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MOZ is critical for the development of *MOZ/MLL* fusion–induced leukemia through regulation of *Hoxa9/Meis1* expression

Takuo Katsumoto,[1] Yoko Ogawara,[1] Kazutsune Yamagata,[1] Yukiko Aikawa,[1] Ryo Goitsuka,[2] Takuro Nakamura,[3,4] and Issay Kitabayashi[1]

[1]Division of Hematological Malignancy, National Cancer Center Research Institute, Tokyo, Japan; [2]Division of Development and Aging, Research Institute for Biomedical Sciences, Tokyo University of Science, Chiba, Japan; [3]Division of Carcinogenesis, The Cancer Institute, Japanese Foundation for Cancer Research, Tokyo, Japan; and [4]Department of Experimental Pathology, Institute of Medical Science, Tokyo Medical University, Tokyo, Japan

Key Points

- MOZ is critical for *MOZ/MLL* fusion-mediated AML development, *Meis1* induction by *MOZ* fusions, and *Hoxa9/Meis1* induction by *MLL* fusions.
- Endogenous MOZ is required to maintain MOZ-target and active histone modifications at the *Meis1* gene locus.

Monocytic leukemia zinc finger protein (MOZ, MYST3, or KAT6A) is a MYST-type acetyltransferase involved in chromosomal translocation in acute myelogenous leukemia (AML) and myelodysplastic syndrome. MOZ is established as essential for hematopoiesis; however, the role of MOZ in AML has not been addressed. We propose that MOZ is critical for AML development induced by *MLL-AF9*, *MLL-AF10*, or *MOZ-TIF2* fusions. *Moz*-deficient hematopoietic stem/progenitor cells (HSPCs) transduced with an *MLL-AF10* fusion gene neither formed colonies in methylcellulose nor induced AML in mice. *Moz*-deficient HSPCs bearing *MLL-AF9* also generated significantly reduced colony and cell numbers. *Moz*-deficient HSPCs expressing *MOZ-TIF2* could form colonies in vitro but could not induce AML in mice. By contrast, *Moz* was dispensable for colony formation by *HOXA9*-transduced cells and AML development caused by *HOXA9* and *MEIS1*, suggesting a specific requirement for MOZ in AML induced by *MOZ/MLL* fusions. Expression of the *Hoxa9* and *Meis1* genes was decreased in *Moz*-deficient *MLL* fusion-expressing cells, while expression of *Meis1*, but not *Hoxa9*, was reduced in *Moz*-deficient *MOZ-TIF2* AML cells. AML development induced by *MOZ-TIF2* was rescued by introducing *Meis1* into *Moz*-deficient cells carrying *MOZ-TIF2*. *Meis1* deletion impaired *MOZ-TIF2*–mediated AML development. Active histone modifications were also severely reduced at the *Meis1* locus in *Moz*-deficient *MOZ-TIF2* and *MLL-AF9* AML cells. These results suggest that endogenous MOZ is critical for *MOZ/MLL* fusion-induced AML development and maintains active chromatin signatures at target gene loci.

Introduction

Acute myelogenous leukemia (AML) is a hematological malignancy derived from hematopoietic stem cells (HSCs) and myeloid progenitors with acquired fusion genes or oncogenic mutations. Various fusion genes involving transcription factors and epigenetic modulators, such as AML1 (RUNX1), CBFβ, MLL1, or PML, have been found in AML. In addition, mutations in genes involved in transcription, such as *AML1*, *CEBPA*, *DNMT3A*, *TET2*, and *EZH2*, are also observed in AML. These findings imply that many cases of AML arise because of failure of transcription regulation caused by gene mutations or generation of fusion genes.[1-5]

Histone modifications, including acetylation, methylation, or ubiquitination of the histone *N*-terminal tail, are critical epigenetic gene expression regulatory mechanisms.[6] Histone modifications are crucial for

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cancer pathophysiology and molecular targets for therapies, including bromodomain and extraterminal motif or histone deacetylase inhibitors.[7,8] In *MLL* fusion leukemias, DOT1L methyltransferase is critical for leukemia development and induction of aberrant *HOXA9/MEIS1* gene expression.[9] These reports indicate that various histone modification factors are essential in hematological malignancy through target gene regulation.

Monocytic leukemia zinc finger protein (MOZ; also MYST3 or KAT6A) is one of the MYST-type histone acetyltransferases, which catalyze acetylation of histone H3K9 (H3 lysine 9),[10] H3K14 (lysine 14),[11] and H3K23 (lysine 23).[12] *MOZ* is a component of fusion genes, including *MOZ-CREBBP* and *MOZ-TIF2*, found in FAB M4/M5 acute monocytic leukemia and interacts with the AML1 (RUNX1), PU.1, and p53 transcription factors, which are essential in leukemia development.[13] In addition, MOZ forms transcriptional complexes with the bromodomain and PHD finger-containing (BRPF) proteins, ING5, and MEAF6,[14] and is necessary for HSC self-renewal and appropriate hematopoietic cell lineage development.[15-17] Expression levels of the homeobox genes, *Hoxa9* and *Meis1*, and cytokine receptors, *c-Kit*, *c-Mpl,* and *c-Fms*, are reduced in *Moz*-deficient hematopoietic stem/progenitor cells (HSPCs) and B cells.[16,18,19] MOZ localizes to *HOXA* and *MEIS1* loci in normal human CD34$^+$ HSCs and B cells,[19,20] while expression of the tumor suppressor gene, *p16^{Ink4a}*, is elevated in *Moz*-null HSCs, neural stem cells, and mouse embryonic fibroblasts.[21,22] In leukemia, aberrant expression of *HOXA9* and *MEIS1* is observed in *MOZ*, *MLL*, and *NUP98* fusion leukemias and *NPM1*-mutated AML.[3,23-25] *HOXA9* is upregulated in approximately 50% of patients with AML and is essential for *MLL* fusion-induced transformation and AML development.[26,27] *MEIS1* is also critical for *MLL* fusion carrying leukemia[28] through its roles in cell proliferation,[29] homing and engraftment,[30] and protection from oxidative stress.[31] Although endogenous MOZ is required for self-renewal of HSCs and *Hoxa9/Meis1* expression in normal hematopoietic cells, the roles of endogenous MOZ in *MOZ/MLL* fusion AML development and aberrant expression of *Hoxa9/Meis1* in this context have not been addressed. Therefore, we analyzed the role of endogenous MOZ in *MOZ/MLL* fusion leukemia.

Using *Moz*-deficient mouse cells transfected with *MOZ/MLL* fusion genes, we conducted in vitro serial colony replating and in vivo leukemogenesis assays, which demonstrated that endogenous MOZ is essential for *MLL* fusion-induced immortalization and AML development, as well as *MOZ* fusion-induced AML development. Further, gene expression analysis revealed notable decreases in *Hoxa9/Meis1* expression in *Moz*-deficient *MLL* fusion cells and *Meis1* expression in *MOZ* fusion cells. Active histone marks at the *Meis1* locus were significantly reduced in *Moz*-deficient *MOZ/MLL* fusion AML cells. Moreover, the onset of AML development depended on aberrant *Meis1* expression in *MOZ* fusion AML. These results suggest that endogenous MOZ is a critical factor in *MOZ/MLL* fusion AML via its induction of aberrant *MEIS1* and *HOXA9* gene expression.

