Supplementary Materials for

Tumor suppressor CEBPA interacts with and inhibits DNMT3A activity

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Table S1. KEGG pathway analysis reveals CEBPAC-mut genes enriched in multiple signaling pathways.

Table S2. Hypermethylated PRC2 target genes in TCGA-AML patients with CEBPA mutations (a) and C-terminal mutations (b).

Table S3. The individual luciferase raw data for the mammalian two-hybrid assay.

Table S4. List of oligonucleotides used in this study.
Figure S1. CEBPA physically interacts with DNMT3A

a, Flag-tagged full-length and different truncates of DNMT3A were co-expressed with HA-CEBPA in HEK293T cells. Protein-protein interaction was examined by IP-western using the indicated antibodies.

b, Proximity ligation assay using the indicated antibodies in K562 cells. Scale bar, 10 μm.

c, Endogenous CEBPA protein was purified by IP with an anti-CEBPA antibody from the indicated leukemia cell lines, followed by western blot to detect DNMT3A. Normal rabbit IgG was used as a negative control. * refers to the protein band of the long splice isoform DNMT3A.

d-f, In vitro pull-down assay shows the direct interaction between DNMT3A and CEBPA. Recombinant CEBPA proteins and Flag-DNMT3A were purified and detected by Coomassie brilliant blue staining (d). Bound proteins were examined after pull-down on Flag-beads (e) or GST-beads (f) as indicated.
Figure S2. DNMT3A physically interacts with CEBP proteins

a, Multiple sequence alignment of the C-terminal regions of CEBP proteins was performed using the Jalview software. As shown, all contain the basic region-leucine zipper (BR-LZ) domains and are highly conserved and thus are colored in blue.

b, Schematic representation of human CEBP proteins.

c, Sequence alignment of the N-terminal region (residues 1-291) of human and mouse DNMT3A was performed using the Jalview software.

d-e, HA-tagged DNMT3A$^{N291}$ or Dnmt3a$^{N291}$ was co-expressed with individual Flag-tagged CEBP in HEK293T cells. Protein-protein interaction was examined by IP-western using the indicated antibodies.
Figure S3. AML-derived mutations in CEBPA C-terminus commonly disrupt DNMT3A binding

a, Schematic representation of the CEBPA-DNA complex structure (PDB: 1NWQ) by Pymol software analysis. As shown, two residues (A296 and R297, marked in red in basic region) are located in the DNA-binding interface, while other two residues (Q311 and N321, marked in red in LZ region) are located in the interface for p42-p42 homodimer formation.

b, HA-tagged CEBPA mutant proteins were each co-expressed with Flag-CEBPA in HEK293T, and CEBPA homodimer formation was examined by IP-western using the indicated antibodies.

c, Schematic illustration of AML-associated hotspot mutations in the bZIP domain of CEBPA, according to the TCGA database. AML-derived mutations in CEBPA tested in this study are indicated.

d- f, Flag-DNMT3A was transiently co-overexpressed with HA-tagged wild-type CEBPA or AML-derived mutants in HEK293T cells. Protein-protein interaction was examined by IP-western using the indicated antibodies.
Figure S4. CEBPA, but not DNMT3A N-terminus, has DNA-sequence binding preference

a, Detection of recombinant GST-DNMT3A N-terminus (residues 1-291, purified from *E. coli*) by Ponceau S staining.

b, EMSA shows the DNA binding ability of CEBPA and DNMT3A N-terminus using random DNA substrate or CEBPA-binding motif (GCAAT) containing DNA. Data are representative of three independent experiments.
Figure S5. CEBPA\textsuperscript{N321D} mutant cannot affect the DNA binding ability of DNMT3A N-terminus

a, EMSA shows the DNA binding ability of CEBPA and CEBPA\textsuperscript{N321D} mutant proteins to its DNA binding motif (GCAAT).

b, EMSA shows the DNA binding ability of DNMT3A N-terminus (residues 1-291) with the presence of increased amounts of recombinant CEBPA\textsuperscript{N321D} mutant protein, using random DNA substrate.

c, EMSA shows the DNA binding ability of DNMT3A N-terminus (residues 1-291) in the absence or presence of wild-type CEBPA or N321D mutant protein.

d, EMSA shows the DNA binding ability of full-length DNMT3A in the absence or presence of wild-
type CEBPA or N321D mutant protein.

