Supplementary Experimental Procedures:

1.1 Cell culture exposed to DNA methyltransferase inhibitors

Cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FBS, penicillin G (50 units/ml), and streptomycin (50 μg/ml) in a humidified incubator containing 5% CO_2 in air. The DNA methyltransferase inhibitors (DNMT-is), decitabine (Dac) and zebularine (Zeb) (Sigma-Aldrich-Japan, Tokyo, Japan), were dissolved in phosphate-buffered saline. To adjust the biological effects of Dac and Zeb, we analyzed their growth suppressive effects in 5 AML cell lines categorized in the MPOa-L group (KG-1, KG-1a, THP-1, CMK-86, and K562). A total of 5×10^5 cells were seeded on day 0, media containing DNMT-is were freshly added on days 1 and 3, and cells were harvested on day 5, as previously described [1]. The final concentrations of Dac in the cultures were 0.3, 1.0, 3.0, and 10.0 μM; those of Zeb were 10.0, 20.0 50.0, and 100.0 μM. The number of viable cells was determined daily by the exclusion of Trypan blue. DNMT-is inhibited cell proliferation in dose- and time-dependent manners in all cell lines tested. The exposure to Dac at 1.0 μM and Zeb at 50.0 μM led to comparable growth suppression in MPOa-L AML cell lines cultures at day 5 (Figure S1d). To assess the DNA-hypomethylating activity of Dac and Zeb, the methylation status of the 5' region of the *MPO* gene was quantified by bisulfite sequencing. Demethylation was

present at 1.0 μM of Dac and 50.0 μM of Zeb in all 5 AML cell lines with MPOa-L. On the basis of these results, 1.0μM of Dac and 50.0 μM of Zeb were considered biologically equivalent in this study for these 5 AML cell lines.

1.2 Flow cytometry analysis

The expression of cytoplasmic MP) protein was examined using flow cytometry (FACSCalibur flow cytometer and Cellquest software, BD Biosciences). The antibody against MPO conjugated with FITC (DAKO) was used after th fixation and permeabilization of cells using FIX&PERM cell permeabilization reagents (Invitrogen, Carlsbad, CA, USA) following the manufacturer's protocol.

1.3 Direct sequencing of FLT3, NPM1, and CEBPA genes

Mutations in the *FLT3*, *NPM1*, and *CEBPA* genes were detected by direct sequencing after the amplification of genomic DNA by PCR. The exons tested were as follows: *FLT3*, exons 14 and 15; *NPM1*, exon 12; *CEBPA*, exon 1. The primers for sequencing were previously described (see Table below) [2-4]. DNA sequencing of exons was performed as follows. PCR reactions were run in a final volume of 50 μL containing 10 ng template DNA, 5× buffer, 0.2 mmol/L of each deoxynucleotide triphosphate,

primers (0.3mmol/L of each), nucleotides (0.2 mmol/L of each), and 1 U of KOD-Plus-Neo polymerase (TOYOBO, Osaka, Japan). The mixture was initially heated at 94°C for 2 min before being subjected to 35 cycles of denaturation at 94°C for 10 s and annealing and extension at 68°C for 1 min. The amplified products were fractionated by gel electrophoresis (1.2% agarose gel), cut from the gel, and purified with the MinElute Gel extraction kit (QIAGEN, Germany). To screen gene mutations, PCR products were sequenced in both directions with the previously published primers, using the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100 ×1 Genetic Analyzer (Applied Biosystems, CA, USA). Heterozygous or homozygous mutations in *NPM1* and *CEBPA* genes were confirmed by cloning PCR products using the StrataClone Blunt PCR Cloning Kit (Stratagene, CA, USA) following the manufacturer's recommendations. Plasmid DNA was prepared using a QIAprep spin plasmid miniprep kit (Qiagen, Hilden, Germany), and its integrity was confirmed by the sequence of both strands using T3 and T7 primers.

1.4 Quantitative reverse transcriptase-polymerase chain reaction *MPO* and *DNMT3B* genes

Quantitative reverse transcriptase-polymerase chain reaction (QRT-PCR) was

performed using LightCycler TaqMan Master (Roche Diagnostics, Mannheim, Germany) following the manufacturer's instructions. PCR amplification was performed using a LightCycler 350S instrument (Roche). Thermal cycling conditions were as follows; 10 min at 95°C, followed by 45 amplification cycles at 95°C for 10 seconds, 60°C for 30 seconds, and 40°C cooling cycle for 30 seconds. These primers for DNMT3B could detect 6 alternative spliceoforms; 3 catalytic forms (3B1, 3B2, and 3B6) and 3 non-catalytic forms (3B3, 3B7, and 3B8). Primers and the TaqMan probe for the sequence of the *ABL1* gene were those published in the EAC network protocol for RQ-PCR [5].

