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Runx1 repression by histone deacetylation is critical for *Setbp1*-induced mouse myeloid leukemia development

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Abstract

Abnormal activation of *SETBP1* through overexpression or missense mutations is highly recurrent in various myeloid malignancies; 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* overexpression also causes transcriptional repression of critical hematopoiesis regulator gene *Runx1* and this effect is crucial for *Setbp1*-induced transformation. *Runx1* repression is induced by Setbp1-mediated recruitment of a nucleosome remodeling deacetylase (NuRD) complex to *Runx1* promoters and can be reversed by treatment with histone deacetylase (HDAC) inhibitors Entinostat and Vorinostat. Moreover, treatment with these inhibitors caused efficient differentiation of *Setbp1* activation-induced leukemia cells *in vitro*, and significantly extended the survival of mice transplanted with such leukemias, suggesting that HDAC inhibition could be an effective strategy for treating myeloid malignancies with *SETBP1* activation.

Keywords

Setbp1; Runx1; HDAC inhibitors; Myeloid leukemia

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Introduction

SETBP1 is a large nuclear protein first identified through its interaction with oncoprotein SET.[1] Growing evidence suggests its abnormal activation may play an important role in the development of multiple myeloid malignancies. Overexpression of *SETBP1* was previously found in over 27% AML patients of old age,[2] suggesting its common involvement in AML development. Increased *SETBP1* expression was also later detected in a subset of CML blast crisis patients. More recently, we and others have found highly recurrent missense mutations of *SETBP1* in patients of atypical chronic myeloid leukemia,[3] chronic myelomonocytic leukemia (CMML),[4] secondary AML,[4] chronic neutrophilic leukemia,[5, 6] and juvenile myelomonocytic leukemia (JMML),[7] which stabilize SETBP1 protein through decreasing its degradation.[3] Multiple mechanisms could contribute to the involvement of *SETBP1* in leukemia development. SETBP1 may promote inhibition of PP2A through physical interaction with SET.[2] Setbp1 can also function as an AT-hook transcription factor to activate the transcription of oncogene *Hoxa9* and *Hoxa10*.[8] We have also shown that 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.[8, 9] 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. It is also critical to identify targeted therapies for leukemias with *SETBP1* activation due to their association with poor prognosis.[2, 4]

Chromatin remodeling is a critical step for proper control of gene transcription and is dynamically regulated by recruitment of chromatin associated proteins that can be categorized into epigenetic ‘writers’, ‘erasers’, and ‘readers’.[10] Different chemical marks can be added to DNA or histones by ‘writers’ such as DNA and histone methyltransferases, removed by ‘erasers’ including histone deacetylases (HDACs) and demethylases, and bound to by ‘readers’ to directly regulate transcription. Abnormal epigenetic regulation plays an important role in leukemia development as many of these writers, erasers, and readers have been found mutated in leukemias such as MLL and EZH2 or gets recruited by leukemic fusion proteins including AML1/ETO and PML/RAR.[11–15] The presence of three AT-hook DNA-binding motifs in Setbp1 suggest that it may be involved in epigenetic regulation as proteins with such motifs are known to be important components of large chromatin-remodeling complexes.[16–18] However, this possibility has not been examined.

Here we showed that overexpression of *Setbp1* in 5-FU-treated bone marrow progenitor cells is capable of inducing myeloid leukemia development in recipient mice. Before leukemia development, increased expression of *Setbp1* dramatically enhanced self-renewal of hematopoietic stem cells (HSCs) and promoted the expansion of GMPs. We also identified a novel function of Setbp1 as a transcriptional repressor through the recruitment of the Nucleosome Remodeling Deacetylase (NuRD) complex. Through this mechanism, Setbp1 directly represses the transcription of tumor suppressor gene *Runx1*, which is critical for *Setbp1*-induced transformation. Our results further showed that treatment with class I histone deacetylase (HDAC) inhibitors can relieve *Runx1* repression by *Setbp1* and

represents a promising strategy for treating human myeloid leukemias with *SETBP1* activation.

Materials and Methods

Mice

C57BL/6 and B6-*Ly5.2* female mice (7–12 weeks old; Charles River, Frederick, MD) were maintained in the animal facility of Center for 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.

Retrovirus generation

The *pMYs-Setbp1-IRES-GFP* retroviral construct was described previously[8]. The murine *Runx1* cDNA from pcDNA3.1-Flag-Runx1FL[19](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, Indianapolis, IN). Viral titer was assessed by serial dilution and infection of NIH-3T3 cells.

Retroviral transduction and bone marrow transplantation

5-FU-treated bone marrow cells from C57BL/6 mice were extracted and expanded in culture as described.[20] These expanded BM cells were subsequently infected three times with high-titer retrovirus carrying *Setbp1* cDNA (*pMYs-Setbp1-IRES-GFP*) or GFP only (*pMYs-IRES-GFP*) on retronectin-coated plates. For transplantation, 0.7–1.3 × 10^6 transduced BM cells were injected into the tail vein of each lethally-irradiated (1100 rads from ^{137}Cs source) B6-*Ly5.2* female mouse along with 7.5 × 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 at 4, 8 and 16 weeks to analyze the short-term and long-term engraftment of the donor cells by fluorescence-activated cell sorting (FACS). For secondary transplantation, 1 × 10^6 spleen cells from primary recipients with leukemia were injected into lethally irradiated secondary recipients, along with 7.5 × 10^5 supporting bone marrow cells. See supplementary information for details on serial transplantation of LSK cells.

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 red blood cells, 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, Carlsbad, CA). For serial transplantation of LSK cells, 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 for 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 (lin$^-$IL-7Rα^- Sca-1$^-$c-Kit$^+$Fc-γR-II/III^{Hi}CD34$^+$) population in bone marrow was analyzed at 3 months after transplantation using 5-FU-treated cells. lin$^-$ cells were obtained 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.

Chromatin immunoprecipitation (ChIP)

Mouse myeloid progenitors immortalized by FLAG-tagged *Setbp1* were generated as described[8]. 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)[21], rabbit polyclonal anti-acetylated histone H3 (#39139, Active Motif)[22], rabbit polyclonal anti-Mta2 (sc-28731, Santa Cruz), rabbit polyclonal anti-Mbd3 (A302-528A, Bethyl), mouse IgG (G3A1, #5415, Cell Signaling Technologies), rabbit polyclonal anti-Chd4 (14173-1, Proteintech), and rabbit IgG (#P120-101, Bethyl Laboratories). Chromatin DNA was purified using MinElute PCR Purification Kit (QIAGEN) and quantified by real-time PCR. See See supplementary information for *Runx1* promoter-specific primer sequences.

