Author Manuscript Published OnlineFirst on March 20, 2015; DOI: 10.1158/0008-5472.CAN-14-2200
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Fig.1

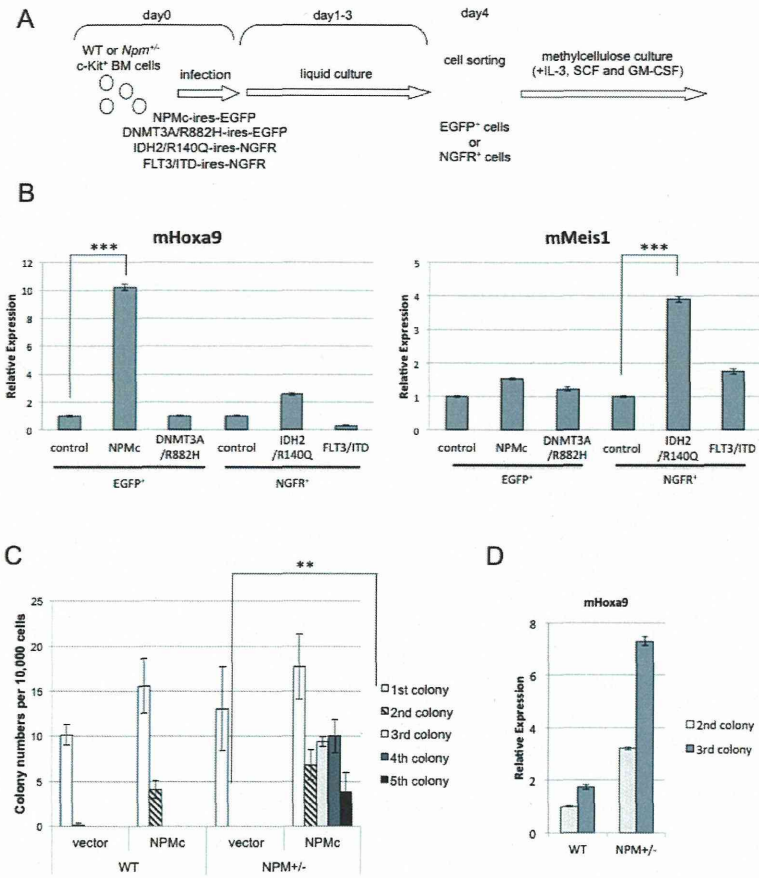

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Fig.2

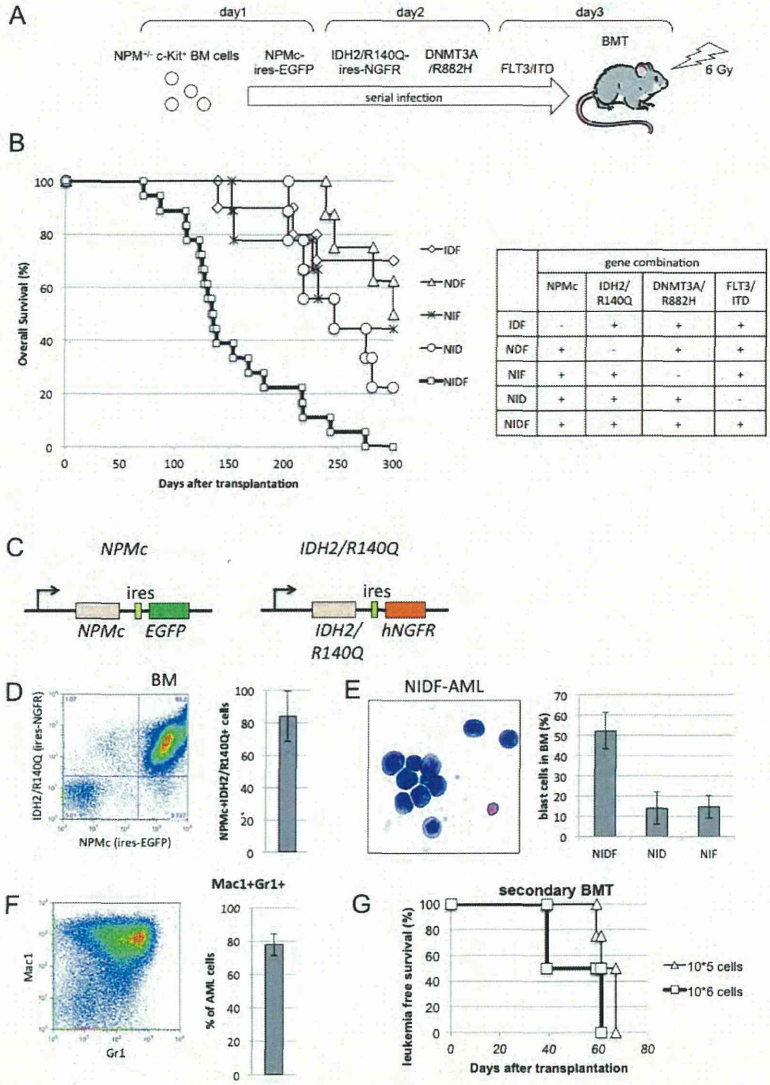

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Fig.3

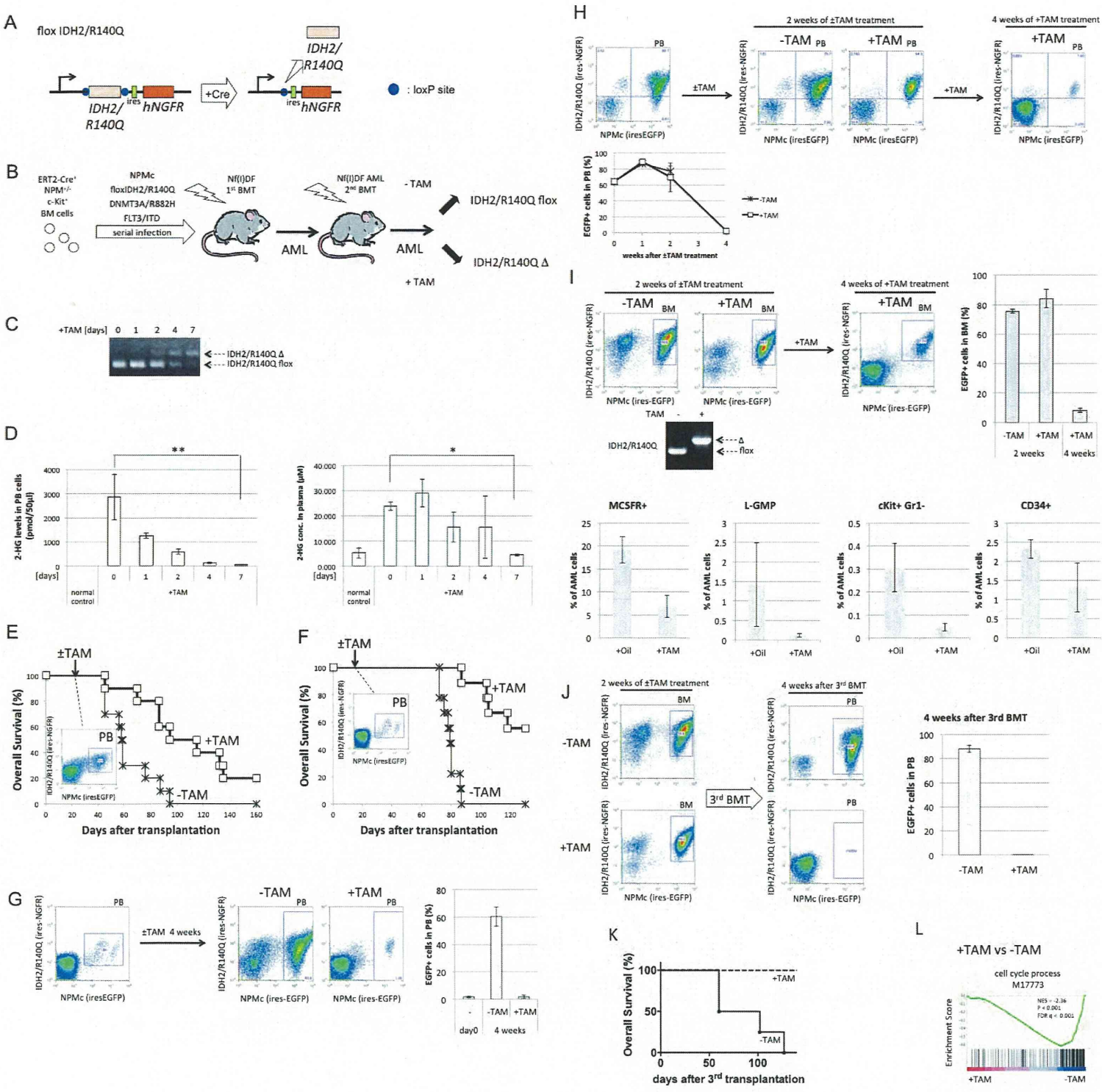

