

# **Role of *Setbp1* in Myeloid Leukemia Development**

by

Bandana Ajay Vishwakarma, M.Sc., M.Tech

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#### DISSERTATION AND ABSTRACT APPROVED:

DATE:

9-5-14

Dr. Chou-Zen Giam  
Department of Microbiology & Immunology  
Committee Chairperson

9/5/14

Dr. Yang Du  
Department of Pediatrics  
Dissertation Advisor

9/5/14

Dr. Thomas Darling  
Molecular and Cell Biology Program  
Committee Member

9/5/14

Dr. Andrew Snow  
Department of Pharmacology  
Committee Member

9-5-14

Dr. Jonathan Keller  
NCI, Frederick  
Committee Member

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## **DEDICATION**

I dedicate this dissertation to my parents, Meena and Munni Lal Sharma, my husband, Ajay and my son, Prithviraj, for being source of inspiration throughout my life.



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Bandana Ajay Vishwakarma  
MCB Program, Department of Pediatrics  
Uniformed Services University

## ABSTRACT

**Title of Dissertation:**    **Role of *Setbp1* in Myeloid Leukemia Development.**

**Author:**                    **Bandana Ajay Vishwakarma, PhD, 2014**  
                                     **Uniformed Services University of the Health Sciences**

**Thesis directed by:**    **Dr. Yang Du, PhD,**  
                                     **Assistant Professor,**  
                                     **Department of Pediatrics,**  
                                     **Uniformed Services University of the Health Sciences**

SETBP1, an AT-hook transcription factor, was first identified through its interaction with SET. Since then it has been implicated in development of myeloid leukemias either through overexpression or missense mutation. We have found previously that overexpression of *Setbp1* can immortalize mouse myeloid progenitors in culture through activation of Homeobox genes, *Hoxa9* and *Hoxa10* both *in vitro* and *in vivo*. However, it is not known whether activation of *Setbp1* alone is sufficient to induce myeloid leukemia development. Here we show that *Setbp1* overexpression in murine bone marrow progenitors through retroviral transduction is capable of inducing myeloid leukemia development in irradiated recipient mice. In pre-leukemia stage, overexpression of *Setbp1* enhances the self-renewal of hematopoietic stem cells (HSCs) and expands granulocyte macrophage progenitors (GMPs). Interestingly, *Setbp1* activation also causes transcriptional repression of tumor suppressor gene *Runx1* and this effect is crucial for *Setbp1*-induced transformation. *Runx1* repression is induced by *Setbp1*-mediated recruitment of Hdac1 to *Runx1* promoters and can be relieved by treatment with histone

deacetylases (HDAC) inhibitors entinostat and vorinostat. Moreover, treatment with these inhibitors caused efficient differentiation of *Setbp1*-induced myeloid leukemia cells and immortalized myeloid progenitors in culture and significantly extended the survival of mice with *Setbp1*-induced myeloid neoplasm, suggesting that HDAC inhibition could be an effective strategy for treating myeloid malignancies with *SETBP1* activation.

Previous observations demonstrated that overexpression of *Setbp1* in mouse bone marrow cells is capable of inducing myeloid leukemia development in mice. However, only 50% of the mice receiving *Setbp1*-transduced cells developed leukemia in 10 months, suggesting that additional cooperating mutations may be required for *Setbp1*-induced leukemia development. To identify such mutations, we cloned retroviral insertions from a total of 16 *Setbp1*-induced leukemias. Interestingly, two such leukemias contained independent viral integrations at *Mllt3* that activated its expression, strongly suggesting that *Mllt3* may cooperate with *Setbp1* to induce leukemia development. To test this hypothesis, we co-transduced BM progenitors with retroviruses expressing *Setbp1* and *Mllt3*, and compared their leukemia induction potential to cells singly infected with either virus by transplantation into irradiated recipient mice. When aged for 6 months, only 2 out of 8 mice receiving cells singly transduced with *Setbp1* virus developed leukemia and none of the mice transplanted with *Mllt3*-transduced cells fell ill. In contrast, 100% of the mice transplanted with co-transduced cells developed myeloid leukemia within 92 days, confirming cooperation between *Mllt3* and *Setbp1* in inducing myeloid leukemia development. Moreover, we also found that co-transduction induced leukemia cells expressed significantly higher levels of *Meis1* compared to leukemia cells induced by

*Setbp1* alone. Given that *Setbp1* activates *Hoxa9*, which is known to cooperate with *Meis1* in leukemic transformation, this finding further suggests that *Meis1* activation by *Mllt3* may be responsible for the cooperation between *Setbp1* and *Mllt3*.

Taken together our studies indicate that *Setbp1* is a novel oncogene capable of inducing myeloid leukemia development.

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## LIST OF ABBREVIATIONS

5-FU	5-fluorouracil
ALL	Acute Lymphoblastic Leukemia
AML	Acute Myeloid leukemia
AUL	Acute Undifferentiated Leukemia
BM	Bone marrow
CGD	Chronic Granulomatous Disease
CHIP	Chromatin Immunoprecipitation
CML	Chronic Myeloid Leukemia
CMML	Chronic Myelomonocytic leukemia
CSCs	Cancer Stem Cells
DOT1L	DOT1-like histone H3K79 ethyltransferase
FACS	Fluorescence –Activated Cell Sorting
GSK3	Glycogen Synthase Kinase 3 beta
HDAC	Histone Deacetylases
Hoxa10	Homeobox protein Hox-A10
Hoxa9	Homeobox Protein Hox-A9
HSCs	Hematopoietic Stem Cells
IL11	Interleukin 11
IL3	Interleukin 3
IL6	Interleukin 6
JMML	Juvenile Myelomonocytic leukemia
LICs	Leukemia Initiating Cells
LSCs	Leukemic Stem Cells
LT- HSC	Long Term HSC
LTR	Long Terminal Repeat
MDR1	Multi Drug Resistance 1
MDS	Myeloid Dysplastic Syndrome
Meis1	Myeloid Ecotropic Viral Integration Site 1

MLL	Mixed lineage leukemia
MLLT1	Mixed Lineage Leukemia Translocated to 1
MLLT3	Mixed Lineage Leukemia Translocated to 3
MPN	Myeloproliferative Neoplasm
MSCV	Murine Stem Cell Virus
NOD/SCID	Non Obese Diabetic/Severe Combined immunodeficient
PCG	Polycomb group of genes
PP2A	Protein Phosphatase Type 2A
SCF	Stem Cell Factor
SET	SET nuclear proto-oncogene
Setbp1	SET binding protein 1

## CHAPTER 1: INTRODUCTION, HYPOTHEISIS AND AIMS

The conventional model of cancer states that all cells in a tumor have the capacity to propagate malignancy. Alternately, the cancer stem model posits that cancer is maintained by a small population of cells, cancer stem cells (CSCs), which can regenerate themselves (self-renew) and also gives rise to more differentiated cells that constitute the bulk of the disease. In leukemia, such cells with capability to self-renew are referred to as leukemic stem cells (LSCs) or leukemia initiating cells (LICs). They are the source of initiation and maintenance of leukemia. Targeting the self-renewal pathway of LSCs could be a potential therapeutic approach to cure the disease. The Mechanism of self-renewal in LSCs is still not well understood. Initially it was thought that LSCs arose from transformation of hematopoietic stem cell (HSC), as they share a common feature: self-renewal. However, recent studies have shown that committed progenitors which lack self-renewal property can also acquire, through mutations, the capability to self-renew and give rise to LSCs. Hence, characterization of the mutations conferring self-renewal properties to committed progenitors would help to understand the mechanism of self-renewal and provide rational targets for development of specific therapeutics.

SET-binding protein1 (*Setbp1*), an AT-hook transcription factor, is a gene identified in our lab through retroviral insertional mutagenesis and was activated through viral insertions in two different hematopoietic progenitor clones. When expressed ectopically *Setbp1* is able to immortalize myeloid progenitor cells. The morphology of the

immortalized cells resembles immature myeloid blast cells. Knockdown of *Setbp1* in *Setbp1*-immortalized cell line reduces the colony forming capability of these cells. Homeobox genes, *Hoxa9* and *Hoxa10*, implicated in self-renewal of LSCs, are direct downstream targets of *Setbp1*. They are down regulated upon *Setbp1* knock down, suggesting that *Setbp1* might regulate the Hox genes. *Setbp1* also promotes self-renewal *in vivo*. When co-expressed with *BCR/ABL*, it could transform the myeloid progenitors and induce leukemia similar to blast crisis in recipient mice (88). *SETBP1* has been reported to be overexpressed in 27.6% of human acute myeloid leukemia and its overexpression predicts shorter overall survival (27). Moreover, abnormal activation of *SETBP1* through overexpression or missense mutations is highly recurrent in various myeloid malignancies (12; 69; 72; 92); however, it is unclear whether such activation alone is able to induce leukemia development. Thus, **we hypothesize that *Setbp1* is a novel oncogene which can promote self-renewal of HSC and myeloid progenitors during myeloid leukemia development.**

The specific aims to test the hypothesis proposed are as follows:

**Specific Aim1: To determine the leukemogenic potential of *Setbp1* and identify the cooperating mutations through oncogenic retrovirus induced insertional mutagenesis.**

5-fluorouracil (5-FU) treated murine bone marrow cells will be infected with high titer retrovirus carrying *Setbp1* cDNA and subsequently will be transplanted into lethally irradiated congenic recipients to examine whether the transduced stem and progenitor cells can induce leukemia. Taking advantage of insertional mutagenesis by the *Setbp1*

expressing virus in this system, we will also identify the insertional mutations that cooperate along with *Setbp1* to contribute to leukemogenesis by cloning the retroviral integrations present in the developed leukemia.

**Specific Aim 2: To determine the cell types to which Setbp1 can promote self-renewal.**

Different myeloid progenitors and HSCs will be sorted based on cell surface markers, using FACS, and infect with retrovirus carrying *Setbp1* and then assess for the self-renewal property *in vitro* and in transplanted recipients. My study will reveal the cellular compartment in hematopoietic hierarchy which can be altered to LSCs by Setbp1.

The first step to target leukemic stem cells for therapy is to identify and understand the role of self-renewal pathways involved in maintaining LSCs. Preliminary studies in our laboratory have identified *Setbp1* as a novel gene regulating LSC self-renewal. The proposed studies will further characterize the capacity of *Setbp1* to confer self-renewal capacity and its leukemogenic potential. This would give us insight into the underlying mechanism of LSC self-renewal and reveal potential therapeutic target to inhibit LSCs.

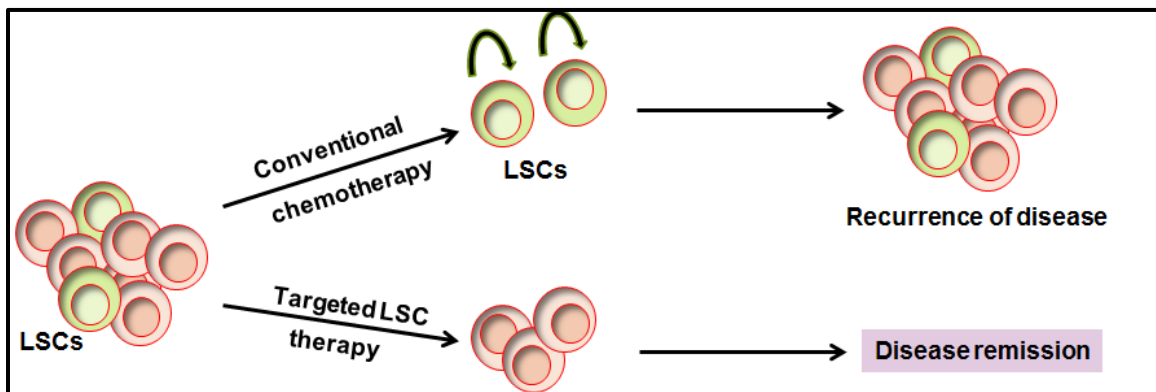
## CHAPTER 2: BACKGROUND INFORMATION

### LEUKEMIC STEM CELL AND ITS ORIGIN IN MYELOID LEUKEMIA

Human myeloid leukemias are classified into two types based on the latency of the disease: acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). LSCs have been identified in both AML and CML and share functional properties with normal stem cells (52; 59). Signaling pathways normally involved in regulation of stem cells are found to be deregulated in LSCs, suggesting that stem cells can be the target of transformation in some cancers (104). It was first demonstrated using non obese diabetic/severe combined immunodeficient (NOD/SCID) mice that AML-LSCs arose from primitive cells with phenotype CD34<sup>+</sup> CD38<sup>-</sup>, termed as SCID leukemic initiating cell (SL-IC) ;with cell surface markers similar to SCID repopulating cells or HSCs (9). Most of the leukemic cells were unable to proliferate extensively and only a small subset (.2-1%) could transmit the malignancy to recipient mice. The most frequent fusion transcript *AML1-ETO*, associated with AML, is detected in leukemic blast cells as well as in normal HSC of AML patients in remission, suggesting that translocation occurred in HSC and later additional mutation in a group of HSCs or its progeny generated leukemia (77; 119). *AML1-ETO* expressing HSCs were Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>Thy<sup>+</sup> whereas LSCs were Lin<sup>-</sup>CD34<sup>+</sup>CD38<sup>-</sup>Thy<sup>-</sup> signifying that subsequent mutation might have occurred in Thy1<sup>-</sup> progeny of HSCs or could have lost the expression of Thy1. A similar report was published, where LSCs of AML are Thy1<sup>-</sup> (CD90) (8). In CML, the *BCR-ABL* oncogenic fusion transcript is present in all the blood lineages, suggesting translocation occurs in HSCs (23). It has been debated that LSCs can arise only from primitive stem cell as they

have the machinery to self-renew and can accumulate mutations as they persist for longer period. The restricted progenitor cells are less likely to transform as they lack in self-renewal, proliferate for short period and require more mutations for the neoplastic change(13; 98). However, LSCs can arise from more committed progenitors by acquiring the capacity to self-renew and explain why phenotypic differences exist with leukemia of same molecular abnormality (31; 41). Fusion protein *MOZ-TIF2* resulting from *inv(8)(p11q13)* has been implicated in AML, where *MOZ* a chromatin remodeling gene and *TIF2* nuclear receptor transcriptional co-activator regulate target genes through abnormal histone acetylation(18; 35). Using retroviral gene transfer, followed by serial replating assay and transplantation into lethally irradiated mice, it has been shown that *MOZ-TIF2* fusion protein can bestow self-renewal property to highly purified common myeloid progenitors(CMPs) and granulocyte-monocyte progenitors (GMPs)(53), whereas *BCR-ABL* and mutant form of *MOZ-TIF2* could not. *BCR-ABL* can only confer proliferative and survival advantage to stem and progenitor cells(32; 101) but *MOZ-TIF2* have more oncogenic effect as, beside conferring self-renewal can block differentiation and cause leukemia on transplantation. The results imply that progenitor cells require additional mutations to acquire self-renewal property to become a LSCs. *MLL-ENL* and *MLL-AF9* fusion protein too can transform progenitors to LSCs (26; 64). Murine model of acute promyelocytic leukemia (APL) , M3 subtype of AML, was used to show that *PML-RAR $\alpha$*  can confer properties of self-renewal to committed promyelocytic progenitors supporting the concept that leukemic stem cells can arise from committed progenitors which lack stem cell properties (116). The differing transforming ability of the oncoprotein could be due to differing ability to induce self-renewal properties (40).

Higher frequencies of LSCs have also been found in several congenic transplantation mouse models. The discrepancy between the studies may be explained by lower engraftment efficiency in xenotransplantation models due to a different microenvironment (95). The frequency of LSCs could also be affected by oncogenic mutation, as different mutation causes transformation in these studies (100). Besides unlimited self-renewal capacity, another important characteristic of LSCs in AMLs is that it exists in quiescent non cycling stage. This characteristic explains the frequent relapse of the disease, as conventional therapies are mostly designed to kill proliferating cells and thus may not be able to effectively target LSCs (Fig1)(44; 102). Therefore new treatments capable of eliminating LSCs have to be developed to cure AML.



**Figure 1. Targeting leukemic stem cell to eradicate the disease**

Upper panel- Conventional chemotherapy kills cycling cell, while sparing LSCs causing recurrence of the disease. Lower panel- LSCs- targeting therapeutic approach destroy LSCs. The surviving tumor cells lack self-renewal property and cannot maintain the tumor, resulting into cure of the disease.

CML, which accounts for 20% of adult leukemia, is characterized by biphasic clinical course in which the initial chronic phase resembles a benign myeloproliferative disorder with high level of granulocytes and progresses into blast crisis with immature blast cells(15; 17). It is induced by expression of *BCR-ABL* fusion protein which



constitutively activates tyrosine kinase. The origin of LSC population in CML is different in different phase. In chronic phase *BCR-ABL* transcript is detected in all hematopoietic lineages except natural killer cells implying that LSCs in chronic phase arises from HSCs (48; 62). During blast crisis a second hit in the committed progenitors give rise to new set of self-renewing LSCs (59; 63). Imatinib mesylate (Gleevec) is a revolutionary drug in the treatment of CML. It induces remission in patients but does not eliminate LSCs which remain a potential threat for relapse of the disease (51; 82). As in AML, in CML too it is necessary to target LSCs for complete cure of the disease.

### **SELF-RENEWAL ASSOCIATED SIGNALING**

Though many genes and pathways implicated in self-renewal of HSCs are found to be deregulated in LSCs, but there should be some different molecular requirements in both suggesting different self-renewal program in normal and malignant stem cells which could be exploited to develop therapies (118). There can be a possibility that the mechanism of self-renewal might be overlapping but some unique self-renewal signatures might be involved in induction of leukemia (67). NF-KB pathway has been seen to be activated in LSCs rather than HSCs, revealing LSC specific phenomenon. But this is not the only mechanism for the development of leukemia (46).