Materials and methods

Mice

Moz$^{-/-}$ mice[16] were backcrossed with C57BL/6 mice more than 10 times. In addition, *Meis1* conditionally-deficient (*Meis1*$^{f/f}$)[32] and ROSA26-Cre-ERT2 knock-in (*Cre-ERT2*) (TaconicArtemis GmbH; Cologne, Germany) mice were maintained on a C57BL/6 background. C57BL/6J mice were purchased from CLEA Japan (Tokyo, Japan). Animals were kept under specific pathogen-free, temperature-controlled conditions, according to institutional guidelines. Written approval of all animal experiments was obtained from the local Animal Experiments Committee of the National Cancer Center Research Institute. Genotyping was performed using genomic DNA prepared from mouse tail or AML cells boiled in 50 mM NaOH solution at 96°C for 30 minutes. Primers used for genotyping are shown in supplemental Table 1 in the data supplement.

Reagents

Reagents used in this study were: APC-eFluor 780-conjugated streptavidin, anti-CD16/32 (93), anti-CD34-FITC (RAM34), anti-CD115-PE (AFS98), anti-B220-biotin (RA3-6B2), anti-CD11b-PE-Cy7 (M1/70), anti-CD117-APC (2B8), and anti-Gr-1-APC (RB6-8C5) (eBioscience; San Diego, CA); anti-CD3ε-biotin (145-2C11), anti-Gr-1-biotin (RB6-8C5), anti-TER119-biotin (TER-119), anti-CD127-biotin (A7R34), anti-CD16/32-PE-Cy7 (93), and anti-hNGFR-APC (ME20.4) (Biolegend; San Diego, CA); anti-Sca-1-PE (E13-161.7) (BD Biosciences; Franklin Lakes, NJ); polyclonal antibodies against Histone H3 (ab1791), H3K9ac (ab4441), H3K27ac (ab4729), and H3K79me2 (ab3594) (Abcam; Cambridge, United Kingdom); polyclonal antibodies against H3K4me3 (39159), H3K9ac (39137), and H3K23ac (39132) (Active Motif Inc.; Carlsbad, CA); polyclonal antibody against H3K23ac (07-355) (Merck Millipore; Billerica, MA); recombinant murine interleukin-3 (IL-3; 213-03), murine stem cell factor (SCF; 250-03), and murine granulocyte/monocyte-colony stimulating factor (GM-CSF; 315-03) (PeproTech; Rocky Hill, NJ); recombinant murine oncostatin M (OSM; 495-MO-025) (R&D Systems; Minneapolis, MN); and tamoxifen (TAM), 4-OHT (4-hydroxy tamoxifen), corn oil, and rat serum-IgG (Sigma Aldrich; St. Louis, MO).

Purification of HSPCs, c-Kit$^+$, Sca-1$^+$ lineage$^-$ cells, and common myeloid progenitors

Fetal liver (FL) samples from E14.5 *Moz*$^{+/+}$, *Moz*$^{+/-}$, and *Moz*$^{-/-}$ embryos were dispersed into single-cell suspensions in 2% fetal bovine serum in phosphate-buffered saline. Erythrocytes were lyzed with tris-buffered 0.83% ammonium chloride solution. Whole FL cells were incubated with anti-CD16/32 antibody or rat serum IgG to prevent nonspecific antibody binding. FL cells were then incubated with biotin-labeled antibodies against lineage markers (Ter119, CD3ε, B220, Gr-1, and CD127) (30 min, 4°C), followed by incubation with streptavidin-conjugated magnetic beads (Miltenyl Biotech; Bergisch, Gladbach, Germany) (20 min, 4°C). Cell suspensions containing magnetic bead-bound cells were loaded onto a magnetic column (Miltenyl Biotech) to remove lineage marker-positive magnetic bead-bound cells. The flowthrough of unbound cells was collected (lineage$^-$ HSPCs).

For purification of c-Kit$^+$, Sca-1$^+$ lineage$^-$ (KSL), and chronic myeloid progenitor (CMP) fractions, lineage$^-$ HSPCs were incubated with streptavidin-APC-eFluor 780, c-Kit-APC, Sca-1-PE, PE-Cy7-CD16/32, and CD34-FITC (45 min, 4°C). KSLs and CMPs were distinguished as lineage$^-$, c-Kit$^+$, and Sca-1$^+$, or lineage$^-$ c-Kit$^+$, Sca-1$^-$, CD16/32^{lo}, and CD34$^+$, respectively, using a JSAN flow cytometer (Bay Bioscience; Kobe, Japan). Sorted lineage$^-$ HSPCs, KSLs, and CMPs were cultured in StemPro34 medium (Thermo Fisher Scientific; Waltham, MA) supplemented with

recombinant murine SCF (50 ng/mL), IL-3 (10 ng/mL), OSM (10 ng/mL), tylosin (8 μg/mL) (Sigma Aldrich), penicillin/streptomycin (100 U/mL) (Nacalai Tesque; Kyoto, Japan), and L-glutamine (2 mM; Sigma Aldrich).

In vitro serial colony formation assay

Human *MOZ-TIF2*, *MLL-AF9*, *MLL-AF10*, or *HOXA9* genes were introduced into cells using a retrovirus system and cultured for 5 days. Green fluorescent protein (GFP) marker-positive cells were sorted by JSAN flow cytometry. Sorted GFP^+ cells (5×10^4) were cultured in Methocult M 3234 methylcellulose medium (Stemcell Technologies; Vancouver, BC, Canada) containing IL-3 (20 ng/mL), SCF (50 ng/mL), GM-CSF (10 ng/mL), and 100 U/mL penicillin/streptomycin. After 3 to 5 days, colonies and total cells were counted, and 3×10^4 or 1×10^5 cells were cultured in methylcellulose medium 3 or 5 times. Cultures were maintained at 37°C in a 5% CO_2 humidified atmosphere.

In vivo leukemogenesis assay

Cells transduced with the *MOZ/MLL* fusion, human *MEIS1*, or human *HOXA9/MEIS1* genes were prepared as described above. Cells (MOZ-TIF2, MLL-AF10, and HOXA9/MEIS1, 2×10^5; MEIS1, 5×10^5) were transplanted into sublethally γ-irradiated (600 rad) recipient mice via the tail vein. *MOZ-TIF2* + *MEIS1* or mock-transduced cells were generated by the introduction of the *MOZ-TIF2* gene into $Moz^{+/-}$ or $Moz^{-/-}$ FL HSPCs. After 3 days of culture, a vector expressing human *MEIS1* or empty vector (Mock) was introduced into *MOZ-TIF2*–infected cells, and these cells were then cultured for 5 days. After infection, MOZ-TIF2 + MEIS1 or MOZ-TIF2 + Mock cells (5×10^5) were transplanted into sublethally γ-irradiated (600 rad) recipient mice. Peripheral blood was collected from recipient mice every 4 weeks and analyzed for populations of GFP^+ cells expressing *HOXA9*, *MOZ*, or *MLL* fusion genes and for Mac-1 (myeloid cell marker)-positive cells to monitor AML development, which was defined as a proportion of GFP^+ cells >80% in recipient mouse bone marrow (BM), with hepatosplenomegaly and lymphadenopathy.

Statistical analysis

A 2-tailed unequal-variance *t* test was used to assess the significance of differences among samples. Differences in survival curves among groups were evaluated using the log-rank test in GraphPad Prism 6.0 (GraphPad Software; San Diego, CA).

