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

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## Abstract

Leukemia stem cells (LSC) are a rare population capable of limitless self-renewal and are responsible for the initiation, maintenance, and relapse of leukemia. Elucidation of the mechanisms underlying the regulation of LSC function could provide novel treatment strategies. Here, we show that TWIST1 is extremely highly expressed in the LSC of MLL-AF9$^+$ acute myeloid leukemia (AML), and its upregulation is positively regulated by KDM4C in a H3K9me3 demethylation-dependent manner. We further demonstrate that TWIST1 is essential for the viability, dormancy, and self-renewal capacities of LSC, and that it promotes the initiation and maintenance of MLL-AF9-mediated AML. In addition, TWIST1 directly interacts and collaborates with HOXA9 in inducing AML in mice. Mechanistically, TWIST1 exerts its oncogenic function by activating the WNT5a/RAC1 axis. Collectively, our study uncovers a critical role of TWIST1 in LSC function and provides new mechanistic insights into the pathogenesis of MLL-AF9$^+$ AML.

## Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with a multitude of chromosomal aberrations, transcriptional deregulation and epigenetic changes.[1] Chromosomal translocations involving the mixed lineage leukemia (*MLL*) gene cause aggressive leukemia, namely MLL-rearranged (MLL-r) leukemia that accounts for 5-10% of adult AML and 50% of infant AML.[2,3] Patients with MLL-r AML have unfavorable outcomes due to refractoriness to current chemotherapy regimens and a shorter period to relapse.[4] MLL-r leukemia exhibits unique biological and clinical characteristics that are distinct from those of non-MLL-r AML.[5] Multiple chromatin-associated proteins and epigenetic regulators interact with MLL-fusion protein to form large multisubunit complexes, which are involved in chromatin modification and transcriptional elongation.[5,6] More than 130 partner genes that fuse with MLL have been identified, and one of the most common driver fusion genes in AML is *AF9*.[7] A better understanding of the molecular mechanisms underlying the pathogenesis of MLL-AF9-mediated AML will lead to the development of new therapeutic strategies. MLL-AF9 protein transforms hematopoietic stem and progenitor cells (HSPC) through induction of aberrant expression of stem-cell-associated gene programs.[8] The most well-known targets of MLL-AF9 protein include HOXA9 and its co-factors MEIS1 and PBX3, all of which are important for MLL-AF9$^+$ AML development.[9-12] HOXA9 is overexpressed in more than one half of AML cases and is a strong predictor of poor prognosis.[13,14] Overexpression of HOXA9 induces aberrant self-renewal capability and blocks differentiation of HSC, leading to myeloproliferative phenotypes in mice.[15] Co-expression of HOXA9 with either MEIS1 or PBX3 induces fatal AML.[12,15-17]

TWIST1, a key epithelial-to-mesenchymal transition (EMT)-related transcription factor, is shown to play a crucial role in generation and maintenance of cancer stem cells in a number of human solid tumor types.[18,19] In the blood system, TWIST1 is required for normal HSC maintenance.[20,21] Although TWIST1 expression was overall increased in AML,[22-24] among AML subtypes, it has only been reported to be implicated in the progression of acute promyelocytic

leukemia, which arises from committed promyelocytic progenitors rather than leukemic stem cell (LSC).[23] The role of TWIST1 in LSC of AML remains unknown. Recently Luo *et al.* reported that, in MLL-AF9$^+$ AML, HOX gene loci-associated long non-coding RNA HOTTIP recruits WDR5/MLL complex to promote HOXA gene expression and contributes to disease progression.[25,26] HOTTIP aberration in mice leads to an increased LSC pool.[25,26] Interestingly, TWIST1 expression positively correlates with HOTTIP, and HOTTIP deletion reduces the recruitment of MLL1 to TWIST1 and leads to a significant decrease in TWIST1 expression in MLL-AF9$^+$ AML cells.[25] These data indicate that TWIST1 may be involved in the pathogenesis and LSC regulation of MLL-AF9$^+$ AML. Here we report for the first time that TWIST1 is required for the LSC function of MLL-AF9$^+$ AML through activation of the WNT5a/RAC1 axis, and it directly interacts and collaborates with HOXA9 in malignant transformation.

# Methods

### Mice

*ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ mice were generated as previously described.[27] *Cre* expression was induced by daily intraperitoneal injection of tamoxifen (75 mg/kg; Sigma-Aldrich, St. Louis, MO, USA) for 5 days. C57BL/6-Ly5.1 or NOD/SCID mice were purchased from the animal facility of the State Key Laboratory of Experimental Hematology (SKLEH). All animal care and experimental procedures complied with the animal care guidelines and were approved by the Institutional Animal Care and Use Committees of SKLEH.

