Twist family BHLH transcription factor 1 is required for the maintenance of leukemia stem cell in MLL-AF9^{+} acute myeloid leukemia

Nan Wang,[1,2,3*] Jing Yin,[1,2,3*] Na You,[1,2,3*] Wenqi Zhu,[1,2,3] Nini Guo,[1,2,3] Xiaoyan Liu,[1,2,3] Peiwen Zhang,[1,2,3] Wanling Huang,[1,2,3] Yueqiao Xie,[1,2,3] Qian Ren[1,2,3] and Xiaotong Ma[1,2,3]

[1]State Key Laboratory of Experimental Hematology, National Clinical Research Center for Blood Diseases, Haihe Laboratory of Cell Ecosystem, Institute of Hematology & Blood Diseases Hospital, Chinese Academy of Medical Sciences & Peking Union Medical College; [2]Tianjin Institutes of Health Science and [3]Center for Stem Cell Medicine, Chinese Academy of Medical Sciences, Tianjin, China

NW, JY and NY contributed equally as first authors.

Correspondence: X. Ma
maxt@ihcams.ac.cn

Received: Janury 15, 2023.
Accepted: September 20, 2023.
Early view: September 28, 2023.

https://doi.org/10.3324/haematol.2023.282748

Supplementary File

Supplementary Methods

Supplementary Table S1-S3

Supplementary Figure S1-S14 and Figure Legends

Supplementary Methods

Cell lines

Human THP-1 and MOLM-13 AML cells were maintained in Roswell Park Memorial Institute 1640 medium (RPMI-1640) supplemented with 10% FBS. 293T cells were maintained in DMEM with 10% FBS.

Plasmids

FLAG-tagged murine *Twist1* was subcloned into a pMSCV-IRES-GFP or pMSCV-PGK-BFP retroviral vector. The full-length cDNAs of constitutively activated mutant Rac1 (Q61L), dominant negative mutant Rac1 (T17N), and murine Wnt5a were purchased from Addgene, and were cloned into the pMSCV-PGK-BFP vector. A cDNA coding for human TWIST1 was cloned into the pCDH-CMV-EF1-copGFP vector, a cDNA coding for murine TWIST1 was cloned into pGEX-4T-1 vector, and a cDNA coding for murine HOXA9 was cloned into the pMAX-C2X vector. The resulting constructs were verified by sequencing. pMSCV-Hoxa9-IRES-GFP plasmid was purchased from Addgene. *TWIST1* shRNA or scramble control shRNA were designed and cloned into the pLL3.7 vector. The shRNA against *KDM4C* and scramble shRNA were designed and cloned into the pLKO.1 vector. The shRNA targeting *Hoxa9* or a nontargeting sequence was cloned into the doxycycline (Dox)-inducible YSH-LV010 lentiviral vector (Ubigene, Shanghai, China), wherein *Hoxa9* knockdown is under the control of a doxycycline-inducible promoter. All the target sequences for shRNA are listed in

Supplementary Table 2.

Virus production

For retrovirus production, pMSCV-MLL-AF9-IRES-GFP, pMSCV-Hoxa9-IRES-GFP, pMSCV-Twist1-PGK-BFP, MSCV-Twist1-IRES-GFP, pMSCV-Q61L-PGK-BFP, pMSCV-T17N-PGK-BFP, pMSCV-Wnt5a-PGK-BFP or pMSCV-PGK-BFP was co-transfected with the packaging vectors pCMV-VSVG and pKat into 293T cells by using Lipofectamine 2000 (Thermo Fisher Scientific, Grand Island, NY, USA). Lentiviruses were produced in 293T cells by co-transfection of individual expression constructs with the pMD2.G:pMDLg/pRRE:pRSV-Rev packaging mix or the psPAX2 and pMD2.G packaging plasmids. The supernatant containing viruses was collected and concentrated 48 and 72 hours later and used for transduction. MOI was calculated for fluorescently tagged virus experiments by dividing the amount of transducing units (TU) added by the number of cells plated. The virus titer was determined by transducing 293T cells with limiting dilutions of virus stocks and analyzing them on a flow cytometer 48 hours after transduction. TU/mL was calculated using the following formula: ((Percent GFP-positive cells × number of cells)/100)/ volume viral stock added). Equivalent viral titers were used for all vectors in each experiment, and cell transduction efficiencies were assessed by flow cytometry to ensure that they were similar in all groups.

Chromatin immunoprecipitation qPCR (ChIP-qPCR)

ChIP assays were performed using the Magna ChIP™ A/G Chromatin Immunoprecipitation Kit (Millipore, Massachusetts, USA) according to the manufacturer's instruction. Immunoprecipitation was carried out with antibodies against trimethyl-Histone H3 (Lys9; Abcam, Cambridge, MA, USA), KDM4C (Thermo Fisher Scientific), murine TWIST1 (Sigma-Aldrich) or an equal amount of isotype IgG (Abcam) as a background control. Immunoprecipitated DNA was eluted and amplified by quantitative real-time PCR (qPCR). Primers for the ChIP-qPCR are listed in Supplementary Table 2.

Quantitative real-time PCR

Briefly, total RNA was isolated using the TRIzol extraction reagent (Thermo Fisher Scientific) or a RNeasy Mini Kit (Qiagen, Hilden, Germany). cDNA was synthesized with M-MLV Reverse Transcript reagent (Thermo Fisher Scientific). qPCR was performed with SYBR Green PCR kit (TaKaRa Bio Inc, Otsu, Shiga, Japan) on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The expression level of each gene was normalized to that of GAPDH. The sequences of the qPCR primers are listed in Supplementary Table 2.

Western blot analysis

Western blot analysis was done by standard techniques as previous described. The antibodies used were listed in Supplementary Table 3.

Leukemic transplants

For primary transplant of MLL-AF9-transduced c-kit$^+$ cells, 1 × 10^5 transduced cells along with 2 × 10^5 wildtype BM cells were transplanted into lethally (9.5 Gy) irradiated female recipients. Mice were monitored for leukemia development. For secondary transplantation, 5 × 10^5 GFP$^+$ leukemia cells from BM of primary recipient mice were transplanted into sub-lethally (4.75 Gy) irradiated recipients. To induce deletion of *Twist1* in established leukemia, *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ or *ER-Cre*$^{+/-}$;*Twist1*$^{wt/wt}$ mice were sacrificed to generate MLL-AF9-transduced cells. 5 × 10^5 GFP$^+$ BM cells of primary AML mice were transplanted into the secondary recipients. Seven days after transplant, recipients were intraperitoneally injected with tamoxifen (75 mg/kg; Sigma-Aldrich) for 5 days to delete *Twist1*.

The limiting dilution assay was performed by injecting 100 to 100,000 FACS-sorted GFP$^+$ cells into sub-lethally irradiated C57BL/6J mice. The frequencies of LSCs were calculated using ELDA software.

To obtain the TWIST1-overexpressing AML cells, GFP$^+$ cells were sorted from AML mice and transduced with control vector or pMSCV-TWIST1 retrovirus. Cells co-expressing both GFP and BFP were sorted and transplanted into sub-lethally irradiated C57BL/6 mice.

For AML xenograft experiments, MOLM-13 cells expressing firefly luciferase (MOLM-13-luc2) were transduced with lentivirus expressing scramble shRNA or TWIST1 shRNA. 8 × 10^5 GFP$^+$ cells were engrafted into the sub-lethally irradiated (2

Gy) NOD/SCID mice. Bioluminescence imaging was used to monitor and quantify *in vivo* engraftment of human AML cells.

For the TWIST1 restoration assay, *Twist1*-deficient or control c-kit$^+$ cells were harvested from the MLL-AF9-driven AML mice, and infected with control vector or pMSCV-TWIST1 retrovirus twice. Cells co-expressing both GFP and BFP were sorted and transplanted into sub-lethally irradiated C57BL/6 mice.