Results

Overexpression of *Setbp1* is capable of inducing myeloid leukemia development

To examine the oncogenicity of *Setbp1* overexpression, we transduced 5-FU treated C57BL/6 mouse bone marrow progenitors with high titer pMYs retrovirus expressing *Setbp1* and marker *GFP* (*pMYs-Setbp1-IRES-GFP*) or empty virus (*pMY-IRES-GFP*) and subsequently transplanted transduced cells along with healthy supporting bone marrow cells into lethally irradiated syngeneic B6-*Ly5.2* recipient mice. Interestingly, mice receiving *Setbp1* virus infected cells began 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 (Figure 1B). In contrast, mice receiving cells infected by empty virus at higher infection efficiencies remained healthy during the same period (Figure 1B and Supplementary Figure 1). Moribund animals displayed enlarged spleens and livers (Figure 1C and data not shown). Cytospin analysis of bone marrow and spleens of these animals revealed high prevalence of immature myeloid blasts (Figure 1D), which represent 30 ± 8.2% (Mean ± SD) of total bone marrow nucleated cells. These abnormal cells also frequently infiltrated into the livers and lungs of affected animals (Figure 1E). The myeloid identity of these abnormal cells was confirmed by 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 (Figure 1F). Less than 10% of the cells are also positive for immature markers including c-kit and Sca-1(Figure 1F). These phenotypic examinations indicate the development of myeloid leukemias in the moribund animals. These leukemias are also transplantable as irradiated recipient mice receiving 1×10^6 cells from the spleens of primary leukemic mice developed the same diseases within 21 days (Figure 1B). As expected, leukemia cells expressed increased levels of *Setbp1* and its known

targets *Hoxa9* and *Hoxa10* (Supplementary Figure 2). To examine the clonality of the leukemias, we carried out Southern blotting analysis on genomic DNA from the leukemic spleens using a *GFP*-specific probe. Integration bands of similar intensity were observed in the majority of the leukemias, suggesting that they are mostly monoclonal (Supplementary Figure 3). Cell lines can be efficiently established from these leukemias by culturing leukemia cells in the presence of SCF and IL-3. Knockdown of *Setbp1* or its known critical targets *Hoxa9* and *Hoxa10* in these leukemia cell lines dramatically reduced their colony formation on methylcellulose (Supplementary Figure 4 and 5), suggesting that their expression are critical for the maintenance of leukemia cells. Collectively, these results suggest that *SETBP1* is a potent oncogene capable of inducing myeloid leukemia development.

Setbp1 overexpression promotes the expansion of GMPs and self-renewal of HSCs

In order to study the early effects of *Setbp1* overexpression on normal hematopoiesis before leukemia development, we analyzed the engraftment of *Setbp1* virus transduced cells versus cells infected with empty virus in the peripheral blood of recipient mice at 4, 8 and 16 weeks after transplantation. While there was a gradual decrease of GFP positive donor cells from about 50% at 4 weeks to around 20% at 16 weeks of total nucleated cells in the blood of mice receiving empty virus infected cells, *Setbp1* virus transduced cells increased gradually from representing less than 40 % to over 50% of blood nucleated cells during the same period despite of lower transduction efficiencies by the *Setbp1* virus (Figure 2A), suggesting that *Setbp1* overexpression may promote the expansion of hematopoietic stem and progenitor cells. Lineage analysis of the infected donor cells further showed dramatically increased contribution of *Setbp1*-expressing cells to the myeloid lineage and concomitant reductions in their contributions to the B and T cell lineages (Figure 2B). Consistent with this expansion of myeloid compartment, the GMP population (lineage$^-$IL-7Rα^-Sca-1$^-$c-Kit$^+$FcγR-II/III^{Hi}CD34$^+$) was significantly expanded for *Setbp1*-expressing cells compared to control virus infected cells (Figure 2C). To further examine the possibility that *Setbp1* expression may also promote the self-renewal of HSCs, we transduced purified mouse lineage$^-$Sca1$^+$c-kit$^+$ (LSK) cells enriched for HSCs with the same viruses and compared their long-term HSC populations after transplantation and also their serial engraftment potentials. An average of over 5-fold expansion of the long-term HSC population (lineage$^-$Sca-1$^+$c-kit$^+$CD48$^-$CD150$^+$) in the LSK compartment after *Setbp1* expression was observed at 2 months after transplantation (Figure 2D). Concomitantly, a greater engraftment by *Setbp1*-transduced cells than control virus-infected cells was observed beginning at 8 weeks despite lower transduction efficiencies by the *Setbp1* virus (Figure 2E, Supplementary Figure 6). Furthermore, an average of over 80-fold higher engraftment potential was detected for *Setbp1*-transduced cells over control cells in secondary recipients receiving 500 GFP$^+$ LSK cells purified from the primary recipients at 16 weeks after transplantation (Figure 2E). These results support the notion that increased expression of *Setbp1* significantly enhances the self-renewal capability of HSCs.

Setbp1 represses *Runx1* transcription through recruitment of Hdac1

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 oncogenes *Hoxa9* and *Hoxa10* during leukemia induction. Human AMLs with high *SETBP1* expression display significantly lower mRNA levels of tumor suppressor gene *RUNX1* compared to AMLs with low *SETBP1* expression (Supplementary Figure 7). Similar to *Setbp1* overexpression, *Runx1* loss has also been shown to induce expansion of HSCs and GMPs. These evidences suggest that *SETBP1* may suppress *RUNX1* expression. In supporting this notion, *Runx1* mRNA levels were significantly reduced in primary myeloid progenitors from C57BL/6 mice at 48 hours after infection with *Setbp1* virus (Figure 3A). Meanwhile, knockdown of *Setbp1* using a *GFP*-specific lentiviral shRNA in *Setbp1*-immortalized S3 cells[8] induced substantial increases in *Runx1* mRNA and protein levels (Figure 3B). The repression of *Runx1* by *Setbp1* is also critical for *Setbp1*-induced immortalization and transformation as ectopic *Runx1* expression in S3 cells and *Setbp1*-induced leukemia cell line BL12 dramatically inhibits their colony-forming capability (Supplementary Figure 8).

