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ORIGINAL ARTICLE

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

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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

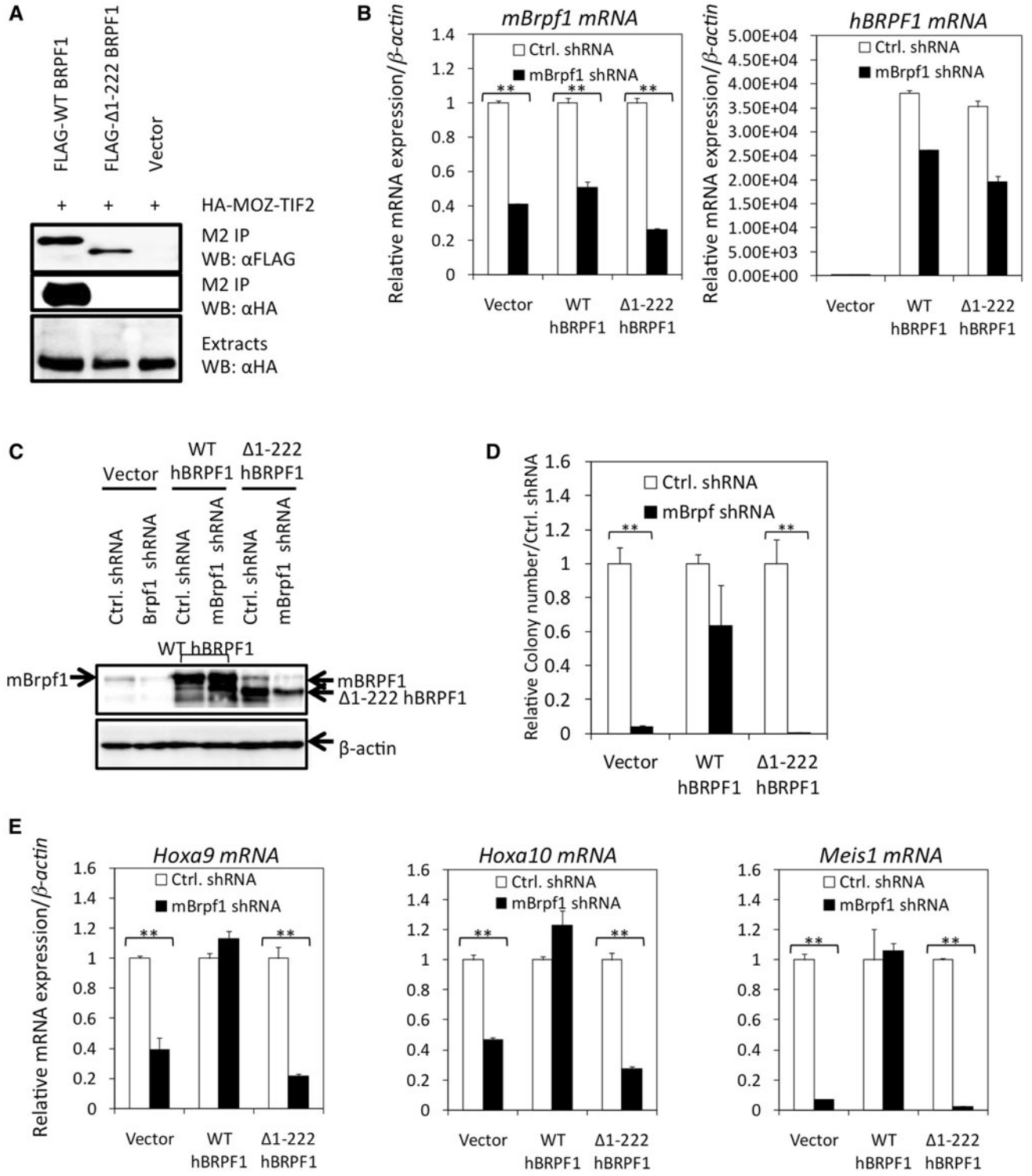
Plasmid DNA was transfected into PlatE packaging cells using the FuGENE 6 reagent (Roche Diagnostics), and supernatants containing retrovirus were collected 48 h after transfection. c-kit$^+$ progenitors were obtained from BM mononuclear cells using anti-CD117 beads (Miltenyi Biotec.) and incubated with 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

