Role of \textit{Setbp1} in Myeloid Leukemia Development

by

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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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ABSTRACT

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

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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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<tr>
<td>5-FU</td>
<td>5-fluorouracil</td>
</tr>
<tr>
<td>ALL</td>
<td>Acute Lymphoblastic Leukemia</td>
</tr>
<tr>
<td>AML</td>
<td>Acute Myeloid leukemia</td>
</tr>
<tr>
<td>AUL</td>
<td>Acute Undifferentiated Leukemia</td>
</tr>
<tr>
<td>BM</td>
<td>Bone marrow</td>
</tr>
<tr>
<td>CGD</td>
<td>Chronic Granulomatous Disease</td>
</tr>
<tr>
<td>CHIP</td>
<td>Chromatin Immunoprecipitation</td>
</tr>
<tr>
<td>CML</td>
<td>Chronic Myeloid Leukemia</td>
</tr>
<tr>
<td>CMML</td>
<td>Chronic Myelomonocytic leukemia</td>
</tr>
<tr>
<td>CSCs</td>
<td>Cancer Stem Cells</td>
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<tr>
<td>DOT1L</td>
<td>DOT1-like histone H3K79 ethyltransferase</td>
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<td>FACS</td>
<td>Fluorescence – Activated Cell Sorting</td>
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<td>GSK3</td>
<td>Glycogen Synthase Kinase 3 beta</td>
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<td>HDAC</td>
<td>Histone Deacetylases</td>
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<tr>
<td>Hoxa10</td>
<td>Homeobox protein Hox-A10</td>
</tr>
<tr>
<td>Hoxa9</td>
<td>Homeobox Protein Hox-A9</td>
</tr>
<tr>
<td>HSCs</td>
<td>Hematopoietic Stem Cells</td>
</tr>
<tr>
<td>IL11</td>
<td>Interleukin 11</td>
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<tr>
<td>IL3</td>
<td>Interleukin 3</td>
</tr>
<tr>
<td>IL6</td>
<td>Interleukin 6</td>
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<tr>
<td>JMML</td>
<td>Juvenile Myelomonocytic leukemia</td>
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<td>LICs</td>
<td>Leukemia Initiating Cells</td>
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<tr>
<td>LSCs</td>
<td>Leukemic Stem Cells</td>
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<tr>
<td>LT-HSC</td>
<td>Long Term HSC</td>
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<tr>
<td>LTR</td>
<td>Long Terminal Repeat</td>
</tr>
<tr>
<td>MDR1</td>
<td>Multi Drug Resistance 1</td>
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<td>MDS</td>
<td>Myeloid Dysplastic Syndrome</td>
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<td>Meis1</td>
<td>Myeloid Ecotropic Viral Integration Site 1</td>
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<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>MLL</td>
<td>Mixed lineage leukemia</td>
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<td>MPN</td>
<td>Myeloproliferative Neoplasm</td>
</tr>
<tr>
<td>MSCV</td>
<td>Murine Stem Cell Virus</td>
</tr>
<tr>
<td>NOD/SCID</td>
<td>Non Obese Diabetic/Severe Combined Immunodeficient</td>
</tr>
<tr>
<td>PCG</td>
<td>Polycomb group of genes</td>
</tr>
<tr>
<td>PP2A</td>
<td>Protein Phosphatase Type 2A</td>
</tr>
<tr>
<td>SCF</td>
<td>Stem Cell Factor</td>
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<tr>
<td>SET</td>
<td>SET nuclear proto-oncogene</td>
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<td>Setbp1</td>
<td>SET binding protein 1</td>
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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⁺ CD38⁻, 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⁻CD34⁺CD38⁻Thy⁺ whereas LSCs were Lin⁻CD34⁺CD38⁻“Thy⁻” signifying that subsequent mutation might have occurred in Thy1⁻ progeny of HSCs or could have lost the expression of Thy1. A similar report was published, where LSCs of AML are Thy1⁻ (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 is 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-RARα 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-/- 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/β-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). β-catenin, the downstream molecule of Wnt signaling pathway has been shown to be necessary for HSC development as β-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. β-catenin is required for the maintenance of self-renewal in LSCs. Activation of β-catenin has been shown to occur in CML-blast crisis LSCs (59). This hyper activation of β-catenin is due to aberrant splicing of glycogen synthase kinase 3 beta (GSK3), an inhibitor of β-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-HOXD13 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-XI 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.
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 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

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**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 (JML)(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 cytospin 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-Scal-ec-kit+ (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+ 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

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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). Cytopsin 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/RARa,* 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-fluourouracil (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-IRE$S$-GFP) or GFP only ($pMYs$-IRE$S$-GFP) on retronectin coated plates.

