Elevated TWIST1 expression in myelodysplastic syndromes/ acute myeloid leukemia reduces efficacy of hypomethylating therapy with decitabine

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Materials and methods

Cell lines and cell culture

Human leukemia-derived cell line KG1a was propagated in the laboratory of H.J. Deeg (Fred Hutchinson Cancer Research Center; Seattle, WA, USA). Human cell line SKM1 was a gift from Prof. Xiao Li (Shanghai Jiaotong University, Sixth People's Hospital, Shanghai, China). AML cell line OCI-AML3 was a gift from Prof. Jiang Zhu (Hematology Institute, RuiJin Hospital, Shanghai, China). All cells were cultured in RPMI 1640 medium (HyClone; Provo, UT, USA) at 37°C in 5% CO_2 atmosphere.

Primary hematopoietic cells were derived from bone marrow aspirates of MDS patients who were 30-70 years old, represented broad MDS categories, and had international prognostic scoring system (IPSS) scores of 0 to 3.0. Patient and disease characteristics are summarized in *Online Supplementary Table 1*. Healthy control subjects were 24-79 years old.

Primary $CD34^+$ cord blood cells were derived from cord blood of healthy infants from People's Hospital of Shaanxi Province. Blood was aseptically aspirated from placenta and umbilical cord blood veins immediately following normal obstetrical deliveries, and stored in blood bags containing an anticoagulant (citrate, phosphate, dextrose, and adenine-1). Samples were processed within 8 h of collection. Informed consent was obtained from each participant included in the study.

Mononuclear cells were separated by Ficoll-Hypaque gradient centrifugation. $CD34^+$ cells were isolated by magnetic-activated cell sorting (MACS) as per manufacturer's protocol (Miltenyi Biotec; Bergisch Gladbach, Germany), and cultured in medium from STEMCELL Technologies (Vancouver, BC, Canada).

Global DNA methylation assay

Global DNA Methylation Assay Kit (Abcam; Cambridge, MA, USA) was used for this assay, in which methylated fraction of DNA is recognized by a 5-mC antibody (Ab), and amount of methylated DNA (proportional to optical density intensity) is quantified through an ELISA-like reaction.

Real-time polymerase chain reaction (PCR)

Total RNA was isolated using RNA Pure Tissue & Cell Kit (CW Biotech; Beijing, China), and cDNA was synthesized using ReverTra Ace qPCR RT Kit (Toyobo; Osaka, Japan), as per manufacturer's protocol. Power SYBR Green Master Mix (CW Biotech) and ABI PRISM 7500 system (Applied Biosystems; Foster City, CA, USA) were used for amplification and detection. Primers are listed in *Online Supplementary Table 2*. For quantitative analysis, copy numbers of DNMT3a, TWIST1, CDKN1A, and CDKN1C were normalized to GAPDH expression.

SDS-PAGE and western blotting assay

Cells were collected and lysed with lysis buffer (10 µL PMSF in 1 mL RIPA). Protein concentration was determined using BCA Protein Assay Kit (Beyotime Biotechnology; Haimen, China). Equal amounts of protein (25 µg) from each lysate

were diluted in Laemmli SDS sample buffer, resolved by electrophoresis in 10% polyacrylamide resolving gel, and transferred onto PVDF membranes. Gels were blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich; St. Louis, MO, USA) in Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h at room temperature, and incubated overnight at 4°C in 5% nonfat dry milk/ TBS-T containing either anti-TWIST1 Ab (1:500; Santa Cruz Biotech; Santa Cruz, CA, USA), anti-DNMT3a Ab (1:1,000; Cell Signaling Technology; Beverly, MA, USA), or anti-tubulin Ab (1:1,000; Cell Signaling Technology), followed by addition of HRP-conjugated secondary Ab (1:5,000; Beyotime).

Construction of stable overexpressing cell lines

Construction of lentivirus expression plasmids and packaging of lentivirus were performed by Applied Biological Materials, Inc. (Richmond, BC, Canada). Virus packaging was performed in 293T cells by cotransfection with plasmid pHelper 1.0 vector and envelope plasmid pHelper 2.0 vector. Lentiviral vectors encoding short hairpin RNAs (shRNAs) targeting TWIST1 were generated using piLenti-siRNA. A scrambled piLenti-siRNA was used as negative control. shRNA insert sequences are shown in *Online Supplementary Table 3*. Stable TWIST1-overexpressing cells were generated by lentiviral transduction using pLenti-GIII-CMV vector or pCeMM NTAP. Cells were screened by puromycin for 48 h.

Immunofluorescence staining

Cells were centrifuged onto glass coverslips at 1,500 × g for 5 min, washed three times with PBS, fixed with 2% paraformaldehyde in PBS, permeabilized with 0.2% Triton X-100 in PBS for 30 min, incubated with blocking buffer (3% BSA in PBS) for 2 h, incubated with mouse anti-TWIST1 or anti-DNMT3a Ab (1:500; Cell Signaling Technology) overnight at 4°C, incubated with fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG (Abcam) for 1 h, washed with PBS, incubated with 4,6-diamidino-2-phenylindole (DAPI) (Abcam), washed again with PBS, and mounted on glass slides using Vectashield Mounting Medium (Vector Labs; Burlingame, CA, USA). Fluorescent images were obtained using confocal microscopy (model TCS SP8; Leica; Mannheim, Germany).