The rapid down-regulation of *Runx1* mRNA levels by *Setbp1* overexpression suggests that *Runx1* may be a direct transcriptional target of *Setbp1*. To test this idea, we carried out chromatin immunoprecipitation (ChIP) assays in mouse myeloid progenitors immortalized by FLAG-tagged Setbp1 (Figure 3C). In 2 independently immortalized myeloid progenitor populations, genomic regions within the two alternative promoters of *Runx1* were significantly enriched in immunoprecipitates prepared using FLAG M2 antibody over control IgG immunoprecipitates (Figure 3C), indicating that Setbp1 may directly repress *Runx1* transcription in myeloid progenitors.[23]

Proteins containing AT-hook motifs are known to participate in chromatin remodeling.[16–18] To investigate whether epigenetic regulation is involved in this transcriptional repression of *Runx1* induced by *Setbp1*, we examined changes in histone modifications at *Runx1* promoters after *Setbp1* knockdown in the same cells. We detected significant increases in histone H3 acetylation levels at *Runx1* promoters after *Setbp1* knockdown while no significant changes in total acetylated histone H3 levels were observed (Figure 4A), suggesting that *Setbp1* may repress *Runx1* transcription by inducing histone H3 deacetylation at its promoters. In support of this concept, significant bindings of Hdac1 in a similar pattern as Setbp1 bindings could be detected at *Runx1* promoters by ChIP analysis (Figure 4B). Moreover, *Hdac1* knockdowns significantly increased *Runx1* mRNA levels in these cells, which were also accompanied by significant reductions in their colony-forming capability (Supplementary Figure 9). In addition, Hdac1 bindings at *Runx1* promoters were dramatically reduced by *Setbp1* knockdown while total Hdac1 protein levels were not affected (Figure 4B), indicating that Setbp1 may be responsible for recruiting Hdac1 to *Runx1* promoters. Collectively, these results suggest that Setbp1 may repress *Runx1* transcription through recruiting Hdac1, which induces histone deacetylation at its promoters.

The NuRD complex is recruited to *Runx1* promoters

HDAC1 has been frequently identified in distinct multi-protein complexes including the Sin3, NuRD, and CoREST complexes.[24] Particularly, the NuRD complex was shown previously to repress gene transcription in myeloid progenitors.[15] Therefore, we first tested the possibility that Hdac1 may be recruited to *Runx1* promoters as part of the NuRD

complex. By ChIP analysis, significant bindings of Chd4, Mbd3 and Mta2, representing the unique components of the NuRD complex, were all detected at both *Runx1* promoters in *Setbp1*-immortalized myeloid progenitors (Figure 5A), suggesting that *Setbp1*-induced *Runx1* repression is mediated by the NuRD complex. Consistent with this idea, knockdown of *Mbd3* or *Mta2* significantly increased *Runx1* mRNA levels in *Setbp1*-induced leukemia cells (Supplementary Figure 10 and 11). To further test the possibility that Setbp1 may physically associate with the NuRD complex, we transiently expressed 3xFLAG tagged Setbp1 in 293T cells and examine the association of endogenous NuRD complex components with Setbp1 by immunoprecipitation using FLAG M2 antibody. NuRD complex components including MBD3, HDAC1, MTA2 and CHD4 could be readily detected in FLAG M2 immunoprecipitates prepared from transfected cells but not in that of non-transfected cells or precipitates prepared using control IgG (Figure 5B). Setbp1 along with HDAC1 and CHD4 can also be significantly precipitated from the transfected cells using a MTA2 specific antibody (Figure 5C). These results suggest that Setbp1 may directly recruit the NuRD complex to *Runx1* promoters through physical interaction.

HDAC inhibitors are effective in treating myeloid leukemias with *Setbp1* activation

HDAC inhibitors have been shown to exert inhibitory effects against myeloid leukemias induced by *AML/ETO, PLZF/RARa*, or *Hoxa9/Meis1*.[25–27] 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* overexpression. As expected, *Runx1* mRNA and protein levels were significantly up-regulated in *Setbp1*-induced BL3 and BL12 leukemia cell lines by treatment with HDAC inhibitors Entinostat and Vorinostat (Supplementary Figure 12). Treatment with these inhibitors also completely ablated colony formation by these leukemia cells and *Setbp1*-immortalized S3 cells (Figure 6A). Cytospin analysis of treated cells in liquid culture further suggests induction of myeloid differentiation (Figure 6B), which was confirmed by significantly increased expression of differentiation markers including *Cd11b, Lyz2*, and *Csf1r* (Supplementary Figure 13). Since *SETBP1* missense mutations were thought to cause *SETBP1* activation by increasing its protein stability[3] and were also found to associate with high *SETBP1* mRNA levels,[4] we additionally tested effects of HDAC inhibitors on mouse myeloid progenitors immortalized by ectopic expression of mutant *Setbp1* carrying such a mutation in leukemia patients. As expected, both HDAC inhibitors also induced identical effects in these cells to cells immortalized by wild-type *Setbp1* (Supplementary Figure 14). To further test the potential efficacy of HDAC inhibition in treating leukemias with *SETBP1* overexpression *in vivo*, we transplanted mice with 2 independent primary 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 (Figure 6C). All recipient mice treated with vehicle become moribund due to leukemia development after 2 weeks. In contrast, Entinostat treatments significantly prolonged the survival of all leukemic mice with up to 90% extension of their survival time. Collectively, these results suggest that HDAC inhibition may represent a promising therapeutic strategy for treating myeloid leukemias with *SETBP1* activation.

Discussion

Several lines of evidence suggested the involvement of *SETBP1* in myeloid leukemia development including its frequent activation by overexpression or mutations in various myeloid malignancies,[2–4, 7] capabilities to promote PP2A inhibition and self-renewal of myeloid progenitors by up-regulating *Hoxa9* and *Hoxa10,*[2, 8] and cooperation with *BCR/ABL*[8] and *ASXL1* mutations[28] in leukemia development; however, it was unclear whether its activation is critical for the initiation or progression of myeloid leukemia. Here we show that *SETBP1* overexpression can be a 'driver' mutation in the initiation of AML development as over half of mice receiving mouse bone marrow progenitors transduced by a *Setbp1*-expressing retrovirus developed myeloid leukemia within 10 months. Most of these leukemias also express physiologically relevant levels of *Setbp1* as similarly increased levels of *SETBP1* mRNA were observed in human myeloid leukemias with overexpression of wild-type *SETBP1* or *SETBP1* missense mutations when compared to normal bone marrow controls.[2, 4] The relatively long disease latency and over 50% disease penetration in our model further suggest that *Setbp1* overexpression by itself is not sufficient to cause full transformation, which likely requires additional mutations. Since *Hoxa9* is a critical target of *Setbp1* and is known to cooperate with *Meis1* in leukemia induction, it is possible that one such cooperating mutation for *Setbp1* is activation of *Meis1*. However, *Meis1* overexpression was only detected in one out of 6 *Setbp1*-induced leukemias (data not shown), suggesting that other unknown cooperating mutations/pathways likely exist for *Setbp1*. Identification of these mutations/pathways, which may be achieved by cloning the virus insertion sites from these leukemias,[20] should help advance our understanding of the molecular mechanisms underlying *SETBP1*-induced leukemia development.