Supplemental materials and methods

Materials and methods for immunofluorescent staining and flow cytometric analysis, retrovirus production and infection, RNA purification, quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) analysis, chromatin immunoprecipitation (ChIP) assay, ChIP-Seq analysis, and conditional deletion of the *Meis1* gene in AML cells in vitro and in vivo are provided as supplemental materials on the *Blood Advances* website.

Results

Endogenous MOZ is essential for *MOZ/MLL* fusion-induced immortalization and AML development

To explore the roles of endogenous MOZ in AML development, we used *Moz* knockout mice.[16] FL HSPCs from E14.5 $Moz^{+/+}$, $Moz^{+/-}$, and $Moz^{-/-}$ embryos were transduced with *MOZ-TIF2*, *MLL-AF9, MLL-AF10*, or *HOXA9* using retrovirus vectors and their abilities to form colonies in vitro tested (Figure 1A). $Moz^{-/-}$ HSPCs transduced with either *MOZ-TIF2* or *HOXA9* serially formed colonies in methylcellulose medium ≥5 times, whereas $Moz^{-/-}$ HSPCs transduced with *MLL-AF10* formed fewer colonies and did not show continuous growth and, although $Moz^{-/-}$ HSPCs transduced with *MLL-AF9* transiently formed colonies, their number decreased significantly with successive replating. In addition, $Moz^{-/-}$ HSPCs expressing *MLL-AF9* generated fewer cell colonies than $Moz^{+/+}$ and $Moz^{+/-}$ HSPCs expressing *MLL-AF9*. To investigate the roles of endogenous MOZ in AML development, $Moz^{+/+}$, $Moz^{+/-}$, and $Moz^{-/-}$ FL HSPCs transduced with *MOZ-TIF2*, *MLL-AF10*, or *HOXA9*, together with *MEIS1*, were transplanted into γ-irradiated recipient mice (Figure 1B). Although $Moz^{-/-}$ HSPCs expressing both *HOXA9* and *MEIS1* induced AML in recipient mice, $Moz^{-/-}$ HSPCs with either *MOZ-TIF2* or *MLL-AF10* did not. Further, mice transplanted with $Moz^{+/-}$ *MLL-AF10* cells survived longer than those transplanted with $Moz^{+/+}$ *MLL-AF10* cells. These results indicate that endogenous MOZ is essential for *MOZ/MLL* fusion-induced AML development.

Endogenous MOZ is required for *MOZ/MLL* fusion-induced *Meis1* and *Hoxa9* expression, dependent on fusion gene and origin

To investigate the molecular mechanisms underlying the phenotypes observed in $Moz^{-/-}$ *MOZ-TIF2, MLL-AF9,* and *MLL-AF10* AML cells, we analyzed *Hoxa9* and *Meis1* concentrations, which are aberrantly expressed in *MOZ/MLL* fusion AML. *Hoxa9/Meis1* concentrations were strikingly higher in *MOZ/MLL* fusion AML cells than in *AML1-ETO* AML cells that did not exhibit aberrant *Hoxa9/Meis1* expression. *Hoxa9* and *Meis1* concentrations were similar between $Moz^{+/+}$ and $Moz^{+/-}$ AML cells and between *MOZ* and *MLL* fusion AML cells (Figure 2A). Although mean *Hoxa9* expression in $Moz^{-/-}$ *MOZ-TIF2* AML cells was similar to that in $Moz^{+/+}$ or $Moz^{+/-}$ *MOZ-TIF2* AML cells, expression of the *Meis1* gene was dramatically lower in $Moz^{-/-}$ *MOZ-TIF2* AML cells. Furthermore, *Hoxa9* and *Meis1* concentrations were significantly lower in $Moz^{-/-}$ *MLL-AF9* AML cells than in $Moz^{+/+}$ or $Moz^{+/-}$ *MLL-AF9* AML cells (Figure 2A).

We also investigated *Hoxa9/Meis1* expression in $Moz^{-/-}$ cells bearing *MLL-AF10* and found that both *Hoxa9* and *Meis1* concentrations were significantly lower in *MLL-AF10*–expressing $Moz^{-/-}$ cells than those in *MLL-AF10*–expressing $Moz^{+/+}$ or $Moz^{+/-}$ cells, similar to our findings for *MLL-AF9* (Figure 2B). These results demonstrate that endogenous MOZ is essential for aberrant *Meis1* induction in *MOZ* fusion AML cells and both *Hoxa9* and *Meis1* expression in *MLL* fusion AML cells.

MOZ or MLL fusions can transform HSCs and myeloid progenitors, CMPs, and granulocyte monocyte progenitors,[33] implying that the roles of endogenous MOZ differ between HSCs and myeloid progenitors in *MOZ/MLL* fusion leukemia. Therefore, we sorted KSL (containing HSCs) and CMP fractions and transduced each with *MOZ-TIF2* and *MLL-AF9*. After transfection, we sorted *MOZ* or *MLL* fusion-expressing cells and subjected them to serial colony replating assays and gene expression analysis. Only *MOZ-TIF2*–expressing $Moz^{-/-}$ cells derived from the KSL fraction could continuously form colonies, which those from CMPs could not.