AML fusion genes *AML1-ETO*, *MLL-ENL*, *MOZ-TIF2* and *NUP98-HOXA9* confer self-renewal to LSCs in leukemia. Evidences indicate that the polycomb groups of genes (PCG) are involved in both normal and leukemic hemopoiesis through epigenetic regulation of HSCs and progenitor self-renewal and proliferation. Bmi1, a polycomb group of protein is essential for the self-renewal of HSCs and LSCs (68). It maintains

stem cell pool population by either repressing genes involved in senescence or by inducing telomerase to prevent shortening of telomere (58). Induction of telomerase by Bmi1 is cell specific because it failed to induce in fibroblast. Expression of Bmi1 has been found to be higher in AML cells than in normal bone marrow cells. It regulates self-renewal through suppression of cyclin dependent inhibitors (CDK), P16ink4a and p19Arf (68). Besides, in *Bmi1*<sup>-/-</sup> mice AML is produced but cannot be serially transplanted suggesting that Bmi1 is important for self-renewal in LSC (91). Bmi-1 has been implicated in human AML (115) where it was upregulated in cord blood cells transformed *in vitro*.

Wnt/ $\beta$ -catenin pathway has been implicated in different types of cancer (107). In hematopoiesis it is required in the bone marrow niche to regulate proliferation and self-renewal of HSCs (42).  $\beta$ -catenin, the downstream molecule of Wnt signaling pathway has been shown to be necessary for HSC development as  $\beta$ -catenin knockout mice were deficient in long term HSC(LT-HSC) (121) but, in adult HSC it is not indispensable for HSC maintenance indicating a different requirement for development versus maintenance of HSC.  $\beta$ -catenin is required for the maintenance of self-renewal in LSCs. Activation of  $\beta$ -catenin has been shown to occur in CML-blast crisis LSCs (59). This hyper activation of  $\beta$ -catenin is due to aberrant splicing of glycogen synthase kinase 3 beta (GSK3), an inhibitor of  $\beta$ -catenin(1).

Notch signaling is another pathway suggested in the regulation of self-renewal. Notch receptors are found to be activated and expressed in HSCs (38) and as

differentiation occurs it is downregulated. In both AML and CML LSCs, Notch has not been demonstrated very convincingly. However, Hes1 downstream molecule of Notch 1 is upregulated in CML blast crisis. Retroviral co-expression of *BCR-ABL* and *Hes1* resulted in aggressive acute leukemia (83).

Homeobox (Hox) genes are regulators of hematopoiesis and are downregulated during differentiation. Mixed lineage leukemia (MLL) rearrangements accounts for 5-6% in AML and 20% in acute lymphoblastic leukemia (ALL) (70). Fusion proteins involving MLL: MLL-ENL, MLL-AF9 and MLL-AF4, deregulate *HOXA9* and *MEIS1* in AML. Acute lymphoblastic leukemia's with MLL rearrangement display higher expression of *HOXA7*, *HOXA9* and *MEIS1* (6). Overexpression of *Hoxa9* along with *Meis1* immortalizes cells, blocks myeloid differentiation and subsequently causes AML on transplant (65). These results indicate that *HOXA9* and *MEIS1* are regulators of transformation by MLL fusion proteins. Direct involvement of HOX genes as fusion proteins, *NUP98-HOXA9* and *NUP98-HOXA13* have been reported in AML (2). *NUP98-HOXA9* confers self-renewal property to GMPs in a mouse model of CML blast crisis. (34; 71; 85)

Translocations targeting the core binding factor, *RUNX1-ETO* confer an immortalization phenotype to the progenitors and can be propagated in serial transplantation assays *in vitro*. Further, translocations including *RUNX1-EVII* and *RUNX1-PRDM16* and activating mutations in *GATA-2* during CML progression suggest

that they could be also involved in regulating LSC self-renewal in CML blast crisis (28; 29; 76) .

Self-renewal of LSCs is a complex process which is still not very well understood. Many genes and regulatory pathways have been discovered and many more are required to be identified to have a clear idea of the mechanism involved in self-renewal of LSCs and in the development of therapies to target LSCs towards elimination of the disease from source.

#### **RETROVIRAL INSERTIONAL MUTAGENESIS –A TOOL TO IDENTIFY COOPERATING MUTATION IN CANCER**

Multiple genetic and epigenetic alterations confer growth advantage to a cell which leads to carcinogenesis (49). Several mutations involving cancer have been identified in human and animal. These mutations are cell type specific and involve specific genetic cooperation in the multistep evolution of cancer. Retroviral insertional mutagenesis is a powerful forward genetic strategy to identify genes involved in carcinogenesis /leukemogenesis (112).

Retrovirus is a RNA virus with a unique ability to integrate their genome into the host genome. Integration of retrovirus in the genome may either activate a proto-oncogene or inactivate tumor suppressor gene. Cell with such integrations acquire growth advantage and is clonally selected to grow into tumor. The integrations are identified using the provirus as a molecular tag. Proviral tagging technique has been useful to

discover oncogene, tumor suppressors and genes worth examining for their role in cancer (74). Transgenic mice susceptible to cancer have been used for identification of cooperative genetic events through retroviral mutagenesis (106). *NUP98-HOXD13* transgenic mice were infected with MOL4070LTR retrovirus to study the collaborating gene, which transformed myelodysplastic syndrome to acute leukemia (105). This technique is time consuming as well as it requires meticulous development of a correct transgenic model. Moreover, using replication competent virus has other drawbacks too. Tumors from replication competent virus are oligoclonal. If two genes are mutated in the same cancer, it is difficult to tell whether they are in the same cell. Retroviruses also often target many genes infrequently than a few genes more frequently. The present concept is to transfer gene using replication incompetent retrovirus into primary bone marrow cell and transplant into myeloablated mice to analyze for oncogenesis (84). It was thought that replication incompetent retrovirus, very rarely causes insertional mutagenesis because they only integrate into the genome at the time of initial infection. However, it was found during retroviral gene therapy that retrovirus carrying *IL2RG* could induce T cell lymphomas in patients with SCID-X1 mutation by insertionally mutating *LMO2* gene. Subsequent studies revealed that *IL2RG* act as oncogene when expressed through retroviral LTR and cooperate with *LMO2* to induce leukemia (47). Bone marrow cells infected with replication defective retroviruses carrying multidrug resistance 1(*MDR1*) transplanted into mice developed leukemia and had multiple integration which likely represent cooperating cancer genes (79). When murine stem cell retrovirus (MSCV) carrying Sox4 gene was used to infect bone marrow cells and transplanted in lethally irradiated mice, it developed myeloid leukemia due to insertional

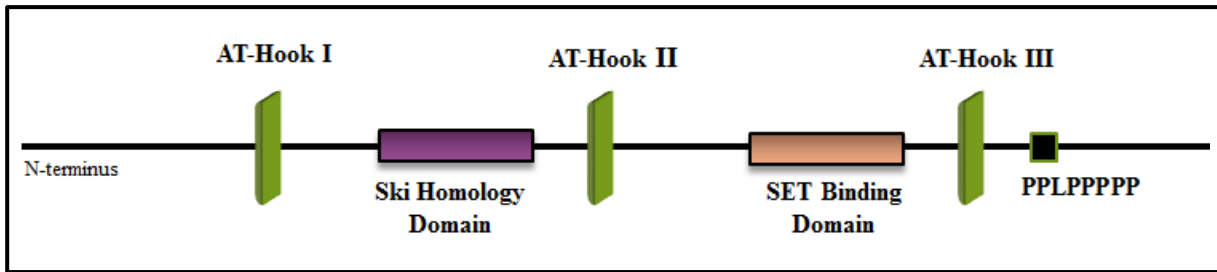
mutation in *Mef2c* gene(2). It was first time shown in this study that replication defective retrovirus carrying oncogene can induce leukemia through insertional mutagenesis. Recently, in various studies HSCs and progenitor cells have been infected with replication defective viruses *in vitro* and cells, either grown in culture or transplanted into recipient to select the transforming events and identify the genes involved in inducing leukemia (61; 73). Retrovirus insertional mutagenesis also provides information to understand the genetic interaction involved in the mechanism of leukemogenesis (84).

Insertional mutagenesis can also discover genes which can immortalize primary bone marrow cells and probably be candidate genes for self-renewal in LSCs. It has been shown in our lab that increased expression of *Evi1* and *Prdm16* due to viral insertions could immortalize the myeloid progenitors which normally lack self-renewal capacity. These cells had a phenotype similar to LSCs and could self-renew indefinitely and also differentiate into granulocytes and macrophages (2). Deregulation of *MDS1/Evi1* through retroviral insertion has been reported to immortalize nonhuman primate myeloid progenitors (16). *Evi1* and *Prdm16* are involved in various fusion proteins in human AML, CML blast crisis and myelodysplastic syndrome (28; 29). They confer growth advantage to myeloid progenitors through viral insertion activation during gene therapy trial of chronic granulomatous disease (CGD) patient (20). During the screen of *Evi1* and *Prdm16* in our lab, SET binding protein (*Setbp1*) was also identified as a retroviral insertion site (RIS) and was activated due to insertion. Activation of *Setbp1* immortalized myeloid progenitors in the presence of SCF and IL3. *SETBP1* has been reported to be activated by vector insertions in myeloid clones in a patient for CGD gene therapy trial

(20). Thus, it indicates that Setbp1 can be a possible novel regulator of self-renewal in myeloid progenitors and HSCs during myeloid leukemia development.

## **SETBP1**

SETBP1, located on chromosome 18q21, encodes 170kda protein of 1542 amino acids with unknown functions (75). Both mouse and human SETBP1 proteins display 90% homology and most likely have conserved functions. The peptide sequence has 3 AT-hook motifs which are conserved in both mouse and human. These AT-hook motifs are positively charged stretch of amino acid containing the unchanging repeat Arg-Gly-Arg-Pro (R-G-R-P) flanked by other positively charged residues and bind to AT rich sequences in the minor groove of B-form of DNA (97). It has been demonstrated that AT-hook containing proteins play an important role in chromatin structure, act as transcription factor or cofactors and also serve as DNA-binding domains for other transcription factors (5; 22). This implies that most probably SETBP1 might also have chromatin remodeling functions. Besides AT-hook, the peptide sequence contain Ski homology domain ( 652 – 863 AA) , six PEST sequence , three bipartite Nuclear localization (NLS) motifs , three proline rich repeats PPLPPPPP at the C-terminus and SET binding domain at the C-terminal end extending from 1238 -1434 AA. The three nuclear localization signal sequence(462-477,1370-1384 and 1383-1399 amino acids) might help in translocating the protein into the nucleus where it predominantly resides (Fig2)(50).



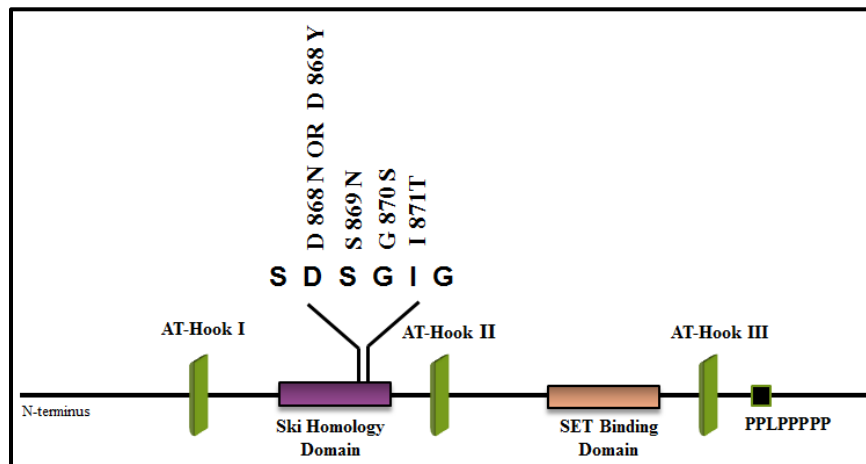
**Figure 2. Schematic diagram of SETBP1 protein**

SETBP1 is ubiquitously expressed and shown to bind to SET protein through SET binding domain. SET, a nucleophosphoprotein, implicated in leukemogenesis, inhibit protein phosphatase 2A (PP2A) which is involved in regulation of cell proliferation, differentiation and transformation (25). Fusion of *SET* with *CAN/NUP214* has been reported in AML, AUL and T- cell acute lymphoblastic leukemia (4). *SET-NUP214* fusion protein binds to the promoter region of HOXA genes and elevates its expression which contributes to the pathogenesis of T-ALL (114). MLL fusion proteins have been found to form complex with SET and PP2A, MLL-SET-PP2A, suggesting that SET can play a role in leukemia through MLL fusion proteins (3). As CAN and SET both are associated with myeloid leukemogenesis, it indicates that SETBP1 which binds to SET can also play a role in leukemia.

SETBP1 has been implicated in other diseases too. In Schinzel-Giedion syndrome, characterized by mental retardation, skeletal deformity and high occurrence of tumor, missense mutations have been observed in highly conserved Ski homology domain of SETBP1 [2]. Fusion of SETBP1 and NUP98 has been identified in pediatric



acute lymphoblastic leukemia (90). In 27.6% of elderly patients with acute myeloid leukemia, recurrent overexpression of *SETBP1* has been reported and is associated with poor prognosis for overall survival (27). Overexpression is due to translocation involving *ETV6*. Besides, it is found to be associated with other markers such as monosomy 7 and increased expression of *EVII* (27). Activation of SETBP1 through retroviral insertion during gene therapy in CGD patients imparts a growth advantage to myeloid progenitor cells (20). Recently, somatic gain of function mutations identical to germline mutations found in Schinzel-Giedion syndrome has been reported in atypical chronic myeloid leukemia, secondary AML, chronic myelomonocytic leukemia and juvenile myelomonocytic leukemia (Fig3). Growing evidence suggests its abnormal activation through overexpression or missense mutations may play an important role in the development of multiple myeloid malignancies (27; 69; 88; 92). Though SETBP1 has been shown to be involved in leukemia and other diseases but very little is known about the physiological function of the same and its role in the development of leukemia.



**Figure 3. Somatic SETBP1 mutations in Ski homology domain.**

Somatic missense mutations (D868N, D868Y, S869N, G870S, and I871T) identified in the highly conserved Ski-homology domain of Setbp1 in myeloid malignancies.

We have shown previously shown that overexpression of *Setbp1* immortalizes myeloid progenitors *in vitro* and *in vivo* (88), suggesting that it could confer self-renewal capability to LSCs in AML. Thus characterizing the role of Setbp1 in self-renewal of LSCs and its leukemogenic potential will help us to understand the molecular mechanism of LSC self-renewal.

## CHAPTER 3: Manuscript 1

(Submitted)

### ***Setbp1* induces leukemia development through repression of *Runx1***

Bandana A. Vishwakarma<sup>1</sup>, Kristbjorn O. Gudmundsson<sup>1</sup>, Hideki Makishima<sup>2</sup>, Naoko Honsono<sup>2</sup>, Nhu Nguyen<sup>1</sup>, Vijay Negi<sup>1</sup>, Kevin Oakley<sup>1</sup>, Bartłomiej Przychodzen<sup>2</sup>, Jarosław P. Maciejewski<sup>2,3</sup>, and Yang Du<sup>1,3</sup>

<sup>1</sup>Department of Pediatrics, Uniformed Services University of the Health Sciences, Bethesda, MD, USA, <sup>2</sup>Department of Translational Hematology and Oncology Research, Taussig Cancer Institute, Cleveland Clinic, Cleveland, OH, USA, <sup>3</sup>These authors contributed equally to this work.

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Corresponding authors: Yang Du, Ph.D.

Uniformed Services University of the Health Sciences,  
4301 Jones Bridge Road, Bethesda, MD, USA 20814

Phone: 301-295-9714, FAX: 301-295-3898

E-mail: [yang.du@usuhs.edu](mailto:yang.du@usuhs.edu)

Jaroslav P. Maciejewski MD., Ph.D., FACP

Taussig Cancer Institute/R40, 9500 Euclid Avenue,  
Cleveland, OH, USA 44195

Phone: 216-445-5962, FAX: 216-636-2498

E-mail: [maciejj@ccf.org](mailto:maciejj@ccf.org)

## ABSTRACT

Abnormal activation of *SETBP1* through overexpression or missense mutations is highly recurrent in various myeloid malignancies (27; 69; 88; 92); however, it is unclear whether such activation alone is able to induce leukemia development. Here we show that *Setbp1* overexpression in mouse bone marrow progenitors through retroviral transduction is capable of initiating leukemia development in irradiated recipient mice. Before leukemic transformation, *Setbp1* overexpression significantly enhances the self-renewal of hematopoietic stem cells (HSCs) and expands granulocyte macrophage progenitors (GMPs). Interestingly, *Setbp1* activation also causes transcriptional repression of tumor suppressor gene *Runx1* and this effect is crucial for *Setbp1*-induced transformation. *Runx1* repression is induced by *Setbp1*-mediated recruitment of Hdac1 to *Runx1* promoters and can be relieved by treatment with histone deacetylases (HDAC) inhibitors entinostat and vorinostat. Moreover, treatment with these inhibitors caused efficient differentiation of *Setbp1*-induced myeloid leukemia cells and immortalized myeloid progenitors in culture and significantly extended the survival of mice with *Setbp1*-induced myeloid neoplasm, suggesting that HDAC inhibition could be an effective strategy for treating myeloid malignancies with *SETBP1* activation.

