Supplementary materials

Inactivation of PBX3 and HOXA9 by down-regulating H3K79 methylation represses NPM1-mutated leukemic cell survival

Wu Zhang, Chen Zhao, Junmei Zhao, Yongmei Zhu, Xiangqin Weng, Qiusheng Chen, Huiping Sun, Jian-Qing Mi, Junmin Li, Jiang Zhu, Zhu Chen, Pier Paolo Pandolfi, Saijuan Chen, Xiaojing Yan, Jie Xu

Figure S1. NPMc+ AML patients have high PBX3 and HOXA9 expression.

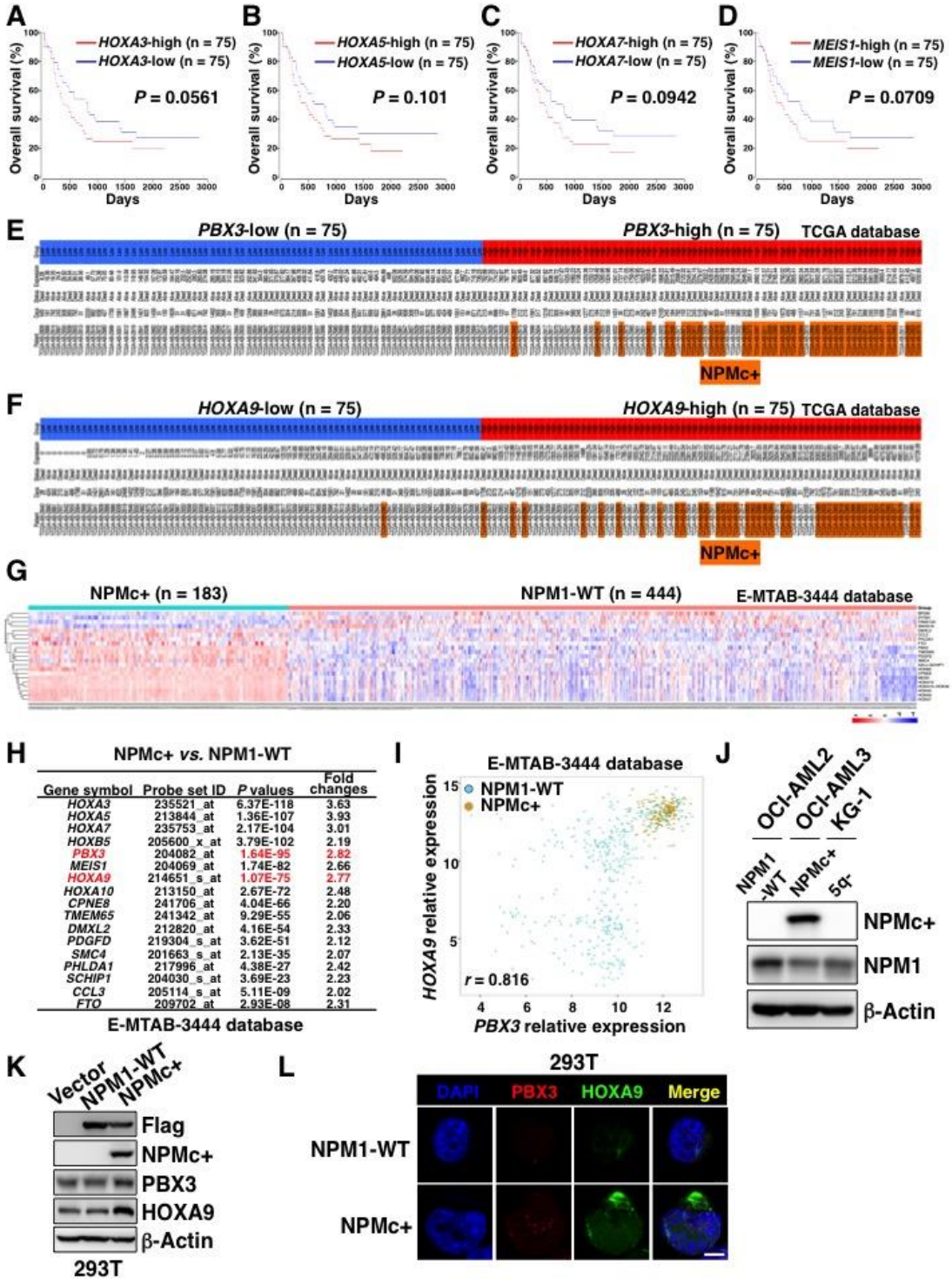
Figure S2. NPMc+ drives myeloproliferation in murine hematopoietic progenitors and aberrant H3K79 modification in hematopoietic cells.