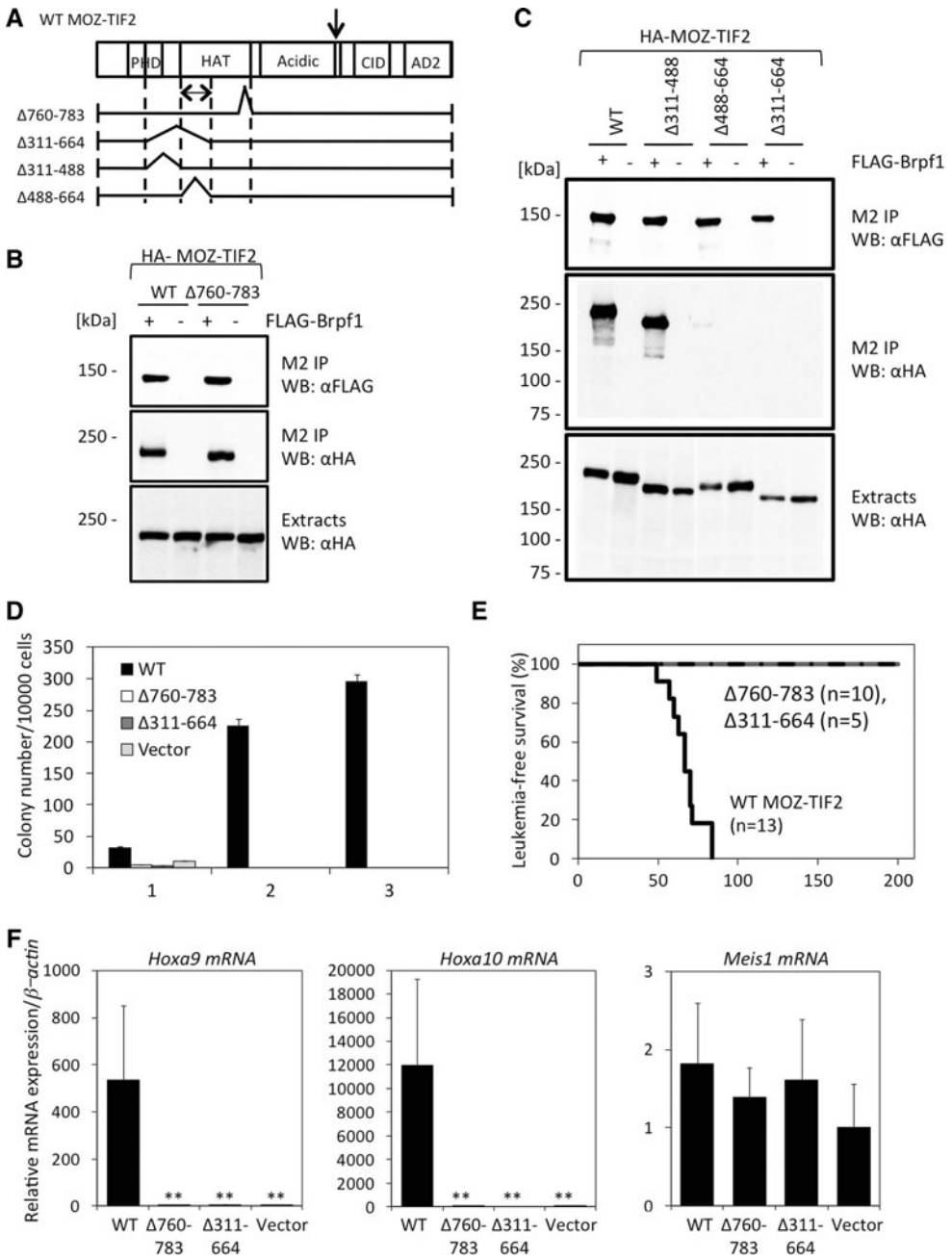


Fig. 2 Interacting domain of MOZ–TIF2 with Brpf1. **a** Structure of MOZ–TIF2 and its deletion mutants. **b** HA-tagged WT or Δ760–783 MOZ–TIF2 was cotransfected into 293FT cells together with FLAG-tagged Brpf1. Immunoprecipitates with anti-FLAG antibody (M2 IP) or cell lysates (Extracts) were subjected 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′-GAGAGGTGGGCAAAGAGTGG-3′, Reverse primer (R)/5′-CCGACAACCTCATACCTATTCCTG-3′

Hoxa7 coding:

F/5′-CTGGACCTTGATGCTTCTAACT-3′, R/5′-AGCCAGAGAAAGAGGGATTCTA-3′

Hoxa9 promoter:

F/5′-GAGCGGTTCAGGTTTAATGC-3′, R/5′-TGCCTGCTGCAGTGTATCAT-3′

Hoxa9 coding:

F/5′-GGTCCCGTGTGAGGTACATGT-3′, R/5′-CAAAACACCAGACGCTGGAA-3′

Hoxa10 promoter:

F/5′-CGGCCTTTGAGCCATAGGT-3′, R/5′-GCCCGCGATTGATATAAATATGT-3′

Hoxa10 coding:

F/5′-TTCGGGCATCCCACTAAATG-3′, R/5′-GGCCACTCGGGCTGTATG-3′

Hoxa13 promoter:

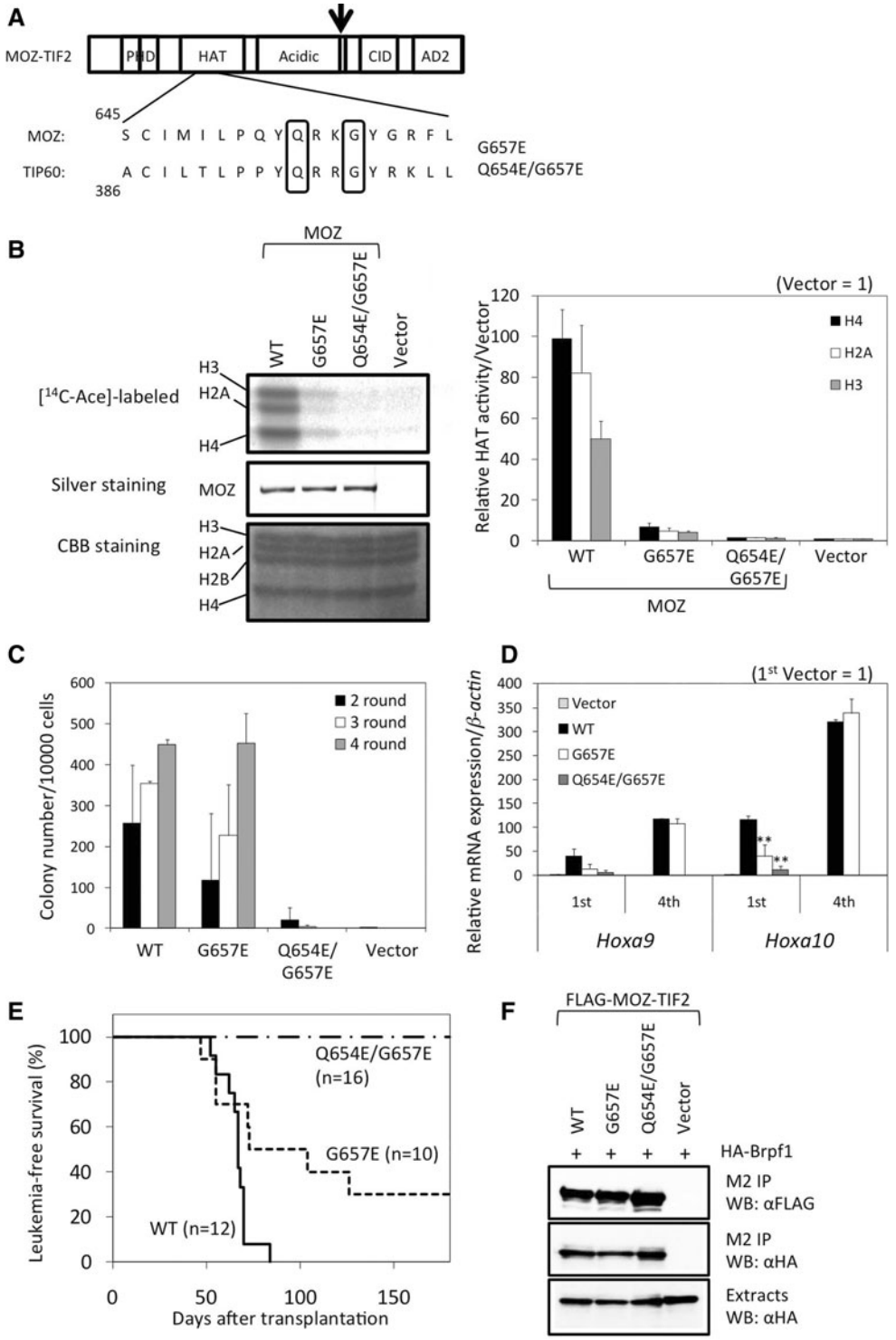
F/5′-TCCTTGGATGAGCGTTCTCT-3′, R/5′-TGCATGTTAAGTGCCTGCTC-3′

β-actin promoter:

F/5′-GCAGGCCTAGTAACCGAGACA-3′, R/5′-AGTTTTGGCGATGGGTGCT-3′

Myf5 promoter:

F/5′-GGAGATCCGTGCGTTAAGAATCC-3′, R/5′-CGGTAGCAAGACATTAAAGTTCCGTA-3′



The antibodies used in these experiments are the same as written above for MOZ and Brpf1, and anti-acetyl-Histone H3 (Upstate), and anti-Histone H3 (abcam).

Statistical analysis

Statistical significance was determined by two-tailed Student *t* test.