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Fig. 4

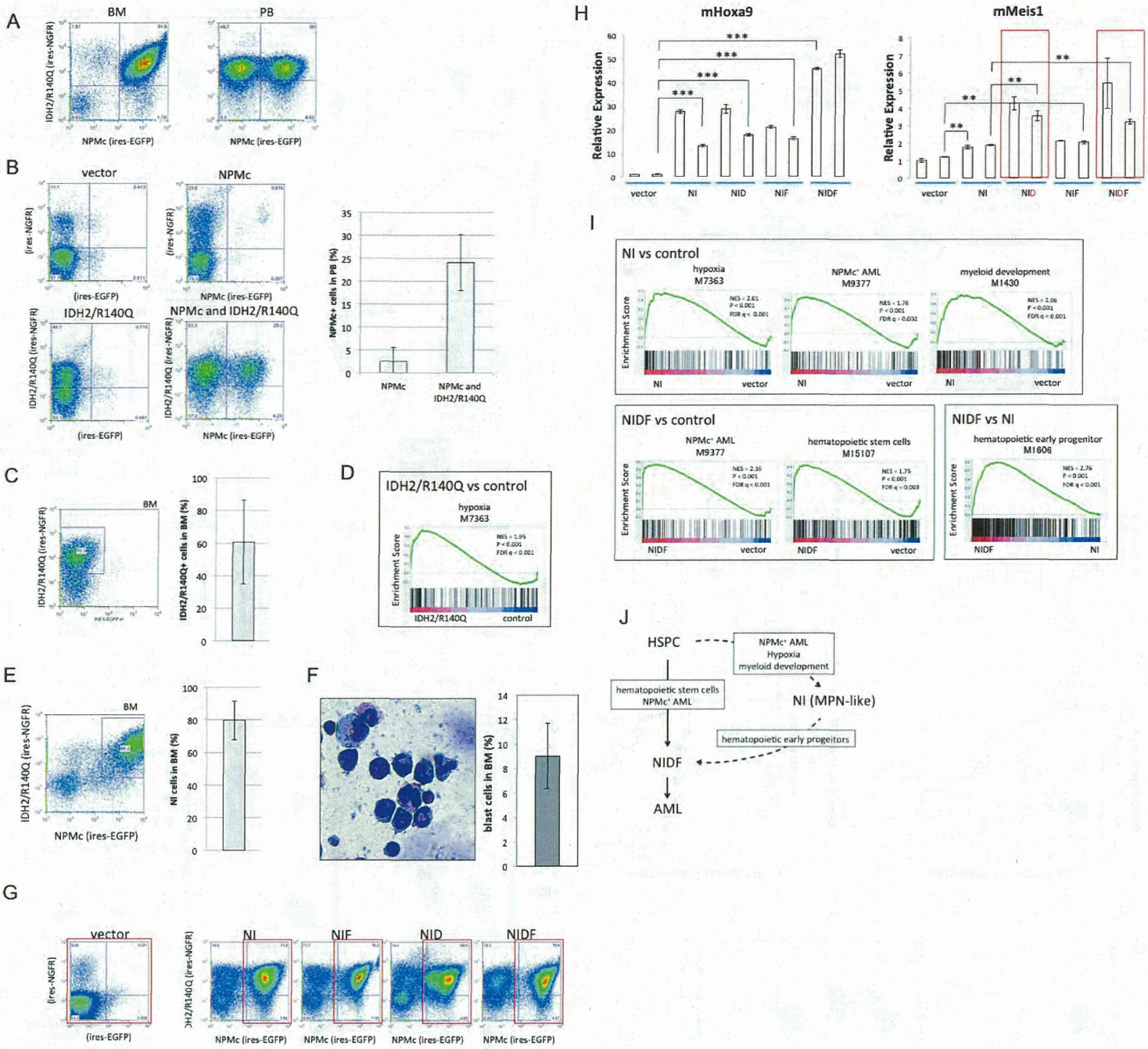

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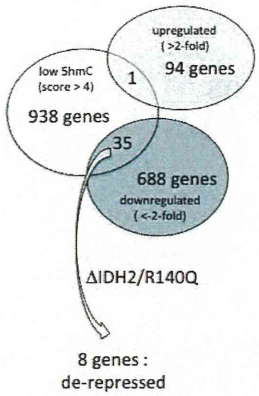
Fig. 5

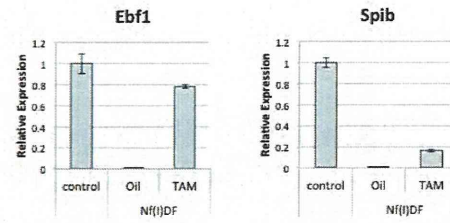
A



B

gene symbol	low5hmc score	FC NIDF vs NBM	FC Nf(I)DF TAM vs Oil
Ank1	13.243509	-3.3333268	3.877751