Flow cytometric analysis

Apoptosis, cell cycle phase, and proliferation were analyzed using flow cytometry. For apoptosis analysis, cells were stained with Annexin V-PE kit (BD Biosciences; San Jose, CA, USA). For cell cycle analysis, cells were fixed with cold 70% ethanol overnight at 4°C and mixed with propidium iodide (50 μg/mL; Beyotime) and RNase A (50 μg/mL; Beyotime) at 37°C for 30 min. For proliferation analysis, cells were stained with EdU Alexa Fluor 647 kit (Keygen; Jiangsu, China) as per manufacturer's protocol. Stained cells were analyzed on a Calibur flow cytometer (BD Biosciences).

DNMT activity assay

Total DNMT activities were measured using an EpiQuik Methyltransferase Activity/ Inhibition Assay Kit (Epigentek Group; Brooklyn, NY, USA). Experiments were performed as per manufacturer's protocols. In brief, nuclear extracts were bound to specific substrates to form a complex, and the complex was captured using a capture Ab and developed using a specific detection Ab. Enzymatic activity was measured calorimetrically via an ELISA-like reaction. DNMT activity was calculated as per manufacturer's instructions.

Protein purification and mass spectrometric analysis

Protein was purified and immunoprecipitated by tandem affinity purification (TAP) tag as described previously (25). In brief, KG1a cells stably expressing TAP-tag-TWIST1 were subjected to nuclear extraction from 40 mL suspension culture medium. TAP tag was cleaved by addition of 10 μL TEV protease (Gibco; San Diego, CA, USA). Final eluted immunocomplexes were separated on SDS-PAGE, and bound protein was excised from the gel and subjected to LC-MS/MS mass spectrometric analysis (Applied Biomics; Hayward, CA, USA). Proteins analyzed by LC-MS/MS were identified, and acetylation sites were determined by Ingenuity Pathway Analysis (Qiagen GmbH; Hilden, Germany).

Co-immunoprecipitation (co-IP)

Protein was extracted from cells as described previously (26). In brief, cell lysates were incubated with anti-TWIST1 or anti-DNMT3a Ab overnight at 4°C, then incubated with *Protein A/G* agarose (Santa Cruz) for 12 h at 4°C. Agarose was washed with PBS, and collected by centrifugation at 2,000 x *g* for 5 min. Samples were collected by boiling in 1× SDS loading buffer for 20 min, and subjected to western blotting analysis.

Pull-down assay

Pull-down assays were performed using Glutathione Sepharose 4B (GE Healthcare; Little Chalfont, UK). In brief, 1 mg bait protein (GST-TWIST1) was incubated on glutathione beads at 4°C for 2 h with gentle mixing, and washed. 100 μg prey protein (His-DNMT3a) was incubated for 1 h at 4°C with gentle rocking on a rotating platform, washed, and eluted. Degree of interaction was analyzed by SDS-PAGE and western blotting.

Epitope mapping assay

This assay was performed as described previously (23). In brief, synthetic peptides were spotted on PEG-NC membrane using AutoSpot peptide synthesizer (Intavis Bioanalytical Instruments AG; Cologne, Germany).The peptide sequences were list in *Online Supplementary Table 4*. Prior to incubation, membrane was saturated with 2 mg of the recombinant protein of interest for 1 h at room temperature. Positive peptides were revealed using secondary Abs coupled to HRP. Chemiluminescence intensity was measured using Bio-Rad ChemiDoc XRS+ (Becton-Dickinson; Franklin Lakes, NJ, USA). Binding intensities of recombinant

proteins to spotted peptides were quantified using ImageJ software program and converted to sequence-specific normalized units. Individual intensities obtained for peptides covering a given amino acid were added and divided by the number of peptides.

Enzyme-linked immunosorbent assay (ELISA)

100 μL purified His-DNMT3a protein (10 μg/mL) in coating buffer was placed in each well of a microtiter plate, and incubated overnight at 4°C. Coating solution was removed and the plate was washed three times with 100 μL 0.05% Tween 20 in PBS. 100 μL synthetic peptides were added to each well and incubated overnight at 4°C. Peptides were removed, the plate was washed three times with 100 μL PBS-0.05% Tween 20 and incubated at 37°C for 1 h, and 100 μL purified GST-TWIST1, anti-GST Ab (Cell Signaling Technology), and HRP-conjugated secondary Ab were added sequentially. 100 μL tetramethylbenzidine (TMB; Beyotime) was added, and color intensity of chromogenic reaction was determined at 490 nm with a plate reader (Bio-Rad Laboratories, Hercules, CA, USA).

Methylation chip sequencing

Genome-wide methylation was detected by the Illumina Human Methylation 850K Bead chip (Genergy biotechnology, Shanghai, China). Briefly, the genomic DNA was extracted using QIAamp DNA MiniKit (Qiagen, Hilden, Germany) and treated with bisulfite to convert cytosine to uracil. Converted DNA was denatured with sodium hydroxide prior to whole genome amplification on the MSA4 plate. Probes were designed for the converted sequence and hybridized with the chip. The methylation status was determined by calculating the ratio of fluorescent signals. Relative methylation level was the ratio of Methylation/ (Methylation + Unmethylation).