SETBP1 is a large nuclear protein first identified through its interaction with oncoprotein SET (75). Growing evidence suggests its abnormal activation through overexpression or missense mutations may play an important role in the development of multiple myeloid malignancies including primary acute myeloid leukemia (AML) (27), chronic myeloid leukemia blast crisis (CML-BC) (88), atypical chronic myeloid leukemia (92), chronic myelomonocytic leukemia (CMML) (69), secondary AML (69), and juvenile myelomonocytic leukemia (JMML)(69). Multiple mechanisms could contribute to the involvement of SETBP1 in leukemia development. SETBP1 may promote inhibition of PP2A through physical interaction with SET (27). *Setbp1* can also function as an AT-hook transcription factor to activate the transcription of oncogenes *Hoxa9* and *Hoxa10* (88). Overexpression of *Setbp1* can promote the self-renewal of myeloid progenitors *in vitro* and *in vivo*, further suggesting that *Setbp1* could play a direct role in conferring unlimited self-renewal capability to leukemia-initiating cells in myeloid leukemias (20; 88). However, it remains unclear whether *SETBP1* is a potent oncogene capable of inducing leukemia development and whether additional mechanism(s) may be important for its leukemia promoting effects.

To examine the oncogenicity of *SETBP1* overexpression, we transduced 5-fluorouracil (5-FU) treated C57BL/6 mouse bone marrow progenitors with high titer pMYs retrovirus expressing *Setbp1* and *GFP* (*pMYs-Setbp1-IRES-GFP*) or empty virus (*pMYs-IRES-GFP*) and subsequently transplanted transduced cells into lethally irradiated syngeneic B6-Ly5.2 recipient mice. Interestingly, mice receiving *Setbp1* virus infected cells started to fall ill starting from about 4 months after transplantation and by 10 months over 50% of the mice had to be euthanized due to sickness (Fig. 1b). In contrast, mice that received

empty virus infected cells with higher infection efficiencies remained healthy during the same period (Fig. 1b and Supplementary Fig. 1). Moribund animals displayed enlarged spleens and livers (Fig.1c and data not shown) and cytopsin analysis of their bone marrow and spleens revealed high prevalence of immature myeloid blasts (Fig. 1d), suggesting the development of myeloid leukemias. This was confirmed by histopathological examinations and flow cytometry analyses showing that over 70% of the expanded cells are positive for both Gr-1 and negative for other lineage markers including CD19, CD3 and Ter119 (Fig.1e and 1f). These leukemias were also transplantable as irradiated secondary recipient mice died of the same disease within 21 days (Fig. 1b). As expected, leukemia cells expressed high levels of *Setbp1* and its targets *Hoxa9* and *Hoxa10* (Supplementary Fig. 2). Southern blotting analysis on genomic DNA from the leukemic spleens using a *GFP*-specific probe further suggests that these leukemias are mostly monoclonal (Supplementary Fig. 4). Cell lines can also be readily established from these leukemia cells by culturing in the presence of SCF and IL-3. Knockdown of *Setbp1* in these leukemia cell lines dramatically reduced their colony formation on methylcellulose (Supplementary Fig. 3), suggesting that *Setbp1* overexpression is also critical for the maintenance of leukemia cells. These results suggest that *SETBP1* is a potent oncogene capable of inducing myeloid leukemia development. The variable leukemia latencies and incomplete penetrance observed further suggest that additional mutations are likely required for leukemic transformation.

To study the early effects of *Setbp1* overexpression before leukemia development, we analyzed the engraftment of transduced cells in the peripheral blood of recipient mice at 4, 8 and 16 weeks after transplantation. The engraftment of *Setbp1* virus transduced

cells increased gradually over time while a gradual decline of empty virus infected cells was detected (Fig. 2a), suggesting that *Setbp1* overexpression may promote the expansion of hematopoietic stem and progenitor cells. Lineage analysis of the donor cells further showed dramatically increased contribution of *Setbp1*-expressing cells to the myeloid lineage and concomitant reduction in their contribution to the B and T cell lineages (Fig. 2b). Consistent with this expansion of myeloid compartment, the GMP population was significantly expanded after *Setbp1* expression (Fig. 2c). The gradually increased engraftment of *Setbp1*-expressing cells also suggests that *Setbp1* expression may also promote the self-renewal of HSCs. To test this notion, we transduced purified mouse lineage<sup>-</sup>Sca1<sup>+</sup>c-kit<sup>+</sup> (LSK) cells enriched for HSCs with the same viruses and compared their engraftment potential by serial transplantation. In line with results using 5-FU treated progenitors, a significantly greater engraftment by *Setbp1* transduced cells than control virus infected cells was observed starting from 8 weeks after transplantation despite of lower transduction efficiencies by the *Setbp1* virus (Fig. 2d and Supplementary Fig. 5). Furthermore, an average of over 80-fold higher engraftment potential was detected for *Setbp1* transduced cells than control cells in secondary recipients receiving GFP<sup>+</sup> LSK cells purified from the primary recipients 16 weeks after transplantation (Fig. 2d). These data support the notion that increased expression of *Setbp1* significantly enhances the self-renewal capability of HSCs.

As both activation of proto-oncogenes and suppression of tumor suppressors are likely required for cancer transformation, we were interested to learn whether *Setbp1* may additionally induce repression of tumor suppressor gene(s) besides activating proto-oncogenes *Hoxa9* and *Hoxa10* during leukemia induction. Human AMLs with high



*SETBP1* expression display significantly lower mRNA levels of tumor suppressor gene *RUNX1* compared to AMLs with low *SETBP1* expression (Supplementary Fig. 6), suggesting that *SETBP1* may suppress *RUNX1* expression. This regulation would also be consistent with increased HSC self-renewal and GMP expansion associated with loss of *Runx1*(43; 54; 57). In supporting this notion, *Setbp1* overexpression in primary myeloid progenitors significantly reduced *Runx1* mRNA levels while its knockdown in *Setbp1*-immortalized cells induced substantial increases in *Runx1* mRNA and protein levels (Fig. 3a and 3b). Such repression is also critical for *Setbp1*-induced transformation as ectopic *Runx1* expression in *Setbp1*-induced BL3 and BL12 leukemia cells dramatically inhibit their colony-forming capability (Fig. 3c). Interestingly, chromatin immunoprecipitation (ChIP) analysis using FLAG M2 antibody in myeloid progenitors immortalized by FLAG-tagged *Setbp1* (88) showed that *Setbp1* directly binds to *Runx1* promoters in myeloid progenitors (Fig. 3d)(1), further suggesting that *Runx1* is a direct transcriptional target of *Setbp1*. Proteins with AT-hook DNA-binding motifs are known to be important chromatin-remodeling factors (11; 14; 40; 117). In search of potential epigenetic changes induced by *Setbp1* for the repression of *Runx1*, we found significant increases in histone H3 acetylation at *Runx1* promoters after *Setbp1* knockdown in cells immortalized by FLAG-tagged *Setbp1* (Fig. 3e), suggesting that *Setbp1* may repress *Runx1* transcription by preventing histone H3 acetylation at its promoters. In line with this notion, significant binding of Hdac1 to *Runx1* promoters can be detected by ChIP assay in these cells (Fig. 3f). This binding is also critical for *Runx1* repression as *Hdac1* knockdown in these cells significantly increased *Runx1* mRNA levels (Supplementary Fig. 7). Moreover, significant reductions in Hdac1 binding to the *Runx1* promoters were detected after

*Setbp1* knockdown in the same cells (Fig. 3f). These results suggest that *Setbp1* recruits Hdac1 to the *Runx1* promoters causing histone H3 deacetylation and subsequent transcriptional repression of *Runx1*.

Given that *Runx1* repression by *Setbp1*-mediated Hdac1 recruitment is required for efficient colony formation by *Setbp1*-induced leukemia cells, we explored the therapeutic potential of HDAC inhibitors for treating leukemias induced by *Setbp1* activation. As expected, *Runx1* mRNA and protein levels were significantly up-regulated in *Setbp1*-induced leukemia cells by treatment with HDAC inhibitors entinostat and vorinostat (Fig. 4a). Treatment with these inhibitors also completely ablated colony formation by these leukemia cells and *Setbp1*-immortalized S3 cells (Fig. 4b). Cytospin analysis of treated cells in liquid culture further suggests induction of myeloid differentiation, which was confirmed by significantly increased expression of differentiation markers including *Cd11b*, *Lyz2*, and *Csf1r* (Fig. 4c and 4d). Both HDAC inhibitors also induced identical effects on myeloid progenitors immortalized by mutant *Setbp1* carrying a recurrent mutation in leukemia patients (Supplementary Fig. 8). Similarly, HDAC inhibitors also caused significant growth inhibition and differentiation of primary human leukemia cells harboring an activating *SETBP1* mutation (Fig. 4e and Supplementary Fig. 9). To further test therapeutic potential of HDAC inhibitors in vivo, we transplanted mice with 2 independent mouse myeloid leukemias induced by *Setbp1* overexpression, and treated the recipient mice with entinostat or vehicle once every three days for 21 days starting from 7 days post transplantation (Fig. 4f). While all vehicle-treated recipient mice become moribund after 2 weeks, significant survival extensions were observed for mice treated with entinostat. HDAC inhibitors including vorinostat and romidepsin have been

recently approved by FDA for the treatment of cutaneous T-cell lymphoma (39; 89; 93). Studies have also suggested that myeloid leukemias induced by *AML/ETO*, *PLZF/RAR $\alpha$* , or *Hoxa9/Meis1* are sensitive to HDAC inhibitors (6; 10; 96). Our results suggest that HDAC inhibitors are likely effective for treating human myeloid malignancies with *SETBP1* activation.

Taken together, our results establish *SETBP1* activation as a ‘driver’ mutation capable of initiating myeloid leukemia development partly through histone deacetylation mediated transcriptional repression of *RUNX1*, and identify HDAC inhibition as a rational and likely effective therapeutic strategy for various myeloid malignancies with SETBP1 activation.

## **METHODS**

### **Mice**

C57BL/6 and B6-*Ly5.2* mice (7-12 weeks old; Charles River, Frederick, MD) were maintained in the animal facility of Laboratory of Animal Medicine at Uniformed Services University of the Health Sciences (USUHS, Bethesda, MD). All mouse experiments were carried out according to protocols approved by the USUHS Institutional Animal Care and Use Committee.

### **Patient samples**

Primary human AML cells were collected after signing the informed consent, according to the protocols approved by the Institutional Review Board of Cleveland Clinic in accordance with the Declaration of Helsinki.

### **Retrovirus generation**

The *pMys-Setbp1-IRES-GFP* retroviral construct was described previously (88). The murine *Runx1* cDNA from pcDNA3.1-Flag-Runx1FL(60)(Addgene plasmid 14585) was cloned into *MSCV-neo* using *EcoRI* and *XhoI* sites to generate *MSCV-Runx1-neo*. High titer retroviruses were produced by transient transfection of Plat-E cells using Fugene-6 (Roche). Viral titer was assessed by serial dilution and infection of NIH-3T3 cells.

### **Retroviral transduction and bone marrow transplantation**

C57BL/6 mice (7-12 weeks old) were injected intraperitoneally with 5-fluorouracil (150 mg/kg of body weight) 4 days before harvest of their bone marrow (BM) cells. The

harvested BM cells were grown in media [DMEM with 15% fetal bovine serum containing SCF (100ng/ml), IL-3 (6ng/ml) and IL-6 (10ng/ml)] for 2 days to induce proliferation of hematopoietic stem cells (HSCs). These expanded BM cells were subsequently infected three times with high-titer retrovirus carrying *Setbp1* cDNA (*pMYs-Setbp1-IRES-GFP*) or GFP only (*pMYs-IRES-GFP*) on retronectin coated plates. For transplantation,  $0.7-1.3 \times 10^6$  transduced BM cells were injected into the tail vein of each lethally irradiated (1100 rads from  $^{137}\text{Cs}$  source) B6-*Ly5.2* mouse along with  $7.5 \times 10^5$  supporting bone marrow cells from un-irradiated B6-*Ly5.2* mice. Transplanted mice were aged and closely monitored for signs of leukemia development. Retro-orbital bleeding was performed in 4, 8 and 16 weeks to analyze the short term and long term engraftment of the donor cells by FACS. For secondary transplantation,  $1 \times 10^6$  spleen cells from primary recipients with leukemia were injected into lethally irradiated secondary recipients along with  $7.5 \times 10^5$  supporting bone marrow cells.

For serial transplantation of LSK cells,  $1 \times 10^5$  LSK cells transduced twice with *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus were first transplanted into each lethally irradiated primary B6-*Ly5.2* recipient. At 4 months after primary transplantation,  $5 \times 10^2$  GFP<sup>+</sup> LSK cells purified from the primary recipients by FACS were transplanted into each lethally irradiated secondary B6-*Ly5.2* recipient along with supporting bone marrow.

### **Flow Cytometry**

Flow cytometry analysis of mouse peripheral blood, bone marrow and spleen samples were performed using BD LSRII flow cytometer. After sample collection and ACK lysis

of RBCs, spleen and bone marrow cells were blocked by incubation with anti-Fc $\gamma$ R-II/III and subsequently stained with antibodies against markers for myeloid (Gr-1, Mac-1), erythroid (Ter-119), B (CD19) and T (CD3) lineages. Dead cells were excluded by staining with Sytox Blue (Invitrogen). For serial transplantation of LSK cells, first mononuclear cells were isolated from the bone marrow of C57BL/6 mice (7-12 weeks old) by density centrifugation through lymphocyte separation medium. Lineage positive cells were labeled by incubation with a cocktail of purified rat anti-mouse antibodies specific to Gr-1, Mac-1, CD4, CD8, B220, CD127, and Ter-119 and were subsequently removed by incubation with sheep anti-rat IgG conjugated magnetic beads (Invitrogen) and exposure to a magnet. The isolated lin<sup>-</sup> cells were then stained with anti-Sca-1-APC, and anti-c-Kit-PE antibodies and LSK cells were sorted using a FACS Aria cell sorter. The GMP (IL-7R $\alpha$ <sup>-</sup> Sca-1<sup>-</sup>c-Kit<sup>+</sup>Fc $\gamma$ R-II/III<sup>Hi</sup>CD34<sup>+</sup>) population in bone marrow was analyzed at 3 months after transplantation using 5-FU treated cells. Lin<sup>-</sup> cells were obtained similarly as mentioned above and subsequently stained with anti-Sca-1-APC, anti-CD34-Alexa fluor-700, anti-c-Kit-PE, and anti-FcR-II/III-PE-Cy7 and analyzed using BD LSRII flow cytometer.

### **In vitro HDAC inhibitor treatment**

5 x 10<sup>5</sup> *Setbp1*-induced leukemic cells (BL3 and BL12) plated in media (IMDM, 20% horse serum and 1x pen/strep) with SCF (50ng/ml) and IL3 (10ng/ml) were treated with 1 $\mu$ M of Entinostat (LC Laboratories Woburn, MA and Selleck Chemicals, Houston, TX),

Vorinostat (LC Laboratories) or equal volume of control DMSO for 48hrs. Treated cells were subsequently subjected to cytospin, RNA extraction and Western blotting analysis. For colony formation assay,  $2 \times 10^4$  BL3 and BL12 cells were plated in IMDM methylcellulose medium supplemented with 20% horse serum, mouse SCF (50ng/ml), IL-3 (10ng/ml) and 1 $\mu$ M of Entinostat, Vorinostat, or DMSO. Colony numbers were counted after 7 days.

Primary human AML cells were cultured in IMDM medium supplemented with 10% fetal bovine serum, SCF(10ng/ml) , IL-3 (10ng/ml), TPO (10ng/ml) and FLT3 ligand (10ng/ml) at a density of  $1 \times 10^5$  cells/ml and treated with 1  $\mu$ M Vorinostat or equal volume of control DMSO for 72hrs.

#### **In vivo entinostat treatment**

Spleen cells from Setbp1-induced leukemic mice (BL12 and BL19) were transplanted into lethally irradiated secondary recipients ( $1 \times 10^6$  cells/animal) for inducing leukemia development. Beginning from 7days after transplantation, recipient mice were injected intraperitoneally with either 30 mg/kg of Entinostat (dissolved in 20  $\mu$ l of DMSO and 180  $\mu$ l of 50% polyethylene glycol) or vehicle once every 3 days for 21 days.

#### **Lentiviral production, infection, and analysis**

pLKO.1 lentiviral constructs containing shRNA were purchased from Sigma ( NC-sh, SHC002; GFP-sh, SHC005; St. Louis, MO) and infectious lentivirus were generated as described previously (88). Colony formation assays were performed at 48 hours after infection using  $2 \times 10^4$  puromycin resistant cells on IMDM methylcellulose medium

supplemented with 20% horse serum, mouse SCF (50ng/ml) and IL-3 (10ng/ml), and puromycin (2 µg/ml). Colony numbers were counted after 7days.

### **Western blotting analysis**

For Western blotting analysis the cells were washed twice with cold PBS and then whole cell lysates were prepared by direct lysis of cell pellets in heated 2 x SDS sample buffer. Samples were resolved on 4-12% tris-glycine gels (Life Technologies, Carlsbad, CA) before transferring onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Primary antibodies used include anti-Setbp1 (16841-1AP, Proteintech, Chicago, IL) (88) Runx1 (19555-1-AP, Proteintech) and  $\beta$ -actin (MAB1501R, Millipore). Secondary antibodies used include goat anti-rabbit (SC-2004, Santa Cruz Biotechnology, Dallas, TX) and anti-mouse IgG-HRP (a-9044, Sigma Aldrich). Protein bands were visualized by incubation with SuperSignal West chemiluminescent substrate (Pierce, Thermo Fisher Scientific, Rockford, IL) and quantified using Quantity One data analysis software (Bio-Rad).

