibition activated p27^KIP1 expression, which may also contribute to inhibition of CML LSCs. Collectively, the increased p15^INK4B and p27^KIP1 expression by PRMT5 inhibition may facilitate eradication of LSCs in CML.

PRMT5 is an oncogenic protein in CML LSCs. The body of evidence we present here supports that PRMT5 is required for survival and self-renewal of CML LSCs, and that inhibition of PRMT5 eliminated the quiescent LSCs and impaired their self-renewal in human CML LSCs and in a CML mouse model. The effectiveness of targeting PRMT5 on LSCs may have particular implications for AP-CML patients, in which additional oncogenic drivers are assumed to be involved. The underlying mechanism may be associated with β-catenin, a central component of the Wnt/β-catenin pathway that regulates stemness of CSCs in a wide range of malignancies (e.g., leukemia, breast cancer, and colorectal cancer) (13, 52, 53).

Regarding the mechanism whereby PRMT5 elevates β-catenin levels, our data support that PRMT5 may execute this function in an indirect manner, i.e., via regulating the level of DVL3. Among the 3 isoforms of DVL protein, DVL1 was undetectable in K562 cells (data not shown), and DVL2 was not altered in PRMT5-silenced CML cells (Figure 9). However, PRMT5 inhibition markedly decreased DVL3 mRNA and protein levels (Figure 9). Our results suggest that increased PRMT5 in CML CD34+ cells may promote transcription of the DVL3 gene, which elicited increased stability of β-catenin in the absence of Wnt. Hence, decreased β-catenin and increased p15^INK4B levels by PRMT5 knockdown or inhibition may contribute to the elimination of CML LSCs.

PRMT5 inhibitors. Among the 11 members of the PRMT family, only PRMT5 has a solved crystal structure (33). We identified a potent PRMT5 small-molecule inhibitor, PJ-68, that was effective in the LSCs and impaired their self-renewal in human CML LSCs (49). Monitoring the evolution of xenografted human BCR-ABL lymphoblastic LSCs in SCID mice indicates that loss of CDKN2A/B is associated with increased LSC proportion and shortened survival (50). PRMT5 knockdown or pharmacological inhibition of PRMT5 activity increased p15^INK4B mRNA levels, in association with a mechanism by which enrichment of H4R3SDM and DNMT3A is reduced at the promoter of p15^INK4B in CML cells.
Chemical reagents and DNA constructs

DAPI was from Invitrogen. The TOPflash/FOPflash system and ChIP Kit were from Upstate Biotechnology. MG132, cycloheximide (CHX), propidium iodide (PI), human and murine PRMT5 shRNA, human BCR-ABL shRNA, human STAT5A, and STAT5B shRNA were from Sigma-Aldrich. pCMV-2B-Flag and pCMV-2B-Flag-PRMT5 constructs were provided by Shilai Bao (Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beijing, China) (55); the MSCV-BCR-ABL-IRES-EGFP construct was described previously (56); pcDNA3, pcDNA3-BCR-ABL, pSG5-BCR-ABL (p210), HA-tagged BCR, pSG5-ABL and pSG5-BCR-ABL (p190), lentiviral-TOP-dGFP-reporter, lentiviral-FOP-dGFP-reporter, pCMV-dR8.2, and pCMV-VSVG constructs were from Addgene.

Cell lines

The KBM5 and KBM5-T315I cells were from Sai-Ching J. Yeung (Department of Emergency Medicine, University of Texas M.D. Anderson Cancer Center, Houston, Texas, USA) and cultured in Iscove’s modified Dulbecco’s medium (Invitrogen) supplemented with 10% FBS (57). K562 and M2-10B4 cells were purchased from totally different from the previously reported PRMT5 inhibitors EPZ015666 and HLCL-61 (24, 54). PJ-68 effectively and selectively inhibited the methyltransferase activity of type II PRMT5 over type I PRMTs at nanomolar concentrations. Our PK studies showed that plasma concentrations (of about 3.57 ± 0.08 to 7.88 ± 0.31 μM) of PJ-68 are postulated to be sufficient to block in vitro histone methyltransferase activity of PRMT5. Our findings that PJ-68 decreased the in vitro and in vivo self-renewal capacity of CML LSCs indicate that further investigation of this lead compound and the therapeutic strategy of combining TKIs with relevant inhibitors of epigenetic modifiers is warranted.