1.5 Bisulfite sequencing for the 5' region of the MPO gene

Genomic DNA (1μg) was chemically modified with the Cells-to-CpG Bisulfite Conversion Kit (Applied Biosystems) according to the manufacturer's recommendations. Bisulfite-treated DNA was amplified in PCR with *MPO*-specific primers. PCR reactions were run in a final volume of 50 μL containing 100 ng DNA, 10× buffer, 2.5 mmol/L of each deoxynucleotide triphosphate, primers (0.2 mmol/L of each), $MgCl_2$ (25 mmol/L of each), and 2.5 U of TaKaRa EpiTaq HS (TaKaRa, Ohtsu, Japan). The mixture was initially heated at 95°C for 3 min, before being subjected to 49

cycles of denaturation at 95°C for 1 min, annealing at 55°C for 2 min, and extension at 72°C for 2 min. The amplified products were gel-purified using the MinElute Gel extraction kit (QIAGEN, Germany) and ligated into the pMD20T-Vector using the Mighty TA-cloning Kit (TaKaRa). Eighteen to thirty clones per sample were sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit and ABI Prism 3100 ×1 Genetic Analyzer. Analysis of the obtained sequences confirmed a complete bisulfite reaction in all samples. The average methylation of the *MPO* promoter fragment was assessed by the Student *t*-test.

1.6 Pyrosequencing for *long interspersed nuclear element-1*

DNA methylation (%5-mC) of *long interspersed nuclear element-1 (LINE-1)* was quantified using PCR-pyrosequencing of the bisulfite-treated DNA (EpigenDx Laboratory Service (Worcester, MA)), as previously described [6]. In brief, bisulfite-treated DNA was amplified by PCR using primers designed toward a consensus *LINE-1* sequence. PCR was performed in a 50μL reaction mixture containing 25μL GoTaq Green Master mix (Promega, Madison, WI, USA), 1 pmol of the forward and biotinylated reverse primers, 50 ng of bisulfite-treated genomic DNA, and water. Biotin-labeled final PCR products (amplified by biotin-labeled primers) bound to

Streptavidin Sepharose HP (Amersham Biosciences, Uppsala, Sweden) were washed using the Pyrosequencing Vacuum Prep Tool (Pyrosequencing Inc., Westborough, MA, USA), as recommended by the manufacturer. A total of 0.3μM of the pyrosequencing primer was then annealed to the purified single-stranded PCR products, and pyrosequencing was performed using the PSQ-HS 96 Pyrosequencing System (Pyrosequencing Inc.). The relative 5-mC content was expressed as a percentage of methylated cytosines divided by the sum of methylated and unmethylated cytosines (5-mC / [5-mC + unmethylated cytosine] = %5-mC). Built-in controls were used to verify bisulfite conversion efficacy. To increase precision, each sample was tested four times for *LINE-1* methylation, and the mean of 4 independent experiments was used in statistical analyses. The coefficient of variation (CV) among 48 blinded replicate samples was 5.7%, and the inter-plate CV was 2.9%. Differences in the percentage of DNA methylation (%5-mC) of *LINE-1* among patients in different categories (MPOa-H group, MPOa-L group, Ph+ALL group, and healthy donor group) were compared using the non-parametric Kruskal-Wallis test and followed by Dunn's multiple comparison post-test.

1.7 Primer Sequences

Primers for direct sequencing

Gene	Primer	Sequencing
FLT3	forward	5’-GCAATTTAGGTATGAAAGCCAGC-3’
	reverse	5’-CTTTCAGCATTTTGACGGCAACC-3’
NPM1	forward	5’-GGTTGTTCTCTGGAGCAGCGTTC-3’
	reverse	5’-CCTGGACAACATTTATCAAACACGGTA-3’
CEBPA	forward1	5’-TGCCGGGTATAAAAGCTGGG-3’
	reverse1	5’-CTCGTTGCTGTTCTTGTCCA-3’
	forward2	5’-TGCCGGGTATAAAAGCTGGG-3’
	reverse2	5’-CACGGTCTGGGCAAGCCTCGAGAT-3’