Ebf1	6.6217546	-5.912092	10.749922
Gm4759	11.477708	-8.610808	1.0092947
H2-Ab1	4.708803	-5.815544	10.272903
Hivep2	5.1208234	-3.7377193	2.193409
Rras2	5.297404	-2.8384004	6.244185
Scd1	4.767663	-21.843945	4.0045795
Spib	9.123306	-13.987195	6.639536

C



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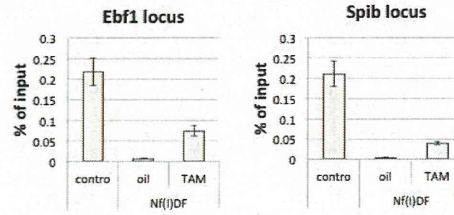
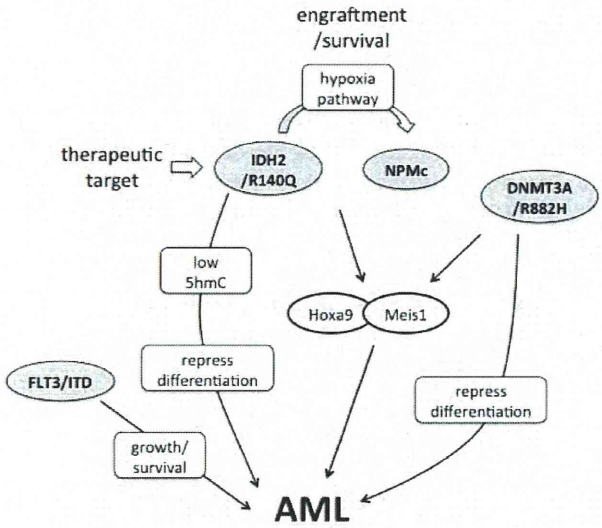


