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SETBP1 Mutations Drive Leukemic Transformation in ASXL1-Mutated MDS

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Abstract

Mutations in *ASXL1* are frequent in patients with myelodysplastic syndrome (MDS) and associated with adverse survival yet the molecular pathogenesis of *ASXL1* mutations are not fully understood. Recently it has been found that deletion of *Asx1l* or expression of C-terminal-truncating *ASXL1* mutations (*ASXL1*-MT) inhibit myeloid differentiation and induce MDS-like disease in mice. Here, we find that *SETBP1* mutations (*SETBP1*-MT) are enriched among patients with *ASXL1*-mutated MDS patients and associated with increased incidence of leukemic transformation as well as shorter survival, suggesting *SETBP1*-MT play a critical role in leukemic transformation of MDS. We identify that *SETBP1*-MT inhibit ubiquitination and subsequent degradation of *SETBP1*, resulting in increased expression. Expression of *SETBP1*-MT, in turn, inhibited Pp2a activity, leading to Akt activation and enhanced expression of posterior *Hoxa* genes in *ASXL1* mutant cells. Biologically, *SETBP1*-MT augmented *ASXL1*-MT-induced differentiation block, inhibited apoptosis, and enhanced myeloid colony output. *SETBP1*-MT collaborated with *ASXL1*-MT in inducing AML *in vivo*. The combination of *ASXL1*-MT and *SETBP1*-MT

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Conflict of Interest

The authors report no competing conflicts of interest.

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activated a stem cell signature and repressed the TGF- β signaling pathway, in contrast to the ASXL1-MT-induced MDS model. These data reveal that SETBP1-MT are critical drivers of ASXL1-mutated MDS and identify several deregulated pathways as potential therapeutic targets in high-risk MDS.

INTRODUCTION

Myelodysplastic syndromes (MDS) are heterogeneous clonal diseases of hematopoietic stem cells (HSCs) characterized by dysplastic myeloid cells, ineffective hematopoiesis associated with increased apoptosis, and a predisposition to acute myeloid leukemia (AML).¹⁻³ Transformation of MDS to AML is one of the largest contributors of mortality in MDS patients and a number of studies have suggested genetic alterations which contribute to leukemic transformation of MDS.^{1, 3, 4} However, the underlying mechanisms for transformation of MDS to AML remain largely elusive and genetically accurate preclinical models for leukemic transformation of MDS are lacking.

With the continuous innovation in the field of next-generation sequencing, a variety of mutations have recently been identified that shed light on the molecular pathogenesis of MDS.⁵ Several genetic alterations related to epigenetic regulation (including *ASXL1*, *TET2*, and *EZH2*), splicing machinery (including *SF3B1*, *SRSF2*, and *U2AF1*), and cohesion complex (*STAG2*, *RAD21*, and *SMC3*) have been identified in MDS patients in addition to those of transcription factors and oncogenes.⁶⁻¹³ Recent data suggests that even a single alteration of one epigenetic regulator may induce myeloid malignancies such as MDS or MPN (myeloproliferative neoplasms) *in vivo*.¹⁴⁻²⁰ Using a mouse bone marrow transplantation (BMT) model, we have recently found that expression of C-terminal-truncating ASXL1 mutants (ASXL1-MT), which are found in 15–20% of MDS patients, inhibit myeloid differentiation and induce an MDS-like disease after a long latency by abrogating polycomb repressive complex 2 (PRC2)-mediated methylation of histone H3K27.¹⁴ These mice mimicked human MDS in terms of multilineage myelodysplasia, pancytopenia, and hypercellular bone marrow, as well as occasional leukemic transformation. However, given that *ASXL1* mutations have been linked to high-risk MDS and leukemic transformation of MDS, further studies to identify the mechanistic basis for transformation of *ASXL1*-mutated low-risk MDS to high-risk MDS/AML are needed.²¹⁻²³

In multiple clinical studies with large cohorts of MDS samples, *ASXL1* mutations have been reported to be statistically associated with mutations in *RUNX1*, *EZH2*, *SRSF2*, *STAG2*, *NRAS*, and *SET binding protein 1 (SETBP1)*.^{4, 22, 24} In particular, mutations in *NRAS* and *SETBP1* appear to occur after initial establishment of MDS, suggesting that these mutations contribute to disease progression or evolution.^{4, 25-27} Consistent with this, we previously revealed that ASXL1-MT or ASXL1-knockdown collaborated with *NRAS* mutations to induce AML.^{14, 16} The biologic contribution of *SETBP1* mutations, in contrast, have not been fully investigated, although several groups have reported that *SETBP1* mutations seem to cause gain of function and play oncogenic roles in myeloid malignancies via protection of the protein from degradation by ubiquitination including MDS, chronic myelomonocytic leukemia (CMML) and atypical chronic myeloid leukemia (aCML) and that its mutation is a

poor prognostic factor.^{25, 26, 28, 29} Moreover, *in vivo* models reflecting oncogenic function of *SETBP1* mutation have not been presented. *SETBP1* encodes a predominantly nuclear-localized protein that binds the SET oncoprotein and the resulting heterodimer interacts with PP2A, a major serine/threonine phosphatase and tumor suppressor regulating cell proliferation. Formation of a SETBP1-SET-PP2A complex results in PP2A inhibition and AKT activation.^{25, 30, 31} In addition, Oakley et al. demonstrated that overexpression of *Setbp1* promotes the self-renewal of murine progenitors via binding to *Hoxa9* and *Hoxa10* promoters.³²

In this study, we identify genetic and functional evidence for a collaborative association between *ASXL1* and *SETBP1* mutations among patients with advanced MDS. These data present a novel murine model for leukemic transformation of MDS, provide evidence to support a new role for *SETBP1* mutation in leukemic transformation, and identify a number of pathways necessary for leukemic progression of *ASXL1/SETBP1*-mutated myeloid malignancies.

MATERIALS AND METHODS

Patients

Adult patients (n = 386) diagnosed with *de novo* MDS according to the 2008 WHO classification at the National Taiwan University Hospital (NTUH) and had cryopreserved bone marrow cells for study were recruited for gene mutation analyses.^{22, 26} All patients signed informed consents for sample collection in accordance with the Declaration of Helsinki. The clinical portion of this study was approved by the Institutional Review Board of the NTUH (approval 201207075RIB).

Mutation analysis

The coding region of *ASXL1* from exon 12 until the stop codon was amplified by three pairs of primers and sequenced by another set of six internal primers. The primer sequences and PCR conditions were described previously.^{26, 33} Mutation analyses of *SETBP1* were performed as described previously.²⁶

Cell culture and differentiation assay

HEK293T and HL60 cells were cultured in DMEM supplemented with 10% FBS and in RPMI-1640 supplemented with 10% FBS, respectively. The murine myeloid cell line 32Dcl3 was grown in RPMI-1640 medium supplemented with 10% FBS and 1 ng/mL IL-3. Before assays for apoptosis and differentiation, transduced 32Dcl3 or HL60 cells were GFP-sorted or subjected to drug selection with 1 µg/mL puromycin and/or 10 µg/mL blasticidin, if necessary.

Analyses of cell growth and apoptosis

The relative rate of viable cells was estimated using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI, USA). Cells were stained with PE-conjugated annexin V (R&D systems, Minneapolis, MN, USA) and apoptosis was determined using flow cytometry. All data with error bars represent the mean ± SEM.