Figure S3. H3K79me2, HOXA9 and PBX3 are highly expressed in NPMc+ MEF cells.

Figure S4. Knockdown of HOXA9 and PBX3 in NPM1 WT or NPMc+ leukemic cell lines.

Figure S5. The anti-H3K79 methylation agent EPZ5676 inhibits the HOXA9/PBX3 axis and promotes apoptosis of NPMc+ leukemia cells.

.



Gene symbol	Probe set ID	*P* values	Fold changes
HOXA3	235521_at	6.37E-118	3.63
HOXA5	213844_at	1.36E-107	3.93
HOXA7	235753_at	2.17E-104	3.01
HOXB5	205600_x_at	3.79E-102	2.19
PBX3	204082_at	1.64E-95	2.82
MEIS1	204069_at	1.74E-82	2.66
HOXA9	214651_s_at	1.07E-75	2.77
HOXA10	213150_at	2.67E-72	2.48
CPNE8	241706_at	4.04E-66	2.20
TMEM65	241342_at	9.29E-55	2.06
DMXL2	212820_at	4.16E-54	2.33
PDGFD	219304_s_at	3.62E-51	2.12
SMC4	201663_s_at	2.13E-35	2.07
PHLDA1	217996_at	4.38E-27	2.42
SCHIP1	204030_s_at	3.69E-23	2.23
CCL3	205114_s_at	5.11E-09	2.02
FTO	209702_at	2.93E-08	2.31

Figure S1. NPMc+ AML patients have high PBX3 and HOXA9 expression.
(**A-D**) The overall survival curves of AML patients with high (n=75) or low (n=75) transcription levels of *HOXA3* **(A)**, *HOXA5* **(B)**, *HOXA7* **(C)** and *MEIS1* **(D)**. The follow-up AML patient survival data was obtained from the TCGA database. The *P* value was calculated using Kaplan-Meier analyses. (**E-F**) Detailed information of AML patients with high (n=75) or low (n=75) *PBX3* **(E)** and *HOXA9* **(F)** levels in the TCGA database (see also Figure 1A-B). The orange-filled boxes indicate the NPMc+ AML cells, which mostly harbor high *PBX3* and *HOXA9* gene expression. (**G**) A heat

map shows the mRNA levels of a cluster of genes expressed differentially (P<0.01, fold change>2) between AML patients with NPMc+ (n=183) and AML patients with WT NPM1 (n=444) from the E-MTAB-3444 database. These top 17 differentiated genes were listed for their significant differentiation between two groups. The selection criterion was based on their P values. The z-score bar measures the color expression intensity. (**H**) Detailed information on the differentiated genes listed in Figure S1G. The red annotations indicate relative transcription levels of *PBX3* and *HOXA9*. **(I)** A Pearson correlation analysis on the relative mRNA levels of *PBX3* and *HOXA9* in WT NPM1 or NPMc+ samples obtained from the E-MTAB-3444 dataset. **(J)** NPMc+ and NPM1 protein expression in OCI-AML2, OCI-AML3 and KG-1 cells. These cell lines carry WT NPM1, NPMc+ and NPM1 haploinsufficiency (5q-), respectively. Three independent western blotting replicates were performed. **(K)** Flag-tagged NPM1, NPMc+, PBX3 and HOXA9 protein expression in 293T cells stably transfected with the Flag-tagged vector, WT NPM1 or NPMc+. The measurement of protein level was from three independent western blotting replicates. **(L)** Representative immunofluorescence staining of PBX3 and HOXA9 in 293T cells transfected with lentivirus carrying WT NPM1 or NPMc+. Images were representative from three independent experiments of at least six random fields.