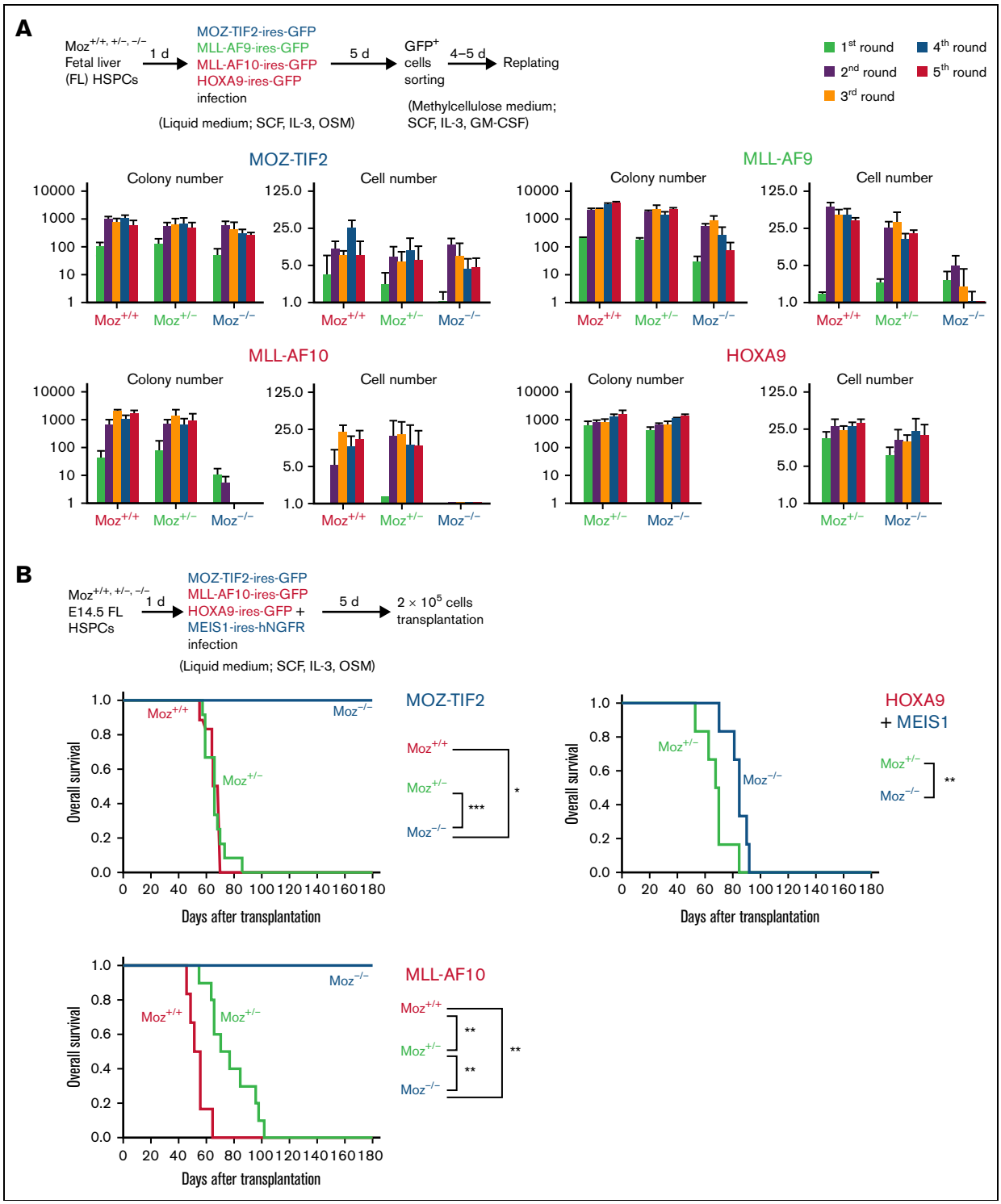

Figure 1. Endogenous MOZ is essential for *MOZ/MLL* fusion-induced leukemia development. (A) Serial colony replating assays were performed in *Moz*$^{+/+}$, *Moz*$^{+/-}$, and *Moz*$^{-/-}$ HSPCs bearing *MOZ-TIF2*, *MLL-AF9*, *MLL-AF10*, or *HOXA9*. A scheme of the experimental workflow is shown at the top. First, *Moz*$^{+/+}$, *Moz*$^{+/-}$, or *Moz*$^{-/-}$ FL lineage$^-$ HSPCs (2×10^5 cells) were transduced with *MOZ-TIF2*, *MLL-AF10*, *MLL-AF9*, or *HOXA9* and cultured in a liquid medium. Subsequently, GFP$^+$ cells (5×10^4) were sorted and cultured in a methylcellulose medium. Every 4 to 5 days, colony and cell numbers ($\times 10^5$ cells) were counted, and then 1×10^5 cells were serially replated 5 times. Mean values were calculated from 3 to 4 independent experiments. Error bars represent mean ± standard deviation (SD). (B) Overall survival of recipient mice transplanted with *Moz*$^{+/+}$, *Moz*$^{+/-}$, or *Moz*$^{-/-}$ HSPCs (2×10^5) bearing *MOZ-TIF2*, *MLL-AF10*, or *HOXA9* and *MEIS1* (*MOZ-TIF2*, *Moz*$^{+/+}$ and *Moz*$^{-/-}$ [n = 6], *Moz*$^{+/-}$ [n = 12]; *MLL-AF10*, *Moz*$^{+/+}$ [n = 6], *Moz*$^{+/-}$ [n = 10], *Moz*$^{-/-}$ [n = 4]; *HOXA9* + *MEIS1*, [n = 6]). Differences in survival were compared using the log-rank test; *$P < .05$, **$P < .01$, and ***$P < .005$.