Vector construction

Human *SETBP1*-WT and *SETBP1*-D868N cDNA with TY1 or FLAG tags were subcloned into the pMYs-IRES-GFP (pMYs-IG) retrovirus vector. We constructed and used the retrovirus vectors pMYs-SETBP1-WT-3×TY1-IG, pMYs-SETBP1-D868N-3×TY1-IG, pMYs-SETBP1-D868N-3×FLAG-IG, and pMYs-ASXL1-MT-IRES–nerve growth factor receptor (pMYs-ASXL1-MT-INGFR), in which *SETBP1*-WT or *SETBP1*-D868N tagged with a TY1 or FLAG epitope at the C terminus and *ASXL1*-MT (1900–1922del;E635RfsX15) tagged with a FLAG epitope at the N terminus was inserted upstream of the IRES-EGFP/NGFR cassette of pMYs-IG/pMYs-INGFR.

Western blot analysis

Cell lysates were subjected to immunoblotting using the following antibodies: TY1 (clone MAb-054-050; Diagenode, Denville, NJ, USA), tubulin (clone B-5-1–2; Santa Cruz Biotechnology, Dallas, TX, USA), HA (clone 3F10; Roche, Penzberg, Germany), Akt1 (clone C-20; Santa Cruz Biotechnology), Phospho-Akt (Thr308) (clone D25E6; Cell Signaling Technology, Danvers, MA, USA), Phospho-Akt (Ser473) (clone D9E; Cell Signaling Technology), PP2A (clone 1D6; Millipore, Billerica, MA, USA), Phospho-PP2A alpha (clone E155; Abcam, Cambridge, UK), FLAG (M2 FLAG; Sigma-Aldrich, St. Louis, MO, USA), ERK1/2 (clone C-16/C-14; Santa Cruz Biotechnology), and I2PP2A (SET) (clone E-15; Santa Cruz Biotechnology). Immunoprecipitation was performed in an immunoprecipitation buffer (150 mM NaCl, 50 mM Tris pH 7.5, 1 mM EDTA, 1% Triton, 2 mM sodium orthovanadate, 2 mM PMSF, 50 mM sodium fluoride) as previously reported.¹⁴

Flow cytometric analysis

Briefly, cells were stained with the indicated phycoerythrin (PE)-conjugated antibodies (eBioscience, San Diego, CA, USA). Flow cytometric analysis of the stained cells was performed with FACSCalibur Flow (BD Biosciences, San Jose, CA, USA) equipped with FlowJo Version 7.2.4 software (Tree Star, Ashland, OR, USA). All data with error bars represent the mean ± SEM.

Colony-forming assay

Lineage-negative Sca1⁺ c-Kit⁺ (LSK) cells were isolated from bone marrow (BM) of C57BL/6 mice according to published methods.^{34, 35} Retrovirus-infected LSK cells were sorted at 60 hours from the initiation of infection with a FACSAria cell sorter (Becton Dickinson, Franklin Lakes, NJ, USA) for use in the Methocult 3231 colony-forming assay (StemCell Technologies, Vancouver, Canada) supplemented with 100 ng/mL mouse SCF and 20 ng/mL mouse IL-3. A total of 1,000 cells were cultured in duplicate in 2.5-cm dishes. The colony-forming cells were harvested and replated every 7 days and scored for colony formation.

Mice

C57BL/6 (Ly5.1) mice (Sankyo Labo Service Corporation, Tokyo, Japan) and C57BL/6 (Ly5.2) mice (Charles River Laboratories Japan, Yokohama, Japan) were used for BMT experiments. Mouse BMT was performed as described previously.³⁶ Briefly, BM cells

derived from 5-fluorouracil-treated Ly5.2 mice were transduced with the retrovirus vectors described above, and the transduced cells were transplanted into sublethally-irradiated Ly5.1 mice.

Transfection and retrovirus production

Retroviral production was done as described previously.^{36, 37} Briefly, retroviruses were generated by transient transfection of Plat-E packaging cells using the calcium-phosphate co-precipitation method. An alternative method was used for the retroviral production of SETBP1-WT or SETBP1-D868N to obtain an adequate viral titer. The retrovirus vectors and pcDNA3/VSV-G were transfected into a 293gp packaging cell line using Lipofectamine 2000 (Life Technologies, Carlsbad, CA, USA) and the subsequent viral supernatants were collected. These supernatants were used to infect 293gp cells, which were then sorted by FACS AriaII (Becton Dickinson) twice to enrich for cells that expressed EGFP at high levels. VSV-G pseudotyped retroviruses were produced by removing tetracycline from the culture media and the viruses were concentrated by centrifugation at $6,000 \times g$ for 16 h. Cell lines 32Dcl3 and HL60 cells were infected with retroviruses as previously described.³⁷

qRT-PCR

As previously described, total RNAs were treated with deoxyribonuclease I (Invitrogen) and reverse transcribed using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, USA).³⁸ qRT-PCR was performed using SYBR Premix EX Taq (Takara Bio, Otsu, Japan) and Rotor-Gene Q (Qiagen, Venlo, Netherlands). Primer sets are described in Supplementary Table 1.

ChIP assay

Chromatin immunoprecipitation (ChIP) assays were performed using anti-TY1 antibodies as described previously.³⁹ Quantitative PCR was performed with a Rotor-Gene Q (Qiagen) using SYBR Premix EX Taq (Takara). Primer sets are described in Supplementary Table 1. All data with error bars indicate the mean \pm SEM. Data are representative of three independent experiments.

PP2A phosphatase assay

A phosphatase assay from whole cell lysates was carried out using the PP2Ac Immunoprecipitation Phosphatase Assay kit (Millipore) according to the manufacturer's protocol as previously described.⁴⁰

BM serial transplantation and drug model of FTY720

GFP/NGFR-positive BM cells (1×10^5 cells) from leukemic mice were transplanted into sublethally irradiated C57BL/6 (Ly5.2) mice. After confirmation of engraftment (2 weeks after transplant), mice were treated with FTY720 (Selleck, Houston, TX, USA, 10 mg/kg/d, i.p.) or vehicle for 5 days.

Transcriptome analysis

Library preparation for whole transcriptome analysis was performed according to the manufacturer's instructions (SureSelect Strand Specific RNA Preparation Kit, Agilent Technologies, Santa Clara, CA, USA). Briefly, poly(A) RNA purified from 2 µg of total RNA was chemically fragmented to appropriate sizes. First-strand cDNA was synthesized from the fragmented poly(A)-selected mRNA, followed by the synthesis of second-strand cDNA. Then, adaptor oligo-DNA was ligated to both ends of the double-stranded cDNA. Libraries were prepared with 12 cycles of PCR amplification of adaptor-ligated cDNA using primers that are complimentary to the adaptors. The libraries were sequenced by GAIIX next generation sequencer (Illumina, San Diego, CA, USA) using the single-end 36 bp sequencing protocol. The generated sequence tags were mapped onto the murine genomic sequence (mm9, UCSC Genome Browser) and mRNA expression levels were normalized as reads per kilobase per million (RPKM).

RESULTS

Clinical significance of *SETBP1* mutation among *ASXL1*-mutated MDS patients

We first investigated a possible genetic interaction between *SETBP1* and *ASXL1* mutations in MDS patients through analysis of *ASXL1* and *SETBP1* in a cohort of 368 patients with *de novo* WHO-defined MDS. *ASXL1* mutations were detected in 17.39% (64/368) of the cohort. Patients with an *ASXL1* mutation had a significantly higher incidence of concurrent *SETBP1* mutations than *ASXL1*-wildtype MDS patients (6/64 (9.38%) vs. 2/304 (0.66%); $p=0.0005$) (Figure 1a). The mutation patterns of six MDS patients with concurrent *ASXL1* and *SETBP1* mutations are shown in Supplementary Table 2. Consistent with recent reports, *SETBP1*D868N ($n=3$), E858K ($n=1$), G870S ($n=1$), and S867R ($n=1$) mutations, all of which are within the SKI homologous region, were identified (Figure 1b and Supplementary Table 2). Other than E858K, these mutations were found in the consensus binding region for β -TrCP1, the substrate recognition subunit for E3 ubiquitin ligase.²⁹

Next, we analyzed the impact of *SETBP1* mutations on the prognosis of *ASXL1*-mutated cases. Among *ASXL1*-mutated patients, those harboring *SETBP1* mutations had a higher incidence of leukemic transformation than those without ($p=0.042$; Figure 1c). Furthermore, MDS patients with both mutations had a significantly shorter overall survival compared to those without *SETBP1* mutations (median, 10.5 vs. 22.5 months, $p=0.046$; Figure 1d). We also performed multivariate analysis for the overall survival in 64 MDS patients with *ASXL1* mutations and found that the *SETBP1* mutation was an independent poor prognostic factor (relative risk 3.340, 95% CI 1.150–9.698, $p=0.027$) irrespective of age, 2008 WHO classification and IPSS (International Prognostic Scoring System) classification (Supplementary Table 3).