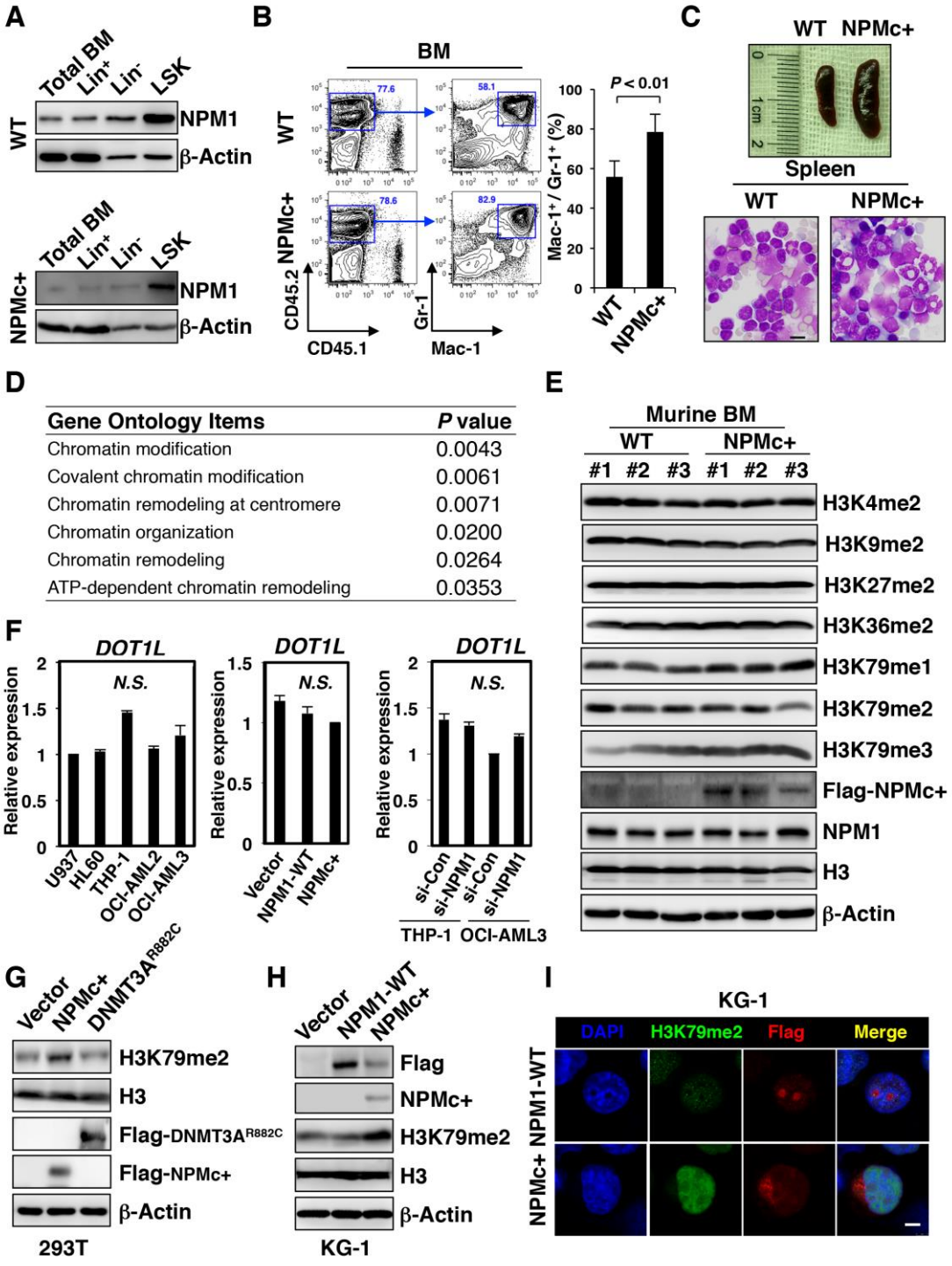


Figure S2. NPMc+ drives myeloproliferation in murine hematopoietic progenitors and aberrant H3K79 modification in hematopoietic cells.

(A) NPM1 protein expression in different compartments of bone marrow (BM) cells sorted from WT (upper) or NPMc+ transgenic (bottom) mice, as detected by western blot. Lin: cell population labeled by the Lineage antibody; LSK: Lin-Sca-1^{+}c-Kit^{+} group. Three independent western blotting replicates were performed. **(B)** CD45.2^{+} WT or NPMc+ murine BM LSKs were purified and transplanted into lethally irradiated CD45.1^{+} WT mice. Representative scatter plots show the percentage of

myeloid populations (Mac-1^+/Gr-1^+) in the murine BM cells collected 12 weeks after transplantation with CD45.2^+ WT or NPMc+ LSKs. Column diagram showing the percentage of myeloid populations (Mac-1^+/Gr-1^+) in the murine BM cells collected 12 weeks after transplantation with CD45.2^+ WT or NPMc+ LSKs. The values are presented as the mean ± SEM (n=6 per group). Data are representative of two independent transplant experiments. **(C)** A comparison of the size of the spleens in the WT and NPMc+ mice 12 weeks after transplantation (upper). Wright's staining of the splenic cells in the WT and NPMc+ mice 12 weeks post-transplantation (bottom). Data are representative of six WT and NPMc+ mice, respectively. The scale bar represents 10 μm. **(D)** A gene ontology (GO) analysis of the differentially expressed genes in