For transplant of HOXA9-, TWIST1- or TWIST1/ HOXA9-transduced cells, infected cells (8×10^5) were injected into the lateral tail vein of lethally (9.5 Gy) irradiated female recipients.

For the rescue experiment, *Twist1*-deficient or control c-kit$^+$ cells were collected from the MLL-AF9-driven AML mice and transduced with pMSCV-Q61L-PGK-BFP, pMSCV-T17N-PGK-BFP, pMSCV-Wnt5a-PGK-BFP or pMSCV-PGK-BFP-expressing retroviruses. 8×10^5 GFP$^+$BFP$^+$ cells sorted by FACS were transplanted into sub-lethally irradiated C57BL/6 mice.

To generate *Hoxa9* knockdown AML mice, c-kit$^+$ cells from MLL-AF9-driven AML mice were transduced with a lentivirus expressing TetO-shHoxa9 or TetO-shCtrl along with rtTA. 8×10^5 transduced cells were transplanted into sub-lethally irradiated C57BL/6 mice. Immediately after cell injection, doxycycline was added to drinking water (1 mg/ml supplemented with 7.5% sucrose) for 14 days to induce *Hoxa9* knockdown.

Flow cytometry analysis and cell sorting

Sing-cell suspensions were prepared from peripheral blood, bone marrow and spleen. Analysis and cell sorting were carried out on a BD FACS Canto II or FACS Aria III (BD Biosciences, Franklin lake, NJ, USA). The antibodies are provided in Supplementary Table 3.

Mouse pathology and blood cell analysis

Dissected spleen and Liver tissue samples were fixed with 4% paraformaldehyde, then dehydrated and embedded in paraffin. Sections were stained with haematoxylin-eosin (H&E) for the analysis of the infiltration of leukemia cells. Spleen and bone marrow cytospins (Thermo Fisher Scientific) and peripheral blood smears were stained first with May-Grünwald dye and then Giemsa stain (both from Sigma-Aldrich) by following manufacturer's manual. PB cellularity was enumerated manually by automated counter KX-21 (Sysmex Co., Kobe, Japan).

Colony-forming cell assay (CFC)

The transduced HSPCs were plated in MethoCultTM M3434 (Stemcell, Vancouver, BC, Canada) methylcellulose medium according to the manufacturer’s instructions. For serial replating, cells were collected from the methylcellulose, washed, counted and replated in the methylcellulose medium in the same way as in the 1st round. Colonies were counted and identified based on morphological examination every 7-9 days post seeding.

Cell cycle analysis and apoptosis staining

To assess apoptosis levels, cells were processed according to manufacturer's instructions (Annexin V Apoptosis detection kit, BD Biosciences). For cell-cycle analysis, GFP^+ BM and GFP^+ spleen cells were sorted by FACS and subjected to LSC staining. Cells were fixed/permeabilized and stained with an antibody against Ki67 (BD Biosciences) and Hoechst 33342 (Thermo Fisher Scientific). For BrdU incorporation assays *in vivo*, mice were injected intraperitoneally with 50 mg/kg BrdU 16 hours prior to BM and spleen harvest. GFP^+ leukemia cells were sorted using FACS Aria III. Incorporated BrdU was processed with BrdU Flow Kit (BD Biosciences).

Leukemic cell homing

A total of 5×10^5 GFP^+ leukemia cells were intravenously injected into C57BL/6J-recipient mice. Eighteen hours after transplant, BM cells were collected and analyzed by FACS.

Cell viability assessment

Cell growth was assessed by a growth curve, which was generated by seeding 5,000 cells per well into a 96-well cluster plate and counting cells using a hemocytometer under light microscopy by trypan blue exclusion method at 24 hours intervals for 7 days.

RNA-seq and analysis

Total RNA was extracted from FACS-sorted L-GMPs ($Lin^-cKit^+Sca1^-CD127^-CD34^+CD16/32^+$) by RNeasy Mini Kit (Thermo Fisher Scientific). Sequencing libraries were prepared using NEBNext® UltraTM RNA Library Prep Kit for Illumina (NEB, Ipswich, MA) following manufacturer's recommendations. Library was qualified on an Agilent Bioanalyzer 2100 and sequenced on an Illumia Hiseq 4000 platform. GO or Pathway enrichment analysis was performed using the Gene Ontology Consortium or DAVID.

RAC1 and RAC2 Activities Assay

GTP-Rac1 or GTP-Rac2 activities were measured using the Rac1 or Rac2 activation assay kit (Millipore) following the manufacturer's instructions. Briefly, 1×10^7 GFP^+ or GFP^+ BFP^+ leukemia cells were lysed in 1ml MLB lysis buffer. Equal amounts of lysates were incubated with 10 μg PAK/PBD beads at 4 ℃ for 1 hour. The beads were then washed, and bead-bound protein was eluted in boiling loading buffer and subjected to anti-RAC1 or anti-RAC2 immunoblotting. Western blot analysis was done by standard techniques.

Wnt5a promoter construct and Luciferase reporter assays

A DNA fragment for the murine Wnt5a promoter (-500/-1000/-1500/-1800 to +200) was synthesized and then inserted into the pGL3-basic luciferase reporter plasmid. For luciferase reporter assays, the protein expression plasmid (pcDNA3.1-TWIST1 or

pcDNA3.1 empty vector), luciferase reporter plasmid and pRL-TK Renilla reporter plasmid were cotransfected into 293T cells. Forty-eight hours after the transfection, cells were collected and the firefly and Renilla luciferase activities were measured using the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) according to the manufacturer's protocols.

Co-immunoprecipitation and liquid chromatography-mass spectrometry

Co-immunoprecipitation (Co-IP) assays were carried out using the Pierce™ Classic Magnetic IP/Co-IP Kit (Thermo Fisher Scientific) according to the manufacturer's protocol. Ten micrograms of purified anti-TWIST1 (Sigma-Aldrich) or anti-HOXA9 (Millipore) antibodies were used for each IP. Samples were analyzed by liquid chromatography tandem mass spectrometry. Western blot analysis was performed using standard procedures. Proteins were detected with antibodies against HOXA9 (Santa Cruz Biotechnology, Santa Cruz, CA) or TWIST1 (Thermo Fisher Scientific).

Recombinant protein purification and pull-down assays

The GST or MBP fusion proteins were expressed in *Escherichia coli* BL21 (DE3), and purified by Glutathione Sepharose 4B beads (GE Healthcare, Little Chalfont, UK) or Amylose resin (GE Healthcare). For GST pull-down assays, purified HOXA9 protein was incubated with GST or GST-TWIST1 fusion protein at 4℃ overnight. After incubation, the beads were pelleted and washed with pull-down buffer, followed by protein elution and western blot analysis. The antibodies are listed in Supplementary Table 3.