For transplantation, 0.7-1.3 x 10$^6$ transduced BM cells were injected into the tail vein of each lethally irradiated (1100 rads from $^{137}$Cs source) B6-Ly5.2 mouse along with 7.5 x 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 x 10$^6$ spleen cells from primary recipients with leukemia were injected into lethally irradiated secondary recipients along with 7.5 x 10$^5$ supporting bone marrow cells.

For serial transplantation of LSK cells, 1x10$^5$ LSK cells transduced twice with $pMYs$-Setbp1-IRE$S$-GFP or $pMYs$-IRE$S$-GFP virus were first transplanted into each lethally irradiated primary B6-Ly5.2 recipient. At 4 months after primary transplantation, 5 x10$^2$ GFP$^+$ 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-FcγR-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⁻ 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.

The GMP (IL-7Rα⁻ Sca-1⁻c-Kit⁺FcγR-II/III⁺CD34⁺) population in bone marrow was analyzed at 3 months after transplantation using 5-FU treated cells. Lin⁻ 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 10⁵ 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 1μM 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, 2x10⁴ BL3 and BL12 cells were plated in IMDM methylcellulose medium supplemented with 20% horse serum, mouse SCF (50ng/ml), IL-3 (10ng/ml) and 1μM 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 x 10⁵ cells/ml and treated with 1 μM 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 (1x10⁶ 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 μl of DMSO and 180 μl 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 x10⁴ 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 7 days.

**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 β-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:

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**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:
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. ^32^P-labeled GFP-specific probe was synthesized by random primer labeling using the Prime-IT II kit (Stratagene. LoJolla, 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.

Conflict of interest disclosure: The authors declare no competing financial interests.

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**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 x 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^+ cells in peripheral blood at 4^{th}, 8^{th} and 16^{th} weeks after transplantation. (b) FACS analysis of indicated lineage specific markers on GFP^+ 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^{th}, 8^{th} and 16^{th} week after transplantation. (c) Left panel, FACS analysis of GMP populations of GFP^+ 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^+ cells in peripheral blood of recipient mice at 4^{th}, 8^{th} and 16^{th}
week after primary (1 x 10⁵ cells/mouse) and secondary transplantation (5 x 10² cells/mouse) of LSK cells transduced with \(pMYs\text{-}Setbp1\text{-}IRES\text{-}GFP\) (black bars) or \(pMYs\text{-}IRES\text{-}GFP\) 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 \(pMYs\text{-}Setbp1\text{-}IRES\text{-}GFP\) or \(pMYs\text{-}IRES\text{-}GFP\) 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.

**Figure 4. 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 1µM of Entinostat or Vorinostat in comparison to DMSO treated control. Relative expression levels were calculated by normalizing to β-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 1µM 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 1µM 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 1µM 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

(a) Diagram showing the experimental setup: injection of cells into a mouse, followed by infection and transplantation.

(b) Graph showing percent survival over time.

(c) Images of spleen sections.

(d) Images of SP and BM sections.

(e) Images of liver, lung, and spleen sections.

(f) Flow cytometry analysis showing percentages of GFP, Gr-1, CD19, CD3, and Ter119.
Figure 2

a) Engraftment (%) of pMYs-IRES-GFP and pMYs-Setbp1-IRES-GFP over 4, 8, and 16 weeks.

b) Expression of Gr-1, CD19, CD3, and FcyRIII/II in pMYs-IRES-GFP and pMYs-Setbp1-IRES-GFP.

c) Flow cytometry analysis of CD34 and FcyRIII/II expression in GMP cells.

d) Engraftment (%) of pMYs-IRES-GFP and pMYs-Setbp1-IRES-GFP post 1st and 2nd transplantation over 4, 8, and 16 weeks.
Figure 3

(a) Relative expression of Runx1 in pMYs and Setbp1 KD cells.