Primers for the quantitative reverse transcriptase-polymerase chain reaction

Gene	Primer	Sequencing
MPO	forward	5’-CTGCATCATCGGTACCCAGTTC-3’
	reverse	5’-GCCTGTCGCTGCTGCATG-3’
	probe	5’-CTCCCACCAAAACCGATCACCATCCCG-3’
DNMT3B	forward	5’-TTGGCGATGGCAAGTTCTCC-3’
	reverse	5’-AGACGAGCTTATTGAAGGTGGC-3’
	probe	5’- TGAACAGCCCCAGTGCCACCAGTTTG-3’
ABL1	forward	5’- GATACGAAGGGAGGGTGTACCA-3’
	reverse	5’- CTCGGCCAGGGTGTTGAA -3’
	probe	5’- TGCTTCTGATGGCAAGCTCTACGTCTCC-3’

Primers for bisulfite sequencing

Promoter	Primer	Sequencing
MPO	forward	5’-AGTTTTTTTTAGTTTAATTTG-3’
	reverse	5’-TACAAAAATTACTTCTTACCTAAAAAA-3’

Primers for pyrosequencing

Target	Primer	Sequencing
LINE-1	forward	5’-TTTTGAGTTAGGTGTGGGATATA-3’
	reverse	5’- biotin-AAAATCAAAAAATTCCCTTTC-3’

Supplemental References:

1. Moriguchi K, Yamashita S, Tsujino Y, Tatematsu M, Ushijima T. Larger numbers of silenced genes in cancer cell lines with increased de novo methylation of scattered CpG sites. Cancer Lett. 2007;249(2):178-187.

2. Kiyoi H, Naoe T, Nakano Y, Yokota S, Minami S, Miyawaki S, et al. Prognostic implication of FLT3 and N-RAS gene mutations in acute myeloid leukemia. Blood. 1999;93(9):3074-3080.

3. Falini B, Mecucci C, Tiacci E, Alcalay M, Rosati R, Pasqualucci L, et al. Cytoplasmic nucleophosmin in acute myelogenous leukemia with a normal karyotype. N Engl J Med. 2005;352(3):254-266.

4. Tominaga-Sato S, Tsushima H, Ando K, Itonaga H, Imaizumi Y, Imanishi D, et al. Expression of myeloperoxidase and gene mutations in AML patients with normal karyotype: double CEBPA mutations are associated with high percentage of MPO positivity in leukemic blasts. Int J Hematol. 2011;94(1):81-89.

5. Gabert J, Beillard E, van der Velden VH, Bi W, Grimwade D, Pallisgaard N, et al. Standardization and quality control studies of ‘real-time’ quantitative reverse

transcriptase polymerase chain reaction of fusion gene transcripts for residual disease detection in leukemia – a Europe Against Cancer program. Leukemia. 2003;17(12):2318-2357.

6. Bollati V, Baccarelli A, Hou L, Bonzini M, Fustinoni S, Cavallo D, et al. Changes in DNA methylation patterns in subjects exposed to low-dose benzene. Cancer Res. 2007;67(3):876-880.

Figure S1

(a) DNA methylation status of the *MPO* gene promoter in 10 hematological cell lines. Methylation of the cytosine-phosphate-guanine (CpG) dinucleotide was assessed by sequencing the multiple cloned alleles of PCR products on bisulfite-treated genomic DNA. Each horizontal line represents an individual allele. Filled circles represent methylated CpG sites; open circles, unmethylated CpG sites. The position of each cytosine nucleotide relative to the transcriptional start site is indicated at the top. Kasumi-1 and SKM-1 showed high MPO enzymatic activity (MPOa-H), whereas the 8 other cell lines had low MPO enzymatic activity (MPOa-L).

(b) Effects of the DNMT-i treatment on methylation of the 5’ promoter region of the *MPO* gene.

Open squares, represent the percentage of methylated CpG sites in the *MPO* gene of each cell line without the DNMT-i treatment; filled squares, with the decitabine (Dac) treatment; gray squares, with the zebularine (Zeb) treatment.

(c) Induction of the MPO protein by the treatment with DNMT-i.

The shared histogram shows the level of intracellular MPO as measured in flow cytometry. The black curves represent each cell line without the DNMT-i treatment as the control; gray curves, with the DNMT-is treatment.

(d) Inhibitory effect of DNMT-is on cell proliferation.

A total of 5×10^5 cells were cultured in the presence or absence of Dac (1.0 μM) and Zeb (50.0 μM). The number of living cells after 5 days was counted by the Trypan blue dye exclusion method. Error bars indicate one standard deviation (three replicate experiments).