Our study identifies *Runx1* as a critical transcriptional repression target of *Setbp1* in promoting myeloid leukemia development. Similar to *Hoxa9* and *Hoxa10* knockdowns, increased *Runx1* expression dramatically inhibited colony formation of *Setbp1*-induced leukemia cells. The repression of *Runx1* by *Setbp1* is also supported by the similar effects on normal adult hematopoiesis before leukemia development shared by *Setbp1* overexpression and loss of *Runx1*. Our transplantation studies of LSK cells transduced by *Setbp1* virus suggest that *Setbp1* overexpression significantly enhanced the self-renewal capability of HSCs. *Setbp1* overexpression may also partially promote the self-renewal of GMPs *in vivo* as a significantly expanded GMP population was detected after *Setbp1* transduction. Similar effects were also elicited by *Runx1* loss. Although essential for the onset of definitive hematopoiesis,[29, 30] *Runx1* is a negative regulator of HSC self-renewal.[31] Conditional deletion of *Runx1* in adult mice caused significant increase in the number of HSCs, though these cells are less competitive than wild-type HSCs in reconstituting irradiated recipient mice due to defective niche interaction.[32, 33] Loss of *Runx1* is also associated with partial block of myeloid differentiation leading to expansion of GMPs potentially through its regulation of *Sfpi1*.[32, 34] These similarities support that *Runx1* repression by *Setbp1* is a significant contributor to the leukemia promoting effect of *Setbp1*.

In contrast to the known function of Setbp1 in activating transcription, our current study suggests that it could also function as a transcriptional repressor to directly repress *Runx1* transcription through recruitment of a repressive NuRD complex to *Runx1* promoters. A role

of the same NuRD complex in repressing tumor suppressor genes in leukemia development has been previously shown by its recruitment by PML-RARα for the repression *RARβ2* in acute promyelocytic leukemia cells.[15] The recruitment of this repressive NuRD complex by Setbp1 could be dependent on promoter context, and therefore, may not occur at *Hoxa9* and *Hoxa10* due to their activation by Setbp1. It will be interesting to determine in the future if additional critical repression targets exist for Setbp1 by mapping and comparing the genome-wide chromatin binding profiles of Setbp1 and this NuRD complex in *Setbp1*-induced leukemia cells.

Consistent with the repression of *Runx1* by histone deacetylation induced by the NuRD complex in *Setbp1*-induced mouse leukemia cells, we found that *Runx1* expression can be significantly increased in these cells by HDAC inhibitors, which also efficiently induced their differentiation. HDAC inhibitors including Vorinostat and Romidepsin have been recently approved by FDA for the treatment of cutaneous T-cell lymphoma, which has validated the usage of HDAC inhibitors for cancer treatment.[35–37] Studies have also shown that myeloid leukemias induced by *AML/ETO, PLZF/RARa*, or *Hoxa9/Meis1* are sensitive to HDAC inhibitors.[25–27] Our finding that Entinostat treatment significantly extended the survival time of mice with *Setbp1*-induced leukemias suggest that HDAC inhibitors could be considered for the treatment of acute myeloid leukemias with *SETBP1* overexpression in clinic, which were associated poor prognosis and could represent up to 27% of primary AML patients.[2] Missense mutations of *SETBP1* thought to cause stabilization of its protein have also been frequently found in various myeloid malignancies.[3, 4, 7] We found that HDAC inhibitors are equally efficient in inducing differentiation of mouse myeloid progenitors immortalized by one such *SETBP1* mutation, further suggesting that HDAC inhibitors could be potentially effective in treating myeloid malignancies with *SETBP1* missense mutations. In summary, these studies establish *Setbp1* as a potent oncogene for myeloid leukemia development partly through its repression of *Runx1* and identify HDAC inhibition as a potentially effective therapeutic strategy for the treatment of human leukemias with *SETBP1* activation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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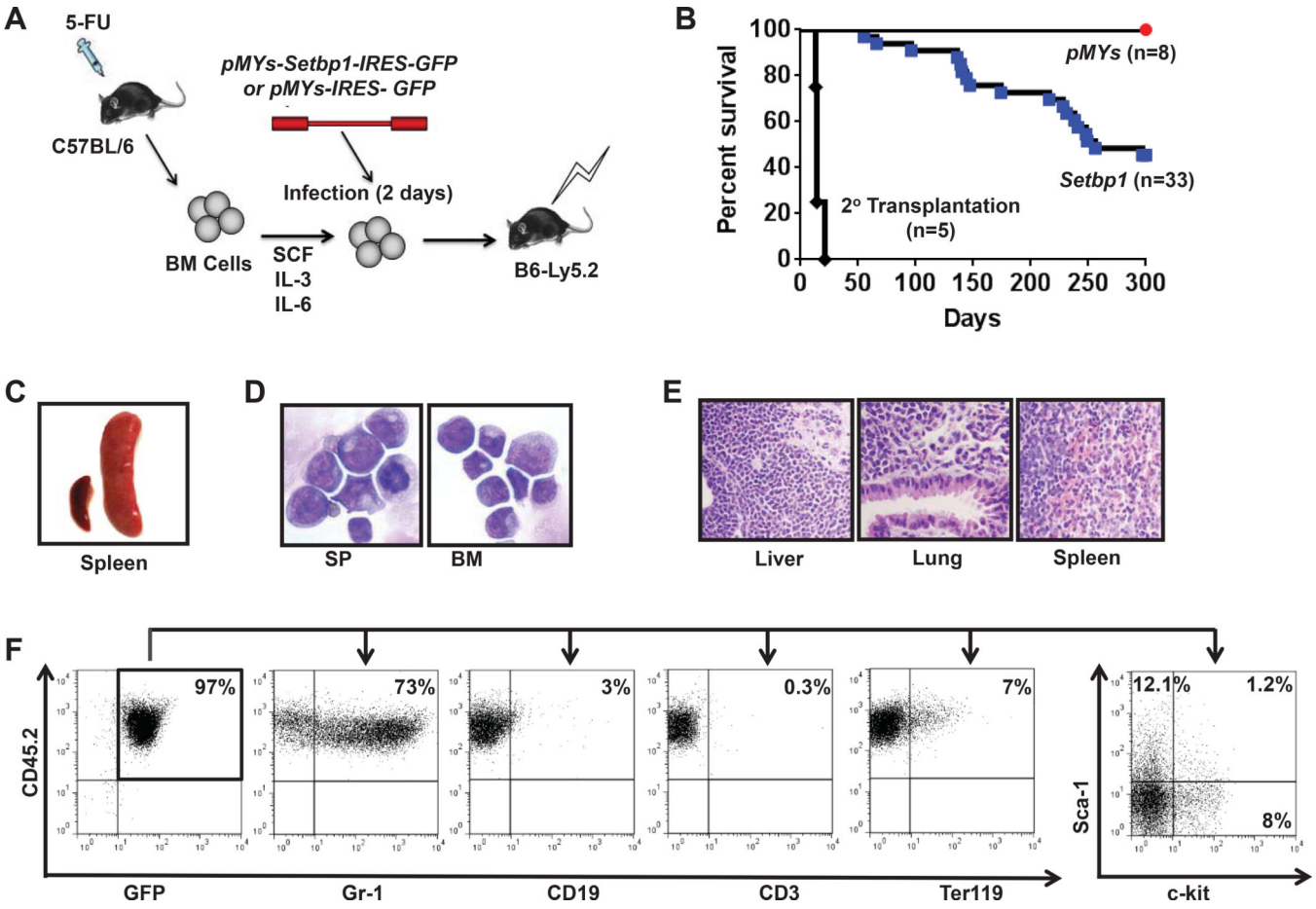