NPMc+ murine BM LSKs revealed that a series of functional genes involved in chromatin modification is up- or down-regulated. **(E)** Expression levels of methylated H3K4, H3K9, H3K27, H3K36, H3K79, mutated NPM1 and total NPM1 in BMs from WT or NPMc+ transgenic mice. **(F)** Real-time PCR detection of *DOT1L* in U937, HL-60, THP-1, OCI-AML2 and OCI-AML3 human leukemia cell lines. OCI-AML3 cells bear NPMc+ (left panel). *DOT1L* mRNA expression in 293T cells overexpressing the Flag-tagged control vector, WT NPM1 or NPMc+ (middle panel). *DOT1L* mRNA levels in THP-1 and OCI-AML3 cells with or without NPM1 knocked down (right panel). NC: scramble siRNA. siNPM1: siRNA targeting *NPM1* mRNA. Three independent assays were performed in each group. The data are expressed as the mean ± SEM. **(G)** Western blot detection of methylated H3K79 in 293T cells overexpressing NPMc+ or the DNMT3A R882C mutant. The measurement of protein level was from three independent western blotting replicates. **(H)** KG-1 cells were stably transfected with a Flag-tagged vector, WT NPM1 or NPMc+. Western blot data showed the expression levels of Flag-tagged NPM1, NPMc+ and H3K79me2 in the transfected cells. Three independent western blotting replicates were performed. **(I)** Representative immunofluorescence staining of H3K79me2 and Flag-tagged NPM1 in KG-1 cells transfected with lentivirus carrying WT NPM1 or NPMc+. Images were representative from three independent experiments of at least six random fields.