Supplementary Table S1

Enriched gene sets within down-regulated in *Twist1*-deleted leukemic stem cells

Gene set name	NES	P value
PID_WNT_NONCANONICAL_Pathway	-1.00	0.0

Supplementary Table S2

List of oligonucleotides

Name	Forward Sequence (5'→ 3')	Reverse Sequence (5'→ 3')
shRNA sequences used for constructs		
pLL3.7-shTWIST1-1	GATCCGATGGCAAGCTGCAGCTATTTCAAGAGAATAGCTGCAGCTTGCCATCTTTTTG	AATTCAAAAAGATGGCAAGCTGCAGCTATTCTCTTGAAATAGCTGCAGCTTGCCATCG
pLL3.7-shTWIST1-2	GATCCGCTGAGCAAGATTCAGACCTTCAAGAGAGGTCTGAATCTTGCTCAGCTTTTTG	AATTCAAAAAGCTGAGCAAGATTCAGACCTTCAAGAGAGGTCTGAATCTTGCTCAGCG
pLL3.7-shCtrl	GATCCATGCGAGACGTACGCATTGTTCAAGAGACAATGCGTACGTCTCGCATTTTTTC	AATTCAAAAAATGCGAGACGTACGCATTGTCTCTTGAACAATGCGTACGTCTCGCATG
pLKO.1-shKDM4C-1	CCGGGCTGTAAGATAATGACCTTCACTCGAGTGAAGGTCATTATCTTACAGCTTTTTG	AATTCAAAAAGCTGTAAGATAATGACCTTCACTCGAGTGAAGGTCATTATCTTACAGC
pLKO.1-shKDM4C-2	CCGGGCAAGTACTGGAAGAACTTAACTCGAGTTAAGTTCTTCCAGTACTTGCTTTTTG	AATTCAAAAAGCAAGTACTGGAAGAACTTAACTCGAGTTAAGTTCTTCCAGTACTTGC
pLKO.1-shCtrl	CCGGGTCACCGTAGTCTCGTACACTCTCGAGAGTGTACGAGACTACGGTGACTTTTTTG	AATTCAAAAAGTCACCGTAGTCTCGTACACTCTCGAGAGTGTACGAGACTACGGTGAC
YSH-LV010-shCtrl	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGACCTAAGGTTAAGTCGCCCTCGTAGTGAAGCCACAGATGTACGAGGGCGACTTAACCTTAGGCTGCCTACTGCCTCGGACTTCAAGGGGCTAG	AATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCAGCCTAAGGTTAAGTCGCCCTCGTACATCTGTGGCTTCACTACGAGGGCGACTTAACCTTAGGTCGCTCACTGTCAACAGCAATATACCTTC
YSH-LV010-shHOXA9	TCGAGAAGGTATATTGCTGTTGACAGTGAGCGAGTGGTTCTCCTCCAGTTGATATAGTGAAGCCACAGATGTATATCAACTGGAGGAGAACCACCTGCCTACTGCCTCGGACTTCAAGGGGCTAG	AATTCTAGCCCCTTGAAGTCCGAGGCAGTAGGCAGGTGGTTCTCCTCCAGTTGATATACATCTGTGGCTTCACTATATCAACTGGAGGAGAACCACTCGCTCACTGTCAACAGCAATATACCTTC
Primers used for real-time PCR		
hTWIST1	GTCCGCAGTCTTACGAGGAG	GCTTGAGGGTCTGAATCTTGCT
hKDM4C	CGAGGTGGAAAGTCCTCTGAA	GGGCTCCTTTAGACTCCATGTAT
hHOXA9	TACGTGGACTCGTTCCTGCT	CGTCGCCTTGGACTGGAAG
mTwist1	CGGGTCATGGCTAACGTG	CAGCTTGCCATCTTGGAGTC
mTwist2	CGCTACAGCAAGAAATCGAGC	GCTGAGCTTGTCAGAGGGG
mWnt5a	CAACTGGCAGGACTTTCTCAA	CCTTCTCCAATGTACTGCATGTG
mWnt3a	CTCCTCTCGGATACCTCTTAGTG	GCATGATCTCCACGTAGTTCCTG

mWnt1	GGTTTCTACTACGTTGCTACTGG	GGAATCCGTCAACAGGTTCGT
mWnt7a	CCTTGTTGCGCTTGTTCTCC	GGCGGGGCAATCCACATAG
Primers used for ChIP-qPCR		
mTwist1-site 1	AGATGGACTCGGTTGTTGAAA	CTGTGACAGCAGTAGTGGCAA
mTwist1-site 2	AAATGTATGGACTACCCAAGGT	GCTAGGGAAAGATAGAGCAGC
mTwist1-site 3	TGGTTCGCAGGACGAATTG	TGTCATTGGCCTGACGTGG
mTwist1-site 4	CAGCGGGTCATGGCTAACG	CTTGTCCGAGGGCAGCGT
hTWIST1	TCTACCAGGTCCTCCAGAGC	CTCCATCCTCCAGACCGAGA
mWnt5a-site 1	CCTTCTACCCTTGAACGTTGGA	CCCTATCCTCCGGTATGCCA
mWnt5a-site 2	GGTGTATGTGGTATGTAGGGGG	CCATCTTCCAACGTTCAAGGG