Methods

Compounds

IM (imatinib, STI571) and the DNMT3A inhibitor SGI-1027 were purchased from Selleckchem. PJ-68 [N-(9-ethyl-9H-carbazol-3-yl) methyl]-1,2,3,4-tetrahydronaphthalen-1-amine, Figure 2G] was obtained from Aurora Fine Chemicals and stored at -20°C as a 100 mM stock solution in DMSO.
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Centrifugation (density 1.077; Sigma-Aldrich) (59). CD34+ cells were isolated by use of the CD34 MicroBead Kit (Miltenyi Biotec). CD34+CD38– and CD34+CD38+ cell populations were further sorted by flow cytometer (BD FACS Aria II) after staining with CD38-PE antibody (catalog 130-098-907, Miltenyi Biotec). These primary cells were incubated in IMDM medium supplemented with 10% FBS in the presence of granulocyte-macrophage colony-stimulating factor (GM-CSF, 100 ng/ml), stem cell factor (SCF, 100 ng/ml), IL-3 (20 ng/ml), and IL-6 (20 ng/ml), which were purchased from PeproTech Inc. (60, 61).

Measurement of intracellular PRMT5 and active β-catenin
CML or NBM CD34+ cells or PJ-68–treated K562 cells were harvested, fixed in 4% formaldehyde for 10 minutes at 37°C, and perme-
PRMT5 inhibition elicited TCF4/LEF1-dependent luciferase activity in CML CD34+ cells. Lenti, lentivirus. β capability between CML or NBM CD34+ cells (1 × 10^6 cells/ml) were infected by spinoculation (1,500 g, 90 minutes, 32°C) with virus-containing supernatants from Sca1+CD45+ progenitor cells (Supplemental Table 3).

Western blot analysis
The whole cell lysates were prepared in RIPA buffer (57, 61). Antibodies against c-ABL (C-19, catalog sc-887), H2A (H-124, sc-10807), H3 (FL-136, sc-10809), CRKL (C-20, sc-319), DVL3 (4D3, sc-8027), cyclin D1 (H-295, sc-753), BCL-X, (S-18, sc-634), p15 (C-20, sc-612), p27 (C-19, sc-528), and DNMT3A (H-295, sc-20703) were from Santa Cruz Biotechnology Inc. Antibodies against p–BCR-ABL (Y245) (catalog 2861), BCR (catalog 3902), p–CRKL (Y207) (catalog 3181), DVL2 (catalog 3224), LEF1 (C18A7, catalog 2286), and TCF4 (C48H11, catalog 2569) were from Cell Signaling Technology. Antibody against HA (16B12, catalog MMS-101P-200) was from Covance Inc. Antibodies against PRMT5 (catalog 07-405), H4R2SDM (catalog ABE460), H3K4me3 (catalog 07-473), and p–BCR-ABL (Y245) (catalog 2861), BCR (catalog 3902), p–CRKL (Y207) (catalog 3181), DVL2 (catalog 3224), LEF1 (C18A7, catalog 2286), and TCF4 (C48H11, catalog 2569) were from Cell Signaling Technology. Antibody against HA (16B12, catalog MMS-101P-200) was from Covance Inc. Antibodies against PRMT5 (catalog 07-405), H4 (clone 62-141-13, catalog 05-858), p–STAT5A/B (Y694/699) (catalog 07-586), STAT5A (catalog 06-553), STAT5B (catalog 06-969), p–STAT3 (Y705) (clone 9E12, catalog 05-485), STAT3 (catalog 06-596), active β-catenin (clone 8E7, catalog 05-665), p75 (catalog 06-556), H3R2SDM (catalog ABE460), H3K4me3 (catalog 07-473), and WDR5 (catalog 07-706) were from EMD Millipore. Antibodies against H4R2SDM (catalog ab5823), H3R8SDM (catalog ab130740), H2AR2SDM (catalog ab22397), and H4R3DM (catalog ab94683) were from Abcam. Antibodies against β-catenin (clone 14/β–catenin, catalog 610153), c–MyC (catalog 551101), and p16 (catalog 551153) were from BD Biosciences. Antibodies against β-actin (clone AC-15, catalog A5441) and α-tubulin (clone B-5-1-2, catalog T5168) were from Sigma-Aldrich. Detailed information about the secondary antibodies for Western blot analysis is shown in Supplemental Table 3. See the uncropped gels in Supplemental Figures 8–16.

Lentiviral transduction in CML CD34+ cells
Lentiviruses were produced by transient transfection in 293T cells with control shRNA (Scramble) or specific shRNA together with the pCMV-dR8.2 packing construct and the pCMV-VP65 envelope construct. CML or NBM CD34+ cells (1 × 10^6 cells/ml) were infected by spinoculation (1,500 g, 90 minutes, 32°C) with virus-containing supernatants twice (63). Cells were harvested 48 hours later, and the knockdown effect was examined by Western blot analysis.