Increased stability of SETBP1-MT due to decreased ubiquitination

Next, we evaluated the protein expression levels of SETBP1-WT and SETBP1-D868N by Western blot analysis after transfecting HEK293T with empty vector, 3×TY1-SETBP1-WT, or 3×TY1-SETBP1-D868N (Supplementary Figure 1A and 1B). As Piazza et al. showed using G870S mutant, another mutant SETBP1-D868N also exhibited a dramatically

increased stability compared to SETBP1-WT. Treatment with MG-132, a protease inhibitor, increased the expression levels of SETBP1-WT.²⁹ These data suggest that SETBP1-D868N can escape ubiquitination and subsequent degradation by inhibiting its binding to the E3 ligase β -TrCP1, as has been previously identified for SETBP1-G870S mutations.²⁹ These results indicate that D868N acts as a gain-of-function mutation.

Expression of the *SETBP1* mutant impairs apoptosis and differentiation

In order to understand the biological contribution of *SETBP1* mutations to *ASXL1* mutations, we first examined the effect of SETBP1 mutant on apoptosis or differentiation of an *ASXL1*-mutated clone. We produced IL-3-dependent murine 32Dcl3 myeloid cells overexpressing mock/mock, ASXL1-MT (*ASXL1*E635RfsX15)/mock, mock/SETBP1-WT, mock/SETBP1-D868N, ASXL1-MT/SETBP1-WT, or ASXL1-MT/SETBP1-D868N using pMys-IRES-Puro and pMys-IRES-GFP vectors, whose expressions were confirmed using qRT-PCR (Supplementary Figure 2A and 2B). As shown in Figure 2a and 2b and Supplementary Figure 2C, expression of both ASXL1-MT and SETBP1-D868N attenuated apoptosis of 32Dcl3 cells induced by IL-3 withdrawal, although it did not induce factor-independent growth nor change the growth rate of 32Dcl3 cells in the presence of IL-3 (data not shown). Cell cycle analysis of the transfectants revealed that simultaneous expression of ASXL1-MT and SETBP1-D868N resulted in a slight increase of cells in G2/M and S phases and concomitant reductions of cells in the G1 phase (Supplementary Figure 2D), suggesting that the additional expression of SETBP1-D868N induces cell cycle progression.

We previously reported that ASXL1-MT inhibited G-CSF-induced differentiation of 32Dcl3 toward neutrophils.¹⁴ Interestingly, the acquisition of SETBP1-D868N augmented the differentiation block in ASXL1-MT-expressing 32Dcl3 cells (Figure 2c and 2d). In accordance with this, all-trans retinoic acid-induced (ATRA-induced) granulocytic differentiation of HL60 cells, a human AML cell line, was also synergistically attenuated by the expression of ASXL1-MT and SETBP1-D868N (Supplementary Figure 2E and 2F).

SETBP1-MT enhances clonogenic capacity of myeloid progenitor cells

To determine whether expression of SETBP1-D868N has an influence on differentiation or self-renewal in hematopoietic stem cells, we compared colony formation ability between LSK cells transduced with mock/mock, ASXL1-MT/mock, mock/SETBP1-WT, mock/SETBP1-D868N, ASXL1-MT/SETBP1-WT, or ASXL1-MT/SETBP1-D868N using pMys-IRES-GFP and pMys-IRES-hNGFR vectors. ASXL1-MT/SETBP1-D868N-transduced LSK cells produced significantly more colonies with a relatively immature morphology, although overexpression of SETBP1-D868N or SETBP1-WT did not immortalize LSK cells (Figure 3a and 3b), contrary to previous reports.²⁵

SETBP1-D868N collaborates with ASXL1-MT in inducing AML

We next examined the *in vivo* effect of simultaneous expression of ASXL1-MT and SETBP1-D868N using mouse BMT model. Intriguingly, in 3 independent experiments, all of the mice transplanted with BM cells expressing both ASXL1-MT and SETBP1-D868N died of acute leukemia with remarkable hepatosplenomegaly after a short latency (median survival, 73 days), while all of the mice transplanted with BM cells expressing either

ASXL1-MT or SETBP1-D868N survived for 6 months after transplantation (Figure 4a and 4b). We confirmed high expression levels of both mutants in the BM cells of AML mice (Supplementary Figure 3A and 3B). Morphological analysis of BM or spleen cells in leukemic mice displayed frequent immature blasts with slight differentiation toward myeloid lineage. The blasts were positive for GFP and NGFR (Figure 4c), indicating they expressed SETBP1-D868N and ASXL1-MT. The phenotypic markers of the blast were CD11b–positive, and Gr1, c-kit, ScaI, and CD34–weak positive (Figure 4d). These leukemic mice exhibited remarkable leukocytosis, anemia, thrombocytopenia, macrocytosis, hepatosplenomegaly, and hypercellular BM compared to other control mice (Figure 4e–4k). Consistent with our previous report, mice with ASXL1-MT alone developed mild pancytopenia and hepatosplenomegaly with hypercellular BM mimicking early stages of MDS.¹⁴ On the other hand, we observed no significant change other than BM cellularity or splenomegaly in mice with SETBP1-D868N alone up to 6 months (Figure 4e–4k) or up to 12 months (data not shown). In addition, leukemic cells from mice with both ASXL1-MT and SETBP1-D868N were serially transplantable in sublethally irradiated recipients (data not shown) and grew rapidly without a change in phenotype *in vitro*, while those of the mice with ASXL1-MT or mock could not survive under the same conditions (Supplementary Figure 3C–3E).

Acquisition of *SETBP1* mutation results in Pp2a inhibition

To determine whether SETBP1-D868N also interacts with SET and inactivates PP2A as previously reported for other SETBP1 mutants,²⁹ coimmunoprecipitation analysis using the anti-TY1 antibody was carried out in HEK 293T cells transfected with mock, SETBP1-WT, or SETBP1-D868N vectors. Given that none of the *SETBP1* mutations occurs within the SET-binding domain (Figure 1b), SETBP1-D868N and SETBP1-WT were shown to associate with SET and PP2A (Figure 5a). To further investigate whether SETBP1-D868N induced PP2A inactivation in BM cells *in vivo*, we analyzed the phosphorylation levels of Pp2a and Akt in FACS-purified c-kit-positive cells from the BM of mice transplanted with BM cells expressing empty vector (control), *ASXL1*-MT (MDS model), or both *ASXL1*-MT and *SETBP1*-D868N (AML model). As expected, we observed phosphorylation of tyrosine 307 of Pp2a in AML mice. Consistent with this finding, threonine 308 and serine 473 residues of Akt were highly phosphorylated in AML mice, without affecting their expression levels, relative to control or MDS mice, indicating that impairment of Pp2a phosphatase activity, at least in part, resulted in leukemogenesis in our mice model as well as previously reported *in vitro* experiments (Figure 5b and 5c).^{29–31} Next, to assess the therapeutic potential of targeting Pp2a against disease progression, we treated the AML mice with FTY720, a Pp2a activator, *in vitro* and *in vivo* (Figure 5d and Supplementary Figure 3A). As expected, FTY720 reduced phosphorylation of tyrosine 307 of Pp2a and inhibited, in a dose dependent manner, the proliferation of cell lines transduced with both ASXL1-MT and SETBP1-D868N (Figure 5d and Supplementary Figure 4A). However, the therapeutic effect of FTY720 on AML mice was limited in spite of decreased phosphorylation of Pp2a, suggesting that pathways other than Pp2a inhibition also contribute to leukemogenesis in these mice (Supplementary Figure 4A and 4B).