### Mouse acute myeloid leukemia model

Bone marrow (BM) cells were harvested from C57BL/6-Ly5.1, *Twist1*-knockout, *ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ or *ER-Cre* mice. c-kit-positive BM cells were enriched by positive selection using CD117 microbeads (Miltenyi Biotec, Bergisch Gladbach, Germany) and incubated overnight in Iscove's modified Dulbecco medium supplemented with 5% fetal bovine serum, 50 ng/mL murine SCF (Peprotech, Rocky Hill, NJ), 10 ng/mL murine IL-6 (Peprotech), and 10 ng/mL murine IL-3 (Peprotech). After prestimulation, cells were transduced with retroviruses expressing MLL-AF9, Hoxa9, Twist1, or Hoxa9 and Twist1 at a multiplicity of infection (MOI) of 15-25 in the presence of 8 μg/mL polybrene (Sigma-Aldrich) on retronectin-coated plates. Seventy-two hours after beginning the transduction, infected cells were sorted for *in vitro* culture and *in vivo* transplantation studies.

### Statistical analysis

All data are expressed as the mean ± standard deviation (SD) and are representative of at least two trials with **P*<0.05, ***P*<0.01, and ****P*<0.001. Statistical significance was calculated using a paired or unpaired Student's *t* test. In the survival experiments, mouse survival rates were analyzed using the log-rank test. Details of other experimental procedures are described in the *Online Supplementary Appendix*.

### Data availability

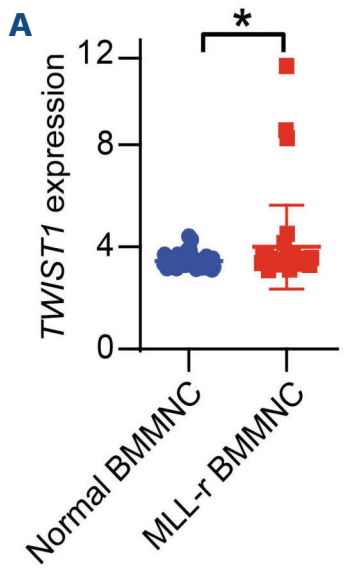
RNA-sequencing data have been deposited in NCBI's Gene Expression Omnibus (GEO accession: GSE215026). The mass spectrometry proteomics data are available at the ProteomeXchange Consortium via the iProx partner with the dataset identifier PXD037217.

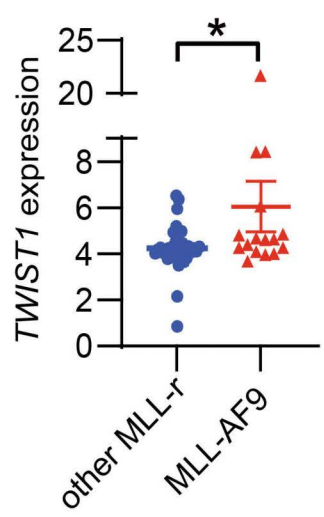
# Results

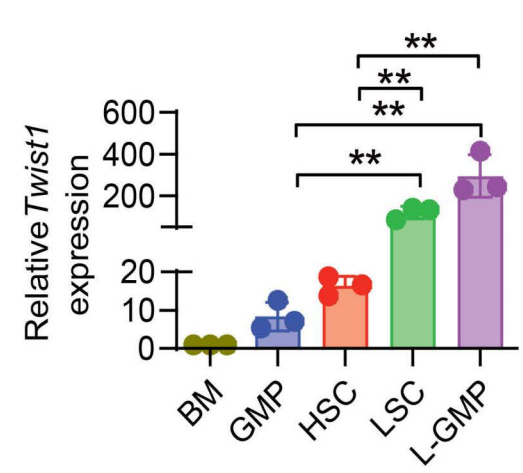
### TWIST1 is highly expressed in MLL-AF9$^+$ acute myeloid leukemia stem cells and its expression is regulated by KDM4C