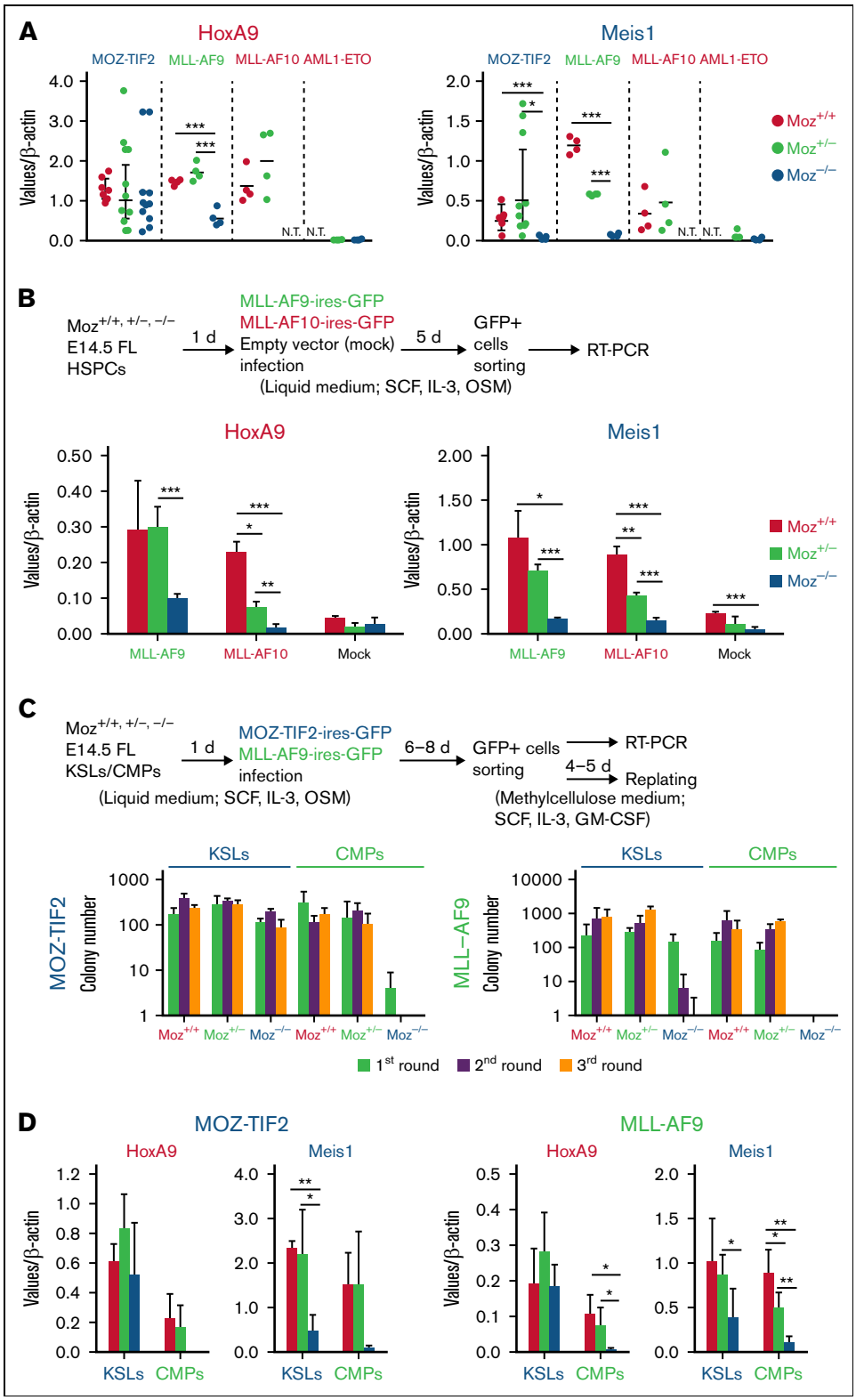

Figure 2. Endogenous MOZ is critical for *MOZ/MLL* fusion-mediated induction of target gene expression. (A) Gene expression concentrations of the *Hoxa9/Meis1* genes in *MOZ-TIF2*, *MLL-AF9*, *MLL-AF10*, and *AML1-ETO* AML cells. Colonies of $Moz^{+/+}$, $Moz^{+/-}$, or $Moz^{-/-}$ AML cells that were serially replated >3 times were harvested and analyzed for *Hoxa9*, *Meis1*, and *β-actin* expression by quantitative reverse transcriptase-PCR (qRT-PCR). Expression levels of *Hoxa9* and *Meis1* were normalized to those of *β-actin* (n = 4 to 8). Error bars represent mean ± SD. Expression levels were compared using the Student *t* test; $^{*}P < .05$ and $^{***}P < .005$. N.T., not tested. (B) Expression of the *Hoxa9/Meis1* genes in $Moz^{+/+}$, $Moz^{+/-}$, or $Moz^{-/-}$ cells bearing *MLL-AF9* or *MLL-AF10*. The experimental scheme is shown at the top. $Moz^{+/+}$, $Moz^{+/-}$, or $Moz^{-/-}$ FL lineage^{-} HSPCs were transduced with *MLL-AF9*, *MLL-AF10*, or empty vector (Mock) and cultured in a liquid medium. GFP^{+} cells were

Although *Moz*$^{-/-}$ *MLL-AF9* cells derived from KSLs formed colonies in the first round, colony-forming activity was markedly decreased with each successive generation. Further, *Moz*$^{-/-}$ *MLL-AF9*–expressing cells derived from CMPs could not generate colonies (Figure 2C), while *Moz*$^{-/-}$ *MLL-AF9*–expressing cells derived from HSPCs only showed colony formation activity in the first round, when they were replated at 3 × 10^4 cells (supplemental Figure 1). Gene expression analysis showed that induction of both *Meis1* and *Hoxa9* was decreased in *MOZ-TIF2*– or *MLL-AF9*–expressing *Moz*$^{-/-}$ cells derived from CMPs (Figure 2D), whereas only *Meis1* expression was markedly reduced in KSL-derived *MOZ-TIF2*– or *MLL-AF9*–expressing cells (Figure 2D). These results indicate that endogenous MOZ is also essential for immortalization and induction of the *Hoxa9* gene by the *MOZ/MLL* fusion in myeloid progenitors.

Endogenous MOZ is required for the maintenance of an active chromatin landscape at target gene loci

To reveal the molecular mechanisms underlying the involvement of endogenous MOZ in fusion gene induction of target gene expression, we analyzed the acetylation of H3K9 and H3K23 (target sites for acetylation by MOZ) at the *Hoxa* cluster and *Meis1* loci in *MOZ-TIF2* AML cells by ChIP-Seq analysis. In *Moz*$^{-/-}$ *MOZ-TIF2* AML cells, both H3K9 and H3K23 acetylation were reduced at the *Meis1* gene locus but not at the *Hoxa* gene cluster (Figure 3A and supplemental Figure 2A). Other types of active histone marks (H3K4me3, H3K27ac, and H3K79me2) were significantly reduced at the *Meis1* gene locus and modestly decreased at the *HoxA9* locus in *Moz*$^{-/-}$ *MOZ-TIF2* AML cells (Figure 3B). In *Moz*$^{-/-}$ *MLL-AF9* AML cells, active histone marks were remarkably reduced at the *Meis1* locus. In addition, H3K4me3 modification was strikingly decreased at the *Hoxa9* locus. Moreover, concentrations of H3K23ac, H3K4me3, and H3K27ac modification were higher in *Moz*$^{+/+}$ *MLL-AF9* AML cells than in *Moz*$^{+/-}$ and *Moz*$^{-/-}$ *MLL-AF9* AML cells at the *Hoxa9/Meis1* loci (supplemental Figure 2B).

These data suggest that endogenous MOZ is essential for maintaining an active chromatin landscape at the *Meis1* gene locus in *MOZ-TIF2* and *MLL-AF9* AML cells.

MEIS1 is a critical factor for *MOZ* fusion-induced AML development but not for immortalization

To confirm whether the failure to develop AML in *Moz*$^{-/-}$ *MOZ* fusion AML cells was because of an inability to induce aberrant *Meis1* expression, we investigated the role of MEIS1 in *MOZ* fusion-mediated AML development. Both the *MOZ-TIF2* fusion and human *MEIS1* genes were introduced into *Moz*$^{-/-}$ HSPCs, and these cells were then transplanted into recipient mice. Expression of the endogenous *Meis1* and ectopic *MEIS1* genes were significantly higher in HSPCs transduced with *MOZ-TIF2* + *MEIS1* than those transduced with *MOZ-TIF2* + mock vector (supplemental Figure 3A). Most *MOZ-TIF2*–transduced *Moz*$^{-/-}$ cells coexpressing the *MEIS1* gene caused AML development in recipient mice (Figure 4A). In *Moz*$^{+/-}$ *MOZ-TIF2* AML cells, *MEIS1* overexpression did not affect the onset of AML development in recipient mice, similar to that in mice transplanted with *Moz*$^{+/-}$ *MOZ-TIF2* AML cells transduced with the empty vector (Mock control) (supplemental Figure 3B). *MEIS1*–overexpressing *Moz*$^{-/-}$ *MOZ-TIF2* AML cells expressed myeloid markers, such as Mac-1, Gr-1, and M-CSFR (supplemental Figure 3C). Further, recipient mice transplanted with *Moz*$^{+/-}$ or *Moz*$^{-/-}$ HSPCs transduced with *MEIS1* did not develop AML, as shown in previous reports[34-36] (supplemental Figure 3D).

We also checked the requirement for MEIS1 in *MOZ* fusion-induced AML development using a *Meis1* conditional deficient mouse. *Meis1*$^{f/+}$ or *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells were transplanted into recipient mice. One week after transplantation, recipient mice were intraperitoneally administered corn oil or TAM to induce Cre recombinase-mediated excision of the *Meis1* allele, resulting in alteration of *Meis1* genetic status in *MOZ-TIF2* AML cells from *Meis1* floxed (*Meis1*$^{f/f}$) to *Meis1* deficient (*Meis1*$^{\Delta/\Delta}$). With transplantation of *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells, AML development was significantly delayed or absent in recipient mice injected with TAM compared with those administered with corn oil (Figure 4B). Recipient mice transplanted with *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells and treated with TAM showed no sign of AML development and had only 18.46% GFP$^+$ cells in their BM at 120 days after transplantation (Figure 4B).

We also checked the *Meis1* genotypes of BM cells from recipient mice transplanted with *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells and injected with TAM. GFP$^+$ BM cells from the recipient mice showed no sign of AML development, and only *Meis1*$^{\Delta/\Delta}$ *MOZ-TIF2* AML cells were detected. In 1 mouse that developed AML, the BM contained *Meis1*$^{\Delta/\Delta}$ and *Meis1*$^{f/f}$ *MOZ-TIF2* AML cells. By contrast, only *Meis1*$^{f/f}$ *MOZ-TIF2* AML cells were detected in another mouse, indicating that *MOZ*-TIF2 AML cells that escaped deletion of the *Meis1* gene were mainly responsible for AML development. GFP$^+$ BM cells from the recipient mice showed no sign of AML development, and only *Meis1*$^{\Delta/\Delta}$ *MOZ-TIF2* AML cells were detected except in 1 sample (Figure 4B). Furthermore, AML development was observed in recipient mice transplanted with *Meis1*$^{f/+}$ *Cre-ERT2 MOZ* fusion AML cells treated with either corn oil or TAM (supplemental Figure 3E). These results indicate that MEIS1 is essential for *MOZ* fusion-mediated AML development.