SETBP1-D868N activates *Hoxa9* and *Hoxa10* expression in mice

It has been demonstrated that overexpression of wildtype *Setbp1* activates *Hoxa9* and *Hoxa10* in myeloid progenitors and induces self-renewal of immortalized cells.³² Moreover, we previously reported that ASXL1-MT derepressed the expression of *Hoxa9* and *Hoxa10* in ASXL1-MT-induced MDS mice.¹⁴ Here, we observed further up-regulation of posterior *Hoxa* genes in ASXL1-MT/SETBP1-D868N-induced AML mice (Figure 5e). In addition, SETBP1-WT and SETBP1-D868N, both of which contain three highly conserved AT-hook motifs (Figure 1b), were shown to directly and specifically bind to these *HOXA9* and *HOXA10* regions using anti-TY1 ChIP (Figure 5f and 5g and Supplementary Figure 4C and 4D).

Combination of ASXL1-MT and SETBP1-D868N activates a stem cell signature and represses the TGF- β signaling

To elucidate the mechanism for leukemogenesis induced by the combination of ASXL1-MT and SETBP1-D868N, we compared the gene expression profile of CD3-B220-Ter119- BM cells of AML mice induced by both ASXL1-MT and SETBP1-D868N versus those induced by ASXL1-MT alone. Gene set enrichment analysis (GSEA) revealed that addition of SETBP1-D868N significantly altered the expression profile of ASXL1-MT MDS (Figure 6a-6e). GSEA indicated that the gene set induced by simultaneous expression of ASXL1-MT and SETBP1-D868N correlated with that enriched in HSC or early progenitors, and negatively correlated with that of mature cells or lineage markers when compared to the gene set induced by ASXL1-MT alone (Figure 6a-6e and Supplementary Figure 5A-5G). This suggested that the mutation of *SETBP1* contributes to acquisition of self-renewal of HSCs or leukemic stem cells and inhibition of differentiation toward neutrophils, consistent with our *in vitro* results (Figure 6a-c and Supplementary Figure 5A-5C). Interestingly, GSEA also indicated global repression of the Tgf- β signaling pathway and reciprocal up-regulation of Myc-activated genes in leukemic mice (Figure 6d and 6e and Supplementary Figure 5D-F), while the MDS mice exhibited up-regulation of Tgf- β signaling (Supplementary Figure 5G). The repressed genes encoded for the receptors of Tgf- β , Smad proteins, and the major targets of Tgf- β signaling, which were confirmed by qRT-PCR (Figure 6f).

DISCUSSION

New insights into the genetic basis of MDS were obtained in the last decade using next-generation sequencing technology, shedding light on the founding or driver mutations of MDS and the mutations acquired at the later phases of the disease.³⁻⁵

Of importance, MDS patients at early stages present with the paradox of a variable cytopenia in spite of hypercellular BM, associating with ineffective hematopoiesis in which MDS cells tend to be apoptotic.⁴¹⁻⁴⁴ In fact, the incidence of apoptosis in the BM is higher in patients with low-risk MDS compared to a normal counterpart. On the contrary, in more advanced stages, MDS clones acquire the potential of resistance to apoptosis, leading to transformations and therapeutic tolerance.^{43, 45} As for the underlying mechanism, involvement of mutations in *TP53* and *NRAS* genes, both associated with poor prognosis,

has been well investigated.^{8, 46} For example, *NRAS* mutations, present in 10% of MDS patients, exhibit an anti-apoptotic effect by increasing sensitivities to cytokines or growth factors leading to the progression of MDS.¹ To further investigate how *ASXL1*-mutated MDS clones transform into leukemia, we examined co-existing mutations of 64 *ASXL1*-mutated MDS patients. Interestingly, mutations of *ASXL1* and *SETBP1* not only co-occurred significantly more often than by chance, but also resulted in shorter overall survival and a higher incidence of leukemic transformation when compared with *SETBP1* non-mutated cases, indicating that combined mutation of these genes provides a selective advantage and that an additional *SETBP1* mutation plays a pivotal role in disease progression.

In vitro expression of SETBP1-D868N enhanced myeloid colony formation of ASXL1-MT-transduced LSK cells and augmented the ASXL1-MT-induced differentiation block of 32Dcl3 cells and primary BM cells. Of note, SETBP1-D868N collaborated with ASXL1-MT to induce AML with a short latency in a mouse BMT model. To the best of our knowledge, this is the first report concerning an *in vivo* model of the *SETBP1* mutation and the first report revealing disease progression of an ASXL1-mutant MDS model. The escape of SETBP1-D868N from ubiquitination and subsequent degradation is most likely a gain-of-function mutation, combined with the observation that overexpression of SETBP1-WT exhibited milder effects than that of SETBP1-D868N. *SETBP1* mutations were found in late phase MDS patients, indicating that the acquisition of the *SETBP1* mutation is a critical event for *ASXL1*-mutated MDS during disease progression.²⁶

The formation of the SETBP1-SET-PP2A complex has been shown to result in PP2A inhibition.³¹ PP2A is a tumor suppressor that inhibits cellular transformation by regulating several signaling activities critical for malignant transformation, including AKT and ERK1/2 pathways.^{30, 47, 48} Indeed, we showed that both SETBP1-WT and SETBP1-D868N could interact with SET or PP2A. Moreover, BM cells from leukemic mice displayed activation of Akt, and phosphorylation of Pp2A, leading to decreased activity. However, while administration of FTY720, a Pp2a activator, did efficiently repress the growth rate *in vitro*, the *in vivo* effect of FTY720 against the ASXL1-MT/SETBP1-D868N MDS/AML model was marginal. This suggests that signaling pathways other than SETBP1-SET-PP2A play a predominant role in the ASXL1-MT/SETBP1-D868N-induced MDS/AML model. In this paper, we have identified potentially dysregulated pathways that could contribute to leukemic transformation.

First, both SETBP1-WT and SETBP1-D868N can bind to the promoter regions of *Hoxa9* and *Hoxa10*, leading to remarkable up-regulation of these genes. This is consistent with previous reports that overexpression of *Hoxa9* and *Hoxa10* induced immortalization of myeloid progenitors and disease progression.^{49, 50} Further upregulation of *Hoxa9* and *Hoxa10* by the combination of ASXL1-MT and SETBP1-D868N may play important roles in the leukemic transformation of MDS.

Second, using RNA-seq and GSEA with the *in vivo* mouse model,⁵¹ we identified several deregulated pathways. Among them, we focused on the TGF- β signaling pathway, which is a potent negative regulator of proliferation,⁵² since there is evidence showing that resistance to the growth inhibitory and apoptotic effects of TGF- β promotes clonal expansion and

down-regulation of TGF- β signaling plays a pivotal role in the pathogenesis of AML.^{53–55} For example, it has been demonstrated that AML1/ETO and PML/RAR α prevent DNA binding of Smad3 and Smad2/3 phosphorylation, respectively.^{53, 54} In accordance with the down-regulation of the TGF- β pathway, we observed up-regulation of the Myc pathway that would also contribute to leukemogenesis and the global down-regulation of Smad2/3 targets as well as TGF- β receptors shown in Figure 6F.^{56–58} Intriguingly, Zhou et al. reported that SMAD2, a downstream mediator of TGF- β receptors, is constitutively activated and overexpressed in MDS BM precursors and that shRNA-mediated down-regulation or pharmacologic inhibition of TGF- β receptor 1 leads to enhanced hematopoiesis in a variety of MDS subtypes *in vitro*.⁵⁹ Thus, it is tempting to consider that the TGF- β signaling pathway inhibits leukemic transformation of MDS and that *SETBP1* mutations contribute to the down-regulation of this important pathway.