Results

Brpf1 is required for immortalization of MOZ–TIF2 leukemic cells

Previous studies have suggested that BRPF1 forms complex with MOZ, and interacts directly to MOZ at N-terminal

◀ **Fig. 3** HAT activity of MOZ is required for transformation activity by MOZ–TIF2. **a** Structure of MOZ–TIF2 and its point mutants, Q654E/G657E and G654E. **b** HAT activity of WT and point mutants of MOZ. WT, G654E, Q654E/G657E MOZ or empty vector were cotransfected into 293FT cells. Immunoprecipitates were collected and cultured with histone H2A/B, H3, H4 and C^{14} labeled-acetyl-coenzyme A. C^{14} labeled acetylated histone was detected by BAS. Although HAT activity was reduced in both point mutants of MOZ compared to WT MOZ, G657E MOZ retained subtle HAT activity. Data are mean ± SD ($n = 3$). **c** Colony numbers of cells transduced with WT, G654E, Q654E/G657E MOZ–TIF2 or empty vector. WT and G657E MOZ–TIF2 showed transformation activity, which was not observed in cells transduced with Q654E/G657E MOZ–TIF2 or empty vector. **d** First round and 4th round colonies of cells transduced with WT, G654E, Q654E/G657E MOZ–TIF2 or empty vector were collected and analyzed for expression levels of *Hoxa9, Hoxa10* by qRT-PCR analysis. Expression level of *Hoxa10* was significantly lower in colony cells transduced with both mutants compared to WT MOZ–TIF2 at 1st round, but reached to the similar levels at 4th round in cells with Q654E/G657E MOZ–TIF2. Data are mean ± SD ($n = 3$). $^{**}P < .01$. **e** Kaplan–Meier survival curve analysis of mice transplanted with WT, G657E or Q654E/G657E MOZ–TIF2. All of the mice transplanted with WT MOZ–TIF2 and 7 out of 10 mice transplanted with G657E MOZ–TIF2 developed AML, while none of the mice transplanted with Q654E/G657E MOZ–TIF2 developed leukemia. **f** FLAG-tagged WT, G654E, Q654E/G657E MOZ–TIF2 or empty vector were cotransfected into 293FT cells together with HA-tagged Brpf1. Immunoprecipitates with anti-FLAG antibody (M2 IP) or cell lysates (Extracts) were subjected to immunoblotting with anti-HA or anti-FLAG antibodies. WT and both point mutants of MOZ–TIF2 were able to interact with Brpf1

domain [18]. Thus, we firstly constructed deletion mutant of BRPF1 (Δ1–222) which lacks interacting domain with MOZ, and performed immunoprecipitation assay. This mutant BRPF1 consistently lacked potential to form complex with MOZ (Fig. 1a). Using wild-type (WT) and deletion mutant of BRPF1, we next examined the effect of *Brpf1* knockdown in MOZ–TIF2 leukemic cells. MOZ–TIF2-tranduced mouse BM cells were subsequently transduced with Brpf1 shRNA by lentivirus system. Knockdown efficiencies were confirmed by qRT-PCR and western blotting (Fig. 1b, c). *Brpf1* knockdown resulted in marked decrease of colony numbers, which was rescued by WT human BRPF1 but not by mutant human BRPF1 (Fig. 1d). We further examined the effect of *Brpf1* knockdown on transcriptional activation induced by MOZ–TIF2. As expected, *Brpf1* knockdown led to decrease of *Hoxa9*, *Hoxa10* and *Meis1* expression (Fig. 1E). This decrease was again restored by WT human BRPF1 but not by mutant human BRPF1. These data suggest that Brpf1 contributes to *Hoxa9, Hoxa10* and *Meis1* transcription, which are overexpressed in AML patients with MOZ fusions [15].

Interacting domain of MOZ with Brpf1 is essential for initiation of MOZ–TIF2 leukemia

To investigate the importance of MOZ interaction with Brpf1, we firstly constructed several MOZ–TIF2 mutants as shown in Fig. 2a, and performed immunoprecipitation assay. The 760–783 region of MOZ was previously reported to be the interacting domain with BRPF1 [18]. However, deletion of the region 760–783 did not affect interaction of MOZ–TIF2 with BRPF1 in our condition (Fig. 2b). We previously found that the 760–783 region is required for histone acetyltransferase (HAT) activity [1]. Deletion analysis showed that there is another interacting domain that localized at HAT domain (N488–664) (Fig. 2c). For the following assay, we selected two deletion mutants of MOZ–TIF2, previously reported mutant Δ760–783, and Δ311–664 which lacks the novel interacting domain. We transduced these two deletion mutants, WT MOZ–TIF2 or empty vector to BM progenitors and performed colony formation assay. While WT MOZ–TIF2 cells were capable of forming colonies for several times of replating, others including two deletion mutants of MOZ–TIF2 lost their ability to form colonies in the 2nd round (Fig. 2d). 1st round colony cells were collected and analyzed for expression levels of *Hoxa9*, *Hoxa10* and *Meis1* by qRT-PCR. Corresponding to the results of colony formation assay, transcriptional activation of *Hoxa9* and *Hoxa10* induced by WT MOZ–TIF2 was not observed by two deletion mutants (Fig. 2f). Furthermore, none of the mice injected with BM cells transduced with either of the deletion mutants of MOZ–TIF2 developed AML (Fig. 2e).