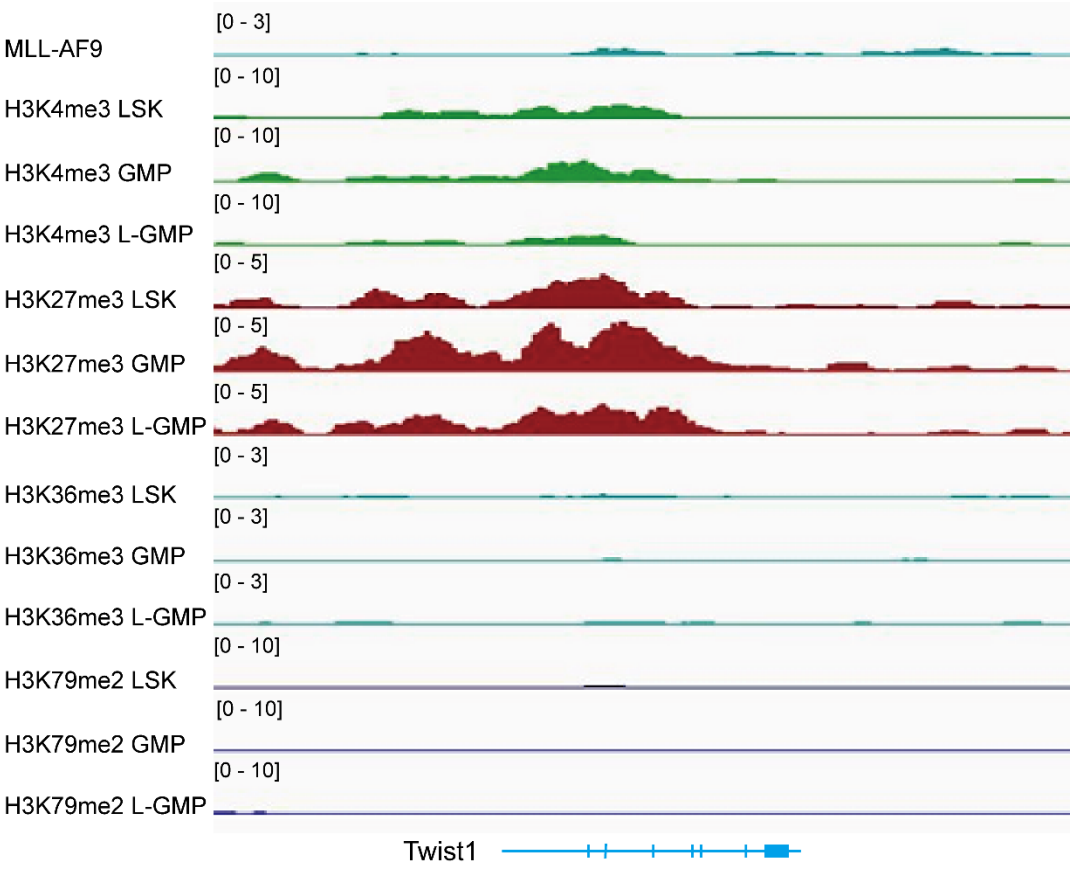
Supplementary Table S3

Related antibodies used in the experiments

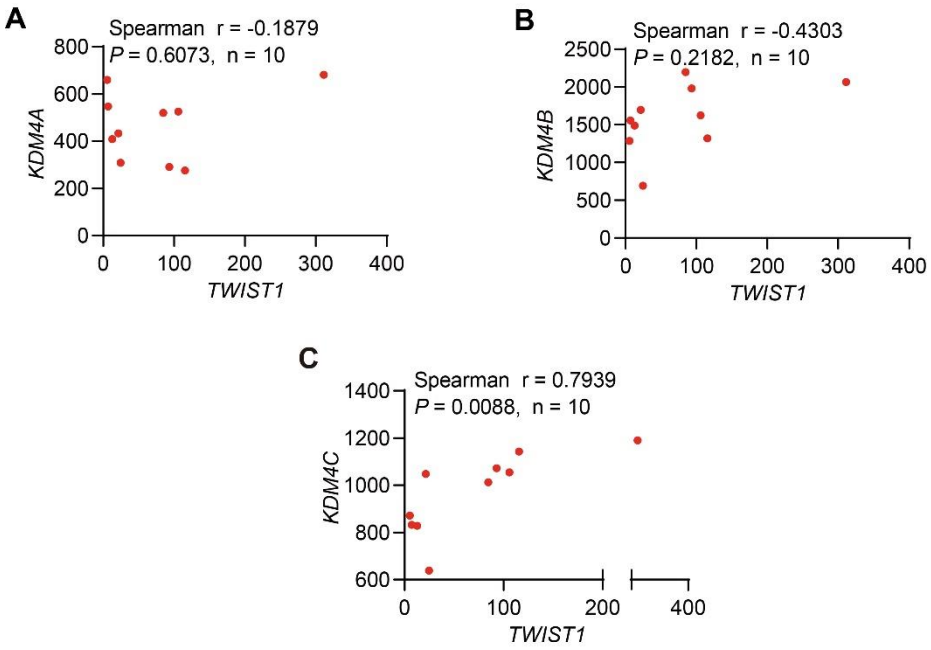
CATEGORY	SOURCE	CATALOG NUMBER
Antibodies		
c-Kit-APC	eBioscience	17-1171-81
c-Kit-PE-Cy7	eBioscience	25-1172-82
c-Kit-PerCP-Cy5.5	eBioscience	46-1171-82
Sca-1-PE-Cy7	eBioscience	25-5981-82
CD34-FITC	eBioscience	11-0341-85
CD34-eFluor 660	eBioscience	50-0341-82
IL-7R-PE	eBioscience	12-1271-81
CD16/32-PE	eBioscience	12-0161-81
CD16/32-APC	eBioscience	17-0161-82
Gr-1- PE	eBioscience	12-9668-82
CD11b-APC	eBioscience	17-0112-83
CD11b-PE-Cy7	eBioscience	25-0118-42
CD14-PE-Cy7	eBioscience	25-0149-42
CD3-PE	eBioscience	12-0031-82
B220-APC	eBioscience	17-0452-82
CD11b-biotin	eBioscience	13-0112-82
B220-biotin	eBioscience	13-0452-82
CD3-biotin	eBioscience	12-0031-82
Ter119-biotin	eBioscience	13-5921-82
Gr-1-biotin	eBioscience	13-5931-82
CD4-biotin	eBioscience	13-0041-81
CD8-biotin	eBioscience	13-0081-81
CD45-PE-Cy7	eBioscience	25-0451-82
hCD11b-PE	eBioscience	12-0113-42
Streptavidin-APC-Cy7	BD Biosciences	554063
Ter119-PE-Cy7	BD Biosciences	557853
CD16/32-PerCP-Cy5.5	BD Biosciences	560540
CD34-BV421	BD Biosciences	562608
Gr-1-APC-Cy7	BioLegend	108424
CD11b-APC-Cy7	BioLegend	101226
B220-APC-Cy7	BioLegend	103224
CD3-APC-Cy7	BioLegend	100330
Ter119-APC-Cy7	BioLegend	116223
CD4-APC-Cy7	BioLegend	100414
CD8-APC-Cy7	BioLegend	100714
hCD14-APC	BioLegend	301808
TWIST1	Thermo Fisher Scientific	MA5-38652
TWIST1	Sigma-Aldrich	T6451

RAC1	Abcam	ab155938
RAC2	Abcam	Ab154711
GAPDH	Abcam	Ab8245
GAPDH	Cell Signaling Technology	97166S
WNT5a	Abcam	Ab229200
IgG	Abcam	Ab172730
H3K9me3	Abcam	Ab8898
KDM4C rabbit polyclonal	Thermo Fisher Scientific	PA5-23065
HOXA9	Santa Cruz Biotechnology	Sc-81291
HOXA9	Millipore	07-178
GST	Abcam	Ab111947
Maltose Binding Protein	Abcam	Ab65
Drugs		
Tamoxifen	Sigma-Aldrich	T5648
SD70	Xcessbio	M60194
Doxycycline	MCE	WC2031
puromycin	Merck	540411

Supplementary Figures and Figure Legends

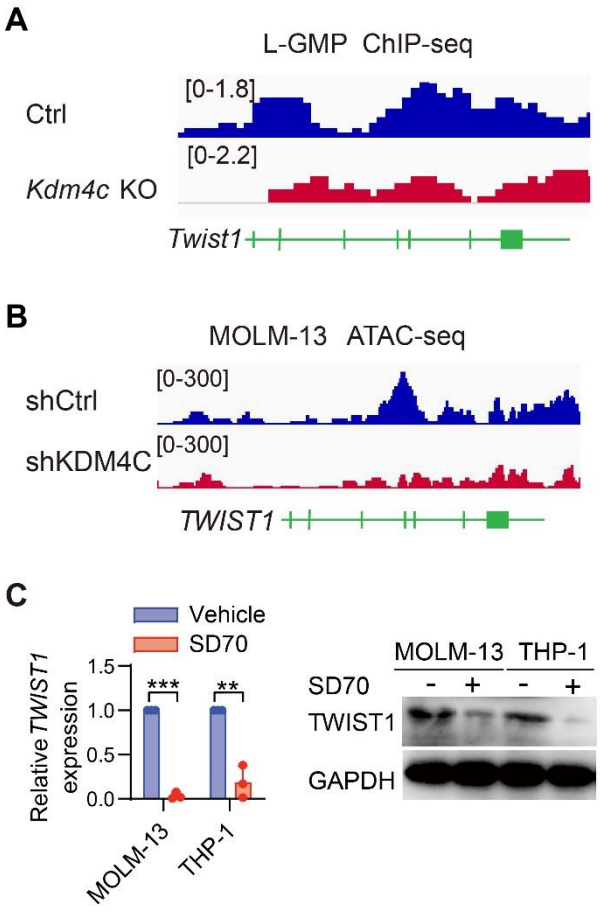


Supplementary Figure S1. Epigenetic profiles at *Twist1* locus in murine leukemic stem cells. Analyses of published chromatin immunoprecipitation sequencing (ChIP-seq) datasets for MLL-AF9, H3K4me3, H3K27me3, H3K36me3 and H3K79me2 marks of *Twist1* in sorted leukemic granulocyte/macrophage progenitor (L-GMP) cells from MLL-AF9^{+} acute myeloid leukemia mice and Lineage^{-}Sca1^{+}cKit^{+} (LSK) and GMP from wildtype mice. ChIP-seq data were obtained from GSE29130.



Supplementary Figure S2. A positive correlation between the expression of *TWIST1* and *KDM4C* in patient cohorts with MLL-rearranged acute myeloid leukemia.

(A-C) Expression correlation between *TWIST1* and *KDM4A* (A), *KDM4B* (B) or *KDM4C* (C) in MLL-rearranged acute myeloid leukemia samples (GSE15013, n = 10). Correlation coefficient (r) and *P*-value were calculated by Spearman rank correlation test.