Taken together, we detected gain-of-function *SETBP1* mutations enriched among *ASXL1*-mutated MDS and characterized an *in vivo* MDS/AML model expressing *SETBP1* mutations for the first time. Our results indicate that the *SETBP1* mutations play critical roles in the leukemic transformation of MDS involving anti-apoptosis, differentiation block, and increased self-renewal through several mechanisms including PP2A inactivation, up-regulation of posterior *HOXA*, and inhibition of the TGF- β signaling pathway. Given that *SETBP1* seems to be directly or indirectly involved in transcription, the binding partners among transcriptional factors and epigenetic modifiers should be elucidated to understand the precise mechanism of the transformation. Our data also suggests deregulated pathways induced by *SETBP1* mutations could be potential targets for future therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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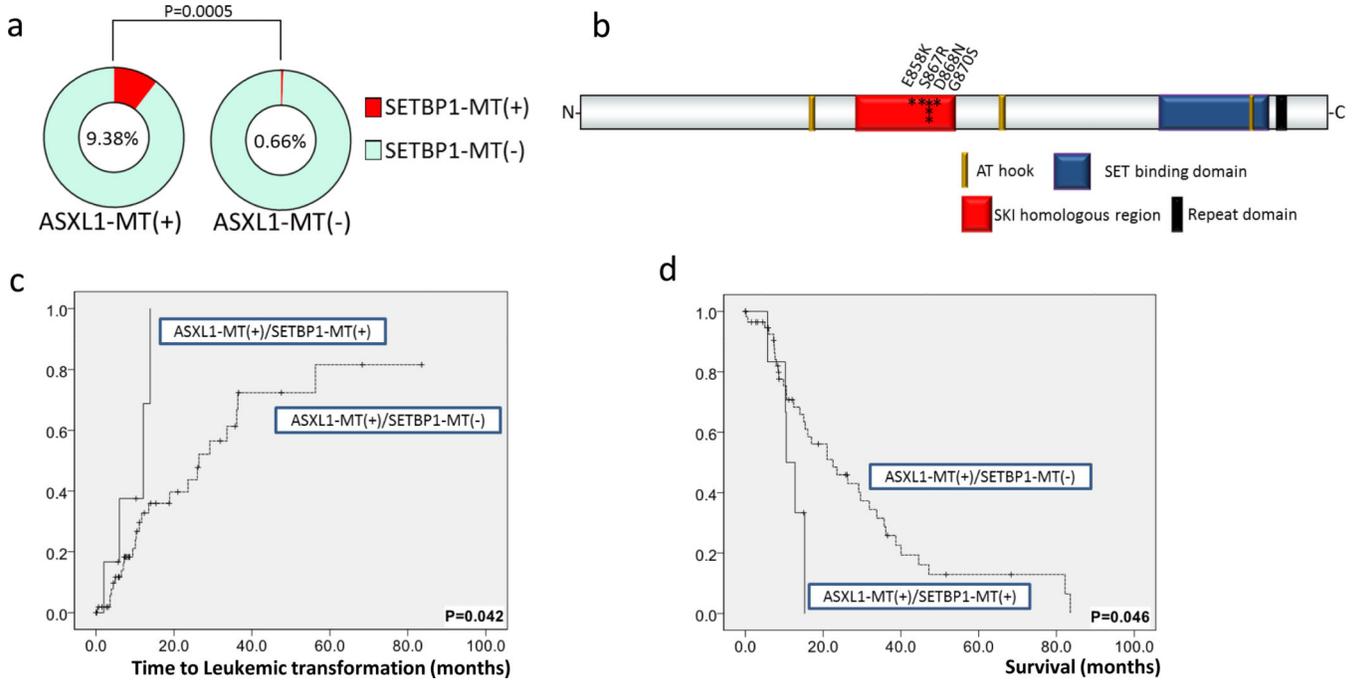
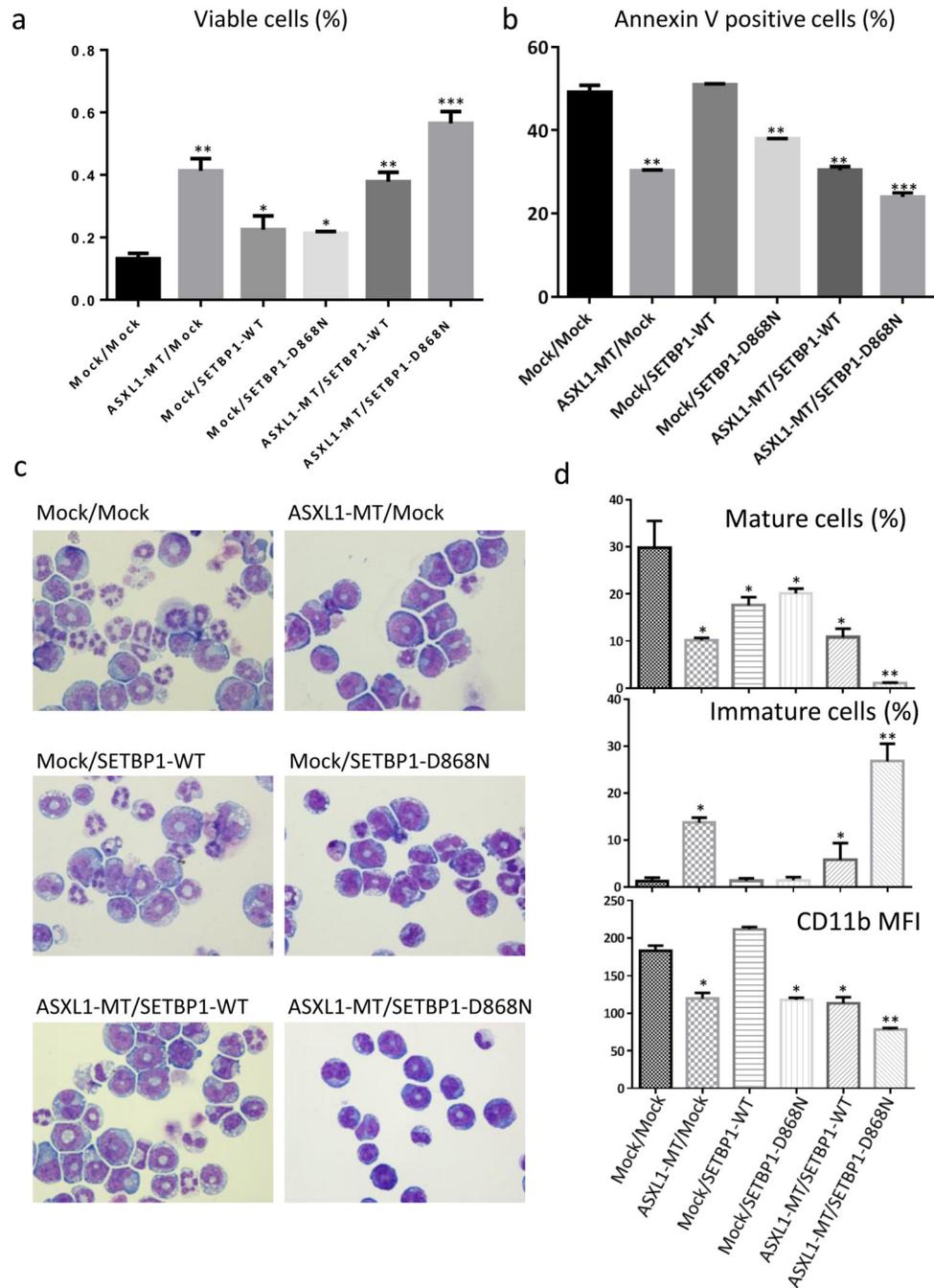


Figure 1. *SETBP1* mutations are enriched in *ASXL1*-mutant *de novo* MDS patients and inhibit ubiquitination and subsequent degradation of *SETBP1*. (a) Proportion of *SETBP1* mutations among *ASXL1*-mutated (left, n=64) or *ASXL1*-wild type (right, n=304) MDS patients are indicated. Red portion shows *SETBP1*-mutational frequency. (b) Schematic diagram of *SETBP1* protein and *SETBP1* mutations (asterisk) identified in *ASXL1*-mutated MDS patients. Three AT hook domains, SKI homologous region, SET binding domain and repeat domain are indicated. (c–d) Cumulative incidence of leukemic transformation (c) and Kaplan-Meier analysis for survival (d) in *ASXL1*-mutated MDS patients with or without *SETBP1* mutations (n=6, solid line, n=58, dashed line, respectively; *p*-values calculated using a log-rank test).