E



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ORIGINAL ARTICLE

Bromodomain-PHD finger protein 1 is critical for leukemogenesis associated with MOZ–TIF2 fusion

Haruko Shima · Kazutsune Yamagata · Yukiko Aikawa · Mika Shino · Haruhiko Koseki · Hiroyuki Shimada · Issay Kitabayashi

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Abstract Chromosomal translocations that involve the monocytic leukemia zinc finger (MOZ) gene are typically associated with human acute myeloid leukemia (AML) and often predict a poor prognosis. Overexpression of HOXA9, HOXA10, and MEIS1 was observed in AML patients with MOZ fusions. To assess the functional role of HOX upregulation in leukemogenesis by MOZ–TIF2, we focused on bromodomain-PHD finger protein 1 (BRPF1), a component of the MOZ complex that carries out histone acetylation for generating and maintaining proper epigenetic programs in hematopoietic cells. Immunoprecipitation analysis showed that MOZ–TIF2 forms a stable complex with BRPF1, and chromatin immunoprecipitation analysis showed that MOZ–TIF2 and BRPF1 interact with HOX genes in MOZ–TIF2-induced AML cells. Depletion of BRPF1 decreased the MOZ localization on HOX genes, resulting in loss of transformation ability induced by MOZ–TIF2. Furthermore, mutant MOZ–TIF2 engineered to lack histone acetyltransferase activity was incapable of deregulating HOX genes as well as initiating leukemia. These data indicate that MOZ–TIF2/BRPF1 complex upregulates HOX genes mediated by MOZ-dependent histone acetylation, leading to the development of leukemia. We suggest that activation of BRPF1/HOX pathway through MOZ HAT activity is critical for MOZ–TIF2 to induce AML.

Keywords MOZ–TIF2 · BRPF1 · *HOX* genes · AML

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Introduction

Monocytic leukemia zinc finger protein (MOZ) is a MYST (MOZ, Ybf2 (Sas3), Sas2, Tip60)-type histone acetyltransferase (HAT), and interacts with AML1, PU.1 or p53 to activate transcriptions of their target genes [1–3]. While MOZ plays a crucial role in the maintenance of normal hematopoietic stem cells [4], MOZ is also involved in chromosomal translocations such as t(8;16)(p11;p13), t(8;22)(p11;q13) and inv(8)(p11;q13), resulting in fusions of MOZ–CBP, MOZ–p300 and MOZ–TIF2, respectively, which are associated with acute myelomonocytic leukemia [5–8]. MOZ-related translocations are observed in approximately 1–6.5 % of AML, and indicate poor prognosis [9, 10]. We have previously shown that upregulation of M-CSFR (CSF1R) mediated by PU.1 is crucial for the maintenance of AML stem cells in MOZ–TIF2 leukemia [2]. However, although deletion of *CSF1R* delayed the onset of MOZ–TIF2 leukemia in vivo, approximately half of the mice transplanted with *CSF1R*-deleted, MOZ–TIF2-transduced cells developed leukemia in the long term. This may suggest the presence of another pathway involved in the generation of MOZ–TIF2 leukemia.

To pursue other pathways independent of PU.1/M-CSFR pathway, we focused on *HOX* genes. Enforced

expression of *HOXA9* or *HOXA10* immortalizes bone marrow (BM) progenitors in vitro [11, 12]. However, HOXA9 or HOXA10 overexpressed cells require relatively long latency period to induce leukemia in recipient mice. This may suggest that another complementing mutation would be needed for dominant outgrowth of transplanted cell [13, 14].

HOX genes are upregulated in BM samples of patients with MOZ-related leukemias as well as in BM cells of MOZ–TIF2-induced AML mouse model [2, 15]. MOZ forms complex with ING5 (inhibitor of growth 5), EAF6 (homolog of Esa1-associated factor 6), and BRPF1/2/3 (bromodomain-PHD finger protein 1, 2 or 3), and MOZ is the catalytic component of this HAT complex [9]. Recently, it was reported that BRPF1, a component of the HAT complex is required for the maintenance of HOX genes expression [16, 17]. Because HAT domain is intact in most of the fusions and sufficient for forming HAT complex [9], MOZ fusions may also form complex and deregulate *HOX* genes mediated by BRPF1. This study was performed to determine the role of BRPF1 in the regulation of *HOX* genes, and in the generation of MOZ–TIF2 leukemia.