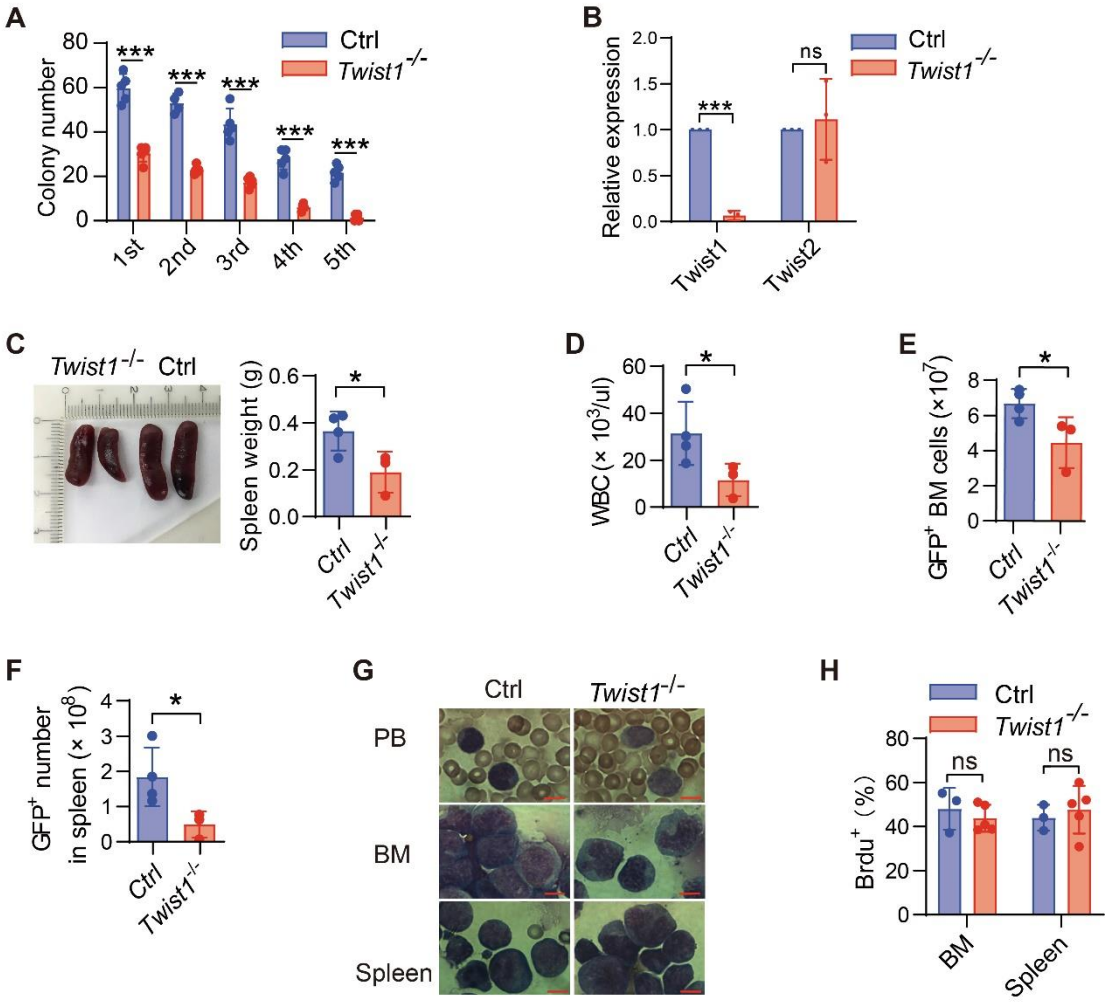


Supplementary Figure S3. KDM4C regulates expression of TWIST1 in MLL-AF9^{+} acute myeloid leukemia cells.

(A) Chromatin immunoprecipitation sequencing (ChIP-seq) signals for KDM4C at *Twist1* loci in *Kdm4c* knockout leukemic granulocyte/macrophage progenitor (L-GMP) cells (GSE81299).

(B) ATAC signals for KDM4C at *TWIST1* loci in *KDM4C* knocked down MOLM-13 cells (GSE146831).

(C) qRT-PCR and western blot analysis of TWIST1 expression in MOLM-13 and THP-1 cells treated with or without 3 μM SD70 for 72 hours. Data are represented as mean ± SD. **, $p < 0.01$; ***, $p < 0.001$, by Student's *t-test*.



Supplementary Figure S4. Loss of *Twist1* impairs the initiation of MLL-AF9-induced acute myeloid leukemia.

(A) Colony number as determined by serial replating assays using 300 GFP^+ cells (n = 4-5).

(B) Relative expression of *Twist1* and *Twist2* mRNA in control and *Twist1*-deleted acute myeloid leukemia cells from primary recipient mice (n = 3).

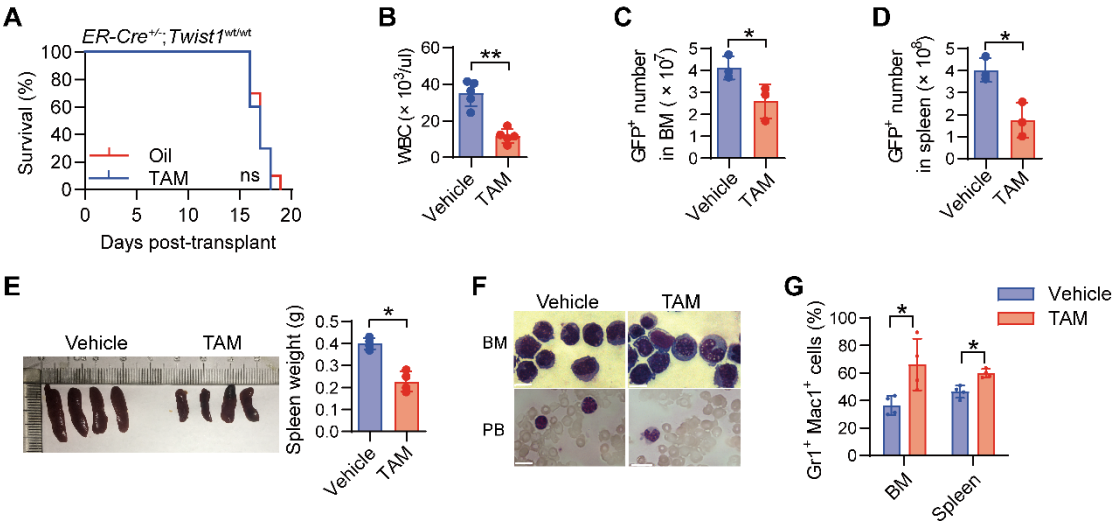
(C-F) One hundred thousand MLL-AF9-transduced $c\text{-}kit^+$ cells from wildtype or *Twist1*-knockout mice were transplanted into lethally irradiated recipients. The leukemic burden was examined 1 month after transplantation. Spleen weight (right) and representative images of spleens (left) (C, n = 3-4). White blood counts (WBC)

counts (D, n = 3-4). Total numbers of GFP^+ cells in bone marrow (BM) or spleens (E-F, n = 3-4).

(G) May-Grünwald-Giemsa staining of peripheral blood (PB) smears, BM and spleen cells from primary recipient mice at 1 month after transplantation, Scale bars, 100 μm.

(H) Proliferation analysis of GFP^+ cells in BM and spleen from primary recipient mice at 1 month after transplantation (n = 3-5).

Data are shown as mean ± SD. *, $p < 0.05$; ***, $p < 0.001$, by Student's *t-test* (A-F and H).



Supplementary Figure S5. *Twist1* depletion leads to reduced tumor burden within established MLL-AF9$^+$ acute myeloid leukemia.

(A) Kaplan-Meier survival curves of mice transplanted with *ER-Cre*$^{+/-}$;*Twist1*$^{wt/wt}$ leukemic cells, treated with tamoxifen (TAM) or vehicle (n = 7).

(B) White blood counts (WBC) of secondary *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ bone marrow transplantation (BMT) recipient mice (n = 5).