### **Real-time RT-PCR**

For real-time RT-PCR, total RNA was extracted from cells using RNAeasy Plus mini kit (QIAGEN). Oligo-dT-primed cDNA samples were prepared using Superscript III (Invitrogen), and real-time PCR analysis was performed in triplicates using SYBR green detection reagents (Invitrogen) on a 7500 real time PCR system (Applied Biosystems). Relative changes in expression of *Setbp1*, *Hoxa9*, *Hoxa10*, *Runx1*, *Cd11b*, *Lyz2* and *Csf1r* were calculated according to the  $\Delta\Delta$ Ct method. The cycling conditions are 50°C for 2



minutes followed by 95°C for 2 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene-specific primer sequences are:

<i>Setbp1</i>	5' CTG CTC ACT GTG GAG ACG ATT C 3' 5' TTC TTA TCC AGC ACA CCA AGC TT 3'
<i>Hoxa9</i>	5' TGT CTC CTC TCC CCC AAA CC 3' 5' GAG ATG AGG CCT GGG ATTTAG A 3'
<i>Hoxa10</i>	5' CCA GCC CTG GGT AAA CTT AGC 3' 5' CATTGA CCT CAG GCC AGA CA 3'
<i>Runx1</i>	5' GCA GGC AAC GAT GAA AAC TAC T 3' 5' GCA ACT TGT GGC GGA TTT GTA 3'
<i><math>\beta</math>-Actin</i>	5' CCT CCC TGG AGA AGA GCT A 3'; 5' TCC ATA CCC AAG AAG GAA G 3'
<i>Rpl4</i>	5' ATG ATG AAC ACC GAC CTT AGC A 3' 5' CGG AGG GCT CTT TGG ATT TC 3'
<i>Cd11b</i>	5' GAA GCT GCC CCC CAA GAC 3' 5' GGT CAA TGC ATG GAG AAA AGG 3'
<i>Csf1r</i>	5' TGG ACT TCG CCC TCA GCT T 3' 5' CCC CAG ACC CCT CAT GTT C 3'
<i>Lyz2</i>	5' TGT GAG CTG CAG GGC TTT G 3' 5' CCC ACC ACA GAG GCT GTT CT 3'

### Chromatin immunoprecipitation (ChIP)

Mouse myeloid progenitors immortalized by FLAG-tagged Setbp1 were generated as described (88). ChIP analyses were performed using ChIP-IT Express kit (Active Motif). Immunoprecipitations were performed using FLAG M2 (Sigma Aldrich), mouse monoclonal anti-HDAC1 antibody (10E2, #5356, Cell Signaling Technologies), rabbit polyclonal anti-acetylated histone H3 (#39139, Active Motif) and mouse IgG (G3A1, #5415, Cell Signaling Technologies) and rabbit IgG (#p120-101, Bethyl Laboratories). Chromatin DNA was purified using MinElute PCR Purification Kit (QIAGEN) and quantified by real-time PCR. The following *Runx1* promoter-specific primers were used:

<i>Runx1-P1</i>	S 5' ACAGGATCTGAAAGCCACCAA 3' AS 5' CCTGCCTCAGTCTTTTCTTGCT 3'
<i>Runx1-P2</i>	S 5'CCGTCGGTCTCCTCTATGCA 3' AS 5' GCCCGACCCGAGGAATT 3'

### **Southern blotting analysis**

7 ug of DNA from leukemic spleens were digested with *EcoRI*, resolved on 0.75% agarose gel, and transferred to nylon membrane using standard procedures. <sup>32</sup>P-labeled *GFP*-specific probe was synthesized by random primer labeling using the Prime-IT II kit (Stratagene, LaJolla, CA) and hybridization was carried out in MiracleHyb buffer (Stratagene) following manufacturer's instructions.

### **Statistical analysis**

Sample sizes and animal numbers were determined by previous experiences. No samples were excluded from analyses. All data were analyzed by two-tailed Student's t-test except that survival curves were compared by Log-rank test. The researchers were not blinded during sample collection and analysis.

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### **Authorship**

B.A.V. designed experiments, performed experiments, analyzed results, and wrote the manuscript. K.O.G. and H.M. designed experiments, performed experiments, and analyzed results. N.H., N.N., V.N., and K.O. performed experiments and analyzed results. B.P. analyzed data. J.P.M. and Y.D. designed research, analyzed data, and wrote the manuscript.

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Corresponding authors:

Yang Du, Ph.D., Uniformed Services University of the Health Sciences, 4301 Jones Bridge Road, Bethesda, MD, USA 20814. e-mail: [yang.du@usuhs.edu](mailto:yang.du@usuhs.edu)

Jaroslav P. Maciejewski MD., Ph.D., FACP, Taussig Cancer Institute/R40, 9500 Euclid Avenue, Cleveland, OH, USA 44195. E-mail: [maciejj@ccf.org](mailto:maciejj@ccf.org)

## FIGURE LEGENDS

### **Figure 1 Setbp1 overexpression induces myeloid leukemia development.**

(a) Schematic diagram of bone marrow transduction transplantation assay. (b) Survival curves of irradiated C57BL6-Ly5.2 mice receiving bone marrow progenitors transduced with pMYs-Setbp1-IRES-GFP or pMYs-IRES-GFP virus, or  $1 \times 10^6$  spleen cells from primary leukemic mice. (c) Enlarged leukemic spleen (right) compared to a normal spleen (left). (d) Cytospin of Spleen (SP) and bone marrow (BM) cells from leukemic mice. (e) H&E staining showing infiltration of myeloid blasts in liver, lung and spleen of a Setbp1-induced leukemic mouse. (f) FACS analysis of lineage specific markers on bone marrow cells of a leukemic mouse.

### **Figure 2 Overexpression of Setbp1 promotes self-renewal of HSCs and expansion of GMPs.**

(a) Engraftment of indicated transduced 5-FU treated bone marrow cells in recipient mice analyzed by FACS analysis of percentage of GFP<sup>+</sup> cells in peripheral blood at 4<sup>th</sup>, 8<sup>th</sup> and 16<sup>th</sup> weeks after transplantation. (b) FACS analysis of indicated lineage specific markers on GFP<sup>+</sup> donor cells in peripheral blood of mice receiving 5-FU treated bone marrow cells transduced with *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus at 4<sup>th</sup>, 8<sup>th</sup> and 16<sup>th</sup> week after transplantation. (c) Left panel, FACS analysis of GMP populations of GFP<sup>+</sup> donor cells in the bone marrow of mice transplanted with 5-FU treated bone marrow cells transduced with *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus at 3 months after transplantation. Right panel, quantification of results on the left. (d) FACS analysis of GFP<sup>+</sup> cells in peripheral blood of recipient mice at 4<sup>th</sup>, 8<sup>th</sup> and 16<sup>th</sup>

week after primary ( $1 \times 10^5$  cells/mouse) and secondary transplantation ( $5 \times 10^2$  cells/mouse) of LSK cells transduced with *pMYs-Setbp1-IRES-GFP* (black bars) or *pMYs-IRES-GFP* virus (white bars).

**Figure 3 Setbp1 directly represses Runx1 transcription through recruitment of Hdac1.**

(a) Real-time PCR analysis of *Runx1* mRNA levels in total RNA from mouse primary myeloid progenitor 48 hours after infection with *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus. (b) Left panel, real-time PCR analysis of *Runx1* mRNA levels in S3 cells at 72hrs after infection with a lentiviral shRNA targeting Setbp1 (Setbp1 KD) or control shRNA (NC). Right panel, Western blotting analysis of Runx1 and  $\beta$ -actin protein levels in S3 cells at 96hrs after lentiviral shRNA infections. (c) Upper Panel, mean and SD of colony formation potential of S3 and BL12 cells after infection with MSCV-Runx1 (Runx1) or control empty MSCV virus (MSCV). Lower panel, representative western blotting analysis of Runx1 and  $\beta$ -actin protein levels at 72 hrs. after infection. (d) Left panel, ChIP analysis of *Runx1* promoters (P1 and P2) in myeloid progenitors immortalized by FLAG-tagged Setbp1 (88) using FLAG M2 antibody or control IgG. Right panel, schematic diagram showing P1 and P2 promoters at *Runx1*. (e) ChIP analysis of *Runx1* promoters in FLAG-tagged Setbp1 immortalized cells after infection with a lentiviral shRNA targeting Setbp1 (Setbp1 KD) or control shRNA (NC) using acetylated H3 specific antibody or control IgG. (f) ChIP analysis of *Runx1* promoters in FLAG-tagged Setbp1 immortalized cells after infection with a lentiviral shRNA targeting

Setbp1 (Setbp1 KD) or control shRNA (NC) using Hdac1 specific antibody or control IgG.

**Figure4. Histone H3 deacetylation is essential for Setbp1-induced *Runx1* repression, immortalization and transformation.**

(a) Upper panel, real-time RT-PCR analysis of *Runx1* mRNA levels using total RNA from indicated Setbp1-induced leukemic cell lines 48 hours after treatment with 1 $\mu$ M of Entinostat or Vorinostat in comparison to DMSO treated control. Relative expression levels were calculated by normalizing to  $\beta$ -*Actin* mRNA levels. Lower panel, representative western blotting analysis of *Runx1* and b-Actin protein levels in the same cells. (b) Mean and SD of colony formation potential of S3, BL3 and BL12 cells in the presence of 1 $\mu$ M entinostat (ENT), vorinostat (VOR), or control DMSO. (c) Cytospin of S3 cells and *Setbp1*-induced leukemic cell lines BL3 and BL12 after 48hrs of treatment with 1 $\mu$ M of entinostat (ENT), vorinostat (VOR), or control DMSO. (d) Real-time RT-PCR analysis of total RNA from BL3 and BL12 cells at 48 hours after treatment with 1 $\mu$ M entinostat (ENT), vorinostat (VOR) or DMSO (C) using primers specific for myeloid differentiation marker genes *Cd11b*, *Lyz2* or *Csf1r*. (e) Expansion of primary bone marrow mononuclear cells from a leukemia patient with *SETBP1* activation mutation G870S at 48 hours after treatment with vorinostat and entinostat. (f) Survival curves of irradiated B6-Ly5.2 mice transplanted with 2 independent *Setbp1*-induced leukemias and treated with entinostat (30 mg/kg of body weight) or vehicle. Animals were injected intraperitoneally every 3 days starting from 7 days after transplantation till 21 days after transplantation.

