

**The Role of Protein Arginine Methyltransferase - 4 (PRMT4)
in Normal and Malignant Hematopoiesis**

by

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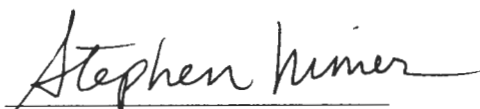
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Dedication

This thesis is dedicated to my husband - my soul mate and best friend - who has always been there for me.

Abstract

A transcription regulatory network involving transcription factors, target genes and microRNAs as well as epigenetic regulators controls the differentiation program in hematopoietic system. Recently, defining the role of histone modifying enzymes in normal myeloid differentiation has become critically important, as recurrent mutations as well as an aberrant expression of a wild type protein have been identified in hematological malignancies. The group of protein arginine methyltransferases (PRMTs) has emerged to play important roles in hematopoiesis. Although PRMT4 (aka CARM1), a type I PRMT, is known as a positive regulator of several biological processes, its function in the hematopoietic system is unknown.

In our studies, we shown that PRMT4 is a negative regulator of myeloid differentiation of human hematopoietic stem/progenitor cells using the primary cord-blood derived CD34+ cells system. Knockdown of PRMT4 accelerates myeloid differentiation while overexpression of PRMT4 blocks the process. We demonstrated that PRMT4 regulates myeloid differentiation, (at least by part), though repressing the expression of a myeloid-specific microRNA- miR-223. We also found that PRMT4 expression is downregulated during normal myeloid differentiation. Interestingly, miR-223 post-transcriptionally down regulates PRMT4 expression, thus forming a reciprocal regulatory loop with PRMT4 to foster the differentiation process. Mechanistically, we established that PRMT4 interacts with and arginine methylates RUNX1 (aka AML1), a critical transcription factor in hematopoiesis. This results in the recruitment of a novel interacting partner – DPF2, which in turn, controls miR-223 expression and myeloid differentiation. In summary, our work has identified a critical regulatory axis, comprising of PRMT4, microRNA-223,

transcription factor RUNX1 and a transcriptional effector DPF2, in myeloid differentiation. Given that the differentiation process is often compromised in leukemia cells, this prominent regulatory role of PRMT4 points to a potential involvement of PRMT4 in hematopoietic malignancies. Indeed, we found that PRMT4 is overexpressed in AML patient samples. Furthermore, loss of PRMT4 functions results in the differentiation of myeloid leukemia cells in vitro and their decrease proliferation in vivo, implicating PRMT4 as a potential therapeutic target in AML therapy. Overall, our study has shed light into the uncovered function of PRMT4 in the hematopoietic system while providing a basis for further study of the role of PRMT4 in leukemogenesis.

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Table of Contents

List of Figures	x
List of Abbreviations	xi
1. INTRODUCTION	ii
1.1 General Principles in Normal and Malignant Hematopoiesis.....	1
1.1.1 Normal hematopoiesis	1
1.1.2 Malignant hematopoiesis and myeloid malignancies	6
1.2 Transcription regulation of Myeloid Differentiation	9
1.3 RUNX1 and post-translational modification of RUNX1.....	20
1.4 Targeting histone modifying enzymes for leukemia therapy	25
1.5 Arginine Methylation and the Family of Protein Arginine Methyltransferases	38
1.6 Protein Arginine Methyltransferase 4 – PRMT4 and a potential role in hematopoiesis?.....	42
2. MATERIALS AND METHODS	47
2.1 Purification and culture of human primary hematopoietic CD34+ cells	47
2.3 Flow cytometry and cell sorting	48
2.4 Hematopoietic functional assays.....	48
2.4.1 Colony forming unit (CFU) assay.....	48
2.4.2 Cobblestone area forming cell (CAFC) assay	48
2.4.3 In vitro lineage-specific liquid culture.....	49
2.4.4 Morphology analysis.....	49
2.5 RNA extraction and quantitative real time PCR (qRT-PCR)	49
2.6 Cellular transient transfection	49
2.7 Peptide pull-down assay	50
2.8 Co-Immunoprecipitation and Western blot assays	50
2.9 Chromatin Immunoprecipitation (ChIP) assays.....	51
2.10 Antibodies and reagents.....	51
2.11 In vitro and ex vivo treatment of PRMT4 inhibitor.....	52
2.12 Cell viability assays	52
2.12.1 CellTiter – Glo Luminescent cell viability assay –Promega	52
2.12.2 Apoptotic analysis.....	53
2.13 In vivo transplantation of leukemia cells.....	53
2.14 RNA sequencing and DNA microarray for gene expression profiling.....	53
2.15 Gene expression pathways analysis.....	53
2.16 Statistical analysis.....	54
3. RESULTS	55
3.1 PRMT4 Blocks Myeloid Differentiation by Assembling a Methyl-RUNX1- Dependent Repressor Complex (<i>manuscript to be resubmitted to Cancer Cell</i>).....	55
3.1.1 PRMT4 regulates myeloid differentiation	56
3.1.2 PRMT4 is regulated post-transcriptionally by miR-223 during myeloid differentiation.....	60
3.1.3 PRMT4 represses miR-223 expression.....	65
3.1.4 RUNX1 is methylated by PRMT4 on arginine 233 (R223) residue.....	68
3.1.5 Methylation of RUNX1 at R223 regulates its interaction with DPF2	72

3.1.6 MiR-223 expression is regulated by a RUNX1-methylation dependent repressor complex	75
3.2 PRMT4 is a Therapeutic Target for Leukemia Treatment.....	79
3.2.1 Knock down of PRMT4 is sufficient to induce myeloid differentiation and apoptosis in AML cell lines	79
3.2.2 Knock down of PRMT4 reduces leukemia burden in vivo.....	80
3.2.3 Small molecule inhibitors of PRMT4 induces myeloid differentiation and apoptosis in AML cell lines	84
3.2.4 Ex vivo treatment with PRMT4 inhibitor reduces leukemia burden in vivo...	89
3.2.5 Loss of PRMT4 functions activates myeloid differentiation and cell death transcription programs in leukemia cells	91
4. DISCUSSION	96
References	108

List of Figures

Figure 1. Development of HSCs and lineage determination in the adult human hematopoietic hierarchies.	2
Figure 2. Development regulation of hematopoiesis in the mouse.....	5
Figure 3. Deregulated pathways leading to leukemia.....	8
Figure 4. Arginine methylation and the family of Protein Arginine Methyltransferases .	40
Figure 5. PRMT4 regulates myeloid differentiation of HSPCs.....	57
Figure 6. PRMT4 regulates myeloid differentiation of HSPCs.....	59
Figure 7. PRMT4 is a potential target gene of miR-223 during myeloid differentiation of HSPCs.....	62
Figure 8. PRMT4 is a potential target gene of miR-223 during myeloid differentiation of HSPCs.....	64
Figure 9. PRMT4 regulates miR-223 expression.....	66
Figure 10. PRMT4 regulates miR-223 expression.....	67
Figure 11. RUNX1 is arginine methylated by PRMT4 on R223 residue.....	70
Figure 12. RUNX1 is arginine methylated by PRMT4 on R223 residue.....	71
Figure 13. Methylation of RUNX1 at R223 residue regulates its interaction with DPF2	74
Figure 14. The RUNX1 methylation dependent repressor complex regulates miR-223 expression.	76
Figure 15. Knock down of DPF2 promotes myeloid differentiation and miR-223 expression.....	78
Figure 16. Knock down of PRMT4 is sufficient to induce myeloid differentiation and apoptosis in AML cell lines.....	81
Figure 17. Knock down of PRMT4 reduces leukemia burden in vivo.....	83
Figure 18. PRMT4i inhibits leukemia cell growth.....	86
Figure 19. Effects of PRMT4i on Kasumi-1 cells.....	88
Figure 20. Ex vivo treatment using PRMT4 inhibitor reduces leukemia burden in vivo.	90
Figure 21. MiR-223 upregulation is dispensable for myeloid differentiation triggered by loss of PRMT4 function.....	92
Figure 22. Biological function analysis of PRMT4KD in CD34+ cells dataset.....	94
Figure 23. Biological function analysis of PRMT4KD in Kasumi-1 cells dataset.....	95
Figure 24. A schematic model showing PRMT4 regulates myeloid differentiation of human HSPCs.	97

List of Abbreviations

2-HG	2-Hydroxyglutarate
5caC	5-Carboxylcytosine
5fC	5-Formylcytosine
5hmC	5-Hydroxymethylcytosine
5mC	5-Methylcytosine
ABL	Abelson Tyrosine-Protein Kinase
AE	AML1-ETO
AE9a	AML1-ETO9a
ALL	Acute Lymphoid Leukemia
AML	Acute Myeloid Leukemia
AML1	Acute Myeloid Leukemia 1
AML1-ETO	Acute Myeloid Leukemia 1-Eighth Twenty One
AP1	Activator Protein 1
APL	Acute Promyelocytic Leukemia
BCR	Breakpoint Cluster Region
BCR-ABL	Breakpoint Cluster Region-Abelson Tyrosine-Protein Kinase
BRG	Brahma-Related Gene
BRM	Brahma Chromodomain 1
C/EBP	CCAAT/Enhancer Binding Protein
CAFC	Cobblestone Area-Forming Cell
CBP	CREB Binding Protein
CBS	Consensus Binding Site
CFU	Colony Forming Unit
CML	Chronic Myelomonocytic Leukemia
CMP	Common Myeloid Progenitors
CSFR	Colony Stimulating Factor 3
DNMT	DNA Methyltransferase
DOT1L	DOT1-like
DPF2	D4, Zinc and Double PHD Fingers Family 2
E2F1	E2F Transcription Factor 1
ETO	Eighth Twenty One
ETS	E-Twenty Six
EZH2	Enhancer of Zeste Homolog 2
FTL3	Fms-Related Tyrosine Kinase 3
FTL3-ITD	Fms-Related Tyrosine Kinase 3 -Internal Tandem Duplication
G-CSFR	Granulocyte colony-stimulating factor receptor
GADD45A	Growth Arrest And DNA-Damage-Inducible, alpha
GATA1	GATA Binding Protein 1

GFI-1	Growth Factor Independent 1
GFP	Green Fluorescent Protein
GM-CSFR	Granulocyte-Macrophage Colony-Stimulating Factor
GMP	Granulocyte Macrophage Progenitors
GRIP-1	Glutamate Receptor Interacting Protein 1
HATs	Histone Acetyltransferases
HDACs	Histone Deacetylase
HELLS	Helicase, Lymphoid-Specific
hox	homeobox
HSC	Hematopoietic Stem Cell
HSPC	Human Stem/Progenitor Cells
IDH	Isocitrate Dehydrogenase
IL-3	Interleukin-3
IL-6	Interleukin-3
IL-7R	Interleukin-7 Receptor
INI1	Integrase Interactor 1
ISWI	Imitation Switch
JAK2	Janus Kinase 2 Transcription Factor
JMJD6	Jumonji Domain Containing 6
KAT	Lysine Acetyltransferases
KIT	Tyrosine-Protein Kinase Kit
KLF-4	Kruppel-Like Factor 4
LSC	Leukemic Stem Cells
LSD1	Lysine-Specific Histone Demethylase 1
M-CSFR	Macrophage Colony-Stimulating Factor
MDS	Myelodysplastic Syndromes
MEF2C	Myocyte Enhancer Factor 2C
Meis1	Myeloid Ecotropic Viral Integration Site 1 Homolog
MEP	Megakaryocyte Erythroid Progenitors
MEP50	Methylosome Protein 50
miRNA	microRNA
MLL	Myeloid/Lymphoid or Mixed-Lineage Leukemia
MLP	Myeloid Lymphoid Progenitors
MOZ	Monocytic Leukemia Zinc Finger Protein
MPN	Myeloproliferative Neoplasms
MPO	Myeloperoxidase
MPP	Multipotent Progenitors
MYC	Myelocytomatosis Viral Oncogene Homolog
NE	Neutrophil Elastase
NF-IA	Nuclear Factor I/A

NF- κ B	Nuclear Factor kappa-B
NSD1	Nuclear Receptor Binding SET Domain Protein 1
NUP98	Nucleoporin 98kDa
NuRD	Nucleosome Remodelling and Histone Deacetylase
p300	Histone Acetyltransferase p300
PAD4	Peptidylarginine deiminase 4
PKMT	Lysine Methyltransferases
PML	Promyelocytic Leukemia
PML-RAR α	Promyelocytic Leukemia-Retinoic Acid Receptor, alpha
PRMT	Protein Arginine methyltransferase
PTM	Posttranslational Modifications
RAR α	Retinoic Acid Receptor, alpha
RFP	Red Fluorescent Protein
RHD	Runt Homologous Domain
ROS	Reactive Oxygen Species
RUNX1	Runt-Related Transcription Factor 1
SCF	Stem Cell Factor
SETD7/9	SET Domain Containing (Lysine Methyltransferase) 7/9
SFFV	Spleen Focus Forming Virus
SIN3A	SIN3 Transcription Regulator Homolog A
SPI1	Spleen Focus Forming Virus Proviral Integration Oncogene
STAT	Signal Transducer and Activator of Transcription
SWI/SNF	Switch/Sucrose NonFermentable
TAF	TATA Box Binding Protein-Associated Factor
TBP	TATA Box Binding Protein
TET	Ten-Eleven Translocation
TF	Transcription Factor
TP53	Tumor Protein p53
UTR	3' Untranslated Region
YAP	Yes-Associated Protein
α KG	alpha-Ketoglutarate

1. INTRODUCTION

1.1 General Principles in Normal and Malignant Hematopoiesis

1.1.1 Normal hematopoiesis

Hematopoietic development is a delicately orchestrated process that results in maturation of immature hematopoietic stem and progenitor cells into a variety of terminal differentiated functional cells of the blood system [1]. This regenerative process is essential for maintenance of the blood system, which consists of about one trillion cells newly generated everyday in an adult human bone marrow. The view of hematopoiesis as a cellular hierarchy derived from a common precursor, a hematopoietic stem cell (HSC), can be dated back to as early as 1909 with Russian biologist A. Maximow postulating their existence to explain the diversity of bone marrow anatomy [2]. Over the last 100 years, advancements in development of functional assays for the regenerative potential of HSCs both in vivo and in vitro as well as the identification of cell surface markers used to characterize various cell types by flow cytometry have brought about a finely detailed view of the blood system with multipotent HSCs and terminally differentiated cells (Figure 1 from left to right). HSCs are defined as cells with capacity to self-renew and proliferate and the potential to differentiate to all cellular lineages. In an in vivo functional assay, it is defined by the ability of HSCs to regenerate the entire blood system upon transplantation of HSCs into recipients. As HSCs differentiate, they give rise to progenitor cells, which in turn undergo a step-wise commitment process to become mature blood cells. Characterization of these linear processes has provided scientists with a “roadmap” to follow the development of hematopoietic systems (Figure 1).

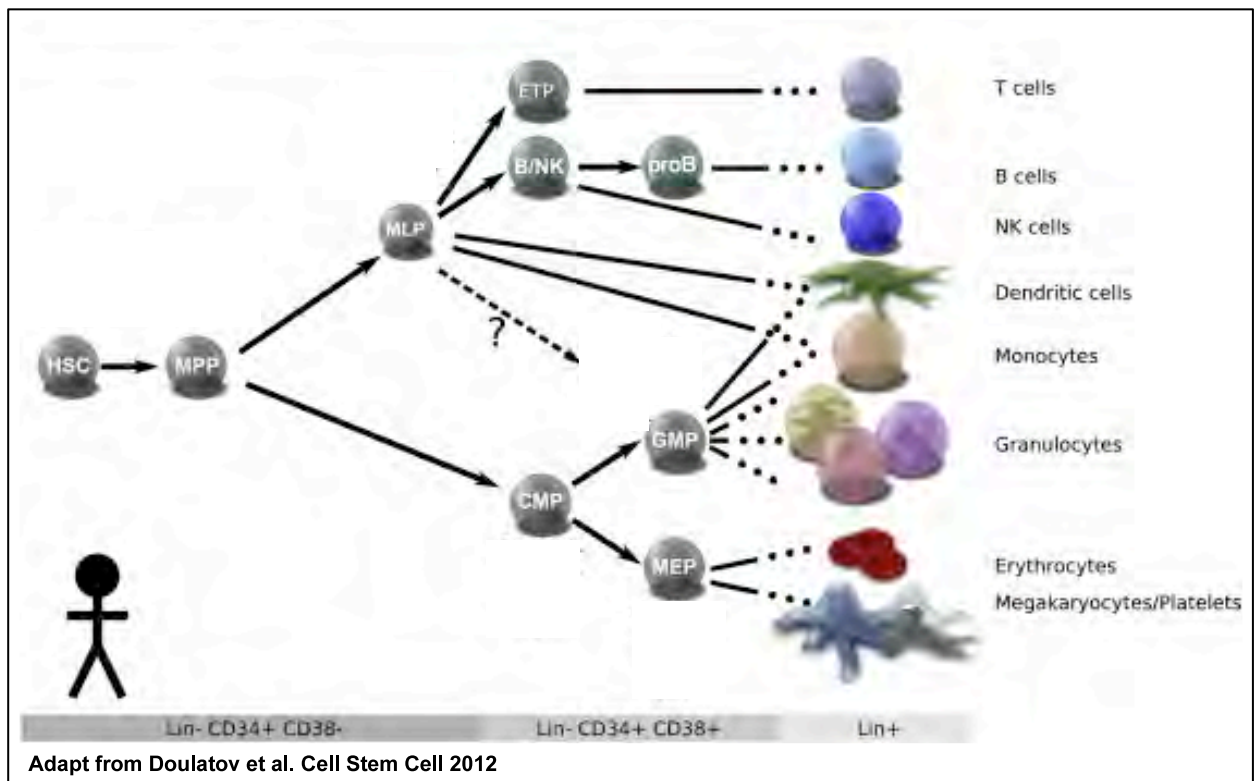


Figure 1. Development of HSCs and lineage determination in the adult human hematopoietic hierarchies.

Hematopoietic stem, progenitor and terminally differentiated cells can be distinguished by cell surface markers (as depicted in figure). The hierarchy starts from Hematopoietic Stem Cell (HSCs) and Multipotent Progenitors (MPP), which give rise to Myeloid Lymphoid Progenitors (MLP) and Common Myeloid Progenitors (CMP). CMP in turn gives rise to Granulocyte Macrophage Progenitors (GMP) and Megakaryocyte Erythroid Progenitors (MEP), which are responsible for the generation of granulocyte, macrophages, red blood cells and platelets. MLP generates T and B cells. Both MLP and GMP can give rise to monocytes and dendritic cells.

Much of our understanding of hematopoiesis comes from the mouse as it provides the experimental system to operationally test the reconstitution potential of HSCs. Furthermore, the power to manipulate with genetic perturbations in a defined compartment and/or at a particular developmental period has brought about our vast knowledge of the development of hematopoiesis. Multiple waves of hematopoiesis during development were identified and characterized. The first wave of hematopoiesis – the primitive hematopoiesis – happens in mammalian yolk sac to produce red blood cells. The next wave of hematopoiesis – the definite hematopoiesis- starts in the aorta-gonad mesonephros (AGM) region and placenta and subsequently occurs in fetal liver and ultimately the bone marrow, where all cell lineages are generated (Figure 2) [3].

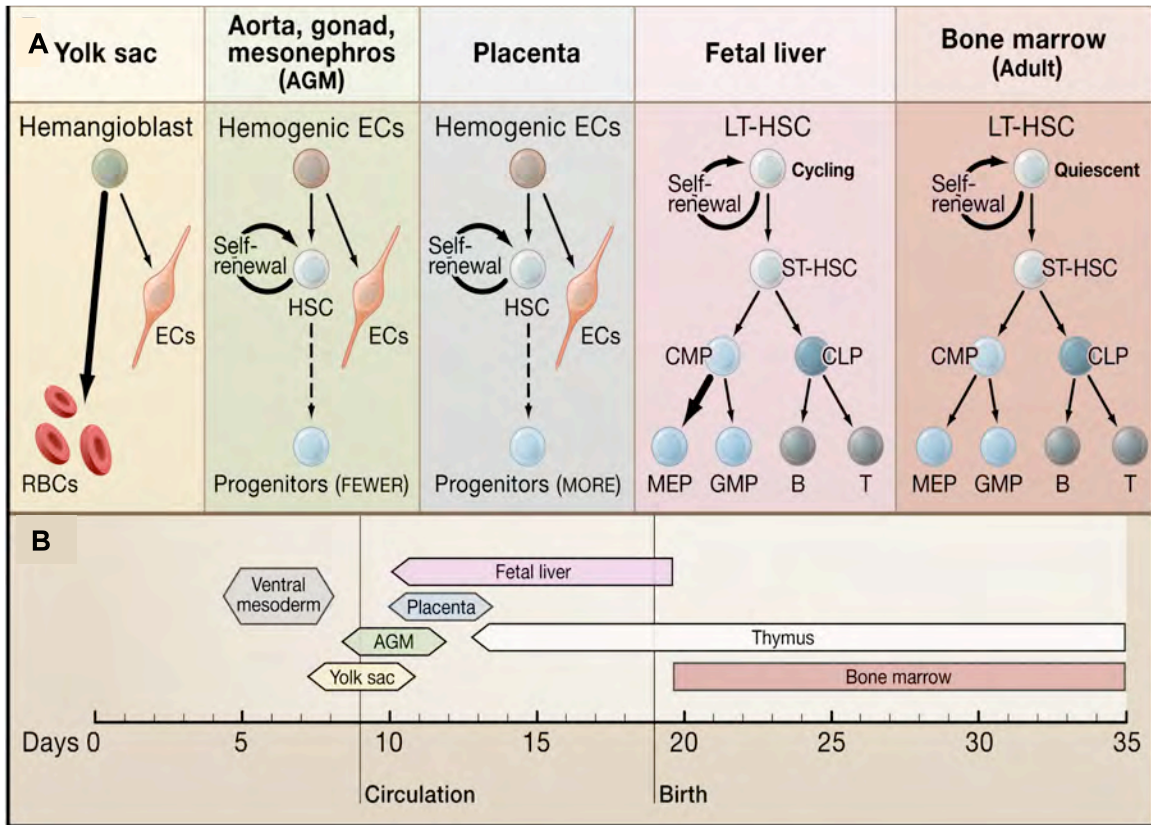
Despite being a powerful system to study hematopoiesis, the mouse model or other animal models reach their limit when it comes to the species – specific differences and their relevance to therapeutic development in human. Hence, it is crucial to also study hematopoiesis with the complementary knowledge obtained from human genetics, population statistics and clinical insights from patients as well as using primary human cells for functional and mechanistic studies. This approach was made possible due to the purification of human HSCs using cell surface markers and the use of viral system that enables genetic manipulation in primary cells. CD34, expressed on less than 5% of all blood cells, was the first marker found to enrich and mark human HSCs and progenitors [2, 4, 5]. Several other markers including CD38 [6], Thy1 (CD90) [7] and CD45RA [8] have later on been introduced to further distinguish HSCs from its progenitors with $CD34^+CD38^-Thy1^+CD45RA^-$ cells considered to be the most primitive population of

HSCs. These primary cells can be transduced with viruses carrying DNA sequences for gene overexpression or shRNAs for gene silencing. Transduced cells are sorted and examined for stem cell potentials in multiple functional assays such as in vitro surrogate colony forming units, stromal long-term culture initiating cells (LTC-IC) or Cobblestone area assays, and liquid culturing and in vivo xenotransplantation.

Studies using those complementary systems have shed light into the molecular basis of hematopoiesis. The fundamental issue in hematopoiesis is to understand the regulation of HSCs self-renewal, proliferation and differentiation. The decision to self-renew or to differentiate and what determines the cell fate remains the prime interest in the field. A wealth of evidence gained through tremendous number of studies has indicated regulation of gene expression via transcription and epigenetic regulation as the common mechanism that governs the hematopoietic system. These studies led to the identification of numerous transcription factors and epigenetic regulators to play essential roles in hematopoiesis and leukemogenesis [9, 10]. In addition, gene expression profiling in mouse and human hematopoiesis [11-13] revealed different gene signatures in HSCs and cells at various stages of differentiation. These results strongly supported a central role of gene expression regulation in controlling hematopoietic differentiation and lineage commitment. Despite these successes, the questions of the exact regulatory networks and epigenetic landmarks that shape these developmental stages and what drives the changes in genes regulatory networks leading to a next differentiation event still remain.

The blood system is arguably the best well studied human organ that has served as a paradigm for understanding stem cell biology as well as the implications of stem cell principles in diseases and particularly in oncogenesis. Knowledge of the normal

development is undoubtedly important to understand the impact of molecular aberrations found in hematological malignancies.



Adapted from Stuart H. Orkin and Leonard I. Zon. *Cell* 2008

Figure 2. Development regulation of hematopoiesis in the mouse.

(A). Hematopoiesis occurs first in the yolk sac (YS) blood islands and later at the AGM region, placenta, fetal liver and bone marrow in adult.

ECs, endothelial cells; RBCs, red blood cells; LT-HSC, long-term hematopoietic stem cell; ST-HSC, short-term hematopoietic stem cell; CMP, common myeloid progenitor; CLP, common lymphoid progenitor; MEP, megakaryocyte/erythroid progenitor; GMP, granulocyte/macrophage progenitor.

(B). Developmental time windows for shifting sites of hematopoiesis.

1.1.2 Malignant hematopoiesis and myeloid malignancies

Malignant hematopoiesis is characterized by the abnormal development of blood tissue accompanying a block in differentiation and/or aberrant proliferation of leukemia cells. The development of leukemia, like other cancers, is a step-wise process in which accumulation of mutations give rise to a clonal population of transformed cells. The deregulation of hematopoiesis can happen in both lymphoid and myeloid lineages. Even though there are variations in lymphoid and myeloid leukemia, the general principles discussed in details below for myeloid malignancies are shared by both.

Myeloid malignancies are disorders in the myeloid lineages including: erythroid, megakaryocyte, granulocyte, and monocyte (which are usually referred to as myeloid cells). Myeloid malignancies are clonal diseases comprising of diseases including myelodysplastic syndromes (MDS), myeloproliferative neoplasms (MPN), chronic myelomonocytic leukemia (CMML) and an acute phase in acute myeloid leukemia (AML). In myeloid malignancies, the activity of genes involved in key processes of self-renewal, proliferation, survival/apoptosis, and differentiation are perturbed [14] (Figure 3A). In many cases, impairments in DNA damage responses and oxidative DNA damage resulting from elevated ROS production creates a genomic instability environment predisposing cells to acquire mutations [15, 16]. Even though leukemia, like other cancers, is a heterogeneous disease comprising of numerous cell types with distinct features, the leukemic potentials reside in a small population of cells characterized as leukemic stem cells (LSCs) [17, 18]. One critical issue is to define the origin of LSC; in particular to answer the question of where at the hematopoietic hierarchy does the transformation take place. The current paradigm in the field recognizes both stem cells

and committed progenitors as potential “cells of origin”. According to the “stem cell” model, LSCs inherit self-renewal potential directly from HSCs and heterogeneity is the result of variables in the development program of LSCs influenced by specific transformation events. On the other hand, the “progenitor” model postulates that the phenotype of leukemia is dependent on the degree of lineage commitment of targeted progenitors and self-renewal is gain through acquisition of mutations. While there are evidences to support and dispute one versus the other [19], both models place emphasis on self-renewal, a unique feature of LSCs that is essential for sustainment of the disease.

A two hit model was first proposed by Gilliland in 2001 to explain the molecular pathogenesis of hematopoietic malignancies using acute leukemia as a proof of concept [20]. The heart of the model lies in the notion that acute leukemia is caused by two classes of mutations: Class I oncoprotein mutations confer a proliferative and/or survival advantage, whereas class II mutations impair hematopoietic differentiation. The two together cause the acute leukemic phenotype of enhanced proliferation and survival with impaired differentiation. Classical examples of those mutations are BCR-ABL and FLT3-ITD in class I and AML1-ETO, PML-RAR α and C/EBP α loss of function in class II. Oncogenic cooperation has been observed in BCR-ABL- positive CML progressing to AML upon acquisition of AML1-EV1 fusion [21], in AML1-ETO driven leukemia with mutations in tyrosine kinases FLT3 or KIT [22-24], or in JAK2-NPM progressing to AML with the addition of AML1-ETO [25]. The later evidence came from a clinical study showing a sequential acquirement of JAK2V617F mutation and AML1-ETO at two distinct disease phases: NPM and later on in AML. Even though the concept is somewhat simplified and there is no doubt of variations from the theme (such as the addition of

another class of mutations in epigenetic regulators), the paradigm does capture the essence of the multistep model of tumorigenesis, during which oncogenic cooperativity are required for leukemia progression of a full blown malignant disease. A view of the two hit model is depicted in Figure 3B.

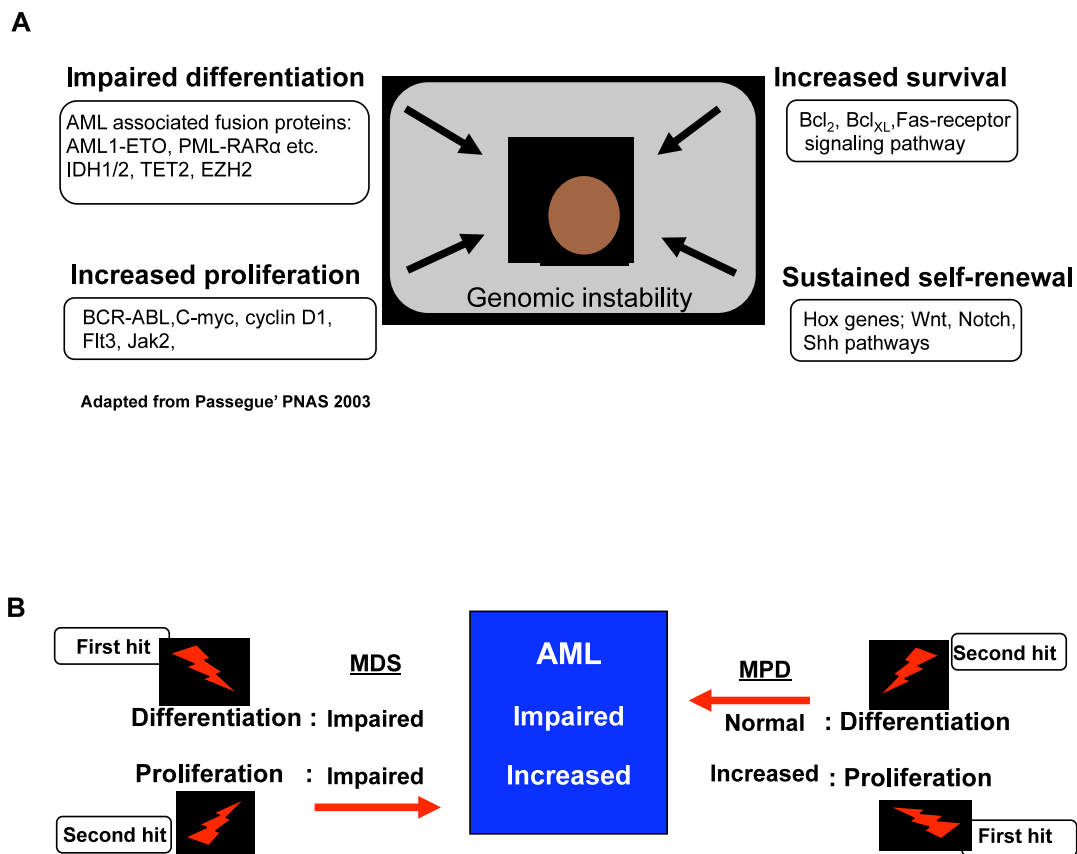


Figure 3. Deregulated pathways leading to leukemia.

(A). General mechanisms underlying leukemic transformation: impaired differentiation, increased in proliferation, cell survival and self-renewal. Few examples of such deregulated pathways found in various types of leukemia are depicted.

(B). The two-hit model for the transformation of acute myeloid leukemia.

1.2 Transcription regulation of Myeloid Differentiation

Granulocytes and monocytes, referred to as myeloid cells (together with their committed progenitors), are key mediators of the innate immunity system. Myeloid differentiation-myelopoiesis - is the process during which these myeloid cells arise from HSCs through a number of sequential lineage specification and maturation steps. Understanding the normal differentiation process is critical for the study of myeloid leukemia, where normal development is blocked. The regulation of myeloid differentiation involves a network of regulatory factors in transcription and epigenetic regulation, and cytokine and signaling cascades. The intricate interaction and precise coordination between these regulatory elements are essential to ensure the proper generation of myeloid cells.

It is well established in the field that transcription regulation governs the process of myeloid differentiation. The network of key transcription factors orchestrates the expression of myeloid gene signatures corresponding to the differentiation program. Epigenetic regulation including DNA methylation, histone modifications and chromatin remodeling and gene expression silencing via small non-coding RNAs add another layer to transcriptional control, in which the establishment and modulation of epigenetic marks as well as the involvement of these regulatory factors allows for dynamic regulation of gene expression. The central role of transcription factors (TFs) and epigenetic regulators is highlighted by the fact that numerous genetic lesions as well as aberrant expression patterns of those factors are observed in myeloid malignancies.

Transcription factors in myelopoiesis

There is no single master myeloid transcription factor that can single-handedly drive myeloid lineage commitment and specification. Instead, the formation of myeloid cells is controlled by a number of transcription factors [26] including runt-related transcription factor-RUNX1 (described in more details in a following section), PU.1 [27], CCAAT/enhancer binding proteins (C/EBPs) [28, 29], growth factor independent 1 (GFI-1) [30], KLF4 [31], MYC [32] and AP1 proteins [33]. Two proteins PU.1 and C/EBP α , provide excellent examples of transcriptional regulation by TFs in myeloid differentiation. Mutations of in both *sp1/pu.1* and *c/ebp α* genes have been found in AML [34, 35].