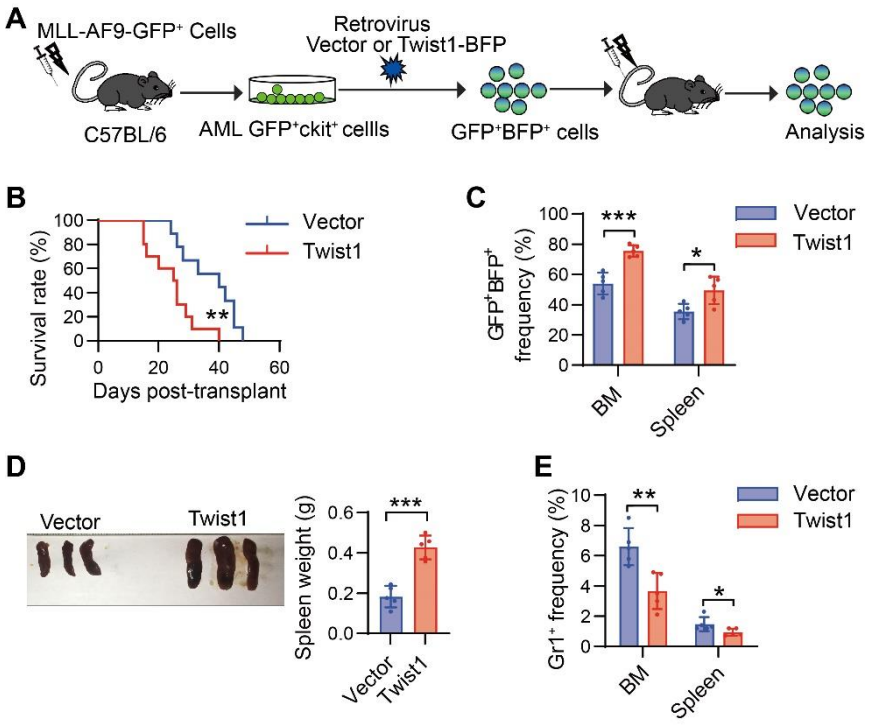
(C-D) Total number of leukemic GFP$^+$ cells in BM (C) and spleen (D) of secondary *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ BMT recipient mice (n = 3).

(E) Spleen weight index of secondary *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ BMT recipient mice (right) and representative images of spleens (left) (n = 4).

(F) May-Grünwald-Giemsa staining of peripheral blood (PB) smears and BM cells from secondary *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ BMT recipient mice, Scale bars, 50 μm.

(G) Frequency of GFP$^+$Gr1$^+$Mac-1$^+$ cells in BM and spleen cells from secondary *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ BMT recipient mice (n = 3-4).

Data shown in (B-G) were analyzed at 5 days after mice treated with TAM or vehicle. Data are shown as means ± SD. *, $p < 0.05$; **, $p < 0.01$, by Student's *t-test* (B-E and G) or log-rank test (A).



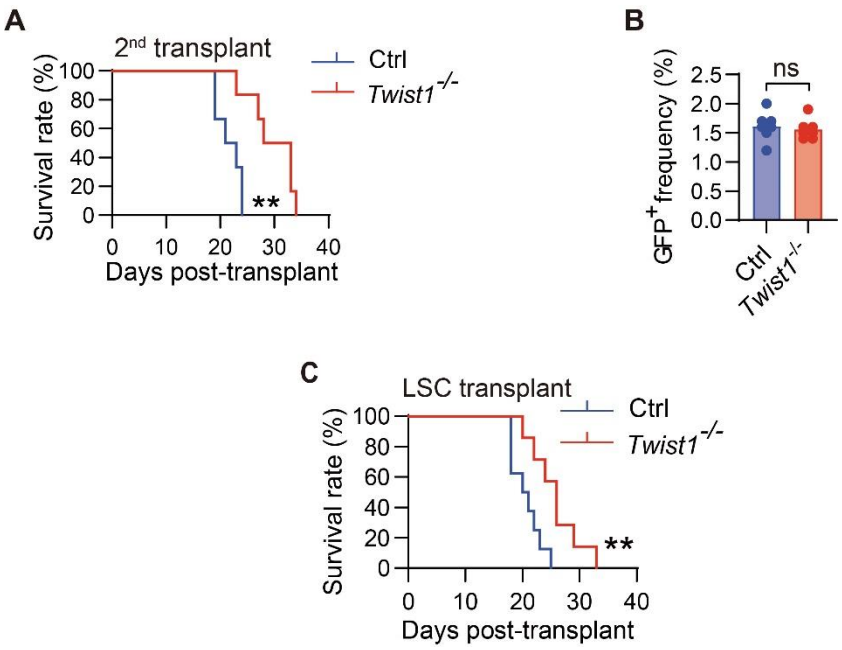
Supplementary Figure S6. Overexpression of *Twist1* accelerates MLL-AF9-mediated acute myeloid leukemia development.

(A) Schematic of strategy for the establishment of Twist1 overexpressing murine acute myeloid leukemia (AML) models.

(B) Kaplan-Meier survival plot of mice transplanted with *Twist1* overexpressing or control AML cells (n = 7).

(C-E) c-kit$^+$ cells from MLL-AF9$^+$ AML mice were transduced with vectors expressing Twist1 or vector control and engrafted into irradiated recipients. Leukemia burden was examined 14 days after secondary transplantation. Percentages of leukemia cells in bone marrow (BM) and spleen (C, n = 5). Spleen weights (right) and representative images of spleens (left) (D, n = 5). Frequency of GFP$^+$BFP$^+$Gr-1$^+$ cells in BM and spleen (E, n = 5).

Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Student's *t-test* (C-E) or log-rank test (B).



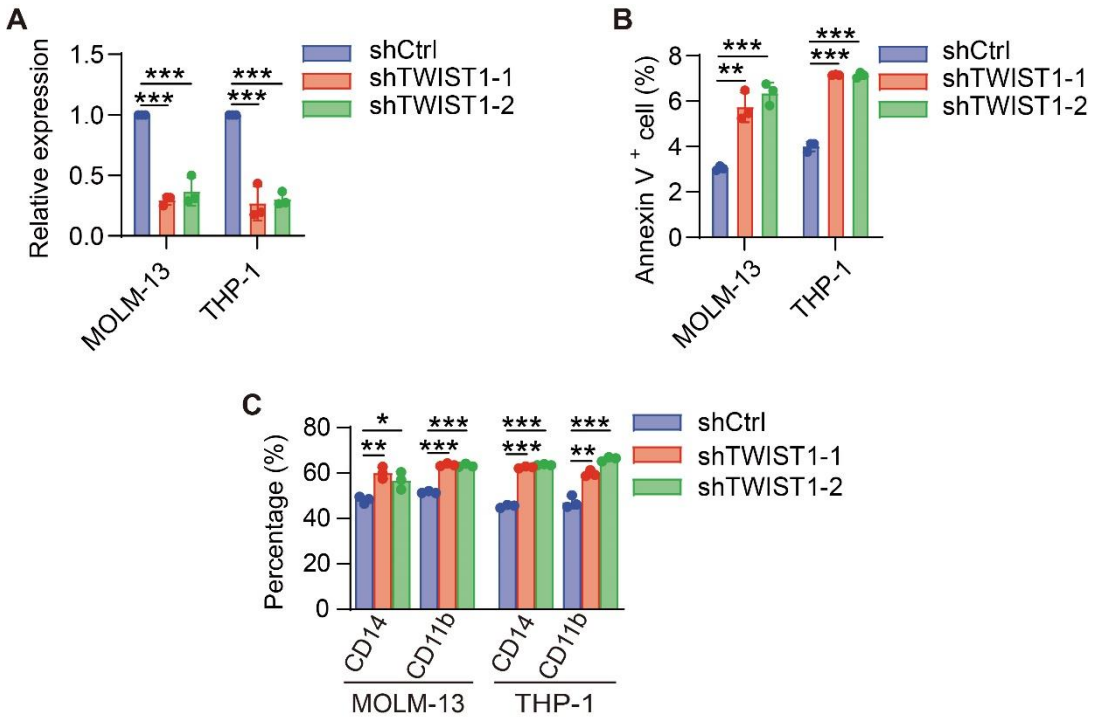
Supplementary Figure S7. Loss of *Twist1* impairs the function of leukemic stem cells and does not affect the homing ability of MLL-AF9$^+$ leukemia cells.