Figure 1

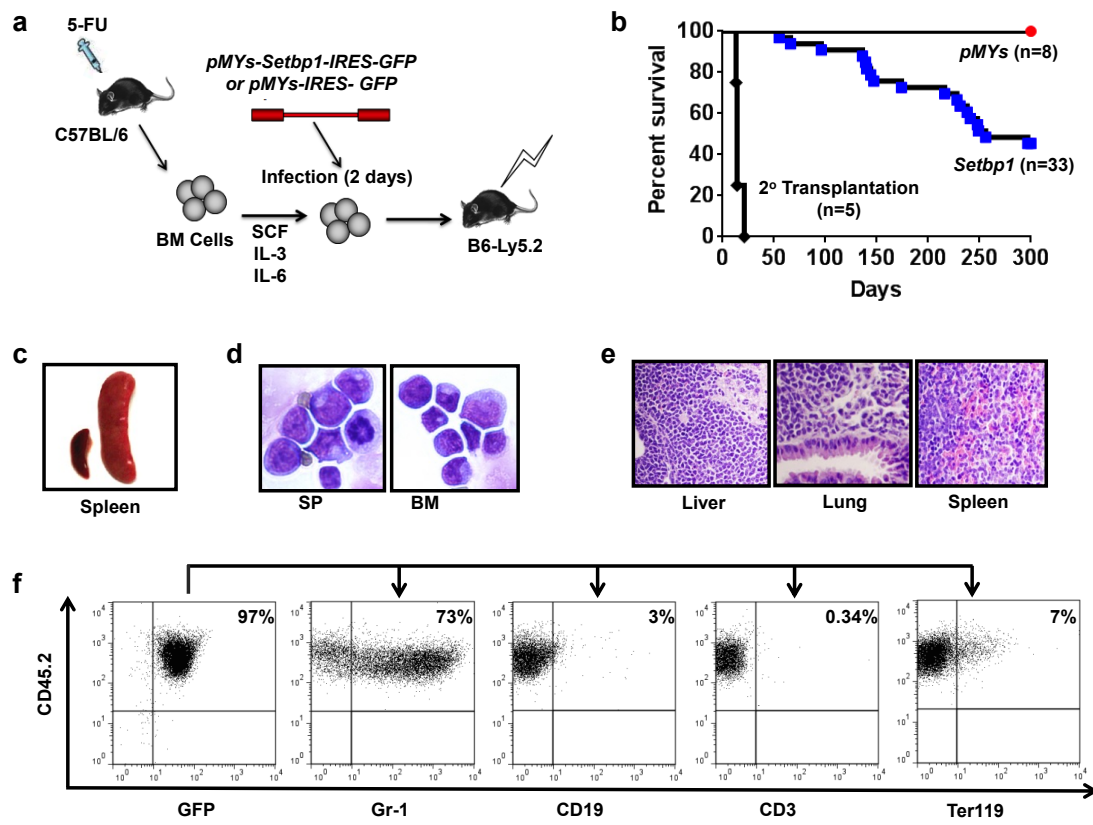


Figure 2

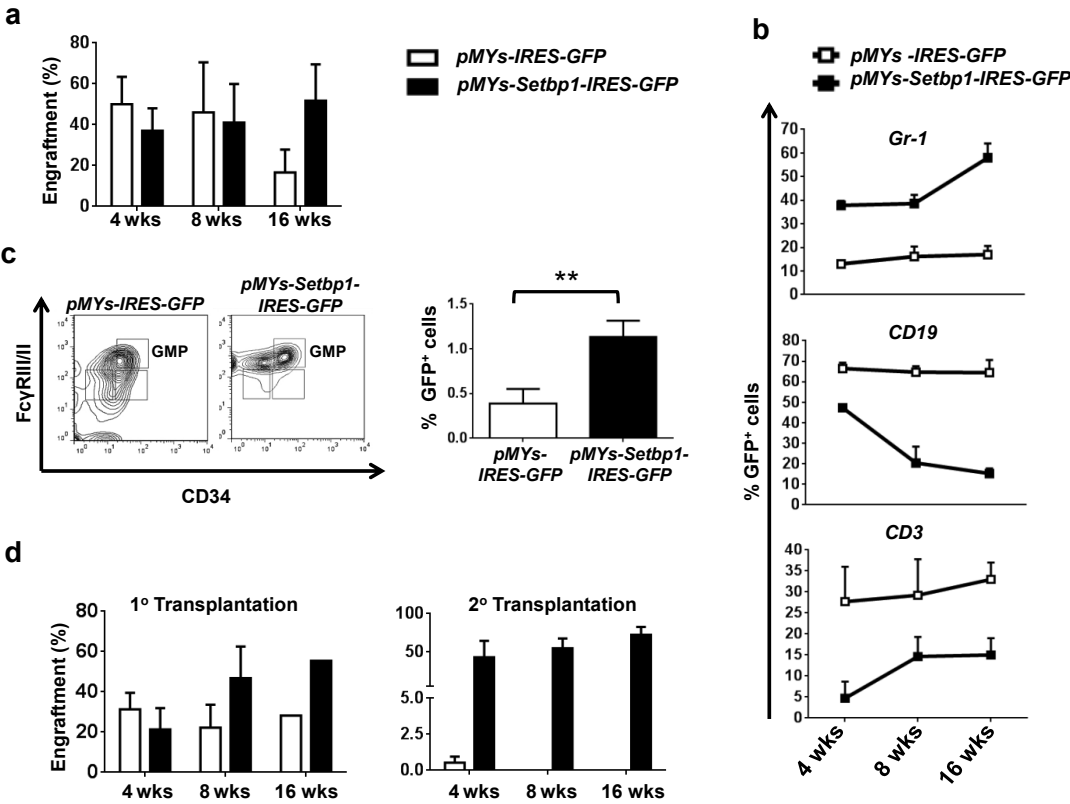




Figure 3

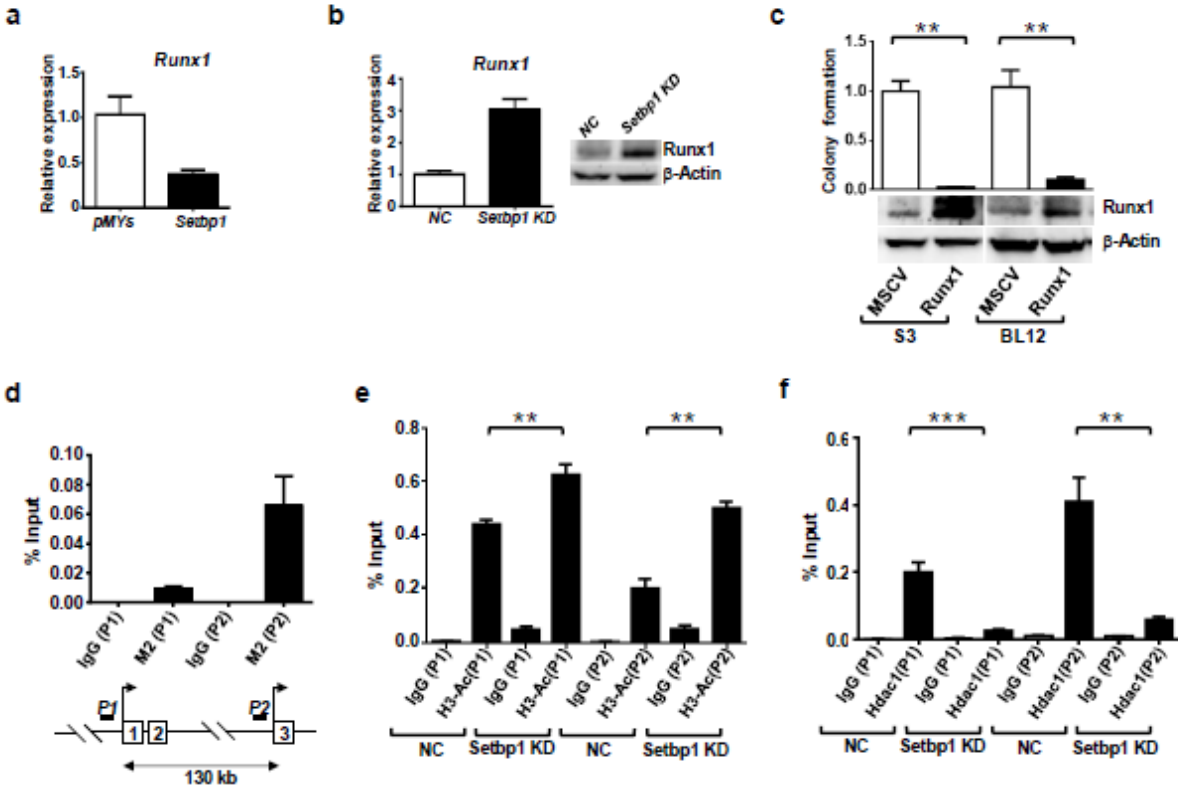
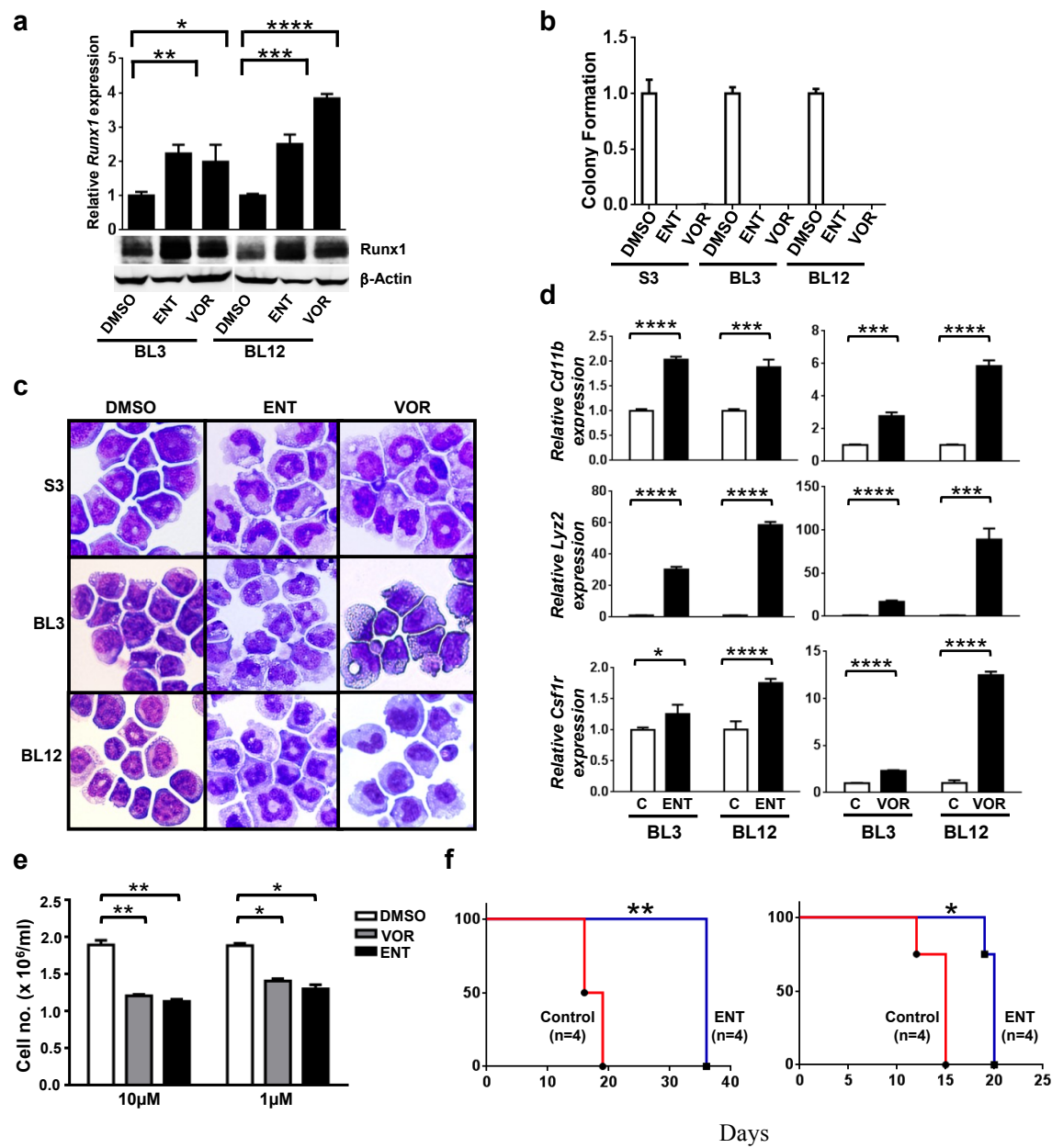
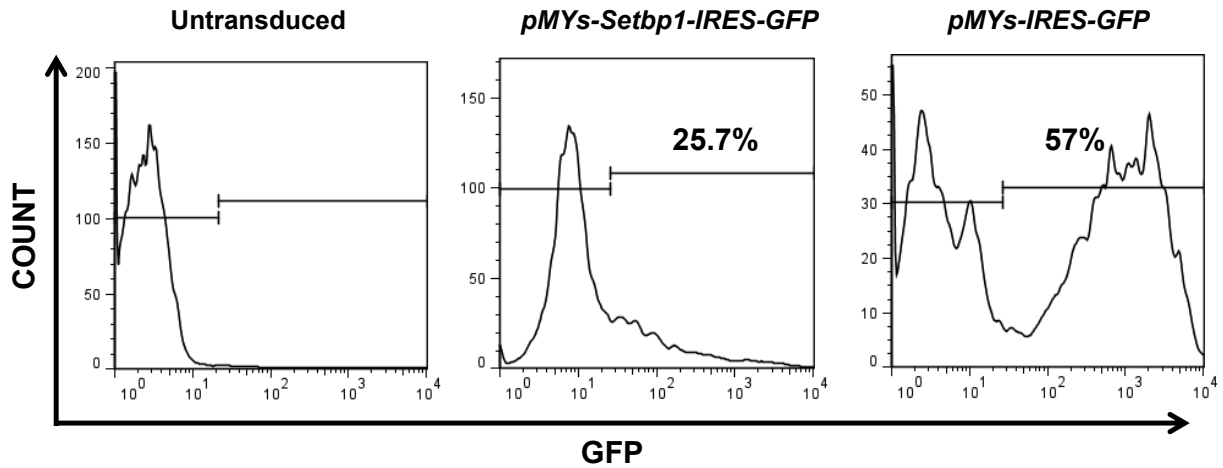


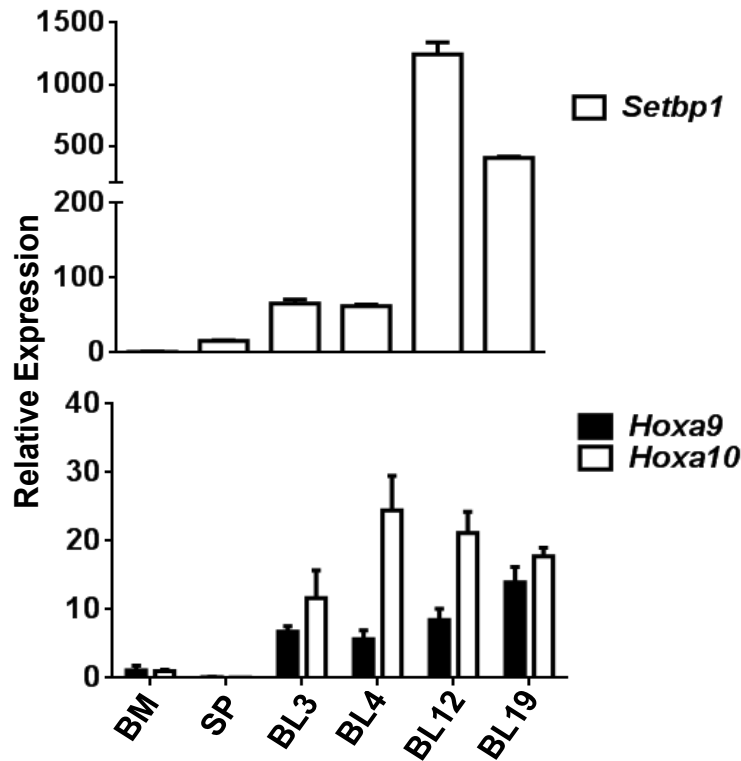
Figure 4



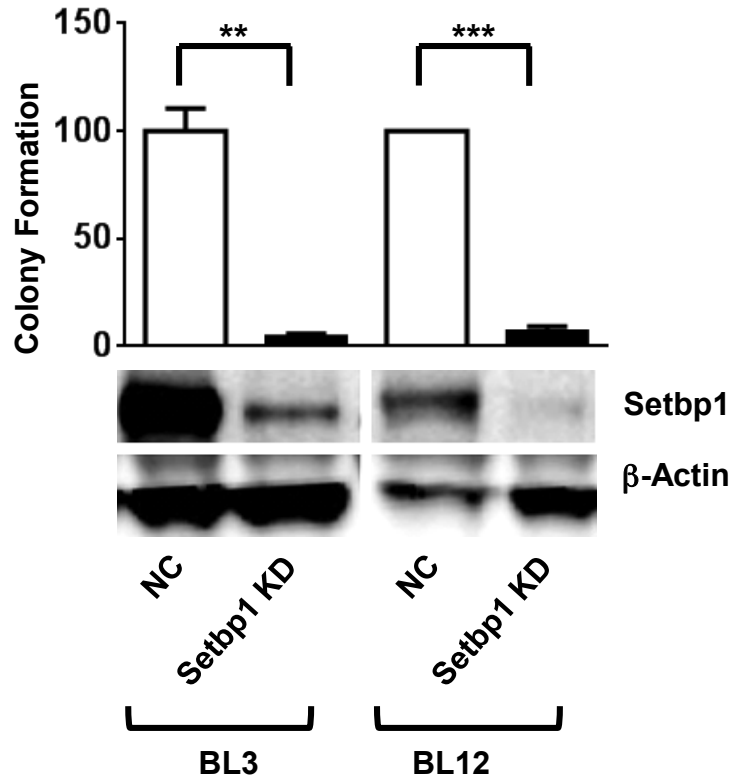
## Supplementary Figures



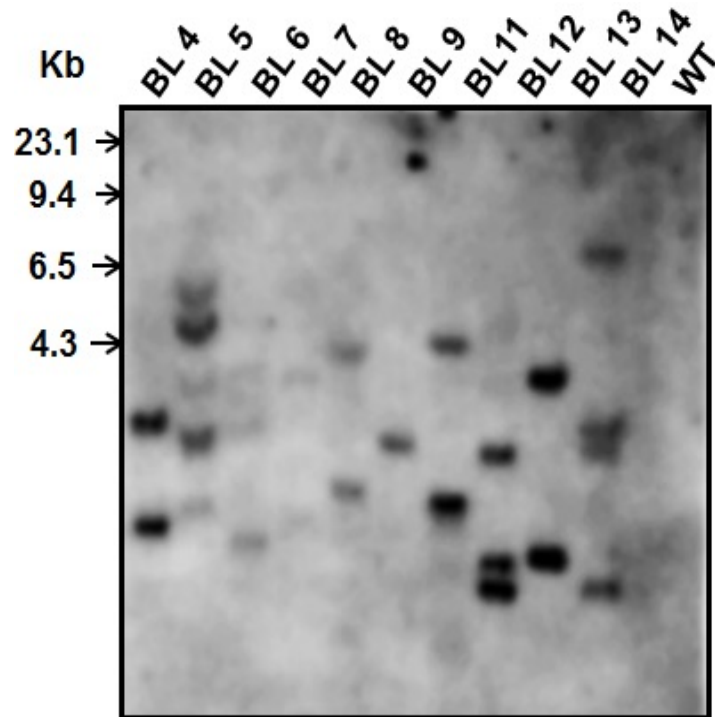
**Supplementary Figure 1.** Transduction efficiencies for 5-FU treated mouse bone marrow progenitors. Representative transduction efficiencies in indicated transduction groups determined by GFP fluorescence are shown. 20-50% and 55-72% infection efficiencies were observed for *pMYs-Setbp1-IRES-GFP* and *pMYs-IRES-GFP* virus respectively. Samples were analyzed at 48 hours after infection. Numbers represent the percentages of GFP positive cells.



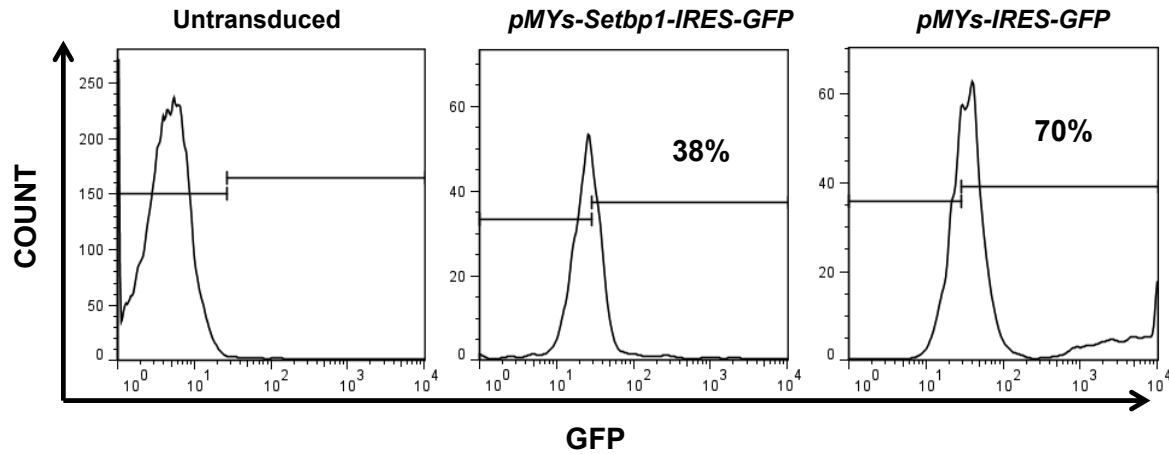
**Supplementary Figure 2.** Increased expression of *Setbp1*, *Hoxa9* and *Hoxa10* in *Setbp1*-induced myeloid leukemias Real-time RT-PCR analysis of total RNA extracted from spleens of *Setbp1*-induced leukemic mice (BL3, BL4, BL12, and BL19) and control normal bone marrow (BM) and spleen (SP) using gene-specific primers (n=3). Relative expression levels were calculated by normalizing to *Rpl4* mRNA levels in the same sample and also wild-type bone marrow. The mean and SD of each relative expression level is shown.



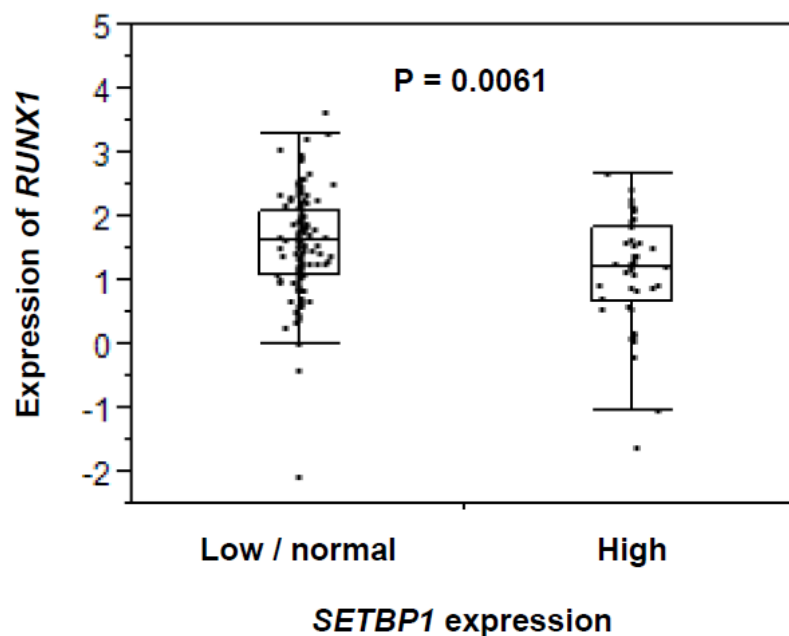
**Supplementary Figure 3.** *Setbp1*-induced leukemia cells are dependent on *Setbp1* expression for maintenance. Upper panel, mean and SD of colony-forming potential of *Setbp1*-induced leukemia cell lines BL3 and BL12 in the presence of SCF and IL-3 at 48 hours after infection with GFP-specific lentiviral shRNA (Setbp1KD) or control lentiviral shRNA (NC). Lower panel, representative Western blotting analysis of Setbp1 and β-Actin protein in the infected cells of the top panel at 72 hours after infection \*\*,  $P < 0.01$ ; \*\*\*,  $P < 0.001$  (two-tailed Student's  $t$  test)



**Supplementary Figure 4.** *Setbp1*-induced leukemias are mostly clonal. Southern blotting analysis of viral integrations present in 10 *Setbp1*-induced myeloid leukemias (BL4-9, BL11-14) using a *GFP*-specific probe. Seven ug of genomic DNA from each leukemic spleen was digested with *EcoRI*, resulting the generation of a single *GFP*-containing DNA fragment from each provirus. Each band represents an independent integration. Same amount of genomic DNA from wild-type spleen (WT) was included as negative control

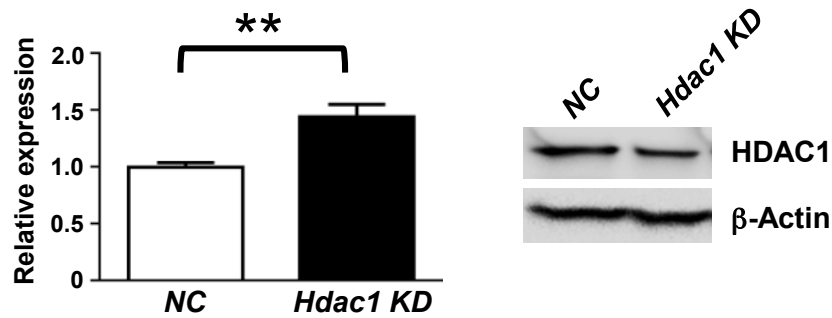


**Supplementary Figure 5.** Transduction efficiencies for purified mouse LSK cells. Representative transduction efficiencies in indicated transduction groups were determined by GFP fluorescence. 37-75% and 70-85% infection efficiencies were observed for *pMYs-Setbp1-IRES-GFP* and *pMYs-IRES-GFP* virus respectively. Samples were analyzed at 48 hours after infection. Numbers represent the percentages of GFP positive cells.