PU.1 is a member of the ETS family of transcription factors that contain a winged helix-turn-helix- type DNA binding domain. PU.1 expression is not restricted to the myeloid lineage as it is also detected in lymphoid cells [36, 37]. Indeed, PU.1 expression fluctuates dynamically to regulate various lineage differentiation processes [36]. PU.1 deficient mice exhibit defects in neutrophil, macrophage and B cell development [38, 39], indicating that PU.1 is required for the generation of those cells. Moreover, the regulation of differentiation by PU.1 is dose-dependent in lymphoid cells [40, 41]. It has been shown that distinct levels of PU.1 expression determine cell fate; low levels of PU.1 favor MPPs to differentiate to B cells while high level of PU.1 generates macrophages. In myeloid lineages, PU.1 expression at high level results in production of macrophages while low PU.1 expression supports granulocytes [42, 43]. Furthermore, altered expression of PU.1 leads to different outcomes, PU.1 expression levels 20% of wild type levels blocks myeloid differentiation, while a 50% reduction in PU.1 expression is

compatible with normal hematopoiesis[43, 44]. The requirement of a precise expression level of PU.1 expression for normal myelopoiesis reflects the importance of PU.1 in regulating myeloid gene expression profiles. PU.1 regulates a larger number of myeloid specific genes. Notable target genes are genes encoding cytokines receptors: M-CSFR, GM-CSFR and G-CSFR; myeloid antigens: CD11b, CD18 [45]; primary granule enzymes: myeloperoxidase (MPO) [46], neutrophil elastase (NE) [47], TFs: PU.1 [47], JUNB [47], EGR-2 [48], KLF4 [31] and the microRNAs: miR-223 [49], miR-424 [50] and others.

C/EBP α belongs to a TF family, which has 6 members of related leucine-zipper transcription factors. C/EBP α is predominantly expressed in myeloid cells with a high level in immature HSCs, CMP and GMP cells [51]. Compelling evidence from the study of C/EBP α loss of function in mouse models indicate a central role of C/EBP α in myeloid differentiation. Deletion of C/EBP α in mice results in a complete lack of neutrophils while retaining all other lineages [28, 52]. The phenotype is accompanied with a block in CMP to GMP transition in adult stage [52]. Like PU.1, C/EBP α regulates expression of various important myeloid genes such as CSFR3 (the G-CSF receptor)[45]. This network of target genes can be one of the reasons for the lack of myelopoiesis when C/EBP α is depleted. Another way for C/EBP α to regulate the differentiation process is through modulating cell cycle progression and proliferation. An example for such regulatory mechanism is the repression of E2F1 function by C/EBP α via direct interaction, which results in growth inhibition and terminal differentiation of granulocyte [53, 54].

Even though each of those myeloid TFs plays a distinct, non-redundant role in myeloid differentiation, it is an interplay between them that forms a transcriptional circuit

directing the differentiation program. The relationship between PU.1 and C/EBP α is an excellent example. The decision to differentiate to either macrophages or neutrophils is regulated by PU.1 and C/EBP α ratio as due to their antagonistic effects on the activity of other proteins, which can tip the scale between granulocyte and monocyte differentiation[42]. On the other hand, PU.1 and C/EBP α play a synergistic role in regulating monocyte function in response to NF- κ B activation[55]. Moreover, upregulation of PU.1 expression by C/EBP α is important for aspects of granulocyte maturation [56]. Data from genome wide studies have revealed a complex network of multiple TFs, which mutually coordinate their activities to drive the differentiation process [57]. To achieve such delicate control, multiple layers of regulation have been employed to connect and fine-tune the activity of these transcription factors. Moreover, the ability of TFs to modulate gene transcription is dependent on their association with transcriptional co-activator(s) or co-repressor(s) and epigenetic regulators. Regulation of transcriptional factor function via post-translational modification and protein-protein interactions will be discussed in greater detail, for RUNX1 in a later section (*1.3. RUNX1 and post-translational modifications of RUNX1*).

Epigenetic regulation of myeloid differentiation

Epigenetic regulation of gene transcription refers to stable and inheritable patterns of gene expression that do not involve alternations in DNA sequence. This regulation is brought about through multiple mechanisms that include post-translational histone modifications, DNA methylation and (recently recognized) RNA interference via small noncoding RNAs. Epigenetic regulators have captured interest and attention of scientists

in the field due to their involvement in chromosomal translocations and in somatic mutations that are frequently identified in myeloid malignancies [10, 58, 59]. Subsequent functional studies, in both mouse and human models, have helped bring to light the central role of those factors in regulation of hematopoiesis, and myelopoiesis in particular. Importantly, emerging therapeutic interventions have focused on targeting these regulators to exploit the reversible nature of epigenetic modifications.

Nucleosomes and histone modifications

Transcription in eukaryote is regulated in a chromatin-dependent context. Chromatin is an organized nucleoprotein complex in which DNA is packaged. The chromatin structure is based upon the nucleosome – a basic repeating unit, which comprises 147 bp of DNA wrapped around a core of eight histones including two molecules each of H2A, H2B, H3 and H4, and linker histone (H1). This compressed structure provides a mean to compact the basic DNA strand while at the same time poses a barrier to direct accessibility of DNA to TFs and their cofactors. Therefore, the ability to regulate and alter the chromatin structure via chromatin remodeling and histone modifications is extremely important in transcription regulation [60, 61].

The structure of chromatin can be reconfigured via ATP-dependent remodeling processes. Three groups of chromatin-remodeling complexes, which all contain DNA-dependent ATPase activity, have been identified: the SNF2 subfamily with the catalytic subunits BRG-1 and BRM; the ISWI subfamily with the SNF2-homology or SNF2-like subunits and the chromodomain 1 (CHD1) or Mi-2 α /NuRD subfamily. These proteins function in multi-subunit machinery to mobilize and rearrange nucleosomes[62, 63]. These processes result in changes in nucleosome position, and subsequently chromatin

structure, which correspond to either an “open” conformation to facilitate transcription or a “closed” configuration favoring transcriptional silencing. An important role of chromatin-remodeling complexes in development and oncogenesis has been well established [60, 64-67]. Disruption of SNF2 ATPases or subunits in the complexes (except for BRM) results in embryonic lethality while haploinsufficiency of INI1 or BRG1 leads to an increased incidence of cancer[64, 68]. Several studies have also suggested an involvement of these proteins in myeloid differentiation. Expression of a subset of myeloid genes controlled by the transcription factors C/EBP β and MYB requires the recruitment of BRM via physical interaction with C/EBP β [69]. This was the first report of endogenous genes regulated by the SWI/SNF complex. Later studies, using a more biologically relevant system, revealed the direct involvement of Brg1 in myeloid differentiation. Forced expression of a dominant negative mutant of Brg1 in murine myeloid progenitor cells delays G-CSF induced granulocytic differentiation[70].

Another line of evidence comes from studies of oncogenic fusion proteins associated with chromatin-modifying factors. ENL, a fusion partner of MLL (myeloid/lymphoid or mixed-lineage leukemia in MLL-ENL) was identified as a subunit in SWI/SNF complexes. The fusion protein MLL-ENL cooperates with the remodeling complexes to trans-activate the HoxA7 promoter[71].

Chromatin structure is also regulated by covalent modifications of histones. Histones, especially their extending tails, are subjected to numerous post-translational modifications including methylation, acetylation, phosphorylation, ubiquitylation and SUMOylation. These modifications bring about intrinsic changes in chromatin structure which would enhance or inhibit the binding of cofactors, thereby affecting gene

expression through both transcription activation and repression [72]. Enzymes that can either add, or remove those specific chemical groups in histones are called histone-modifying enzymes. A role for those proteins in the transcriptional regulation of myeloid differentiation and in myeloid malignancies will be comprehensively reviewed in the following section (*1.4. Targeting histone-modifying enzymes for leukemia therapy*) using several examples as proof of concept.

DNA methylation

DNA methylation is an epigenetic mark involving addition or removal of a methyl group at the fifth position of cytosine in CpG dinucleotides. DNA methylation occurs predominantly in repetitive regions across the genome and at dense CG regions, termed CpG islands. These CpG islands are highly prevalent near transcriptional regulatory regions of housekeeping and essential development regulator genes[73]. In normal cells, while most of the CpGs (70-80%) in the genome are hypermethylated, CpG islands, especially those associated with promoters, are generally hypomethylated[74]. These methylation patterns are perturbed in many cancers, including leukemia, which is characterized by overall genome wide DNA hypomethylation and aberrant hypermethylation at promoters of several tumor repressor genes such as *p15^{INK4b}* and *p16^{INK4a}* [75]. The transcriptional effect of DNA methylation is generally stable gene silencing. DNA methylation can interfere with transcription via two main mechanisms[73]. The presence of the methyl group itself at CpG sequences hinders the binding of factors required for transcription[76]. On the other hand, methylation at the CpG dinucleotides creates a docking site for the binding of methyl-CpG-binding proteins

and their associated repressors, resulting in suppression of gene expression. During hematopoietic differentiation, the regulation of DNA methylation has been employed to coordinate changes in gene expression. Upon differentiation of progenitor cells, promoters of genes whose functions are to maintain stemness, such as *Meis1*, are silenced and marked with methylation while genes responsible for myeloid lineage differentiation such as *Gadd45a* are demethylated and actively transcribed [12, 77]. In addition, distinct DNA methylation signatures of AML subtypes were identified via genome-wide profiling of AML patients, suggesting a direct contribution of aberrant epigenetic regulation by DNA methylation to the pathogenesis of myeloid malignancies [78]. Thus, properly established and maintained DNA methylation patterns are essential for the normal development of myeloid cells.

DNA methylation is mediated by three conserved DNA methyltransferase (DNMT) enzymes: DNMT1, DNMT3A and DNMT3B. While DNMT3A and DNMT3B are responsible for de novo methylation[79], DNMT1 helps maintain DNA methylation patterns. The functions of these DNMTs are absolutely required for normal development as knockout of *dnmt1* and *dnmt3b* result in embryonic lethality while mice that lack *dnmt3a* die shortly after birth[79, 80]. Studies of conditional *dnmt1* knockout mice reveal a profound defect in HSC self-renewal and specific impacts on myeloid progenitor cells with their differentiation potential skewed toward myeloid fates [81, 82]. On the other hand, loss of DNMT3A or DNMT3B results in minimal but definitive phenotypic effects on HSCs as HSC function impairments was clearly observed after serial transplantation[83, 84]. Interestingly, among the DNMT proteins, DNMT3A is the only member with mutations frequently observed in myeloid malignancies[59, 85]. Although much work is

still needed to explain the relevance of DNMT3A deregulation in AML, it is worthy to note that serially transplanted *dnmt3a* null HSCs exhibit aberrant DNA methylation patterns with prominent CpG hypermethylation. The *dnmt3a* null differentiated hematopoietic cells show a more global hypomethylation with an increased expression of several stem cell associated genes. This landscape is similar to that of transformed cells, suggesting that abnormal DNMT3A function could provide a favorable epigenetic setting for leukemic transformation.

DNA methylation was thought to be irreversible and considered as a permanent epigenetic mark. However, it was later shown that is the Ten-eleven translocation (TET) protein family members could convert 5-methylcytosine (5mC) to 5-hydroxymethylcytosine (5hmC) [86]. After this discovery, 5-formylcytosine (5fC) and 5-carboxylcytosine (5caC) were found as continuous products from a stepwise oxidation of 5mC catalyzed by TET enzymes [87, 88]. These two variants exist in much lower abundance compared to 5hmC and can be removed by thymine DNA glycosylase[89]. While DNA methylation is generally viewed as a “silencing” mark, the conversion of 5mC to 5hmC does not always result in gene activation. It appears to have dual roles in transcriptional regulation as a genome-wide profiling of 5hmC revealed the presence of 5hmC at both active and repressed genes[90]. Even though it was firstly identified as an intermediate of the DNA demethylation process, 5hmC is also an independent epigenetic modification. 5hmC itself can block the binding of methyl-binding protein[91] while also recruiting nucleosomal remodeling and deacetylase complexes[92]. Interestingly, somatic deletion or inactivating mutations of TET2 is quite common in myeloid leukemia[59, 93]. The levels of 5hmC in bone marrow samples of patients with TET2 mutations is

significantly lower than those of healthy control[94]. Studies of TET2 function in normal hematopoiesis reveal a role for TET2 in HSC self-renewal and myelopoiesis. Expansion of myeloid compartments and in particular monocyte lineage was observed upon targeted deletion of TET2 both in vitro[94] and in vivo[95].

MicroRNAs in myelopoiesis

A number of studies have established the essential role of the network of microRNAs (miRNA) on transcriptional regulation in myeloid cell development and function[96-98]. The list of those microRNAs is undoubtedly going to expand as our understanding of microRNA expression and function matures. MicroRNAs are 20-22 nucleotide (nt) small regulatory RNAs that bind to the 3' untranslated region (UTR) of target mRNAs, and regulate gene expression via mRNA degradation and/or translational repression[99]. MicroRNAs biogenesis involves the transcription of primary precursors called pri-miRNA from miRNA encoding regions, followed by a processing step mediated by RNase Drosha to generate a 70-120 nucleotide hairpin structure - pre-miRNA. This precursor is exported to cytoplasm where it undergoes another step of processing by Dicer to generate a mature miRNA that is later on incorporated into RNA interfering silencing complex (RISC)[100]. The regulation mediated by microRNAs provides an additional level of control beyond transcriptional regulation by TFs. By modulating the expression of their target genes, miRNAs exert their roles in fine-tuning the differentiation of myeloid cells (and all other cell types).

The first microRNA found to play critical role in myeloid differentiation was miR-223. MiR-223 is predominantly expressed in myeloid cells[101]. Loss of miR-223 impairs

granulocytic maturation[102], while miR-223 overexpression promotes myeloid differentiation[103]. MiR-223 expression has been shown to be transcriptionally regulated by several critical myeloid TFs including NF-IA[103], C/EBPs and PU.1[49] and by E2F1[104]. Fazi et al. reported that the AML1-ETO fusion protein represses miR-223 expression by binding to a RUNX CBS located upstream of the pre-miR-223[105]. They, and others, have found that miR-223 expression is downregulated in AML patient samples[104-106]. Many miR-223 bona fide target genes are several TFs critical for myelopoiesis: NF-IA, MEF2C and E2F1; this forms a close regulatory loop where a microRNA and a regulatory TF reciprocally control expression of each other. A number of miRNAs regulated during RA-induced granulocytic differentiation of APL cells was identified using a miRNA microarray platform[107]. The data suggests that regulation of differentiation process requires complementary activities of a network of miRNAs.

Using the same approach of miRNA microarray screening, Velu et al. identified miR-21 and miR-196b as important target genes of Gfi1 in the control of myelopoiesis[108]. Gfi1 repressed the expression of these microRNAs upon differentiation as miR-21 and miR-196b negatively regulate myeloid differentiation. The complete block in granulopoiesis that recapitulate the phenotype observed in Gfi1 knockout mice is achieved only when both microRNAs are overexpressed, suggesting that the two microRNAs work cooperatively to modulate the differentiation process. During monocytopoiesis, a regulatory loop consisting of the miRNA 17-5p-20a-106a cluster, RUNX1 and M-CSF is critical for monocyte differentiation and maturation[109]. Several recent studies have identified roles for microRNAs in both normal myelopoiesis and leukemia such as: miR-29a[110], miR-328[111] etc. As many more miRNAs will be shown to play importance

roles in myeloid differentiation and in hematopoiesis, the next challenge will be to integrate miRNAs into the network of hematopoietic regulators as well as to decipher the regulation of miRNA expression and functions. We can then apply our knowledge of their biology to help develop novel therapeutic strategies in leukemia.

1.3 RUNX1 and post-translational modification of RUNX1

RUNX1: a pivotal transcription factor in hematopoiesis

RUNX1 (also known as acute myeloid leukemia - AML1, CBF α 2 or polyoma enhancer-binding protein 2 α B - PEPB2 α B) belongs to the core-binding factor (CBF) family of transcriptional regulators. The RUNX1 binding sequence -PyGpyGGTPy (Py = pyrimidine) is present in promoter and enhancer regions of various genes known to play important roles in development. There are three members of the family including RUNX1, RUNX2 and RUNX3. They share a highly conserved region of 128 amino acids, designated as the Runt homologous domain (RHD), which mediates DNA binding and interacts with CBF β ; this interaction with CBF β is required for RUNX1 function in vivo[112, 113].

RUNX1 plays a crucial role in hematopoiesis. RUNX1 knock out mice die during embryonic day [E] 11.5 - [E] 13.5 from hemorrhaging into the central nervous system and soft tissues; additionally, there is a complete lack of fetal liver-derived definitive hematopoiesis[114, 115]. However, conditional deletion of RUNX1 in adult mice revealed that RUNX1 function is dispensable for the maintenance of hematopoietic stem cells (HSCs). Disruption of RUNX1 function resulted in several lineage-specific abnormalities, including a block in lymphoid development, reduced platelet production

and development of a myeloproliferative phenotype[116, 117]. Notably, no spontaneous leukemia was observed in the RUNX1 ^{-/-} adult mice.

Several studies have demonstrated an essential role of RUNX1 in monocytopoiesis. Apoptosis of myeloid colony-forming cells in RUNX1 knockout mice demonstrates that a certain degree of maturation arrest occurs in the absence of RUNX1. Moreover, knock out of RUNX1 in embryonic stem cells impairs monocytic differentiation in culture. Fontana L. et al. (2007) demonstrated that in human CD34⁺ hematopoietic stem progenitor cells an increase in RUNX1 protein level during monocytic differentiation is achieved by the concurrent down regulation of RUNX1 – targeting miRNAs. Impairments in RUNX1 upregulation resulted in a block of monocytic differentiation and maturation[109]. RUNX1 controls transcription of many critical lineage specific factors in hematopoiesis including: TCR α , IL-3, CD41, GM-CSF, M-CSF receptor, CBF α , and Pu.1[118, 119].

RUNX1 in leukemogenesis

RUNX1 is one of the most frequently targeted genes in leukemia. RUNX1 was first identified based on its involvement in the fusion protein RUNX1 (AML1)-ETO, a product of the t (8; 21) chromosomal translocation that is found in about 40% of AML subtype M2. To date, over 30 different chromosomal translocations involving RUNX1 have been identified in leukemia or MDS patients [119-121].

The mechanism of RUNX1 dysregulation by RUNX1-ETO in leukemogenesis is well studied. In RUNX1- ETO, the N-terminus of RUNX1, including the RHD, is fused in frame with the active ETO – the Eight-Twenty-One coding region. Expression of the

fusion protein RUNX1-ETO results in an impairment of myeloid differentiation and an increased self-renewal capacity of HSCs [122, 123]. The intact RUNX1 –ETO fusion protein can out-compete the endogenous RUNX1 protein for DNA target sequence binding. Moreover, RUNX1-ETO is able to recruit co-repressors including SIN3A, nuclear receptor co-repressor-NCOR, histone deacetylase-HDACs[124, 125] and potentially DNA methyltransferase – Dnmt1[126] via the ETO portion of the fusion protein, in order to repress RUNX1-mediated gene transcription.

Our group has recently reported also a transcriptional activation effect of RUNX-ETO. This function is dependent on its interaction and acetylation at the N-terminal by p300, resulting in potential recruitment of bromodomain containing proteins[127]. Zhang, et al. (2004) demonstrated that RUNX1-ETO, as well as ETO but not RUNX1, stably interacts with E proteins, a key transcription factor in the regulation of cell growth, differentiation and cell death. This stable interaction precludes recruitment of p300/CREB-binding protein (CBP) co activators, resulting in silencing of E proteins transcriptional activation[128]. However, other studies suggest that the E protein interaction appears to contribute relatively little to RUNX1-ETO leukemia promoting effects. Yan, et al. (2009) showed that deletion of the E-protein interaction domain in RUNX1-ETO9a (an isoform of RUNX1-ETO, which can induce leukemia de novo has no effect on its leukemic activity[129]. Park, et al (2009) has been able to identify key residues in RUNX1-ETO that mediate its interaction with the E protein- HEB. Mutations of these residues do not impair RUNX1-ETO's ability to enhance clonogenic capacity and to repress differentiation of primary mouse bone marrow cells[130]. However, given the importance of the interaction with p300 and the acetylation mediated by p300 in leukemia promoting

functions of RUNX1-ETO, it appears that the association with E-proteins is still critical. Further studies are needed to definitely address these complex relationships between RUNX1-ETO and its interacting partners.

In addition to chromosomal translocation, inherited or acquired mutations of RUNX1 represent a second mode by which RUNX1 can be dysregulated[131]. Frame shift and nonsense mutations can result in a trans-activation domain deficient RUNX1. Missense mutations, clustering in RHD of RUNX1, result in a loss of function-DNA binding deficient RUNX1. Point mutations in RUNX1 are implicated in familial platelet disorder with propensity to AML (FPD/AML) and identified in about 10% of sporadic myeloid malignancies[132]. Studies of heterozygous RUNX1 +/- mice demonstrated that loss of one allele of RUNX1 led to haploinsufficiency and RUNX1 function might indeed be dose -dependent. Therefore, fine-tuning of the expression level and activity of RUNX1 is pivotal for proper hematopoietic development.

It is noted that dysregulation of RUNX1 function alone is not sufficient to cause leukemia. Additional genetic changes are required for the development of a full-blown disease[133].

RUNX1 transcriptional activity is regulated by protein-protein interactions and post-translational modifications

Although RUNX1 is a pivotal transcription factor, the transcriptional activity of the RUNX1 proteins is highly context dependent. While being a weak activator of several in vitro transcriptional reporter constructs, in cellular conditions, RUNX1 can act either as an activator or a repressor, depending on the promoter and its interacting partners.

RUNX1 acts synergistically to activate specific target genes with many transcription factors, including GATA-1[134], C/EBP α [135], Pu.1[136] etc. Besides these, RUNX1 is able to interact with several co-activators such as YAP[137], ALY[138], p300/CBP[139], MOZ[140] and PRMT1[141] and with co-repressors, including SIN3A[142] and a mammalian homolog of Drosophila Groucho complex-TLE complex[143]. RUNX1 also has been shown to physically and functionally interact with the SWI/SNF chromatin remodeling complex[144, 145]. Overall, RUNX1 seems to act as a DNA binding organizer, recruiting other transcriptional regulatory factors. These factors can either activate or repress transcription through their direct effects on basal transcription machinery and/or their ability to alter chromatin structure.

Notably, interaction of RUNX1 with different partners during hematopoietic differentiation appears to be lineage-specific. RUNX1 and GATA-1 cooperate particularly in megakaryocytopoiesis, while RUNX1 and C/EBP α specifically function in directing myeloid differentiation. RUNX1 also interacts with Cdk6 in moderating proliferation and differentiation during myeloid development. Modulation of RUNX1's interaction network can modulate the switch between proliferation, self-renewal and lineage- differentiation. However, how RUNX1 selects particular partners over others to assemble a functional complex is largely unknown.

Posttranslational modifications (PTMs) have been implicated as a critical regulatory mode of RUNX1 transcriptional function. Posttranslational modifications of RUNX1 include: ubiquitination, phosphorylation, acetylation and methylation. Ubiquitinated RUNX1 is targeted to proteasome-mediated degradation[146]. RUNX1 is phosphorylated by Erk (extracellular signal regulated kinase) in response to IL- 3[147], phorbol

ester[148] and thrombopoietin[149]. ERK-dependent phosphorylation results in shedding of RUNX1 from the SIN3A complex further activating its transcriptional activity[150]. Phosphorylation of RUNX1 by cyclin-dependent kinase-cdk1 and cdk2 destabilizes RUNX1 during G2/M[151]. It also regulates RUNX1 transactivation in a cell cycle-dependent manner[152]. Moreover, RUNX1 is acetylated by the histone acetyltransferases p300/53 and MOZ; RUNX1 acetylation results in enhanced DNA binding and increased transcriptional activation[153].

Recently, our lab demonstrated that RUNX1 is arginine methylated on multiple sites[141]. Methylation of RUNX1 by PRMT1 on an RTAMR motif abrogates SIN3A binding, and potentiates RUNX1 transcriptional activity. PRMT1 regulates RUNX1 transcription activation of CD41 and Pu.1 during early myeloid differentiation of primary human hematopoietic CD34+ cells. The study demonstrated for the first time that RUNX1 function is regulated by arginine methylation. We wish to explore whether arginine methylation is a universal regulatory pathway controlling RUNX1 function during hematopoiesis.

1.4 Targeting histone modifying enzymes for leukemia therapy ¹

Histone modifying enzymes catalyze the addition or removal of covalent, post-translational modifications (PTMs) in histone and non-histone proteins. These modifications include methylation, acetylation, phosphorylation, ubiquitination and sumoylation, and can regulate protein function by altering the protein's enzymatic activity, localization within the cell, and protein-protein interactions. Histone modifying enzymes can be classified as “writers” which add PTMs or “erasers”, proteins that can

¹ *This section is reproduced here verbatim from reference 149*

remove or alter the presence of specific PTM on histones. Another level of regulation is provided by “readers” of chromatin structure, those proteins whose domains recognize specific histone residues, generally based on the presence or absence of specific PTMs. In addition to histones, these enzymes have a broader range of substrates and as such, can regulate numerous cellular processes, including gene expression, RNA processing and the DNA damage response. Accumulating evidence has shown that these histone-modifying enzymes play an important role in regulating virtually all aspects of hematopoiesis. Furthermore, many of these “writers, readers, or erasers” have been shown to be abnormally regulated in cancer. The epigenetic landscape is clearly altered in acute leukemia, due to a variety of acquired lesions in chromatin modifier genes, or changes in their level of expression. This provides the rationale for exploring how these abnormalities can be targeted by new therapeutic approaches. Several examples are discussed below as proof of concept for the potential of these new approaches [154].

Protein Methylation

There are two families of histone methyltransferases, the lysine methyltransferases (PKMTs) and the protein arginine methyltransferases (PRMTs). The side chain of lysine residues can be mono-, di- or tri- methylated, while the nitrogens in arginine residues can be monomethylated or symmetrically or asymmetrically dimethylated. Unlike acetylation or phosphorylation, methylation does not change the overall charge of the molecule, however, the bulkiness of the methyl group can either promote or inhibit protein-protein interactions. These methyl marks are recognized by specific binding motifs, which include the Tudor domains, chromo domains, MBT (malignant brain tumor) domains and PHD fingers; proteins containing these motifs can distinguish the target residue (lysine

vs. arginine) as well as the state of methylation. Crystal structures now exist for many of these interactions, which highlight their specificity. Recognition of combinations of PTMs dictates their output in terms of gene expression and cell behavior. Histone lysine methylation can be reversed by demethylases, which are grouped into 2 classes: (1) the amine-oxidase type lysine specific demethylases (LSD1 and LSD2), and (2) the Jumanji (JmJ) C-domain containing histone demethylases. Not all histone lysine methyl marks appear to be susceptible to rapid reversibility, and for arginine methylation, the reversibility has not been clearly established. Rather, arginine methylation can be further chemically converted into citrulline (by the protein arginine demethylase PAD4). It is unclear what additional modifications add further complexity to this dynamic process.

The regulation of histone methylation has been shown to be important in numerous hematopoietic processes. Alteration of a number of proteins involved in the methylation of histone and non-histone substrates, have now been reported in leukemia, and many other hematologic and non-hematologic cancers [155] [156].

Protein lysine methyltransferases

The two families of protein lysine methyltransferases are characterized by the presence or absence of a SET domain: The SET domain-containing PKMTs include MLL, EZH2, NSD1 and SET7/9 (G9a), that methylate numerous substrates including histone H3 (K4, K9, K27 and K36) and H4 (K20), as well as a number of non-histone proteins, such as p53, TAF10, E2F, STAT3 and NF- κ B. The PKMTs that lack a SET domain include hDOTL1, a PKMT that methylates histone H3K79; and histone lysine methylation is intimately involved in gene regulation, influencing chromatin structure, a key element of the transcriptional status of a gene. Thus, H3K9me3 and H3K27me3 are typically

associated with heterochromatin and gene repression, while H3K4me, H3K36me and H3K79me are associated with transcriptionally active regions, which are primarily located in regions of euchromatin.

Methylation of transcription factors can alter their function, and profoundly influence the expression of their target genes. Histone methyltransferases are also components of large, multi-protein nuclear complexes that contain other histone modifying enzymes and other regulatory proteins including histone acetyltransferases (HATs), histone deacetylases (HDACs), DNA methyltransferases (DNMTs) and SWI/SNF complex components. The complex nature of these interactions ensures the appropriate regulation of transcription during the execution of multiple differentiation programs that are required for normal hematopoiesis. Impairment at any step can promote the process of malignant transformation.

MLL

The *MLL* (Mixed lineage leukemia) gene encodes a PKMT that is the mammalian homolog of the *Drosophila trithorax* (*Trx*) gene. The methyltransferase (SET) domain of *MLL* is involved in methylating H3K4, a mark usually associated with gene activation. Chromosomal rearrangements involving the *MLL* gene, which is located at 11q23, are seen in both AML and ALL. *MLL* is fused to more than 50 different partner genes. These distinct fusions are associated with unique clinical characteristics and often a poor outcome [157]. Many of these *MLL* fusions result in loss of the SET domain, although the fusion proteins often retain their DNA-binding domain and can positively regulate *MLL* target genes, including the *Hox* genes, a class of proteins critical for the regulation of differentiation and self-renewal. In many cases, the *MLL* fusion partner brings gain-of-

functions, for example the AF10 portion of MLL-AF10 fusion protein recruits hDOT1L, a H3K79 PKMT. H3K79 methylation is generally associated with high level expression of MLL target genes such as *HoxA9*, which promote leukemic cell transformation [158]. Other MLL fusion partners also interact with hDOT1L including AF9 [159], AF4[160], and ENL [161] [157], among others. In the Okuda study, the expression of an enzymatically dead form of hDOT1L, or knock down of hDOT1L using siRNA, abrogated the leukemia promoting activity of MLL fusions.

Several therapeutic approaches have been taken to target MLL-induced leukemia, including blocking interactions between MLL fusion proteins with functional effectors or targeting the downstream targets and regulatory pathways [162]. Given the role of hDOT1L in MLL driven leukemia, efforts to target hDOT1L have been prioritized [163, 164]. Bernt et al. provided direct evidence for the essential function of DOT1L in MLL-driven leukemia, as they found a significant reduction in the in vivo transformation of Dot1L *-/-* cells by MLL-AF9 [164]. In the study by Daigle et.al, the authors identified a highly potent and selective inhibitor of DOT1L, EPZ004777 that selectively killed MLL-driven leukemic cells with minimum effect on non-MLL-rearranged cells. EPZ004777 also significantly reduced the growth of subcutaneously injected MV4-11 cells in tumor bearing mice, suggesting that small molecule inhibitors of DOT1L may be useful in the treatment of MLL-induced acute leukemia.

EZH2

The maintenance of gene activation promoted by the TrxG proteins is counteracted by the activity of the polycomb (PcG) proteins, which maintain gene repression. EZH2 is a catalytic component of the PcG repressive complex (PRC2), which mediates the

trimethylation of H3K27 (H3K27me3) [165]. The H3K27me3 mark serves as the docking site for the Polycomb proteins, such as PC3, that are contained within PRC1, promoting the silencing of repressed target genes. EZH2 mutations are found in several hematologic malignancies, with loss-of-function mutations identified in patients with myelodysplastic syndromes (MDSs), myeloproliferative neoplasms (MPNs) [166] [167] and T-acute lymphoblastic leukemia [168] [169]. Patients with these mutations appear to have a poorer than average prognosis, and these events identify EZH2 as a tumor suppressor protein. In contrast, gain-of-function mutations in EZH2, at a single tyrosine residue (Tyr 641) in the SET domain, are found in large B-cell lymphoma patients [170], supporting the notion that EZH2 can also function as an oncogene, consistent with prior reports that EZH2 is overexpressed in breast cancer and prostate cancer [171]. Furthermore, two recent studies demonstrated a role for EZH2 in promoting MLL-AF9 driven leukemia [172] [173]. Given the potential opposing roles for EZH2 in these disorders, it will be important to assess which malignancies are dependent on EZH2 function for their maintenance.

NSD1

Another PKMT involved in AML is NSD1 (nuclear receptor-binding SET domain protein 1), which is fused to NUP98 by the cryptic t(5;11) translocation, which is seen in childhood AML [174] and adult AML [175] [176], and generally confers a poor prognosis. NSD1 methylates H3K36, which is generally an activation mark, and the NUP98-NSD1 fusion protein retains the PhD fingers and the SET domain from NSD1. Target genes of NUP98-NSD1 include the *Hox* and *MEIS1* genes, which are normally repressed by H3K27 methylation. De-regulation of the target genes of NSD1 presumably

leads to the transformation of myeloid progenitor cells and the development of AML [177].

Readers of methylation: PhD fingers

An example of a methyl-lysine “reader” that is altered in cancer, is the NUP98-JARID1A chimeric protein [178], which contains the PHD-containing domain of JARID1A. Wang et al. demonstrated that the PHD domains of either the NUP98-JARID1A-PHD3 or a NUP98-PHF23-PHD chimeric protein were essential for their ability to induce leukemia in several model systems. This effect seems to be induced by the sustained expression of several Hox genes, and also Meis1, Gata3 and Pbx1. The PHD domain in these proteins recognizes the H3K4me3 mark, and when mutated there is no activation of these target genes, or a leukemic potential, when the fusion proteins are expressed. As these two translocations were identified in patients with AML [179] [180], this work implicates both the writers and the readers of activating histone marks. These studies also support attempts to target these interactions in novel therapies for acute leukemia.

Protein lysine demethylases

Until the discovery of LSD1 (lysine-specific histone demethylase-1) and the JmJC-domain-containing histone demethylases, histone methyl marks were regarded as part of the permanent “epigenetic” signature [181]. However, the ability of demethylases to remove methyl groups from histone substrates identified this mark as a dynamic one. The role of these demethylases in normal and malignant hematopoiesis has triggered great scientific interest.

LSD1

LSD1/ KDM1A was the first demethylase to be identified [182]. It was shown to specifically demethylate mono- and di-methyl lysine in an amine oxidation reaction that uses flavin adenine dinucleotide (FAD) as a cofactor. Targets of LSD1 include H3K4 and H3K9, and its effects on these two critical substrates give it a central role in transcriptional regulation. LSD1 is highly expressed in AML [183, 184], suggesting that it could function as an oncogene, thereby representing a potential therapeutic target. Several recent studies identified a role for LSD1 in acute leukemia, as well as suggesting that LSD1 inhibitors could be useful in its treatment [185] [186] [183]. Using two different LSD1 inhibitors, (tranylpromine – TCP and a biguanide polyamine analog), Shenk et.al showed that inhibiting LSD1 activity promoted ATRA-driven differentiation of non-APL leukemic cells. Ex vivo treatment of primary AML samples with ATRA and an LSD1 inhibitor (but not ATRA or the LSD1 inhibitor alone) ex vivo diminished the potential of these cells to cause leukemia in a NOD-SCID mouse model. Harris et al. analyzed 23 MLL rearranged leukemias and found a strong correlation between LSD1 expression and clonogenic or leukemia stem cell like features[187]. Knock down of LSD1 expression in MLL-AF9+ AML reduced the expression of MLL-AF9 target genes, which was coupled to an increase in the H3K4me2/H3K4me3 ratio and a loss of leukemic potential. The effectiveness of two TCP analog inhibitors of LSD1 was shown using both murine leukemia models, and primary human AML patient samples.