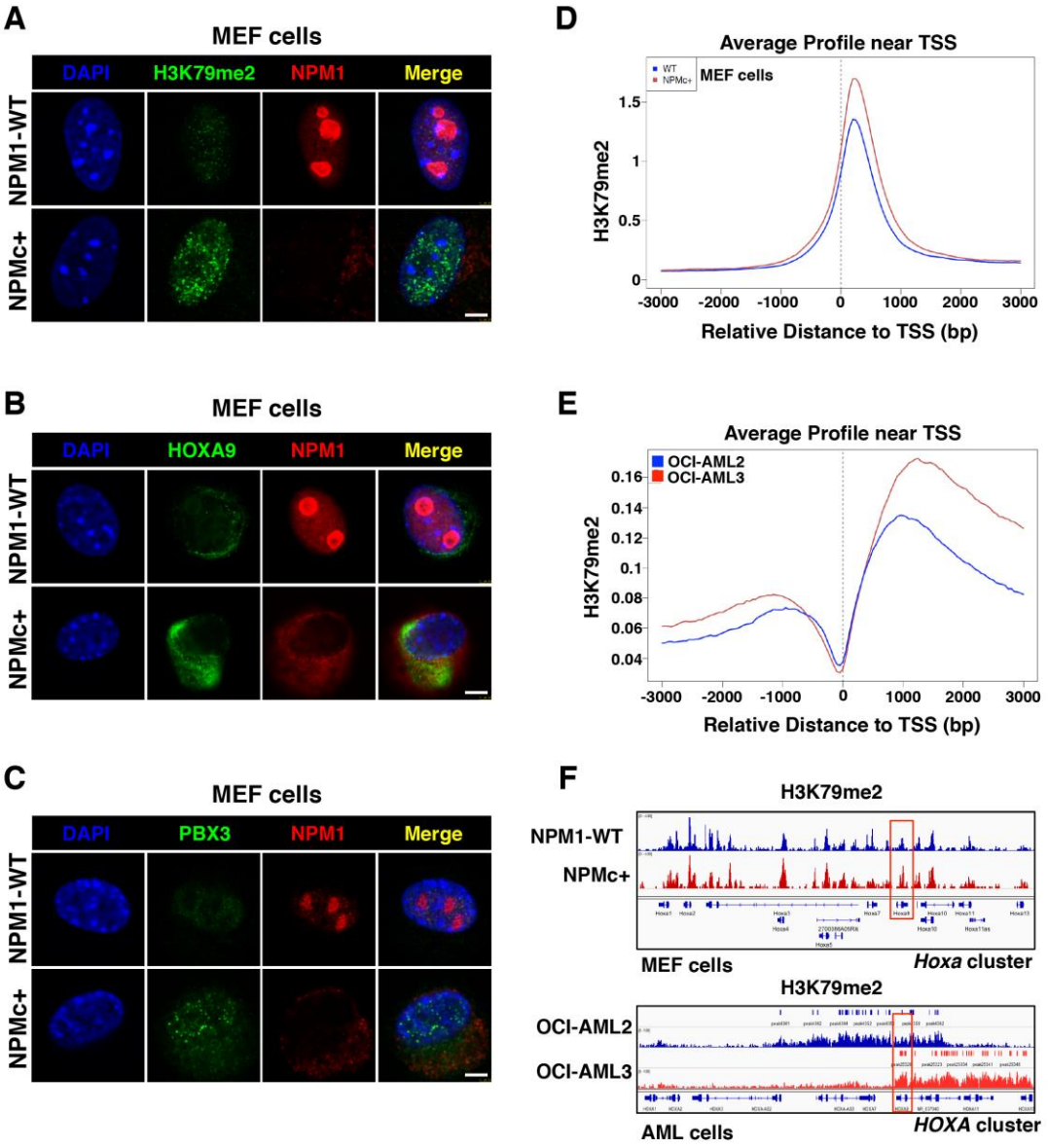


Figure S3. H3K79me2, HOXA9 and PBX3 are highly expressed in NPMc+ MEF cells.