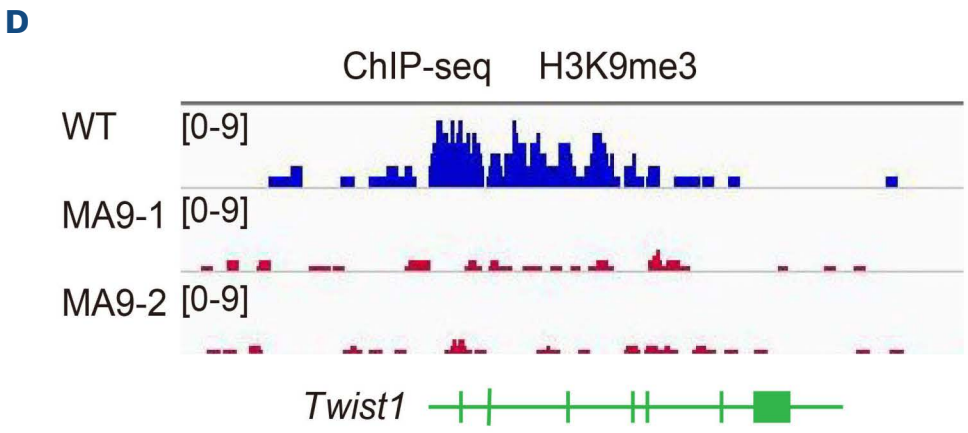
In order to uncover the role of TWIST1 in MLL-r AML, we first analyzed the expression level of *TWIST1* mRNA in patients with MLL-r AML by surveying publicly available datasets. We found that *TWIST1* expression was significantly increased in BM mononuclear cells (BMMNC) from patients with MLL-r AML compared with those from healthy volunteers (Figure 1A). In addition, the expression levels of TWIST1 were substantially heterogeneous among MLL-r AML patients, and patients with t (9; 11) *MLL*-AF9 fusion gene exhibited significantly higher TWIST1 expression than those with other translocations (Figure 1B). We then examined the expression of *Twist1* in leukemic granulocyte/macrophage progenitors (L-GMP) and c-kit$^+$Gr1$^-$ cells, both of which have been identified as LSC in mice with MLL-AF9-driven AML.[3,28] GMP and HSC from normal mice were used as controls. Interestingly, the expression level of *Twist1* in L-GMP was extremely high, approximately 35- and 18-fold higher than those in normal GMP and HSC, respectively (Figure 1C), and its level in c-kit$^+$Gr1$^-$ cells was 14- and 7-fold higher than those in GMP and HSC, respectively (Figure 1C). These findings point to a potential pathogenetic role of TWIST1 in MLL-AF9-induced AML.

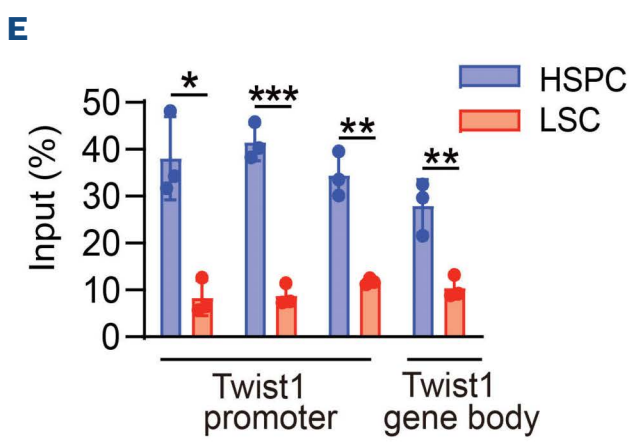
We next sought to understand the upstream epigenetic mechanisms controlling TWIST1 expression in MLL-AF9$^+$ AML. In order to clarify whether TWIST1 is directly upregulated by the MLL-AF9 protein, we analyzed published MLL-AF9 chromatin immunoprecipitation sequencing (ChIP-seq) data generated from MLL-AF9 transformed murine hematopoietic cells, and found that MLL-AF9 exhibited no binding to the *Twist1* locus (*Online Supplementary Figure S1*). Aberrant histone modification has been identified as a regulatory mechanism of altered expression of various oncogenes, such as Hox clusters, that are responsible for MLL-AF9$^+$ AML development.[29,30] In order to determine whether TWIST1 expression is regulated by histone modification, we analyzed the publicly available ChIP-seq data of H3K9me3, H3K4me3, H3K27me3, H3K36me3, and H3K79me2 in LSC from MLL-