(A) Survival of the secondary recipient mice transplanted with GFP$^+$ bone marrow (BM) cells isolated from the primary recipient mice (n = 5).

(B) Homing of *Twist1*-deficient or control MLL-AF9$^+$ leukemia cells to the BM of recipient mice at 18 h post-transplant (n = 7).

(C) Kaplan-Meier survival curves of recipient mice receiving control or *Twist1*-deficient c-kit$^+$Gr1$^-$ leukemic stem cells after the first transplantation (n = 6-7).

Data are represented as mean ± SD. By log-rank test (A and C) or Student's *t-test* (B).

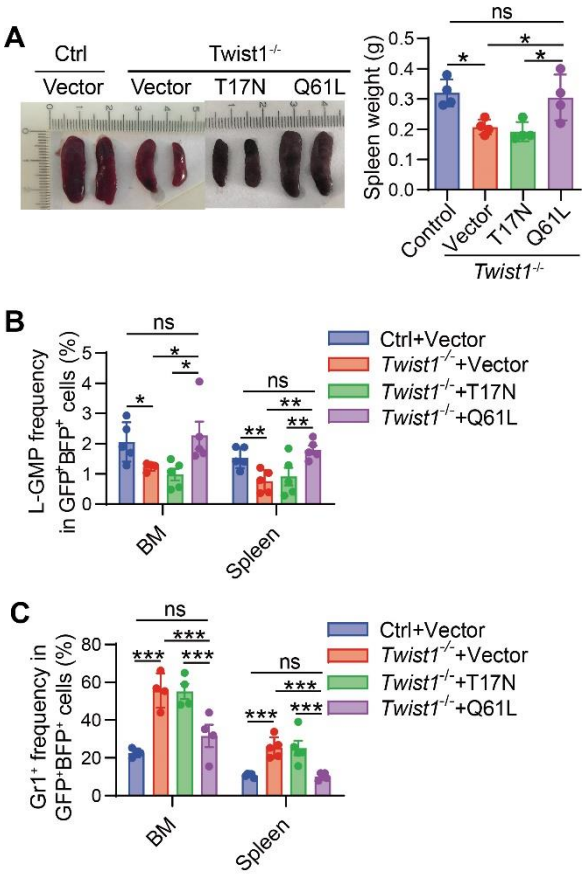


Supplementary Figure S8. Knockdown of *TWIST1* induces apoptosis and myeloid differentiation of human MLL-AF9$^+$ acute myeloid leukemia cells.

(A) qPCR analysis of *TWIST1* mRNA levels in MOLM-13 and THP-1 cells expressing non-targeting control or 2 independent shRNAs (n = 3).

(B-C) Effects of TWIST1 knockdown on cell apoptosis (B), and differentiation (C) in MOLM-13 and THP-1 cells, as determined by flow cytometric analysis of Annexin V$^+$ staining, and CD11b$^+$ and CD14$^+$ cell subsets, respectively (n = 3-5).

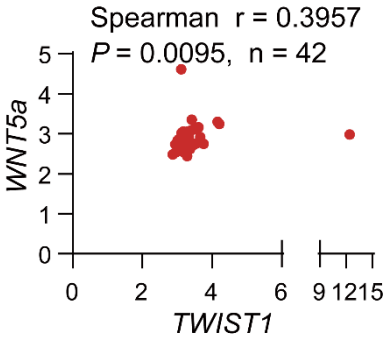
Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Student's *t-test* (A-C).



Supplementary Figure S9. RAC1 mediates the oncogenic role of TWIST1 in MLL-AF9$^+$ acute myeloid leukemia development.

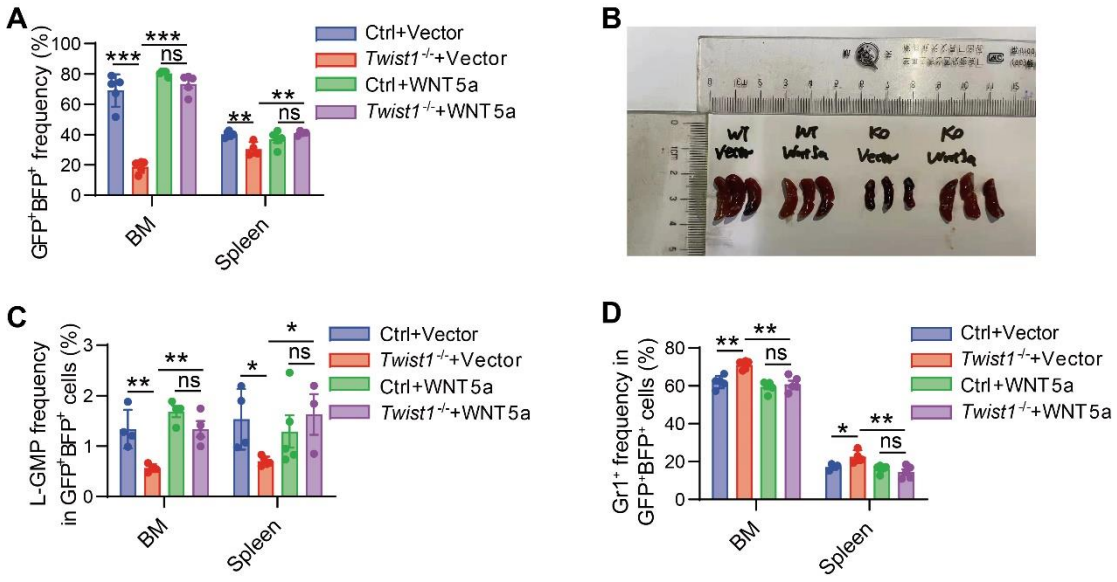
(A-C) *Twist1*-deficient c-kit$^+$ cells or control cells from MLL-AF9$^+$ acute myeloid leukemia mice were transduced with vectors expressing Rac1-Q61L, Rac1-T17N, or vector control, and engrafted into recipient mice. Leukemia burden was examined 14 days after transplantation. Spleen weights (right), and representative images of spleens (left) (A, n = 4). Frequency of leukemic granulocyte/macrophage progenitor (L-GMP) in bone marrow (BM) and spleen cells (B, n = 5). Frequency of GFP$^+$BFP$^+$Gr1$^+$ cells in BM and spleen (C, n = 4-5).

Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Student's *t-test* (A-C).



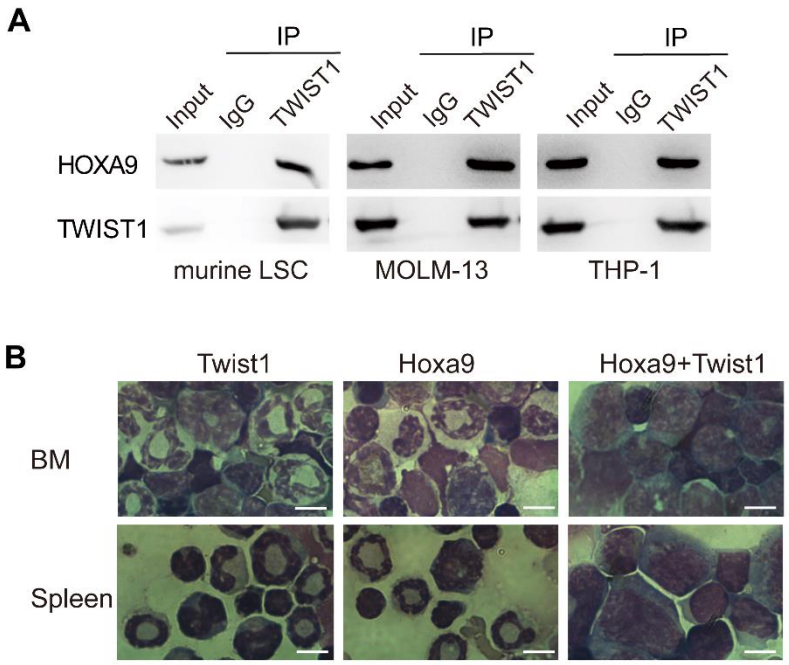
Supplementary Figure S10. A positive correlation between the expression of *TWIST1* and *Wnt5a* in patient cohorts.