Figure S2

DNA methylation status of the *MPO* gene promoter in CD34-positive cells obtained from 15 AML patients. CpG dinucleotide methylation was assessed by sequencing multiple cloned alleles obtained from PCR on bisulfite-treated genomic DNA. Numbers on the left of each figure show the percentage of MPO positive blasts on

bone marrow smears. MPOa-H and -L indicate a high percentage (>50%) and low percentage (≤50%) of MPO-positive myeloblasts, respectively.

Figure S3

Methylation levels of *LINE-1* in clinical samples.

LINE-1 methylation was determined as a surrogate for global methylation in CD34-positive cells from clinical samples (i.e. MPOa-H AML, MPOa-L AML, Ph+ALL, and healthy donor groups). No significant differences were observed in the median levels (horizontal dotted line) between the MPOa-H AML group and other groups (Kruskal-Wallis test).

Table S1 Expression of MPO and *FLT3*, *NPM1*, and *CEBPA* Mutations in cell lines

Cell line	Origin	Fusion gene	MPO/ABL1 mRNA ratio	MPO enzymatic activity	*FLT3-ITD*	*NPM1*	*CEBPA*
SKM-1	Myeloid leukemia	-	137.45	Positive	Negative	wt	wt
Kasumi-1	Myeloid leukemia	*RUNX1-RUNX1T1*	293.65	Positive	Negative	wt	wt
KG-1	Myeloid leukemia	-	0.03	Negative	Negative	wt	wt
KG-1a	Myeloid leukemia	-	0.01	Negative	Negative	wt	wt
THP-1	Monocytic leukemia	-	0.54	Negative	Negative	wt	wt
CMK-86	Megakaryoblastic leukemia	-	0.01	Negative	Negative	wt	Single mutation
K562	Erythroblastic leukemia	*BCR-ABL1*	0.00	Negative	Negative	wt	wt
CML-T1	T-cell leukemia	*BCR-ABL1*	0.72	Negative	Negative	wt	wt
BV173	B-cell leukemia	*BCR-ABL1*	0.01	Negative	Negative	wt	wt
SU-DHL-6	B-cell lymphoma	-	0.00	Negative	Negative	wt	wt

Abbreviations: MPO represents myeloperoxidase; wt, wild type.

Quantitative analysis of MPO and ABL1 mRNA was performed by quantitative reverse-transcriptional PCR amplifications after RNA isolation and complementary DNA synthesis. MPO enzymatic activity was evaluated using the diaminobenzidine method. The mutation status of *FLT3*, *NPM1*, and *CEBPA* was determined using direct sequencing.