**Figure 2.**

SETBP1-MT impairs differentiation and apoptosis *in vitro*. (a–b) IL-3 dependent murine myeloid 32Dcl3 cells transduced with pMYs-IP/pMYs-IG, pMYs-IP-ASXL1-MT/mock, mock/pMYs-IG-SETBP1-WT, mock/pMYs-IG-SETBP1-D868N, pMYs-IP-ASXL1-MT/pMYs-IG-SETBP1-WT, or pMYs-IP-ASXL1-MT/pMYs-IG-SETBP1-D868N were cultured in RPMI-1640 supplemented with 10% FBS. Proportions of viable cells (a) and Annexin V positive cells (b) 24 hours after IL-3 withdrawal are shown. All data with error bars indicate the mean + SEM from duplicated experiments. (c–d) The transduced 32Dcl3 cells were

cultured with 50 ng/ml of G-CSF for 6 days. Cytospin preparations of these cells assessed by Wright-Giemsa staining (c) are shown. Original magnification, $\times 400$. Proportions of mature (segmented) cells (% , top) and immature (blastic) cells (% , middle) or mean fluorescent intensity (MFI) of CD11b estimated by FACS analysis (bottom) are shown. All data with error bars indicate the mean + SEM from duplicated experiments. Compared to the control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t* test.

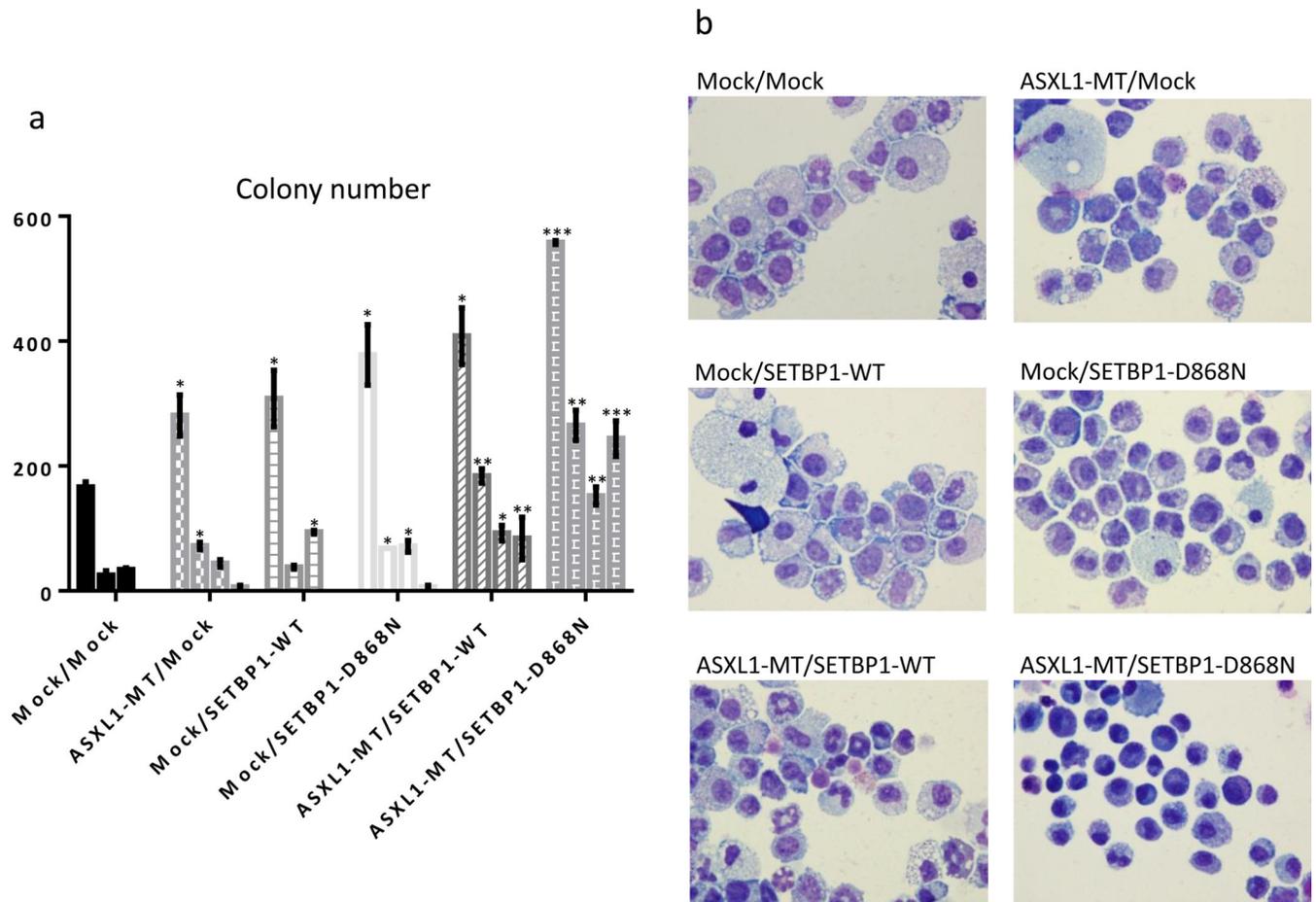


Figure 3.

Expression of both ASXL1-MT and SETBP1-D868N increased myeloid colony output. Colony-forming potentials of LSKs transduced with indicated plasmids in methylcellulose supplemented with SCF (100 ng/ml) and IL-3 (20ng/ml) are shown. Four columns represent numbers of serially replated colonies from the first (left) to the fourth (right) plating (a). All data with error bars indicate the mean \pm SEM from duplicated cultures. Cytospin preparations of cultured cells in the second replating colonies assessed by Wright-Giemsa staining are shown (b). Compared to the control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t* test.