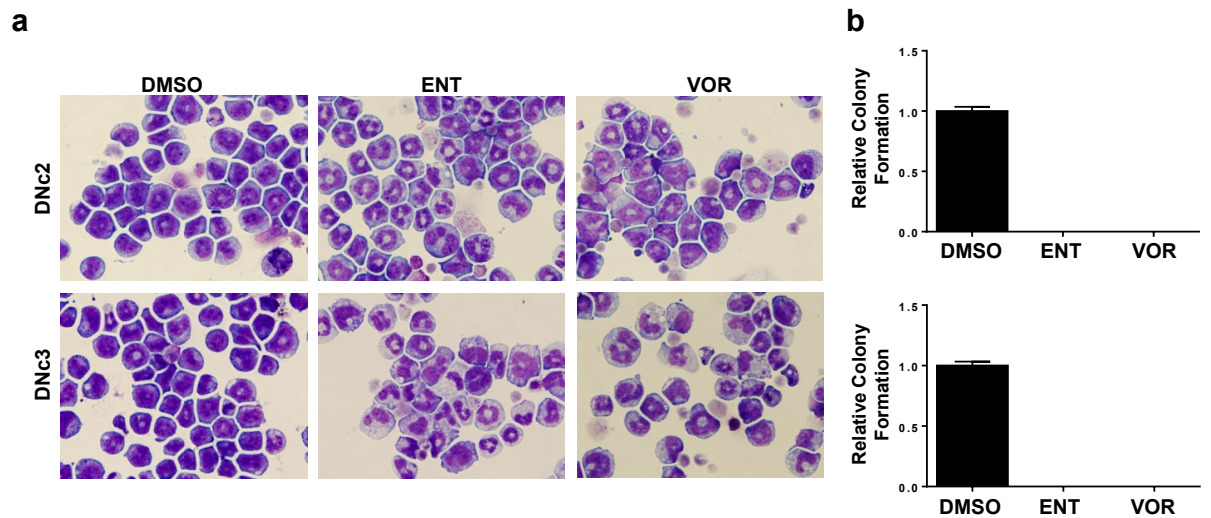


**Supplementary Figure 6.** Correlation between *SETBP1* and *RUNX1* expression in human AMLs. Expression array values were extracted from Oncomine dataset<sup>1</sup>. P-values were calculated by comparisons between indicated 2 groups with AML (without *RUNX1* mutations) using Mann-Whitney *U* test. Cut off value of high (n=42) and low / normal (n=140) expression of *SETBP1* was mean+0.5 standard deviation.

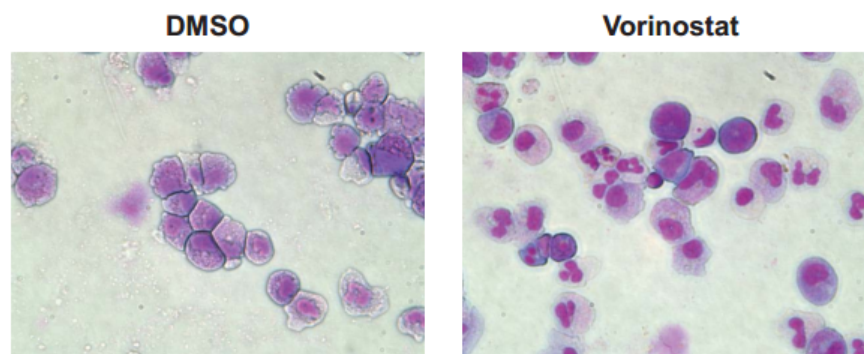




**Supplementary Figure 7.** *Hdac1* knockdown induces *Runx1* transcription. Left panel, real-time PCR analysis of *Runx1* mRNA levels in FLAG-tagged *Setbp1*-immortalized myeloid cells at 72hrs after infection with a lentiviral shRNA targeting *Hdac1* (*Hdac1* KD) or control (NC) shRNA (n=3 for each infection). Right panel, representative Western blotting analysis of Hdac1 and β-Actin protein in the infected cells of left panel at 72 hours after infection \*\*,  $P < 0.01$  (two-tailed Student's *t* test)



**Supplementary Figure 8.** HDAC inhibitors induced differentiation of myeloid progenitors immortalized by SETBP1 activation mutation identified in leukemia patients. (a) Representative cytopsin of myeloid progenitor cells (DNC2 and DNC3) immortalized by mutant *Setbp1* (harboring activation mutation D868N) after 48hrs of treatment with 1 $\mu$ M of entinostat (ENT), vorinostat (VOR), or control DMSO. (b) Mean and SD of colony formation potential of DNC2 (upper panel) and DNC3 (lower panel) cells in the presence of 1 $\mu$ M entinostat, vorinostat or DMSO (n=3 for each treatment).



**Supplementary Figure 9.** Vorinostat induced differentiation of human myeloid leukemia cells with SETBP1 activation mutation G870S. Representative cytopsin of leukemia cells after 72hrs of treatment with 1 $\mu$ M of vorinostat, or control DMSO (N=3).

## CHAPTER 4: Manuscript 2

### ***Mllt3* cooperates with *Setbp1* in inducing myeloid transformation**

Bandana A Vishwakarma<sup>1</sup> and Yang Du<sup>1</sup>

<sup>1</sup>Department of Pediatrics, Uniformed Services University of the Health Sciences,  
Bethesda, MD, USA.

#### **ABSTRACT**

We showed previously that overexpression of *Setbp1* in mouse bone marrow (BM) progenitors, through retroviral transduction is capable of inducing myeloid leukemia development in irradiated recipient mice. However, only 50% of the mice receiving *Setbp1*-transduced cells developed leukemia in 10 months, suggesting that additional cooperating mutations may be required for *Setbp1*-induced leukemia development. To identify such mutations, we cloned retroviral insertions from a total of 16 *Setbp1*-induced leukemias. Interestingly, two such leukemias contained independent viral integrations at *Mllt3* that activated its expression, strongly suggesting that *Mllt3* may cooperate with *Setbp1* to induce leukemia development. To test this hypothesis, we co-transduced BM progenitors with retroviruses expressing *Setbp1* and *Mllt3*, and compared their leukemia induction potential to cells singly infected with either virus by transplantation into irradiated recipient mice. When aged for 6 months, only 2 out of 8 mice receiving cells singly transduced with *Setbp1* virus developed leukemia and none of the mice transplanted with *Mllt3*-transduced cells fell ill. In contrast, 100% of the mice transplanted with co-transduced cells developed myeloid leukemia within 92 days, confirming cooperation between *Mllt3* and *Setbp1* in inducing myeloid leukemia development. Moreover, we also found that co-transduction induced leukemia cells

expressed significantly higher levels of *Meis1* compared to leukemia cells induced by *Setbp1* alone. Given that *Setbp1* activates *Hoxa9*, which is known to cooperate with *Meis1* in leukemic transformation, this finding further suggests that *Meis1* activation by *Mllt3* may be responsible for the cooperation between *Setbp1* and *Mllt3*. In summary, our studies identify cooperation between *Mllt3* and *Setbp1* in leukemia induction by simultaneous activation of *Hoxa9* and *Meis1*.

## **Introduction**

Recurrent somatic activating mutations in *SETBP1* or its overexpression has been reported in various human leukemias (12; 27; 33; 69; 92). SETBP1 is known to bind to nuclear protein SET (75) and inhibit the activity of tumor suppressor PP2A through the formation of heterotrimeric complex SETBP1-SET-PP2A (27). Furthermore, its overexpression promotes self-renewal in murine myeloid progenitors through activation of Homeobox genes, *Hoxa9* and *Hoxa10*, both *in vitro* and *in vivo* suggesting that *Setbp1* could be an oncogene (88). Recently, we showed that overexpression of *Setbp1* in mouse bone marrow progenitors through retroviral transduction, is capable of inducing myeloid leukemia in mice. However, only 50% of the mice receiving the *Setbp1*-transduced cells developed leukemia in 10 months. Given that multiple cooperating genetic and epigenetic alterations are required for carcinogenesis (49), this result suggests that additional cooperating mutations are required for the development of leukemia. Identification of these cooperating mutations will be critical for designing combinatorial therapies for *Setbp1*-induced leukemia.

Insertional mutagenesis using retroviral vectors is a powerful tool for identifying cooperating mutations in leukemia development (37; 84; 110; 112). Oncogene of interest is inserted into a replication incompetent retrovirus and is used for infection of bone marrow cells. The retrovirus incorporates into the genome in a largely random fashion and frequently causes activation of the gene in its vicinity. Cells with the viral integration activate a cooperating gene and acquire higher growth advantage and develop faster into leukemia. Retroviral integrations in individual tumor are cloned and sequenced to identify the genes that cooperate with oncogene of interest to induce leukemia (2; 61).

In the present study, we attempted to identify *Setbp1*-cooperating partner by cloning integrations from the leukemias induced by transplant of mouse bone marrow progenitors with a *Setbp1*-expressing retroviral vector. Mixed-Lineage Leukemia Translocated to 3 (*Mllt3*) was identified as a potential cooperating mutation for *Setbp1*. Co-transduction of *Mllt3* and *Setbp1* in BM progenitors accelerated the development of *Setbp1*-induced leukemia. Furthermore, we found that deregulated expression of *Mllt3* upregulated the expression of *Meis1* oncogene, which is known to cooperate with *Hoxa9* in leukemic transformations (65), and may be responsible for the acceleration of *Setbp1*-induced leukemia. Taken together, our studies indicate cooperation between *Mllt3* and *Setbp1* in myeloid leukemia induction by simultaneous activation of *Hoxa9* and *Meis1*.

## **METHODS**

### **Mice**

C57BL/6 and B6-Ly5.2 mice (Charles River Laboratories, Frederick, MD) were maintained in the animal facility of the Laboratory of Animal Medicine at Uniformed Services University of the Health Sciences (USUHS). All mouse experiments were carried out according to protocols approved by the USUHS Institutional Animal Care and Use Committee.

### **Splinkerette PCR**

Viral integrations were cloned using splinkerette PCR as previously described (113). Briefly, genomic DNA prepared from spleens of animals with *Setbp1*-induced leukemia was digested with *NlaIII* or *MseI* and ligated to the splinkerette linker overnight. Nested PCR was done to amplify the genomic sequence between the insertion and annealed splinkerette, using primers specific to splinkerette and long terminal repeat (LTR) of pMYs. PCR products were separated using 2% agarose gel and purified using Mini Elute Columns (Qiagen, Valencia, CA). Amplified fragments were directly sequenced.

### **Retrovirus generation**

The *pMYs-Setbp1-IRES-GFP* retroviral construct was generated as described previously (88). The murine *Mllt3* cDNA was amplified and cloned into *MSCV-puro* using *XhoI* and *EcoRI* sites to generate *MSCV-Mllt3-puro*. High titer retroviruses were produced by transient transfection of Plat-E cells using Fugene-6 (Roche, Indianapolis, IN). Viral titer was assessed by serial dilution and infection of NIH-3T3 cells.

### **Retroviral transduction and bone marrow transplantation**

C57BL/6 mice (7-12 weeks old) were injected intraperitoneally with 5-fluorouracil (150 mg/kg of body weight), 4 days before harvest of their bone marrow cells. The harvested BM cells were grown in media [DMEM with 15% fetal bovine serum containing SCF (100ng/ml), IL-3 (6ng/ml) and IL-6 (10ng/ml)] for 2 days to induce proliferation of hematopoietic stem cells (HSCs). These expanded BM cells were subsequently infected two times with high-titer retrovirus carrying *Setbp1* cDNA (*pMYs-Setbp1-IRES-GFP*) and *MSCV-Ml1t3-puro*, *pMYs-Setbp1-IRES-GFP* or *MSCV-Ml1t3-puro* on retronectin coated plates. For transplantation,  $0.4-0.6 \times 10^6$  transduced BM cells were injected into the tail vein of each lethally irradiated (1100 rads from  $^{137}\text{Cs}$  source) B6-*Ly5.2* mouse along with  $7.5 \times 10^5$  supporting bone marrow cells from un-irradiated B6-*Ly5.2* mice. Transplanted mice were aged and closely monitored for signs of leukemia development.

### **Flow Cytometry**

Flow cytometry analysis of BM and spleen samples of moribund mice was performed using BD LSRII flow cytometer. After sample collection and ACK lysis of RBCs, spleen and bone marrow cells were blocked by incubation with anti-Fc $\gamma$ R-II/III and subsequently stained with antibodies against markers for myeloid (Gr-1, Mac-1), erythroid (Ter-119), B (CD19) and T (CD4 & CD8) lineages. Dead cells were excluded by staining with Sytox Blue (Invitrogen). For LSK cell purification, mononuclear cells were isolated from the BM of C57BL/6 mice (7-12 weeks old) by density centrifugation through lymphocyte separation medium. Lineage positive cells were then labeled by



incubation with a cocktail of purified rat anti-mouse antibodies specific to Gr-1, Mac-1, CD4, CD8, B220, CD127, and Ter-119 and were subsequently removed by incubation with sheep anti-rat IgG conjugated magnetic beads (Invitrogen) and exposure to a magnet. The isolated lin<sup>-</sup> cells were then stained with anti-Sca-1-APC, and anti-c-Kit-PE antibodies and LSK cells were sorted using a FACSAria cell sorter.

### **Colony Formation Assay**

Colony formation assay was performed, at 72 hours after infection of LSK cells with *pMYs-Setbp1-IRES-GFP* and *MSCV-Mllt3-puro*, *pMYs-Setbp1-IRES-GFP* or *MSCV-Mllt3-puro*, using 3000 cells on methylcellulose medium supplemented with 15% Fetal Bovine Serum, mouse SCF (50ng/ml) and IL-3 (6ng/ml), and IL6 (10ng/ml). LSK cells positive for *Mllt3* c-DNA were selected by puromycin, whereas *Setbp1* cells were sorted for GFP<sup>+</sup>. Colony numbers were counted after 7days.

### **Western blotting analysis**

For western blotting analysis, the cells were washed twice with cold PBS and whole cell lysates prepared by direct lysis of cell pellets in heated 2 x SDS sample buffer. Cell lysates were resolved on 4-12% tris-glycine gels (Life Technologies) before transferring onto nitrocellulose membranes (Bio-Rad). For protein detection following antibodies were used: anti-Mllt3 (A300 595A-Bethyl), anti-Meis1 (ab19867-abcam) anti-Setbp1 (16841-1AP, Proteintech), anti-Hoxa9 (07-178, Millipore) and  $\beta$ -actin (MAB1501R, Millipore). Secondary antibodies used include goat anti-rabbit (SC-2004, Santa Cruz) and anti-mouse IgG-HRP (a-9044, Sigma Aldrich). Protein bands were visualized by

incubation with SuperSignal West chemiluminescent substrate (Pierce) and quantified using Quantity One data analysis software (Bio-Rad).