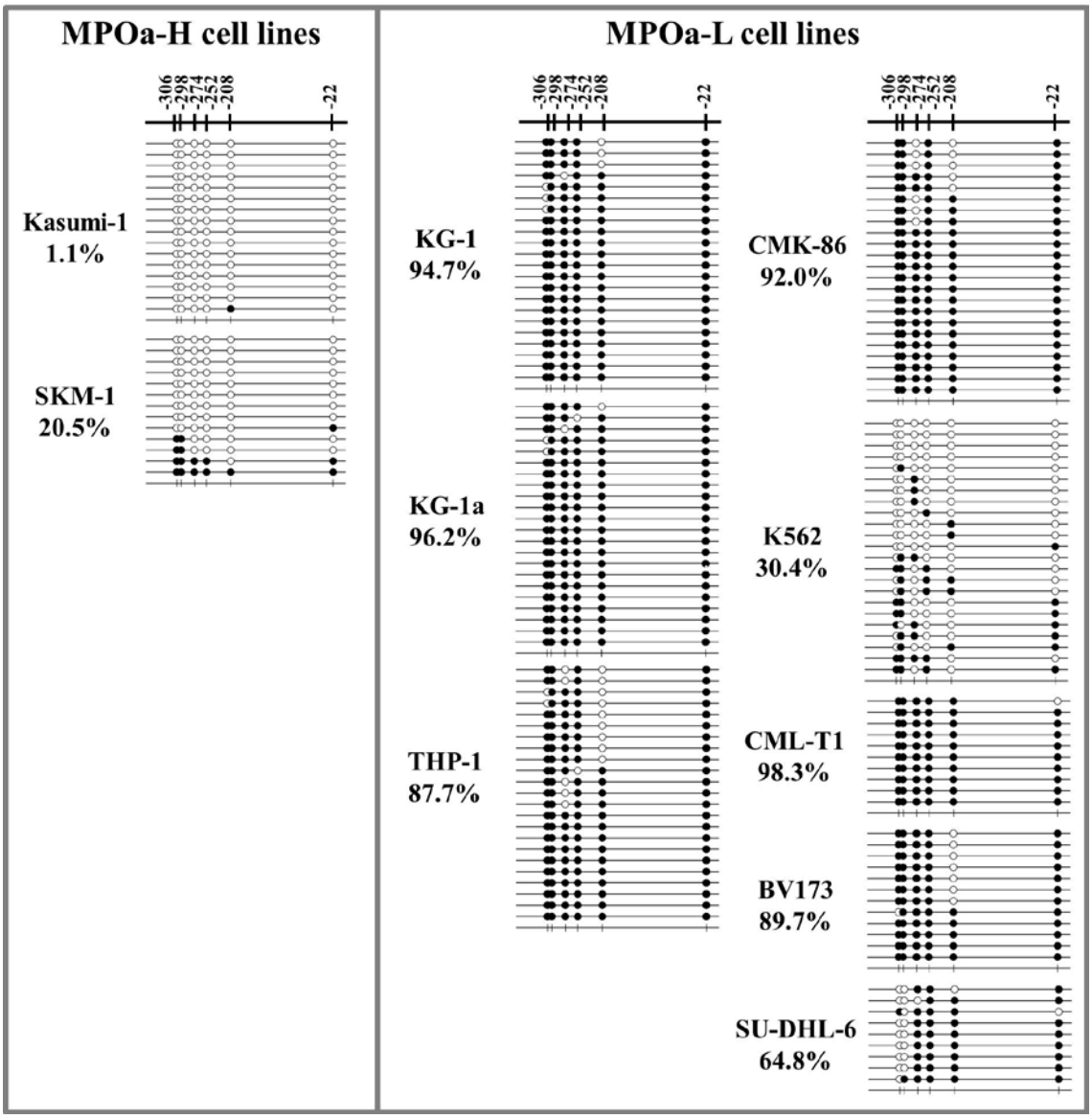
Table S2 Expression of DNMT3B in CD34-positive cells obtained from clinical samples

UPN	DNMT3B/ABL1 mRNA ratio
1	0.072
2	0.026
3	0.018
4	0.093
5	0.003
6	0.081
7	0.033
8	0.057
9	0.074
10	0.044
11	0.352
12	0.153
13	0.085
14	0.030
15	0.045

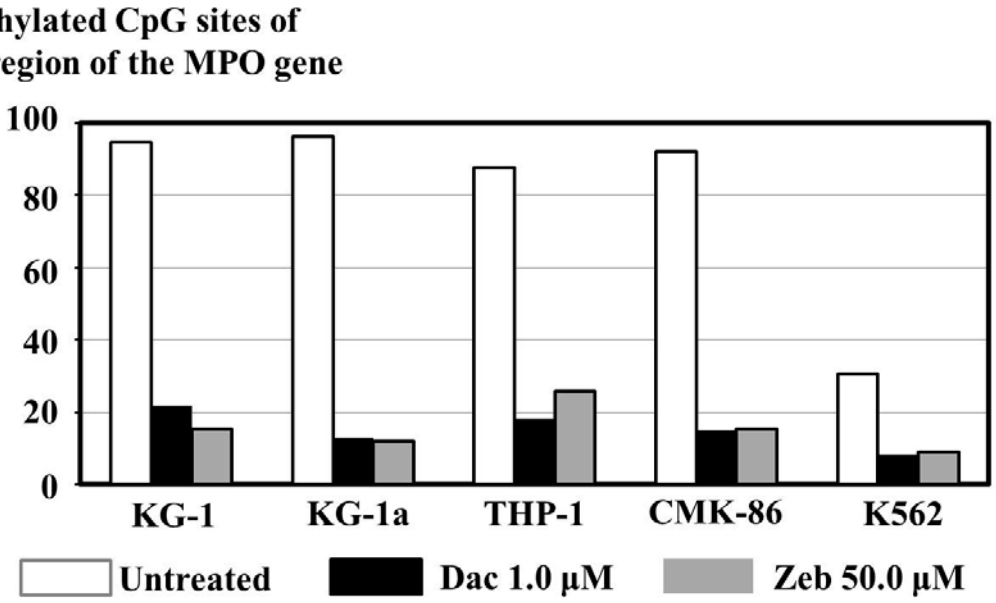
Quantitative analysis of DNMT3B mRNA was also performed in CD34-positive cells.