Figure 2 (continued) then sorted, and the expression levels of *Hoxa9*, *Meis1*, and *β-actin* were analyzed by qRT-PCR (n = 3 to 4). Expression levels of *Hoxa9* and *Meis1* were normalized to those of *β-actin*. Error bars represent mean ± SD. Expression levels were compared using the Student *t* test; **P* < .05 and ****P* < .005. (C) Colony formation of *Moz*$^{+/+}$, *Moz*$^{+/-}$, or *Moz*$^{-/-}$ KSLs/CMPs bearing *MOZ-TIF2* or *MLL-AF9*. The experimental scheme is shown at the top. First, *Moz*$^{+/+}$, *Moz*$^{+/-}$, or *Moz*$^{-/-}$ KSLs/CMPs (1 × 10^4 cells) were transduced with *MOZ-TIF2* or *MLL-AF9* fusion genes and cultured in a liquid medium. Subsequently, GFP$^+$ cells (5 × 10^4) were sorted and cultured in a methylcellulose medium. Colony numbers were counted every 4 to 5 days, and then 3 × 10^4 cells were subsequently serially replated 3 times. The mean numbers of colonies formed by *MOZ-TIF2*– or *MLL-AF9*–expressing *Moz*$^{+/+}$, *Moz*$^{+/-}$, and *Moz*$^{-/-}$ cells derived from KSL or CMP fractions were calculated from 3, 4, or 5 independent experiments. (D) *Hoxa9* and *Meis1* expression in *MOZ-TIF2*–expressing cells derived from KSLs or CMPs. *Moz*$^{+/+}$, *Moz*$^{+/-}$, or *Moz*$^{-/-}$ KSLs and CMPs were transduced with the *MOZ-TIF2* or *MLL-AF9* fusion genes and cultured in a liquid medium. Then GFP$^+$ cells were sorted, and gene expression levels of *Hoxa9*, *Meis1*, and *β-actin* were analyzed by qRT-PCR (n = 3 to 5). Expression levels of *Hoxa9* and *Meis1* were normalized to those of *β-actin*. Error bars represent mean ± SD; **P* < .05, ***P* < .01, and ****P* < .005.

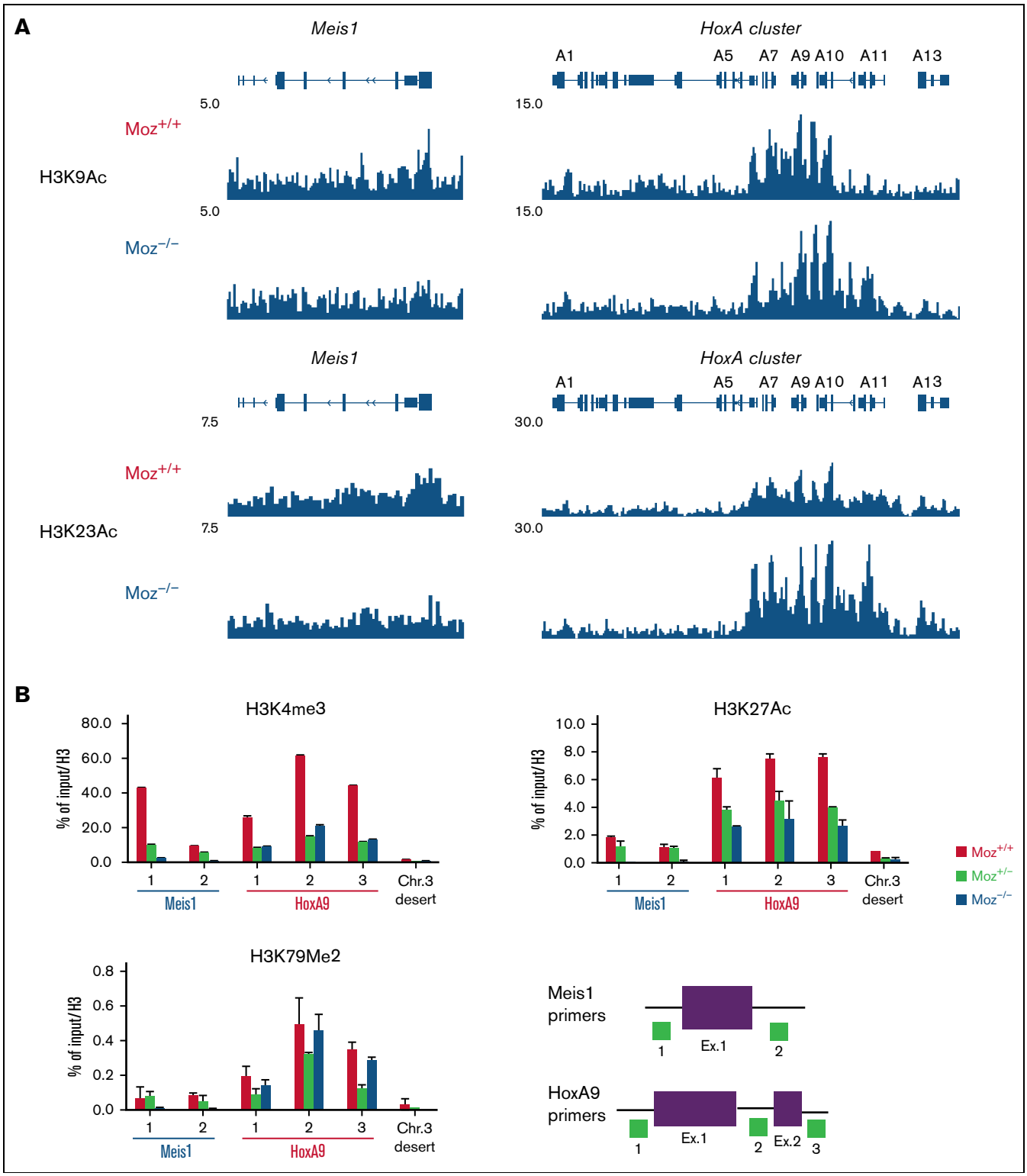

Figure 3. Endogenous MOZ is required for active histone modifications at the *Meis1* locus. *Moz*$^{+/+}$, *Moz*$^{+/-}$, or *Moz*$^{-/-}$ *MOZ-TIF2* and *MLL-AF9* AML cells were fixed with formalin, and ChIP-Seq or ChIP-qPCR assays were performed. (A) ChIP-Seq analysis of Histone H3 K9 and K23 acetylation at the *Hoxa* cluster and *Meis1* loci in *Moz*$^{+/+}$ and *Moz* $^{-/-}$ MOZ-TIF2 AML cells. (B) Active histone modifications at the *Meis1*, *Hoxa9* loci, and a mouse chromosome 3 desert (Chr. 3 desert) (negative control) loci in *MOZ-TIF2* AML cells. Histone modifications at *Meis1*, *Hoxa9* loci, and a Chr. 3 desert loci were measured by quantitative PCR analysis. Representative results of ChIP assays are shown. Seven independent experiments were conducted in *MOZ-TIF2* AML cells. Primer sets used to amplify the *Meis1* and *Hoxa9* loci are indicated at the bottom. Levels of each histone modification were normalized to input DNA and total histone H3 concentrations. Error bars represent mean ± SD.