Fig. 1 Effects of *Brpf1* knockdown on MOZ–TIF2 leukemic cells in vitro. **a** HA-tagged wild-type MOZ–TIF2 was cotransfected into 293FT cells together with FLAG-tagged WT BRPF1, deletion 1–222 (Δ1–222) BRPF1, or empty vector. Immunoprecipitates with anti-FLAG antibody (M2 IP) or cell lysates (Extracts) were subjected to immunoblotting with anti-HA or anti-FLAG antibodies. Δ1–222 BRPF1 lost its ability to coprecipitate with MOZ–TIF2. **b** Efficiency of *Brpf1* knockdown by Brpf1 shRNA on MOZ–TIF2 leukemic cells. RT-PCR analysis for mouse *Brpf1* (*left*) and human *BRPF1* (*right*) of MOZ–TIF2 leukemic cells expressing WT human BRPF1, Δ1–222 BRPF1 or empty vector, after transduction with mouse Brpf1 shRNA. Brpf1 shRNA significantly suppressed the expression level of mouse Brpf1 but not human BRPF1. Data are mean ± SD ($n = 3$). $^{**}P < .01$. **c** Efficiency of *Brpf1* knockdown by Brpf1 shRNA on MOZ–TIF2 leukemic cells. Western blots for both mouse Brpf1 and human BRPF1 in cell lysates from MOZ–TIF2 leukemic cells expressing WT human BRPF1, Δ1–222 BRPF1 or empty vector, after transduction with mouse Brpf1 shRNA. Brpf1 shRNA significantly suppressed the expression level of mouse *Brpf1* but not human *BRPF1*. **d** Relative colony number of MOZ–TIF2 leukemic cells transduced with control shRNA or Brpf1 shRNA. Knockdown of *Brpf1* resulted in reduction of colony formation. Overexpression of WT BRPF1 but not Δ1–222 BRPF1 restored the colony formation activity of cells with downregulated Brpf1. Data are mean ± SD ($n = 3$). $^{**}P < .01$. **e** Effects of Brpf1 shRNA on expression of *Hoxa9, Hoxa10, Meis1* and *BRPF1* in MOZ–TIF2 leukemic cells expressing WT BRPF1, Δ1–222 BRPF1 or empty vector. qRT-PCR showed that the expression level of *Hoxa9, Hoxa10* and *Meis1* was significantly suppressed by Brpf1 shRNA, which was restored by overexpression of WT BRPF1 but not Δ1–222 BRPF1. Data are mean ± SD ($n = 3$). $^{**}P < .01$

Methods

Mice

C57/BL6 mice were purchased from CREA Japan. Mouse experiments were performed in a specific pathogen-free environment at the National Cancer Center animal facility according to institutional guidelines with approval of the National Cancer Center Animal Ethics Committee.

Plasmids

MOZ and MOZ–TIF2 plasmids used here were described previously [1, 2]. Human BRPF1 cDNA was purchased from Openbiosystems and inserted into MSCV-neo vector. Human HOXA9 cDNA was isolated by cloning K562 cells and inserted into MSCV-neo vector. Deletion mutants of MOZ–TIF2 and BRPF1 and point mutants of MOZ were constructed by ligation of the cDNA fragments made by restriction enzymes and PCRs.