Chromatin immunoprecipitation (ChIP) assay

TWIST1-overexpressing KG1a cells were cross-linked with 1% formaldehyde. DNA was sheared to average size 200-500 bp using a sonicator. IP was performed using anti-TWIST1 Ab (Abcam), anti-DNMT3a Ab, and anti-IgG Ab (Santa Cruz). Protein A/G conjunct agarose was added for 4 h. Samples were washed, treated with proteinase K and RNase A (Beyotime), extracted with phenol/ chloroform, and analyzed by PCR. PCR primers used for ChIP analysis are listed in *Online Supplementary Table 2***.**

Methylation-specific PCR

Genomic DNA was obtained from cells (KG1a, KG1a-TWIST1, CD34+ cell, mononuclear) by Genomic Extraction Kit (Qiagen GmbH) as per manufacturer's protocol, and bisulfite-modified using Methylamp One-Step DNA Modification Kit (Epigentek Group; Brooklyn, NY, USA) as per manufacturer's protocol. Modified DNA was used for methylation amplification. Relative methylation level was the ratio of

Methylation/ (Methylation + Unmethylation). PCR primers used for methylation-specific PCR are listed in Online Supplementary Table 2.

In vivo mouse experiment

Six- to eight-week-old B-NSG™ mice (NOD-*Prkdc*^{scid}*IL2rg*^{tm1}/Bcgen, NSG) were irradiated with 180 cGy. 2×10^6 KG1a or KG1a-TWIST1 cells were injected via tail vein as described previously (28). Xenotransplanted mice were i.p. injected with DAC (0.2 g/kg BW) twice per week. Peripheral blood was collected following three DAC injections. Mononuclear cells from peripheral blood were stained with anti-human CD45 Ab (BD Biosciences) for flow cytometric analysis. Spleen and bone marrow were collected following six DAC injections. The above Ab was used to detect engraftment in spleen and bone marrow.

Statistical analyses

Prism 5.0 Statistical Software program (GraphPad Software; La Jolla, CA, USA) was used for statistical analyses. The diagnostic accuracy of the TWIST1 gene for DAC therapy were evaluated by receiver-operating-characteristic (ROC) analysis. ROC curve analysis was performed using SPSS version 21.0 (SPSS, Chicago, IL, USA). Intergroup means were compared using Student's t-test, and differences with $p< 0.05$ were considered statistically significant. Each experiment was performed in triplicate. Data are presented as mean ± SEM.

Study approval

All patients and healthy subjects in this study gave informed consent as required by the Institutional Review Board of the People's Hospital of Shaanxi Province (IRB), in accordance with the Declaration of Helsinki. All protocols were reviewed and approved by the IRB. All animal experiments were approved and monitored by the Animal Ethics Committee of Northwest University.

Supplementary Table 1. Patient and disease characteristics.

Diagnosis	Age (yr)	Gender	Cytogenetics	IPSS	BM cellularity	Marrow blast count	DAC response	FLT3-ITD	Ct(GAPDH)-t(TWIST1)	WBC (10^9/L)
AML	59	M	normal	≥2.5	normal	33%	NR	negative	-9.00333	4.77
AML	60	M	normal	0.5	hyper	58%	NR	negative	-6.51	6.01
MDS	58	M	+8	1	normal	6.5%	NR	negative	-15.1333	11.2
AML	69	M	normal	0.5	hyper	41%	NR	negative	-14.0867	2.56
AML	50	F	t(8, 21)	0	hyper	63.5%	NR	negative	-7.64333	1.31
AML from MDS	80	M	t(8, 21)	0	hyper	36%	NR	negative	-10.67	6.76
MDS	54	M	normal	≥2.5	normal	16.8%	NR	negative	-11.6433	59.03

AML	73	F	normal	≥2.5	hyper	43%	NR	negative	-13.6467	6.17
AML	48	M	normal	≥2.5	hyper	78%	NR	negative	-4.85667	4.6
AML	48	M	normal	0.5	hyper	45%	NR	negative	-15.83	0.25
AML	64	M	normal	≥2.5	hyper	91%	NR	negative	-2.97	0.15
AML	31	F	normal	0.5	hyper	53%	NR	positive	-7.88667	0.14
AML	48	F	normal	0.5	hyper	61%	NR	negative	-11.71	0.43
MDS	35	M	normal	≥2.5	hyper	12%	NR	negative	-16.83	2.65
MDS	31	M	normal	2	hyper	14%	NR	negative	-16.75	1.19
MDS	56	M	normal	0.5	hype	13%	NR	negative	-16.0133	1.66
AML	77	F	normal	0.5	hype	24%	NR	negative	-9.13	2.54
AML	64	M	normal	1	normal	35%	NR	negative	-10.73	4.12
MDS	72	F	normal	1	normal	17%	NR	negative	-15.0567	3.10
AML	53	F	normal	1	hype	27%	NR	negative	-15.0867	2.63
AML	62	F	t(8, 21)	0.5	normal	30%	NR	negative	-8.6433	1.76
MDS	65	M	normal	0.5	hype	15%	NR	negative	-17.75	1.66
AML from MDS	78	F	normal	0.5	normal	40%	R	negative	-12.38	0.57
MDS	65	M	normal	0.5	normal	6.5%	R	negative	-19.09	1.88
MDS	62	M	normal	1	normal	8%	R	negative	-18.1933	1.61
AML	48	M	t(8, 21)	0	hyper	23.5%	R	negative	-18.8533	0.2
AML	58	M	normal	0.5	hyper	29%	R	negative	-16.8933	4.07
MDS	66	F	complex	≥2.5	normal	23%	R	negative	-18.66	10.72
AML	43	F	t(8, 21), +8	0.5	hyper	29%	R	positive	-16.2333	0.71
AML	58	F	normal	0.5	normal	32%	R	negative	-12.8133	5.88
AML	32	F	t(8, 21)	0	hyper	36%	R	negative	-16.8333	0.14
AML	54	M	normal	0.5	hyper	25%	R	negative	-11.902	3.12