Figure S1

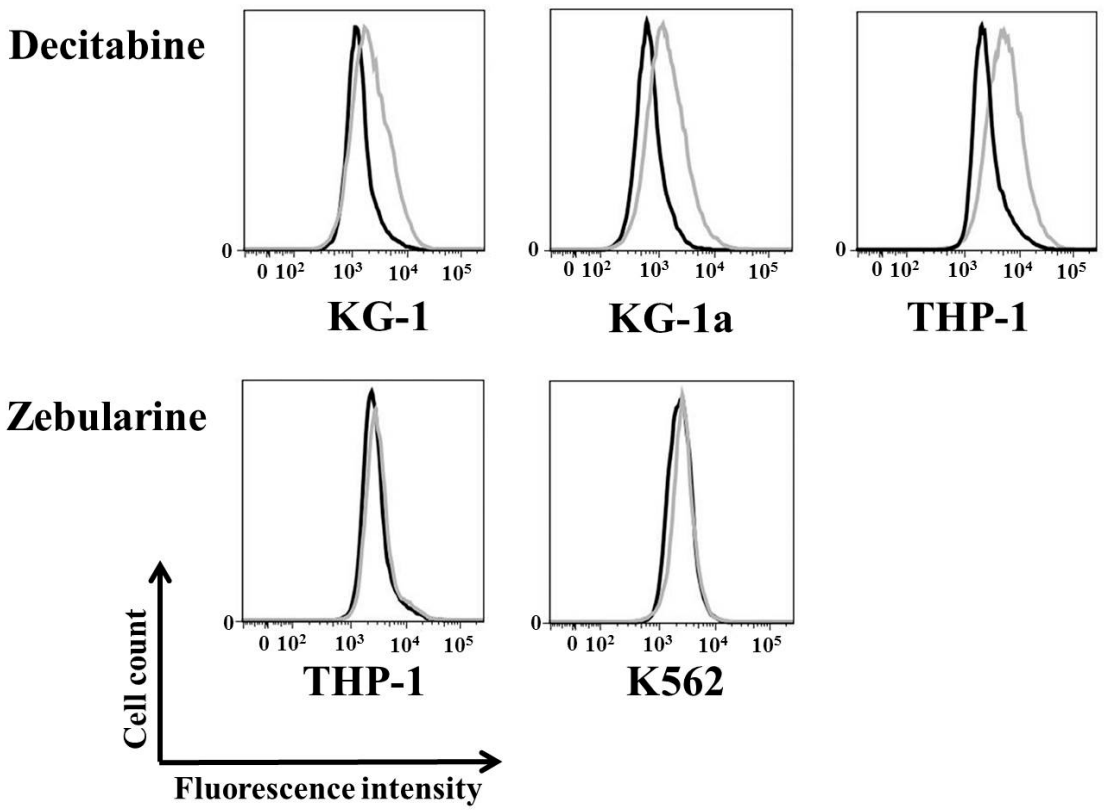
a


MPOa-H cell lines
MPOa-L cell lines
-306
-298
-274
-252
-208
-22
Kasumi-1
1.1%
SKM-1
20.5%
KG-1
94.7%
KG-1a
96.2%
THP-1
87.7%
CMK-86
92.0%
K562
30.4%
CML-T1
98.3%
BV173
89.7%
SU-DHL-6
64.8%

b


% methylated CpG sites of
the 5' region of the MPO gene
100
80
60
40
20
0
KG-1
KG-1a
THP-1
CMK-86
K562
Untreated
Dac 1.0 µM
Zeb 50.0 µM

c


Decitabine
KG-1
KG-1a
THP-1
Zebularine
THP-1
K562
Cell count
Fluorescence intensity