Retrovirus transduction and AML mouse model

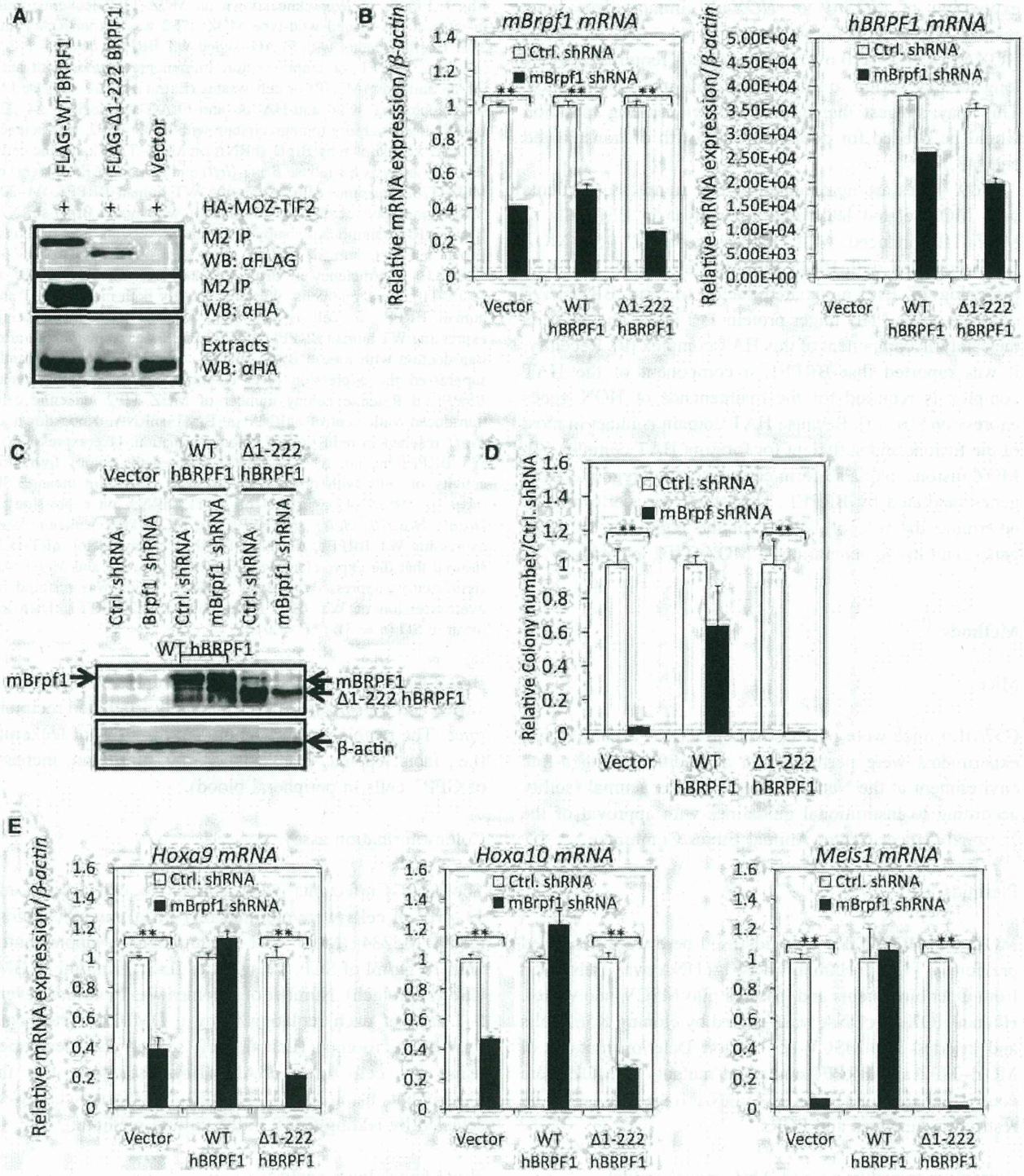
Plasmid DNA was transfected into PlatE packaging cells using the FuGENE 6 reagent (Roche Diagnostics), and supernatants containing retrovirus were collected 48 h after transfection. c-kit$^+$ progenitors were obtained from BM mononuclear cells using anti-CD117 beads (Miltenyi Biotec.) and incubated with retrovirus in a retronectin (Takara Bio)-coated plate. After 24 h of culture, cells were harvested and transplanted into sublethally irradiated recipient mice. The recipients were monitored for signs of leukemia (i.e., facial edema, lymphadenopathy, moribund, increase of GFP$^+$ cells in peripheral blood).

Colony formation assay

The GFP$^+$ infectants were sorted by cell sorter, and 1×10^4 of cells were cultured in 1 ml of methylcellulose media M3234 (Stem Cell Technologies) supplemented with 10 ng/ml of SCF, 10 ng/ml of IL-3, 10 ng/ml of GM-CSF (Peprotech). Number of colonies was monitored every 5–7 days of each replating using a DMIL inverted contrasting microscope (Leica). Cell sorting was performed using the cell sorter JSAN (Baybioscience). For the experiments using MSCV-neo vectors, infected cells were selected by adding G418 in the culture medium.

Brpf1 knockdown analysis

Brpf1 shRNA in lentiviral vectors was purchased from Openbiosystems. Viral particles were generated by co-transfection of 293FT cells with lentiviral packaging plasmids using Gene Juice (MERCK4Biosciences). $1–2 \times 10^5$ of MOZ–TIF2 leukemic cells were transduced with pLKO.1 vector or pLKO.1-Brpf1 vector by



spinoculation at 2500×*g* for 2 h at 32 °C in virus containing medium supplemented with 8 ng/ml of polybrene. The cells were resuspended in StemPro-34 SFM medium (Invitrogen) containing cytokines (20 ng/ml SCF, 10 ng/ml OSM, 10 ng/ml IL-3) for 24 h. The infectants were plated in methylcellulose media supplemented with 10 ng/ml of SCF, 10 ng/ml of IL-3, 10 ng/ml of GM-CSF, in the presence of puromycin (4 μg/ml) for selection of infected cells. Three days after selection with puromycin, 1×10^4 cells were plated in 1 ml of methylcellulose media and were proceeded to colony formation assay. The redundant infectants were used for qRT-PCR assay and