IDH

While not itself a chromatin reader, writer or eraser, the recent link between metabolic processes and epigenetics has been cemented by the discovery of mutations in the isocitrate dehydrogenase (IDH) 1 and 2 enzymes in brain tumors [188] and subsequently

in AML [189, 190]. These mutations generate a neomorphic enzymatic activity, which converts α -ketoglutarate (α KG) to 2-hydroxyglutarate (2-HG), the first identified oncometabolite. Reduction of α KG levels impairs the function of enzymes that require it as cofactor; these include the TET family of methylcytosine hydroxymethylases, and the JmJc-demethylases. Moreover, high levels of 2-HG competitively inhibit the catalytic activity of these dioxygenases [191]. IDH1/2 mutations were first connected to aberrant TET2 regulation in AML [192], but recently an effect of these mutations on demethylase was shown [193]. This work suggests that inhibitors of the mutant IDH1/2 enzymes could be useful in treating cancer and that blocking the effects of or production of 2-HG could similarly have a positive therapeutic effect.

Histone acetyltransferases

Lysine acetylation involves the transfer of an acetyl group from acetyl-CoA to lysine residues, to form ϵ -N-acetyl lysine. The lysine acetyltransferases (KAT), or histone acetyltransferases (HAT), are called writers, while the histone deacetylases (HDAC) are the erasers; the “readers” of ϵ -N-acetyl lysine containing motifs are the bromodomains, an evolutionary conserved, protein-interaction module. Histone acetylation is associated with a more accessible chromatin state; acetylation of lysine neutralizes its positive charge thereby diminishing its interaction with (negatively charged) DNA. Less compact chromatin state, i.e. euchromatin, is more accessible to transcription factor binding and is generally associated with gene transcriptional activation.

Seventeen histone acetyltransferases have been identified in humans thus far; they are members of five different families of proteins: the Gcn5-related acetyltransferases (GNATs), the MYST acetyltransferases (MOZ, Ybf2/Sas3, Sas2, Tip60), p300 and CBP,

the transcription factor TAF250, and the nuclear hormones SRC1 and SRC3. Mutations in the HATs have been identified in B-cell lymphoma and relapsed acute lymphoblastic leukemia and aberrant acetylation of cancer-related genes has been observed in hematological malignancies [194, 195]. Bromodomain proteins have recently been described as new therapeutic targets in acute myeloid leukemia [196]. We will focus on the MYST and p300/CBP proteins, in this review, for the role they play in hematological malignancies.

p300/CBP

The p300/CBP (CREB Binding Protein) family of HATs generally contains three cysteine-histidine rich regions, a bromodomain, HAT domain and PHD motif. p300/CBP proteins are usually associated with gene activation [197] and are referred to as co-activators. They interact with aspects of the basal transcriptional machinery, to induce a more open chromatin conformation as a result of the acetylation of lysines within the histone tails (K12 and K15 in H2B, K14 and K18 in histone H3, and K5 and K8 in histone H4). Regulation of transcription can also be achieved through the acetylation of non-histone proteins.

Presumably inactivating mutations in the human *CREBBP* and *EP300* genes have been identified in non-Hodgkin lymphoma, both in follicular lymphoma (FL; ~41% of cases) and diffuse large B-cell lymphoma (DLBCL; in ~39% of cases); these mutations primarily affect the histone acetyltransferase (HAT) domain. These missense mutations prevent p300/CBP from acetylating the BCL6 transcriptional repressor, a PTM that diminishes its function, and from acetylating pro-apoptotic p53 molecule, a PTM that promotes its function. As a consequence, BCL6 becomes constitutively active, repressing

p53 function in the germinal centers [198]; p53 activity is further reduced by the lack of its acetylation. Restoring the acetylation of BCL6 and p53, using HDAC inhibitors, may represent a promising therapy for p300 or CBP mutant B-cell non-Hodgkin lymphoma[194].

In another study, mutations in the CBP gene were found in 18.3% of relapsed acute lymphoblastic leukemia (ALL) patients. These mutations generally affect the HAT domain, reducing CBP's acetyltransferase activity, which leads to impaired histone acetylation (H3K18) and diminished expression of CREB target genes, including the glucocorticoid-receptor-responsive genes. Current ALL therapy includes administration of the glucocorticoids and in fact, ALL cell lines harboring CBP mutations are resistant to treatment with dexamethasone. The class I/II HDAC inhibitor vorinostat increases the sensitivity of these cells to dexamethasone, suggesting that HDAC inhibitor treatment may be useful for some patients with ALL[195].

Among the non-histone substrates of p300, we recently showed that acetylation of the AML1-ETO oncogene plays a crucial role in its ability to trigger the development of AML. AML1-ETO and p300 physically and functionally interact, leading to the acetylation of AML1-ETO on K24 and K43, which promotes the activation of AML1-ETO target genes involved in self-renewal. Mutation of lysine 43, but not K24, abrogates the ability of AML1-ETO to induce leukemia in vivo. Ex-vivo treatment of AML1-ETO9a, but not MLL-AF9 driven leukemia cells with the p300 inhibitor Lys-CoA-Tat prolongs their survival. It appears that the acetylation of K43 provides a docking platform for bromodomain containing proteins, which can help promote gene activation by AML1-ETO [199]. This data suggests that targeting p300 or the docking of proteins to K43

acetylated AML1-ETO could be a promising mechanism for developing novel therapeutics for some patients with AML.

The bromodomains

The bromodomain (BRD) is the only protein module that can recognize and bind ϵ -N-acetyl lysines within histones. It is widely conserved among species, taking its name from the *Drosophila* Brahma protein structural domain [200]. Bromodomains contain 110 amino acids that are folded in a left-handed bundle of four alpha helices, with an external hydrophobic pocket that reads the acetylation mark. Flanking marks, such as acetylation or phosphorylation, can modulate the binding of bromodomains to combinations of PTMs present on ϵ -N-acetyl lysines and other histone marks [201]. So far 61 bromodomain containing proteins have been identified, which either have HAT activity or are factors involved in chromatin remodeling.

Targeting of the epigenetic machinery has recently gained favor [202] and the bromodomains are undoubtedly attractive targets. They have a “druggable” functional pocket and they powerfully translate the histone marks they recognize by recruiting transcriptional coactivators. Thus far, the best example of targeting a bromodomain in cancer has come from studies of BRD4, a member of the bromodomain and extra-terminal (BET) family of proteins, that is involved in a translocation in the rare NUT midline carcinoma [203]. BRD4 has also been implicated in promoting the growth of MLL-AF9 driven AML [196], based on an shRNA library screen. A crucial role for Brd4 in the survival and proliferation of MLL-AF9+, NrasG12D+ murine leukemic cells was demonstrated. Recipient mice transplanted with these cells survives longer if treated with the BET protein inhibitor JQ1[202]. Interestingly, leukemia cells are more sensitive to

JQ1 than normal cells, suggesting some specificity of the drug for proliferating cells. JQ1 represents the first drug therapy to target a chromatin reader; it is a membrane soluble molecule that binds the hydrophobic pocket that recognizes acetylated lysines. Inhibition of Brd4 rapidly reduces the expression of c-Myc, and its target genes, inducing differentiation and slowing cell proliferation.

Additional studies have also demonstrated efficacy of JQ1 in the treatment of hematological malignancies. JQ1 induces the death of B-ALL cell lines, downregulating c-Myc and its target genes and the expression of IL-7R. IL-7R and CRLF2 form heterodimers, which promote cell survival via triggering of JAK/STAT5 signaling. Given the reduction in IL-7R expression, it is interesting that the most sensitive ALL cell lines were those carrying CRLF2 rearrangements; this suggests that JQ1 could be efficacious in a variety of malignant diseases [204].

Histone deacetylases

Histone deacetylases (HDAC) are grouped into three distinct families of proteins. Class I HDACs are widely expressed and include HDAC1, 2, 3 and 8. Class II HDACs include HDAC 4,5,6,7,9a, 9b, and 10; they are expressed in a cell-specific manner. Class III HDACs are Sir2/Hst homologues; they are called Sirtuins (1,2,3,4,5,6,7) and their structure and enzymatic mechanism is totally different from class I and Class II HDACs. They are NAD⁺ dependent and are not inhibited by class I or class II inhibitors. HDAC proteins counteract HAT activity; they are associated with transcriptional repression, forming macromolecular complexes with corepressor molecules, such as N-COR, but also transcription factors and nuclear receptors that sit on gene regulatory elements [205].

Histone acetylation is deregulated in transformed cells, resulting in downregulation of tumor suppressor gene expression and oncogene over-expression [206].

HDAC1 has been implicated in the overexpression of the mutant c-KIT found in a subset of acute myeloid leukemias. HDAC inhibitors, particularly of HDAC class I and II, are currently under study for the treatment of AML due to their powerful ability to negatively affect cell proliferation and induce apoptosis[207].

Recent studies have found overexpression of or mutations in a number of epigenetic modifying enzymes, including those impacting directly on histones, and also those that impact on DNA itself. Learning how these abnormalities contribute to the malignant process will help guide our strategies to target the epigenetic abnormalities that characterize cancer, as these may be much more amenable to changes, than are the fixed genetic abnormalities.

1.5 Arginine Methylation and the Family of Protein Arginine Methyltransferases

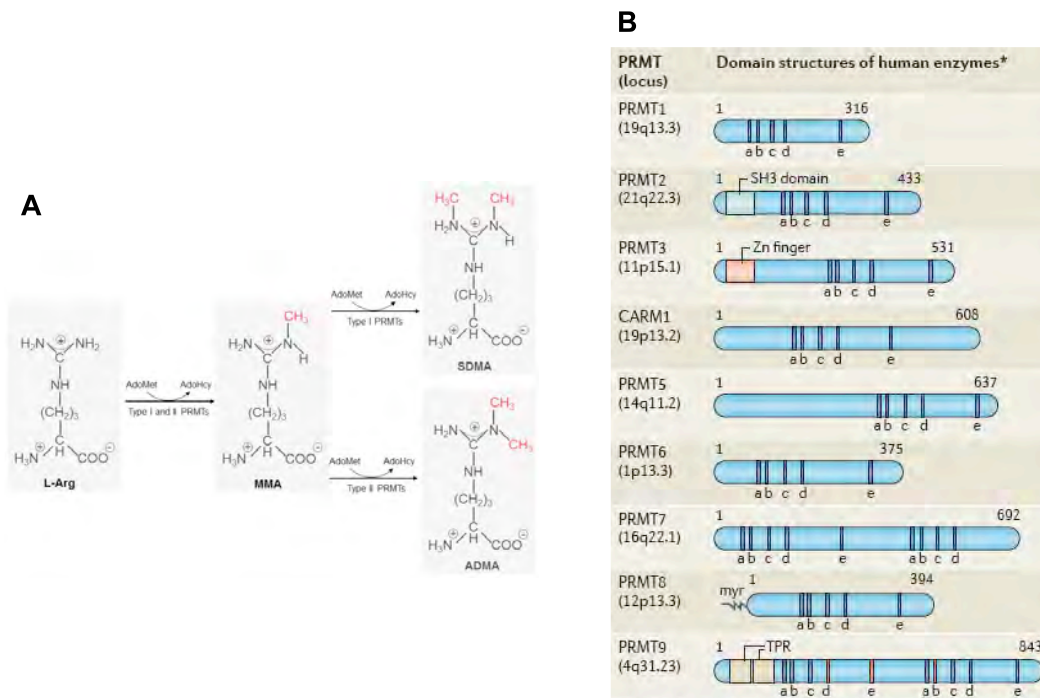
Protein arginine methylation is a widespread post-translational modification. Paik WK first discovered this unique modification at the arginine residue in the calf thymus in 1967[208]. Since its identification, arginine methylation has been extensively studied, indicating its great importance in regulation of protein functions in a variety of fundamental cellular processes. There are three types of methylarginine species: ω - N^G -monomethylarginine (MMA), ω - N^G, N^G -asymmetric dimethylarginine (ADMA) and ω - N^G, N^G -symmetric dimethylarginine (SDMA)[209]. An addition of the methyl group does not change the cationic charge of an arginine residue but results in dramatic changes in its shape and removes a potential hydrogen bond donor of the guanidino group. These

alternations can introduce bulkiness and hydrophobicity to a protein, thus impact protein-protein interactions[210, 211].

Arginine methylation is catalyzed by a family of Protein arginine methyltransferases (PRMTs). PRMT family members share the signature motifs of seven- β -strand methyltransferase and “double E” and “THW” sequences. To date, ten mammalian PRMTs have been identified, but only eight of them have been shown to be enzymatically active. Based on their activity the PRMTs are classified into two groups: Type I PRMTs, which include PRMT 1,3, 4, 6 and 8 and catalyze the formation of asymmetric dimethylarginine, and type II PRMTs, which include PRMT 5, 7 and 11 and catalyze the formation of symmetric dimethylarginine (Figure 4)[211].

PRMTs are widely expressed and they often methylate within glycine and arginine rich (GAR) motifs in substrates (except for CARM1/PRMT4). Even though multiple PRMTs can methylate a common substrate in vitro, they play non-redundant roles in vivo with distinct substrates. The cellular substrates of PRMTs include histones, and non-histone proteins such as transcription factors and regulators, heterogeneous nuclear ribonucleoproteins (hnRNP), proteins involved in DNA damage repair (Mer11), and signal transducers (e.g. growth factor receptors and their downstream effector molecules). Similarly, arginine methylation is implicated in regulation of many cellular processes including signal transduction, DNA repair, RNA processing, transcription regulation, translation and apoptosis [211]. Loss of functions of major PRMTs such as PRMT1 and PRMT5 in mice result in embryonic lethality[212, 213], suggesting that arginine methylation is critical for normal physiological development. Arginine methylation can

potentially be modified or removed by protein arginine demethylating enzymes, which include peptidylarginine deiminase 4 (PAD4) and possibly JMJD6 [214] [215].



Yanzhong Yang and Mark T. Bedford Nature Review Cancer 2013; Dariusz Zakrzewicz et al. Int J Mol Sci. 2012

Figure 4. Arginine methylation and the family of Protein Arginine Methyltransferases

(A). The mechanism of protein arginine methylation. The arginine residue can be monomethylated by all PRMTs. Type I PRMTs generate asymmetric dimethylarginine while type II PRMTs catalyze the formation of symmetric dimethylarginine.

(B). The family of PRMTs. Members of the PRMT family share structural similarity and also possess unique sequences. Vertical dark blue lines indicate signature PRMT motifs with good sequence similarity to the indicated PRMT motif: a, Motif I: VLD/EVGXGXG; b, Post I: V/IXG/AXD/E; c, Motif II: F/I/VDI/L/K; d, Motif III: LR/KXXG; e, THW loop. Red vertical lines indicate poor sequence similarity to the indicated PRMT motif.

Several studies suggest a role for PRMTs in cancer as PRMTs have been found to be overexpressed in numerous solid tumors and lymphoma[209]. In leukemia, PRMT1 has been shown to be a critical component of the MLL-oncogenic transcriptional complex [216]. Direct fusion of MLL with PRMT1 enhanced the self-renewal capacity of primary hematopoietic cells, while knockdown of PRMT1 suppresses MLL-mediated transformation. Furthermore, during myeloid differentiation, PRMT1 methylates RUNX1 (aka AML1) within an RTAMR motif, which abrogates SIN3A binding, promoting the expression of two RUNX1 target genes, CD41 and PU.1, in primary human hematopoietic CD34+ cells [217]. While this site of methylation is lost when RUNX1 is fused to ETO in t(8;21) AML, PRMT1 also interacts with and methylates RUNX1/AML1-ETO, which promotes its effects as an activator of transcription and promoter of cell proliferation [218].

Other PRMTs may also play a role in cancer development by regulating the expression or function of tumor suppressor genes such as p53. p53 can be arginine methylated by PRMT5, and this may trigger p53-dependent G1 arrest, rather than apoptosis, in response to DNA damage due to specific changes in the expression of p53 target genes that are specifically required for each process[219]. A role for PRMT5 in the hematological malignancies was recently highlighted by two research groups: We showed that PRMT5 (aka JAK binding protein 1, JBP1) is a substrate of the mutant, constitutively active JAK2 kinases, such as JAK2V617F, the most common JAK2 mutation found in patients with MPN. Phosphorylation of PRMT5 by JAK2 inhibits its enzymatic activity toward histone H4R3, and knockdown of PRMT5 promoted the erythroid differentiation of the human primary CD34+ cells [220]. This suggests that PRMT5 may block differentiation

and serve as an oncogene. In B cell lymphomas, Aggarwal et al showed that a constitutively active cyclin D kinase can phosphorylate MEP50 – a regulatory factor of PRMT5 thereby enhancing the PRMT5/MEP50 complex activity [221]. Thus, similar to several epigenetic enzymes, dysregulation of PRMT5 functions appears to have opposite effects in leukemia vs. lymphoma, likely reflecting differences in their pathogenesis.

PRMT6 has been shown to antagonize the ability of MLL to methylate H3K4 [222], suggesting its potential involvement in regulating hematopoietic processes. Very few studies have examined the involvement of PRMTs in normal and malignant hematopoiesis; however, insights into the roles of PRMTs may provide new approaches for leukemia prevention, diagnosis and treatment.

1.6 Protein Arginine Methyltransferase 4 – PRMT4 and a potential role in hematopoiesis?

As the fourth PRMT to be identified, PRMT4 was initially isolated from a yeast-two hybrid system using the C-terminal portion of GRIP-1- a member of nuclear receptor (NR) p160 coactivator- as the bait. Because of its ability to transactivate gene expression, PRMT4 is also named co-activator-associated arginine (R) methyltransferase 1 (CARM1). It function as a secondary co-activator in transcription activation of many nuclear receptors[223], such as the estrogen receptor, androgen receptor, thyroid receptor[224] and farnesoid X-receptor. PRMT4 also cooperates with several transcription factors to enhance their transcriptional activity including myocyte enhancer factor 2C (MEF2C)[225], β -catenin[226], p53, nuclear factor (NF)- κ B[227] and the cAMP-responsive element-binding factor[224].

Further studies have provided insights into molecular basis of transcriptional regulation by PRMT4. PRMT4 exerts its effects on transcription via multiple ways. The methyltransferase catalytic function of PRMT4 appears to be required for receptor transactivation[224]. Compared to the major asymmetric methyltransferase PRMT1, PRMT4 displays more restricted substrate specificity. PRMT4 targets a PGM-proline-glycine and methionine rich motif in its substrates instead of the common GAR-glycine and arginine rich motif targeted by other PRMTs. The crystal structure of PRMT4 also reveals a substrate-binding groove supported by the unique post-core region of PRMT4, which is not present in other PRMTs. This particular conformation provides the arginine residue in the substrate a “narrow” access channel to the enzymatically active site in PRMT4[228, 229]. This distinct feature of PRMT4 underscores its indispensable role in a number of biological processes.

PRMT4 substrates include histone H3 (target residues are R17 and R26), proteins involved in transcriptional regulation such as: p300/CBP[230], RNA polymerase II[231], SRC-3[232], C/EBP β [233] and Pax7[234], RNA binding protein HuD[235] and several splicing factors[236]. By modulating their functions, PRMT4 controls gene transcription, turnover of mRNA[235], protein degradation[232] and coupling of transcription and mRNA processing[236]. The methyltransferase catalytic activity is required for PRMT4 capability to transactivate gene expression initiated by nuclear receptors[224]. Further studies have provided more insights into the molecular basis for transcriptional activation by PRMT4. Methylation of H3 at R17 is known to serve as an epigenetic mark associated with transcriptional activation[237, 238]. A tudor domain – containing protein TDRD3 has been reported to recognize the mark, thus potentially functioning as an effector [239].

H3R17me2 also recruits the transcription elongation-associated PAF1 complex (PAF1c) to facilitate transcription[240]. The methylation is found to be more favorable on histones preacetylated by histone acetyltransferase p300/CBP, suggesting a sequential operation between PRMT4 and p300/CBP in a NR responsive complex[241]. Furthermore, PRMT1 and PRMT4 function cooperatively in transcription activation[242]. In cells, PRMT4 also forms a complex with ATP-remodeling (SWI/SNF) complex, in which physical association of PRMT4 and BRG1 mutually facilitates their ATPase and methylase functions to promote transcription[243]. PRMT4 also control activities of transcriptional regulators such as p300/CBP. Methylation of p300/CBP by PRMT4 modulates its transcriptional co-activation function between CREB-regulated and NR-regulated gene expression[230, 244, 245]. Moreover, methylation of p300 at specific R754 residue also promotes the binding of BRCA1, thereby facilitating the cooperation between PRMT4, p300, BRCA1 and p53 to induce expression of p21 in response to DNA damage[246]. Together, these studies suggest that PRMT4 works in concert with other factors in a functional complex to both directly and indirectly mediate transcriptional regulation.

Thus far, PRMT4 has been implicated in the regulation of a number of biological processes. PRMT4 knock out mice are small in size and die perinatally [247]. Phenotypic analysis of these mice demonstrated important roles for PRMT4 in the expression of estrogen- responsive genes, at the stage of DN1 – DN2 transition in early T cell development [245] and adipocyte differentiation[248]. The knockout mice also exhibit a defect in lung development due to the hyperproliferation and impaired maturation of pulmonary epithelial cells [249]. Another independent study showed that PRMT4 regulates the proliferation and differentiation of a neural – PC12 cell line[235]. PRMT4

has also been implicated to play a role in muscle cell differentiation[241, 250]. Notably, Wu et al. demonstrated that PRMT4 is required for the self-renewal and pluripotency of embryonic stem (ES) cells [251]. This activity is correlated with the regulation of H3R17me2 mark by PRMT4. Level of H3 arginine methylation in the ES cells can direct the cell fate as higher level of H3R17me2 biases the cells of the inner cell mass to pluripotent cells [252]. Taken together, these studies suggest a critical role of PRMT4 in the regulation of cell differentiation. Nonetheless, the exact functions of PRMT4 in each tissue can be different, such that PRMT4 can function as a positive regulator in one system while behave as a negative regulator in another.

PRMT4 has been also studied in several cancers. It has been shown that PRMT4 expression is elevated in solid tumors including breast cancer[253, 254], prostate and colorectal cancers[255] where it functions as a coactivator for hormone receptors to regulate expression of critical target genes. As there is a potential value in targeting PRMT4 for cancer therapy, several attempts have been made to create PRMT4 small molecule inhibitors [256-259]. However, while these compounds have been shown activity in vitro, there are no reports describing their cellular activities in vivo.

SUMMARY

Given recent discoveries of the role of the PRMT family members may play in the hematopoietic system and an increasing focus in targeting histone-modifying enzymes in leukemia, it was our prime interest to explore the role of PRMT4 in both normal and malignant hematopoiesis. We aimed to define PRMT4 functions during hematopoiesis, in

particular during the differentiation of myeloid cells, such studies hold the possibility of developing a therapeutic approach targeting PRMT4 in AML.

2. MATERIALS AND METHODS

2.1 Purification and culture of human primary hematopoietic CD34+ cells

CD34+ HSPCs were purified by positive selection using the Midi MACS (magnetic-activated cell sorting) LS+ separation columns and isolation Kit (Miltenyi) starting with mononuclear cells that were isolated from cord blood (CB) by Ficoll-Hypaque Plus density centrifugation. Purity was confirmed by flow cytometry with over 95% of purified cells is CD34 positive.

CD34+ cells were cultured in Iscove's modified Dulbecco's medium (IMDM, Cellgro) containing 20% BIT 9500 medium (Stem Cell Technologies) supplemented with SCF (100 ng/ml), FLT-3 ligand (10 ng/ml), IL-6 (20 ng/ml) and TPO (100 ng/ml) as the basic culture. After 24 hours of growth, CD34+ cells were infected with high-titer lentiviral concentrated suspensions, in the presence of 8µg/ml polybrene (Aldrich).

2.2 Generation and infection of retro- and lenti- viruses

Viruses were produced by transfection of 293T cells with vectors, according to standard protocols (Moffat et al., 2006). Viral supernatant was collected for concentration with 20% sucrose by centrifugation at 25,000 rpm for 2 hours at 4°C. Viral pellet was collected and resuspended in 2% BSA. Viruses then can be used immediately for infection or stored at -80°C.

Cells were seeded at optimal density prior to infection. High titer viruses were used for infection in the presence of 8µg/ml polybrene (Aldrich) with spin inoculation for 45 minutes at 1,400 rpm. Cells were left in culture for 72 hours after transfection prior to sorting.

2.3 Flow cytometry and cell sorting

Transduced cells were sorted based on expression of selection markers (GFP, RFP) using a fluorescence-activated cell sorting (FACS) Vantage cell sorter. CD34⁺ cells were also selected for CD34 expression by staining using an APC-conjugated anti-CD34 antibody (BD Pharmingen). To monitor the differentiation status, cells were stained with the following antibodies: CD11b-PE and CD11b-APC (Invitrogen), CD11b-APC (BD Pharmingen), CD13 (Invitrogen), CD71-APC (BD Pharmingen), Glycophorin A-PE (Invitrogen). Data were analyzed with CellQuest software using a Becton Dickinson FAScan.

2.4 Hematopoietic functional assays

2.4.1 Colony forming unit (CFU) assay

1×10^4 GFP⁺ CD34⁺ transduced cells were plated (in duplicate) in methylcellulose with erythropoietin (5 IU/ml), SCF (50 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), G-CSF (20 ng/ml) and GM-CSF (20 ng/ml). The total number of CFU colonies was scored 14 days after seeding.

2.4.2 Cobblestone area forming cell (CAFC) assay

4×10^5 GFP⁺ CD34⁺ cells were grown on MS-5 stromal cells in IMDM, supplemented with 12.5% horse serum, 12.5% fetal bovine serum (FBS), 4 mmol/L L-glutamine, 100 U/mL penicillin and 100 µg/mL streptomycin and 1 µM hydrocortisone. Medium was half-replenished every week and cobblestone areas were scored at week five.

2.4.3 In vitro lineage-specific liquid culture

To differentiate HSPCs, cells were cultured under the myeloid-promoting conditions: SCF (100 ng/ml), FLT-3 ligand (10 ng/ml), IL-3 (20 ng/ml), IL-6 (20 ng/ml), GM-CSF (20 ng/ml) and G-CSF (20 ng/ml) and the erythroid-promoting conditions: Epo (6 IU/ml) and SCF (100 ng/ml). All cytokines were purchased from Peprotech, NJ. The differentiation status of the cells was examined after 7 days in culture.

2.4.4 Morphology analysis

2×10^5 cells were centrifuged onto slides for 5 minutes at 500 RMP and air-dried prior to GIEMSA staining. The cell morphology was evaluated by light microscopy.

2.5 RNA extraction and quantitative real time PCR (qRT-PCR)

Total RNA was isolated using the Qiagen RNeasy Plus® mini kit (QIAGEN, Germany). cDNA was generated from RNA using SuperScript III kit (Invitrogen) with random hexamers according to the manufacturer's instructions. Real-time PCR reactions were performed using an ABI 7500 sequence detection system. Quantitative PCR for HPRT was performed to normalize for cDNA loading. Qiagen miniRNease Mini Kit was used to purify microRNA enriched RNA. cDNA was generated using TaqMan® MicroRNA Reverse Transcription Kit (ABI #4366596). Quantitative PCR for RNU6 was performed to normalize for microRNA loading. Taqman MicroRNA assays were purchased from ABI (ID# 002098 and ID# 001093 respectively). Relative quantification of the genes was calculated using the method (2^{-Ct}) as described by the manufacturer.

2.6 Cellular transient transfection

Transfection of 293T cells with vectors capable of overexpressing proteins was performed using Lipofectamin 2000, according to the manufacturer's instructions (Invitrogen).

Hematopoietic cell lines and CD34⁺ cells were transiently transfected with siRNAs/microRNAs using the Amaxa Nucleofector Kit, according to the manufacturer's instructions (Lonza). The transfection efficiency was determined by flow cytometric analysis of GFP by transfecting the cells with pmaxGFP.

2.7 Peptide pull-down assay

Methylated (Acetyl-TPNPR (Asymmetric-dimethyl) ASLNHS-C-amide) and non-methylated (Acetyl-TPNPRASLNHS-C-amide) peptides were synthesized, quantified and conjugated to SulfoLink agarose (Pierce, Rockland, IL). For each pull down reaction, 10mg of HEL cell nuclear extract was used with 10 μ g peptide bound beads in H lysis buffer (20 mM Hepes pH 7.9, 150mM NaCl, 1mM MgCl₂, 1% NP40, 10mM NaF, 0.2mM NaVO₄, 10 mM β -glycerol phosphate, 5% glycerol) with freshly added 1mM DTT and proteinase inhibitor cocktail (Roche). After rotating overnight at 4°C, the beads were washed five times with the binding solution. The bound protein was then eluted with 1XSDS sample buffer and analyzed on 4-12% NUPAGE gels.

2.8 Co-Immunoprecipitation and Western blot assays

Protein expression constructs were transiently transfected into 80% confluent 293T cells. After 48h, the cells were lysed in H lysis buffer with 1mM DTT and a proteinase inhibitor cocktail (Roche), followed by sonication for 1 minute at 20% amplitude with 10

seconds on and 10 seconds off. The supernatant was collected and the mix was spun at 16,000xg for 15 minutes. We mixed 400µl of cell extract with 600µl of DTT buffer (20mM HEPES pH 7.9, 100mM KCl, 0.2mM EDTA, 0.5 mM PMSF and 10% glycerol and DNase I) and approximately 40µl of anti-Flag agarose beads (Sigma) in the presence of RNase A (1µg/ml). The reaction proceeded for 4 hours at 4°C then the beads were extensively washed with H lysis buffer and eluted using 1XSDS sample buffer.

HEL cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS). Approximately 4×10^7 cells were harvested and fractionated to collect nuclear extract for use in Co-immunoprecipitation assays. We mixed 200µl of nuclear extract (~2mg) and 800µl of DTT buffer with 40µl of anti-RUNX1 antibody cross-linked beads and IgG control. After rotation over night at 4°C, the beads were washed five times with H lysis buffer, eluted by elution buffer (25mM HEPES pH 7.9, 10% glycerol, 0.1mM EDTA, 150 mM NaCl, 0.8% Sarkosyl) and analyzed on 4-12% NUPAGE gels.

2.9 Chromatin Immunoprecipitation (ChIP) assays

Approximately 4×10^6 cells were used per ChIP reaction (per antibody) after crosslinking with 1% formaldehyde for 10 minutes at room temperature. ChIP assays were performed according the previously reported methodology (Zhao et al. 2000). After purification, the associated DNA was subjected to qRT-PCR to detect specific DNA sequences. Quantitative results are represented as percentages relative to 5% DNA input.

2.10 Antibodies and reagents

We generated a methyl arginine specific anti-RUNX1 polyclonal antibody by immunizing rabbits with the synthetic peptide (Acetyl-TPNPR (Asymmetric-dimethyl) ASLNHS-C-amide) conjugated to KLH. The antiserum was collected and purified using a peptide affinity column. The affinity of the antibody against the methyl-arginine R223RUNX1 was measured by ELISA and dot blot assays.

Antibodies used for immunoblotting, immunoprecipitation and Chip included: anti-Flag M2 antibody (Sigma-Aldrich), anti-PRMT4 (Bethyl, Santa Cruz Biotechnology, Abcam), anti-DPF2 (Santa Cruz Biotechnology), mouse anti-RUNX1 6B4 (MBL International), rabbit anti-cleaved caspase 3 (Cell Signaling). The rabbit anti-RUNX1 N-terminal antibody was generated as reported previously (Zhao et al., 2008). The rabbit anti-RUNX1 C-terminal antibody was raised against the peptide sequences: CPSLPNQSDVVEAEGSHSNSPTNMAPSAR

2.11 In vitro and ex vivo treatment of PRMT4 inhibitor

PRMT4 inhibitor was dissolved in DMSO. Treatment of cells with PRMT4 inhibitor was performed in parallel with DMSO-treated control. Cells were then collected at various time points for various assays. In ex vivo treatment experiment, cells were collected and washed 3 times with PBS prior to injection into recipient mice.

2.12 Cell viability assays

2.12.1 CellTiter – Glo Luminescent cell viability assay –Promega

Assay was performed following manufacturers' protocol. In brief, the assay detects cell viability via measurement of luminescent signal, which is proportional to the amount of ATP present in cell lysis.

2.12.2 Apoptotic analysis

Apoptosis was assayed using Annexin V and 7-AAD staining followed by flow cytometry. Annexin V staining kit was purchased from BD Biosciences.

2.13 In vivo transplantation of leukemia cells

AE9a expressing mouse leukemia cells were generated based on the work of Wang et al.2011[260]. These cells were transduced with lentiviruses expressing RFP and shRNAs against PRMT4 or a scrambled control shRNA. Transduced cells were sorted for RFP positivity and 10⁵-sorted cells were injected via tail vein into female C57Bl/6 recipient mice that had been sublethally irradiated with 475 cGy.

2.14 RNA sequencing and DNA microarray for gene expression profiling

RNA samples were prepared in triplicate for RNA sequencing and in duplicate for microarray. Microarray analysis was performed using Affimetrix GeneChip Human Genome U133 Plus 2.0 Arrays. Microarray hybridization and RNA sequencing were performed at the MSKCC genomic core facility. RNA sequencing data analysis was performed with the assistance from the computational biology core facility. Microarray data analysis was performed using Partek software.

2.15 Gene expression pathways analysis

Relevant biological pathways analysis was performed using Ingenuity Systems (<http://www.ingenuity.com>) and Gene Set Enrichment Analysis (GSEA) (<http://www.broadinstitute.org/gsea/index.jsp>)

2.16 Statistical analysis

Statistical analyses were carried out using Prism 5.0 for Macintosh. All data are shown as mean \pm SD. The mean values of each group were compared by Student's *t*-test.