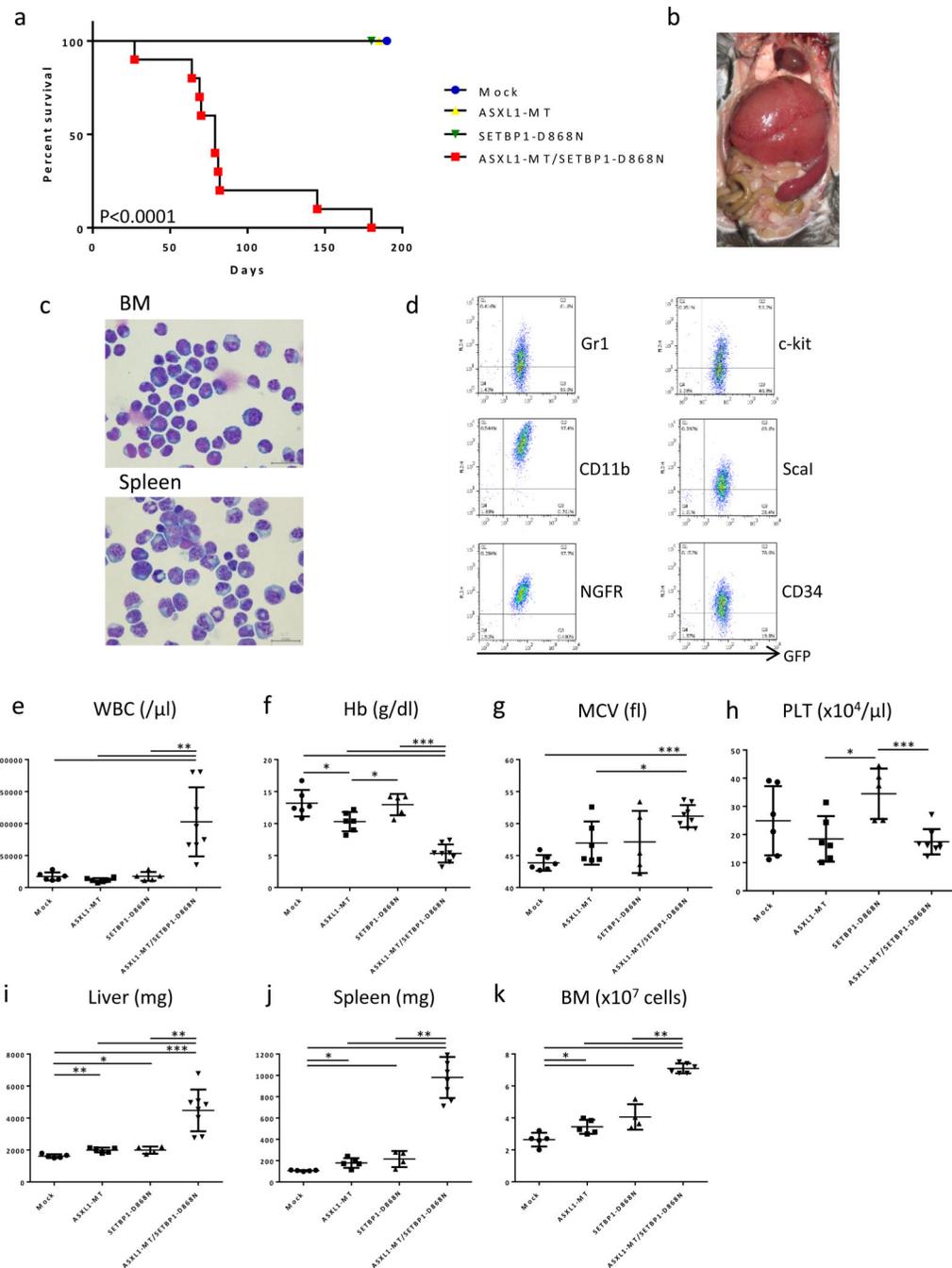
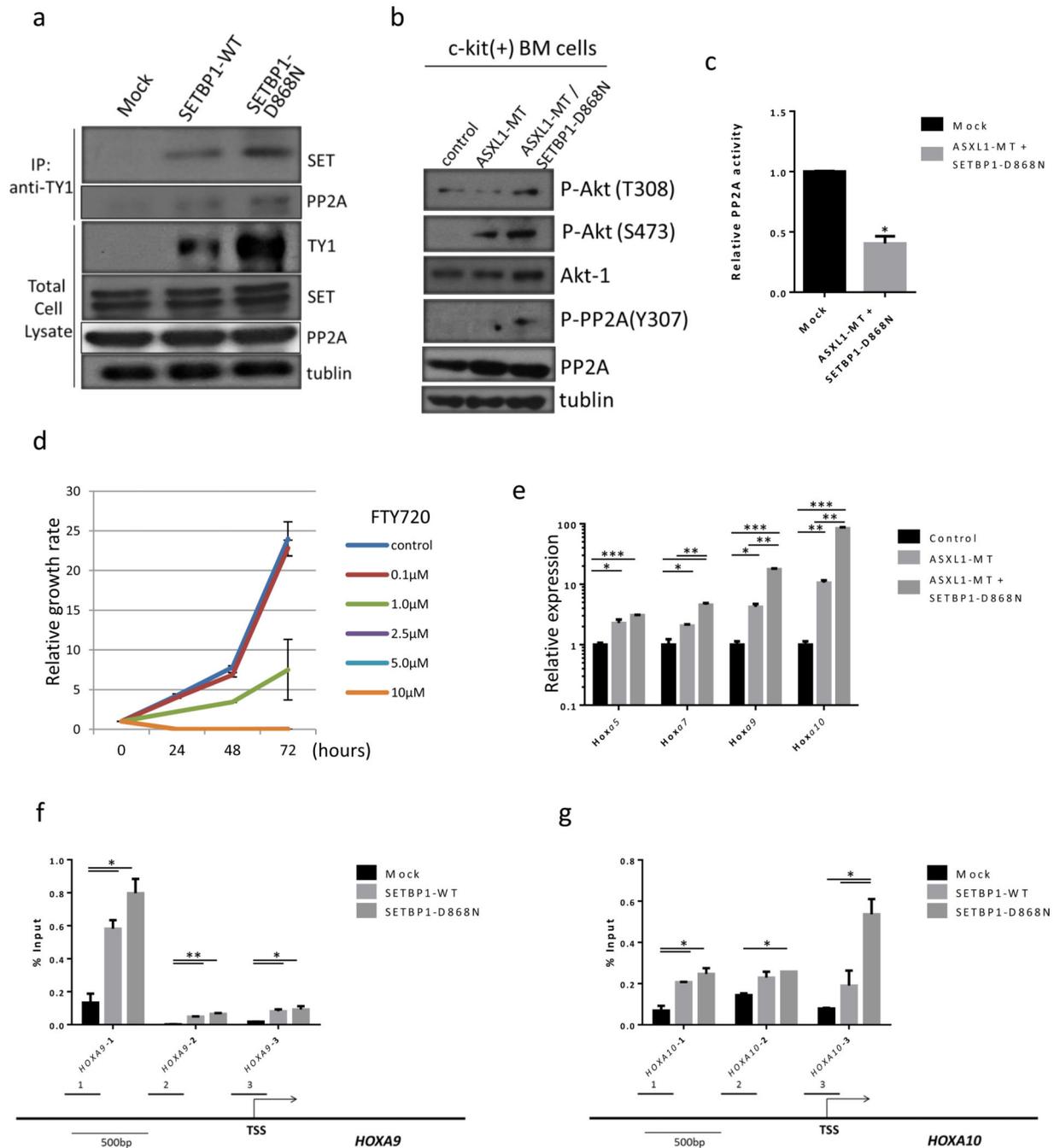


Figure 4. SETBP1-MT collaborated with ASXL1-MT in inducing AML. (a) Kaplan-Meier analysis for the survival of mice transplants with BM cells transduced with empty vector (Mock, n=6, blue circle), pMYS-ASXL1-MT-IG (ASXL1-MT, n=6, yellow triangle), pMYS-SETBP1-D868N-IG (SETBP1-D868N, n=6, green triangle), and pMYS-SETBP1-D868N-IG and pMYS-ASXL1-MT-INGFR (ASXL1-MT/SETBP1-D868N, n=9, red square). P values were calculated using a log-rank test. (b) Macroscopic findings of sacrificed mice transplanted with BM cells transduced with ASXL1-MT and SETBP1-D868N.