Supplementary Table 2. List of Primers used in the study

Primer	Sequence (5' to 3')	Used for
CDKN1A-R-R	GTCGTCGATCGTTATACGTTTTT	methylation PCR
CDKN1A-M-R-F	TCTCCAATTCCCTCCTTCCC	methylation PCR
CDKN1A-NM-R-F	CTCCAATTCCCTCCTTCCT	methylation PCR
CDKN1C-NM-F-R	ACTAAACATTCCACAAACCAAATACA	methylation PCR
CDKN1C-M-F-R	TAAACGTTCCACAAACCAAATACG	methylation PCR

CDKN1C-F-F	TTCGGTCGGTATTTTTCGAGTATAG	methylation PCR
CDKN1A-EBOX-F	CAGGAACATGTCCCAACATGTTGA	ChIP
CDKN1A-EBOX-R	TACCCTGTTCAGAGTAACAGGCTAA	ChIP
CDKN1C-EBOX-F	ATACTACATTATGCTAATCGCGGCC	ChIP
CDKN1C-EBOX-R	CGATGCCTGCTGGCTAG	ChIP
CDKN1A-RT-F	AGGGTAAGGTTCTTGCCCAC	real-time PCR
CDKN1A-RT-R	GCAGGATCCTTCCATTGAGA	real-time PCR
CDKN1C-RT-F	CAGAACCGCTGGGATTACGA	real-time PCR
CDKN1C-RT-R	CACCGAGTCGCTGTCCACTT	real-time PCR
TWIST1-R	CGCAAATGGGCGGTAGGCGTG	TWIST1 overexpression
TWIST1-F	TAGTCAGCCATGGGGCGGAGA	TWIST1 overexpression

Supplementary Table 3. Sequence of shRNA for inhibition of TWIST1

shTWIST1-1	CCAGGGCAAGCGCGGCAAGAAGTCTGCGG
shTWIST1-2	AGCAAGATTCAGACCCTCAAGCTGGCGGC

Supplementary Table 4. Peptide sequences from epitope mapping.

peptide	sequence	peptide	sequence
1	M M Q D V S S S P V S P A D D	49	G G S P Q S Y E E L Q T Q R V
2	Q D V S S S P V S P A D D S L	50	S P Q S Y E E L Q T Q R V M A
3	V S S S P V S P A D D S L S N	51	Q S Y E E L Q T Q R V M A N V
4	S S P V S P A D D S L S N S E	52	Y E E L Q T Q R V M A N V R E
5	P V S P A D D S L S N S E E E	53	E L Q T Q R V M A N V R E R Q
6	S P A D D S L S N S E E E P D	54	Q T Q R V M A N V R E R Q R T
7	A D D S L S N S E E E P D R Q	55	Q R V M A N V R E R Q R T Q S
8	D S L S N S E E E P D R Q Q P	56	V M A N V R E R Q R T Q S L N
9	L S N S E E E P D R Q Q P P S	57	A N V R E R Q R T Q S L N E A
10	N S E E E P D R Q Q P P S G K	58	V R E R Q R T Q S L N E A F A