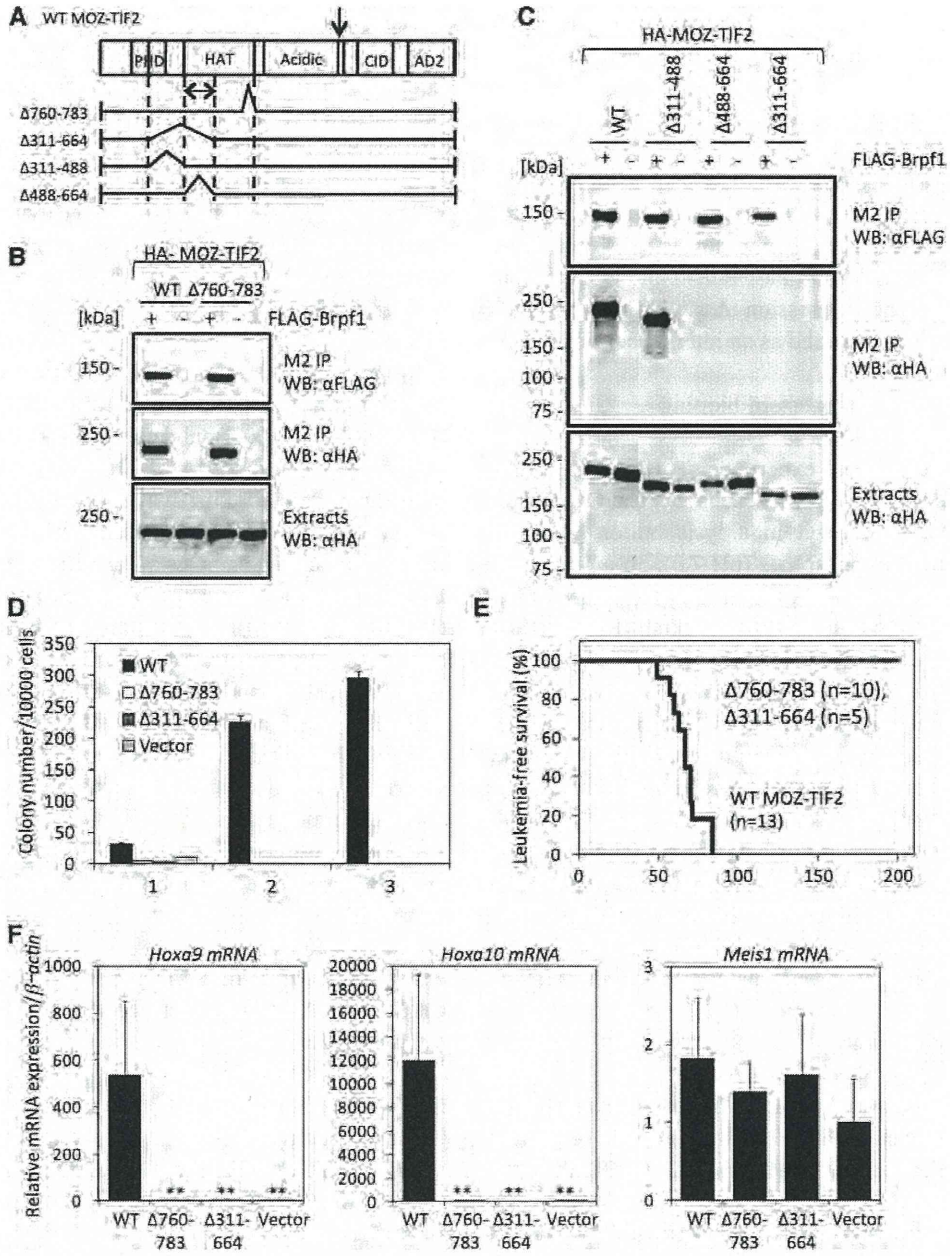


Fig. 2 Interacting domain of MOZ–TIF2 with Brpf1. **a** Structure of MOZ–TIF2 and its deletion mutants. **b** HA-tagged WT or Δ760–783 MOZ–TIF2 was cotransfected into 293FT cells together with FLAG-tagged Brpf1. Immunoprecipitates with anti-FLAG antibody (M2 IP) or cell lysates (Extracts) were subjected to immunoblotting with anti-HA or anti-FLAG antibodies. Both WT and Δ760–783 MOZ–TIF2 interacted with Brpf1. **c** HA-tagged WT or deletion mutants of MOZ–TIF2 were cotransfected into 293FT cells together with FLAG-tagged Brpf1. Immunoprecipitates with anti-FLAG antibody (M2 IP) or cell lysates (Extracts) were subjected to immunoblotting with anti-HA or anti-FLAG antibodies. MOZ–TIF2 containing N-terminal region of MOZ HAT domain (N488–664) interacted with Brpf1. **d** Number of colonies formed by cells transduced with WT, Δ760–783, Δ311–664 MOZ–TIF2 or empty vector. Both of the deletion mutants lost colony formation activity after 2nd round of replating. Data are mean ± SD ($n = 3$). **e** Kaplan–Meier survival curve analysis of mice transplanted with WT, Δ760–783 or Δ311–664 MOZ–TIF2. All of the mice transplanted with WT MOZ–TIF2 developed AML, while all the mice transplanted with deletion mutants of MOZ–TIF2 survived without development of leukemia. **f** First round colonies of cells transduced with WT, Δ760–783, Δ311–664 MOZ–TIF2 or empty vector were collected and analyzed for expression levels of *Hoxa9, Hoxa10* and *Meis1* by qRT-PCR analysis. Increased expression levels of *Hoxa9* and *Hoxa10* were only observed in cells transduced with WT MOZ–TIF2. Data are mean ± SD ($n = 3$). $^{**}P < .01$

immunoblotting to determine the knockdown level of *Brpf1* or chromatin immunoprecipitation (ChIP) assay.

Quantitative real-time PCR (qRT-PCR) analysis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). The cDNA was reverse-transcribed using Superscript® VILO™ (Invitrogen). Expression levels of genes were detected using the ABI 7500 Fast Real-Time PCR System with TaqMan® Gene Expression Assay Mixes (Applied Biosystems). β-actin was used as an internal control.