Figure 1.

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 1×10^6 spleen cells from primary leukemic mice. (C) Enlarged leukemic spleen (right) compared to a normal spleen (left). (D) Representative cytospin of spleen (SP) and bone marrow (BM) cells from leukemic mice. Original magnification × 400. (E) Representative H&E staining showing infiltration of myeloid blasts in liver, lung and spleen of a *Setbp1*-induced leukemic mouse. (F) Representative FACS analysis of indicated cell surface markers on bone marrow cells of a leukemic mouse.

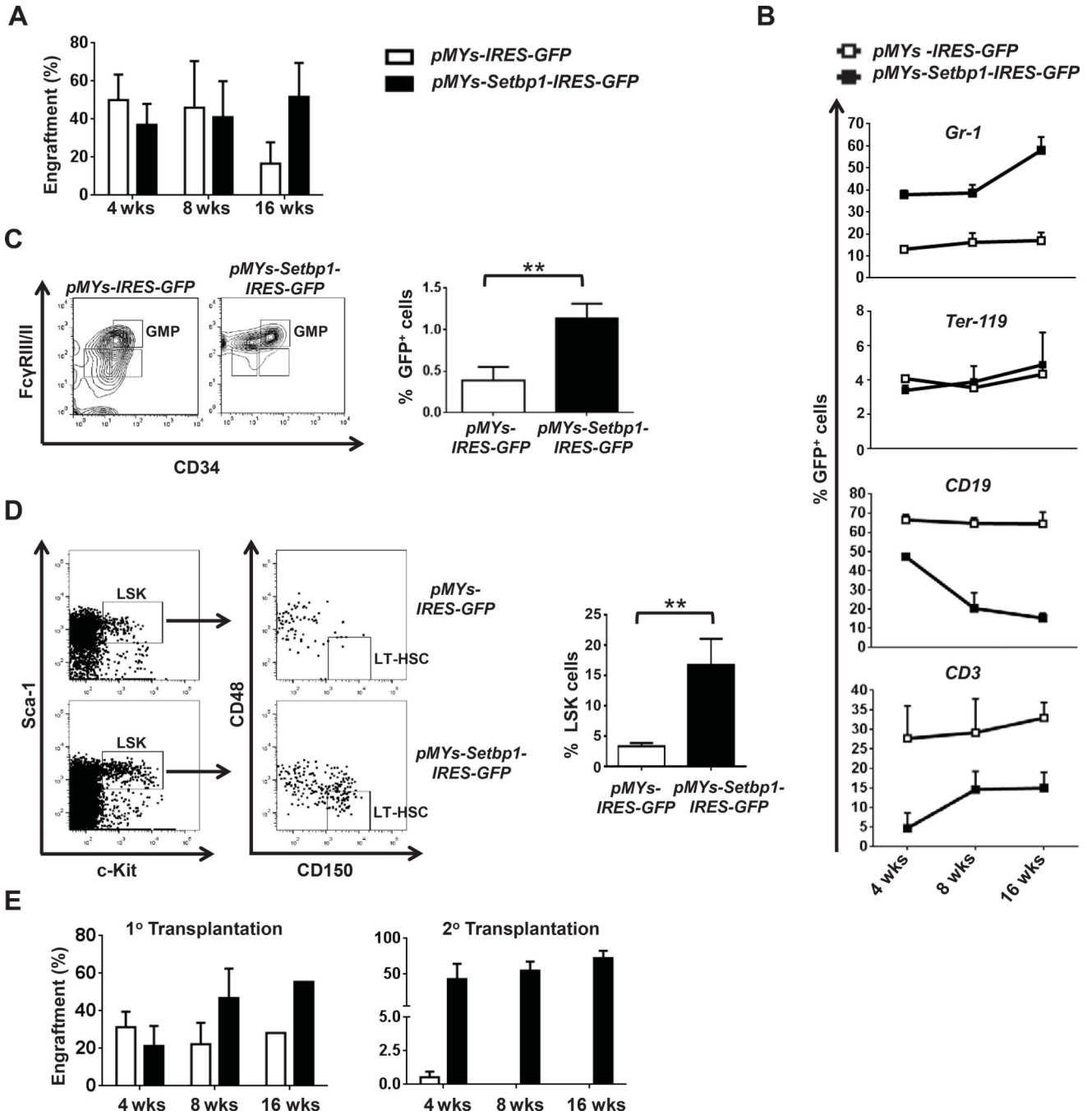

Figure 2.