11	EEEPDRQQPPSGKRG	59	ERQRTQSLNEAFAAL
12	EPDRQQPPSGKRGGR	60	QRTQSLNEAFAALRK
13	DRQQPPSGKRGGRKR	61	TQSLNEAFAALRKII
14	QQPPSGKRGGRKRRS	62	SLNEAFAALRKIIPT
15	PPSGKRGGRKRRSSR	63	NEAFAALRKIIPTLP
16	SGKRGGRKRRSSRRS	64	AFAALRKIIPTLPSD
17	KRGGRKRRSSRRSAG	65	AALRKIIPTLPSDKL
18	GGRKRRSSRRSAGGG	66	LRKIIPTLPSDKLSK
19	RKRRSSRRSAGGGAG	67	KIIPTLPSDKLSKIQ
20	RRSSRRSAGGGAGPG	68	IPTLPSDKLSKIQTL
21	SSRRSAGGGAGPGGA	69	TLPSDKLSKIQTLKL
22	RRSAGGGAGPGGAAG	70	PSDKLSKIQTLKLAA
23	SAGGGAGPGGAAGGG	71	DKLSKIQTLKLAARY
24	GGGAGPGGAAGGGVG	72	LSKIQTLKLAARYID
25	GAGPGGAAGGGVGGG	73	KIQTLKLAARYIDFL
26	GPGGAAGGGVGGGDE	74	QTLKLAARYIDFLYQ
27	GGAAGGGVGGGDEPG	75	LKLAARYIDFLYQVL
28	AAGGGVGGGDEPGSP	76	LAARYIDFLYQVLQS
29	GGGVGGGDEPGSPAQ	77	ARYIDFLYQVLQSDE
30	GVGGGDEPGSPAQGK	78	YIDFLYQVLQSDELD
31	GGGDEPGSPAQGKRG	79	DFLYQVLQSDELDSK
32	GDEPGSPAQGKRGKK	80	LYQVLQSDELDSKMA
33	EPGSPAQGKRGKKSA	81	QVLQSDELDSKMASC
34	GSPAQGKRGKKSAGC	82	LQSDELDSKMASCSY
35	PAQGKRGKKSAGCGG	83	SDELDSKMASCSYVA
36	QGKRGKKSAGCGGGG	84	ELDSKMASCSYVAHE
37	KRGKKSAGCGGGGGA	85	DSKMASCSYVAHERL
38	GKKSAGCGGGGGAGG	86	KMASCSYVAHERLSY
39	KSAGCGGGGGAGGGG	87	ASCSYVAHERLSYAF
40	AGCGGGGGAGGGGGS	88	CSYVAHERLSYAFSV
41	CGGGGGAGGGGGSSS	89	YVAHERLSYAFSVWR
42	GGGGAGGGGGSSSGG	90	AHERLSYAFSVWRME
43	GGAGGGGGSSSGGGS	91	ERLSYAFSVWRMEGA
44	AGGGGGSSSGGGSPQ	92	LSYAFSVWRMEGAWS
45	GGGGSSSGGGSPQSY	93	YAFSVWRMEGAWSMS
46	GGSSSGGGSPQSYEE	94	FSVWRMEGAWSMSAS
47	SSSGGGSPQSYEELQ	95	SVWRMEGAWSMSASH
47	SSSGGGSPQSYEELQ		

Legends to Supplementary Figures

Supplementary Figure S1. (A) TWIST1 mRNA levels in cells from MDS/AML patients were analyzed by real-time PCR. RNAs were prepared from mononuclear cells from healthy individuals (HD) (n=10) and patients unresponsive to DAC (DAC-NR) (n=22) and from

patients responsive to DAC (DAC-R) (n=10). **(B)** ROC curve analysis for TWIST1 expression in DAC-NR group versus DAC-R group to calculate the best cut off value. Area under the curve (AUC) is 0.841, standard error is 0.076 and confidence limit is (0.693–0.989). **(C)** Western blotting analysis of TWIST1 in KG1a and KG1a-DAC-R (DAC-resistant leukemia-derived KG1a) cells, with tubulin as loading control. **(D)** Global DNA methylation analysis of KG1a and KG1a-DAC-R cells. **(E)** Transcriptome changes upon TWIST1 overexpression. The transcriptome was prepared from $CD34^+$-TWIST1 (overexpressing TWIST1). Data and statistical notations as in Figure 1.