**e.** Superimposed fluorescence polarization plots for DNA-binding affinities of full-length DNMT3A in the absence or presence of CEBPA\textsuperscript{N321D} mutant. Random DNA substrate (Forward: 5'-'6-\textsuperscript{FAM}/TGGATATCTAGGGCGCTATGATATCT-3'; Reverse: 5'-AGATATCATA\textsuperscript{CG}CCCCTAGATATCCA-3') was used. See ‘Methods’ for more details.

Data are representative of three independent experiments.
Figure S6. CEBPA inhibits the activity of DNMT3A, but not DNMT3A2 and DNMT3A catalytic domain

a, *In vitro* methyltransferase activity assays for the activity of DNMT3A D529A mutant measured in the presence of wild-type CEBPA or N321D mutant protein, using random DNA substrate and unmethylated histone 3 (H3K4me0). See ‘Methods’ for more details.

b, *In vitro* methyltransferase activity assays for the activity of DNMT3A2 measured in the presence of wild-type CEBPA or N321D mutant protein, using random DNA substrate.

c, *In vitro* methyltransferase activity assays for the activity of DNMT3A AD-CD domain (residues 476-
912) measured in the presence of DNMT3L CD-linker (residues 178-378) proteins as well as wild-type CEBPA or N321D mutant protein, using random DNA substrate. Average values of triplicated results with standard deviation (S.D.) are shown. Asterisks denote statistical significance with two-tailed unpaired Student's t-test. **p < 0.01, and ***p < 0.001 for the indicated comparison; n.s. = not significant.
Figure S7. Overexpression of wild-type CEBPA, but not the N321D mutant, decreases global 5mC in cells

a, b, In HEK293T cells, Flag-DNMT3A was transiently co-overexpressed with HA-tagged wild-type CEBPA or N321D mutant protein, followed by western blot to detect overexpressed proteins (a). In these cells, genomic DNA was isolated and then subjected to dot-blot assay using the antibody specific for 5mC. The amount of DNA in each reaction was examined by methylene blue staining. See ‘Methods’ for more details.

c, DNMT3A were co-overexpressed with HA-tagged wild-type CEBPA or N321D mutant protein in HEK293T cells. DNMT3A homodimer formation was examined by IP-western using the indicated antibodies.

d, Myc-DNMT3L and Flag-DNMT3A were co-overexpressed with HA-tagged wild-type CEBPA or N321D mutant protein in HEK293T cells. DNMT3A-DNMT3L interaction was examined by IP-western using the indicated antibodies.
Figure S8. CEBPA\textsuperscript{N321D} mutation reduces its chromatin/DNA binding

\textbf{a}, HA-tagged wild-type CEBPA or the N321D mutant was transiently expressed in HeLa cells, and their subcellular location was determined by immunofluorescence staining using the indicated antibodies. Representative figures are shown. Scale bar, 5 μm.

\textbf{b}, HA-tagged wild-type CEBPA or the N321D mutant was transiently co-expressed with Flag-DNMT3A in HeLa cells. Different subcellular fractions of these transfected cells were harvested to detect ectopically expressed CEBPA and DNMT3A, including the cytosolic fraction, the nuclear soluble fraction (i.e. Nuclear-S), and the nuclear chromatin binding fraction (i.e., nuclear-CB). See Materials and Methods for more details. Lamin A/C, H3, and β–ACTIN antibodies were used to verify proper cell fractionation.