Overexpression of *Setbp1* promotes expansion of GMPs and self-renewal of HSCs. (A) Engraftment of *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus transduced 5-FU-treated bone marrow cells in recipient mice analyzed by FACS analysis of percentage of GFP^+ cells in peripheral blood at 4, 8 and 16 weeks after transplantation (n=10 for *pMYs-Setbp1-IRES-GFP* group; n=3 for *pMYs-IRES-GFP* group). (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, 8 and 16 weeks after transplantation (n=7 for *pMYs-Setbp1-IRES-GFP* group; n=3 for *pMYs-IRES-GFP* group). (C) Left panel, representative FACS analysis of GMP populations ($Lin^{-}IL\text{-}7R\alpha^{-}$ $Sca\text{-}1^{-}c\text{-}Kit^{+}Fc\text{-}\gamma R\text{-}II/III^{Hi}CD34^{+}$) 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 (n=3 for each group). (D) Left panel, FACS analysis of GFP^{+} LT-HSC population ($lineage^{-}Sca\text{-}1^{+}c\text{-}kit^{+}CD48^{-}CD150^{+}$) in bone marrow of recipient mice at 2 months after transplantation of LSK cells(1×10^5 cells/mouse) transduced with *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus. Right panel, quantification of results on the left (n=3 for each group). (E) FACS analysis of GFP^{+} cells in peripheral blood of recipient mice at 4, 8 and 16 weeks after primary (1×10^5 cells/mouse) and secondary transplantation (5×10^2 cells/mouse) of LSK cells transduced with *pMYs-Setbp1-IRES-GFP* (black bars) or *pMYs-IRES-GFP* (white bars) virus (Primary transplantation, n=5 per group at 4 and 8 weeks and n=1 per group at 16 weeks; secondary transplantation, n=4 and n=3 for *pMYs-Setbp1-IRES-GFP* and *pMYs-IRES-GFP* group respectively at all indicated time points. Data are presented as means ± s.d. **, $P < 0.01$ (two-tailed Student's *t* test).

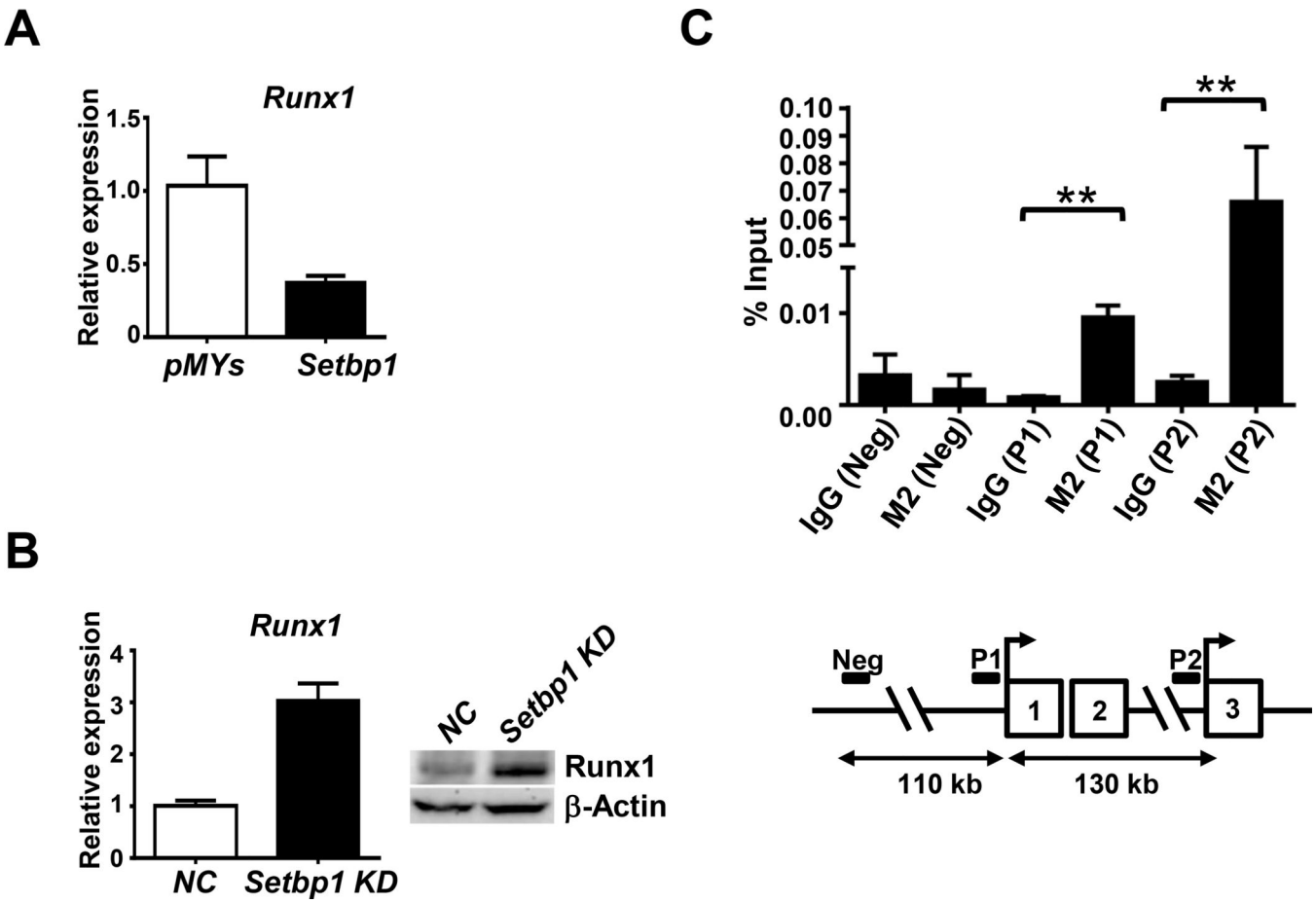

Figure 3.

Setbp1 directly represses *Runx1* transcription. (A) Real-time PCR analysis of *Runx1* mRNA levels in total RNA from mouse primary myeloid progenitor 48 hours after infection with *pMYs-Setbp1-IRES-GFP* or *pMYs-IRES-GFP* virus (n=3 for each group). (B) Left panel, real-time PCR analysis of *Runx1* mRNA levels in *Setbp1*-immortalized S3 cells[8] at 72hrs after infection with a lentiviral shRNA targeting Setbp1 (Setbp1 KD) or control shRNA (NC) (n=3 for each group). Right panel, representative Western blotting analysis of Runx1 and β-actin protein levels in S3 cells at 96hrs after lentiviral shRNA infections. (C) Upper panel, ChIP analysis of *Runx1* promoters (P1 and P2) and a nearby control genomic region (Neg) in myeloid progenitors immortalized by FLAG-tagged Setbp1[8] using FLAG M2 antibody or control IgG (n=3 for each immunoprecipitation). Lower panel, schematic diagram showing P1 and P2 promoters of *Runx1* and Neg region.