d

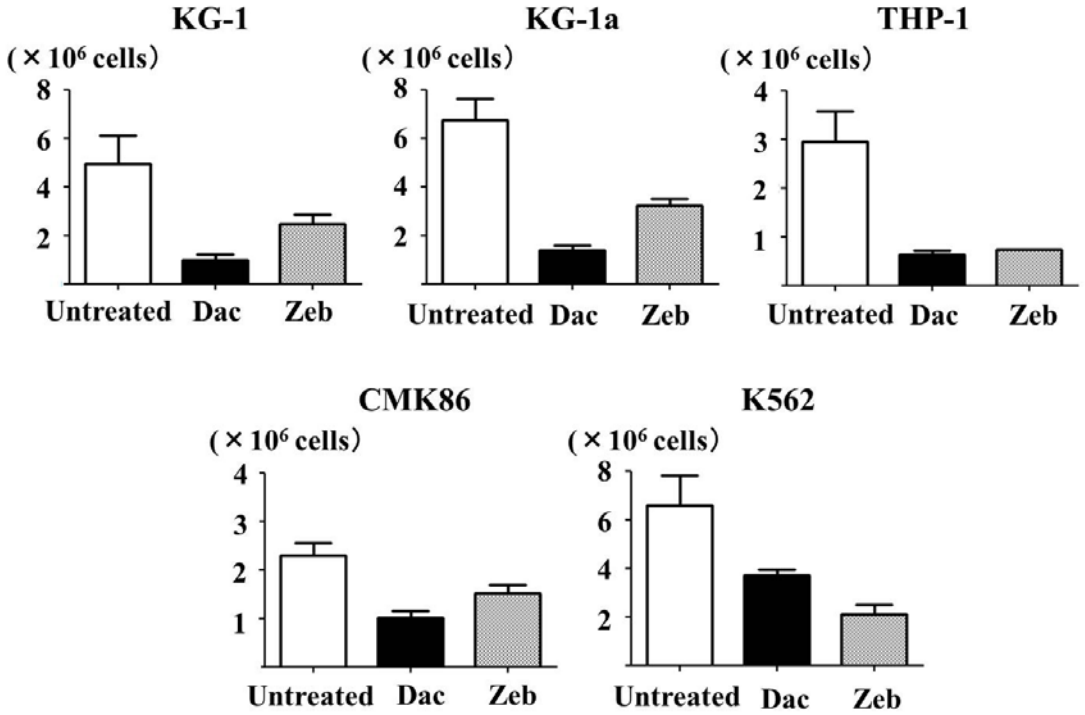

KG-1
(×10⁶ cells)
8
6
4
2
Untreated
Dac
Zeb
KG-1a
(×10⁶ cells)
8
6
4
2
Untreated
Dac
Zeb
THP-1
(×10⁶ cells)
4
3
2
1
Untreated
Dac
Zeb
CMK86
(×10⁶ cells)
4
3
2
1
Untreated
Dac
Zeb
K562
(×10⁶ cells)
8
6
4
2
Untreated
Dac
Zeb