HAT activity of MOZ contributes to leukemic transformation induced by MOZ–TIF2

The 760–783 and 488–664 regions were localized in MOZ HAT domain [1]. Although HAT activity is reported to be dispensable for leukemogenesis by MOZ–TIF2 [10], we evaluated the possible association between lack of MOZ HAT activity and loss of leukemogenic potential in Δ311–664 or Δ760–783 mutant MOZ–TIF2. MOZ is the founding member of the MYST family of HATs, which share the conserved MYST domains [5]. To assess the importance of MOZ HAT activity, we constructed previously reported MOZ HAT-deficient G657E mutant as well as Q654E/G657E mutant according to the HAT-deficient mutant of TIP60 (Fig. 3a) [8, 19, 20]. Firstly, we assessed HAT activity of these two HAT-deficient mutants using immunoprecipitates with anti-FLAG antibody, obtained from 293FT cells transfected with FLAG-tagged WT or mutant MOZ (Fig. 3b). WT MOZ showed HAT activity for Histones H2A, H3, H4. Although both HAT-deficient mutants G657E and Q654E/G657E possessed markedly low HAT activity compared to WT MOZ, HAT activity slightly remained in G657E mutant. Next, we examined the colony formation activity of these two HAT-deficient mutants. BM progenitors were transduced with WT, G657E, Q654E/G657E mutant of MOZ–TIF2 or empty vector and subsequently cultured in methylcellulose media. Through 4 rounds of replating, WT and G657E mutant, but not Q654E/G657E mutant, were capable of