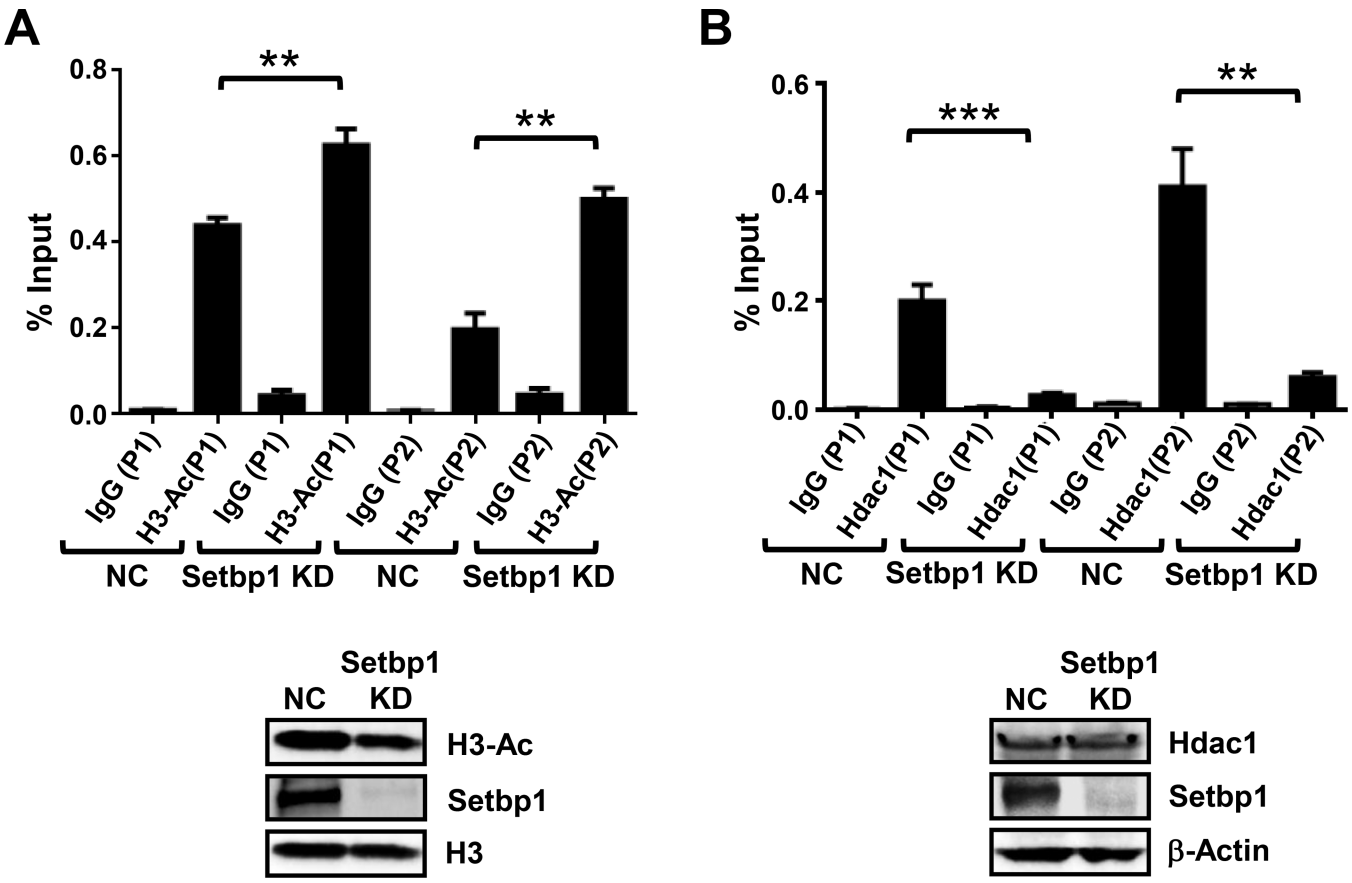

Figure 4.

Setbp1 represses *Runx1* transcription through recruitment of Hdac1. (A) Upper panel, representative 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 (n=3 for each immunoprecipitation). Lower panels, representative Western blotting analyses of indicated proteins in same cells of upper panel. (B) Upper panel, representative 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 (n=3 for each immunoprecipitation). Lower panels, representative Western blotting analyses of indicated proteins in same cells of upper panel. **, $P < 0.01$; ***, $P < 0.001$ (two-tailed Student's *t* test).