(**A-C**) Representative immunofluorescence staining of H3K79me2 **(A)**, HOXA9 **(B)** or PBX3 **(C)** in MEF cells carrying WT NPM1 or NPMc+. Cells with WT NPM1 or NPMc+ were co-stained with an NPM1 antibody. The scale bar represents 5 μm. Images were representative from three independent experiments of at least six random fields. (**D-F**) Average profiles showing ChIP-seq signal of H3K79me2 at TSSs ± 3 kb regions for all genes in MEF cells (**D**) or AML cells (**E**). *HOXA* cluster genes are ranked according to the ChIP-seq signal of H3K79me2 in MEF cells or AML cells (**F**).

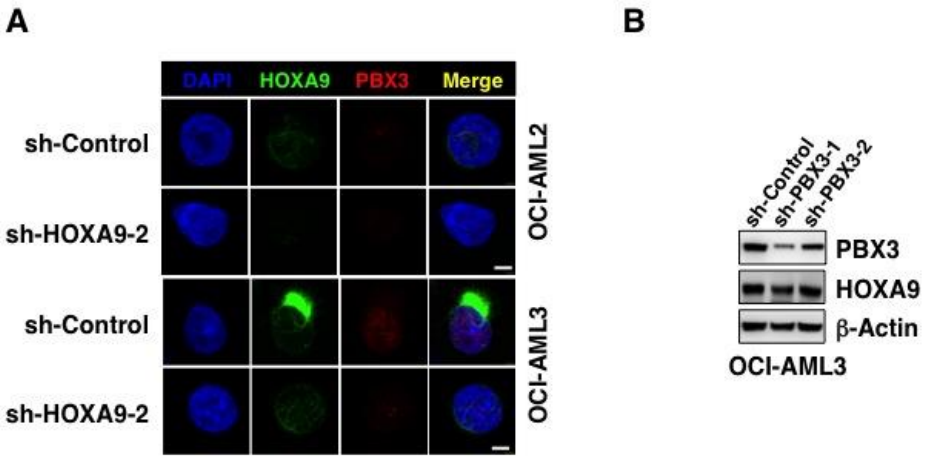


Figure S4. Knockdown of HOXA9 and PBX3 in NPM1 WT or NPMc+ leukemic cell lines.

(**A**) Representative immunofluorescence staining of HOXA9 and PBX3 in the OCI-AML2 or OCI-AML3 cells transduced with control shRNA (shControl) or shRNA targeting HOXA9 mRNA (sh-HOXA9-2). The scale bar represents 5 μm. Images were representative from three independent experiments of at least six random fields. (**B**) Western blot assays of the PBX3 and HOXA9 levels in OCI-AML3 cells exposed to one control shRNA (shControl) or two different shRNAs targeting PBX3 (sh-PBX3-1, sh-PBX3-2) delivered by lentiviral vectors. The measurement of protein level was from three independent western blotting replicates.