Analysis of apoptosis of primary CD34+ cells
CML CD34+ cells or NBM CD34+ cells were labeled with annexin V–FITC and CD38–PE antibodies after treatment; apoptotic cells (CD34–CD38– annexin V+) were detected by flow cytometer (BD FACs Aria II) (61).

CFSE assay. CFSE-labeled cells (CellTrace CFSE Proliferation Kit; Invitrogen) were cultured with PJ-68 for 96 hours, cells were harvested and stained with annexin V–PE (catalog 556422, BD Biosciences), and quiescent cell apoptosis (CD34–CD38–CFSE^−annexin V+) was determined by flow cytometer (BD FACs Aria II) (11, 64, 65).

CFC/replating and LTC-IC assay
CFC/replating. CML CD34+ cells or NBM CD34+ cells were treated with different concentrations of PJ-68 for 24 hours, washed with PBS, and counted; 1,000 cells were plated in methylcellulose medium (MethoCult H4434, Stemcell Technologies). Colonies were counted on day 14. Cells were then harvested and counted, and 1,000 cells were replated for the secondary and tertiary rounds; colonies were counted on day 14 after each replating (11, 64, 65).

LTC-IC assay. 2 × 10^5 CML or NBM cells were seeded in LTC-IC medium onto irradiated (80 Gy) M2-10B4 cells and treated with PJ-68 (25.0 μM) with or without IM (2.5 μM) for 1 week; compound-containing medium was removed, and cells were cultured for 5 weeks in LTC-IC medium (H5100, Stemcell Technologies) by weekly half-medium changes. After 6 weeks, cells were harvested and plated into methylcellulose medium (MethoCult H4435, Stemcell Technologies) (11, 64, 65). LTC-IC-derived colonies were counted after 2 weeks.

CML mouse model
The retrovirus was produced by transient transfection with the MSCV-BCR-ABL-IRES-EGFP construct in Plat-E cells. Viral supernatants were harvested at 48 and 72 hours after transfection and purified with the 0.45-μm filter. BM cells from 6- to 8-week-old donor male C57BL/6 mice (Guangdong Medical Laboratory Animal Center) primed by 5FU were stimulated with cytokines in vitro, then cells were transduced twice with the MSCV-BCR-ABL-IRES-EGFP retrovirus, followed by tail vein injection into irradiated (550 cGy) recipient female C57BL/6 mice. Following transplantation, mice were treated with IM (100 mg/kg/d, p.o.), PJ-68 (25 mg/kg/d and 50 mg/kg/d, i.p.), or both for 2 weeks; then mice were euthanized and BM and spleen cells were collected, labeled with the indicated antibodies, and analyzed by flow cytometer (BD FACs Aria II). BCR-ABL–expressing (GF+) leukemia cells or myeloid cells were labeled with anti-mouse Gr-1–APC and Mac-1–PE antibodies (BD Pharmingen). Antibodies for lineage cocktail APC, Ly-6A/E

bilized in prechilled 90% methanol for 30 minutes on ice (62). After washing and blocking, the cells were incubated with anti-PRMT5 or anti-active β-catenin, then stained with the secondary antibody Alexa Fluor 488–conjugated goat anti-rabbit or anti-mouse IgG. The FITC-conjugated mouse IgG1 isotype control was stained similarly except for the secondary antibody. The samples were analyzed by flow cytometer (BD FACs Aria II).

**Figure 9. PRMT5 activates Wnt/β-catenin signaling through upregulating DVL3 expression in human CML CD34+ cells.** (A) PRMT5 knockdown by shRNA decreased the expression of Wnt/β-catenin–related genes in CML cells. (B and C) Pharmacological abrogation of PRMT5 activity decreased the expression of β-catenin–related genes in K562 cells and in primary human CML CD34+ cells. (D) PRMT5 overexpression activated Wnt/β-catenin signaling. (E) PRMT5 knockdown decreased active intracellular β-catenin levels in CML cells. Flow cytometry of active intracellular β-catenin levels in K562 cells after labeling with anti-active β-catenin (ABC) antibody. Representative flow cytometry plot (left) and bar plots (right) are shown. (F) PRMT5 inhibition elicited TCF4/LEF1-dependent luciferase activity in CML CD34+ cells. Lenti, lentivirus. (G) PRMT5 knockdown disrupted the binding capability between β-catenin and TCF4. (H–K) PRMT5 activated Wnt/β-catenin signaling through DVL3. (H) Ectopic PRMT5 expression increased DVL3 gene transcription. (I) PJ-68 inhibited transcription of the DVL3 gene. (J) PRMT5 knockdown or inhibition reduced the level of H3R2SDM as determined by Western blot analysis in K562 cells. (K) PRMT5 methylated histone H3R2 and recruited WDR5 to promote H3K4 trimethylation and DVL3 activation. ChIP experiments with antibodies specific for PRMT5, WDR5, H3R2SDM, and H3K4me3 showed significant enrichment of PRMT5 and its epigenetic marks at the DVL3 gene promoter, whereas knockdown of PRMT5 led to loss of recruitment of PRMT5 and its epigenetic marks on the DVL3 gene promoter in K562 cells. Two-tailed Student’s t test was performed for F, H, and K; 1-way ANOVA, post hoc intergroup comparisons, Tukey’s test was used for E and I. *P < 0.05; **P < 0.01; ***P < 0.0001.
pEF<sub>Renilla</sub>-luc (10 ng) for 24 hours. K562 cells with Scramble, shPRMT5 #1, or shPRMT5 #2 were transfected with TOPflash or FOPflash constructs (0.5 μg) and pEF<sub>Renilla</sub>-luc (10 ng) by electroporation for 24 hours. Luciferase activity was measured by use of a dual-luciferase assay kit (Promega) (61).