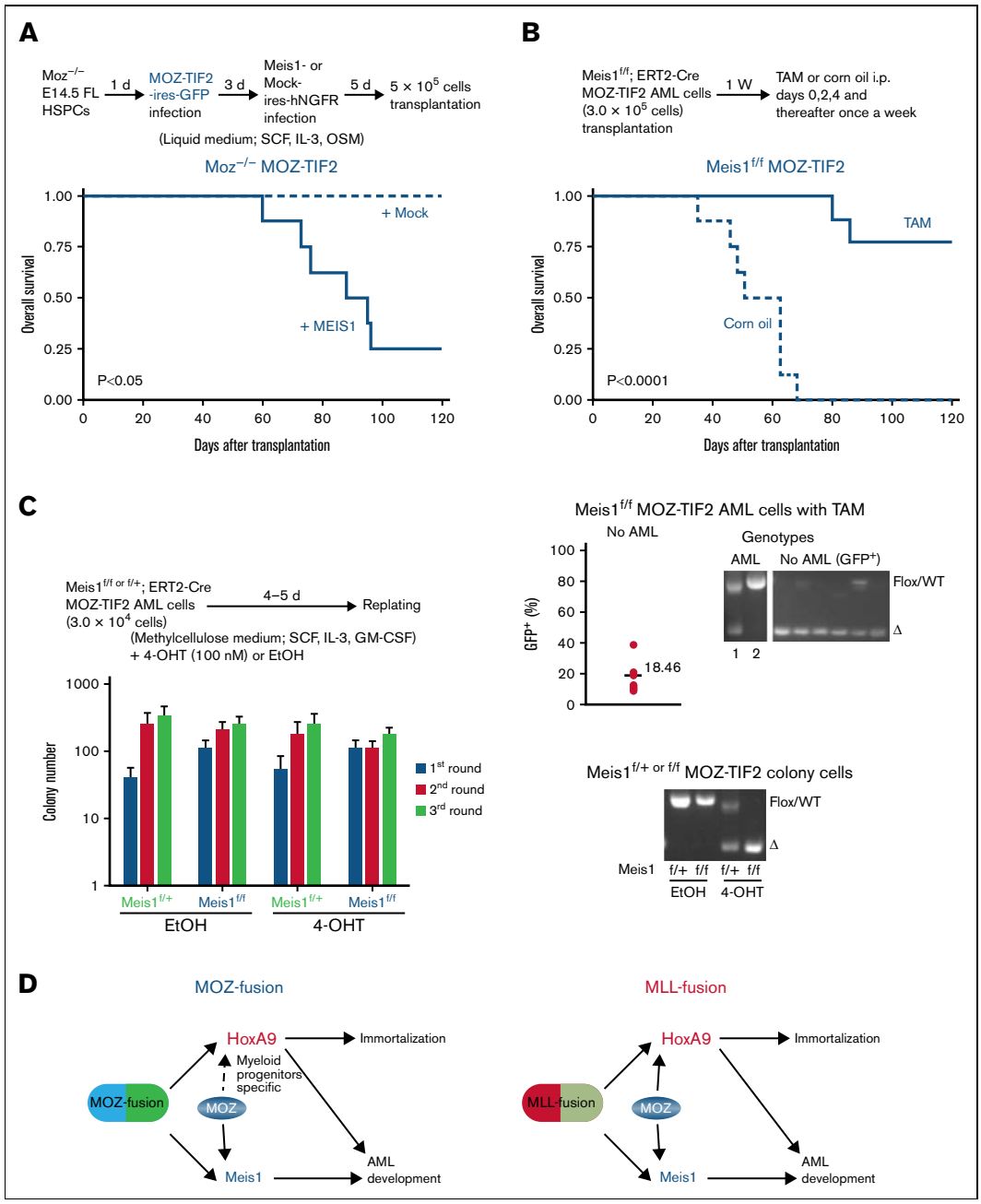

Figure 4. MEIS1 is essential for *MOZ* fusion-mediated AML development but not for immortalization. (A) AML development of *Moz*$^{-/-}$ cells bearing *MOZ* fusion and *MEIS1* genes. The experimental scheme is shown at the top. *Moz*$^{-/-}$ FL lineage^{-} HSPCs were infected with the *MOZ-TIF2* gene, followed by the *MEIS1* gene or empty vector (Mock), and cultured in a liquid medium. After 5 days, these cells (5 × 10^5) were transplanted into recipient mice, and their survival was analyzed (n = 5 to 8 per group). Survival was compared using the log-rank test. (B-C) Conditional deletion of the *Meis1* gene in *MOZ-TIF2* AML cells. (B) Survival of *Meis1*-deleted *MOZ-TIF2* AML cells. The experimental scheme is shown at the top; *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells were transplanted into recipient mice, and then tamoxifen (TAM; 80 mg/Kg) or the same dose of corn oil, administered intraperitoneally (n = 8 to 9 per group). Differences in survival were compared using the log-rank test. The lower right panel shows PCR analysis of *Meis1* genotypes in BM samples or BM GFP$^+$ cells from mice that did and did not develop AML following transplantation with *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells and treatment with TAM. The lower left graph shows the GFP$^+$ cell population in the BM of recipient mice in which no AML development was observed 120 days after transplantation with *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells and treatment with TAM. Values indicate the mean numbers of GFP$^+$ cells in the BM. (C) Colony formation by *Meis1*-deleted *MOZ-TIF2* AML cells. After replating 3 times, *Meis1*$^{f/+}$ or *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells were treated with 4-hydroxy TAM (4-OHT; 100 nM) or the same dose of ethanol (EtOH) and then serially cultured in methylcellulose medium 3 times. The mean number of colonies was calculated from the results of 3 independent experiments. The lower panel shows genotypes of colonies of *Meis1*$^{f/+}$ and *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells treated with EtOH or 4-OHT. (D) Summary of the findings of this study.

Next, we analyzed the effects of *Meis1* deletion on the maintenance of proliferation or immortalization of *MOZ* fusion AML cells. *Meis1*$^{f/f}$ *MOZ-TIF2* AML cells were treated with 4-OHT to induce Cre recombinase activation, resulting in the conversion of the *Meis1*$^{f/+}$ genotype to *Meis1*-heterozygous–deleted (*Meis1*$^{\Delta/+}$) and of *Meis1*$^{f/f}$ to *Meis1*-deleted (*Meis1*$^{\Delta/\Delta}$). *Meis1* deletion did not affect the proliferation and immortalization activity of *MOZ-TIF2* AML cells in vitro (Figure 4C). We confirmed that the *Meis1* gene was deleted entirely in *Meis1*$^{f/f}$ *Cre-ERT2 MOZ-TIF2* AML cells treated with 4-OHT. This result suggests that MEIS1 is unnecessary for the maintenance of *MOZ* fusion AML cells to maintain proliferation and immortalization. These findings provide further evidence that *Moz*$^{-/-}$ *MOZ* fusion cells failed to promote AML development because aberrant *Meis1* expression could not be induced.