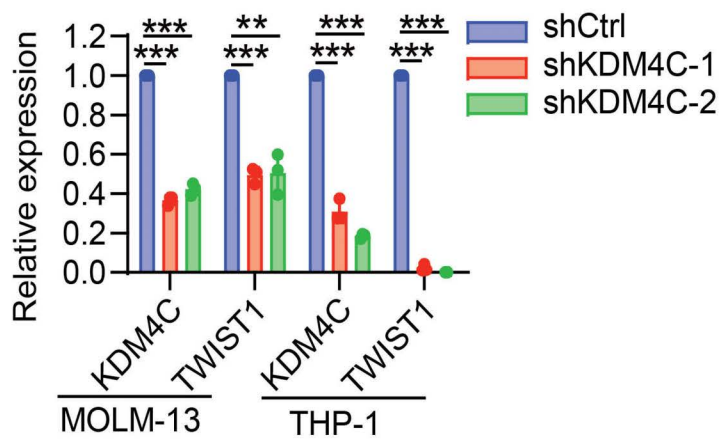


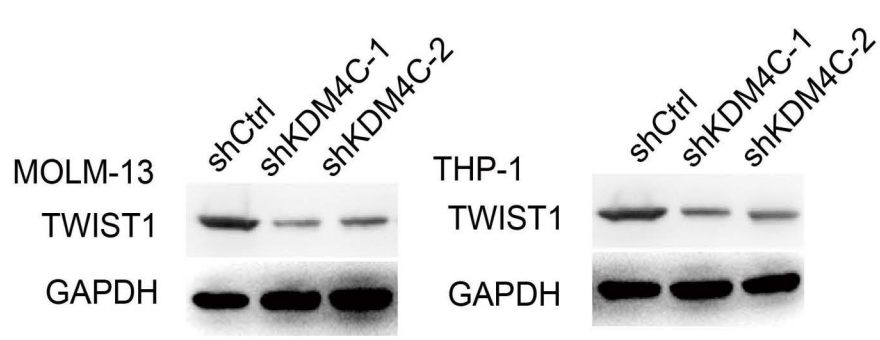



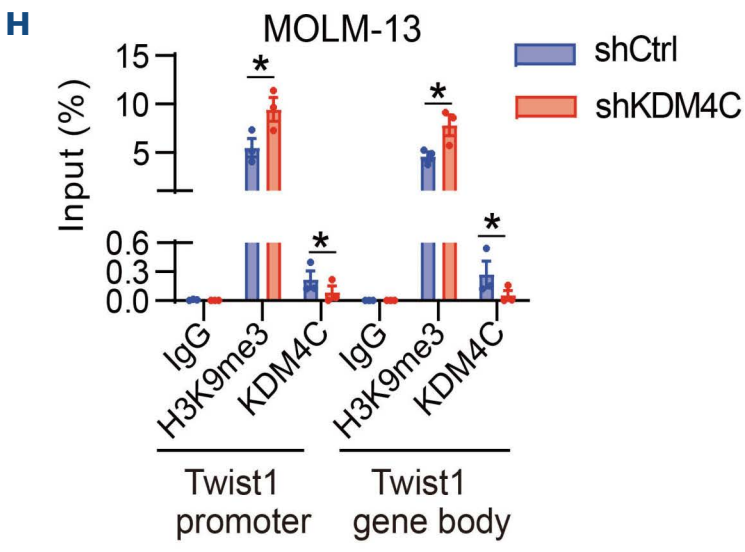



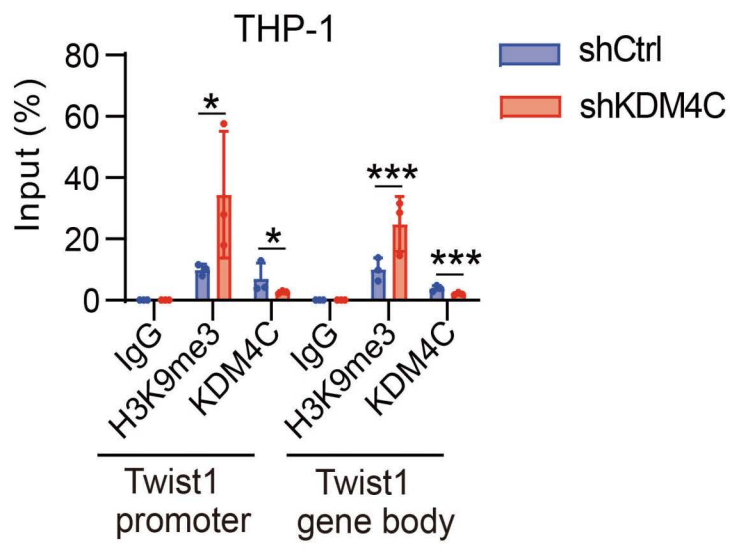




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**Figure 1. TWIST1 is expressed at high levels in leukemic stem cells of MLL-AF9$^+$ acute myeloid leukemia and its expression is positively regulated by KDM4C.** (A) Comparison of *TWIST1* expression between primary bone marrow mononuclear cells (BMMNC) from patients with MLL-rearranged (MLL-r) acute myeloid leukemia (AML, N=38) and those from healthy volunteers (N=73) in acute myeloid leukemia (AML) datasets Microarray Innovations in Leukemia (MILE) (GSE13159). (B) *TWIST1* expression in BMMNC from patients with MLL-AF9 AML (N=16) *versus* all the other subtypes of MLL-r AML (N=36) (GSE61804 and GSE19577). (C) Relative expression levels of *Twist1* in BMMNC, granulocyte/macrophage progenitors (GMP), hematopoietic stem cell (HSC) from wild-type mice, and in GFP$^+$c-kit$^+$Gr1$^-$, and leukemic GMP (L-GMP) cells from MLL-AF9 leukemic mice were evaluated by quantitative polymerase chain reaction (qPCR) (N=3). (D) Integrative genomics viewer plots showing signal tag density of H3K9me3 at the *Twist1* gene in c-kit$^+$ cells from MLL-AF9$^+$ AML (MA9-1 and MA9-2) and wild-type control mice (GSE132175). (E) H3K9me3 chromatin immunoprecipitation (ChIP)-qPCR of Twist1 in c-kit+ cells from MLL$^-$AF9$^+$ AML and wild-type control mice. Genomic DNA was immunoprecipitated with anti-H3K9me3 antibody or immunglobulin (Ig)G control and amplified with primers spanning the *Twist1* promoter and gene body sequences (N=3). (F) The expression of *TWIST1* and *KDM4C* in KDM4C small hairpin RNA (shRNA)-transduced MOLM-13 and THP-1 cells was examined by quantitative real-time PCR (N=3). (G) Analysis of protein expression of TWIST1 by western blot. (H) ChIP-qPCR analysis of H3K9me3 and KDM4C enrichment on the *Twist1* locus in MOLM-13 and THP-1 cells transduced with shCtrl or shKDM4C (N=3). Data are represented as mean ± standard deviation. **P*<0.05; ***P*<0.01; ****P*<0.001, by Student's *t* test (A-C, E, F and H).