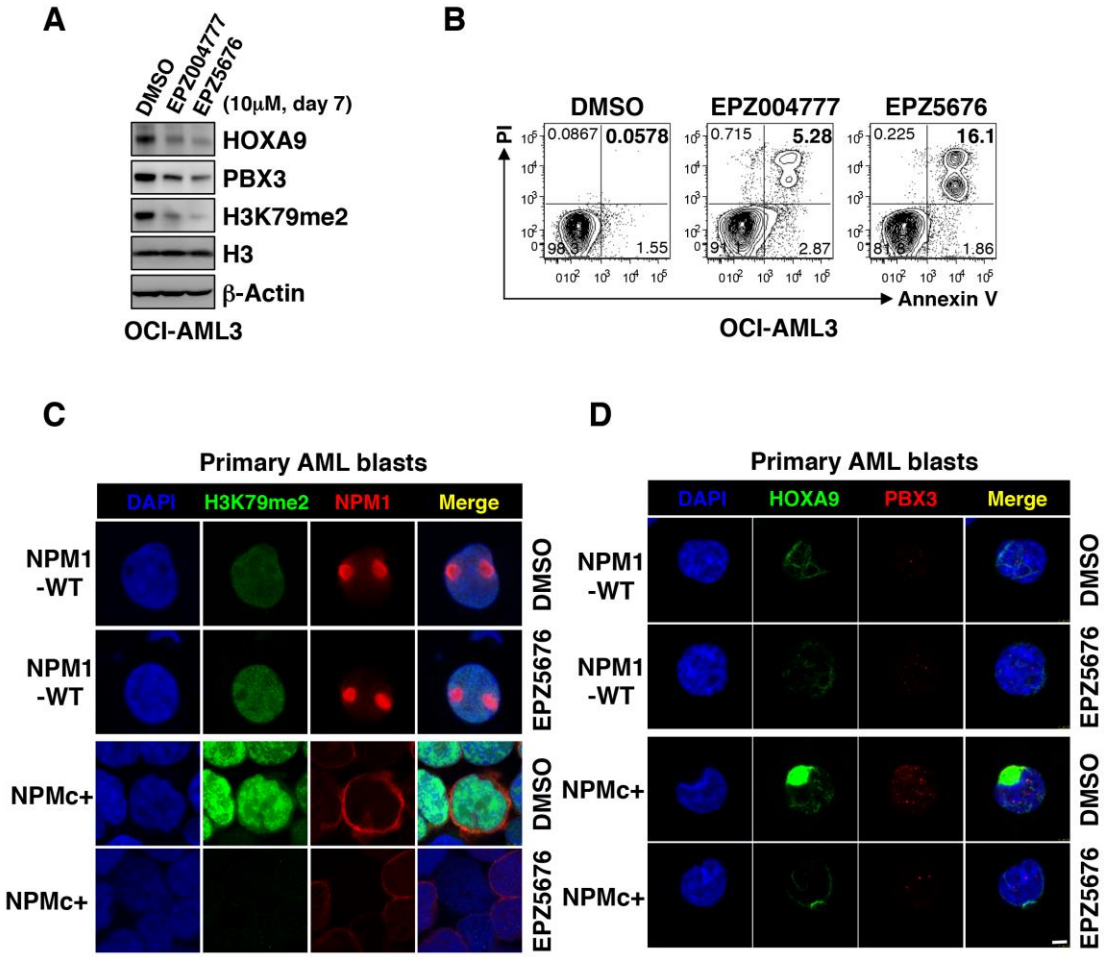


Figure S5. The anti-H3K79 methylation agent EPZ5676 inhibits the HOXA9/PBX3 axis and promotes apoptosis of NPMc+ leukemia cells.

(**A**) OCI-AML3 cells were treated with EPZ004777 (10 μM) or EPZ5676 (10 μM) for 7 days, and HOXA9, PBX3 and H3K79me2 expression was measured by western blot. Three independent western blotting replicates were performed. (**B**) Representative scatter plots of apoptotic OCI-AML3 cells (Annexin V+) treated with EPZ004777 (10 μM) or EPZ5676 (10 μM) for 7 days. Three independent flow cytometric assays were performed for each group. **(C)** Representative immunofluorescence staining of H3K79me2 in the AML patients' blasts treated with DMSO or EPZ5676 (10 μM) for 48 h *in vitro*. Blasts with WT NPM1 or NPMc+ were co-stained with an NPM1 antibody. Scale bar represents 5 μm. The measurement of protein level was from three independent western blotting replicates. **(D)** Representative immunofluorescence staining of HOXA9 and PBX3 in the AML patients' blasts (WT NPM1 or NPMc+) treated with DMSO or EPZ5676 (10 μM) for 48 h *in vitro*. The measurement of protein level was from three independent western blotting replicates.