\textbf{c}, HA- or Flag-tagged wild-type CEBPA and mutant proteins were transiently expressed in HEK293T cells, and the dimer formation of CEBPA was examined by IP-western using the indicated antibodies.

\textbf{d}, In stable cells of K562 and HL-60 with or without DNMT3A deletion, the expression of endogenous CEBPA and re-introduced Flag-tagged wild-type CEBPA or the N321D mutant was verified by western blot using the indicated antibodies. β–ACTIN was detected as a loading control.
Figure S9. Downregulation of CEBPA target genes is associated with promoter CGI hypermethylation in CEBPA-mutated AML patients

a, The mRNA expression of target genes that exhibit DNA hypermethylation in the CEBPA-mutated AML samples compared to CEBPA wild-type AML controls.

b, Heatmap of DNA methylation clustering profile showing CEBPA mutations are linked to hypermethylation of selected target genes in AML patients.

c, Average of DNA methylation levels at individual CpGs within the promoter regions (in pink) of CEBPA target genes that exhibit DNA hypermethylation in the CEBPA-mutated AML samples compared to wild-type controls, measured by using the Infinium HumanMethylation450 BeadChip array. Asterisks denote statistical significance with two-tailed unpaired Student's t-test. *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001 for the indicated comparison.
Supplementary Figure 10

a. CEBPA\textsuperscript{mut} TCGA, GSEA analysis

| Hypermethylated Gene Sets, Top 10 | P-value   | SIZE    |
|-----------------------------------|-----------|---------|
| PRC2\_TARGETS                     | 6.86E-88  | 528/6225|
| SUZ12\_TARGETS                    | 2.23E-102 | 785/6225|
| ES\_WITH\_H3K2\_ME3               | 1.25E-151 | 904/6225|
| EED\_TARGETS                      | 6.63E-98  | 702/6225|
| MEF\_HCP\_WITH\_H3K2\_ME3        | 2.46E-116 | 496/6225|
| METHYLATED\_IN\_LYMPHOMA\_TCL1    | 3.02E-09  | 49/6225 |
| TARGETS\_OF\_PAX3\_FOXO1\_FUSION\_UP | 3.53E-13  | 41/6225 |
| TGF\_1\_SIGNALLING\_VIA\_NFIC\_10hr\_UP | 7.23E-03  | 33/6225 |
| STEM\_CELL\_UP                    | 4.97E-20  | 186/6225|
| IPS\_HCP\_WITH\_H3\_UNMETHYLATED  | 5.14E-04  | 50/6225 |

b. High CpG promoter Genes

CEBPA\textsuperscript{mut} 1219

CEBPA\textsuperscript{mut} 9278

P-value=1.62E-21

1540

c. Down-regulated genes

CEBPA\textsuperscript{mut} 948

CEBPA\textsuperscript{mut} 7623

P-value=7.64E-15

1540

d. Hypermethylated promoters

WT vs. CEBPA\textsuperscript{mut}

97%

Without GCCAAT motif

With GCCAAT motif

e. Hypermethylated promoters

WT vs. CEBPA\textsuperscript{c-mut}

97%

Without GCCAAT motif

With GCCAAT motif
Figure S10. AML-derived CEBPA mutations are associated with DNA hypermethylation of PRC2 targets

a, Summary of gene sets enriched in the list of CEBPA mutation-associated hypermethylated genes (CEBPA\textsuperscript{mut}) from the TCGA dataset. Gene sets were obtained from the Molecular Signatures Database (MSigDB). Hypergeometric test P values are indicated.

b, Venn diagram showing overlap of the CEBPA\textsuperscript{mut} genes from the TCGA dataset and genes with high CpG content in their promoters. Overlap of these gene sets was measured by hypergeometric test.

c, Venn diagram showing overlap of the CEBPA\textsuperscript{mut} genes from the TCGA dataset and genes which are significantly down-regulated in AML patients. Overlap of these gene sets was measured by hypergeometric test.

d, Pie chart showing relative percentage of the CEBPA binding motif (G/CCAAT) in hypermethylated promoters with CEBPA mutations.

e, Pie chart showing relative percentage of the CEBPA binding motif (G/CCAAT) in hypermethylated promoters with CEBPA C-terminal mutations.
Figure S11. Leukemia cells expressing CEBPA<sup>N321D</sup> mutant are hypersensitive to DNMT inhibitors.