### Real-time RT-PCR

For real-time RT-PCR, total RNA was extracted from cells using RNAeasy Plus mini kit (QIAGEN). Oligo-dT-primed cDNA samples were prepared using Superscript III (Invitrogen), and real-time PCR analysis was performed in triplicates using SYBR green detection reagents (Invitrogen) on a 7500 real time PCR system (Applied Biosystems). Relative changes in expression of *Hoxa9*, *Mllt3* and *Meis1* were calculated according to the  $\Delta\Delta C_t$  method. The cycling conditions are 50°C for 2 minutes, followed by 95°C for 2 minutes, and then 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. Gene-specific primer sequences are:

<i>Hoxa9</i>	S 5' TGT CTC CTC TCC CCC AAA CC 3' AS 5' GAG ATG AGG CCT GGG ATTTAG A 3'
<i>Mllt3</i>	S1 5' TCCCAGACCAGGCAACAAG 3' AS1 5' TCGTTGTCATCAGAATGCAGATC3' S5 5' AACATTTCAGCACTTTGTGGAGAAA 3' AS5 5' TTTTGGCCTAGGGAAGCTTT 3'
<i>Meis1</i>	S 5'GCAAAGTATGCCAGGGGAGTA 3' AS 5'TCCTGTGTTAAGAACCGAGGG 3'
<i>Rps29</i>	S 5' CAA CCG CCA CGG TCT GA 3' AS 5'GCA CTG GCG GCA CA TGT 3'

## RESULTS

### **Mllt3 is a common viral insertion site in *Setbp1*-induced leukemias**

To identify genes that cooperate with *Setbp1* in inducing myeloid leukemia development, we cloned and sequenced 26 integrations from 16 *Setbp1*-induced leukemias by

splinkerette PCR (113). The integration site sequences were searched against the mouse genome sequence through BLAT on UCSC genome browser to determine their genomic locations (Table1). Interestingly, 2 independent leukemias (BL6 and BL20) had insertions at *Mllt3* (or *Af9*) gene at two different positions (Fig.1a). In BL6 the insertion was found 0.125kb upstream of the promoter of the *Mllt3* gene in the same direction as the transcription of *Mllt3*, while in BL20 the insertion was present in intron 4 in the opposite direction.

It is known that proviral integrations frequently cause activation of nearby genes due to the strong promoter and enhancer activity of LTR (112). By Real-time RT-PCR, both BL6 and BL20 leukemias had significantly higher expression of *Mllt3* mRNA levels compared to normal BM and other *Setbp1*-induced leukemias, which did not bear any integrations at *Mllt3*, suggesting that both viral integrations activate *Mllt3* transcription (Fig.1b). Given that retrovirus integrate into the host genome in a largely random fashion, frequent identification of insertions at *Mllt3* in *Setbp1*-induced leukemias further suggests that *Mllt3* may cooperate with *Setbp1* to induce myeloid leukemia development.

### **Overexpression of *Mllt3* and *Setbp1* increases the colony forming potential of LSK cells**

To test the possibility that *Mllt3* and *Setbp1* may cooperate in leukemia induction, serial replating assay was performed with Lin<sup>-</sup>Sca1<sup>+</sup>Kit<sup>+</sup> (LSK) cells. LSK cells were sorted and infected with *pMys-Setbp1-IRES-GFP* and *PMSCV-Mllt3-puro* together or only *pMys-Setbp1-IRES-GFP* or *PMSCV-Mllt3-puro*. *Mllt3* positive cells were selected using puromycin, whereas *Setbp1* cells were sorted for GFP<sup>+</sup>. 3000 cells from each group were

plated in methyl cellulose with cytokines SCF, IL3 and IL6 for the formation of colonies (Fig.2a). In the primary colony assay no significant difference was observed in the number of colonies between individually transduced LSK cells and cells co-transduced with *Mllt3* and *Setbp1*. In contrast, in secondary and tertiary plating there was a 50% increase in the number of colonies with cells overexpressing both *Mllt3* and *Setbp1* in comparison to LSK expressing only *Setbp1*(Fig.2b). Very few colonies were observed in secondary and tertiary replating of cells infected with *Mllt3* virus alone (Fig.2b). These data suggest that overexpression of *Mllt3* may cooperate with *Setbp1* to promote cell proliferation *in vitro*.

### **Overexpression of *Mllt3* accelerates leukemia induced by *Setbp1***

To test whether cooperation between *Mllt3* and *Setbp1* in leukemia induction also exists *in vivo*, we co-transduced 5-FU-treated bone marrow progenitors with retroviruses expressing *Mllt3* and *Setbp1* and compared their leukemia inducing capability to that of cells singly transduced with either virus in lethally irradiated B6-*Ly5.2* mice (Fig.3a). To maintain similar exposure of virus in double transduction experiments, half the viral titer was used for each virus. 100% of the mice transplanted with double-infected cells developed leukemia in 92 days than mice that received cells infected with *pMys-Setbp1-IRES-GFP* (Fig.3b). Cytospin of cells from bone marrow and spleen of sick animals of co-transduction group displayed blast like morphology and infiltration of myeloid cells occurred in non-hematopoietic tissues like spleen, lungs and liver (Fig.3c&d), suggesting development of myeloid leukemia. Flow cytometry analysis revealed that 83% of the BM cells were GFP positive and almost all the cells expressed the myeloid marker-Mac1

confirming the development of myeloid leukemia (Fig.3e). In contrast, only 2 mice that received cells infected with *pMys-Setbp1-IRES-GFP* and none of the mice from the *Mllt3* alone cohort developed myeloid leukemia. These results show that *Mllt3* does not induce leukemia on its own, but can accelerate myeloid leukemia development induced by *Setbp1*.

The acceleration of leukemia in double transduction experiment confirms that *Mllt3* and *Setbp1* cooperate to induce leukemia, but it does not indicate whether the cooperation is cell autonomous or non-cell autonomous. If the cooperation is cell autonomous, then both the genes should be present in the same leukemia cell. If non-cell autonomous, the cooperation would have resulted from *Mllt3* and *Setbp1* being overexpressed in two different populations of cells. To verify this, individual colonies developed from single leukemic cells generated on methylcellulose were examined for the presence of GFP and the puromycin resistance gene in the genomic DNA. Both genes can be detected by PCR in the genomic DNA extracted from all the colonies, suggesting that *Setbp1* and *Mllt3* cooperate in a cell autonomous manner to induce myeloid leukemia development (Fig.3f).

#### **Activation of *Meis1* in *Mllt3* -*Setbp1* leukemia**

We reported previously that overexpression of *Setbp1* causes activation of *Hoxa9* and *Hoxa10* in BM progenitors (88). *Hoxa9* is known to cooperate with *Meis1* to induce leukemia development (65). To test the possibility that *Meis1* may be activated by *Mllt3* overexpression (Fig.4a), we performed real-time RT-PCR to examine *Meis1* expression in leukemias developed from co-transduction of *Mllt3* and *Setbp1* in comparison to

*Setbp1*-induced leukemia with integrations other than *Mllt3*. *Meis1* mRNA was barely detected in leukemias induced by *Setbp1* alone, whereas it is expressed at high levels in *Mllt3-Setbp1* leukemia cells (Fig.4a). Significantly higher levels of *Meis1* protein was also detected in the co-transduction induced leukemias than *Setbp1* alone induced leukemia. *Hoxa9* mRNA and protein were also detected at higher levels in the co-transduced leukemia. These results support that activation of *Meis1* and probably increased expression of *Hoxa9* are likely responsible for cooperation between *Mllt3* and *Setbp1* in leukemia induction.

## DISCUSSION

Previously, we showed that overexpression of *Setbp1* in bone marrow progenitors through retroviral transduction is capable of inducing myeloid leukemia development when transplanted in mice. However, it is likely that *Setbp1* alone is insufficient to induce leukemia, as in 10 months only 50% of the transplanted mice could develop leukemia, suggesting that additional cooperating mutations are required for complete transformation. Similar observations have been reported with the *Hoxa9*, *HOXA10* genes (108; 109) and the fusion genes *CALM-AF10*, *NUP98-HOXD13* and *NUP98-HOXA9* (19; 84; 105). This is consistent with the fact that like other cancers, acute myeloid leukemia (AML) is also a consequence of multiple mutations (49). Therefore, we sought to identify the mutations that might cooperate with *Setbp1* in leukemic transformation, by cloning the retroviral integrations in the *Setbp1*-induced leukemias.

We identified 26 integrations from 16 *Setbp1*-induced leukemias. Most of the genes identified are novel mutations and never have been implicated in leukemia or any other cancer. Integrations identified at some of the genes, *Arhgef2*, *IL6*, *Bcl9l* and *Mllt3*, have been implicated in different cancers (30; 36; 45; 78). They are members of signaling pathways which are involved in cell proliferation, apoptosis, differentiation and migration. Evidence shows that *IL6* is deregulated in AML patients and high level of IL6 represents an unfavorable prognosis (103). Apart from this, integration was found in genes involved in splicing, vacuolar trafficking, cytoskeleton and membrane protein. An association of the vacuolar protein sorting family of proteins has been shown in cancer. One of the integrations, *Dnm2* (Dynamin) a GTPase, involved in membrane trafficking, has been reported as recurrent mutation in early T-cell precursor acute lymphoblastic leukemia (45; 86). Loss of function mutation in *Vps37b* is found in gastric and colorectal cancer (21).

Interestingly, 2 independent leukemias (BL6 and BL20) had integration in the *Mllt3* gene. Real-time RT-PCR analysis demonstrated that insertion at *Mllt3* caused its activation, suggesting that *Mllt3* might cooperate with *Setbp1* in inducing leukemia development. MLLT3, also known as AF9, is a homologue of MLLT1 and regulates erythrocyte/megakaryocytes lineage decision (94). During embryogenesis, it is required for controlling embryo patterning. *Af9* knockout mice are perinatal lethal and has no effect on hematopoiesis (24). It was first identified as a fusion protein with mixed lineage leukemia, MLL, in AML as MLL-AF9 (55) and was later shown to generate myeloproliferative disorder, phenotypically similar to human leukemia, when expressed in granulocyte macrophage progenitors (GMPs) in mice (64). This suggests that *Mllt3*

activation could be a potential cooperating mutation for *Setbp1*. To test this hypothesis, serial replating assay was done using LSK cells, either co-transduced with *Mllt3* and *Setbp1* or singly transduced with either virus. Co-expression of *Mllt3* with *Setbp1* increased the colony forming potential of LSK in comparison to cells infected with only *Setbp1* or *Mllt3* virus. The colonies of LSK cells overexpressing *Mllt3* were smaller in size and differentiated in comparison to *Setbp1* transduced or double transduced. This is in line with the previous report, where forced expression of *MLLT3* in total CD34<sup>+</sup> cells resulted in colonies smaller and differentiated than controls (94). Next, *in vivo* study was done to test the leukemia induction potential of co-transduced cells in comparison with singly transduced cells. All the mice transplanted with BM cells expressing *Mllt3* and *Setbp1* fell sick with myeloid leukemia in 92 days. However, only 25% of the mice succumbed to leukemia in *Setbp1* cohort within 8 months, whereas all of the mice remained healthy in *Mllt3* alone. Deregulated expression of *Mllt3* with *Setbp1* accelerated the development of *Setbp1*-induced leukemia.

*Hoxa9* and *Hoxa10* is a direct transcriptional target of *Setbp1* and are activated in *Setbp1*-immortalized myeloid progenitors and in the leukemic cells of *Setbp1*-induced leukemia (88). Thus, *Setbp1* promotes self-renewal through activation of oncogenes, *Hoxa9* and *Hoxa10*. In leukemia induced by co-transduction of *Mllt3* and *Setbp1*, *Hoxa9* expression is enhanced in comparison to *Setbp1*-induced leukemia. Besides, *Meis1* is significantly expressed at higher levels in co-transduction induced leukemia than *Setbp1* alone induced leukemia. *Hoxa9* is known to cooperate with *Meis1* in leukemic transformation (65). It could be possible that *Mllt3* accelerates *Setbp1*-induced leukemia by activating *Meis1*. *Hoxa9* and *Meis1* are the downstream targets of MLL rearranged leukemias and are



required for the transformation and survival of these leukemias (40; 56; 64; 120). It is well established that activation of *HOXA9* and *MEIS1* occurs in MLL-AF9 leukemia. Meis1 is essential for maintaining the stem like features in MLL-AF9 leukemia (66). MLLT3 was earlier shown as a component of ENL associated protein complex (EAP), a transcription elongation complex, which also contains DOT1L and p-TEFb (7; 80; 81). Later it was also identified in another complex, Supra Elongation Complex, with DOT1L, POLII elongation factors, components of p-TEFb kinase and other translocation partners of MLL(4). DOT1L is the only H3K79 methyl transferases and is essential for maintenance of *HOXA9* and *MEIS1* transcription in MLL-AF9 leukemia (87). It might be possible that, when *Mllt3* is overexpressed with *Setbp1*, Mllt3 initiates and maintains the expression of *Hoxa9* and *Meis1* through recruitment of transcription elongation complex at the respective locus and accelerates the development of *Setbp1*-induced leukemia. Thus, our study indicates that *Mllt3* cooperate with *Setbp1* in the development of myeloid leukemia through activation of *Hoxa9* and *Meis1*. Future studies will focus on unraveling any interaction between Mllt3 and Setbp1 in leukemic transformation and identifying *MLLT3* mutation(s) in human leukemias.

## FIGURE LEGENDS.

### Figure1. Viral integration activates *Mllt3* expression.

(a) *pMys-Setbp1-IRES-GFP* integration at *Mllt3* in BL6 (top panel) and BL20 (bottom panel) leukemia. Exon-coding regions are highlighted in yellow and noncoding exons in gray. Arrows indicate transcription start sites. The location and orientation of viral integrations are depicted by red triangles. (b) Real-time RT-PCR analysis of *Mllt3*

expression in BM and spleen cells of leukemic mice with *Mllt3* integration (BL6 and BL20) and other *Setbp1*-induced leukemias (BL2, BL3, BL4, BL7, and BL12) in comparison to wild type BM. Relative expression levels were calculated by normalizing to *Rps29* mRNA levels in the same sample and to WT BM.

**Figure2. Co-transduction of *Mllt3* and *Setbp1* increases the colony forming potential of LSK cells.**

(a) Schematic diagram of colony formation assay. (b) Mean and SD of colony formation potential of *PMSCV-Mllt3-puro*-infected, *pMys-Setbp1-IRES-GFP*-infected or double-infected LSK cells in serial replating assay. (\*\*\*)  $P < 0.001$ , \*\*\*\*\*)  $P < 0.0001$ )

**Figure3. Deregulated *Mllt3* expression accelerates the development of *Setbp1*-induced leukemia.**

(a) Schematic diagram of bone marrow transduction transplantation assay. (b) Survival curves of irradiated C57BL/6-*Ly5.2* mice receiving *pMys-Setbp1-IRES-GFP*-infected, *PMSCV-Mllt3-puro*-infected or double infected bone marrow cells. (c) Cytospin of Spleen (SP) and bone marrow (BM) cells from *Mllt3* and *Setbp1*-induced leukemic mice; MS1, MS2 and MS3. (d) H&E staining showing infiltration of myeloid blasts in liver, lung and spleen of *Mllt3* and *Setbp1*-induced leukemic mice; MS1, MS2 and MS3. (e) FACS analysis of lineage specific markers on bone marrow cells of *Mllt3* and *Setbp1*-induced leukemic mouse. (f) Individual colonies cultured from BM cells of leukemic mice carry both *Mllt3* and *Setbp1* gene. PCR products using primers specific to GFP and puromycin resolved on ethidium bromide gel.

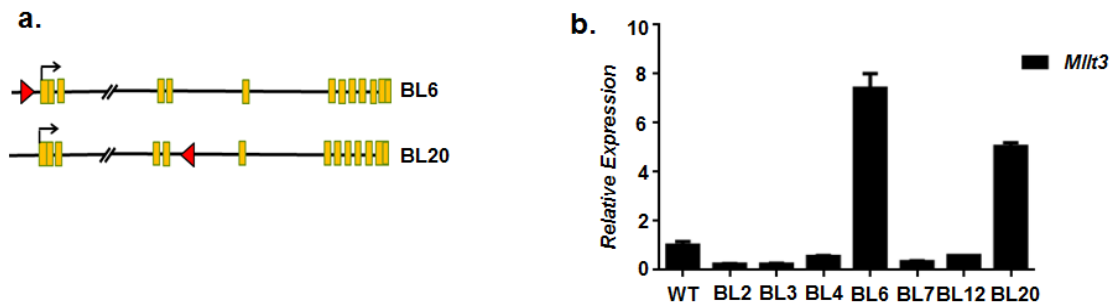
**Figure4. Overexpression of *Mllt3* with *Setbp1* activates *Meis1* expression**

**(a)** Real-time RT-PCR analysis of total RNA extracted from bone marrow of wild-type (WT BM) and spleen cells of *Mllt3-Setbp1* (MS1, MS2, MS3, MS4, and MS6) and *Setbp1* (BL3, BL4, and BL12) -induced leukemic mice using primers specific for *Mllt3*, *Hoxa9* and *Meis1*. Relative expression levels were calculated by normalizing to *Rps29* mRNA levels in the same sample and in WT BM. **(b)** Western blotting analysis of *Hoxa9*, *Meis1*, *Mllt3*, *Setbp1* and beta actin protein level in *Mllt3-Setbp1* and *Setbp1*-induced leukemias.