3. RESULTS

3.1 PRMT4 Blocks Myeloid Differentiation by Assembling a Methyl-RUNX1-Dependent Repressor Complex (*manuscript to be resubmitted to Cancer Cell*)²

Abstract

Defining the role of epigenetic regulators in normal hematopoiesis has become critically important, as recurrent mutations or aberrant expression of these genes has been identified in both myeloid and lymphoid hematological malignancies. We have found that PRMT4, a type I arginine methyltransferase, whose function in normal and malignant hematopoiesis is unknown, is overexpressed in AML patient samples. In support of an oncogenic role for PRMT4, we find that its overexpression blocks the myeloid differentiation of human stem/progenitor cells (HSPCs) while its knockdown (KD) is sufficient to induce myeloid differentiation of HSPCs and multiple AML cell lines. Although classically thought of as a co-activator, we found that PRMT4 functions to repress the expression of miR-223 in HSPCs via the methylation of RUNX1, which triggers the assembly of a multi-protein repressor complex that includes DPF2. As part of a feedback loop, PRMT4 expression is repressed post-transcriptionally by miR-223 during the normal differentiation process. These data reveal an unidentified role of PRMT4 in myeloid differentiation and its unexpected repressive role in transcriptional regulation. Furthermore, depletion of PRMT4 results in the differentiation of myeloid leukemia cells in vitro and their decreased proliferation in vivo. Thus, targeting PRMT4 holds potential as a novel therapy for acute myelogenous leukemia.

² *This section is reproduced here verbatim, with the exception of figure numbers from a manuscript submitted to Cancer Cell*

3.1.1 PRMT4 regulates myeloid differentiation

To examine its function in hematopoiesis, we knocked down PRMT4 in human cord blood (CB) derived – stem/progenitor CD34⁺ cells (HSPCs), using lenti-viral vectors that express GFP and shRNAs directed against PRMT4. We assayed the extent of PRMT4 knockdown (KD) in the GFP positive transduced cells by RT-PCR and western blot analyses, and found a 70%-80% decrease in PRMT4 expression for the KD1 and KD2 short hairpin RNAs, respectively (Figure 5A). The PRMT4-KD cells generated far fewer CFUs when plated in methylcellulose (Figure 5B) and showed enhanced myeloid differentiation after 7 days in myeloid differentiation - promoting liquid culture (which contains SCF, FLT-3 ligand, IL-3, IL-6, GM-CSF and G-CSF) with 60%-70% of the KD cells being CD11b positive and CD13 positive, vs. 40% (and 14%) of the control cells being CD11b (or CD13) positive (Figure 5C and Figure 6B). Consistent with the immunophenotypic evidence, morphologic evidence also showed more mature myeloid cells following PRMT4 KD (Figure 5D). In fact, the KD cells showed condensed nuclear and clear nuclear lobulation. In addition, knockdown of PRMT4 mildly impaired erythroid differentiation under erythroid - promoting culture conditions (which contains: SCF and EPO) (Figure 6D). Consistent with its effect on CFU generation, a decrease in the numbers and size of the cobblestone areas was seen at week 5, reflected an impairment of HSPC self renewal when PRMT4 was knocked down (Figure 6E).

As reduced levels of PRMT4 accelerated myeloid differentiation, we next examined whether PRMT4 overexpression blocks myeloid differentiation (Figure 5D). Indeed, we found a marked reduction in CD11b positive cells generated from PRMT4 overexpressing CD34⁺ cells (compared to the control cells) after 7 days in myeloid

differentiation promoting cultures (Figure 5E). Thus, PRMT4 appears to be an important negative regulator of normal myeloid differentiation.

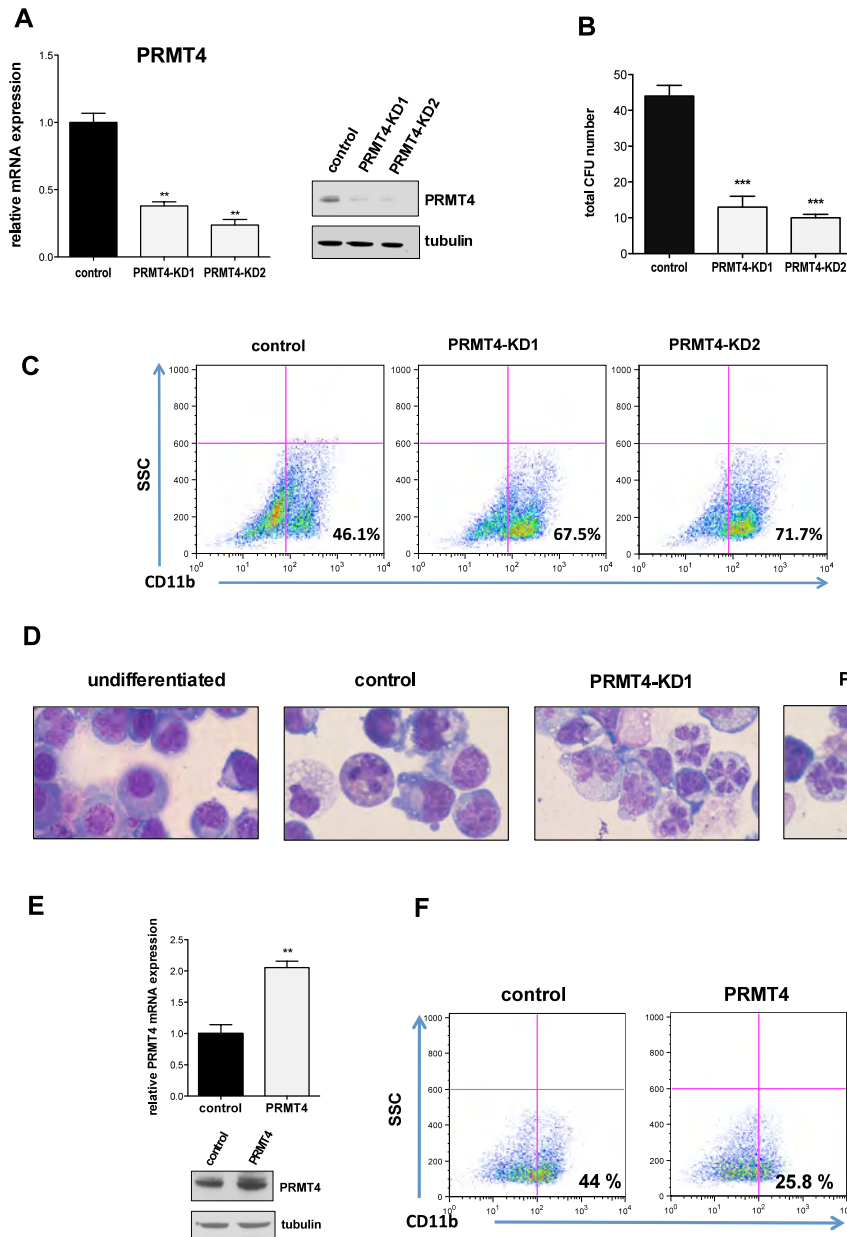


Figure 5. PRMT4 regulates myeloid differentiation of HSPCs

(A). Efficient knock down of PRMT4. Human CB CD34⁺ cells were transduced with lentiviruses expressing a control (scrambled) shRNA or one of two shRNAs directed against PRMT4. GFP-positive cells were sorted 3 days after transfection and collected to perform qRT-PCR and Western blot analyses. mRNA expression levels were normalized to HPRT. The data represents the mean \pm SD of the three independent experiments.

Tubulin served as the loading control. A representative experiment is shown. ** $p < 0.01$ by Student's *t* test.

(B). Downregulation of PRMT4 decreases CFU formation. 1×10^4 of the control or PRMT4 knock down cells were plated in methylcellulose, supplemented with cytokines. The total number of colony forming units (CFUs) was scored 2 weeks after the plating. The data represents the mean \pm SD of the three independent experiments. . *** $p < 0.001$ by Student's *t* test.

(C). Downregulation of PRMT4 promotes the myeloid differentiation of HSPCs. GFP+ CD34+ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression. A representative experiment is shown.

(D). Downregulation of PRMT4 promotes the myeloid differentiation of HSPCs. GFP+ CD34+ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Cellular morphology was evaluated. A representative experiment is shown. Cells growing in basic culture were used as the control for myeloid differentiation.

(E). Overexpression of PRMT4 was demonstrated at the mRNA and protein levels. Human CB CD34+ cells were transduced with retroviruses expressing either control (GFP alone) or GFP together with HA-PRMT4. GFP-positive cells were sorted after 3 days of transfection and collected to perform qRT-PCR and Western blot analyses. mRNA expression levels were normalized to HPRT. Tubulin served as the loading control. A representative experiment is shown.

(E). Overexpression of PRMT4 blocks the myeloid differentiation of HSPCs. GFP+ CD34+ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression. A representative experiment is shown.

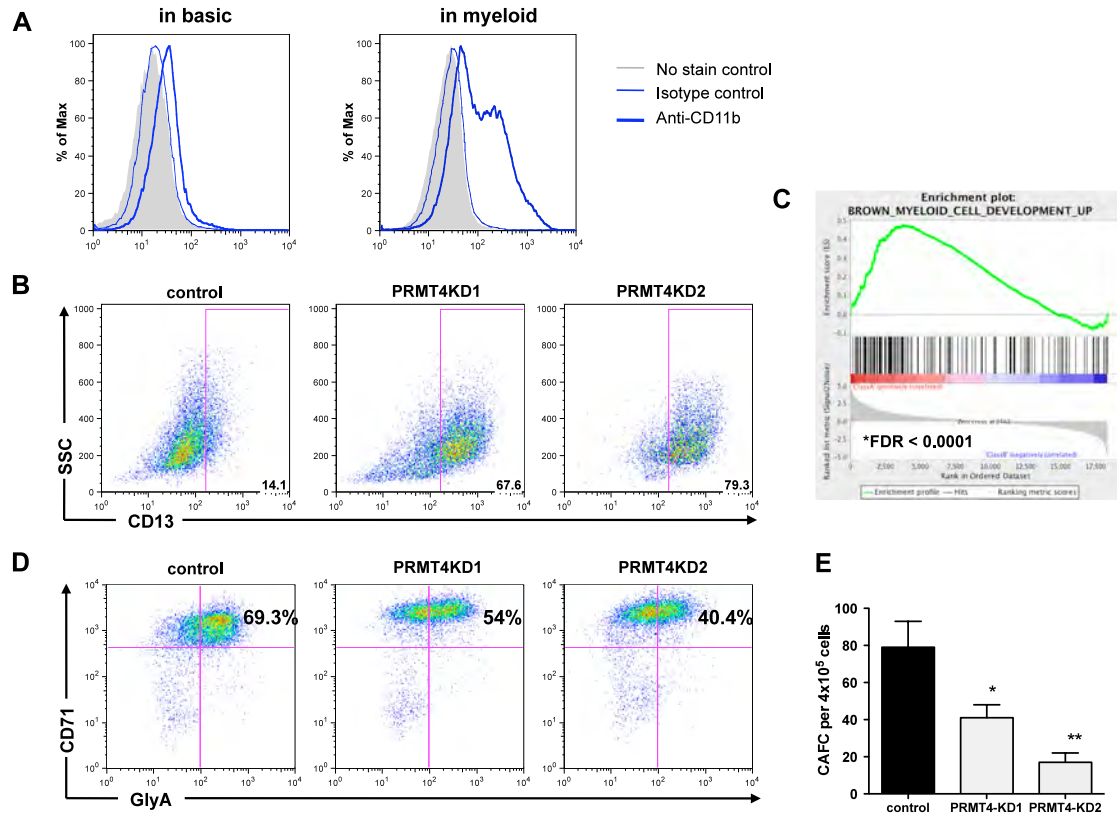


Figure 6. PRMT4 regulates myeloid differentiation of HSPCs

(A). FACS antibody staining for CD11b is highly specific. FACS analysis of no stain control, isotype control and anti-CD11b staining of cells grown in basic culture and myeloid differentiation culture are shown.

(B). Downregulation of PRMT4 promotes the myeloid differentiation of HSPCs. GFP+ CD34+ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD13 expression. A representative experiment is shown.

(C). GSEA on expression datasets of PRMT4 knock down CD34+ cells reveals an enrichment of the “myeloid cell development gene signature” as defined by Brown et al.[261]

(D). Knock down of PRMT4 inhibits the erythroid differentiation of HSPCs. Control and PRMT4 knock down cells were plated in erythroid promoting medium for 7 days, and erythroid differentiation assessed by FACS analysis of Gly-A and CD71 expression. A representative experiment is shown.

(E). Knock down of PRMT4 decreases the number of CAFCs. Human cord blood (CB) CD34+ cells were transduced with lentiviruses expressing GFP and control shRNA or shRNAs against PRMT4. 4×10^5 GFP+ CD34+ cells were grown on MS-5 stromal cells and cobblestone areas were scored at week five. The data represents the average and \pm SD of two independent experiments.

3.1.2 PRMT4 is regulated post-transcriptionally by miR-223 during myeloid differentiation

Given the prominent effect of PRMT4 on myeloid differentiation, we assessed changes in PRMT4 expression during normal in vitro HSPC differentiation. We cultured human CB CD34⁺ cells in myeloid differentiation - promoting liquid culture and observed a significant decrease in PRMT4 protein levels over a seven-day period (Figure 7A). PRMT4 mRNA levels varied only slightly during this process, suggesting that PRMT4 is being regulated post-transcriptionally. MicroRNA target prediction programs (TargetScan release 5.2 (Figure 8A), PITA) suggested that PRMT4 is a potential target of several microRNAs, including miR-223, a myeloid specific microRNA. Interestingly, a seed sequence for miR-223 is found in the 3'-UTR region of PRMT4, which is located adjacent to the stop codon for the PRMT4 open reading frame (ORF) (16-22 nt from the stop codon); this location could confer a strong regulatory effect at the translational level. Indeed, we found that miR-223 expression steadily increases during myeloid differentiation (Figure 7B), concomitant with decreasing PRMT4 protein expression. To determine whether miR-223 regulates PRMT4 expression, we transiently transfected CD34⁺ cells with a short hairpin encoding the mature miR-223 for 24 hours and monitored PRMT4 expression, using siPRMT4 as a positive control and a scrambled short-hairpin as a negative control. PRMT4 protein levels decreased by 50% in the miR-223 overexpressing cells, reaching a level similar to that found in the cells expressing siRNA directed against PRMT4 (Figure 7C).

To validate that PRMT4 is directly targeted by miR-223, we cloned the full length 3'-UTR of the PRMT4 transcript (including the putative miR-223 binding site) into a

luciferase reporter plasmid (UTR- WT), using the same construct with a mutated miR-223 targeting sequence (UTR-mut), which can not bind miR-223, as negative control. We expressed these constructs in 293T cells with either the miR-223 short-hairpin or the control short-hairpin, and found that miR-223 decreased the luciferase activity of the UTR-WT, but had no effect on the UTR-mut reporter plasmid (Figure 7D). Moreover, consistent with the post-transcriptional regulation of PRMT4 expression by miR-223 that we observed in vivo, there was no significant down-regulation in the level of luciferase mRNA (Figure 8C). Thus, miR-223 directly targets PRMT4 by binding to its recognition sequence in the 3'-UTR, suggesting that PRMT4 and miR-223 form a regulatory loop to regulate myeloid differentiation.

To determine how important the regulation of PRMT4 expression by miR-223 is for the effects of PRMT4 on myeloid differentiation, we overexpressed the PRMT4-ORF from an expression vector that contained either the WT or with the mutant PRMT4 3'-UTR in human CB C34+ cells. The presence of the WT 3'-UTR abrogated the PRMT4 imposed block in myeloid differentiation. However, when PRMT4 was expressed either without the 3'-UTR or with the mutant 3'-UTR, the number of CD11b positive cells was reduced indicating that the block in myeloid differentiation persisted (Figure 7E). These effects correlate with the level of PRMT4 protein (Figure 7F), and indicate that the regulation of PRMT4 by miR-223 is a major component of its function during myeloid differentiation.

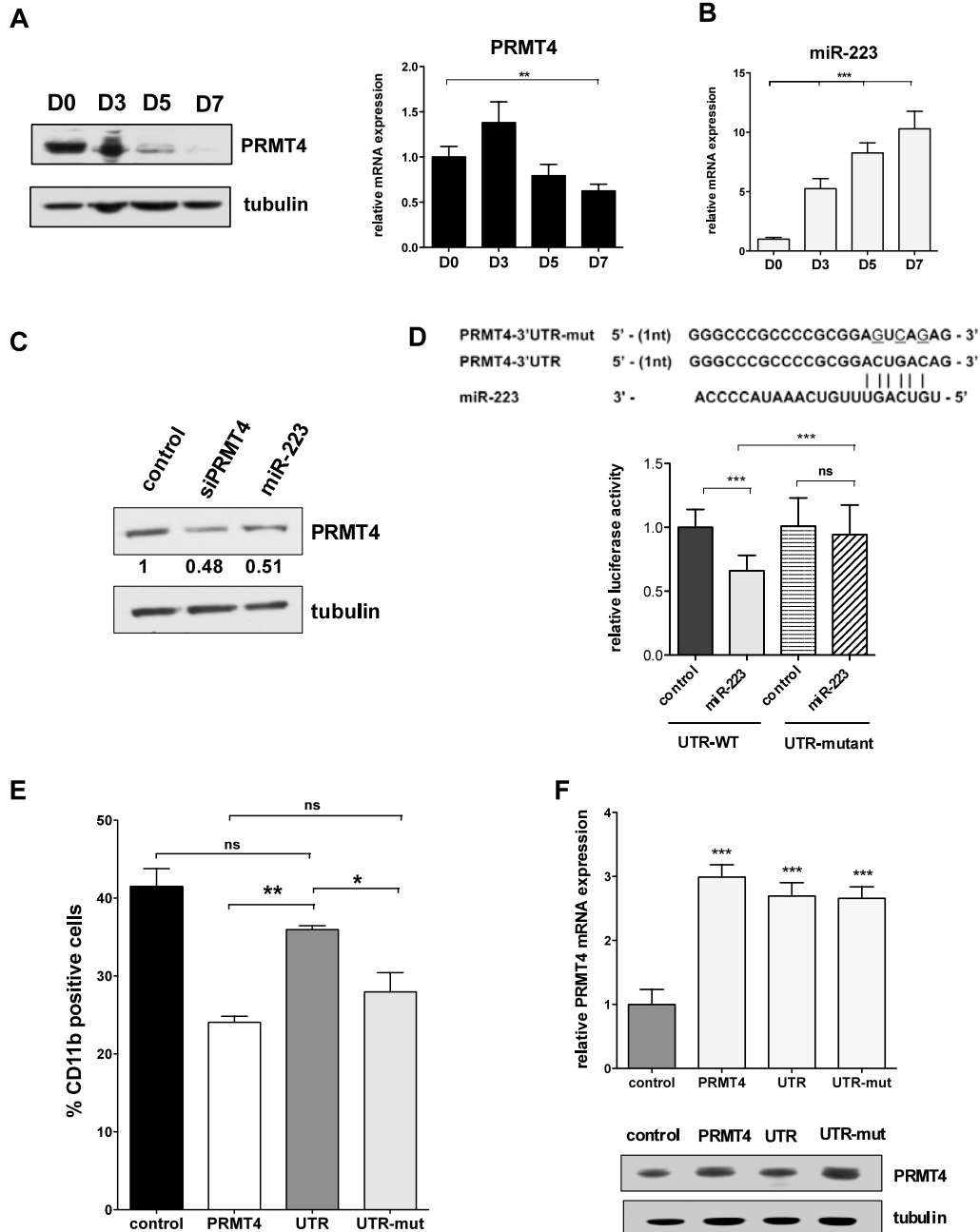


Figure 7. PRMT4 is a potential target gene of miR-223 during myeloid differentiation of HSPCs

(A). PRMT4 protein expression is progressively downregulated during myeloid differentiation (left), while PRMT4 mRNA level decreases modestly during myeloid differentiation (right). Isolated CD34⁺ cells were cultured in myeloid – promoting cytokine containing medium and collected at sequential time points: day (D) 0, 3, 5 and 7. Western blot and qRT-PCR analyses were performed. mRNA expression levels were normalized to HPRT. Tubulin served as the loading control. The data represents the mean \pm SD of three independent experiments. ** $p < 0.01$ by Student's *t* test.

(B). miR-223 expression steadily increases during myeloid differentiation. miR-223 expression was measured by qRT-PCR and normalized to RNU6 expression. The data represents the mean \pm SD of three independent experiments. *** $p < 0.001$ by Student's *t* test.

(C). Overexpression of miR-223 or siRNA directed against PRMT4 lowers PRMT4 protein levels in HSPCs. miR-223, siRNA against PRMT4, and control oligonucleotides were transiently expressed in CD34⁺ cells and 24 hours post-electroporation, the cells were collected and assayed for PRMT4 expression by western blot analyses. Tubulin served as the loading control.

(D). Putative miR-223 binding site in the PRMT4 3'UTR is shown at the top (based on TargetScan.org release 5.2). Luciferase activity in 293T cells co-transfected with a reporter plasmid containing either the wild type 3'-UTR-PRMT4 or the mutated 3'-UTR (3-UTR-mut, which lacks the seed miR-223 sequence) with or without miR-223. Renilla luciferase values are normalized based on the value of firefly luciferase. Mean \pm SD from three independent experiments is shown. *** $p < 0.001$ by Student's *t* test.

(E). Control of PRMT4 expression by miR-223 is essential to regulate PRMT4 function during normal myeloid differentiation. Human CB CD34⁺ cells were transduced with retroviruses expressing control -GFP alone; GFP- PRMT4-ORF or GFP- PRMT4-3'-UTR or GFP-PRMT4- 3'-UTR-mut. Sorted GFP⁺ CD34⁺ cells were cultured in myeloid-promoting cytokine containing medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression. The percentage of CD11b positive cells was quantified as mean \pm SD based on three independent experiments. * $p < 0.05$; ** $p < 0.01$ by Student's *t* test.

(F).qRT-PCR and Western blot analyses of PRMT4 expression in control CD34⁺ cells or CD34⁺ cells expressing PRMT4-ORF, PRMT4-3'-UTR or PRMT4-3'-UTR-mut. mRNA expression levels were normalized to HPRT expression level. Tubulin served as the loading control. A representative experiment is shown.

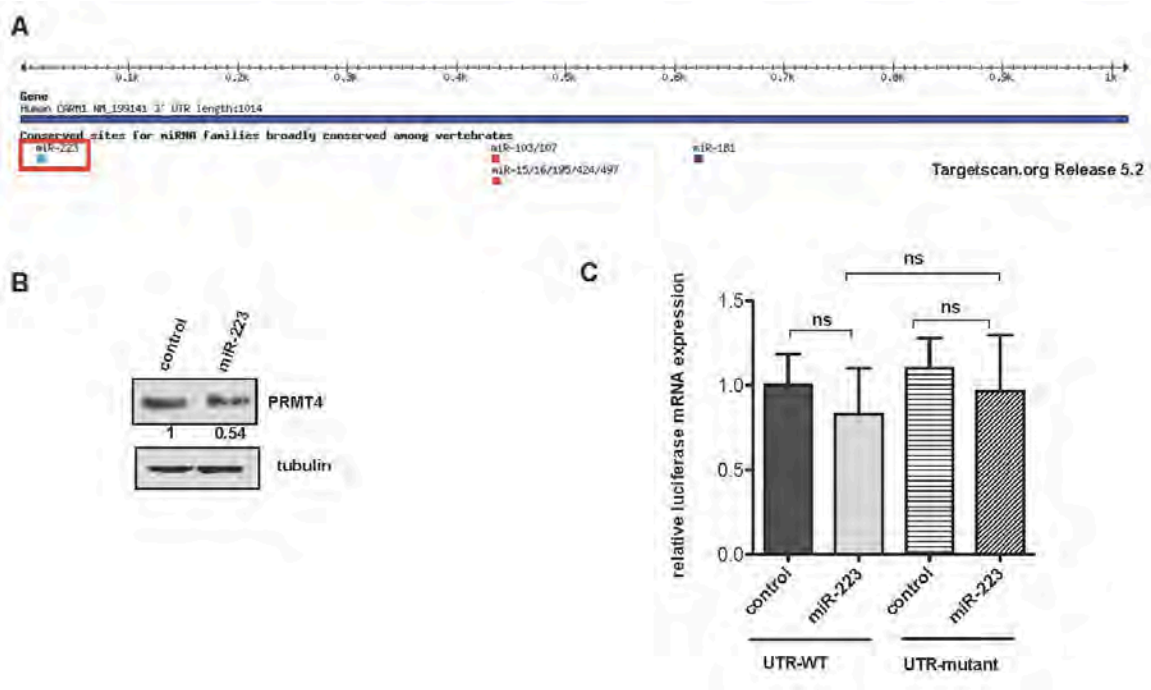


Figure 8. PRMT4 is a potential target gene of miR-223 during myeloid differentiation of HSPCs

(A). MicroRNAs that potentially target the 3'-UTR region of PRMT4 are shown. Data is derived from targetscan.org release 5.2.

(B). Overexpression of miR-223 lowers PRMT4 protein levels in HSPCs. miR-223 were expressed in CD34+ cells using lentiviral-expressing miR-223 vector. 72h after transduction, the cells were collected and assayed for PRMT4 expression by western blot analyses. Tubulin served as the loading control.

(C). q-PCR analysis of mRNA expression level of luciferase in Figure 2D. The data represents the average and \pm SD of three independent experiments.

3.1.3 PRMT4 represses miR-223 expression

Given the known transcription regulatory role of PRMT4, to determine how PRMT4 controls myeloid differentiation, we first examined the expression level of several lineage-differentiation “master” transcription factors, including PU.1, C/EBP α , KLF4 and GATA1 in PRMT4-KD cells. While we found no significant changes (Fig. 10A), we did observe a consistent increase in miR-223 expression in the PRMT4-KD cells (Figure 9A). Because upregulation of miR-223 has been reported to promote the myeloid differentiation of the NB4 and SKNO-1 cell lines, we overexpressed miR-223 in normal CB CD34⁺ cells. We saw a significant increase in CD11b positive cells (51.2% vs. 38.1% for the control cells) (Figure 9B) and a decrease in PRMT4 expression (Figure S2B). Conversely, we found a decrease in miR-223 expression when PRMT4 is overexpressed (Figure 9C). We also knocked down miR-223 expression and found a modest reduction in CD11b positive cells (38.3% vs. 44.8%) (Figure 9D), which suggests that other microRNAs may compensate for miR-223 during myeloid differentiation. This is consistent with the previous *in vivo* study of miR-223 knockout mice, which showed that miR-223 is important for granulocytic maturation and function, but not essential for the differentiation process.

To determine whether PRMT4 regulates the transcription of miR-223, we looked at the level of the miR-223 primary transcript (pri-miR-223) and found that PRMT4 expression does reciprocally regulate pri-miR-223 levels in CD34⁺ cells (Figure 10B). In addition, the gene expression analysis of PRMT4 KD cells revealed a gene signature consistent upregulation of myeloid differentiation (Figure 6C). Thus, PRMT4 regulates myeloid differentiation, at least in part, by modulating miR-223 expression.

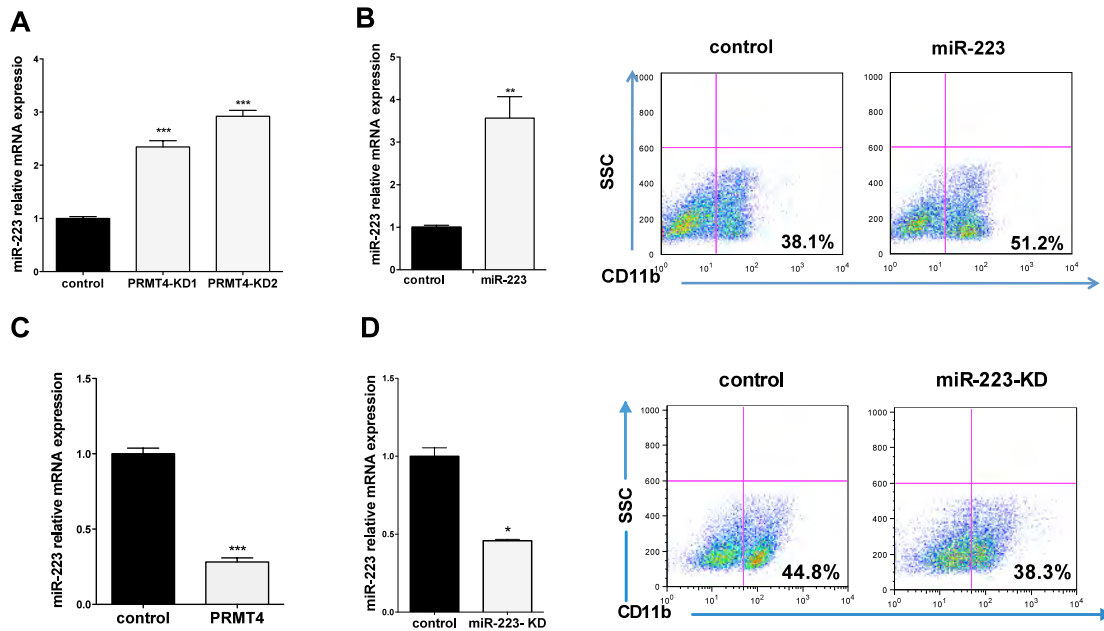


Figure 9. PRMT4 regulates miR-223 expression

(A). PRMT4 downregulation leads to upregulation of miR-223 expression. qRT-PCR analysis of miR-223 levels in control and PRMT4 knock down CD34⁺ cells is shown as Mean \pm SD normalized to RNU6 expression from three independent experiments. *** $p < 0.001$ by Student's *t* test.

(B). Overexpression of miR-223 in HSPCs enhances myeloid differentiation. (Left) qRT-PCR analysis of miR-223 levels in control and miR-223 overexpressing CD34⁺ cells. Results are normalized to RNU6. Mean \pm SD from three independent experiments is shown. ** $p < 0.01$ by Student's *t* test. (Right) Cells were plated in myeloid differentiation promoting culture and assayed for CD11b expression after 7 days. A representative experiment is shown.

(C). Overexpression of PRMT4 diminishes miR-223 expression. qRT-PCR analysis of miR-223 in control and PRMT4 overexpressing CD34⁺ cells. Results are normalized to RNU6. Mean \pm SD from three independent experiments is shown. *** $p < 0.001$ by Student's *t* test.

(D). Knock down of miR-223 in HSPCs slightly impairs myeloid differentiation. (Left) qRT-PCR analysis of miR-223 in control and miR-223 knock down CD34⁺ cells. Results are normalized to RNU6. Mean \pm SD from three independent experiments is shown. * $p < 0.05$ by Student's *t* test. (Right) Cells were plated in myeloid differentiation promoting culture and assayed for CD11b expression after 7 days. A representative experiment is shown.

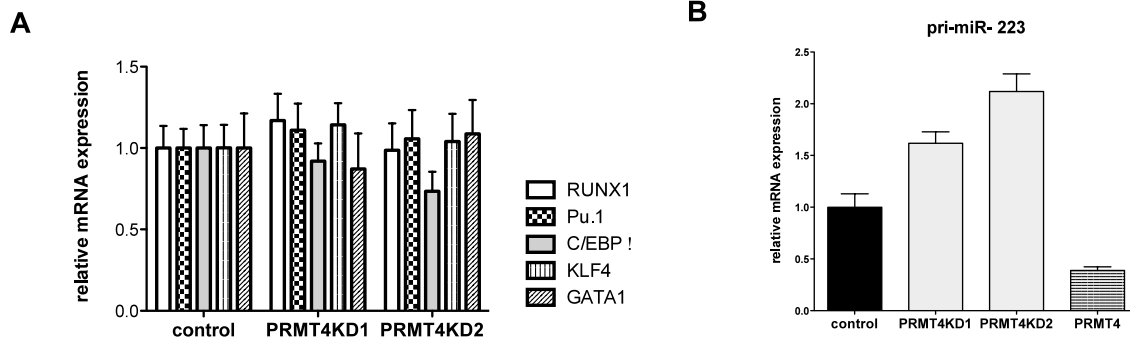


Figure 10. PRMT4 regulates miR-223 expression

(A). Downregulation of PRMT4 does not significantly affect the level of RUNX1, PU.1, C/EBP α , KLF4 or GATA-1 mRNA expression based on qRT-PCR analysis of mRNA expression. mRNA levels were normalized to HPRT. The data represents the average and \pm SD of two independent experiments.

(B). PRMT4 regulates miR-223 expression at the transcriptional level. Knock down of PRMT4 upregulates expression level of pri-miR-223, while overexpression of PRMT4 downregulates its expression. qRT-PCR analysis of pri-miR223 expression in control, PRMT4 knock down and PRMT4 overexpressing CD34⁺ cells is shown, normalized to HPRT. The data represents the average and \pm SD of three independent experiments.

3.1.4 RUNX1 is methylated by PRMT4 on arginine 233 (R223) residue

PRMT4 is generally thought to act as a co-activator in transcription regulation; however, we found that PRMT4 functions as a repressor of miR-223 expression in hematopoietic cells. To determine how this “co-activator” suppresses gene expression, we hypothesized that PRMT4 modulated the activity of a regulatory transcription factor. Based on the report that the AML associated AML1(RUNX1)-ETO fusion protein represses miR-223 expression via a RUNX1 CBS in one of its transcriptional regulatory regions, we examined the miR-223 promoter and found another RUNX1 CBS close to the miR-223 promoter. Given the presence of these consensus-binding sites, we investigated whether wild type RUNX1 also regulates miR-223 transcription. We knocked down RUNX1 in CD34⁺ cells using two different shRNAs and observed an up-regulation of pri-miR-223 and pre-miR-223 expression (Figure 11A), indicating that RUNX1 can transcriptionally inhibit miR-223 expression in HSPCs. Given that both RUNX1 and PRMT4 repress miR-223 transcription, we examined whether RUNX1 (binds and) is arginine methylated by PRMT4. Using an in vitro methylation assay, we identified one specific site in RUNX1, R223, which is methylated by PRMT4. This arginine residue is located just C-terminal to the RUNX1 DNA binding domain (Figure 11B) and is conserved among vertebrates (Figure 11C). To determine whether RUNX1 is methylated at R223 by PRMT4 in vivo, we generated a methylation specific anti-methyl-R223RUNX1 antibody, which recognizes an asymmetric di-methylated R223RUNX1 peptide, but not the unmethylated peptide. We confirmed the specificity of the antibody by overexpressing Flag-RUNX1 or the Flag-RUNX1R223K mutant protein in 293T cells with or without HA-PRMT4, or with an enzymatically dead form of PRMT4 (PRMT4EQ). Methylation of RUNX1 at

R223 was strongly detected when both RUNX1 and PRMT4 were overexpressed (Figure 12A. lane1) or when RUNX1 was overexpressed by itself (lane 5). No methylation was detected when either the mutant R223K-RUNX1 protein was expressed or when PRMT4EQ was overexpressed (Figure 12A, lanes 2,3 and 4). Thus, we conclude that PRMT4 methylates RUNX1 at residue R223 in vivo. The same experiment showed the physical interaction between RUNX1 and PRMT4, using an anti-HA antibody to detect PRMT4 in the RUNX1 containing immunoprecipitates; we detected an interaction between RUNX1 and PRMT4 when RUNX1 and PRMT4EQ were co-expressed or when the R223K-RUNX1 mutant was over expressed with PRMT4 (or PRMT4EQ) (Figure 12A. IP- lane 2, 3, 4). Minimal, if any, interaction was seen when RUNX1 overexpressed with WT-PRMT4 (Figure 12A.IP-lane1), suggesting that PRMT4 preferably associates with the non-methylated form of RUNX1. The physical interaction between PRMT4 and RUNX1 appears to be disrupted once RUNX1 is asymmetrically di-methylated on R223. To determine whether PRMT4 is the major enzyme catalyzing RUNX1R223 methylation in vivo, we knocked down PRMT4 using siRNA in HEL cells; we observed a marked reduction in the methylation of RUNX1 at R223, but no change in RUNX1 methylation at the RTAMR site, which we have previously shown to be methylated by PRMT1 (Figure 12B). Thus, PRMT4 appears to be the major methyltransferase that asymmetrically methylates RUNX1 at R223 in vivo.