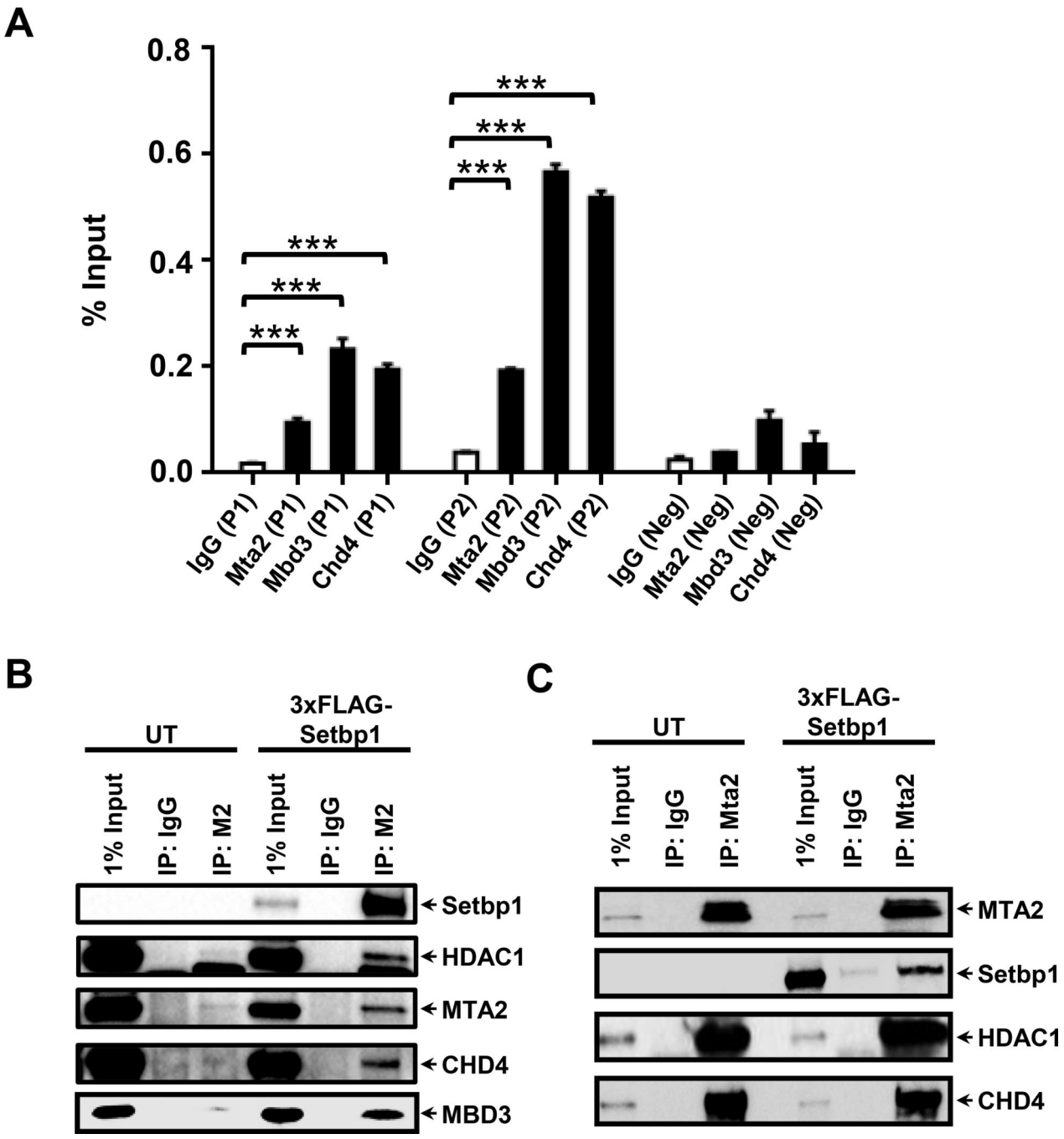

Figure 5.

Setbp1 recruits the NuRD complex to *Runx1* promoters through physical interaction. (A) Representative ChIP analysis of *Runx1* promoters (P1 and P2) and negative control region (Neg) using antibodies specific for Chd4, Mbd3, Mta2, and control IgG (n=3 for each immunoprecipitation). (B) Western blotting analyses of indicated proteins in M2 and IgG immunoprecipitates prepared from nuclear extracts of non-transfected 293T cells (UT) or cells transfected with a construct expressing 3xFLAG-tagged Setbp1 (n=3 for each immunoprecipitation). (C) Western blotting analyses of indicated proteins in

immunoprecipitates prepared using MTA2-specific antibody or control IgG from nuclear extracts of non-transfected 293T cells (UT) or cells transiently expressing 3xFLAG-tagged Setbp1(n=3 for each immunoprecipitation). Data are shown as means ± s.d. ***, $P < 0.001$ (two-tailed Student's *t* test).

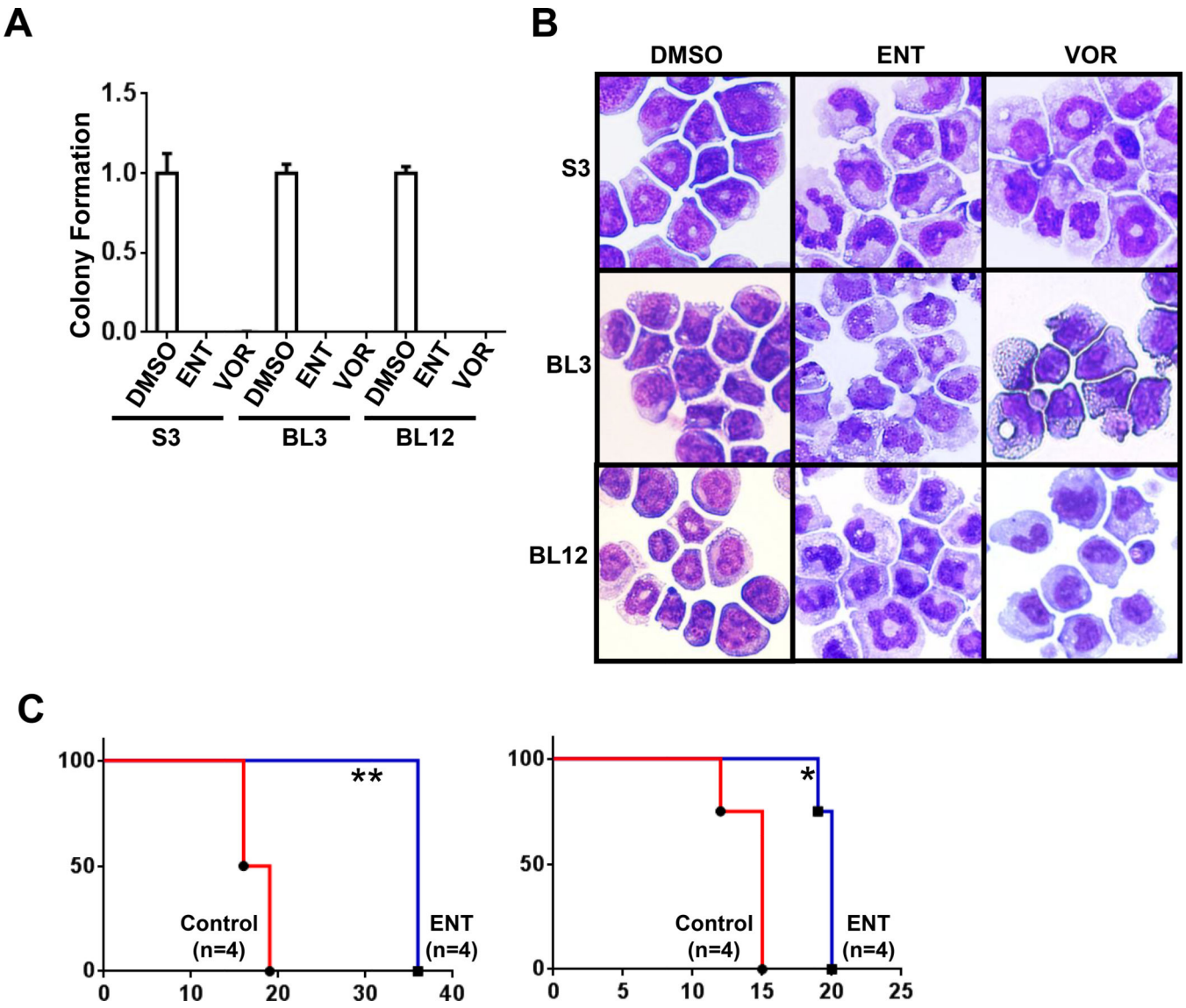

Figure 6.

Histone deacetylation is essential for *Setbp1*-induced immortalization and transformation. (A) Mean and s.d. of colony formation potential of S3, BL3 and BL12 cells in the presence of 1 μM entinostat (ENT), vorinostat (VOR), or control DMSO (n=3 for each treatment). (B) Representative 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. Original magnification × 400. (C) Survival curves of irradiated B6-Ly5.2 mice transplanted with two independent *Setbp1*-induced leukemias and treated with Entinostat (ENT, 30 mg/kg of body weight) or vehicle. Animals were injected intra-peritoneally every 3 days starting from 7 days after transplantation until 21 days after transplantation. *, $P < 0.05$; **, $P < 0.01$ (Log-rank test).