Discussion

This study revealed that endogenous MOZ was essential for AML development and aberrant *Meis1* expression induced by the *MOZ/MLL* fusion and for immortalization and robust *Hoxa9* expression caused by *MLL* fusion genes (Figure 4D). Furthermore, ChIP analysis showed that endogenous MOZ was critical for maintaining an active chromatin landscape at the *Meis1* gene locus. Also, conditional deletion of *Meis1* in *MOZ* fusion AML cells resulted in the absence of AML development or prolonged survival of recipient mice, while *Meis1* overexpression rescued AML development from *Moz*-deficient cells harboring a MOZ fusion.

Previous reports have shown that MOZ and MOZ-related factor (MORF), which share significant sequence similarity, preferentially bind to H3K9ac and H3K14ac sites through their double PHD fingers.[37,38] Furthermore, the MOZ transcriptional complex components, BRPF1 and ING5, colocalize at H3K4me3-enriched *Hoxa* loci via the ING5 PHD finger.[39] Consistently, expression of *Hoxa9* and *Meis1* genes is decreased in *Brpf1*$^{-/-}$ HSPCs[40] and *MOZ* fusion AML cells with *Brpf1* knocked down.[41] Moreover, the binding of MOZ fusion proteins at the *Hoxa9* locus is reduced in *Brpf1* knockdown *MOZ* fusion AML cells.[41] These results suggest that recruitment of a MOZ fusion complex at a target gene locus requires interaction between active histone modifications and the chromatin reader domains of the MOZ transcriptional complex. In addition, H3K4me3 and H3K27ac in *Moz*$^{+/-}$ and *Moz*$^{-/-}$ MOZ-TIF2 AML cells were modestly reduced at the HoxA9 locus (Figure 3B). Considered together with our findings that HoxA9 expression is decreased in *Moz*-deficient *MLL* fusion-expressing cells but not in *Moz*-deficient *MOZ-TIF2* AML cells. These findings suggest that a certain concentration of active histone modifications may be required to recruit the MOZ complex at a target gene locus and to induce aberrant *HoxA9* expression in MOZ-TIF2 AML cells.

Endogenous *Moz* was also required for transformation and AML development by *MLL-AF10*, and for proliferation and colony formation activity by *MLL-AF9*, through aberrant *Hoxa9/Meis1* expression. Also, the onset of *MLL-AF10*–induced leukemia was slower in mice transplanted with *Moz*$^{+/-}$ *MLL-AF10* cells than in those transplanted with *Moz*$^{+/+}$ *MLL-AF10* cells, suggesting that the effect of endogenous *Moz* in MLL fusion-mediated leukemia is dose-dependent.

Although replating of 1 × 10^5 cells *Moz*$^{-/-}$ MLL-AF9 AML cells resulted in successive colony formation activity, there was a significantly decreased cell number of colonies (Figure 1A). By contrast, *MLL-AF9* cells derived from *Moz*$^{-/-}$ KSLs and HSPCs did not show continuous colony formation when replated at 3 × 10^4 cells (Figure 2D and supplemental Figure 1). These results suggest that a large number of cells may be required for the maintenance of continuous colony formation activity of *Moz*$^{-/-}$ *MLL-AF9* cells in culture media because of a need for intercellular support, for example, via paracrine (cytokine or chemokine) signaling. While *HoxA9* expression was similar among MLL-AF9–expressing KSLs with different *Moz* genotypes (Figure 2D), it was significantly decreased in *Moz*$^{-/-}$ MLL-AF9 AML cells (Figure 2A). These data suggest that endogenous *Moz* is required to maintain aberrant *HoxA9* expression in *MLL* fusion AML cells. Taken together, our findings indicate that endogenous Moz is also essential for *MLL* fusion leukemia development.

Previous studies have demonstrated that H3K9 sites at the *HOXA/MEIS1* loci are hyperacetylated in *MLL* fusion AML cells.[42] Indeed, as part of an *MLL* fusion complex, the Yaf9, ENL, AF9, Taf14 and Sas5 (YEATS) domain of eleven nineteen leukemia (ENL) binds to acetylated H3K9 and is required for AML development.[43-45] H3K9ac and other active histone marks were significantly decreased at the *Meis1* locus in *Moz*$^{-/-}$ *MLL-AF9* AML cells. Thus, it is conceivable that endogenous MOZ is required for *MLL* fusion-mediated AML development to facilitate H3K9 acetylation at the *MEIS1* locus. By contrast, although *Hoxa9* expression was remarkably decreased in *Moz*$^{-/-}$ *MLL* fusion AML cells, modification of H3K4me3 was only reduced at the *Hoxa9* locus in *Moz*$^{-/-}$ MLL-AF9 AML cells. H3K4me3 modification by MLL1 is essential for *Hoxa9* expression[46,47]; hence, H3K4me3 appears to be critical for aberrant *Hoxa9* expression in *MLL-AF9* AML cells.

Previous reports showed that MEIS1 is essential for *MLL* fusion-induced transformation of HSPCs and *Hoxa9* expression.[31,36] In this study, we demonstrate that *Meis1* is required for AML development (Figure 4A,B) but not for the colony-forming activity or aberrant *Hoxa9* expression in *MOZ-TIF2* AML cells (Figures 1B, 2A, and 4C). These data suggest that *Meis1* is not critical for *MOZ* fusion-mediated maintenance of colony-forming activity or *Hoxa9* expression. A previous report showed that both *Meis1*- and *Moz*-deficient cells exhibit increased expression of the tumor suppressor *p16*^{Ink4a} and reactive oxygen species (ROS).[21,22,31,48] Introduction of MOZ-TIF2 into HSPCs represses *p16*^{Ink4a} expression.[49] These data suggest that MOZ-TIF2 can suppress transcription induction of the *p16*^{Ink4a} gene and ROS production in *Meis1*-deleted *MOZ-TIF2* AML cells, resulting in maintenance of their colony-forming activity.

In the present study, the role of MOZ in the maintenance of AML cells was not addressed. Conditional deletion of *Moz* in adult hematopoietic cells only engenders defects in the maintenance of quiescent HSCs and lymphocytes,[50] in contrast to the severe defects observed in fetal hematopoiesis in *Moz*$^{-/-}$ mice.[13] Further, a MOZ/MORF inhibitor has recently been shown to block Eμ-Myc–driven B-lymphoma development and induction of *p16*^{Ink4a} expression.[51] These results suggest that the role of MOZ in the maintenance of AML cells may differ from its function in AML development. Additional analyses are now required, using a *Moz* conditional deletion system or an inhibitor, to further explore the

role of MOZ in effects on target gene expression in maintaining AML.

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Authorship

Contribution: T.K., Y.O., K.Y., and I.K. designed the experiments; T.K., Y.O., K.Y., and Y.A. performed experiments; R.G. and T.N. provided materials; and T.K. and I.K. analyzed and interpreted the data and wrote the paper.

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ORCID profiles: R.G., 0000-0002-1137-9760; T.N., 0000-0002-0419-7547; I.K., 0000-0002-8409-0407.

Correspondence: Issay Kitabayashi, Division of Hematological Malignancy, National Cancer Center Research Institute, 5-1-1 Tsukiji, Chuo-ku, Tokyo, 104-0045, Japan; email: ikitabay@ncc.go.jp.

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