Given the downregulation of PRMT4 protein levels during myeloid differentiation, we examined whether RUNX1R223 methylation is similarly down regulated and indeed, as human CD34+ cells differentiate and PRMT4 expression diminishes, the level of methylR223-RUNX1 decreases, even though total RUNX1 protein levels are unchanged

(Figure 12C). This suggests that the effects of PRMT4 on myeloid differentiation may relate to its ability to post-transcriptionally modify RUNX1.

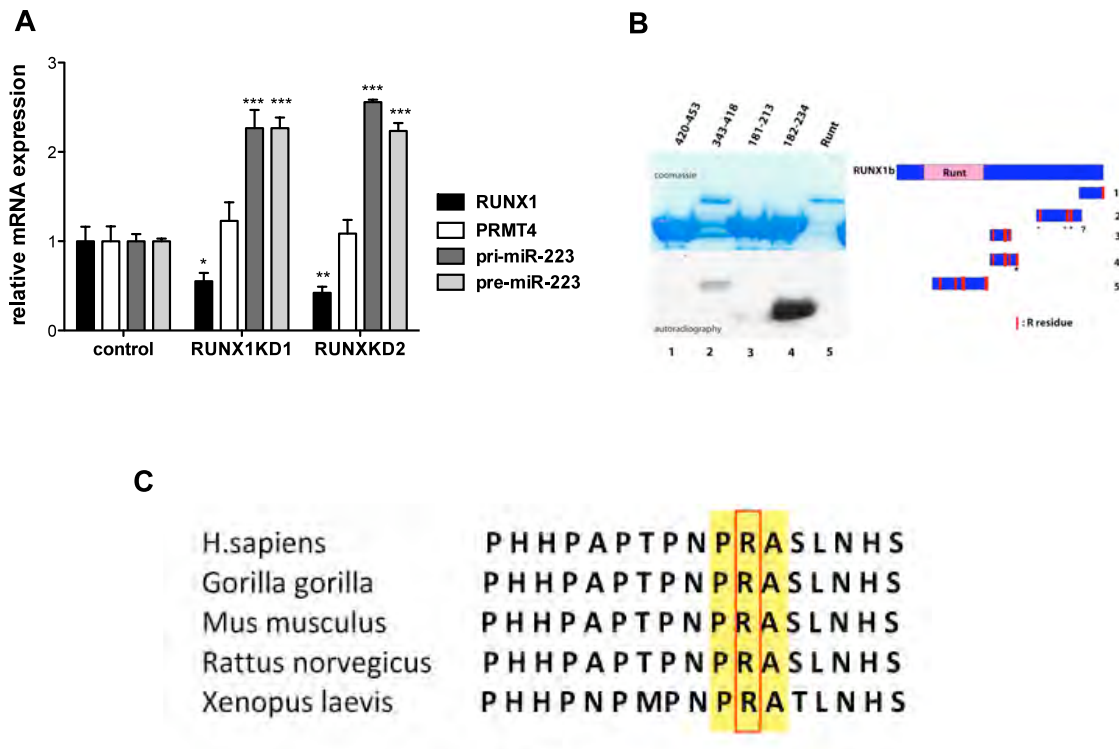


Figure 11. RUNX1 is arginine methylated by PRMT4 on R223 residue

(A). RUNX1 transcriptionally regulates miR-223 expression. Knock down of RUNX1 increases pri-miR-223 and pre-miR-223 expression. qRT-PCR analysis of RUNX1, PRMT4, pri-miR-223 and pre-miR-223 expression in control and RUNX1 knock down CD34+ cells. mRNA level is normalized to HPRT. The data represents the average and ± SD of three independent experiments.

(B). RUNX1 is arginine methylated by PRMT4 in vitro. Purified GST-RUNX1 truncated proteins (as depicted on the right) were incubated with recombinant PRMT4 in the presence of [3H]- SAM. Reaction products were resolved in SDS-PAGE. The gel was stained with Comassie Blue for input and assayed by autoradiography.

(C). Alignments of the R-223 regions of RUNX1 from human, gorilla, mouse, rat and xenopus.

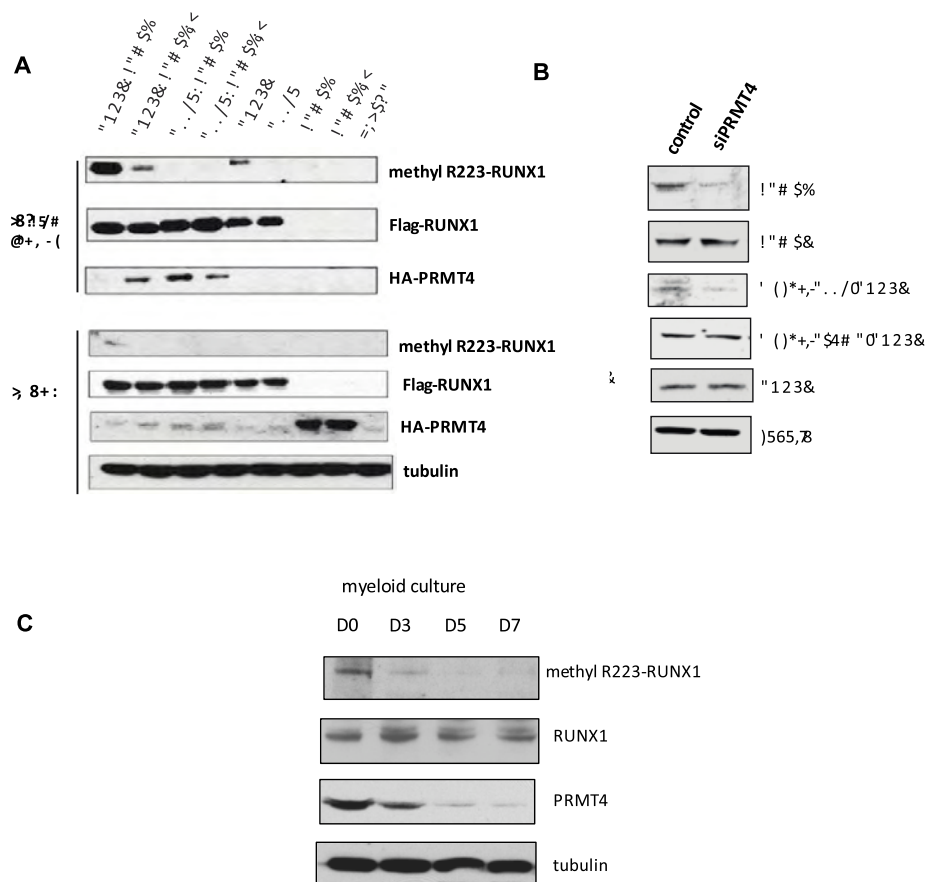


Figure 12. RUNX1 is arginine methylated by PRMT4 on R223 residue

(A). In vivo methylation of RUNX1 by PRMT4 was detected using a methyl-RUNX1 specific antibody. The full-length Flag- RUNX1 or Flag- RUNX1-R223K mutant cDNAs were overexpressed in 293T cells, with or without HA-tagged-WT-PRMT4, or an enzymatically dead PRMT4 mutant (PRMT4EQ). Immunoprecipitation was performed using an anti-Flag antibody and immunoblotting with anti-FLAG or anti-HA antibodies. Wild type RUNX1 (lane 1 and 2), but not the R223K mutant protein (lane 3 and 4) is methylated by wild type PRMT4. The physical interaction between RUNX1 and PRMT4 is detected when RUNX1 is overexpressed with PRMT4EQ, but not WT-PRMT4, and when R223K is overexpressed with PRMT4 or PRMT4EQ (lanes 1-4 in the third row). Tubulin served as the loading control.

(B). Knock down of PRMT4 in HEL cells using siRNA reduces the level of endogenous methylR223-RUNX1, without altering total RUNX1 levels or the methylation of RUNX1 at the RTAMR motif. Tubulin served as the loading control.

(C). The level of RUNX1R223 methylation decreases during myeloid differentiation, without changes in total RUNX1 expression but concordant with changes in PRMT4 protein levels. CB CD34+ cells were cultured in myeloid differentiation promoting medium and collected at sequential time points: day (D) 0, 3, 5 and 7. Western blot analysis was performed. Tubulin served as the loading control.

3.1.5 Methylation of RUNX1 at R223 regulates its interaction with DPF2

Having demonstrated that methylation of the RTAMR motif in RUNX1 by PRMT1 regulates its association with SIN3A, we hypothesized that methylation of R223 in RUNX1 by PRMT4 would also affect its protein-protein interactions. Therefore, we performed peptide pull-downs using both a methyl-R223 RUNX1 peptide and an unmodified RUNX1 peptide as bait, followed by mass spectrometry, to identify proteins that specifically interact with unmethylated or R223methylated RUNX1 protein. We identified several proteins that preferentially interact with the R223 methyl peptide (Table 1), including DPF2 (double PhD Finger 2), a widely expressed member of the d4 protein family, that are characterized by the presence of a tandem plant-homodomain (PHD domain). DPF2 acts as a co-repressor for ER α and it was recently reported to interact with lysine-acetylated histones. We first verified the interaction of DPF2 with the methyl-R223 RUNX1 peptide in a peptide pull-down assay: DPF2 preferentially bound to the arginine methylated peptide, while PRMT4 preferred the non-methylated RUNX1 peptide, as predicted from our characterization of the interaction of PRMT4 with full length RUNX1 (Figure 13A).

To determine whether RUNX1 and DPF2 associate *in vivo*, we performed co-immunoprecipitation assays using two different RUNX1 antibodies and consistently immunoprecipitated the endogenous DPF2 protein, confirming their *in vivo* interaction (Figure 13B). To show that this interaction depends on arginine methylation, we treated HEL cells with Adenosine-2',3'-dialdehyde – AdOx (20 μ M), a pan inhibitor of methyltransferases, which significantly reduced the methylation of RUNX1 after 16h (Figure 13C). This treatment abrogated the interaction of RUNX1 with DPF2 (Figure

13D, compare lane 2 and 4). To further demonstrate that DPF2 interacts specifically with R223methylated RUNX1, we overexpressed Flag-RUNX1 and Flag-RUNX1R223K and performed co-immunoprecipitation studies using anti-Flag beads. DPF2 associated with Flag-RUNX1, but not Flag-RUNX1R223K (Figure 13E, compare lane 2 and 3), demonstrating that the interaction of DPF2 with RUNX1 is dependent on methylation of R223.

Table 1 - Peptide pull-down mass spectrometry representative data		
Proteins	Numbers of Unique Peptide Count	
	Methylated peptide	Non-methylated peptide
<i>SWI/SNF complex</i>		
MLLT1	1	0
DPF2	8	2
BAF170	9	13
BAF155	12	14
BAF250	9	7
BAF60a	2	3
BRG1	3	6
BAF53	2	2
<i>Other proteins relevant to transcription</i>		
RPAP2	1	0
UBP1	1	0
GATAD2B	0	1
CBFA2T3	7	6
TAF15	3	5

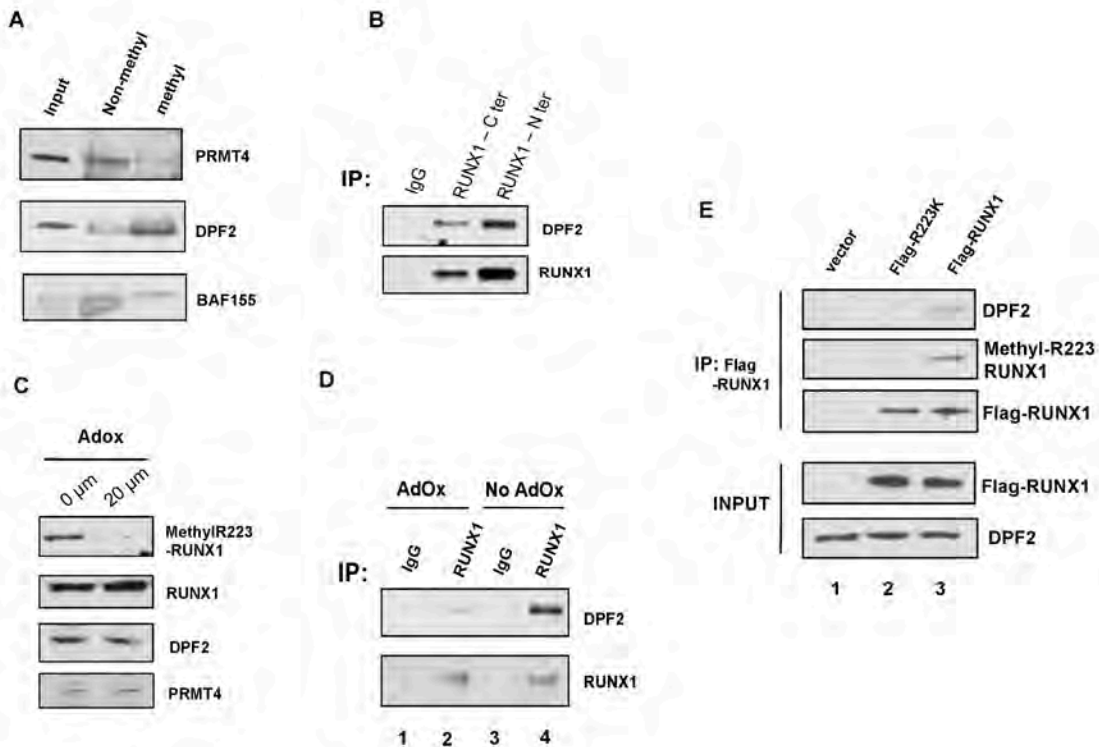


Figure 13. Methylation of RUNX1 at R223 residue regulates its interaction with DPF2

(A). DPF2 is preferably pulled down by a methylR223- RUNX1 peptide, while the unmodified R223-RUNX1 peptide interacts more strongly with PRMT4. BAF155 is used as a control, which shows no preference for binding to either the modified or the unmodified peptide. Input: ten percent of the nuclear extract used for the pulldown assay was loaded.

(B). The endogenous RUNX1 and DPF2 proteins physically interact in vivo. Two anti-RUNX1 antibodies were used to immunoprecipitate DPF2 from HEL cell nuclear extract. DPF2 was detected using an anti-DPF2 antibody. Pre-immune rabbit serum was used as control.

(C). The interaction between RUNX1 and DPF2 is dependent on the RUNX1 methylation status. Overexpressed Flag-RUNX1 is immunoprecipitated using an anti-Flag antibody. DPF2 is co-precipitated with Flag-RUNX1, but not the Flag-R223K mutant. (compare lane 1 vs. lane 3).

(D). Treatment of cells with AdOx reduces the level of RUNX1 methylation (input), abrogating its interaction with DPF2 (compare lane 2 vs. lane 4).

3.1.6 MiR-223 expression is regulated by a RUNX1-methylation dependent repressor complex

Given the decrease in RUNX1R223 methylation during myeloid differentiation and the demonstrated interaction of R223- methylated RUNX1 with DPF2, we hypothesized that the DPF2-RUNX1 interaction regulates miR-223 expression. We examined the binding of RUNX1, PRMT4, and DPF2 to miR-223 regulatory regions using chromatin immunoprecipitation (ChIP) assays, using HSPCs and in vitro differentiated myeloid cells, which correspond to the states where miR-223 is low and RUNX1 R223 methylation high (i.e. the HSPC stage), and where miR-223 is highly expressed, and RUNX1 R223 methylation is low (i.e. in myeloid differentiated cells) (Figure 14A and 14B). Using primer pairs that cover much of the miR-223 putative regulatory regions (as depicted in Figure 14C), we detected RUNX1, methyl-R223 RUNX1 and DPF2 at the pre-miR-223 promoter region, when the cells were at the “stem/progenitor” stage and miR-223 was minimally expressed (Figure 14D). However, in the differentiated cells where miR-223 was actively transcribed, we found RUNX1 and not methyl-R223 RUNX1 (Figure 14E). PRMT4 is found throughout the miR-223 regulatory region in the HSPCs but not in the differentiated cells, with a slight peak at region 4. While this may reflect the level of PRMT4 in these cells, DPF2 protein is clearly expressed in the differentiated cells (Figure 6A), but it is not found at the miR-223 regulatory regions (Figure 14E), suggesting that its recruitment to the miR-223 promoter depends on the methylation status of RUNX1. Thus, recruitment of DPF2 by methyl-R223 RUNX1 dictates the transcriptional effects of RUNX1 on the miR-223 locus.

Vu, et al. Figure 6

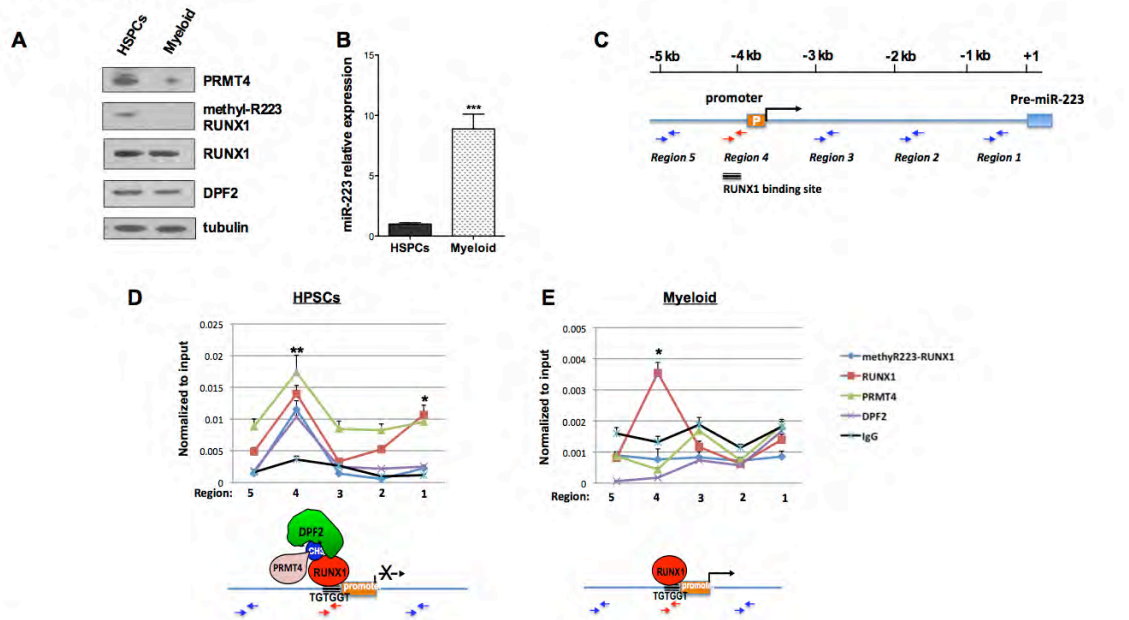


Figure 14. The RUNX1 methylation dependent repressor complex regulates miR-223 expression.

(A). Western blot analysis quantified the levels of expression of PRMT4, methyl R223-RUNX1, RUNX1 and DPF2 in CD34⁺ cells maintained in basic culture (to preserve “stemness”) or in cells cultured in myeloid differentiation-promoting medium for 7 days. Tubulin served as the loading control.

(B). The level of miR-223 expression in the HSPCs and myeloid differentiated cells was evaluated by qRT-PCR. miR-223 expression is normalized to RNU6. Mean ± SD of three independent experiments is shown. *** $p < 0.001$ by Student’s *t* test.

(C). A schematic diagram of the miR-223 promoter. The location of the RUNX1 consensus binding sites and the primers used for the Chromatin-immunoprecipitation assays is shown.

(D). (E). ChIP assays show the presence of a methyl-R223 RUNX1- dependent complex at the promoter of miR-223 in HSPCs (D), but not in myeloid differentiated cells (E).

Upper panel: Enrichment of proteins of interest at miR-223 regulatory regions was assayed by qRT-PCR and shown as percentage of the genomic input DNA. * $p < 0.5$; ** $p < 0.01$ by Student’s *t* test.

Lower panel: diagrams demonstrating the recruitment of RUNX1, methylR223-RUNX1 and DPF2 to the miR-223 promoter.

3.1.7. DPF2 inhibits miR-223 expression and myeloid differentiation

We next examined whether DPF2 can directly regulate miR-223 expression. We achieved a 50% knock down of DPF2 mRNA and protein using shRNA (Figure 15A) and found a 3-fold increase in miR-223 expression (Figure 15B). The DPF2 KD cells also showed enhanced myeloid differentiation (based on CD11b expression, Figure 15C) and decreased clonogenic potential (Figure 15D) similar to what occurred when we knocked down PRMT4. This places PRMT4 and DPF2 in a RUNX1 containing complex that down-regulates miR-223 expression, and impairs the myeloid differentiation of HSPCs.

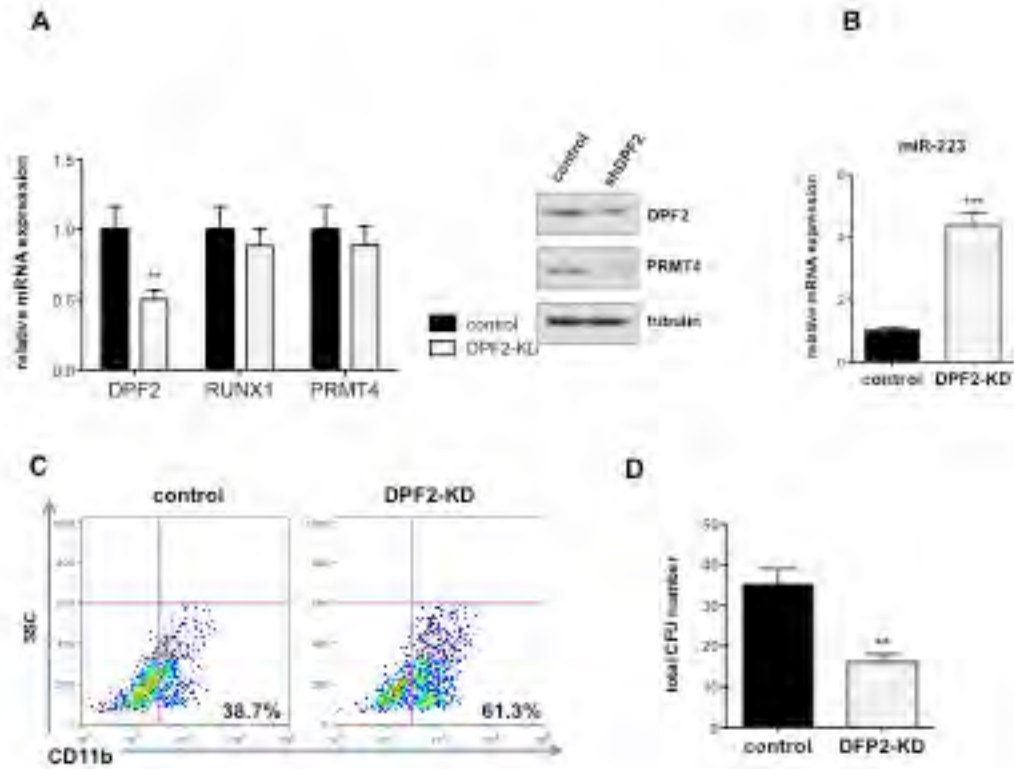


Figure 15. Knock down of DPF2 promotes myeloid differentiation and miR-223 expression

(A). Efficient knock down of DPF2. CD34⁺ cells were transduced with lentiviruses expressing a control (scrambled) shRNA or shRNA directed against DPF2. GFP-positive cells were sorted 3 days after transfection and collected to perform qRT-PCR and Western blot analyses. mRNA expression levels were normalized to HPRT. Tubulin served as the control for protein loading. A representative experiment is shown. ** $p < 0.01$ by Student's *t* test.

(B). Downregulation of DPF2 increases miR-223 expression. qRT-PCR analysis of miR-223 in control and DPF2 knock down CD34⁺ cells. Results are normalized to RNU6. Mean \pm SD of three independent experiments is shown. *** $p < 0.001$ by Student's *t* test.

(C). Downregulation of DPF2 accelerates the myeloid differentiation of HSPCs. GFP⁺ CD34⁺ cells were cultured in myeloid differentiation promoting medium for 7 days. Myeloid differentiation was determined by FACS analysis of CD11b expression. A representative experiment is shown.

(D). Downregulation of DPF2 reduces clonogenic potential of CD34⁺ cells.

3.2 PRMT4 is a Therapeutic Target for Leukemia Treatment

3.2.1 Knock down of PRMT4 is sufficient to induce myeloid differentiation and apoptosis in AML cell lines

As PRMT4 expression impairs the differentiation of human CB CD34⁺ cells, we examined the level of PRMT4 expression in a cohort of 318 AML patient samples (GEO accession number GSE24505). PRMT4 levels were significantly upregulated in the AML samples, compared to the control group (n = 5) (Figure 16A). A high level of PRMT4 expression was seen in core-binding factor (CBF) AMLs (which express either AML1-ETO or CBFβ-SMMHC), which exhibit low-level miR-223 expression. Overall, about 70% of the AML patients had at least a 2-fold increase in PRMT4 expression (Figure 16B).

Several studies have shown that overexpression of miR-223 can promote the granulocytic differentiation of NB4 acute promyelocytic leukemia- (APL) cells. Given that depletion of PRMT4 promotes miR-223 expression and normal myeloid differentiation, we knocked down PRMT4 in NB4 cells and triggered myeloid differentiation with a significant increase in the number of CD11b positive cells (3.3% control vs. 23.8% and 47.3% for the PRMT4-KD1 and PRMT4-KD2 cells) (Figure 16C) as well as changes in cellular morphology (Figure 16D). We then tested whether targeting PRMT4 knock down was also sufficient to induce myeloid differentiation of the ATRA-resistant NB4-R4 cells and found a greater than 10 fold increase in CD11b positive cells, compared to the control (Figure 16C). Because therapeutic targeting of most leukemia fusion proteins, including AML1-ETO remains elusive, we tested whether targeting PRMT4 can promote the differentiation of AML1-ETO expressing cells given the high level of PRMT4

expression in CBF-leukemia (Figure 16A). We knocked down PRMT4 in t(8;21) positive Kasumi-1 cells and found increased differentiation with an increase in CD11b positive cells (Figure 15C). We also saw significant apoptosis of all three cell lines when PRMT4 is knocked down (Figure 16F). This suggests that PRMT4 not only impairs the differentiation of myeloid leukemia cells but it is also critical for their survival.

3.2.2 Knock down of PRMT4 reduces leukemia burden in vivo

To investigate the role of PRMT4 in leukemogenesis in vivo, we used shRNA expressing lentiviruses to knock down PRMT4 in the AML1-ETO9a (AE9a) driven mouse model [262] and [260]. Leukemia cells growing in culture were transduced with 2 different shRNAs and $\geq 70\%$ -80% KD was achieved (Figure 17A). The transduced AE9a-mPRMT4KD and control cells) were injected into sub-lethally irradiated C57Bl/6 mice (day 0). We observed decreased numbers of immature GFP⁺ c-kit⁺ blast cells in the peripheral blood of the AE9a-mPRMT4KD mice compared to control mice during week 3 (Figure 17B). Morphological analysis of the bone marrow (Figure 17C) and peripheral blood (Figure 17D) of the recipient mice also showed a marked reduction in blast cells at week 4, with lower white blood cell counts, less anemia and thrombocytopenia (Figure 17E), compared to the control mice. This translated to a significant increase in median survival, from 28 days for the control group to 51 days and 64.5 days for the AE9a-mPRMT4-KD1 and AE9a-mPRMT4-KD2 groups, respectively ($p < 0.0001$; Figure 17F). This demonstrates an important role for PRMT4 in leukemogenesis, and identifies it as an important therapeutic target.

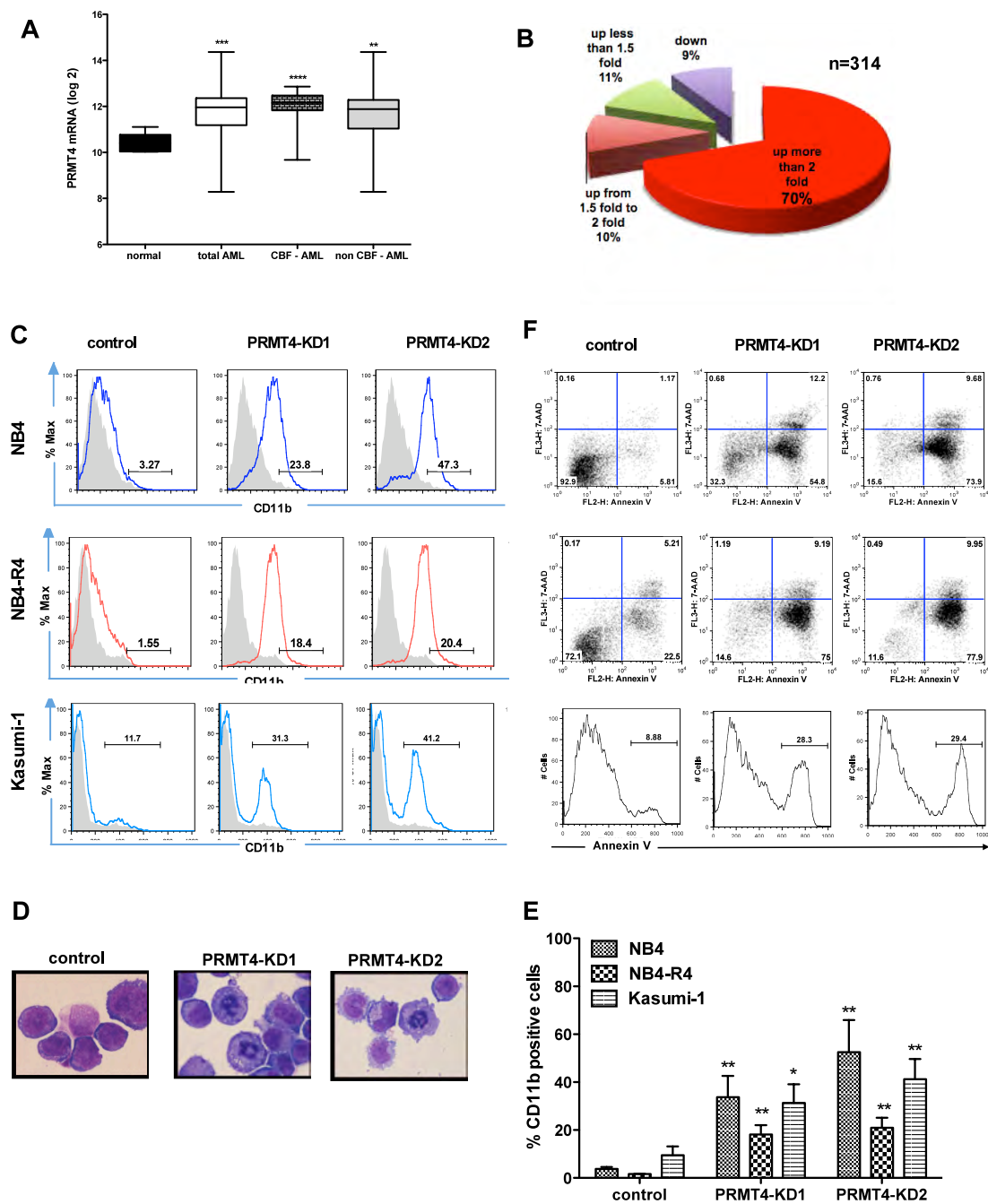


Figure 16. Knock down of PRMT4 is sufficient to induce myeloid differentiation and apoptosis in AML cell lines

(A). PRMT4 expression is upregulated in AML patient samples. The graph shows the log₂ expression of PRMT4 from transcript profiling of CD34⁺ bone marrow cells isolated from 5 healthy donors (normal) or 318 AML patients. CBF: core-binding factor.

CBF-AML n=57. non CBF-AML n=261. **** p < 0.0001 *** p < 0.001 ** p < 0.01 by Student's *t* test.

(B). Detailed analysis of PRMT4 expression patterns in AML patients.

(C). Knock down of PRMT4 triggers the myeloid differentiation of NB4, NB4-R4 and Kasumi-1 cells. Cells were transduced with lentiviruses expressing control (scrambled) shRNA or shRNA directed against PRMT4. Sorted GFP positive cells were cultured for 3 days prior to FACS analysis of CD11b expression. A representative FACS was shown. Quantitative data was presented in supplemental Figure 7D.

(D). Morphology analysis of NB4 cells transduced with scramble shRNA control or shRNA directed against PRMT4 (PRMT4-KD).

(E). Quantitative summary of FACS analysis of CD11b expression in control and shRNA against PRMT4 transduced NB4, NB4-R4 and Kasumi-1 cells. The data represents the average and \pm SD of three independent experiments.

(F). Knock down of PRMT4 in leukemic cells induces apoptosis. Cells transduced with lentiviruses expressing control (scrambled) shRNA or shRNA directed against PRMT4 were sorted for GFP and cultured for 3 days prior to FACS analysis of Annexin V staining.

