Supplementary Files

Supplementary Materials and Methods

Immunofluorescent staining and flow cytometric analysis

One million AML cells were preincubated with anti-CD16/32 antibodies to prevent non-specific binding via FcR interactions. Then, the AML cells were incubated on ice for 45 min with staining reagents, according to standard methods\(^1\). Flow cytometric analysis was performed on a JSAN flow cytometer. The data were analyzed using FlowJo software (Tree Star, Ashland, OR, USA).

Retrovirus production and infection

To generate AML cells, we used pMSCV-ires-EGFP, pMSCV-MOZ-TIF2-ires-EGFP, pMSCV- Flag-MLL-AF10-ires-EGFP, pMSCV-AML1-ETO-ires-EGFP, pMSCV-human HOXA9-ires-EGFP, pGCDN-ires-hNGFR, and pGCDN-human MEIS1-ires-hNGFR retrovirus vectors\(^2-4\). The pMSCV-Flag-MLL-AF9-ires-EGFP vector was created by recombining the AF9 portion of pMSCV-Flag-MLL-AF9-ires-Neo\(^5\) with the AF10 portion of pMSCV-Flag-MLL-AF10-ires-EGFP. Plat-E cells for retrovirus packaging were cultured in DMEM (Nacalai Tesque) containing 10% FBS, 100 U/ml penicillin/streptomycin, 1 \(\mu\)g/ml puromycin (WAKO, Osaka, Japan), and 10 \(\mu\)g/ml blasticidin (Invitrogen, Carlsbad CA, USA). Plasmids were transfected into
Plat-E cells using GeneJuice reagent (Merck Millipore), and retrovirus-containing supernatants were collected 48 h after transfection. For infection, well plates were coated with RetroNectin (Takara Bio., Shiga, Japan) for 2 h at room temperature (RT) or overnight at 4°C. To conjugate retrovirus and RetroNectin onto well plates, supernatants containing retrovirus were added to RetroNectin-coated wells, and plates were then centrifuged four times at 900 g (30 min, 32°C). HSPCs (1 or 2 x 10^5 cells) and KSLs/CMPs (1 x 10^4 cells) were cultured in the resulting retrovirus-coated well plates for 5–8 days.

**RNA purification and qRT-PCR analysis**

For gene expression analysis, total RNA from sorted GFP-positive cells was isolated using Isogen (Nippon Gene, Toyama, Japan) and purified with an RNeasy Micro Kit (Qiagen, Hilden, Germany). Total RNA from AML cells was purified with an RNeasy Mini Kit (Qiagen). cDNA was synthesized using a Superscript III or IV VILO cDNA Synthesis Kit (Thermo Fisher Scientific), following the manufacturer’s protocol. Quantitative reverse transcriptase-PCR (qRT-PCR) analysis was performed using a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) with FastStart Universal SYBR Green Master (ROX; Roche, Mannheim, Germany). The expression values of each gene were calculated using a standard
curve and normalized to $\beta$-actin levels. The primers used are shown in Suppl. Table 1.

Chromatin immunoprecipitation (ChIP) assay

Colonies of Moz$^{+/+}$, Moz$^{+/-}$, and Moz$^{-/-}$ MOZ-TIF2 or MLL-AF9 AML cells were collected and suspended in RPMI 1640 medium (Nacalai Tesque) containing 10% FBS at $5 \times 10^6$ cells/ml, fixed with 1% formalin, and glycine was added to stop the reaction. Fixed AML cells were washed with PBS twice lysed with lysis buffer (50 mM HEPES-KOH (pH 7.55), 1 mM EDTA, 1% TX-100, 150 mM NaCl, 0.1% SDC, and 1% SDS), and fragmented to 150–500 bp using an S220 Focused ultrasonicator (Covaris, Woburn, MA, USA). Chromatin DNA-protein complex fragments were centrifuged at 20,400 g (30 min, 4°C) to remove debris and the insoluble fraction. Supernatants from $0.5 \times 10^6$ cells were diluted 1/10 with FA dilution buffer (20 mM Tris-HCl (pH 8.1), 2 mM EDTA, 150 mM NaCl, and 1% TX-100) and then incubated with antibody against a histone modification at 4°C overnight with slow rotation. The next day, supernatants were incubated with Dynabeads Protein G (Thermo Fisher) (4°C, 6 h, with slow rotation). Histone modification immunoprecipitants were washed with low salt buffer (20 mM Tris-HCl (pH 8.1), 2 mM EDTA, 150 mM NaCl, 1% TX-100, and 0.1% SDS) twice, high salt buffer (20 mM Tris-HCl (pH 8.1), 2 mM EDTA, 500 mM NaCl, 1% TX-100, and 0.1% SDS), and lithium chloride buffer (10 mM Tris-
HCl (pH 8.1), 1 mM EDTA, 250 mM LiCl, 1% NP-40, and 0.1% SDC) (4°C, with rotation). Immunoprecipitants were rinsed with 0.1% Triton-X100 in PBS (twice, 4°C, with rotation) and beads incubated in elution buffer (1.0% SDS, 0.1 M sodium carbonate) (30 min, RT, with shaking) to release immunoprecipitated chromatin protein/DNA complexes from Dynabeads Protein G, which were removed from the elution buffer using the magnetic apparatus. NaCl (final concentration, 200 mM) was added to the elution buffer containing protein/DNA complexes and incubated overnight at 65°C to reverse the DNA and chromatin protein crosslinking. Tris-HCl (pH 6.5), EDTA, and Proteinase K (WAKO) were added to the elution buffer containing de-crosslinked DNA and chromatin proteins at final concentrations of 40 mM, 1 mM, and 80 µg/ml, respectively, and then incubated at 45°C for 2 h. DNA fragments were purified by phenol/chloroform extraction and ethanol precipitation with Ethachinmate (Nippon Gene) and eluted in TE buffer. Antibodies used for ChIP are listed in the ‘Reagents’ section.