ChIP
ChIP was performed using the EZ-ChIP Kit (EMD Millipore) according to the manufacturer’s instructions. K562 or shPRMT5 #2 cells were cross-linked in 1% formaldehyde for 10 minutes at room temperature. Glycine was added to quench unreacted formaldehyde. The cells were washed with cold PBS, harvested, and resuspended in SDS lysis buffer containing a protease inhibitor cocktail. Cross-linked DNA was sheared to 200–1,000 bp by sonication and centrifuged at 10,000 g at 4°C for 10 minutes. Protein A/G agarose (60 μl of a 50% slurry) was then added and incubated at 4°C overnight. Pellets were washed, and protein-DNA complexes were eluted and reversed by overnight incubation at 65°C with proteinase K. DNA was purified with spin columns. The purified DNA was used for PCR reaction. The PCR conditions were 5 minutes at 94°C for initial denaturation, 30 seconds at 94°C for denaturation, 30 seconds at 60°C for annealing, 30 seconds at 72°C for extension, repeat for total 35 cycles, 72°C for 5 minutes for final extension. qRT-PCR was performed as described previously (48). The primers used are listed in Supplemental Table 5.

Luciferase assay
293T cells were transiently cotransfected with a BCR-ABL promoter reporter construct (0.5 μg) and vector, PRMT5, or PRMT5 (R368A) constructs, and pEF<sub>Renilla</sub>-luc (10 ng) for 24 hours. K562 cells with Scramble, shPRMT5 #1, or shPRMT5 #2 were transfected with TOPflash or FOPflash constructs (0.5 μg) and pEF<sub>Renilla</sub>-luc (10 ng) by electroporation for 24 hours. Luciferase activity was measured by use of a dual-luciferase assay kit (Promega) (61).

Engraftment of human cells in immunodeficient mice
CML CD34<sup>+</sup> cells were cultured with PJ-68 (25.0 μM) for 72 hours, and then cells (1 x 10<sup>6</sup> cells/mouse) were transplanted by tail vein injection into NOD-SCID mice.
into sublethally irradiated (300 cGy) 8-week-old NSI mice (Guangzhou Institutes of Biomedicine and Health, Chinese Academy of Sciences) (66). Mice were euthanized after 12 weeks, and BM cells and spleen cells were harvested; the engraftment of human cells was analyzed by flow cytometry (11, 12, 64, 65). Detailed information for generating NSI mice is described in the supplemental material. Detailed information about the antibodies for NSI mice is shown in Supplemental Table 6.

Statistics
All experiments were performed at least 3 times, and data represent mean ± SD. GraphPad Prism 5.0 (GraphPad Software) was used for statistical analysis. Comparisons between 2 groups were analyzed by 2-tailed Student’s t test and comparisons of multiple groups by 1-way ANOVA, post hoc intergroup comparisons, and Tukey’s test. A P value less than 0.05 was considered statistically significant.

Study approval
This study was approved by the Sun Yat-sen University Ethics Committee according to institutional guidelines and Declaration of Helsinki principles, and written informed consent was received from participants prior to inclusion in the study. All animal studies were conducted with the approval of the Sun Yat-sen University Institutional Animal Care and Use Committee.

Author contributions
YLJ designed and performed experiments, analyzed data, and wrote the manuscript; FX performed the molecular docking experiments; BJ, JFZ, LJC, and YW performed experiments; JL and XD provided CML patient samples and clinical data; PL performed the NSI mouse experiments; RBR reviewed data; and JXP designed and guided research, analyzed data, and wrote the manuscript.

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