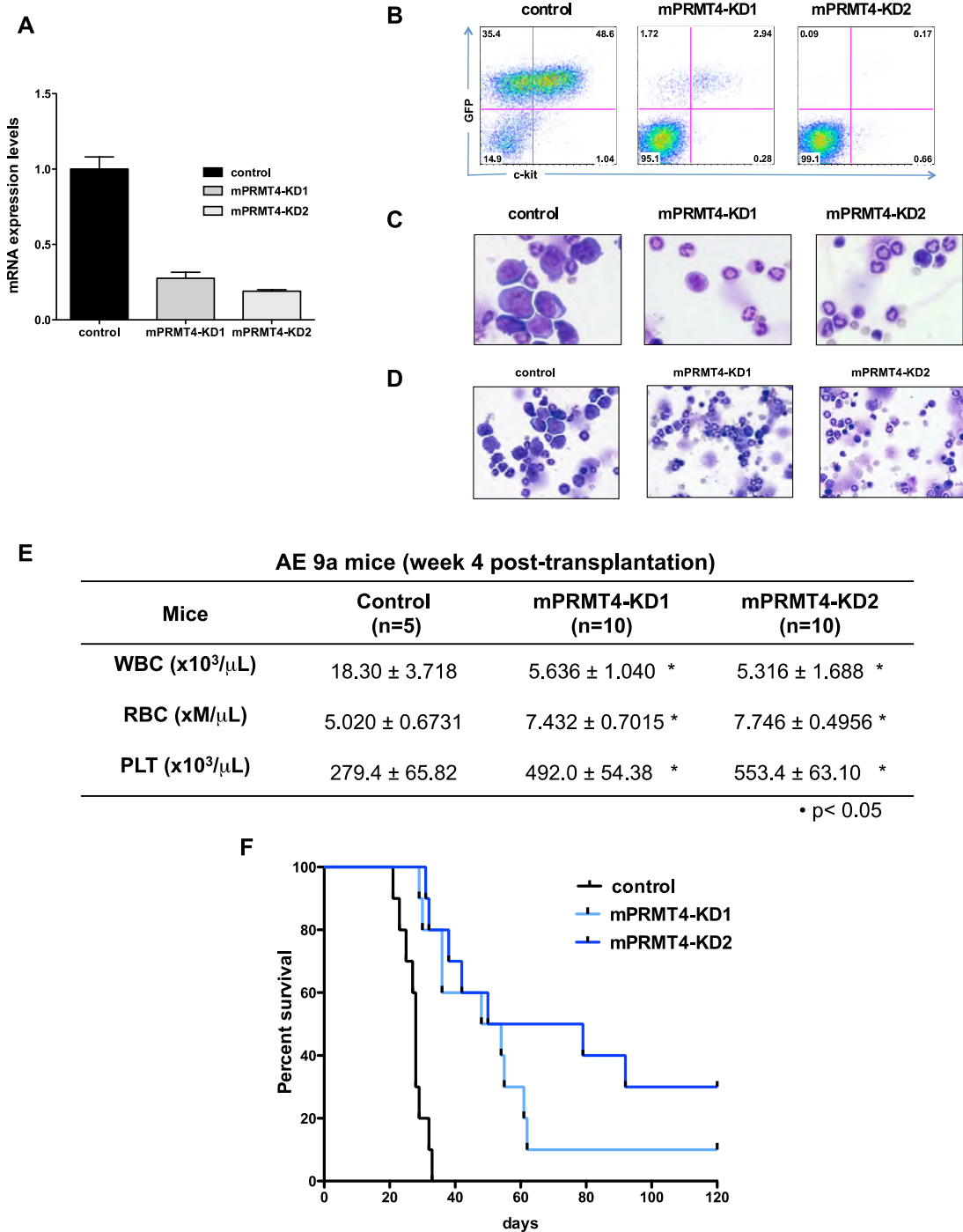


Figure 17. Knock down of PRMT4 reduces leukemia burden in vivo

(A). Efficient knock down of PRMT4 in AE9a expressing mouse leukemia cells. Cells were transduced with lentiviruses expressing a control (scrambled) shRNA or one of two shRNAs directed against PRMT4. RFP-positive cells were sorted 3 days after transfection and collected to perform qRT-PCR analyses. mRNA expression levels were normalized to HPRT. The mean \pm SD of three independent experiments is shown.

(B). The effect of knock down of PRMT4 on AE9a driven acute leukemia. AE9a expressing mouse leukemia cells were transduced with shRNA scramble control (AE9a-control), or shRNAs against PRMT4 (AE9a-mPRMT4-KD1 and mPRMT4-KD2). Transduced cells were sorted for expression of RFP and the sorted cells were injected into recipient mice that had received sublethal irradiation. FACS analysis showed far fewer GFP⁺ ckit⁺ cells in peripheral blood (PB) of the mice transplanted with AE9a – mPRMT4-KD cells in compare to AE9a – control cells at week 3. Representative data is shown.

(C). The effect of knock down of PRMT4 on AE9a driven acute leukemia. Bone marrow (BM) morphology shows marked reduction in the number of leukemic cells in mice transplanted with AE9a -mPRMT4-KD cells, compared to AE9a- control cells. A representative experiment is shown.

(D). The morphology of bone marrow (BM) at 40X showed a marked reduction in the number of leukemic cells in the BM of mice transplanted with AE9a – mPRMT4-KD cells, compared to AE9a – control cells.

(E). CBC counts of mice transplanted with AE9a-mPRMT4KD cells and AE9a control cells.

(F). Knock down of PRMT4 prolongs the survival of AE9a transplanted mice. The median survival was extended in the knock down groups, compared to the control group (54 days and 64.5 days vs. 28 days p <0.0001).

3.2.3 Small molecule inhibitors of PRMT4 induces myeloid differentiation and apoptosis in AML cell lines

By using specific shRNAs to target PRMT4 in leukemic cells both in vitro and in vivo as the first proof of concept, we have demonstrated the therapeutic potential of PRMT4 for treatment of AML. Despite advancements in the field, to develop a small RNA based-therapy, the clinical relevant of this approach is still limited. On the other hand, several small molecule inhibitors of PRMT4 (PRMT4i) have been developed. While these compounds have been shown to be active inhibitors of PRMT4 in vitro at nanomolar range, no in vivo cellular study has been reported. Thus, to more definitely establish PRMT4 as a target in leukemia therapy, we explored the use of small molecule inhibitors of PRMT4 in our established cell and mouse model systems.

Given the growth inhibitory effects of PRMT4 knock down, we first tested whether a PRMT4 inhibitor (PRMT4i – figure 18A[256]) also inhibits cell growth. A panel of lymphoid and myeloid leukemia cell lines with different genetic backgrounds was used. Cells were plated in 96 well plates at an optimal cell density and treated with inhibitors at varying concentrations. After 72h, cell viability was assayed using the CellTiter Glo. We observed that PRMT4i significantly inhibited growth of all leukemic cells tested (Figure 18B). The IC_{50} specific for each cell line was calculated showing a range from 0.5 to 2.2 μ M (Figure 18C). Normal CD34+ cells were the most resistant to PRMT4i's inhibition (IC_{50} =3.3 μ M), suggesting that PRMT4i can be used to specifically target cancer cells. Because PRMT4KD triggered myeloid differentiation and apoptosis in APL cell lines (NB4 and NB4-R4), we examined whether similar effects were achieved using PRMT4i. As we expected, treatment of the cells with PRMT4i resulted in myeloid differentiation, as with an increase in CD11b expression and also increased apoptosis (Figure 18D and 18E). The effect of PRMT4i is more profound than that of retinoic acid, suggesting that PRMT4i is a very potent inducer of differentiation and cell death.

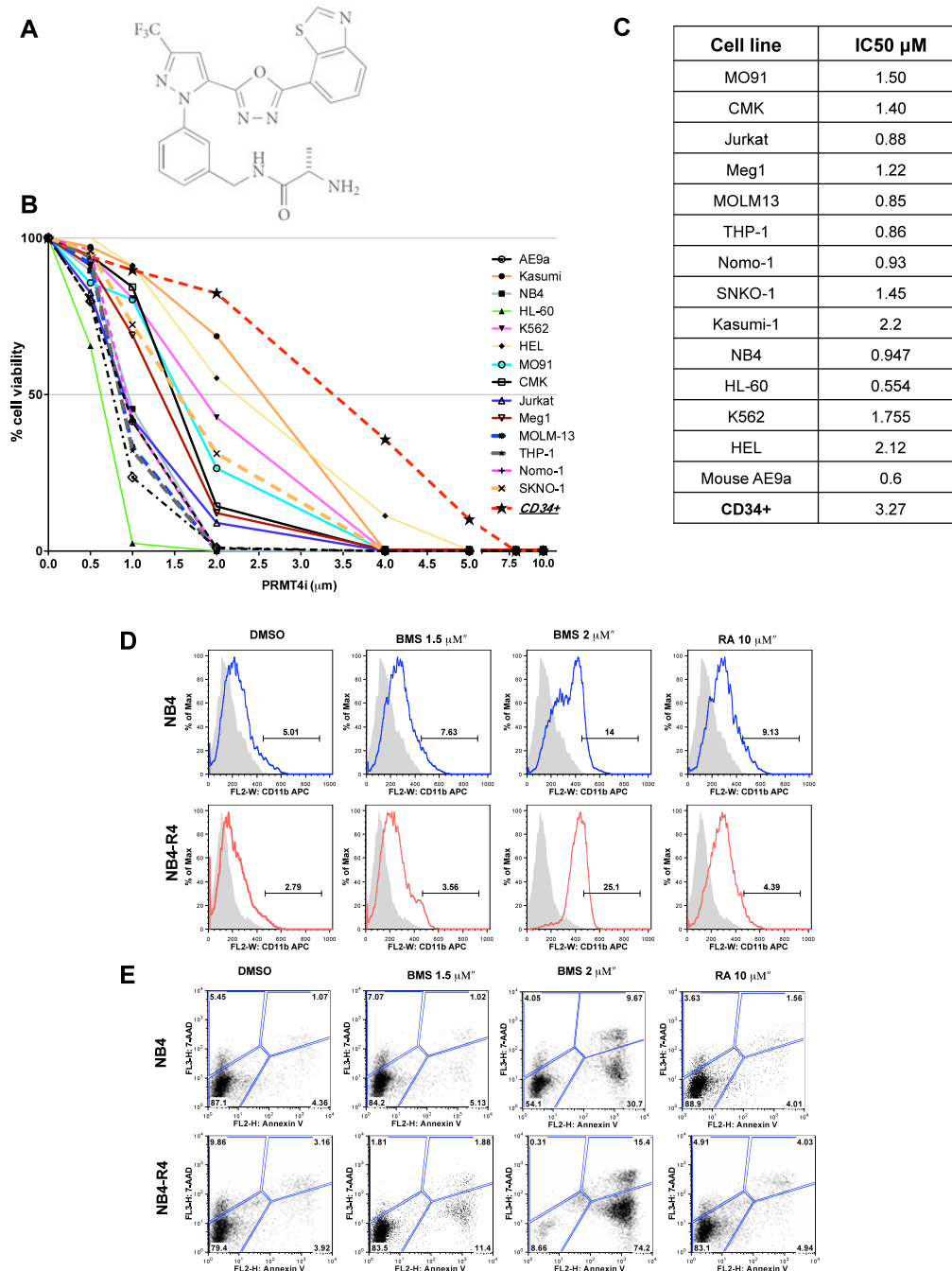


Figure 18. PRMT4i inhibits leukemia cell growth

(A). Structure of PRMT4 inhibitor

(B). Cell viability curve of leukemia cell lines treated with different concentrations of PRMT4i. Cell viability was assayed using CellTiter Glo. % Cell viability value was normalized to control treatment with vehicle only (0 μM).

(C). Calculated IC₅₀ of PRMT4i for each cell line

(D)(E). NB4 and NB4-R4 cells were treated with PRMT4i and RA for 72h and assayed for differentiation by staining for CD11b and for apoptosis by staining for Annexin V

We then further performed a comprehensive analysis of PRMT4 treatment with Kasumi-1 cell line. Kasumi-1 cells express AML1-ETO, as well as a constitutively active mutant form of c-kit. While there are effective therapies for patients with APL, developing a treatment for core-binding factor AML is still a challenge. Therefore, a novel therapeutic approach will have a significant clinical impact.

First, we showed that treatment of cells with PRMT4i inhibited PRMT4 enzymatic activities, reducing the level of RUNX1 methylation and also the methylation of H3R17, two major substrates of PRMT4 (Figure 19A). Mirroring the results of PRMT4KD, PRMT4i treatment also increased the fraction of CD11b⁺ cells by 6 fold. The immunophenotype, correlated with the differentiation-associated morphological changes that we observed in PRMT4i-treated cells (Figure 19B). We again observed a growth inhibitory effect (Figure 19C), as the PRMT4i-treated cells underwent cell cycle arrest, cells at sub-G1 and G2/M phase with a significantly higher fraction of in the PRMT4i-treated samples (Figure 19D), as well as increase in apoptotic cells (Figure 19E). Taken together, treatment with PRMT4i induces myeloid differentiation and inhibits cell growth by triggering cell cycle arrest and apoptosis, which are two of the most desirable features of a cancer drug.

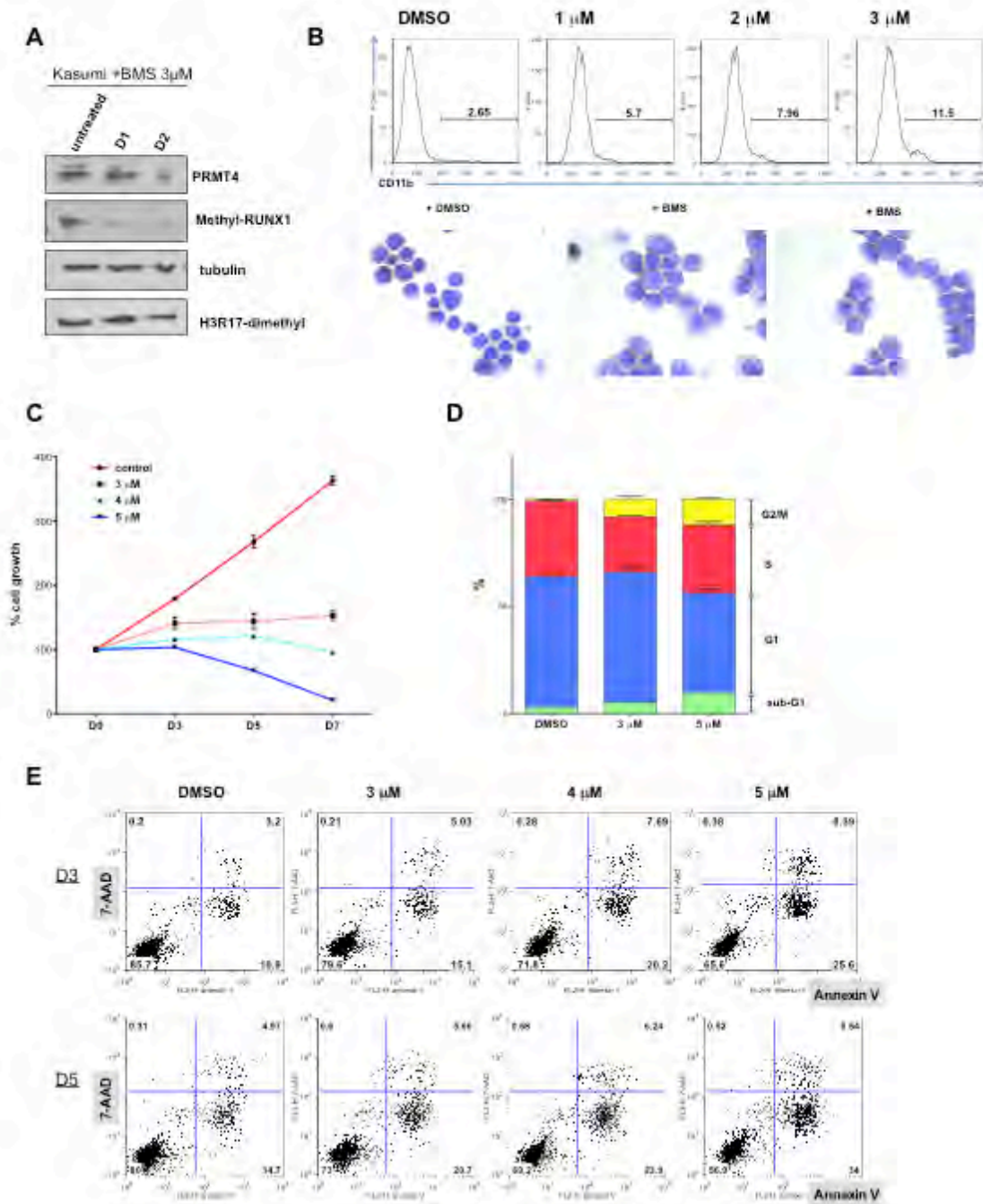


Figure 19. Effects of PRMT4i on Kasumi-1 cells

(A). PRMT4i inhibits PRMT4 enzymatic activity in vivo. Kasumi cells were treated with 3 μ M BMS and collected after 24h (D1) and 48h (D2). Western blot analysis of the whole cell extract showed a decrease in the level of methylation of two PRMT4 substrates, RUNX1 and H3.

(B). PRMT4i induces the differentiation of Kasumi-1 cells. After 5 days of PRMT4i treatment, the cells were collected and assayed by flow cytometry for CD11b expression (and cell morphology) for evidence of myeloid differentiation.

(C). PRMT4i inhibits cell growth. Cells were treated for 7 days with either PRMT4i or vehicle. The number of cells at the different time points was assayed by trypan blue exclusion. The number of cells present was normalized to day 0 (D0).

(D). PRMT4i induces cell cycle arrest. After 5 days of PRMT4i treatment, Kasumi-1 cells were collected, fixed, stained for PI and subjected to cell cycle analysis by flow cytometry.

(E). PRMT4i induces apoptosis. Kasumi-1 cells were treated with PRMT4i or vehicle and collected at day 3 (D3) and day 5 (D5) for apoptotic analysis (by flow cytometry) by staining for 7-AAD and Annexin V.

3.2.4 Ex vivo treatment with PRMT4 inhibitor reduces leukemia burden in vivo

As we observed a profound effect of PRMT4i treatment on cell lines, we next examined the effect of PRMT4i on in vivo leukemia cell growth. We treated 10^5 AE9a expressing murine leukemia cells ex vivo with PRMT4i at $3\mu\text{M}$ and $10\mu\text{M}$ (vs. DMSO as vehicle control) for 1 hour before injecting cells into sub-lethally irradiated C57Bl/6 mice (day 0). We also examined the effect this brief treatment on the cells prior to their injection, and observed a slight increase in apoptotic cells (stained positive for both 7-AAD and Annexin V) after treatment with $10\mu\text{M}$ PRMT4i (Figure 20A). Treatment with PRMT4i also reduced the number of leukemic immature GFP⁺ and GFP⁺c-kit⁺ cells in AE9a mice (Figure 20B, C). This translated to a significant increase in the median survival from 25 days to 30 days ($3\mu\text{M}$ PRMT4i $p < 0.05$) and 43 days ($10\mu\text{M}$ PRMT4i $p < 0.001$) (Figure 20D). Thus, the PRMT4 inhibitor decreases the growth of AE9a expressing leukemic cells in vivo, providing a potential therapeutic approach to t(8;21) AML.

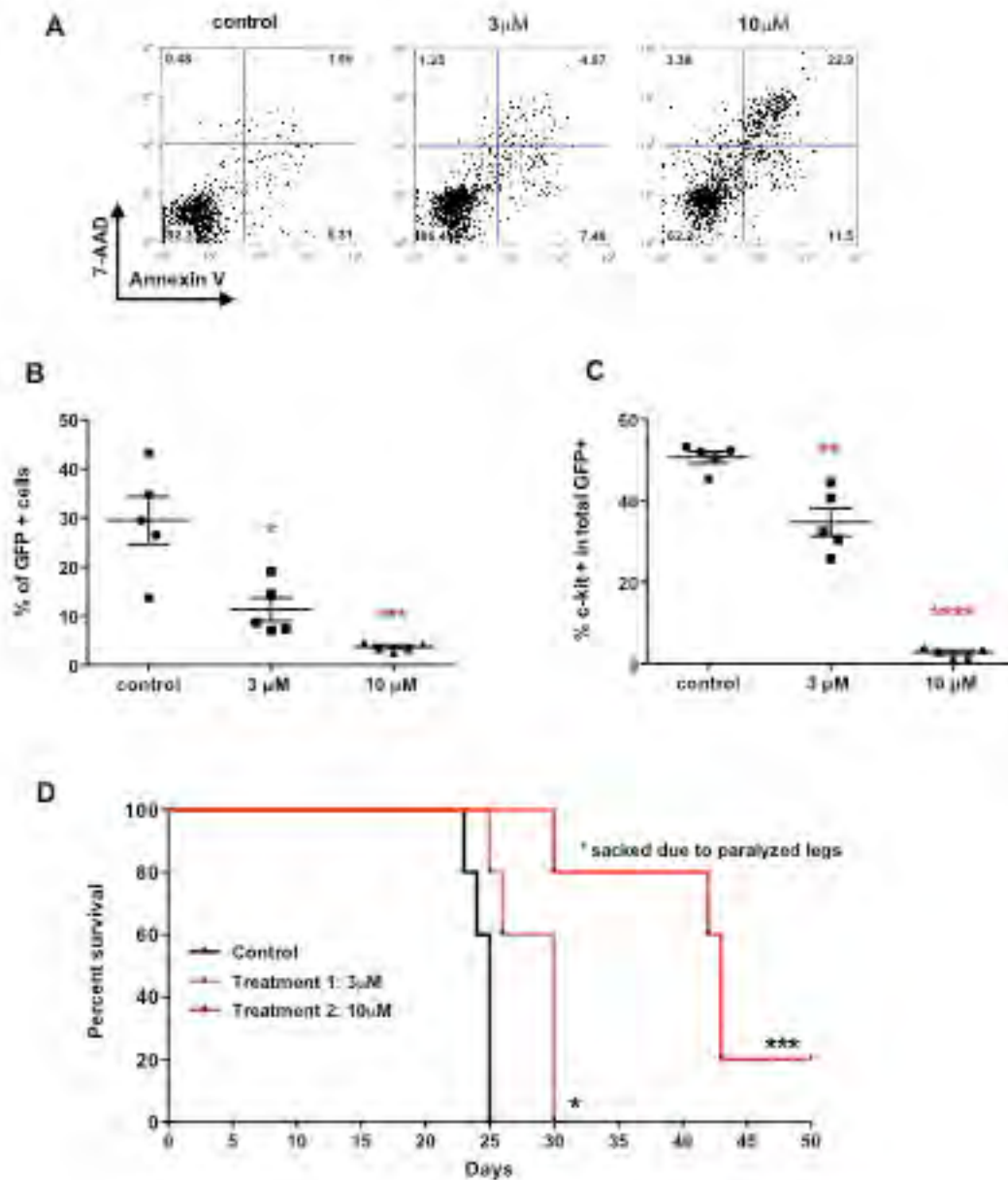


Figure 20. Ex vivo treatment using PRMT4 inhibitor reduces leukemia burden in vivo

(A). Apoptotic analysis of AE9s cells treated with DMSO control and PRMT4i prior to injection

(B), (C). FACS analysis showed far fewer GFP+, GFP+ ckit + cells in peripheral blood (PB) of the mice transplanted with AE9a –PRMT4i treated cells in compare to AE9a –DMSO treated cells at day 19 of transplantation. * p<0.05; ** p<0.01; *** p<0.001; ****p<0.0001

(D). Treatment with PRMT4i prolongs the survival of AE9a- PRMT4i transplanted mice. The median survival was extended in the PRMT4 treatment groups, compared to the control group (30 days and 43 days vs. 25 days p <0.05 and p<0.001)

3.2.5 Loss of PRMT4 functions activates myeloid differentiation and cell death transcription programs in leukemia cells

We have established that PRMT4 regulates myeloid differentiation of human primary CD34⁺ cells partly by modulating miR-223 expression level, and that CD34⁺ cells treated with PRMT4i also exhibit an increase in miR-223 expression (Figure 21A). The similar result was also observed in NB4 cells (data not shown). However, Fazi et al. demonstrated that in t(8;21) positive cells, miR-223 expression is repressed via a different mechanism involving the recruitment of repressors to a different miR-223 promoter through the ETO part of the AML1-ETO fusion protein[105]. We examined miR-223 expression in Kasumi-1 cells and found that miR-223 level was not altered after PRMT4 was knocked down or inhibited by PRMT4i (Figure 21B). This suggests that PRMT4 is a master regulator of myelopoiesis and PRMT4 can control the process via miR-223 independent pathways. To further assess PRMT4 as a major inhibitor of myeloid differentiation we knocked down PRMT4 and also miR-223 (double KD) to see whether the upregulation of miR-223 expression is required for myeloid differentiation in CD34⁺ cells (Figure 21C). CD34⁺ cells were infected with lentiviruses expressing shRNA against PRMT4 and GFP, as well as lentiviruses expressing miR-223 inhibitor and RFP. Transduced cells were sorted based on GFP and RFP positivity and cultured in myeloid differentiation conditions for 7 days prior to FACS analysis. We observed that even without an increase in miR-223 level, the down regulation of PRMT4 expression in the double KD cells is sufficient to promote myeloid differentiation (Figure 21D).

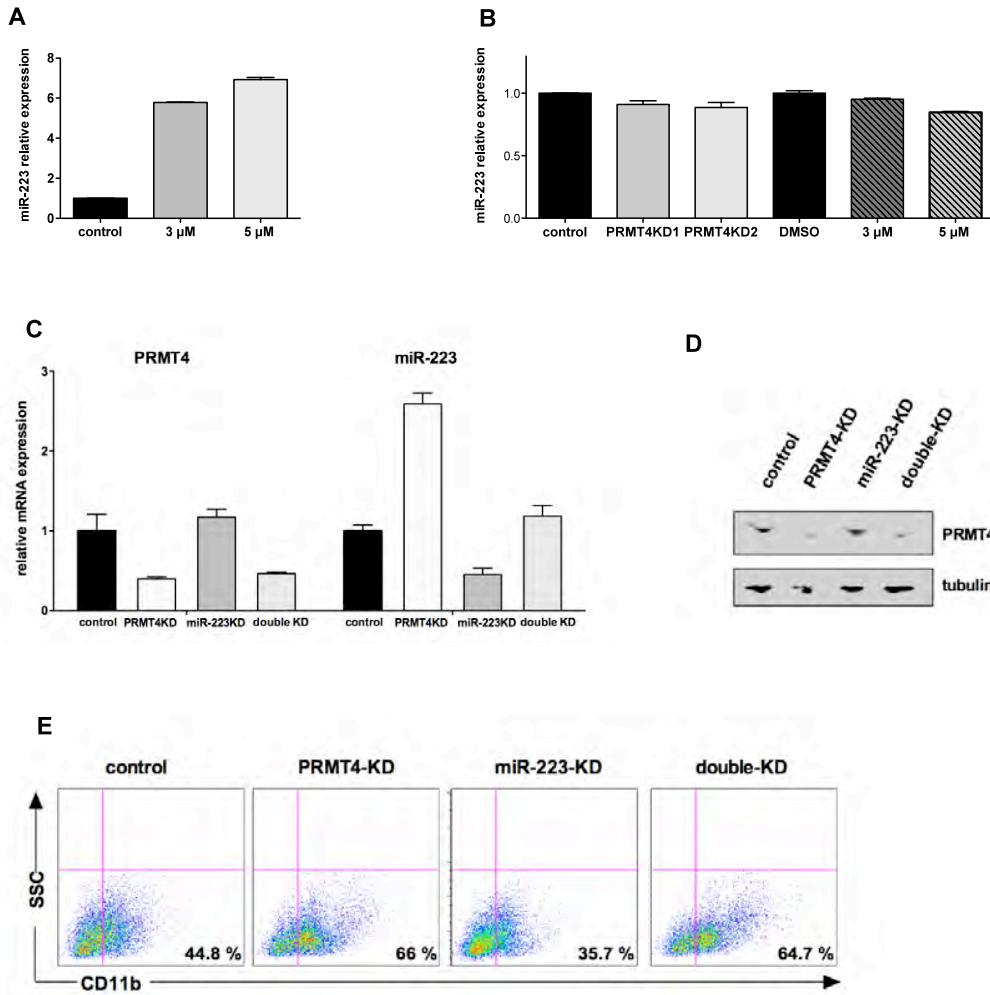


Figure 21. MiR-223 upregulation is dispensable for myeloid differentiation triggered by loss of PRMT4 function

(A). PRMT4i treatment results in increase of miR-223 expression in CD34⁺ cells. qRT-PCR analysis of miR-223 in control and PRMT4i treated CD34⁺ cells. Results are normalized to RNU6. Mean \pm SD of two independent experiments is shown.

(B). Loss of PRMT4 function does not alter miR-23 expression in Kasumi-1 cells. qRT-PCR analysis of miR-223 in control and PRMT4 knock down or PRMT4i treated Kasumi-1 cells. Results are normalized to RNU6. Mean \pm SD of two independent experiments is shown.

(C). Efficient knockdown of miR-223 and PRMT4. qRT-PCR analysis of miR-223 and PRMT4 expression in control, PRMT4 knock down, miR-223 knock down and double knock down CD34⁺ cells. Results are normalized to RNU6 and HPRT. Mean \pm SD of three independent experiments is shown. Tubulin serves as loading control.

(D). Upregulation of miR-223 expression is dispensable for the acceleration in myeloid differentiation when PRMT4 is knocked down. FACS analysis of cells plated in myeloid differentiation promoting culture and assayed for CD11b expression after 7 days. A representative experiment is shown.

Further to identify the regulatory pathways controlled by PRMT4 in normal vs. leukemia cells we performed gene expression profiling of Kasumi-1 cells and compared to normal CD34⁺ cells. Functional analysis using Ingenuity systems (IPA tools) of PRMT4KD CD34⁺ cells (in compare to shRNA scramble control cells) revealed activation of gene signatures indicative of myeloid differentiation (Figure 22A) and also gene signatures corresponding to cell cycle delay (Figure 22B). The result is similar to that using GSEA, indicating the induction of the myeloid differentiation transcription program (Figure 6C). Moreover, the activation of the cell cycle arrest gene sets suggests that the differentiation process is coupled to withdrawal from the cell cycle. On the other hand, the same analysis of PRMT4KD Kasumi-1 cells indicates the upregulation of differentiation gene programs (Figure 23A) and the expression of genes involved in apoptosis and cell death (Figure 23B). These results clearly demonstrate that KD of PRMT4 triggers myeloid differentiation gene expression changes in both normal and leukemia cells. However, unlike normal CD34⁺ cells, leukemia cells exhibit a requirement for PRMT4 in their survival, as loss of PRMT4 leads to activation of apoptotic transcriptional programs and apoptosis. This further suggests that targeting PRMT4 could hold great potential as a novel AML therapy.

A

GEF of PRMT4KD correlates with the gene signature of differentiation			
Functions Annotation	p-Value	Predicted Activation State	Activation z-score
cell movement of myeloid cells	5.12E-04	Increased	3.822
differentiation of blood cells	3.64E-06	Increased	2.594
differentiation of mononuclear leukocytes	2.94E-05	Increased	2.412
differentiation of leukocytes	3.61E-05	Increased	2.281
differentiation of T lymphocytes	1.71E-04	Increased	2.063

B

GEF of PRMT4KD correlates with the gene signature of cell cycle delay			
Functions Annotation	p-Value	Predicted Activation State	Activation z-score
interphase	2.36E-14	Decreased	-3.654
S phase	2.78E-14	Decreased	-3.631
entry into S phase	6.04E-09	Decreased	-3.373
delay in mitosis	1.21E-07	Increased	2.069
delay in cell cycle progression	3.86E-05	Increased	2.073
sister chromatid exchange	6.95E-05	Increased	2.217

Figure 22. Biological function analysis of PRMT4KD in CD34+ cells dataset
 (A). Activation of myeloid differentiation program. (B). Activation of cell cycle delay program. The list of over 1500 genes with at least 2-fold change in KD samples in compared to the control samples (RNA-sequencing FDR <0.05) was used for analysis using IPA tools.

A

GEF of PRMT4KD correlates with the gene signature of myeloid functions			
Functions Annotation	p-Value	Predicted Activation State	Activation z-score
recruitment of leukocytes	1.13E-03	Increased	3.010
cell movement of granulocytes	6.66E-04	Increased	2.953
cell movement of neutrophils	7.92E-04	Increased	2.627
migration of granulocytes	6.99E-03	Increased	2.415
differentiation of blood cells	3.46E-03	Increased	1.616

B

GEF of PRMT4KD correlates with the gene signature of cell death and apoptosis			
Functions Annotation	p-Value	Predicted Activation State	Activation z-score
apoptosis of tumor cell lines	2.79E-04	Increased	2.479
cell death of tumor cell lines	5.20E-04	Increased	2.215
cell death of immune cells	4.02E-07	Increased	1.958
apoptosis of leukemia cells	6.08E-03	Increased	1.932
apoptosis	2.69E-04	Increased	1.904

Figure 23. Biological function analysis of PRMT4KD in Kasumi-1 cells dataset
 (A). Activation of myeloid differentiation program. (B). Activation of cell cycle delay program. The list of over 270 genes with at least 2-fold change in KD samples in compared to the control samples (Microarray FDR <0.05) was used for analysis using IPA tools.

4. DISCUSSION

Using a well-established human primary cell model, we have identified PRMT4 as an inhibitor of myeloid differentiation (Figure 24). We showed that in HSPCs, where PRMT4 is highly expressed in hematopoietic stem/progenitor cells, it methylates RUNX1 at R223, promoting the assembly of a transcriptional co-repressive complex, which contains DPF2, and repressing the transcription of the miR-223 locus. As HSPCs undergo myeloid differentiation, PRMT4 expression decreases, reducing the amount of R223-methyl RUNX1, which in turn, decreases the presence of DPF2 at the miR-223 promoter region, thus allowing miR-223 to be transcribed. The ability of miR-223 to target PRMT4 by binding to its 3'-UTR allows the upregulation of miR-223 expression, which further decreases PRMT4 expression, further sustaining the myeloid differentiation process.