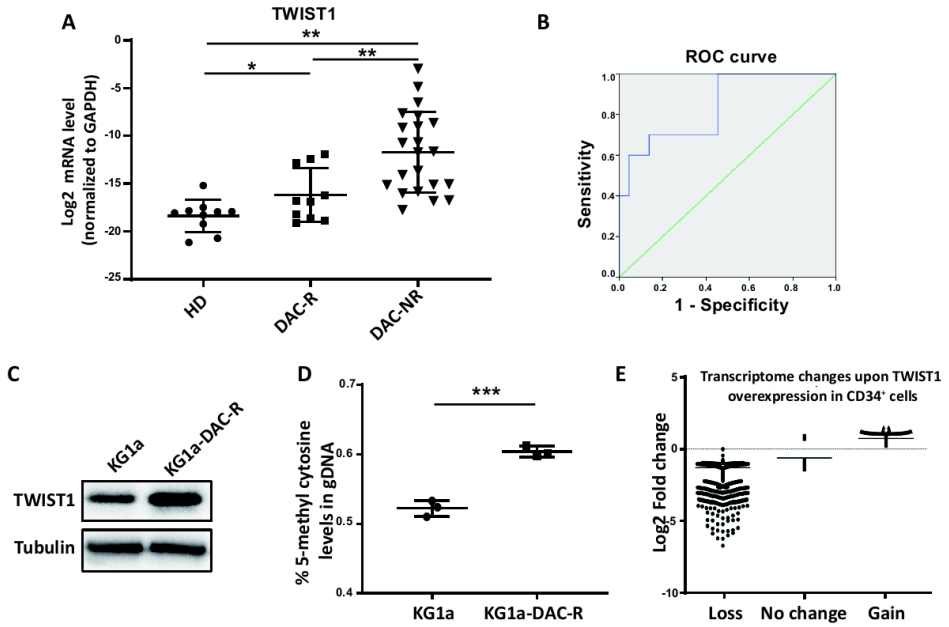
Supplementary Figure S2. (A) Western blotting analysis of TWIST1 in OCI-AML3, SKM1, and KG1a cells. Cell lysates (25 μg) were subjected to SDS-PAGE, and blotted with anti-TWIST1 Abs (anti-tubulin Ab as control). **(B)** Western blotting analysis of TWIST1 in KG1a, KG1a-TWIST1 (TWIST1-overexpressing), SKM1-shNC (SKM1 transfected with scrambled sequences), SKM1-shTWIST1 (TWIST1 knockdown), OCI-AML3-shNC (OCI-AML3 transfected with scrambled sequences) and OCI-AML3-shTWIST1 (TWIST1 knockdown), with tubulin as loading control. **(C)** Cell numbers for the above cell lines. **(D)** Proliferation of the above cell lines, determined by flow cytometry. **(E)** Cell numbers for the above cell lines treated with various DAC concentrations. $1x10^5$ cells were treated with 5, 10, or 20 ng/mL DAC for 96 h. **(F)** Proliferation of the above cell lines under DAC treatment, determined by flow cytometry. **(G)** Establishment (schematic) of xenotransplantation mouse model. Adult NSG mice were injected with $2×10^6$ KG1a or KG1a-TWIST1 cells following 180-cGy irradiation. After one week, mice were injected i.p. with DAC (0.2 g/kg BW) twice per week. Peripheral blood, spleen, and bone marrow (BM) were analyzed by FACS. **(H-I).** Percentages of KG1a and KG1a-TWIST1 cells in peripheral blood, analyzed by FACS, before (H; DAC-) and after (I; DAC+) DAC treatment. **(J-K)** Percentages of KG1a and KG1a-TWIST1 cells (detected by $hCD45^+$ Ab) in spleen and BM, analyzed by FACS, after DAC treatment. Data and statistical notations as in Fig. 1.

Supplementary Figure S3. (A) DNMT activity in KG1a, KG1a-TWIST1, SKM1-shNC, SKM1-shTWIST1, OCI-AML3-shNC and OCI-AML3-shTWIST1. **(B)** Protein interaction network of TWIST1 by TAP/MS on String. **(C)** Identified peptides in KG1a-TWIST1 cells analyzed by Tap-Taq purification and LC-MS. Suppression: peptides were involved in gene silencing. Activation: peptides were involved in gene activation. Chromatin modification: peptides were involved in chromatin modification. **(D)** Immunofluorescence analysis of TWIST1 expression in three cell lines as in Panel A. Cells were stained with anti-TWIST1 Ab (green) and anti-DNMT3a Ab (red). Nuclei were stained with DAPI (blue). The fluorescent intensity along the white arrow was analyzed by software Image J. Scale bar: 10 μm. **(E)** IP analysis of GST-TWIST1 and DNMT3a *in vitro*. GST-TWIST1 (1 mg) and His-DNMT3a (100 μg) were purified from recombinant *E. coli*. GST-TWIST1/His-DNMT3a complex was pulled down by Glutathione Sepharose 4B. **(F)** Epitope mapping scheme. Assay is based on TWIST1 protein translated into 15-aa peptides with peptide-peptide overlap 13 aa. The 220 aa involved in TWIST1 were distributed in 95 peptides. Peptides were synthesized directly on PVDF membrane, and the array was “labeled” with His-DNMT3a that bound to the specific epitope. Binding sites were analyzed following incubation with anti-His Ab and HRP-labeled secondary antibody. **(G)** Quantification of His-DNMT3a and interacting peptides 1 to 95. Intensities of