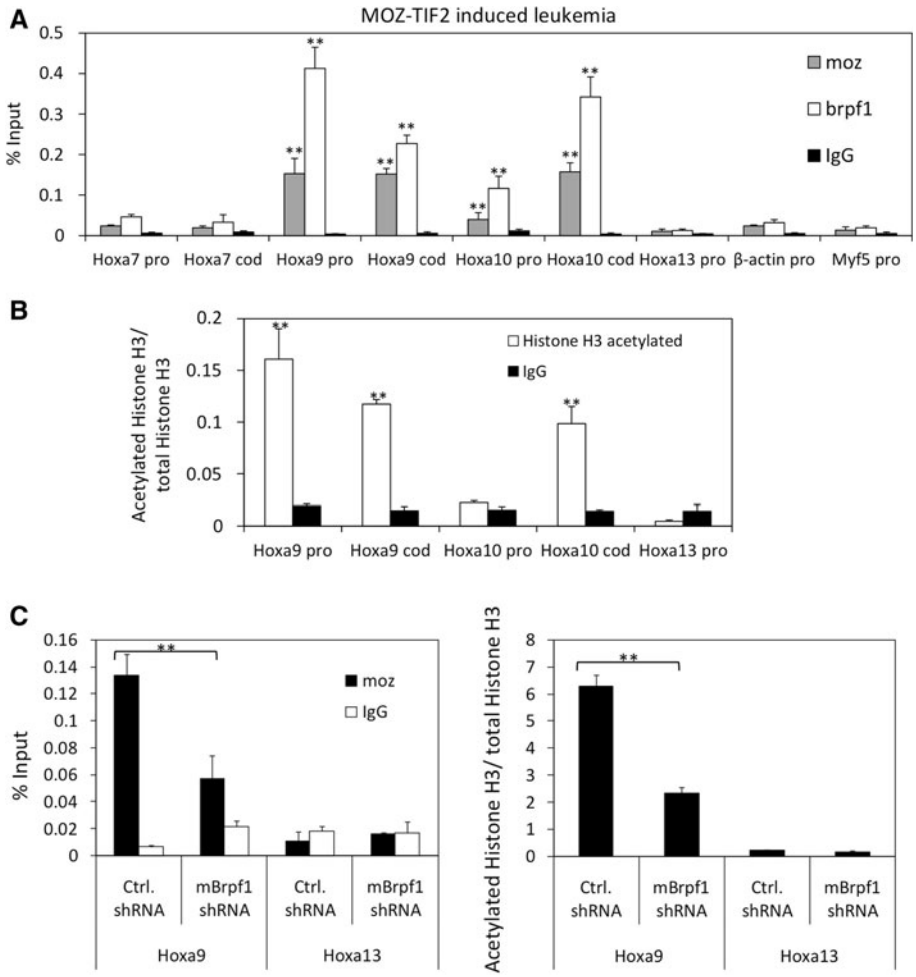


Fig. 4 *Hoxa9* and *Hoxa10* are direct targets of Moz and Brpf1. **a, b** Relative binding of Moz, Brpf1 and acetylated Histone H3 to the promoter and coding region of indicated genes in MOZ–TIF2 leukemic cells. Moz and Brpf1 colocalized at *Hoxa9* and *Hoxa10* genes, in which acetylated Histone H3 levels were higher compared to other tested genes. Data are mean ± SD ($n = 3$). $^{**}P < .01$. **c** Relative binding of Moz and acetylated Histone H3 to the promoter region of *Hoxa9* and *Hoxa13* after *Brpf1* knockdown. Downregulation of Brpf1 reduced the localization of Moz on promoter region of Hoxa9 and also reduced Histone H3 acetylation. Data are mean ± SD ($n = 3$). $^{**}P < .01$

inducing serial replating activity (Fig. 3c). *Hoxa10* expression levels of the 1st round colony cells were decreased by both mutants, but increased to similar level as WT in the 4th round by G657E mutant (Fig. 3d). BM progenitors transduced with WT, G657E or Q654E/G657E mutant of MOZ–TIF2 were also transplanted into sublethally irradiated recipient mice. G657E mutant MOZ–TIF2 led to development of AML in a subset of mice as reported before [8]. However, Q654E/G657E mutant MOZ–TIF2 showed no potential to initiate leukemia (Fig. 3e). These two HAT-deficient mutants were confirmed to be capable of interacting with Brpf1 at similar level as WT MOZ–TIF2 (Fig. 3f). These results indicate that even in a slight level, MOZ HAT activity is required for leukemogenic activity induced by MOZ–TIF2, which was independent of interaction with Brpf1.

Hoxa9 and *Hoxa10* are direct targets of MOZ and BRPF1 complex

To understand the mechanism of leukemic transformation induced by MOZ–TIF2, we performed chromatin immunoprecipitation (ChIP) assay using MOZ–TIF2 colony cells. As shown in Fig. 4a, MOZ and Brpf1 colocalized on chromatin within *Hoxa9* and *Hoxa10* locus, suggesting that these genes are direct targets of MOZ and Brpf1 complex. Indeed, Histone H3 acetylation level was upregulated at target *Hoxa9* and *Hoxa10* genes (Fig. 4b). We also performed ChIP assay using Brpf1 shRNA expressing MOZ–TIF2 colony cells. Depletion of Brpf1 resulted in reduction of MOZ localization on target *Hoxa9* gene, suggesting that Brpf1 enhances the enrichment level of MOZ localization on target genes (Fig. 4c). Together, these data suggest that Brpf1 leads to MOZ localization on the target genes, *Hoxa9* and *Hoxa10*, which enhance histone H3 acetylation and transcriptional activation of these genes, eventually resulting in development of AML.

Hoxa9 overexpression is not substantial for transformation of MOZ–TIF2 in Brpf1 downregulated cells

Previously, HOXA9 alone was reported to be sufficient for leukemic transformation in vitro [11, 13]. Because MOZ–

TIF2 leukemic cells exhibited lower expression level of *Hoxa9,* we assessed the effect of HOXA9 overexpression in MOZ–TIF2 leukemic cells with *Brpf1* knockdown. Unexpectedly, enforced expression of human HOXA9 failed to restore colony formation ability that was impaired by *Brpf1* knockdown (Fig. 5a, b). Downregulation of endogenous *Hoxa9, Hoxa10* and *Meis1* by depletion of Brpf1 was not restored by HOXA9 overexpression (Fig. 5c), suggesting again that Brpf1 contributes to transcriptional activation of not only *Hoxa9* but also *Hoxa10* and *Meis1*, both of which do influence the leukemic transformation activity. [12, 14, 21, 22].

Discussion

HOX genes are deregulated in a subset of AML patients, which strongly correlate with poor prognosis [23, 24]. Human AML with MOZ fusions applies to this subset of group, with high levels of *HOXA9* and *HOXA10* [15]. Herein, upregulation of *HOX* genes may contribute to the leukemogenesis in MOZ-related AML. To assess the mechanism and the role of *HOX* genes deregulation in MOZ-related leukemias, we focused on Brpf1. Brpf1, a component of MOZ complex, possesses PHD finger domain, bromodomain and PWWP domain [16, 17, 25]. PWWP domain and bromodomain of Brpf1 directly bind to Histones and are required for chromatin targeting by Brpf1 [16]. Previous study in zebrafish and medaka suggested that Brpf1 is required for the maintenance of *Hox* genes expression through Moz-dependent histone acetylation. [16, 17] Thus, Brpf1 may contribute to upregulation of *HOX* genes in MOZ-related leukemias.