Figure S2

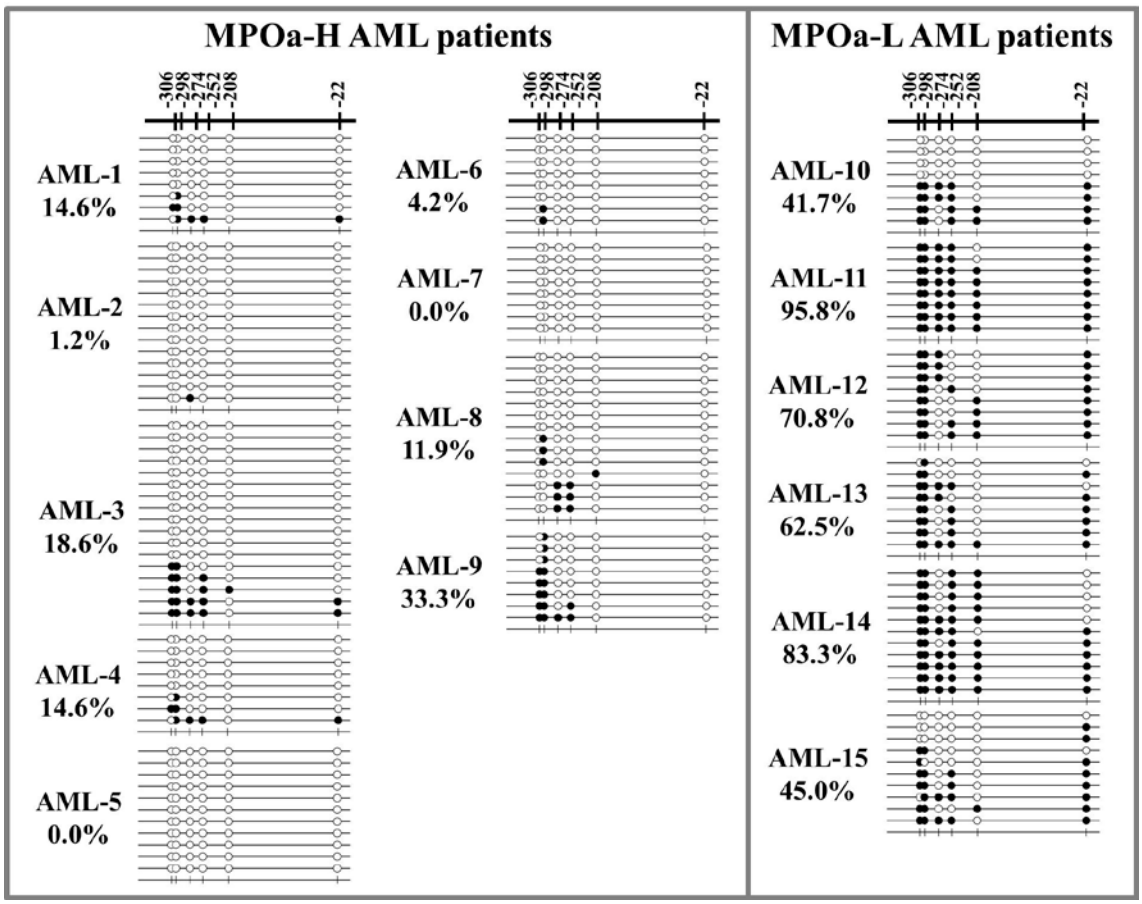
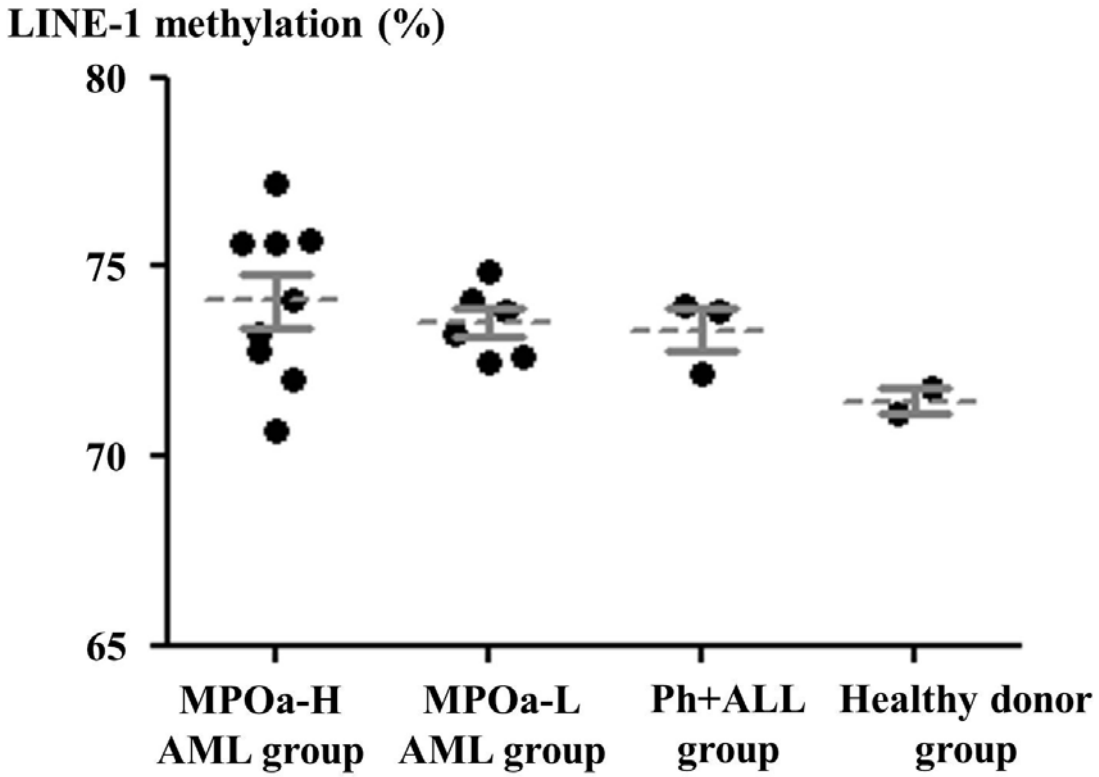

MPOa-H AML patients
MPOa-L AML patients
-306
-298
-274
-252
-208
-22
AML-1
14.6%
AML-2
1.2%
AML-3
18.6%
AML-4
14.6%
AML-5
0.0%
AML-6
4.2%
AML-7
0.0%
AML-8
11.9%
AML-9
33.3%
AML-10
41.7%
AML-11
95.8%
AML-12
70.8%
AML-13
62.5%
AML-14
83.3%
AML-15
45.0%

Figure S3


LINE-1 methylation (%)
80
75
70
65
MPOa-H AML group
MPOa-L AML group
Ph+ALL group
Healthy donor group