The proposed model (Figure 24) demonstrates the underlying mechanism of the regulation of myeloid differentiation by PRMT4. However, it seems clear that PRMT4 impacts many pathways in addition to regulating miR-223 expression. Indeed, we found that loss of PRMT4 not only led to activation of a myeloid development of transcriptional program, but it impacted a variety of developmental and signaling pathways (data not shown). These effects are both miR-223 dependent and miR-223 independent. While we have defined a novel link between an arginine methyltransferase and the expression of a microRNA (miR-223), with PRMT4 and miR-223 forming a regulatory loop to influence myeloid differentiation, we also demonstrated that PRMT4 is the driving force of the process. Down-regulation of PRMT4 activity alone is sufficient to induce differentiation in both human primary and leukemia cells.

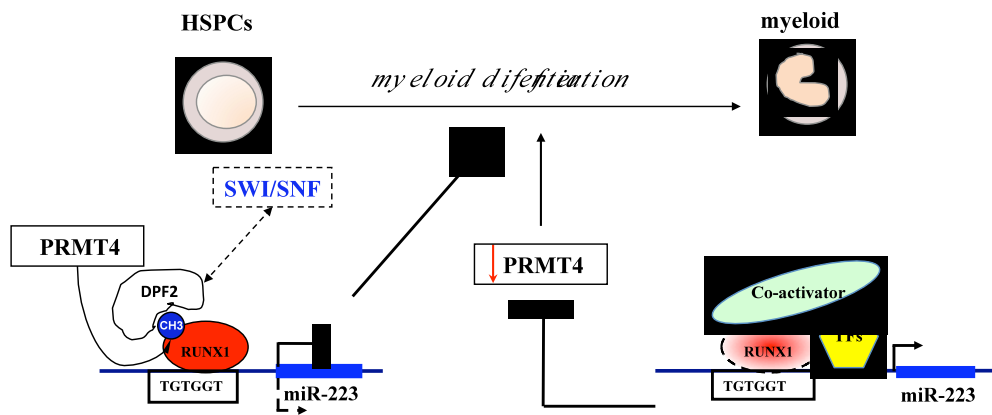


Figure 24. A schematic model showing PRMT4 regulates myeloid differentiation of human HSPCs.

PRMT4 and miR-223 form a regulatory loop that is critical for myeloid differentiation. PRMT4 inhibits myeloid differentiation by assembling a methyl R223-RUNX1-DPF2 repressor complex that suppresses miR-223 expression. When HSPCs undergo myeloid differentiation, PRMT4 expression is downregulated, releasing the miR-223 locus from transcription repression, allowing it to be transcribed. At that stage, the higher expression of miR-223 targets the PRMT4 3'-UTR to further decrease PRMT4 expression, thereby reinforcing the myeloid differentiation process.

Unlike in several other biological systems including T cell, adipocyte and muscle development where PRMT4 has been shown to be a positive facilitator, promoting the differentiation process, in the hematopoietic system, PRMT4 blocks differentiation. This role is actually consistent with its role in embryonic stem (ES) cells. Torres-Padilla et al. demonstrated that the level of histone H3 R17 and R26 methylation, a bone-fide substrates of PRMT4, influence the cell fate determination of mouse embryo cells between the inner cell mass and mural trophectoderm. Elevated expression of PRMT4 (and H3R17 methylation) directs progeny cells to the inner cell mass and results in upregulation of Nanog and Sox2 expression [252]. Given those findings, Wu et al. further showed that PRMT4 is required for the self-renewal and pluripotency of ES cells. Depletion of PRMT4 decreases expression of pluripotency genes, thereby triggering differentiation. On the other hand, overexpression of PRMT4 hampers the cellular response to differentiation signals such as the addition of retinoic acid (RA) or the removal of leukemia inhibitory factor (LIF) from the medium [251]. Thus, it appears that PRMT4 can function to maintain the stemness of HSPCs as depletion of PRMT4 downregulates the HSC gene signature (data not shown). The results suggest that PRMT4 may both preserve the stem cell transcriptional program and suppress the expression of myeloid differentiation promoting genes.

Although the primary human hematopoietic CD34⁺ cells have proven to be a powerful tool in study of hematopoiesis, the system does have several limitations. Thus, it is important to complement the in vitro data with in vivo studies. Although the phenotype of several tissues of the germline PRMT4 knockout (KO) mouse was reported, there has been no comprehensive characterization of its hematopoietic system. PRMT4-KO mice

are born smaller in compare to their littermates and died early after birth due to a defect in lung development [245]. An examination performed at that development stage showed a block in T cell development but no further analysis of the hematopoietic system was reported. To obtain a more through view of the in vivo roles of PRMT4 we have begun to analyze a Vav-Cre-PRMT4-KO mouse, where PRMT4 is deleted specifically in the blood system. Our primary data showed a slight increase in myeloid cells and in granulocyte-macrophage progenitors (data not shown). The result is agreed with our human in vitro data, suggesting that differentiation is skewed toward the myeloid lineage when PRMT4 is lost. However, the phenotype is mild, suggesting that PRMT4 functions are readily compensated for normal cells. Studies are ongoing to carefully evaluate the hematopoietic phenotype of the adult PRMT4 null mice.

A fundamental aspect of transcriptional regulation has been to define how a given protein can function either as an activator or a repressor. We have recently shown that AML1 (RUNX1)-ETO, a well-known leukemia-associated fusion protein generally thought to function as a transcriptional repressor, has activating functions as well, that are critical to its leukemogenic properties [127]. Our study of PRMT4 provides further evidence for a flexible model of how proteins regulate gene expression. PRMT4 has been thought of as a “secondary” co-activator molecule, that helps activate transcription of its target genes. Mechanistic studies suggested that this function, as part of the nuclear receptor-activating pathway, is based on PRMT4 dependent methylation of histone H3, which then recruits additional transcriptional activators [239, 240]. We have not only identified a transcription repressor function for PRMT4 but also provided a molecular basis for this function, which involves the methylation of a non-histone substrate, namely RUNX1,

which triggers the assembly of a R223 methyl RUNX1/DPF2 complex. The interaction of PRMT4 with RUNX1 appears to be transient, i.e. a kind of “hit and run”. However, the recruitment of PRMT4 to the chromatin of its target genes could be more stable, either due to its binding histones or other chromatin associated factors. In our model, we demonstrated the dynamic regulation only at the promoter of miR-223. However, detailed analysis of PRMT4-KD RNA-sequencing data revealed that over 40% of PRMT4 target genes (defined as ≥ 1.5 -fold change upon PRMT4KD) have consensus RUNX1 binding site(s) at their regulatory regions, suggesting that RUNX1 is capable of recruiting PRMT4 to a vast number of genes. Thus, we believe that this mode of regulation is a general mechanism employed by PRMT4 to control transcription.

It is clear that RUNX1 can assemble a variety of multi-protein complexes that affects its transcriptional regulatory functions, allowing it to serve as a scaffold protein. These complexes are regulated by various post-transcriptional modifications: For example, the association of RUNX1 with mSIN3A is disrupted by the PRMT1-dependent methylation of RUNX1 on R206 and R210 [141]. Similarly, the methylation of C/EBP β by PRMT4 interfered with its association with both the SWI/SNF and Mediator complexes [233]. In contrast to that model, we show that the methylation of RUNX1 by PRMT4 actually promotes protein-protein interactions. We found the preferential binding of DPF2 to R223-methylated RUNX1 and that by recruiting DPF2, RUNX1 can repress miR-223 expression. This function of DPF2 is consistent with its ability to act as a co-suppressor of nuclear receptor- mediated transcription regulation, by binding to HDAC1 [265]. In addition, Ho L et al. reported that DPF2/BAF45d is a member of the ES cell-specific Brg-containing complex that interacts with the DNA methylation machinery (including

HELLS, DNMT3B, and DNMT3L)[266]. Among the many interesting candidate proteins that bind preferentially to either methyl- or non-methyl RUNX1 peptides, we examined DPF2 for several reasons in addition to its role as a transcriptional co-repressor. Little is known about its biological functions of DPF2, especially in hematopoietic system. Studies of other BAF45 members in neural stem cell differentiation [267] showed a switch off from BAF45a to BAF45b and BAF45c during the transition from neural progenitors to post-mitotic neurons. The alternation in BAF composition is essential for a proper differentiation process, suggesting that there is a precise requirement for functions of specific BAF45 proteins during each developmental stage. Hence, we hypothesized that DPF2/BAF45d could be a specific BAF subunit that regulates myeloid differentiation. Even though DPF2 is expressed in other tissues and remains expressed during the differentiation of HSPCs, we showed that the recruitment of DPF2 to a RUNX1 target gene promoters is dependent on methylation of RUNX1. Therefore, it appears that DPF2 function can be dynamically regulated by PRMT4 during the differentiation process. Indeed, we identified DPF2 as another important regulator of myeloid differentiation that could cooperate with PRMT4 to maintain the “stemness” of HSPCs.

It is well established that proteins can be subjected to multiple post-translational modifications and the crosstalk between those modifications can help coordinate various regulatory signals. We have previously shown that RUNX1 is arginine methylated by PRMT1 at its RTAMR region, and that this methylation event appears when HSPCs begin to differentiate toward the myeloid lineage. We have discovered another methylation mark of RUNX1, at the R223 residue, by PRMT4 and in contrast to the

RTAMR methylation; the R223-methyl mark decreases during differentiation. In the process of our studies, we found that PRMT5 binds specifically to R233-methylated RUNX1. This interaction allows for the methylation of RUNX1 on other arginine residues by PRMT5 (data not shown). These data strongly implicate an interactive regulatory network of how different PRMT proteins interact to regulate hematopoiesis. A functional connection between different PRMTs has been shown in other biological systems. For example, PRMT1 and PRMT4 can work cooperatively to activate transcription [242] and PRMT4 and PRMT5 are both important for muscle development and differentiation, where they function in a stepwise manner to coordinate the expression of early and late-developmental genes [250]. Thus, it will be important to further decipher such interactions between the different PRMTs in the hematopoietic system and harness this knowledge to develop PRMT-based therapies for leukemia.

Modulating the differentiation and apoptotic processes of cancer cells has become a promising therapeutic approach, especially in the treatment of hematologic malignancies like AML, which are characterized by a block in differentiation. Although clinical advancements in AML have been made, especially in APL, outcomes for patients with non-APL AML remains unsatisfactory. In our studies, we identified PRMT4 as a potent inducer of the differentiation process. In addition, there is a high level of PRMT4 expression in AML patients, suggesting that PRMT4 is involved in AML pathogenesis and identifying PRMT4 as a potential target. Given these findings, we were able to differentiate myeloid leukemic cells by knocking down PRMT4 or treating cells with a PRMT4 inhibitor. The drastic differentiation effects were observed not only in APL cell lines but also in ATRA-resistant APL cell lines and non-APL cell lines. Moreover, by

utilizing the AML1-ETO driven leukemia model, we showed that loss of PRMT4 not only induced myeloid differentiation but also triggered apoptosis leading to improved survival in an in vivo mouse AML model. These results clearly demonstrated that PRMT4 could hold potential as a novel therapy of acute myelogenous leukemia. While these findings are very encouraging, there is still much to be done. While the ex vivo treatment is effective in reduce the leukemia burden, an in vivo treatment using the PRMT4 inhibitors in animals with leukemia will be required to further demonstrate the therapeutic activity of these compounds. Finally, it is desirable for us to be able to bring these drugs to patients in the contact of clinical trials.

The lack of an effect on normal hematopoiesis in PRMT4 null embryonic mouse [245] and different survival responses between normal vs. leukemia cells when PRMT4 is knocked down in human primary cells system (loss of PRMT4 results in leukemic cell death while only differentiating normal primary cells), suggests that PRMT4 specific inhibitor(s) can selectively target leukemia cells. In addition, a mild phenotype has been observed in the hematopoietic system of adult PRMT4 knockout mouse (data not shown), suggesting that PRMT4 functions could be readily compensated for in normal cells. Moreover, PRMT4 appears to play the role of an oncogene when overexpressed in leukemia. It is possible that leukemia cells, which are characterized by a block in differentiation, could become addicted to the high expression level of oncogene PRMT4 to maintain the “leukemic stemness” and survival. Therefore, reduction of PRMT4 expression may lead to cellular differentiation and consequently cell death. The basic concept for targeting PRMT4 is to use PRMT4 inhibitors as an inducer of the differentiation process. Thus, it is interesting to explore whether PRMT4i can be used in

combination with other differentiation compounds such as ATRA and epigenetic modifier LSD1 inhibitors [183,185] to obtain desirable clinical responses. It is anticipated that PRMT4i could act to prime the process, hence potentiate the effects of these specific differentiation agents. While holding great potentials in treatment, the use of PRMT4 based therapy in human context could face several issues. Firstly, we observed that knockdown of PRMT4 blocks the differentiation of erythroid cells and megakaryocytes, thus likely, a complete and sustained inhibition of PRMT4 could lead to thrombocytopenia, and erythropenia. However, it is noted that the effects are reversible since the normal hematopoietic stem cells compartment is intact in comparison to the permanent killing effects of highly toxic chemotherapy drugs. Secondly, since PRMT4 has been shown to play roles in lung, adipocyte and T cell differentiation programs [245-249], inhibition of PRMT4 in vivo could potentially disturb normal functions of those organs. The degree of negative effects on those tissues vs. the benefit in reducing leukemia burden will need to be carefully determined in order to employ PRMT4 based therapy properly in clinics. In addition, the current PRMT4 inhibitor agent displays a potentially high toxicity level. Our data shows that the IC₅₀ of normal CD34⁺ cells is only slightly higher than that of leukemia cells. Treatment of CD34⁺ cells with PRMT4i results in growth arrest and apoptosis, which were not observed in PRMT4-knocked down cells (data not shown), suggesting that there will likely be an off-target toxicity. Therefore, it will be very important to distinguish the side effects of PRMT4 inhibition from outright toxicity when the agent(s) are given to patients. Despite these remaining challenges, we believe that our work has helped in establishing a strong foundation for the development of a novel therapy for AML.

The past decade has witnessed an increasing interest in the study of epigenetic regulators in hematopoiesis. As knowledge of these proteins has grown tremendously, targeting them has become a promising approach in developing cancer therapy. PRMT family members are among the attractive druggable targets, as dysregulation of PRMT activities has been reported in many types of cancers. Furthermore, the identification of small molecule inhibitors specific to PRMTs with high potency in vitro has heightened the interest in establishing PRMTs as potential therapeutic targets in leukemia [263, 264]. Hence, our studies of PRMT4 have significantly advanced the knowledge of PRMTs roles in hematopoietic system while identifying PRMT4 amongst the “master” regulators of the myeloid differentiation. Identifying the key regulators and mapping the key regulatory networks in the cellular hierarchy in human hematopoiesis is one of the central studies of the hematopoietic field. It provides insights into the blood differentiation and lineage commitment processes. As in Waddington’s epigenetic landscape, this developmental process involves global epigenetic and transcription changes in which the initial small changes in functions of a “master” regulator are amplified by downstream regulatory networks, thus bringing about the cascade of gene expression transformations. To define the landmarks and networks that shape the global landscape while determining the fundamental forces that drive the processes at each level of regulation are the two fundamental objections of this view. In our studies, we found that PRMT4 expression is one of the marks that corresponds to “stemness” and the differentiation stages of the cells. The change in PRMT4 protein level appears to be the initial “small” signal that sets off the cascade of gene expression changes driving the differentiation of stem/progenitor cells toward myeloid lineage. While these findings established PRMT4 as a critical

regulator of myelopoiesis, they raised the questions of a time window where the regulation of PRMT4 functions is obligatory for the transition and commitment of the cell to differentiate and whether down-regulation of PRMT4 expression at various sequential stages of differentiation affects the cells similarly. The concept is also important for development of therapeutic approaches. In order to obtain optimal therapeutic effects, it is critical to identify the target leukemic cells, which are highly dependent on PRMT4 functions. In leukemia, several epigenetic regulators are found disrupted through diverse genetic alternations. The possibility to target their enzymatic activities or specific interactions makes these regulators excellent therapeutic pursuits. Moreover, dysregulations of those epigenetic regulators together with genetic lesions involving transcription factors and transcription regulators have been used in patient stratifications and treatment prognosis. While playing a tumor suppressor role in many other cancers, PRMT4 appears to play a role of an oncogene when overexpressed in leukemia. One of possible pathways leading to the abnormal expression of PRMT4 is the suppression of miR-223 expression via AML1-ETO driven epigenetic silencing mechanism. With the development of diagnostic routines to stratify patients based on genetic lesions and abnormal gene expression signature, it is exciting to anticipate that PRMT4-based leukemia therapy can be implemented effectively for patients with aberrant level of PRMT4 expression or/and diagnosed with corresponding genetic lesions.

In conclusion, our work has revealed a critical role for PRMT4 in hematopoietic system. We identified a novel regulatory axis comprising of PRMT4, the microRNA-miR-223, the transcription factor RUNX1 and the transcriptional effector DPF2, which controls

myeloid differentiation. Given that miR-223 expression and RUNX1 transcriptional activities are frequently altered in AML as well as the relevance of PRMT4 high expression level in AML patients, our discovery of PRMT4 as a promising therapeutic target will undoubtedly provide a strong foundation for further clinical developments in the treatment of AML.

References

1. Speck, N.A. and D.G. Gilliland, *Core-binding factors in haematopoiesis and leukaemia*. Nat Rev Cancer, 2002. **2**(7): p. 502-13.
2. Doulatov, S., et al., *Hematopoiesis: a human perspective*. Cell Stem Cell, 2012. **10**(2): p. 120-36.
3. Orkin, S.H. and L.I. Zon, *Hematopoiesis: an evolving paradigm for stem cell biology*. Cell, 2008. **132**(4): p. 631-44.
4. Civin, C.I., et al., *Antigenic analysis of hematopoiesis. III. A hematopoietic progenitor cell surface antigen defined by a monoclonal antibody raised against KG-1a cells*. J Immunol, 1984. **133**(1): p. 157-65.
5. Kang, Y., N.J. Chao, and F. Aversa, *Unmanipulated or CD34 selected haplotype mismatched transplants*. Curr Opin Hematol, 2008. **15**(6): p. 561-7.
6. Bhatia, M., et al., *Purification of primitive human hematopoietic cells capable of repopulating immune-deficient mice*. Proc Natl Acad Sci U S A, 1997. **94**(10): p. 5320-5.
7. Murray, L., et al., *Enrichment of human hematopoietic stem cell activity in the CD34+Thy-1+Lin- subpopulation from mobilized peripheral blood*. Blood, 1995. **85**(2): p. 368-78.
8. Lansdorp, P.M., H.J. Sutherland, and C.J. Eaves, *Selective expression of CD45 isoforms on functional subpopulations of CD34+ hemopoietic cells from human bone marrow*. J Exp Med, 1990. **172**(1): p. 363-6.
9. Miranda-Saavedra, D. and B. Gottgens, *Transcriptional regulatory networks in haematopoiesis*. Curr Opin Genet Dev, 2008. **18**(6): p. 530-5.
10. Rice, K.L., I. Hormaeche, and J.D. Licht, *Epigenetic regulation of normal and malignant hematopoiesis*. Oncogene, 2007. **26**(47): p. 6697-714.
11. Georgantas, R.W., 3rd, et al., *Microarray and serial analysis of gene expression analyses identify known and novel transcripts overexpressed in hematopoietic stem cells*. Cancer Res, 2004. **64**(13): p. 4434-41.
12. Ji, H., et al., *Comprehensive methylome map of lineage commitment from haematopoietic progenitors*. Nature, 2010. **467**(7313): p. 338-42.
13. Novershtern, N., et al., *Densely interconnected transcriptional circuits control cell states in human hematopoiesis*. Cell, 2011. **144**(2): p. 296-309.
14. Passegue, E., et al., *Normal and leukemic hematopoiesis: are leukemias a stem cell disorder or a reacquisition of stem cell characteristics?* Proc Natl Acad Sci U S A, 2003. **100 Suppl 1**: p. 11842-9.
15. Hole, P.S., R.L. Darley, and A. Tonks, *Do reactive oxygen species play a role in myeloid leukemias?* Blood, 2011. **117**(22): p. 5816-5826.
16. Perrotti, D., et al., *Chronic myeloid leukemia: mechanisms of blastic transformation*. J Clin Invest, 2010. **120**(7): p. 2254-64.
17. Reya, T., et al., *Stem cells, cancer, and cancer stem cells*. Nature, 2001. **414**(6859): p. 105-11.
18. Brendel, C. and A. Neubauer, *Characteristics and analysis of normal and leukemic stem cells: current concepts and future directions*. Leukemia, 2000. **14**(10): p. 1711-7.
19. Wang, J.C. and J.E. Dick, *Cancer stem cells: lessons from leukemia*. Trends Cell Biol, 2005. **15**(9): p. 494-501.

20. Gilliland, D.G., *Hematologic malignancies*. Curr Opin Hematol, 2001. **8**(4): p. 189-91.
21. Cuenco, G.M. and R. Ren, *Cooperation of BCR-ABL and AML1/MDS1/EV11 in blocking myeloid differentiation and rapid induction of an acute myelogenous leukemia*. Oncogene, 2001. **20**(57): p. 8236-48.
22. Schessl, C., et al., *The AML1-ETO fusion gene and the FLT3 length mutation collaborate in inducing acute leukemia in mice*. J Clin Invest, 2005. **115**(8): p. 2159-68.
23. Wang, Y.Y., et al., *C-KIT mutation cooperates with full-length AML1-ETO to induce acute myeloid leukemia in mice*. Proc Natl Acad Sci U S A, 2011. **108**(6): p. 2450-5.
24. Wang, Y.Y., et al., *AML1-ETO and C-KIT mutation/overexpression in t(8;21) leukemia: implication in stepwise leukemogenesis and response to Gleevec*. Proc Natl Acad Sci U S A, 2005. **102**(4): p. 1104-9.
25. Schneider, F., et al., *AML1-ETO meets JAK2: clinical evidence for the two hit model of leukemogenesis from a myeloproliferative syndrome progressing to acute myeloid leukemia*. Leukemia, 2007. **21**(10): p. 2199-201.
26. Rosenbauer, F. and D.G. Tenen, *Transcription factors in myeloid development: balancing differentiation with transformation*. Nat Rev Immunol, 2007. **7**(2): p. 105-17.
27. Nerlov, C. and T. Graf, *PU.1 induces myeloid lineage commitment in multipotent hematopoietic progenitors*. Genes Dev, 1998. **12**(15): p. 2403-12.
28. Zhang, D.E., et al., *Absence of granulocyte colony-stimulating factor signaling and neutrophil development in CCAAT enhancer binding protein alpha-deficient mice*. Proc Natl Acad Sci U S A, 1997. **94**(2): p. 569-74.
29. Yamanaka, R., et al., *Impaired granulopoiesis, myelodysplasia, and early lethality in CCAAT/enhancer binding protein epsilon-deficient mice*. Proc Natl Acad Sci U S A, 1997. **94**(24): p. 13187-92.
30. Hock, H., et al., *Intrinsic requirement for zinc finger transcription factor Gfi-1 in neutrophil differentiation*. Immunity, 2003. **18**(1): p. 109-20.
31. Feinberg, M.W., et al., *The Kruppel-like factor KLF4 is a critical regulator of monocyte differentiation*. EMBO J, 2007. **26**(18): p. 4138-48.
32. Johansen, L.M., et al., *c-Myc is a critical target for c/EBPalpha in granulopoiesis*. Mol Cell Biol, 2001. **21**(11): p. 3789-806.
33. Passegue, E., et al., *Chronic myeloid leukemia with increased granulocyte progenitors in mice lacking junB expression in the myeloid lineage*. Cell, 2001. **104**(1): p. 21-32.
34. Mueller, B.U., et al., *Heterozygous PU.1 mutations are associated with acute myeloid leukemia*. Blood, 2003. **101**(5): p. 2074.
35. Pabst, T., et al., *Dominant-negative mutations of CEBPA, encoding CCAAT/enhancer binding protein-alpha (C/EBPalpha), in acute myeloid leukemia*. Nat Genet, 2001. **27**(3): p. 263-70.
36. Back, J., et al., *Visualizing PU.1 activity during hematopoiesis*. Exp Hematol, 2005. **33**(4): p. 395-402.

37. Rosenbauer, F., et al., *Lymphoid cell growth and transformation are suppressed by a key regulatory element of the gene encoding PU.1*. Nat Genet, 2006. **38**(1): p. 27-37.
38. McKercher, S.R., et al., *Targeted disruption of the PU.1 gene results in multiple hematopoietic abnormalities*. EMBO J, 1996. **15**(20): p. 5647-58.
39. Scott, E.W., et al., *Requirement of transcription factor PU.1 in the development of multiple hematopoietic lineages*. Science, 1994. **265**(5178): p. 1573-7.
40. Mak, K.S., et al., *PU.1 and Haematopoietic Cell Fate: Dosage Matters*. Int J Cell Biol, 2011. **2011**: p. 808524.
41. Stirewalt, D.L., *Fine-tuning PU.1*. Nat Genet, 2004. **36**(6): p. 550-1.
42. Dahl, R., et al., *Regulation of macrophage and neutrophil cell fates by the PU.1:C/EBPalpha ratio and granulocyte colony-stimulating factor*. Nat Immunol, 2003. **4**(10): p. 1029-36.
43. Rosenbauer, F., et al., *Acute myeloid leukemia induced by graded reduction of a lineage-specific transcription factor, PU.1*. Nat Genet, 2004. **36**(6): p. 624-30.
44. Scott, E.W., et al., *PU.1 functions in a cell-autonomous manner to control the differentiation of multipotential lymphoid-myeloid progenitors*. Immunity, 1997. **6**(4): p. 437-47.
45. Rosmarin, A.G., Z. Yang, and K.K. Resendes, *Transcriptional regulation in myelopoiesis: Hematopoietic fate choice, myeloid differentiation, and leukemogenesis*. Exp Hematol, 2005. **33**(2): p. 131-43.
46. Ford, A.M., et al., *Regulation of the myeloperoxidase enhancer binding proteins Pu1, C-EBP alpha, -beta, and -delta during granulocyte-lineage specification*. Proc Natl Acad Sci U S A, 1996. **93**(20): p. 10838-43.
47. Nuchprayoon, I., et al., *An enhancer located between the neutrophil elastase and proteinase 3 promoters is activated by Sp1 and an Ets factor*. J Biol Chem, 1999. **274**(2): p. 1085-91.
48. Laslo, P., et al., *Multilineage transcriptional priming and determination of alternate hematopoietic cell fates*. Cell, 2006. **126**(4): p. 755-66.
49. Fukao, T., et al., *An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling*. Cell, 2007. **129**(3): p. 617-31.
50. Rosa, A., et al., *The interplay between the master transcription factor PU.1 and miR-424 regulates human monocyte/macrophage differentiation*. Proc Natl Acad Sci U S A, 2007. **104**(50): p. 19849-54.
51. Akashi, K., et al., *A clonogenic common myeloid progenitor that gives rise to all myeloid lineages*. Nature, 2000. **404**(6774): p. 193-7.
52. Zhang, P., et al., *Enhancement of hematopoietic stem cell repopulating capacity and self-renewal in the absence of the transcription factor C/EBP alpha*. Immunity, 2004. **21**(6): p. 853-63.
53. Porse, B.T., et al., *E2F repression by C/EBPalpha is required for adipogenesis and granulopoiesis in vivo*. Cell, 2001. **107**(2): p. 247-58.
54. Wang, Q.F., et al., *Cell cycle inhibition mediated by the outer surface of the C/EBPalpha basic region is required but not sufficient for granulopoiesis*. Oncogene, 2003. **22**(17): p. 2548-57.

55. Jin, F., et al., *PU.1 and C/EBP(alpha) synergistically program distinct response to NF-kappaB activation through establishing monocyte specific enhancers*. Proc Natl Acad Sci U S A, 2011. **108**(13): p. 5290-5.
56. Wang, X., et al., *C/EBPalpha bypasses granulocyte colony-stimulating factor signals to rapidly induce PU.1 gene expression, stimulate granulocytic differentiation, and limit proliferation in 32D cl3 myeloblasts*. Blood, 1999. **94**(2): p. 560-71.
57. Suzuki, H., et al., *The transcriptional network that controls growth arrest and differentiation in a human myeloid leukemia cell line*. Nat Genet, 2009. **41**(5): p. 553-62.
58. Chen, J., O. Odenike, and J.D. Rowley, *Leukaemogenesis: more than mutant genes*. Nat Rev Cancer, 2010. **10**(1): p. 23-36.
59. Shih, A.H., et al., *The role of mutations in epigenetic regulators in myeloid malignancies*. Nat Rev Cancer, 2012. **12**(9): p. 599-612.
60. Gregory, R.I. and R. Shiekhattar, *Chromatin modifiers and carcinogenesis*. Trends Cell Biol, 2004. **14**(12): p. 695-702.
61. Cairns, B.R., *Emerging roles for chromatin remodeling in cancer biology*. Trends Cell Biol, 2001. **11**(11): p. S15-21.
62. Lusser, A. and J.T. Kadonaga, *Chromatin remodeling by ATP-dependent molecular machines*. Bioessays, 2003. **25**(12): p. 1192-200.
63. Saha, A., J. Wittmeyer, and B.R. Cairns, *Chromatin remodelling: the industrial revolution of DNA around histones*. Nat Rev Mol Cell Biol, 2006. **7**(6): p. 437-47.
64. Bultman, S., et al., *A Brg1 null mutation in the mouse reveals functional differences among mammalian SWI/SNF complexes*. Mol Cell, 2000. **6**(6): p. 1287-95.
65. Roberts, C.W. and S.H. Orkin, *The SWI/SNF complex--chromatin and cancer*. Nat Rev Cancer, 2004. **4**(2): p. 133-42.
66. Reisman, D., S. Glaros, and E.A. Thompson, *The SWI/SNF complex and cancer*. Oncogene, 2009. **28**(14): p. 1653-68.
67. Guidi, C.J., et al., *Disruption of Ini1 leads to peri-implantation lethality and tumorigenesis in mice*. Mol Cell Biol, 2001. **21**(10): p. 3598-603.
68. Stopka, T. and A.I. Skoultschi, *The ISWI ATPase Snf2h is required for early mouse development*. Proc Natl Acad Sci U S A, 2003. **100**(24): p. 14097-102.
69. Kowenz-Leutz, E. and A. Leutz, *A C/EBP beta isoform recruits the SWI/SNF complex to activate myeloid genes*. Mol Cell, 1999. **4**(5): p. 735-43.
70. Vradii, D., et al., *Brg1, the ATPase subunit of the SWI/SNF chromatin remodeling complex, is required for myeloid differentiation to granulocytes*. J Cell Physiol, 2006. **206**(1): p. 112-8.
71. Nie, Z., et al., *Novel SWI/SNF chromatin-remodeling complexes contain a mixed-lineage leukemia chromosomal translocation partner*. Mol Cell Biol, 2003. **23**(8): p. 2942-52.
72. Campos, E.I. and D. Reinberg, *Histones: annotating chromatin*. Annu Rev Genet, 2009. **43**: p. 559-99.
73. Deaton, A.M. and A. Bird, *CpG islands and the regulation of transcription*. Genes Dev, 2011. **25**(10): p. 1010-22.

74. Bird, A., *DNA methylation patterns and epigenetic memory*. Genes Dev, 2002. **16**(1): p. 6-21.
75. Galm, O., J.G. Herman, and S.B. Baylin, *The fundamental role of epigenetics in hematopoietic malignancies*. Blood Rev, 2006. **20**(1): p. 1-13.
76. Becker, P.B., S. Ruppert, and G. Schutz, *Genomic footprinting reveals cell type-specific DNA binding of ubiquitous factors*. Cell, 1987. **51**(3): p. 435-43.
77. Borgel, J., et al., *Targets and dynamics of promoter DNA methylation during early mouse development*. Nat Genet, 2010. **42**(12): p. 1093-100.
78. Figueroa, M.E., et al., *DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia*. Cancer Cell, 2010. **17**(1): p. 13-27.
79. Okano, M., et al., *DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development*. Cell, 1999. **99**(3): p. 247-57.
80. Li, E., T.H. Bestor, and R. Jaenisch, *Targeted mutation of the DNA methyltransferase gene results in embryonic lethality*. Cell, 1992. **69**(6): p. 915-26.
81. Trowbridge, J.J., et al., *DNA methyltransferase 1 is essential for and uniquely regulates hematopoietic stem and progenitor cells*. Cell Stem Cell, 2009. **5**(4): p. 442-9.
82. Broske, A.M., et al., *DNA methylation protects hematopoietic stem cell multipotency from myeloerythroid restriction*. Nat Genet, 2009. **41**(11): p. 1207-15.
83. Tadokoro, Y., et al., *De novo DNA methyltransferase is essential for self-renewal, but not for differentiation, in hematopoietic stem cells*. J Exp Med, 2007. **204**(4): p. 715-22.
84. Challen, G.A., et al., *Dnmt3a is essential for hematopoietic stem cell differentiation*. Nat Genet, 2012. **44**(1): p. 23-31.
85. Ley, T.J., et al., *DNMT3A mutations in acute myeloid leukemia*. N Engl J Med, 2010. **363**(25): p. 2424-33.
86. Tahiliani, M., et al., *Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1*. Science, 2009. **324**(5929): p. 930-5.
87. Ito, S., et al., *Tet Proteins Can Convert 5-Methylcytosine to 5-Formylcytosine and 5-Carboxylcytosine*. Science, 2011. **333**(6047): p. 1300-1303.
88. He, Y.-F., et al., *Tet-Mediated Formation of 5-Carboxylcytosine and Its Excision by TDG in Mammalian DNA*. Science, 2011. **333**(6047): p. 1303-1307.
89. Zhang, L., et al., *Thymine DNA glycosylase specifically recognizes 5-carboxylcytosine-modified DNA*. Nat Chem Biol, 2012. **8**(4): p. 328-30.
90. Wu, H., et al., *Genome-wide analysis of 5-hydroxymethylcytosine distribution reveals its dual function in transcriptional regulation in mouse embryonic stem cells*. Genes Dev, 2011. **25**(7): p. 679-84.
91. Valinluck, V., et al., *Oxidative damage to methyl-CpG sequences inhibits the binding of the methyl-CpG binding domain (MBD) of methyl-CpG binding protein 2 (MeCP2)*. Nucleic Acids Res, 2004. **32**(14): p. 4100-8.
92. Yildirim, O., et al., *Mbd3/NURD complex regulates expression of 5-hydroxymethylcytosine marked genes in embryonic stem cells*. Cell, 2011. **147**(7): p. 1498-510.