In this study, we have demonstrated that Brpf1 plays an important role in leukemogenesis induced by MOZ–TIF2. Our data indicated that *Hoxa9* and *Hoxa10* were direct

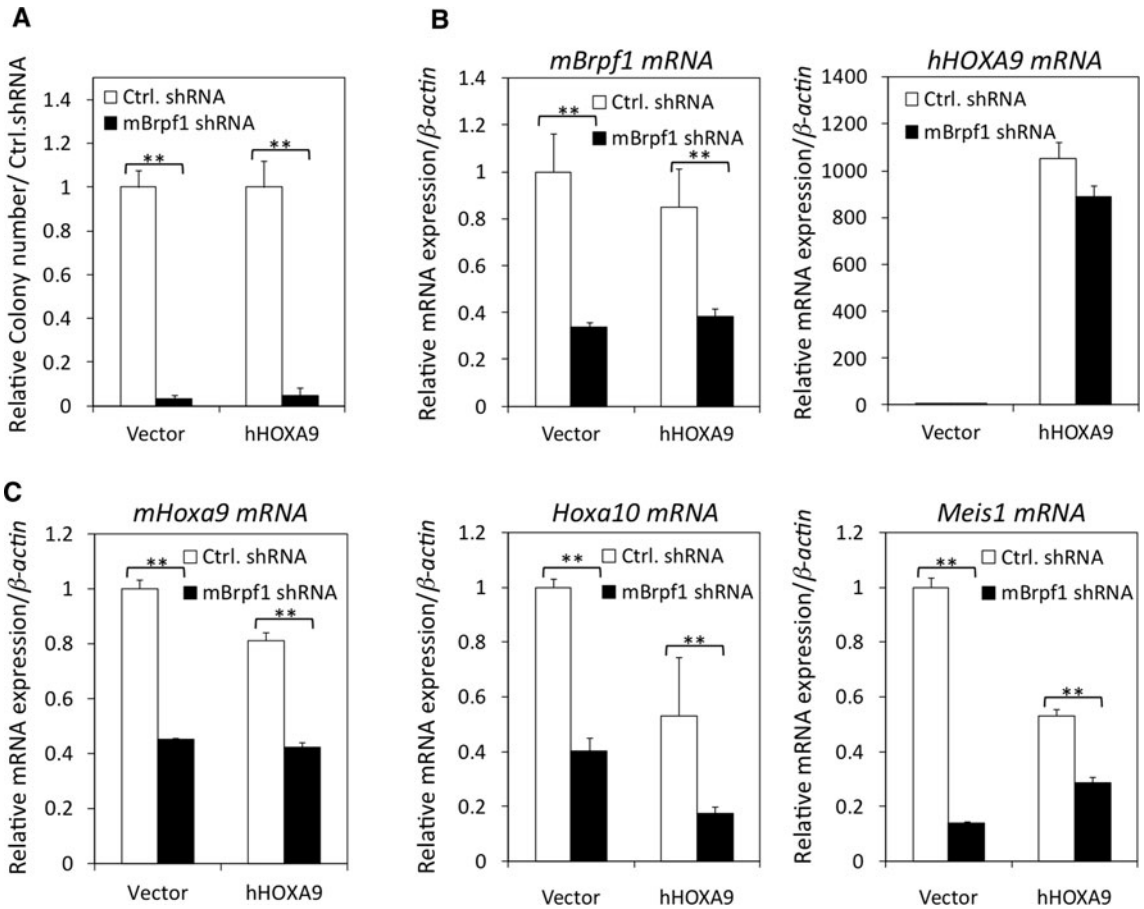


Fig. 5 Effect of HOXA9 overexpression on MOZ–TIF2 leukemic cells with downregulated Brpf1. **a** Relative colony number of MOZ–TIF2 leukemic cells transduced with control shRNA or Brpf1 shRNA. Overexpression of wild-type HOXA9 was not sufficient to restore colony formation activity of MOZ–TIF2 leukemic cells with downregulated Brpf1. Data are mean ± SD ($n = 3$). $^{**}P < .01$. **b** Expression of murine *Brpf1* and human *HOXA9* after Brpf1 shRNA and HOXA9 overexpression. **c** Effects of Brpf1 shRNA and HOXA9 overexpression on expression levels of *Hoxa9, Hoxa10* and *Meis1* in Brpf1 downregulated MOZ–TIF2 leukemic cells. Reduced expressions of *Hoxa9, Hoxa10* and *Meis1* by *Brpf1* knockdown were sustained in cells with HOXA9 overexpression. Data are mean ± SD ($n = 3$). $^{**}P < .01$