Representative photographs are shown. (c) Cytospin preparations of BM cells derived from mice with ASXL1-MT and SETBP1-D868N were stained with Wright-Giemsa. Representative photographs are shown. Original magnification, $\times 400$. (d) Flow cytometric analyses of BM cells derived from mice with ASXL1-MT and SETBP1-D868N. (e–k) Mice transplanted with both ASXL1-MT and SETBP1-D868N (ASXL1-MT/SETBP1-D868N, $n=8$) expressing cells displayed progressive leukocytosis (e), anemia (f), macrocytosis (g), thrombocytopenia (h), hepatosplenomegaly (i–j) and hyperplastic BM (k) compared with the control mice transplanted with empty vectors (mock, $n=6$), ASXL1-MT ($n=6$), or SETBP1-D868N ($n=5$). BM cells were isolated from the femurs and tibias of the sacrificed mice. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$, Student's *t* test.

**Figure 5.**

SETBP1 mutation resulted in Pp2a inhibition and increased *Hoxa9* and *Hoxa10* expression. (a) HEK293T cells were transiently transfected with empty vector, SETBP1-WT-3 \times TY1 and SETBP1-D868N-3 \times TY1, followed by IP of TY1 epitope and Western blotting for SET and PP2A. Cell lysates were also subject to immunoblotting with anti-TY1 Ab, anti-SET Ab, anti-PP2A Ab, or anti-tubulin Ab. (b) Cell lysates from FACS-purified c-Kit⁺ cells from the BM of mice transplanted with BM cells transduced with empty vector (control), ASXL1-MT (MDS model), or both ASXL1-MT and SETBP1-D868N (AML model), were subject to

immunoblotting with anti-phospho-Akt (T308/S473) Ab, anti-Akt Ab, anti-phospho-PP2A (Y307)Ab, anti-PP2A Ab, or anti-tubulin Ab. (c) PP2A phosphatase assay in c-Kit+ cells from a control mouse (Mock) and a leukemic mice (ASXL1-MT/SETBP1-D868N). Results are representative of 3 independent experiments. * $p < 0.05$. (d) Relative growth rate of BM cells from leukemic mice transplanted with BM cells transduced with ASXL1-MT and SETBP1-D868N in the presence of 1ng/ml IL-3 and indicated concentration of FTY720. (e) Relative expression levels of *Hoxa5/Hoxa7/Hoxa9/Hoxa10* were examined by qRT-PCR in CD3- B220- Ter119-negating BM cells derived from normal controls (Control), MDS mice (ASXL1-MT) and AML mice (ASXL1-MT + SETBP1-D868N). The values were normalized by Gapdh mRNA levels. All data with error bars are presented as mean + SEM of 2 independent experiments. (f–g) Quantitative PCR analyses following anti-TY1 ChIP in HEK293T cells transfected with empty vector, SETBP1-WT-3×TY1, SETBP1-D868N-3×TY1 using anti-TY1 antibody. PCR products specific to various regions and diagrams of tested region of *HOXA9* (f) or *HOXA10* locus (g) were shown in bottom panels. Results are representative of 3 independent experiments. Transcriptional start sites are indicated as arrows. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's *t* test.

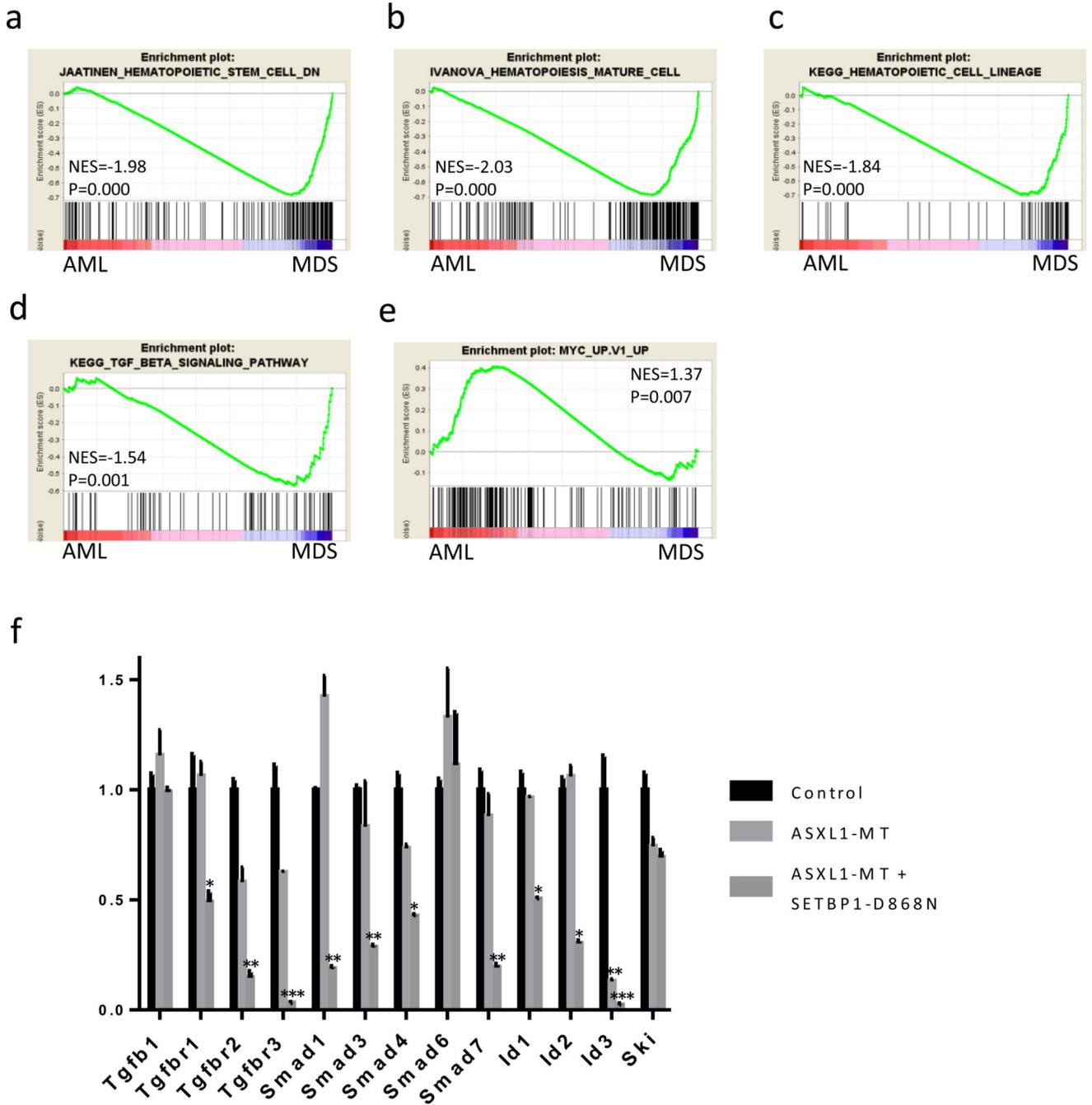


Figure 6. Combined expression of *SETBP1* and *ASXL1* mutations induces a stem cell signature and inhibits expression of genes involved in hematopoietic differentiation and TGF- β signaling. (a–e) Gene set enrichment analysis (GSEA) determining specific gene sets or pathways that are positively or negatively regulated by additional expression of SETBP1-D868N. Compared with MDS mice expressing ASXL1-MT alone (right side), AML mice expressing ASXL1-MT and SETBP1-D868N (left side) negatively correlated with gene sets downregulated in hematopoietic stem cells (a), those of mature hematopoietic cells (b), and

those of cell lineage (c). The expression profile of the AML mice also inversely correlated with gene sets of TGF- β signaling pathway (d) and associated with those of Myc pathway (e). The normalized enrichment scores (NES) and P-values are given. (f) qRT-PCR validation of genes related to TGF- β signaling pathway in the BM of control, MDS (ASXL1-MT) or AML (ASXL1-MT + SETBP1-D868N) mice. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, Student's t test. All data with error bars indicate the mean + SEM from duplicated experiments.