**a,** K562 cells with <i>CEBPA</i> knockdown and put-back were treated with 5-Aza (1 μM) or DAC (1 μM) for 96 hours, and extracted genomic DNA was subjected to LC-MS/MS analysis for global 5mC. See ‘Methods’ for more details.

**b,** K562 cells with <i>CEBPA</i> knockdown and put-back were treated with 5-Aza (1 μM) or DAC (1 μM) or hemin (25 μM, as a positive control) for 96 hours. The percentage of hemoglobin producing cells was assessed by benzidine staining, and representative photographs of benzidine-positive cells are shown (left panel). The quantified results are also shown (right panel). Scale bar, 200 μm. See ‘Methods’ for more details.

**c,** K562 cells with <i>CEBPA</i> knockdown and put-back were treated with increased 5-Aza (1 μM) or DAC (1 μM) for 96 hours, and cell apoptosis was determined by flow cytometry using FITC Apoptosis Detection Kit (left panel). The quantified results are shown (right panel). See ‘Methods’ for more details.

**d,** K562 cells with <i>CEBPA</i> knockdown and put-back were treated with increased concentrations of 5-Aza or DAC for 96 hours, and cell viability was determined by Cell Counting Kit-8 (Beyotime). The IC<sub>50</sub> values for each DNMTi are shown.

Average values of triplicated results with Standard error of mean (S.E.M) are shown. *p < 0.05; **p < 0.01; ***p < 0.001 for the indicated comparison; n.s.= not significant.
Figure S12. Establishment of K562 CDX model expressing CEBPA^{N321D} mutant

a, In stable K562 cells, wild-type CEBPA or the N321D mutant was induced by DOX treatment, and cell proliferation was monitored for 10 days. n.s.= not significant.

b, Verification of DOX-induced wild-type CEBPA or N321D mutant protein (tagged with Flag) in BM samples from NCG-X mice xenografted with stable K562 cells in (a).

c, Representative data shows the strategy used to gate and analyze K562 cells (human CD45^{+}) and mWBCs (mouse CD45^{+}) in peripheral blood samples, as determined by flow cytometry.

d-e, Numbers and percentages of mWBC and K562 cells in peripheral blood samples of xenografted mice without or with DOX treatment (n=5-10 for each group). The results are representative of two independent experiments. Data are analyzed with two-tailed unpaired Student’s test. n.s.= not significant.

f, Body weight of xenografted mice without or with DOX treatment (n=10-16 for each group) was measured daily during the indicated period.

g, Kaplan-Meier survival curves and the Log-rank test in xenografted mice with K562 overexpressing wild-type CEBPA (n=12) or the N321D mutant (n=16) without DOX treatment. The data are representative of two independent experiments. n.s.= not significant.

h, Kaplan-Meier survival curves and the Log-rank test in xenografted mice with K562 overexpressing wild-type CEBPA (n=10) or the N321D mutant (n=9) after DOX treatment. The data are representative of two independent experiments. n.s.= not significant.
Figure 13. A schematic model for DNMT3A regulation by CEBPA at target gene promoters

CEBPA interacts with and decreases the accessibility of DNMT3A to the DNA, while AML-derived mutations in CEBPA C-terminus abolish its DNA binding capacity as well as the inhibitory effect on DNMT3A activity, thereby causing promoter CGI hypermethylation of target genes near CEBPA binding motif.