ChIP-quantitative PCR (ChIP-qPCR) was performed using a QuantStudio 12K Flex Real-Time PCR System (Applied Biosystems) with FastStart Universal SYBR Green Master (ROX; Roche). Amounts of each locus were determined using standard curves and normalized to input DNA levels. For ChIP assays of histone
modification, values were further normalized to those of total histone H3. Primer sequences are shown in Suppl. Table 1.

**ChIP-Seq analysis**

For ChIP-Seq analysis of H3K9ac, supernatants from $1 \times 10^7$ cells were diluted 1/10 with FA dilution buffer and then incubated with 10 µg of H3K9ac antibody (Active Motif) at 4°C overnight with slow rotation. Subsequently, bead conjugation, washing, elution, and DNA purification methods were conducted using the same methods as those for ChIP assay (see above). For analysis of H3K23ac, supernatants from $1 \times 10^7$ cells were diluted 1/10 with modified FA dilution buffer (20 mM Tris-HCl (pH 8.1), 2 mM EDTA, 150 mM NaCl 1% TX-100, and 0.2% SDS) and then incubated with 2.5 µg of H3K23ac antibody (Merck Millipore) at 4°C overnight with slow rotation. The next day, supernatants were incubated with Dynabeads Protein G (4°C, 6 h, with slow rotation). H3K23ac immunoprecipitants were washed twice with high salt buffer and twice with lithium chloride buffer (4°C, with rotation). Immunoprecipitants were rinsed with 0.1% Triton-X100 in PBS (twice, 4°C, with rotation). Subsequently, elution and DNA purification were performed as for ChIP assay (see above).

ChIP DNA was purified using a MinElute PCR purification Kit (Qiagen). Library preparation and sequencing were performed by Azenta Japan using the
Illumina Nova-Seq 6000 system (Illumina Inc., San Diego, CA, USA) to generate 150 bp pair-end reads, resulting in approximately 20 million reads per sample.

Data analysis was performed on the Galaxy Europe website\textsuperscript{6}. Sequence reads were trimmed using the Trimmomatic tool\textsuperscript{7}. then were mapped on the mouse genome (mm10) using Bowtie2 tool\textsuperscript{8,9}. PCR duplicates were removed from aligned sequences, which were sorted into chromosomes using Samtools\textsuperscript{10}. The resulting files were converted to BigWig format using deeptools\textsuperscript{11} and visualized using Integral Genome Viewer\textsuperscript{12}.

**Conditional deletion of Meis1 in AML cells *in vitro* and *in vivo***

For *in vivo* analysis of AML development, *Meis1\textsuperscript{f/+}* or *Meis1\textsuperscript{f/f}* Cre-ERT2 lineage\textsuperscript{-} HSPCs were collected from FL and transduced with MOZ-TIF2 using the retrovirus system described above. Five days after infection, $5.0 \times 10^5$ *Meis1\textsuperscript{f/+}* or *Meis1\textsuperscript{f/f}* Cre-ERT2 MOZ-TIF2 cells were transplanted into sub-lethally irradiated (600 rad) mice. Two to three months after transplantation, *Meis1\textsuperscript{f/+}* or *Meis1\textsuperscript{f/f}* Cre-ERT2 MOZ-TIF2 AML cells were collected from the bone marrow of recipient mice. *MOZ-TIF2* AML cells ($3.0 \times 10^5$) were then re-transplanted into irradiated (300 rad) mice. One week later, TAM (80 mg/Kg) or corn oil (200 µl) was injected intraperitoneally into mice on days 0, 2, 4, and then once each week. AML development was
monitored and confirmed as described above. Bone marrow cells from recipient mice that developed AML were analyzed to determine their *Meis1* genotype and the proportion of GFP marker-positive cells in the bone marrow of survived recipient mice transplanted with *Meis1*\textsuperscript{fl/f} Cre-ERT2 MOZ-TIF2 AML cells treated with TAM was assessed at 120 days after transplantation. Further, 2.0 x 10\textsuperscript{5} GFP-positive cells were sorted, and their *Meis1* genotypes were analyzed.