targets of MOZ and BRPF1 in MOZ–TIF2 leukemic cells. Because depletion of Brpf1 exhibited decreased level of MOZ localization on these target genes, resulting in loss of transformation ability induced by MOZ–TIF2, we suggest that Brpf1 promotes MOZ to localize on chromatin of these target genes. Brpf1 recruits MOZ to the target genes and upregulates transcriptional activation through histone H3 acetylation. Since binding of MOZ–TIF2 to the *Meis1* gene locus is weak compared to its binding to *Hoxa9* and *Hoxa10* (data not shown), MOZ–TIF2 is unlikely to regulate the expression of Meis1 directly. Our data also indicate that HAT activity of MOZ is essential for transformation activity induced by MOZ–TIF2. HAT-deficient MOZ–TIF2 was incapable of not only deregulating *Hox* genes, but also initiating leukemia. We suggest that MOZ or MOZ-fusion/BRPF1 complex upregulates *HOX* genes mediated by MOZ-dependent histone acetylation, which finally leads to the development of leukemia.

Although HOXA9 alone possesses transformation activity, enforced expression of human HOXA9 unexpectedly failed to rescue transformation activity abolished by Brpf1 depletion. There are two possible reasons to be considered. Firstly, *Hoxa10* and *Meis1*, both of which were downregulated in Brpf1 depleted cells remained in lower level compared to control cells despite HOXA9 overexpression. This may suggest that downregulation of several *HOX* genes other than *HOXA9* may result in loss of leukemic transformation. Secondly, Brpf1 may be required for HOXA9 function and its downstream pathway. Further study is required to determine the association of Brpf1 and Hoxa9 in the maintenance of transformation activity in AML cells.

Previously, we have shown that upregulation of M-CSFR is critical for the regulation of AML stem cells in MOZ–TIF2 leukemia, and STAT5, which was highly phosphorylated in M-CSFR high cells but not in low cells, may contribute to the clonal expansion and stem cell maintenance [2]. In this study, we demonstrated that deletion mutant of MOZ–TIF2 lacking interacting domain with Brpf1 lost its transformation activity. However, M-CSFR upregulation was maintained in BM cells transduced with this mutant MOZ–TIF2. Furthermore, Hoxa9 expression was upregulated in both M-CSFR-high cells and low cells [2]. Therefore, MOZ/BRPF1/HOX pathway should be considered as independent of PU.1/M-CSFR pathway (Fig. 6). Collectively, our study reveals that MOZ/BRPF1/HOX pathway plays a crucial role in the development of leukemia induced by MOZ–TIF2. For efficient induction of AML, block in the normal hematopoietic differentiation program together with unrestrained growth is required. Several AML-associated chromosomal translocations require additional mutations for the progenitors to gain both of these functions [26, 27]. However, in terms of MOZ–TIF2 leukemia, although increased expression of *HOX* genes may be insufficient to induce AML in a short period, increased phosphorylation of STAT5 mediated by PU.1/M-CSFR pathway would accelerate the development of leukemia. Thus, both MOZ/BRPF1/HOX pathway and PU.1/M-CSFR pathway are essential for the development of MOZ–TIF2 AML.

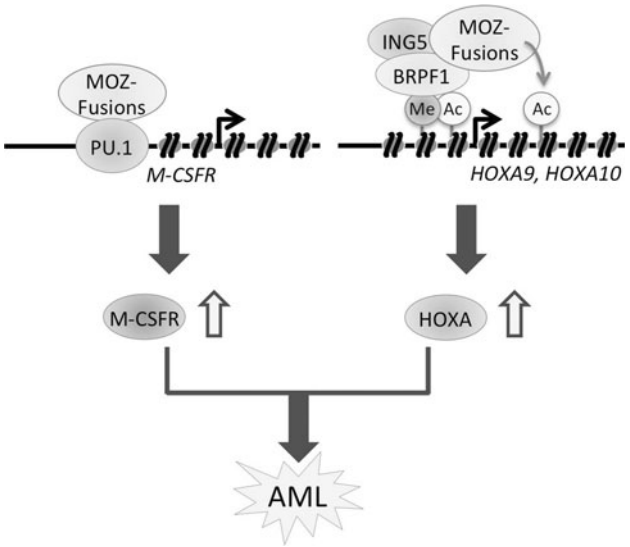


Fig. 6 Model of leukemogenic mechanism induced by MOZ–TIF2. Apart from PU.1/M-CSFR pathway, BRPF1/HOX pathway is essential for leukemogenesis by MOZ–TIF2. BRPF1 enhances the localization of MOZ on target genes such as *HOXA9* or *HOXA10*, and promotes Histone H3 acetylation, which may result in transcriptional upregulation of target genes, finally contributing to the development of leukemia

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Conflict of interest The authors declare no competing financial interests.

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