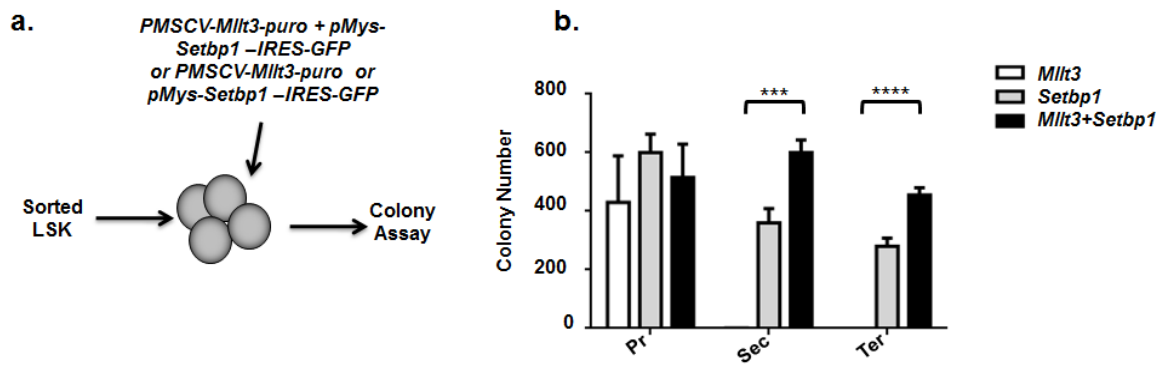
**Table 1- Viral integrations at genes identified in *pMYs-Setbp1* virus induced leukemias**

Leukemia No; Gene	Protein Family	Location and Distance	Orientation	Mouse Chromosome	Human Chromosome	Accession No.	Latency (Days)
BL1 <i>Vps37b</i>	Protein Transport	Intron 1	Inverse	5	12	NM_177876	174
BL2 <i>Rbm8a</i>	RNA Binding	Exon 2	Inverse	3	1	NM_025875	96
BL3 <i>Arhgef2</i>	Dbl Family Rho activator	Intron 1	Same	3	1	NM_001198913	55
BL4 <i>Sh3kbp1</i>	Enzyme	Intron 10	Same	X	X	NM_021389	216
BL5 <i>Lman2</i>	Type1 Membrane Protein	Intron 2	Inverse	13	5	NM_025828	147
<i>IL6</i>	Cytokine	5', 8.4 Kb	Same	5	7	NM_031168	
BL6 <i>Mlt3</i>	Component of Supra Elongation Complex	5', 0.125Kb	Same	4	9	NM_027326	140
<i>Pold4</i>	Enzyme	5', 4.9 Kb	Inverse	19	11	NM_027196	
<i>Cclf1</i>	Cytokine	3', 12.5 Kb	Inverse	19	11	NM_019952	
<i>Foxo3</i>	Transcription Factor	3', 129 Kb	Same	10	6	NM_019740	
<i>Armc2</i>	Armadillo Family Protein	5', 37.9 Kb	Same	10	6	NM_001034858	
BL7 <i>Jmjd1c</i>	Histone Demethylase	5', 14.0 Kb	Inverse	10	10	NM_207221	297
<i>Reep3</i>	Membrane Protein	5', 16.2 Kb	Same	10	10	NM_001204915	
BL8 <i>Hivep3</i>	Transcription Factor	Intron 2	Same	4	1	NM_010657	248
BL9 <i>Ctif</i>	Translation Initiation Factor	Intron7	Same	18	18	NM_201354	249
<i>Arhgdib</i>	Rho Protein Family	Intron1	Same	6	12	NM_007486	
BL11 <i>Mbd3</i>	Subunit of NuRD Complex	Intron1	Same	10	19	NM_013595	241
BL12 <i>Sntb2</i>	Peripheral Membrane Protein	Intron1	Inverse	8	16	NM_009229	136
BL13 <i>Dnm2</i>	GTP Binding Protein	Intron1	Same	9	19	NM_001253894	139
<i>Samsn1</i>	Scaffold Protein	Intron1	Same	21	16	NM_023380	
BL14 <i>Cxxc5</i>	Zinc Finger Protein	3'17.8 Kb	Same	18	5	NM_133687	144
<i>Psd2</i>	Enzyme	5' 85.2 Kb	Same	18	5	NM_028707	
BL17 <i>Cstad</i>	Mitochondrial Membrane Protein	3', 49.7 Kb	Inverse	2	ND	NM_030137	231
<i>Ak138941</i>	ND	3', 54.3 Kb	Same	2	9	Ak138941	
BL19 <i>Bc/9l</i>	Beta –catenin Binding Protein	Intron1	Inverse	9	11	NM_030256	168
BL20 <i>Mlt3</i>	Component of Supra Elongation Complex	Intron4	Inverse	4	9	NM_027326	148

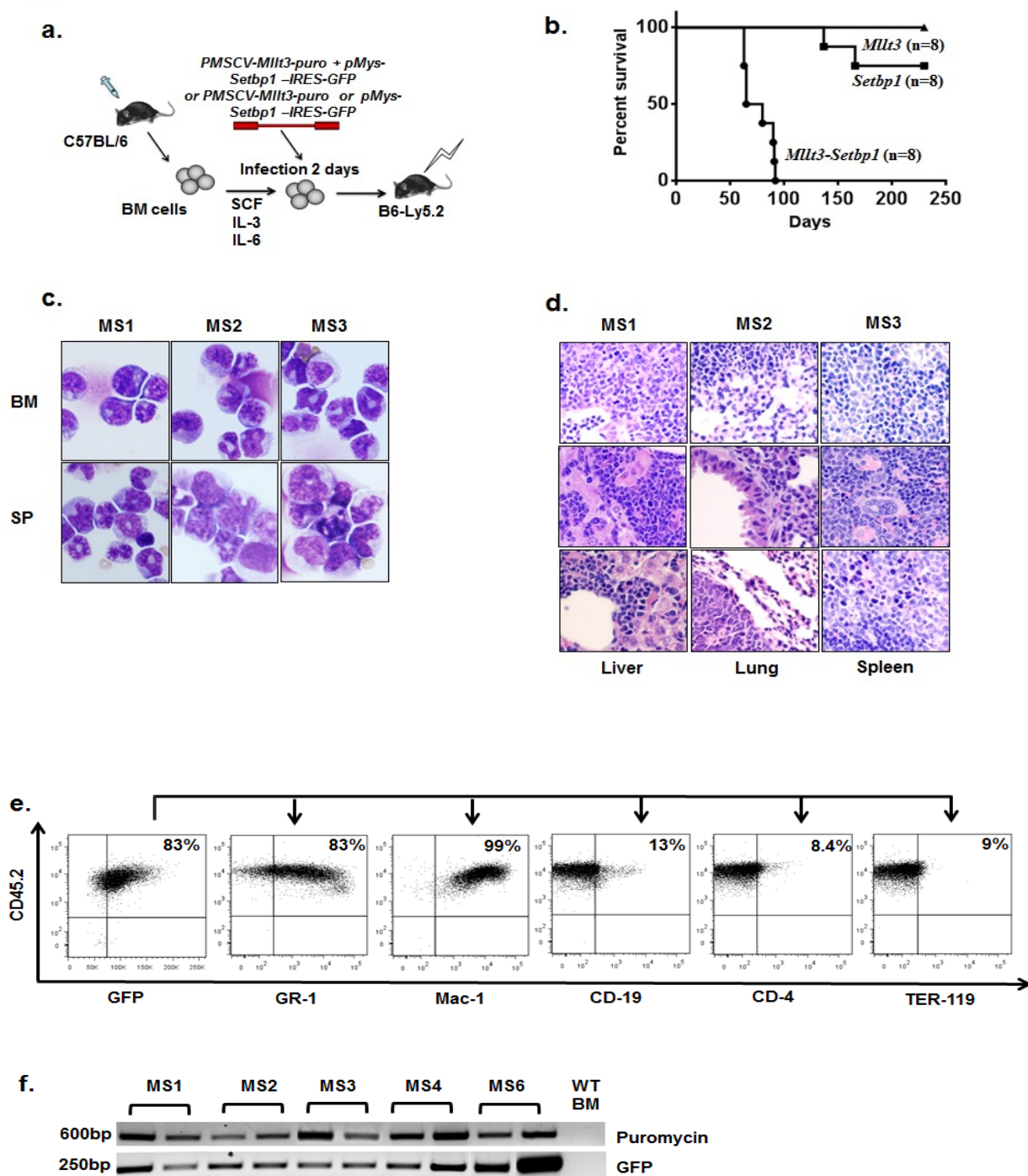
**Fig. 1**



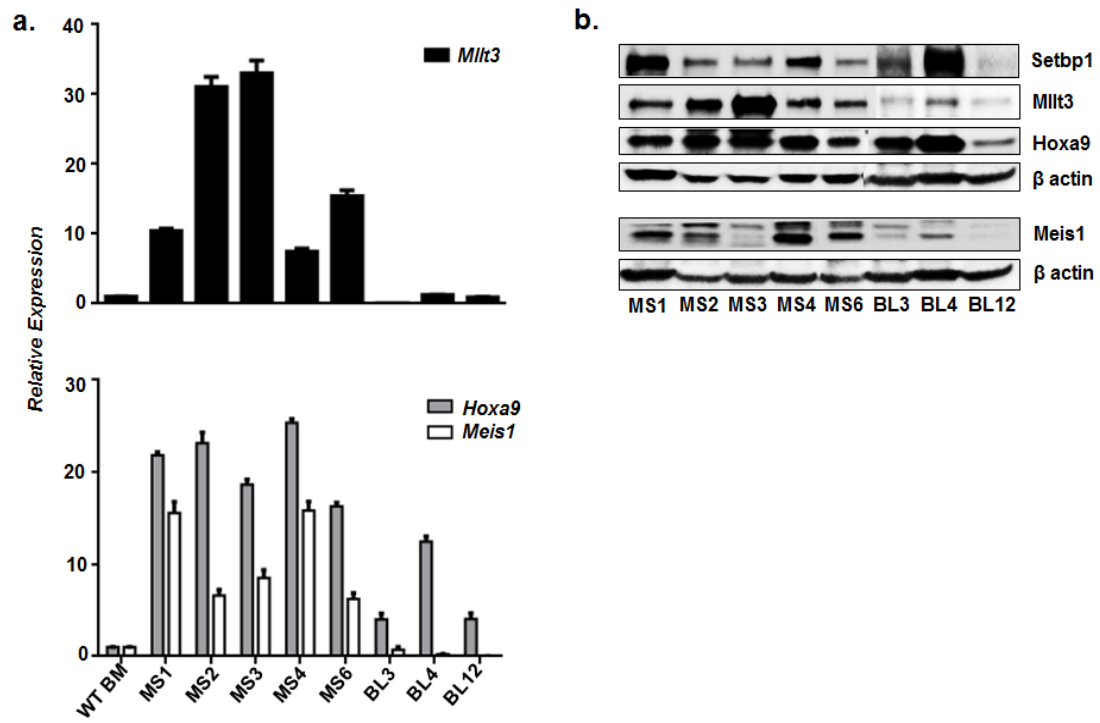
**Fig. 2**



**Fig. 3**



**Fig. 4**



## SUMMARY

Our studies demonstrate that *Setbp1* is a novel oncogene capable of inducing myeloid leukemia development. Retrovirally directed expression of *Setbp1* in murine bone marrow progenitors induces myeloid leukemia development in mice on transplant. Evidence shows that most likely mutations in *SETBP1* occurs after the initial establishment of the disease and SETBP1 contribute to its progression (12; 72; 92; 111). However, our studies indicate that deregulation of *Setbp1* through mutation or overexpression could be a driver mutation during leukemia development. Overexpression of *Setbp1* induces myeloid leukemia as cytopsin of BM and spleen cells from moribund mice displayed myeloid blast like morphology, infiltration of myeloid cells were observed in non-hematopoietic tissues and significantly more infected cells expressed myeloid marker, Gr-1. We found that in the pre-leukemic stage, overexpression of *Setbp1* promotes the expansion of hematopoietic stem and progenitor cells, as the engraftment of *Setbp1* expressing cells increased gradually overtime, while there was a gradual decline of cells infected with empty virus. The gradual increase in the engraftment of the *Setbp1* expressing cells suggests that *Setbp1* expression may promote self-renewal of HSCs. Overexpression of *Setbp1* in hematopoietic stem cells bias their lineage commitment to the myeloid pathway, as we observed expansion of GMP population in mice transplanted with cells overexpressing *Setbp1* in comparison to control group.

Besides activation of oncogenes, suppression of tumor suppressor genes is also essential for leukemic transformations. *Setbp1*, an AT-hook transcription factor, not only activates



proto-oncogenes *Hoxa9* and *Hoxa10* (88), but we found it suppress the expression of tumor suppressor gene *Runx1*. Analysis of human AML revealed that the expression of *RUNX1* is inversely related to *SETBP1* expression. A similar result was observed on overexpression of *Setbp1* in myeloid progenitors. *Setbp1* overexpression inhibited the expression of *Runx1*. Suppression of *Runx1* is critical to *Setbp1*-induced transformation, as the colony forming potential of *Setbp1*-induced leukemic cell line, BL3 and BL12, decreased on ectopic expression of *Runx1*. Loss of *Runx1* is associated with increased HSC self-renewal and GMP expansion (43; 54; 57). CHIP analysis revealed that *Setbp1* binds to *Runx1* promoter and directly regulates its expression. In search of potential epigenetic changes induced by *Setbp1* for the repression of *Runx1*, we found a significant increase in histone H3 acetylation at *Runx1* promoters after *Setbp1* knockdown in cells immortalized by FLAG-tagged *Setbp1*, suggesting that *Setbp1* may repress *Runx1* transcription by preventing histone H3 acetylation at its promoters. Histone deacetylases inhibit transcription by deacetylating histones (99). CHIP data confirmed binding of *Hdac1* at *Runx1* promoter. This binding is also critical for *Runx1* repression, as *Hdac1* knockdown in these cells significantly increased *Runx1* mRNA levels. Moreover, significant reductions in *Hdac1* binding to the *Runx1* promoters were detected after *Setbp1* knockdown in the same cells. These results suggest that *Setbp1* recruits *Hdac1* to the *Runx1* promoters causing histone H3 deacetylation and subsequent transcriptional repression of *Runx1*.

Given that *Runx1* repression by *Setbp1*-mediated *Hdac1* recruitment is required for efficient colony formation by *Setbp1*-induced leukemia cells, we explored the therapeutic potential of HDAC inhibitors for treating leukemias induced by *Setbp1* activation. HDAC

inhibitors, Entinostat and Vorinostat promoted differentiation of *Setbp1*-induced leukemic cell line BL3 and BL12. Treatment of BL3 and BL12 cells with HDAC inhibitor upregulated the expression of *Runx1*. The differentiation observed in HDAC inhibitor treated leukemic cell line suggest that it could be through increased expression of *Runx1*, as studies have shown that *RUNX1* is involved in megakaryocytic and lymphocytic differentiation(54). Chip analysis demonstrated that Setbp1 recruit Hdac1 at the *Runx1* promoter as knockdown of Setbp1 decreased the binding of Setbp1 at the promoter of *Runx1*. This also increased H3 acetylation of the *Runx1* promoter. This result thus confirmed that Setbp1 is responsible for recruitment of Hdac1 at *Runx1* promoter and is involved in epigenetic modification of chromatin.

Interestingly, HDAC inhibitor, Entinostat significantly enhanced the survival of *Setbp1*-induced leukemic mice, suggesting that HDAC inhibition could be a potential therapeutic strategy for leukemia developed by *Setbp1* overexpression. Vorinostat and Entinostat both were effective in *in vitro* studies, but in *in vivo* Entinostat had pronounced effect. Entinostat is better retained in the body because it has longer half- life in comparison to Vorinostat and so is more effective.

Though overexpression of *Setbp1* was capable of inducing myeloid leukemia in mice, but only fifty percent of the transplanted mice fell sick in 10 months, while secondary recipients of spleen cells from leukemic mice developed the same disease with much shorter latency, suggesting that additional mutations may be required for *Setbp1*-induced leukemic transformations. So, we cloned and sequenced 26 integrations from 16 *Setbp1*-induced leukemias, to identify mutations that might have cooperated in the development of AML. Integrations identified are members of signaling pathways involved in cell

proliferation, apoptosis, differentiation and migration. Apart from this, integration was found in genes involved in splicing, vacuolar trafficking, cytoskeleton and membrane protein. Integrations at some of the genes *Arhgef2*, *Bcl9l*, *Il6* and *Mllt3* have been implicated in cancer. A common insertion site was found at *Mllt3* gene, as two independent leukemias, BL6 and BL20, had insertion in *Mllt3*. We selected to study the cooperation of *Mllt3* with *Setbp1*, as two independent leukemias had insertion in *Mllt3* and it has been associated with AML as fusion protein, MLL-AF9. Besides, integration caused activation of *Mllt3*, suggesting that *Mllt3* could cooperate with *Setbp1* in inducing leukemia. *Arhgef2* and *Rbm8a* are also potential candidates to study cooperation with *Setbp1*, as the latency of the leukemias was very short. Beta-catenin binding protein, *Bcl9l* is associated with the canonical Wnt signaling pathway, which has been implicated in cancer and leukemia. Integration in *Bcl9l* also induced leukemia with latency of 168 days, suggesting that it could also be a potential cooperating partner with *Setbp1* in leukemia development.

Co transduction of *Mllt3* and *Setbp1* enhanced the colony forming potential of LSK cells compared to only *Setbp1* expressing LSK cells. The colonies of LSK cells overexpressing *Mllt3* were smaller in size and differentiated in comparison to *Setbp1* transduced or double transduced. This is in congruence with previous study where forced expression of MLLT3 in total CD34<sup>+</sup> cells resulted in colonies smaller and differentiated than controls. *In vivo* study confirmed the cooperation, as co-transduction of *Mllt3* and *Setbp1* accelerated the development of *Setbp1*-induced myeloid leukemia. Moreover, we also found that co-transduction induced leukemia cells expressed significantly higher level of *Meis1* compared to leukemia induced by *Setbp1* alone. *Hoxa9* cooperates with *Meis1* in

leukemic transformations. Our finding thus suggests that Mllt3 accelerates *Setbp1*-induced leukemia through activation of *Hoxa9* and *Meis1*.

It remained to be addressed any protein-protein interaction between Setbp1 and Mllt3 in regulating *Hoxa9* and *Meis1* expression. Besides, future study would focus on identifying crosstalk between the signaling pathway of Mllt3 and Setbp1. Mllt3 has always been implicated as a fusion protein with MLL in leukemia. The direct role played by Mllt3 in inducing myeloid leukemia is unknown. So, identifying Mllt3 mutation(s) in leukemia with *Setbp1* mutation and other leukemia would be a promising area to study.

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