For the *in vitro* replating assay, *Meis1*\textsuperscript{fl/+} or *Meis1*\textsuperscript{fl/fl} Cre-ERT2 MOZ-TIF2-infected cells were prepared as described above. GFP-positive cells (5 x 10\textsuperscript{4}) were sorted and cultured in a methylcellulose medium and replated every 4–5 days. After replicating three times, *Meis1*\textsuperscript{fl/+} or *Meis1*\textsuperscript{fl/fl} Cre-ERT2 MOZ-TIF2 AML cells were replated in a methylcellulose medium containing 4-OHT (100 ng/ml) or the same amount of ethanol (WAKO) a further three times. *Meis1* genotypes of colony cells were analyzed in 1\textsuperscript{st} round colony-forming cells after treatment with 4-OHT or ethanol.
Supplementary References

1. Katsumoto T, Aikawa Y, Iwama A, et al. MOZ is essential for maintenance of hematopoietic stem cells. Genes Dev. 2006;20(10):1321–1330.

2. Ogawara Y, Katsumoto T, Aikawa Y, et al. IDH2 and NPM1 Mutations Cooperate to Activate Hoxa9/Meis1 and Hypoxia Pathways in Acute Myeloid Leukemia. Cancer Res. 2015;75(10):2005–2016.

3. Shima Y, Yumoto M, Katsumoto T, Kitabayashi I. MLL is essential for NUP98–HOXA9–induced leukemia. Leukemia. 2017;31(10):2200–2210.

4. Shima H, Takamatsu–Ichihara E, Shino M, et al. Ring1A and Ring1B inhibit expression of Glis2 to maintain murine MOZ–TIF2 AML stem cells. Blood. 2018;131(16):1833–1845.

5. Yokoyama A, Lin M, Naresh A, Kitabayashi I, Cleary ML. A higher–order complex containing AF4 and ENL family proteins with P–TEFb facilitates oncogenic and physiologic MLL–dependent transcription. Cancer Cell. 2010;17(2):198–212.

6. Galaxy C. The Galaxy platform for accessible, reproducible and collaborative biomedical analyses: 2022 update. Nucleic Acids Res. 2022.

7. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114–2120.
8. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. *Genome Biol.* 2009;10(3):R25.

9. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods.* 2012;9(4):357–359.

10. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics.* 2009;25(16):2078–2079.

11. Ramirez F, Ryan DP, Gruning B, et al. deepTools2: a next generation web server for deep-sequencing data analysis. *Nucleic Acids Res.* 2016;44(W1):W160–165.

12. Robinson JT, Thorvaldsdottir H, Winckler W, et al. Integrative genomics viewer. *Nat Biotechnol.* 2011;29(1):24–26.
Supplementary Figure Legends

Figure S1 related to Figure 2

Colony formation of Moz\textsuperscript{+/−} or Moz\textsuperscript{−/−} KSLs/HSPCs bearing MLL-AF9. First, Moz\textsuperscript{+/−} or Moz\textsuperscript{−/−} KSL cells (1 x 10\textsuperscript{4} cells) and Lineage\textsuperscript{−} HSPCs (1 x 10\textsuperscript{5} cells) were transduced with MLL-AF9 fusion genes and cultured in liquid medium. Subsequently, GFP\textsuperscript{+} cells (5 x 10\textsuperscript{4}) were sorted and cultured in methylcellulose medium. Colony numbers were counted every 4–5 days, and then 3 x 10\textsuperscript{4} cells were serially replated three times. The mean numbers of colonies formed by MLL-AF9-expressing Moz\textsuperscript{+/−} and Moz\textsuperscript{−/−} cells derived from KSLs or HSPCs were calculated from two independent experiments.
Figure S2 related to Figure 3

(A,B) Quantitative PCR ChIP analysis of MOZ-targeted and H3K9 and H3K23 acetylation and active histone modifications at the Meis1 and Hoxa9 loci, and mouse Chr. 3 desert (negative control) loci in MOZ-TIF2 (A) or MLL-AF9 (B) AML cells. Representative results of ChIP assays are shown. Seven and three independent experiments were conducted using MOZ-TIF2 (A) and MLL-AF9 (B) AML cells, respectively. Primer sets used to amplify the Meis1 and Hoxa9 are indicated lower right. Levels of each histone modification were normalized to input DNA and total histone H3 levels. Error bars represent mean ± S.D.
Figure S3 related to Figure 4

(A) Expression of the *HoxA9* and *Meis1* genes in Moz*+/−* or Moz*−/−* cells bearing MOZ-TIF2. Moz*+/−* or 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, the expression levels of both endogenous and ectopic Meis1 and β-actin were analyzed by qRT-PCR (n = 2). Expression levels of HoxA9 and Meis1 were normalized to those of β-actin. Error bars represent mean ± S.D.