Correlation between expression of *WNT5a* and *TWIST1* in bone marrow mononuclear cells from patients with MLL-rearranged acute myeloid leukemia (GSE19577, n = 42). Correlation coefficient (r) and *P*-value were calculated by Spearman rank correlation test.



Supplementary Figure S11. WNT5a overexpression completely rescues the phenotypes caused by *Twist1* depletion in murine MLL-AF9$^+$ acute myeloid leukemia.

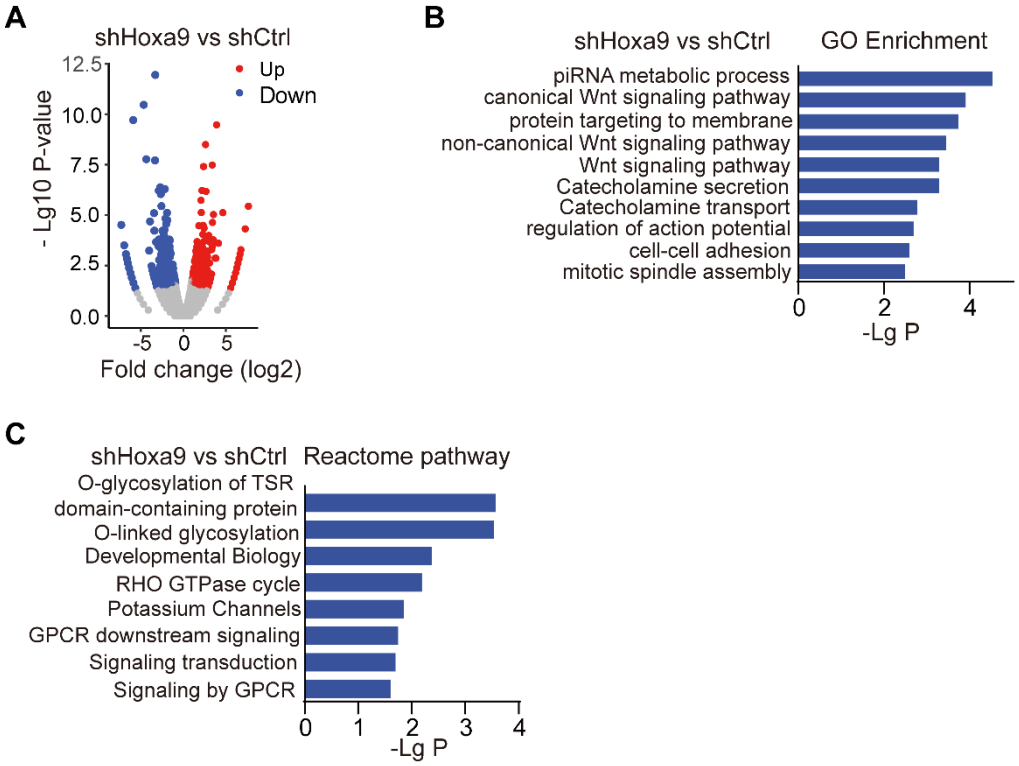
(A-D) *Twist1*-deficient c-kit$^+$ cells or control cells from MLL-AF9$^+$ acute myeloid leukemia mice were transduced with vectors expressing Wnt5a or control, and engrafted into recipient mice. Leukemia burden was examined 14 days after transplantation. Percentages of leukemia cells in the bone marrow (BM) and spleen (A, n = 4-5). Representative images of spleen (B). Frequency of leukemic granulocyte/macrophage progenitor (L-GMP) subpopulation in BM and spleen cells (C, n = 4-5). Frequency of GFP$^+$BFP$^+$Gr1$^+$ cells in BM and spleen cells (D, n = 4-5). Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, by Student's *t-test* (A, C, and D).



Supplementary Figure S12. TWIST1 interacts and cooperates with HOXA9 to induce acute myeloid leukemia.

(A) Co-immunoprecipitation (Co-IP) and immunoblot (IB) analyses in murine leukemic stem cells, MOLM-13, and THP-1 cells using anti-TWIST1 or anti-rabbit IgG for IP and anti-TWIST1 or anti-HOXA9 for IB.

(B) Representative images of cell morphologies. Bone marrow (BM) and spleen cells were stained with May-Grünwald-Giemsa. The length of bars represents 100 μm.

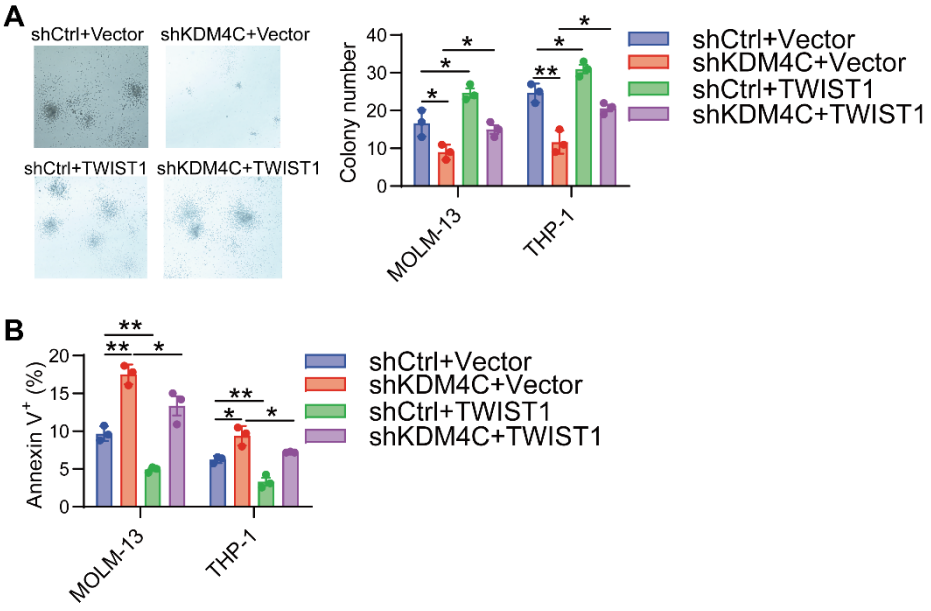


Supplementary Figure S13. Significant enrichment of non-canonical WNT signaling and Rho GTPase signaling in leukemic stem cells after *Hoxa9* knockdown.

(A) Volcano plots showing gene expression differences between leukemic stem cells transduced with shHoxa9 and shCtrl.

(B) Gene ontology (GO) analysis of downregulated genes upon *Hoxa9* knockdown. Only the top ten GO terms are listed.

(C) Reactome pathway analysis of downregulated genes upon *Hoxa9* knockdown. Only the top eight enriched terms are listed.



Supplementary Figure S14. Overexpression of TWIST1 partially abrogated the inhibitory effects of KDM4C knockdown on acute myeloid leukemia cells.

(A-B) shKDM4C or shCtrl transfected MOLM-13 and THP-1 cells were further transduced with vectors expressing TWIST1 or vector. Colony formation (A) and cell apoptosis (B) were determined by colony-forming assay and flow cytometric analysis of Annexin V and 7AAD staining, respectively (n = 3).

Data are represented as mean ± SD. *, $p < 0.05$; **, $p < 0.01$, by Student's *t-test* (A-B).