(b) Relative expression of Runx1 and β-Actin in NC and Setbp1 KD cells.

(c) Colony formation assay showing Runx1 expression with β-Actin as a control.

(d) Genome-wide binding analysis of Runx1 at the indicated genomic loci.

(e) ChIP-qPCR analysis of Runx1 binding at the indicated loci in NC and Setbp1 KD cells.

(f) ChIP-qPCR analysis of Runx1 binding at the indicated loci in NC and Setbp1 KD cells.
Figure 4

(a) Bar graph showing relative Runx1 expression levels in BL3 and BL12 cells treated with DMSO, ENT, and VOR. 

(b) Colony formation assay results for S3, BL3, and BL12 cells treated with different compounds.

(c) Representative images of cell morphology under different treatments.

(d) Relative expression levels of Cd11b, Lyz2, and Csf1r in BL3 and BL12 cells.

(e) Cell proliferation assay results for BL3 and BL12 cells treated with DMSO, ENT, and VOR.

(f) Graphs showing cell number over time for Control (n=4) and ENT (n=4) treatments.

** indicates p < 0.05, *** indicates p < 0.01, **** indicates p < 0.001.
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. 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 cytospin of myeloid progenitor cells (DNc2 and DNc3) immortalized by mutant Setbp1 (harboring activation mutation D868N) after 48hrs of treatment with 1µM 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 1µM 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 cytospin of leukemia cells after 72hrs of treatment with 1µM of vorinostat, or control DMSO (N=3).
CHAPTER 4: Manuscript 2

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

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**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, Fredrick, 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-Mllt3-puro, pMYs-Setbp1-IRES-GFP or MSCV-Mllt3-puro on retronectin coated plates. For transplantation, 0.4-0.6 x 10^6 transduced BM cells were injected into the tail vein of each lethally irradiated (1100 rads from ^{137}Cs source) B6-Ly5.2 mouse along with 7.5 x 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-FcγR-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^- 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^. Colony numbers were counted after 7 days.

**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 β-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 $\Delta\Delta Ct$ 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:

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>AS 5’GAG ATG AGG CCT GGG ATTTAG A 3’</td>
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<tr>
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<td>AS1 5’TGGTGTATCGAATGCAGATGCAGATC3’</td>
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<td>S5 5’AACATTCAGCCTTTGTGGAGAAA 3’</td>
<td>AS5 5’TITGGCCCTAGGGAAGCTTT 3’</td>
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<tr>
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<td>AS 5’TCCGTGTTAAGAACCAGGG 3’</td>
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<tr>
<td><em>Rps29</em></td>
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**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⁻Sca1⁺Kit⁺ (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⁺. 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 overexpressed 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⁺ 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.

**Figure 2. 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)

**Figure 3. 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.
Figure 4. 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

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<th>Orientation</th>
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<th>Human Chromosome</th>
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Fig. 1

a.

b.

Fig. 2

a. PMSCV-Milt3-puro + pMys-Setbp1–IRES-GFP or PMSCV-Milt3-puro or pMys-Setbp1–IRES-GFP

b.

Colony Number

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*** ****
Fig. 3

(a) Schematic representation of the experimental setup for C57BL/6 mice. Transduction of BM cells with PMSCV-Mll3-puro + pMys-Setbp1–IRESGFP or PMSCV-Mll3-puro or pMys-Setbp1–IRESGFP, followed by infection 2 days later with SCF, IL-3, or IL-6.

(b) Percent survival of mice infected with Mll3 or Setbp1. Graph shows survival over time with different conditions.

(c) Images illustrating BM and SP populations for MS1, MS2, and MS3.

(d) Histological images of Liver, Lung, and Spleen from MS1, MS2, and MS3.

(e) Flow cytometry analysis of CD452 expression in GFP, GR-1, Mac-1, CD-19, CD-4, and TER-119 populations, showing percentages for MS1, MS2, MS3, and WT BM.

(f) Western blot analysis showing puromycin and GFP expression in MS1, MS2, MS3, MS4, MS6, and WT BM with bands at 600bp and 250bp.
**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 cytospin 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 Sebp1 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⁺ 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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