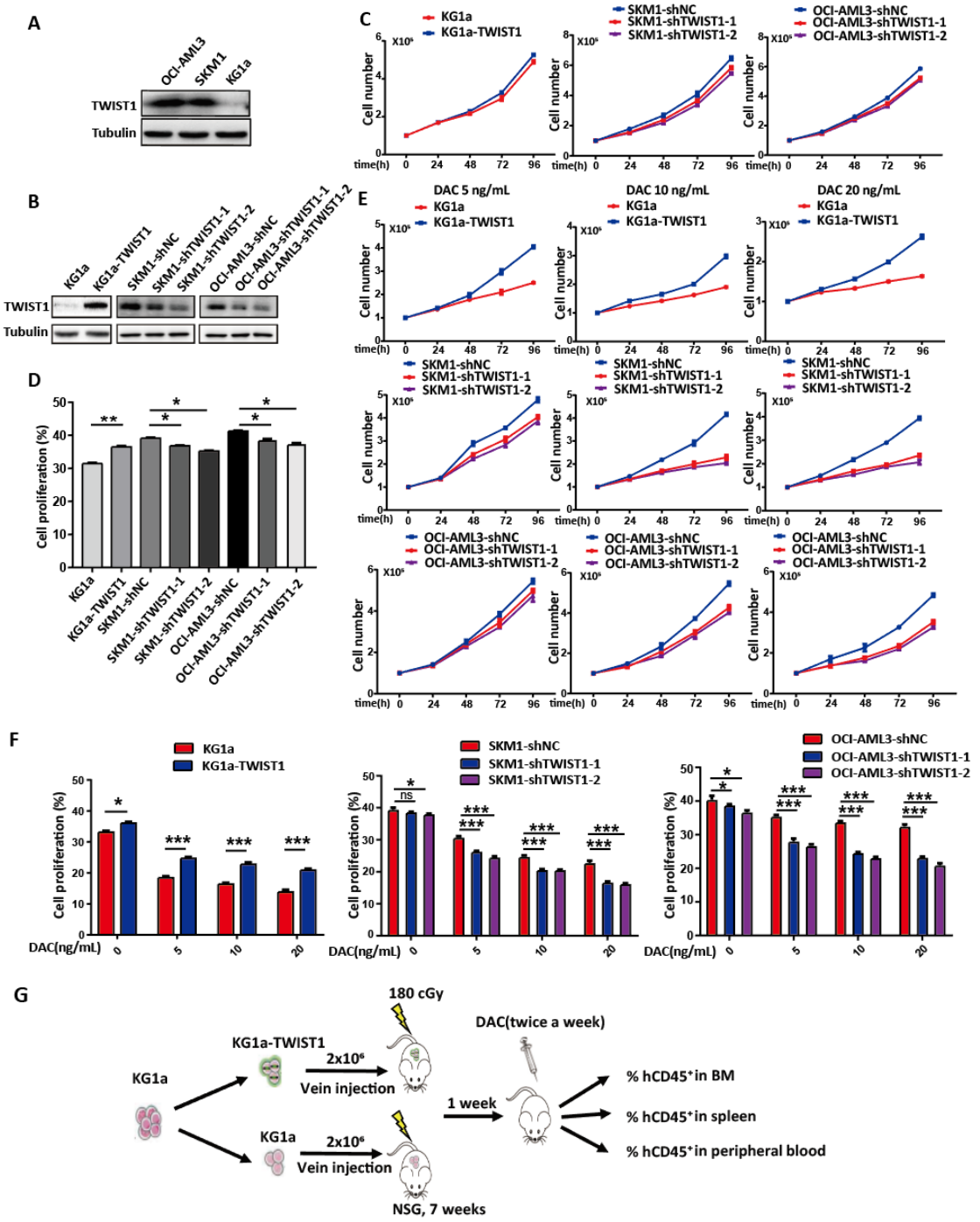
peptide array were determined by epitope mapping assay. Data and statistical notations as in Fig. 1.

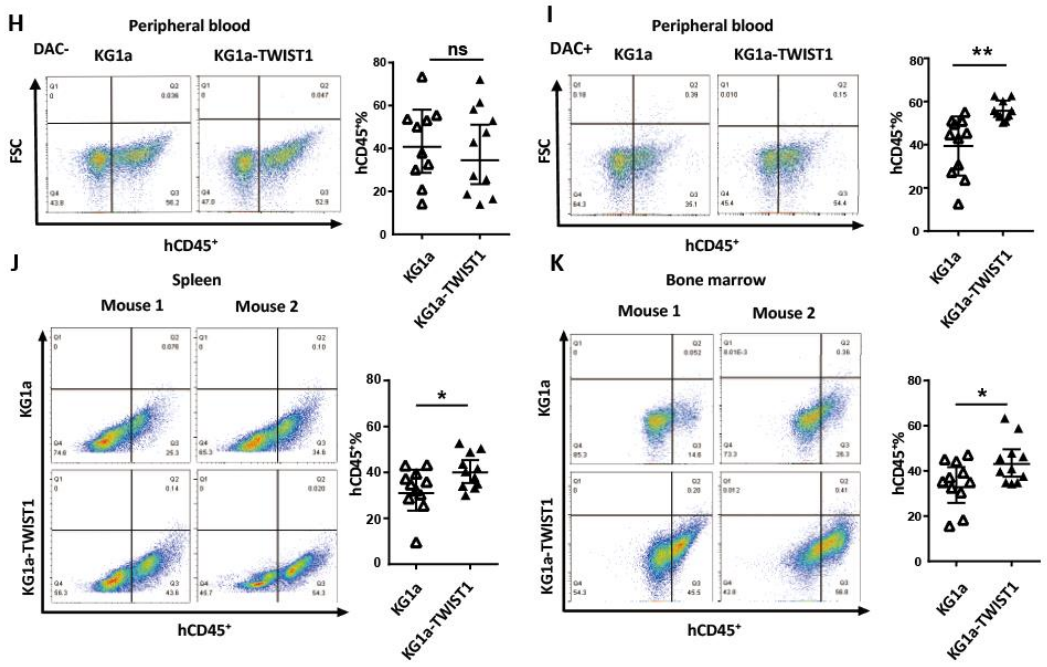
Supplementary Figure S4. Quantification of methylation level in the promoter of CDKN1A and CDKN1C of KG1a and KG1a-TWIST1 cells detected by methylation chip.

Supplementary Figure S1.



Supplementary Figure S2.