(B) AML development of Moz*+/−* cells bearing a MOZ-fusion and MEIS1. 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, cells were transplanted into recipient mice, and their survival was analyzed (n = 8/group). Survival was compared using the log-rank test.

(C) Expression of GFP (MOZ-TIF2) and human NGF receptor (human MEIS1) and immunological phenotypes of MOZ-TIF2 + MEIS1 AML cells. Moz*+/−* or Moz*−/−* cells bearing MOZ-TIF2 and MEIS1 were harvested from the bone marrow of recipient mice that had developed AML and were stained with fluorescently labeled antibodies.
against the Mac-1, Gr-1, M-CSF receptor, or human NGF receptor. Representative
flow cytometry pseudo-color plots are shown.

(D) AML development of Moz+/− or Moz−/− cells overexpressing MEIS1. The
experimental scheme is shown at the top. Moz+/− or Moz−/− FL lineage− HSPCs were
transduced with the MEIS1 gene. After 5 days, these cells (5.0 x 10^5) were
transplanted into sub-lethally irradiated (600 rad.) recipient mice, and their survival
was analyzed until 120 days (n = 6/group).

(E) Conditional heterozygous deletion of the Meis1 gene in MOZ-TIF2 AML cells.
The survival of recipient mice transplanted with Meis1-heterozygous deleted MOZ-
TIF2 AML cells is shown. The experimental scheme is shown at the top; MEis1^+/−Cre-
ERT2 MOZ-TIF2 AML cells were transplanted into recipient mice. TAM (80 mg/kg)
or the same volume of corn oil was administered intraperitoneally (i.p.; n = 4/group).
Survival was compared using the log-rank test.
|                | Lists of primer sequences (5' to 3')                                                                 |
|----------------|-----------------------------------------------------------------------------------------------------|
| **Genotyping** |                                                                                                       |
| MOZKO          | GCCTGCTTGCCGAATATCATGGTGAGAAAT                                                                       |
|                | CTCTGAAGGGTCGCTGTTTCTGC                                                                           |
|                | GATACGACAGAAAAACCACAGGCC                                                                            |
| Meis1/f/f      | CTGGCGCTTCCTACATCACTG                                                                              |
|                | CACTTCACGCTTCAGCTTGAA                                                                             |
| Meis1+/+,f/f, Δ/Δ | CCCCCACCAGTCTGAAGATA                                                                              |
|                | CACTTCACGCTCAGCTTGAA                                                                              |
| **qRT-PCR**    |                                                                                                       |
| mouse          | CTTTCCAGCCCTCTCTTCTTGG                                                                            |
| β-actin        | CAGCACTGTGTTGGCATAGAG                                                                            |
| human/mouse    | GGTGCCTGCTGCAGTGATGT                                                                              |
| HoxA9          | GTCAGCCAAGGAGCGCATAT                                                                               |
| human/mouse    | CCTCGGTCATGACGCTTTAA                                                                               |
| Meis1          | GGTACAAGTAGCTAATTCCATTTTCTCAAAA                                                                    |
| **ChIP-qPCR**  |                                                                                                       |
| mouse          | CTTTGGGAAGAGAGGCTTGAA                                                                              |
| Meis1-1        | CCAGGGCACATGCACACA                                                                                 |
| mouse          | GGTGAAGGATCCTGTGTACTGA                                                                             |
| Meis1-2        | CGGTAACCGGAACGTT                                                                                  |
| mouse          | TGCCCTGTTGTAATCCCTTGAAACAG                                                                        |
| HoxA9-1        | TTCCTCCGGGTTAATTTGAT                                                                               |
| mouse          | GGTCCGCTGTAGGTACATGT                                                                               |
| HoxA9-2        | CAAAACACAGGCAGCTTGAA                                                                              |
| mouse          | AAAGCCGAGGCCTAAGAG                                                                                |
| HoxA9-3        | TCTAAATCCGGCCTCATCTC                                                                               |
| mouse          | ATAGGTACCAAGGACAGTATTAGA                                                                            |
| Chr. 3 desert  | AGTTATCACATTTTCAAGGCA                                                                              |