93. Smith, Z.D. and A. Meissner, *DNA methylation: roles in mammalian development*. Nat Rev Genet, 2013. **14**(3): p. 204-20.
94. Ko, M., et al., *Impaired hydroxylation of 5-methylcytosine in myeloid cancers with mutant TET2*. Nature, 2010. **468**(7325): p. 839-43.
95. Moran-Crusio, K., et al., *Tet2 loss leads to increased hematopoietic stem cell self-renewal and myeloid transformation*. Cancer Cell, 2011. **20**(1): p. 11-24.
96. O'Connell, R.M., et al., *Physiological and pathological roles for microRNAs in the immune system*. Nat Rev Immunol, 2010. **10**(2): p. 111-22.
97. O'Connell, R.M., J.L. Zhao, and D.S. Rao, *MicroRNA function in myeloid biology*. Blood, 2011. **118**(11): p. 2960-9.
98. Baltimore, D., et al., *MicroRNAs: new regulators of immune cell development and function*. Nat Immunol, 2008. **9**(8): p. 839-45.
99. Ambros, V., *The functions of animal microRNAs*. Nature, 2004. **431**(7006): p. 350-5.
100. Bartel, D.P., *MicroRNAs: target recognition and regulatory functions*. Cell, 2009. **136**(2): p. 215-33.
101. Chen, C.Z., et al., *MicroRNAs modulate hematopoietic lineage differentiation*. Science, 2004. **303**(5654): p. 83-6.
102. Johnnidis, J.B., et al., *Regulation of progenitor cell proliferation and granulocyte function by microRNA-223*. Nature, 2008. **451**(7182): p. 1125-9.
103. Fazi, F., et al., *A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis*. Cell, 2005. **123**(5): p. 819-31.
104. Pulikkan, J.A., et al., *Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia*. Blood, 2010. **115**(9): p. 1768-78.
105. Fazi, F., et al., *Epigenetic silencing of the myelopoiesis regulator microRNA-223 by the AML1/ETO oncoprotein*. Cancer Cell, 2007. **12**(5): p. 457-66.
106. Eyholzer, M., et al., *Complexity of miR-223 regulation by CEBPA in human AML*. Leuk Res, 2010. **34**(5): p. 672-6.
107. Garzon, R., et al., *MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia*. Oncogene, 2007. **26**(28): p. 4148-57.
108. Velu, C.S., A.M. Baktula, and H.L. Grimes, *Gfi1 regulates miR-21 and miR-196b to control myelopoiesis*. Blood, 2009. **113**(19): p. 4720-8.
109. Fontana, L., et al., *MicroRNAs 17-5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation*. Nat Cell Biol, 2007. **9**(7): p. 775-87.
110. Han, Y.C., et al., *microRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia*. J Exp Med, 2010. **207**(3): p. 475-89.
111. Eiring, A.M., et al., *miR-328 functions as an RNA decoy to modulate hnRNP E2 regulation of mRNA translation in leukemic blasts*. Cell, 2010. **140**(5): p. 652-65.
112. Bravo, J., et al., *The leukemia-associated AML1 (Runx1)--CBF beta complex functions as a DNA-induced molecular clamp*. Nat Struct Biol, 2001. **8**(4): p. 371-8.

113. Tahirov, T.H., et al., *Structural Analyses of DNA Recognition by the AML1/Runx-1 Runt Domain and Its Allosteric Control by CBF β* . Cell, 2001. **104**(5): p. 755-767.
114. Okuda, T., et al., *AML1, the target of multiple chromosomal translocations in human leukemia, is essential for normal fetal liver hematopoiesis*. Cell, 1996. **84**(2): p. 321-30.
115. Sasaki, K., et al., *Absence of fetal liver hematopoiesis in mice deficient in transcriptional coactivator core binding factor beta*. Proc Natl Acad Sci U S A, 1996. **93**(22): p. 12359-63.
116. Gowney, J.D., et al., *Loss of Runx1 perturbs adult hematopoiesis and is associated with a myeloproliferative phenotype*. Blood, 2005. **106**(2): p. 494-504.
117. Ichikawa, M., et al., *AML-1 is required for megakaryocytic maturation and lymphocytic differentiation, but not for maintenance of hematopoietic stem cells in adult hematopoiesis*. Nat Med, 2004. **10**(3): p. 299-304.
118. Huang, G., et al., *PU.1 is a major downstream target of AML1 (RUNX1) in adult mouse hematopoiesis*. Nat Genet, 2008. **40**(1): p. 51-60.
119. Speck, N.A., et al., *Core-binding factor: a central player in hematopoiesis and leukemia*. Cancer Res, 1999. **59**(7 Suppl): p. 1789s-1793s.
120. Mikhail, F.M., et al., *Normal and transforming functions of RUNX1: a perspective*. J Cell Physiol, 2006. **207**(3): p. 582-93.
121. Blyth, K., E.R. Cameron, and J.C. Neil, *The RUNX genes: gain or loss of function in cancer*. Nat Rev Cancer, 2005. **5**(5): p. 376-87.
122. Higuchi, M., et al., *Expression of a conditional AML1-ETO oncogene bypasses embryonic lethality and establishes a murine model of human t(8;21) acute myeloid leukemia*. Cancer Cell, 2002. **1**(1): p. 63-74.
123. Mulloy, J.C., et al., *Maintaining the self-renewal and differentiation potential of human CD34+ hematopoietic cells using a single genetic element*. Blood, 2003. **102**(13): p. 4369-76.
124. Lutterbach, B., et al., *ETO, a target of t(8;21) in acute leukemia, interacts with the N-CoR and mSin3 corepressors*. Mol Cell Biol, 1998. **18**(12): p. 7176-84.
125. Gelmetti, V., et al., *Aberrant recruitment of the nuclear receptor corepressor-histone deacetylase complex by the acute myeloid leukemia fusion partner ETO*. Mol Cell Biol, 1998. **18**(12): p. 7185-91.
126. Liu, S., et al., *Interplay of RUNX1/MTG8 and DNA methyltransferase 1 in acute myeloid leukemia*. Cancer Res, 2005. **65**(4): p. 1277-84.
127. Wang, L., et al., *The leukemogenicity of AML1-ETO is dependent on site-specific lysine acetylation*. Science, 2011. **333**(6043): p. 765-9.
128. Zhang, J., et al., *E protein silencing by the leukemogenic AML1-ETO fusion protein*. Science, 2004. **305**(5688): p. 1286-9.
129. Yan, M., et al., *RUNX1/AML1 DNA-binding domain and ETO/MTG8 NHR2-dimerization domain are critical to AML1-ETO9a leukemogenesis*. Blood, 2009. **113**(4): p. 883-6.
130. Park, S., et al., *Structure of the AML1-ETO eTAFH domain-HEB peptide complex and its contribution to AML1-ETO activity*. Blood, 2009. **113**(15): p. 3558-67.
131. Osato, M., *Point mutations in the RUNX1/AML1 gene: another actor in RUNX leukemia*. Oncogene, 2004. **23**(24): p. 4284-96.

132. Osato, M., et al., *Point mutations of the RUNX1/AML1 gene in sporadic and familial myeloid leukemias*. Int J Hematol, 2001. **74**(3): p. 245-51.
133. Yuan, Y., et al., *AML1-ETO expression is directly involved in the development of acute myeloid leukemia in the presence of additional mutations*. Proc Natl Acad Sci U S A, 2001. **98**(18): p. 10398-403.
134. Elagib, K.E., et al., *RUNX1 and GATA-1 coexpression and cooperation in megakaryocytic differentiation*. Blood, 2003. **101**(11): p. 4333-41.
135. Zhang, D.E., et al., *CCAAT enhancer-binding protein (C/EBP) and AML1 (CBF alpha2) synergistically activate the macrophage colony-stimulating factor receptor promoter*. Mol Cell Biol, 1996. **16**(3): p. 1231-40.
136. Petrovick, M.S., et al., *Multiple functional domains of AML1: PU.1 and C/EBPalpha synergize with different regions of AML1*. Mol Cell Biol, 1998. **18**(7): p. 3915-25.
137. Yagi, R., et al., *A WW domain-containing yes-associated protein (YAP) is a novel transcriptional co-activator*. EMBO J, 1999. **18**(9): p. 2551-62.
138. Bruhn, L., A. Munnerlyn, and R. Grosschedl, *ALY, a context-dependent coactivator of LEF-1 and AML-1, is required for TCRalpha enhancer function*. Genes Dev, 1997. **11**(5): p. 640-53.
139. Kitabayashi, I., et al., *Interaction and functional cooperation of the leukemia-associated factors AML1 and p300 in myeloid cell differentiation*. EMBO J, 1998. **17**(11): p. 2994-3004.
140. Kitabayashi, I., et al., *Activation of AML1-mediated transcription by MOZ and inhibition by the MOZ-CBP fusion protein*. EMBO J, 2001. **20**(24): p. 7184-96.
141. Zhao, X., et al., *Methylation of RUNX1 by PRMT1 abrogates SIN3A binding and potentiates its transcriptional activity*. Genes Dev, 2008. **22**(5): p. 640-53.
142. Lutterbach, B., et al., *A mechanism of repression by acute myeloid leukemia-1, the target of multiple chromosomal translocations in acute leukemia*. J Biol Chem, 2000. **275**(1): p. 651-6.
143. Aronson, B.D., et al., *Groucho-dependent and -independent repression activities of Runt domain proteins*. Mol Cell Biol, 1997. **17**(9): p. 5581-7.
144. Bakshi, R., et al., *The human SWI/SNF complex associates with RUNX1 to control transcription of hematopoietic target genes*. J Cell Physiol, 2010. **225**(2): p. 569-76.
145. Yu, M., et al., *Direct recruitment of polycomb repressive complex 1 to chromatin by core binding transcription factors*. Mol Cell, 2012. **45**(3): p. 330-43.
146. Huang, G., et al., *Dimerization with PEBP2beta protects RUNX1/AML1 from ubiquitin-proteasome-mediated degradation*. EMBO J, 2001. **20**(4): p. 723-33.
147. Tanaka, T., et al., *The extracellular signal-regulated kinase pathway phosphorylates AML1, an acute myeloid leukemia gene product, and potentially regulates its transactivation ability*. Mol Cell Biol, 1996. **16**(7): p. 3967-79.
148. Zhang, Y., J.R. Biggs, and A.S. Kraft, *Phorbol ester treatment of K562 cells regulates the transcriptional activity of AML1c through phosphorylation*. J Biol Chem, 2004. **279**(51): p. 53116-25.
149. Hamelin, V., et al., *Thrombopoietin regulates IEX-1 gene expression through ERK-induced AML1 phosphorylation*. Blood, 2006. **107**(8): p. 3106-13.

150. Imai, Y., et al., *The corepressor mSin3A regulates phosphorylation-induced activation, intranuclear location, and stability of AML1*. Mol Cell Biol, 2004. **24**(3): p. 1033-43.
151. Biggs, J.R., et al., *AML1/RUNX1 phosphorylation by cyclin-dependent kinases regulates the degradation of AML1/RUNX1 by the anaphase-promoting complex*. Mol Cell Biol, 2006. **26**(20): p. 7420-9.
152. Zhang, L., et al., *Cyclin-dependent kinase phosphorylation of RUNX1/AML1 on 3 sites increases transactivation potency and stimulates cell proliferation*. Blood, 2008. **111**(3): p. 1193-200.
153. Yamaguchi, Y., et al., *AML1 is functionally regulated through p300-mediated acetylation on specific lysine residues*. J Biol Chem, 2004. **279**(15): p. 15630-8.
154. Vu, L.P., L. Luciani, and S.D. Nimer, *Histone-modifying enzymes: their role in the pathogenesis of acute leukemia and their therapeutic potential*. Int J Hematol, 2013. **97**(2): p. 198-209.
155. Zhang, K. and S.Y.R. Dent, *Histone modifying enzymes and cancer: Going beyond histones*. Journal of Cellular Biochemistry, 2005. **96**(6): p. 1137-1148.
156. Dawson, M.A. and T. Kouzarides, *Cancer Epigenetics: From Mechanism to Therapy*. Cell, 2012. **150**(1): p. 12-27.
157. Krivtsov, A.V. and S.A. Armstrong, *MLL translocations, histone modifications and leukaemia stem-cell development*. Nat Rev Cancer, 2007. **7**(11): p. 823-833.
158. Okada, Y., et al., *hDOTIL Links Histone Methylation to Leukemogenesis*. Cell, 2005. **121**(2): p. 167-178.
159. Zhang, W., et al., *Dot1a-AF9 Complex Mediates Histone H3 Lys-79 Hypermethylation and Repression of ENaCa in an Aldosterone-sensitive Manner*. Journal of Biological Chemistry, 2006. **281**(26): p. 18059-18068.
160. Bitoun, E., P.L. Oliver, and K.E. Davies, *The mixed-lineage leukemia fusion partner AF4 stimulates RNA polymerase II transcriptional elongation and mediates coordinated chromatin remodeling*. Human Molecular Genetics, 2007. **16**(1): p. 92-106.
161. Zeisig, D.T., et al., *The eleven-nineteen-leukemia protein ENL connects nuclear MLL fusion partners with chromatin*. Oncogene, 2005. **24**(35): p. 5525-5532.
162. Liedtke, M. and M.L. Cleary, *Therapeutic targeting of MLL*. Blood, 2009. **113**(24): p. 6061-6068.
163. Daigle, Scott R., et al., *Selective Killing of Mixed Lineage Leukemia Cells by a Potent Small-Molecule DOTIL Inhibitor*. Cancer Cell, 2011. **20**(1): p. 53-65.
164. Bernt, K.M., et al., *MLL-Rearranged Leukemia Is Dependent on Aberrant H3K79 Methylation by DOTIL*. Cancer Cell, 2011. **20**(1): p. 66-78.
165. Mills, A.A., *Throwing the cancer switch: reciprocal roles of polycomb and trithorax proteins*. Nat Rev Cancer, 2010. **10**(10): p. 669-682.
166. Ernst, T., et al., *Inactivating mutations of the histone methyltransferase gene EZH2 in myeloid disorders*. Nat Genet, 2010. **42**(8): p. 722-726.
167. Nikoloski, G., et al., *Somatic mutations of the histone methyltransferase gene EZH2 in myelodysplastic syndromes*. Nat Genet, 2010. **42**(8): p. 665-667.
168. Ntziachristos, P., et al., *Genetic inactivation of the polycomb repressive complex 2 in T cell acute lymphoblastic leukemia*. Nat Med, 2012. **18**(2): p. 298-303.

169. Zhang, J., et al., *The genetic basis of early T-cell precursor acute lymphoblastic leukaemia*. Nature, 2012. **481**(7380): p. 157-163.
170. Morin, R.D., et al., *Somatic mutations altering EZH2 (Tyr641) in follicular and diffuse large B-cell lymphomas of germinal-center origin*. Nat Genet, 2010. **42**(2): p. 181-185.
171. Chase, A. and N.C.P. Cross, *Aberrations of EZH2 in Cancer*. Clinical Cancer Research, 2011. **17**(9): p. 2613-2618.
172. Tanaka, S., et al., *Ezh2 augments leukemogenicity by reinforcing differentiation blockage in acute myeloid leukemia*. Blood, 2012. **120**(5): p. 1107-1117.
173. Neff, T., et al., *Polycomb repressive complex 2 is required for MLL-AF9 leukemia*. Proceedings of the National Academy of Sciences, 2012.
174. Cerveira, N., et al., *Frequency of NUP98-NSD1 fusion transcript in childhood acute myeloid leukaemia*. Leukemia, 2003. **17**(11): p. 2244-2247.
175. Hollink, I.H.I.M., et al., *NUP98/NSD1 characterizes a novel poor prognostic group in acute myeloid leukemia with a distinct HOX gene expression pattern*. Blood, 2011. **118**(13): p. 3645-3656.
176. Thol, F., et al., *Analysis of NUP98/NSD1 translocations in adult AML and MDS patients*. Leukemia, 2012.
177. Wang, G.G., et al., *NUP98-NSD1 links H3K36 methylation to Hox-A gene activation and leukaemogenesis*. Nat Cell Biol, 2007. **9**(7): p. 804-812.
178. Wang, G.G., et al., *Haematopoietic malignancies caused by dysregulation of a chromatin-binding PHD finger*. Nature, 2009. **459**(7248): p. 847-851.
179. Reader, J.C., et al., *A novel NUP98-PHF23 fusion resulting from a cryptic translocation t(11;17)(p15;p13) in acute myeloid leukemia*. Leukemia, 2007. **21**(4): p. 842-844.
180. van Zutven, L.J.C.M., et al., *Identification of NUP98 abnormalities in acute leukemia: JARID1A (12p13) as a new partner gene*. Genes, Chromosomes and Cancer, 2006. **45**(5): p. 437-446.
181. Shi, Y. and J.R. Whetstone, *Dynamic Regulation of Histone Lysine Methylation by Demethylases*. Mol Cell, 2007. **25**(1): p. 1-14.
182. Shi, Y., et al., *Histone Demethylation Mediated by the Nuclear Amine Oxidase Homolog LSD1*. Cell, 2004. **119**(7): p. 941-953.
183. Lokken, Alyson A. and Nancy J. Zeleznik-Le, *Breaking the LSD1/KDM1A Addiction: Therapeutic Targeting of the Epigenetic Modifier in AML*. Cancer Cell, 2012. **21**(4): p. 451-453.
184. Berglund, L., et al., *A Genecentric Human Protein Atlas for Expression Profiles Based on Antibodies*. Molecular & Cellular Proteomics, 2008. **7**(10): p. 2019-2027.
185. Schenk, T., et al., *Inhibition of the LSD1 (KDM1A) demethylase reactivates the all-trans-retinoic acid differentiation pathway in acute myeloid leukemia*. Nat Med, 2012. **18**(4): p. 605-611.
186. Harris, William J., et al., *The Histone Demethylase KDM1A Sustains the Oncogenic Potential of MLL-AF9 Leukemia Stem Cells*. Cancer Cell, 2012. **21**(4): p. 473-487.
187. Harris, W.J., et al., *The histone demethylase KDM1A sustains the oncogenic potential of MLL-AF9 leukemia stem cells*. Cancer Cell, 2012. **21**(4): p. 473-87.

188. Parsons, D.W., et al., *An integrated genomic analysis of human glioblastoma multiforme*. Science, 2008. **321**(5897): p. 1807-12.
189. Mardis, E.R., et al., *Recurring Mutations Found by Sequencing an Acute Myeloid Leukemia Genome*. New England Journal of Medicine, 2009. **361**(11): p. 1058-1066.
190. Andersson, A.K., et al., *IDH1 and IDH2 mutations in pediatric acute leukemia*. Leukemia, 2011. **25**(10): p. 1570-1577.
191. Xu, W., et al., *Oncometabolite 2-Hydroxyglutarate Is a Competitive Inhibitor of \pm -Ketoglutarate-Dependent Dioxygenases*. Cancer Cell, 2011. **19**(1): p. 17-30.
192. Figueroa, M.E., et al., *Leukemic IDH1 and IDH2 Mutations Result in a Hypermethylation Phenotype, Disrupt TET2 Function, and Impair Hematopoietic Differentiation*. Cancer Cell, 2010. **18**(6): p. 553-567.
193. Lu, C., et al., *IDH mutation impairs histone demethylation and results in a block to cell differentiation*. Nature, 2012. **483**(7390): p. 474-8.
194. Pasqualucci L, D.-S.D., Chiarenza A, Fabbri G,, et al., *Inactivating mutations of acetyltransferase genes in B-cell lymphoma*. Nature, 2011. **471**(7337): p. 6.
195. Mullighan CG, Z.J., Kasper LH, Lerach S,, et al., *CREBBP mutations in relapsed acute lymphoblastic leukaemia*. Nature, 2011. **471**(7337): p. 4.
196. Zuber J, S.J., Wang E, Rappaport AR, Herrmann H, Sison EA, Magoon D,, B.K. Qi J, Wunderlich M, Taylor MJ, Johns C, Chicas A, Mulloy JC,, and B.P. Kogan SC, Valent P, Bradner JE, Lowe SW, Vakoc CR, *RNAi screen identifies Brd4 as a therapeutic target in acute myeloid leukaemia*. Nature, 2011. **478**: p. 6.
197. Yang XJ, O.V., Nishikawa J, Howard BH, Nakatani Y, *A p300/CBP-associated factor that competes with the adenoviral oncoprotein E1A*. Nature, 1996. **382**(6589): p. 5.
198. Phan RT, D.-F.R., *The BCL6 proto-oncogene suppresses p53 expression in germinal-centre B cells*. Nature, 2004. **432**: p. 4.
199. Wang L, G.A., Sun XJ, Zhao X, Perna F,, et al., *The Leukemogenicity of AML1-ETO Is Dependent on Site-Specific Lysine Acetylation*. Science, 2011. **333**(6043): p. 4.
200. Tamkun JW, D.R., Scott MP, Kissinger M, Pattatucci AM, Kaufman TC, Kennison JA *brahma: A regulator of Drosophila homeotic genes structurally related to the yeast transcriptional activator* Cell, 1992. **68**(3): p. 11.
201. Vollmuth F, G.M., *Interaction of propionylated and butyrylated histone H3 lysine marks with Brd4 bromodomains*. Angew Chem Int Ed Engl. , 2010. **49**(38): p. 4.
202. Filippakopoulos P, Q.J., Picaud S, Shen Y, Smith WB, Fedorov O, Morse EM,, et al., *Selective inhibition of BET bromodomains*. Nature, 2010. **468**: p. 6.
203. Yan J, D.J., Jiao J, Wang R, You J, *Perturbation of BRD4 protein function by BRD4-NUT protein abrogates cellular differentiation in NUT midline carcinoma*. J Biol Chem, 2011. **286**(31): p. 12.
204. Ott CJ, K.N., Bird L, Paranal RM, Qi J, Bowman T, Rodig SJ, Kung AL, Bradner JE, Weinstock DM., *BET bromodomain inhibition targets both c-Myc and IL7R in high-risk acute lymphoblastic leukemia*. Blood, 2012. **120**: p. 9.

205. Gallinari P, D.M.S., Jones P, Pallaoro M, Steinkühler C, *HDACs, histone deacetylation and gene transcription: from molecular biology to cancer therapeutics*. Cell Research, 2007. **17**: p. 16.
206. Minucci S, P.P., *Histone deacetylase inhibitors and the promise of epigenetic (and more) treatments for cancer*. Nature Reviews Cancer, 2006. **6**: p. 13.
207. Liu S, W.L., Pang J, Santhanam R, Schwind S, Wu YZ., et al., *Sp1/NFkB/HDAC/miR-29b Regulatory Network in KIT-Driven Myeloid Leukemia*. Cancer Cell, 2010. **17**: p. 14.
208. Paik, W.K. and S. Kim, *Enzymatic methylation of protein fractions from calf thymus nuclei*. Biochem Biophys Res Commun, 1967. **29**(1): p. 14-20.
209. Yang, Y. and M.T. Bedford, *Protein arginine methyltransferases and cancer*. Nat Rev Cancer, 2013. **13**(1): p. 37-50.
210. Tripsianes, K., et al., *Structural basis for dimethylarginine recognition by the Tudor domains of human SMN and SPF30 proteins*. Nat Struct Mol Biol, 2011. **18**(12): p. 1414-20.
211. Bedford, M.T. and S.G. Clarke, *Protein Arginine Methylation in Mammals: Who, What, and Why*. Mol Cell, 2009. **33**(1): p. 1-13.
212. Nicholson, T.B., T. Chen, and S. Richard, *The physiological and pathophysiological role of PRMT1-mediated protein arginine methylation*. Pharmacol Res, 2009. **60**(6): p. 466-74.
213. Tee, W.W., et al., *Prmt5 is essential for early mouse development and acts in the cytoplasm to maintain ES cell pluripotency*. Genes Dev, 2010. **24**(24): p. 2772-7.
214. Thompson, P.R. and W. Fast, *Histone Citrullination by Protein Arginine Deiminase: Is Arginine Methylation a Green Light or a Roadblock?* ACS Chemical Biology, 2006. **1**(7): p. 433-441.
215. Chang, B., et al., *JMJD6 Is a Histone Arginine Demethylase*. Science, 2007. **318**(5849): p. 444-447.
216. Cheung, N., et al., *Protein arginine-methyltransferase-dependent oncogenesis*. Nat Cell Biol, 2007. **9**(10): p. 1208-1215.
217. Zhao, X., et al., *Methylation of RUNX1 by PRMT1 abrogates SIN3A binding and potentiates its transcriptional activity*. Genes Dev, 2008. **22**(5): p. 640-653.
218. Shia, W.-J., et al., *PRMT1 interacts with AML1-ETO to promote its transcriptional activation and progenitor cell proliferative potential*. Blood, 2012. **119**(21): p. 4953-4962.
219. Jansson, M., et al., *Arginine methylation regulates the p53 response*. Nat Cell Biol, 2008. **10**(12): p. 1431-1439.
220. Liu, F., et al., *JAK2V617F-Mediated Phosphorylation of PRMT5 Downregulates Its Methyltransferase Activity and Promotes Myeloproliferation*. Cancer Cell, 2011. **19**(2): p. 283-294.
221. Aggarwal, P., et al., *Nuclear Cyclin D1/CDK4 Kinase Regulates CUL4 Expression and Triggers Neoplastic Growth via Activation of the PRMT5 Methyltransferase*. Cancer Cell, 2010. **18**(4): p. 329-340.
222. Guccione, E., et al., *Methylation of histone H3R2 by PRMT6 and H3K4 by an MLL complex are mutually exclusive*. Nature, 2007. **449**(7164): p. 933-7.
223. Chen, D., et al., *Regulation of transcription by a protein methyltransferase*. Science, 1999. **284**(5423): p. 2174-7.

224. Kronen-Herzig, A., et al., *Signal-dependent control of gluconeogenic key enzyme genes through coactivator-associated arginine methyltransferase 1*. J Biol Chem, 2006. **281**(6): p. 3025-9.
225. Chen, S.L., et al., *The coactivator-associated arginine methyltransferase is necessary for muscle differentiation: CARM1 coactivates myocyte enhancer factor-2*. J Biol Chem, 2002. **277**(6): p. 4324-33.
226. Koh, S.S., et al., *Synergistic coactivator function by coactivator-associated arginine methyltransferase (CARM) 1 and beta-catenin with two different classes of DNA-binding transcriptional activators*. J Biol Chem, 2002. **277**(29): p. 26031-5.
227. Covic, M., et al., *Arginine methyltransferase CARM1 is a promoter-specific regulator of NF-kappaB-dependent gene expression*. EMBO J, 2005. **24**(1): p. 85-96.
228. Yue, W.W., et al., *Insights into histone code syntax from structural and biochemical studies of CARM1 methyltransferase*. EMBO J, 2007. **26**(20): p. 4402-12.
229. Troffer-Charlier, N., et al., *Functional insights from structures of coactivator-associated arginine methyltransferase 1 domains*. EMBO J, 2007. **26**(20): p. 4391-401.
230. Nishioka, K. and D. Reinberg, *Transcription. Switching partners in a regulatory tango*. Science, 2001. **294**(5551): p. 2497-8.
231. Sims, R.J., 3rd, et al., *The C-terminal domain of RNA polymerase II is modified by site-specific methylation*. Science, 2011. **332**(6025): p. 99-103.
232. Naem, H., et al., *The activity and stability of the transcriptional coactivator p/CIP/SRC-3 are regulated by CARM1-dependent methylation*. Mol Cell Biol, 2007. **27**(1): p. 120-34.
233. Kowenz-Leutz, E., et al., *Crosstalk between C/EBPbeta phosphorylation, arginine methylation, and SWI/SNF/Mediator implies an indexing transcription factor code*. EMBO J, 2010. **29**(6): p. 1105-15.
234. Kawabe, Y., et al., *Carm1 regulates Pax7 transcriptional activity through MLL1/2 recruitment during asymmetric satellite stem cell divisions*. Cell Stem Cell, 2012. **11**(3): p. 333-45.
235. Fujiwara, T., et al., *CARM1 regulates proliferation of PC12 cells by methylating HuD*. Mol Cell Biol, 2006. **26**(6): p. 2273-85.
236. Cheng, D., et al., *The arginine methyltransferase CARM1 regulates the coupling of transcription and mRNA processing*. Mol Cell, 2007. **25**(1): p. 71-83.
237. Ma, H., et al., *Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter*. Curr Biol, 2001. **11**(24): p. 1981-5.
238. Bauer, U.-M., et al., *Methylation at arginine 17 of histone H3 is linked to gene activation*. EMBO Rep, 2002. **3**(1): p. 39-44.
239. Yang, Y., et al., *TDRD3 is an effector molecule for arginine-methylated histone marks*. Mol Cell, 2010. **40**(6): p. 1016-23.
240. Wu, J. and W. Xu, *Histone H3R17me2a mark recruits human RNA polymerase-associated factor 1 complex to activate transcription*. Proc Natl Acad Sci U S A, 2012. **109**(15): p. 5675-80.

241. Daujat, S., et al., *Crosstalk between CARM1 methylation and CBP acetylation on histone H3*. *Curr Biol*, 2002. **12**(24): p. 2090-7.
242. An, W., J. Kim, and R.G. Roeder, *Ordered cooperative functions of PRMT1, p300, and CARM1 in transcriptional activation by p53*. *Cell*, 2004. **117**(6): p. 735-48.
243. Xu, W., et al., *A methylation-mediator complex in hormone signaling*. *Genes Dev*, 2004. **18**(2): p. 144-56.
244. Chevillard-Briet, M., D. Trouche, and L. Vandel, *Control of CBP co-activating activity by arginine methylation*. *EMBO J*, 2002. **21**(20): p. 5457-66.
245. Kim, J., et al., *Loss of CARM1 results in hypomethylation of thymocyte cyclic AMP-regulated phosphoprotein and deregulated early T cell development*. *J Biol Chem*, 2004. **279**(24): p. 25339-44.
246. Lee, Y.H., M.T. Bedford, and M.R. Stallcup, *Regulated recruitment of tumor suppressor BRCA1 to the p21 gene by coactivator methylation*. *Genes Dev*, 2011. **25**(2): p. 176-88.
247. Yadav, N., et al., *Specific protein methylation defects and gene expression perturbations in coactivator-associated arginine methyltransferase 1-deficient mice*. *Proc Natl Acad Sci U S A*, 2003. **100**(11): p. 6464-8.
248. Yadav, N., et al., *CARM1 promotes adipocyte differentiation by coactivating PPARgamma*. *EMBO Rep*, 2008. **9**(2): p. 193-8.
249. O'Brien, K.B., et al., *CARM1 is required for proper control of proliferation and differentiation of pulmonary epithelial cells*. *Development*, 2010. **137**(13): p. 2147-56.
250. Dacwag, C.S., et al., *Distinct protein arginine methyltransferases promote ATP-dependent chromatin remodeling function at different stages of skeletal muscle differentiation*. *Mol Cell Biol*, 2009. **29**(7): p. 1909-21.
251. Wu, Q., et al., *CARM1 is required in embryonic stem cells to maintain pluripotency and resist differentiation*. *Stem Cells*, 2009. **27**(11): p. 2637-45.
252. Torres-Padilla, M.E., et al., *Histone arginine methylation regulates pluripotency in the early mouse embryo*. *Nature*, 2007. **445**(7124): p. 214-8.
253. Al-Dhaheri, M., et al., *CARM1 is an important determinant of ERalpha-dependent breast cancer cell differentiation and proliferation in breast cancer cells*. *Cancer Res*, 2011. **71**(6): p. 2118-28.
254. Frietze, S., et al., *CARM1 regulates estrogen-stimulated breast cancer growth through up-regulation of E2F1*. *Cancer Res*, 2008. **68**(1): p. 301-6.
255. Kim, Y.R., et al., *Differential CARM1 expression in prostate and colorectal cancers*. *BMC Cancer*, 2010. **10**: p. 197.
256. Purandare, A.V., et al., *Pyrazole inhibitors of coactivator associated arginine methyltransferase 1 (CARM1)*. *Bioorg Med Chem Lett*, 2008. **18**(15): p. 4438-41.
257. Huynh, T., et al., *Optimization of pyrazole inhibitors of Coactivator Associated Arginine Methyltransferase 1 (CARM1)*. *Bioorg Med Chem Lett*, 2009. **19**(11): p. 2924-7.
258. Selvi, B.R., et al., *Identification of a novel inhibitor of coactivator-associated arginine methyltransferase 1 (CARM1)-mediated methylation of histone H3 Arg-17*. *J Biol Chem*, 2010. **285**(10): p. 7143-52.

259. Cheng, D., et al., *Small molecule regulators of protein arginine methyltransferases*. J Biol Chem, 2004. **279**(23): p. 23892-9.
260. Wang, L., et al., *The Leukemogenicity of AML1-ETO Is Dependent on Site-Specific Lysine Acetylation*. Science, 2011. **333**(6043): p. 765-769.
261. Brown, A.L., et al., *Genetic regulators of myelopoiesis and leukemic signaling identified by gene profiling and linear modeling*. J Leukoc Biol, 2006. **80**(2): p. 433-47.
262. Yan, M., et al., *A previously unidentified alternatively spliced isoform of t(8;21) transcript promotes leukemogenesis*. Nat Med, 2006. **12**(8): p. 945-9.
263. Copeland, R.A., M.E. Solomon, and V.M. Richon, *Protein methyltransferases as a target class for drug discovery*. Nat Rev Drug Discov, 2009. **8**(9): p. 724-32.
264. Cha, B. and E.H. Jho, *Protein arginine methyltransferases (PRMTs) as therapeutic targets*. Expert Opin Ther Targets, 2012. **16**(7): p. 651-64.
265. Matsuyama, R., et al., *Double PHD fingers protein DPF2 recognizes acetylated histones and suppresses the function of estrogen-related receptor alpha through histone deacetylase 1*. J Biol Chem, 2010. **285**(24): p. 18166-76.
266. Ho, L., et al., *An embryonic stem cell chromatin remodeling complex, esBAF, is an essential component of the core pluripotency transcriptional network*. Proc Natl Acad Sci U S A, 2009. **106**(13): p. 5187-91.
267. Lessard, J., et al., *An essential switch in subunit composition of a chromatin remodeling complex during neural development*. Neuron, 2007. **55**(2): p. 201-15.