Immunoprecipitation and western blotting

For immunoprecipitation experiments, certain plasmids were co-transfected into 293FT cells by $CaPO_4$ co-precipitation. Cells were lysed in a lysis buffer [250 mM NaCl, 20 mM sodium phosphate (pH 7.0), 30 mM sodium pyrophosphate, 10 mM NaF, 0.1 % NP-40, 5 mM DTT, 1 mM PMSF, and protease inhibitor cocktail (Roche)], and cell lysates were incubated with anti-FLAG antibody-conjugated agarose beads (Sigma). After rotation at 4 °C overnight, and washing with lysis buffer, precipitated proteins were eluted by FLAG peptide and dissolved with SDS sample buffer. The blots were probed with anti-FLAG (M2) (Sigma), anti-HA (3F10) (Roche), anti-MOZ [1], anti-Brpf1 (Sigma-Aldrich), or anti-β actin (clone AC-15) (Sigma) as primary antibodies, and horseradish peroxidase-conjugated secondary antibodies (SouthernBiotech). The bands were detected by chemiluminescence using ECL plus Detection Reagents (Amersham Biosciences).

Histone acetylation assay

Immunoprecipitates with anti-FLAG antibody obtained from 293FT cells transfected with FLAG-tagged WT or mutant MOZ were mixed with 50 mM Tris–HCl (pH 8.0), 10 % glycerol, 1 mM dithiothreitol, 0.5 μl of [^{14}C] acetyl-CoA (50 μCi/ml, Amersham), and 1 μg of histone H2A, H2B, H3 and H4, respectively, and incubated at 30 °C for 1 h. After incubation, the samples were subjected to 15 % sodium dodecyl sulfate-PAGE, and the gels were analyzed for the levels of histone acetylation by BAS-2500 (FUJIFILM).

ChIP assay

Cells were fixed with 1 % formaldehyde for 10 min at room temperature and further incubated with 0.125 M glycine for 5 min to stop cross-linking reaction. Cells were then washed with ice-cold PBS containing protease inhibitor cocktail, centrifuged, and the pellets were lysed in lysis buffer (50 mM HEPES pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % sodium dodecyl sulfate, and protease inhibitor cocktail). The lysates were sonicated until the average DNA fragment length was 200–500 bp, using Branson Sonicator, diluted in 10× dilution buffer (1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0, 150 mM NaCl and protease inhibitor cocktail), and incubated with antibodies at 4 °C overnight. On the following day, Dynabeads Protein G (Invitrogen) was added and incubated for another 6 h at 4 °C. The immunoprecipitates were washed twice with low salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0 and 150 mM NaCl), once with high salt buffer (0.1 % SDS, 1 % Triton X-100, 2 mM EDTA, 20 mM Tris–HCl pH 8.0 and 500 mM NaCl) and finally twice with PBS containing 0.1 % Triton X-100. Bound chromatin was eluted in elution buffer (1 % SDS and 0.1 M $NaHCO_3$), and together with input DNA, crosslinking was reversed by overnight incubation at 65 °C with addition of 200 mM NaCl to the elution buffer. The eluted samples were then treated with 10 mM EDTA, 40 mM Tris–HCl (pH 6.5), and proteinase K (Roche) at 45 °C for 2 h. Finally, the immunoprecipitated and input DNA were extracted with phenol/chloroform extraction and ethanol precipitation, and analyzed by qRT-PCR using FastStart Universal SYBR Green Master (Roche) and 7500 Fast Real-Time PCR system.

Primer sequences are as follows.

Hoxa7 promoter:

Forward primer (F)/5′-GAGAGGTGGGCAAAGAGTG G-3′, Reverse primer (R)/5′-CCGACAACCTCATACCTA TTCCTG-3′

Hoxa7 coding:

F/5′-CTGGACCTTGATGCTTCTAACT-3′, R/5′-AGC CAGAGAAAGAGGGATTCTA-3′

Hoxa9 promoter:

F/5′-GAGCGGTTCAGGTTTAATGC-3′, R/5′-TGCCT GCTGCAGTGTATCAT-3′

Hoxa9 coding:

F/5′-GGTCCCGTGTGAGGTACATGT-3′, R/5′-CAAA ACACCAGACGCTGGAA-3′

Hoxa10 promoter:

F/5′-CGGCCTTTGAGCCATAGGT-3′, R/5′-GCCCGC GATTGATATAAATATGT-3′

Hoxa10 coding:

F/5′-TTCGGGCATCCCACTAAATG-3′, R/5′-GGCCA CTCGGGCTGTATG-3′

Hoxa13 promoter:

F/5′-TCCTTGGATGAGCGTTCTCT-3′, R/5′-TGCAT GTTAAGTGCCTGCTC-3′

β-actin promoter:

F/5′-GCAGGCCTAGTAACCGAGACA-3′, R/5′-AGTTT TGGCGATGGGTGCT-3′

Myf5 promoter:

F/5′-GGAGATCCGTGCGTTAAGAATCC-3′, R/5′-CG GTAGCAAGACATTAAAGTTCCGTA-3′