H
Peripheral blood
DAC-
KG1a
KG1a-TWIST1
FSC
hCD45+
hCD45*%
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Peripheral blood
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KG1a
KG1a-TWIST1
FSC
hCD45+
hCD45*%
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KG1a
KG1a-TWIST1
J
Spleen
Mouse 1
Mouse 2
KG1a
KG1a-TWIST1
hCD45+
hCD45*%
*
KG1a
KG1a-TWIST1
K
Bone marrow
Mouse 1
Mouse 2
KG1a
KG1a-TWIST1
hCD45+
hCD45*%
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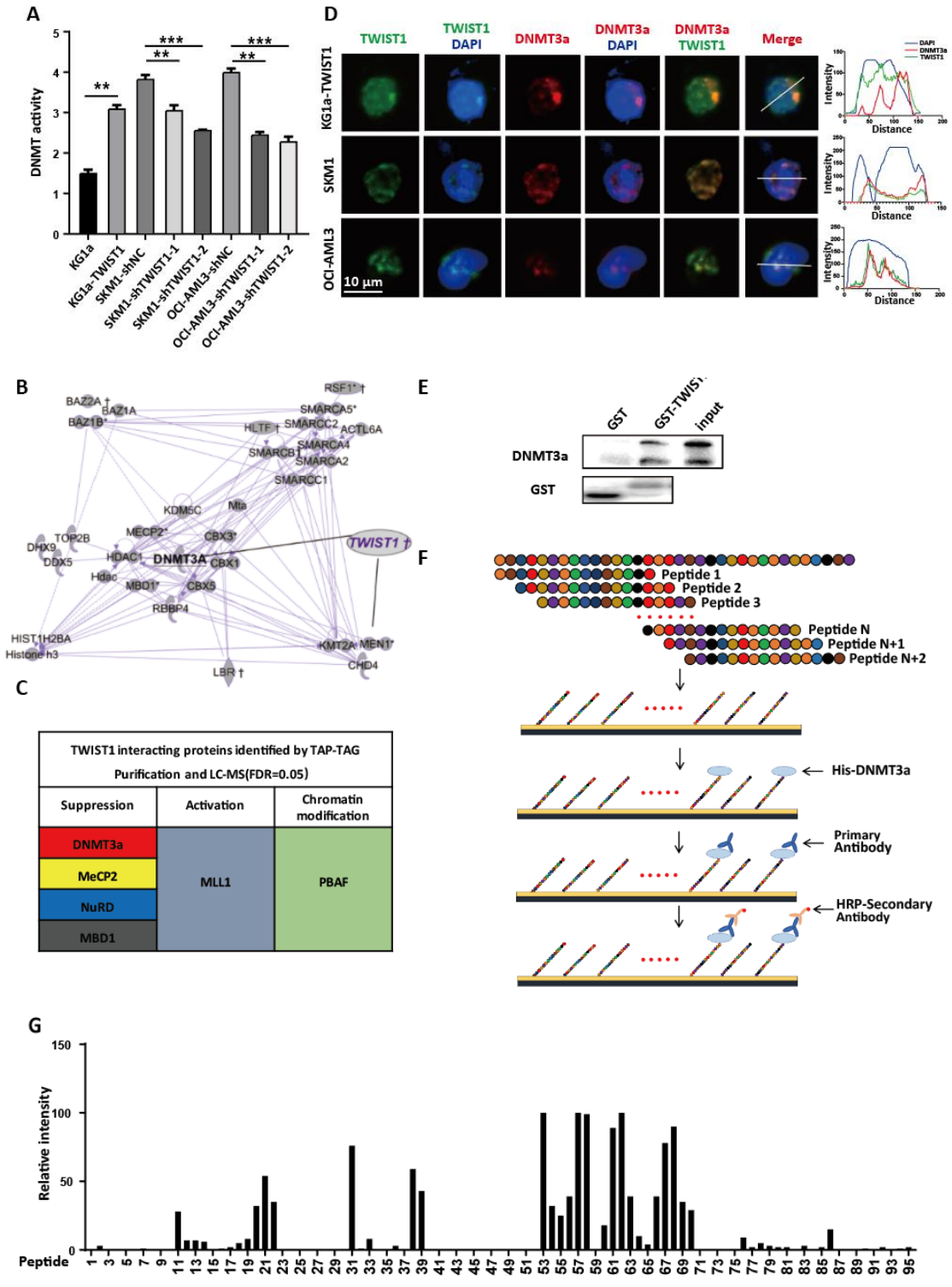
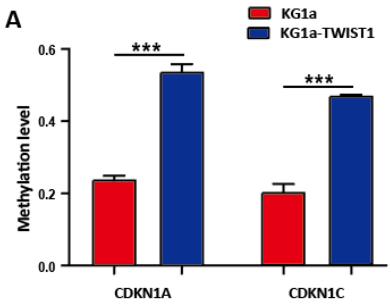
KG1a
KG1a-TWIST1

Supplementary Figure S3.



Supplementary Figure S4.



Methylation level of CDKN1A (The ratio=methylation/methylation+unmethylation)					
KG1a			KG1a-TWIST1		
0.249625	0.251069	0.219396	0.514105	0.560928	0.541154

Methylation level of CDKN1C (The ratio=methylation/methylation+unmethylation)					
KG1a			KG1a-TWIST1		
0.209938	0.224577	0.177805	0.466092	0.465758	0.473294