AF9-driven AML and control cells from wild-type mice. We found that the *Twist1* locus showed a significantly lower level of repressive histone marks H3K9me3 in LSC (Figure 1D; *Online Supplementary Figure S1*). Furthermore, ChIP-quantitative polymerase chain reaction (qPCR) assays demonstrated that the enrichment of H3K9me3 at the promoter and gene body regions of the *Twist1* locus was greatly decreased (by 2.7-4.7-fold) (Figure 1E). These results indicate that reduced repressive mark H3K9me3 of the *Twist1* locus could contribute to the upregulation of *Twist1* expression in LSC of MLL-AF9$^+$ leukemia.

H3K9me3 is tightly controlled by demethylase such as the KDM4 family which can remove H3K9me3 and play a strong regulatory influence on oncogenic gene transcription in AML.[31] We analyzed a published dataset and found that, among the KDM4 family members, only *KDM4C* expression was positively correlated with *TWIST1* in MLL-r AML samples (*Online Supplementary Figure S2A-C*). In addition, analysis of the published KDM4C ChIP-seq data on MLL-AF9-transformed LSC revealed that KDM4C bound to the promoter and spread into the gene body of *Twist1* (*Online Supplementary Figure S3A*), whereas the binding was impaired upon loss of KDM4C (*Online Supplementary Figure S3A*). Published KDM4C ATAC-sequencing datasets from MOLM-13 cells showed a similar binding distribution at the *TWIST1* locus (*Online Supplementary Figure S3B*). In order to verify these data, we knocked down KDM4C in MLL-AF9$^+$ cell lines THP-1 and MOLM-13 or treated these cells with KDM4C inhibitor SD70, and measured TWIST1 expression. Inhibition of KDM4C resulted in significantly reduced expression of TWIST1 at both mRNA and protein levels (Figure 1F, G; *Online Supplementary Figure S3C*). In addition, ChIP-qPCR analysis demonstrated that *KDM4C* was significantly enriched at the transcription sites of the *TWIST1* gene in MOLM-13 and THP-1 cells (Figure 1H). Knockdown of *KDM4C* led to markedly reduced recruitment of endogenous KDM4C at the *TWIST1* loci (Figure 1H), with a corresponding increase in H3K9me3 level (Figure 1H). Taken together, these results indicate that KDM4C is required for TWIST1 expression in a H3K9me3 demethylation-dependent manner.

## TWIST1 promotes the initiation and progression of MLL-AF9-mediated acute myeloid leukemia

In order to explore the role of TWIST1 in MLL-AF9$^+$ AML, we performed transformation assays, both *in vitro* and *in vivo*, using a previously described *Twist1*-knockout mouse model. We transduced c-kit$^+$ BM cells from *Twist1*-knockout and wild-type mice with a retrovirus expressing MLL-AF9 (Figure 2A). The resulting cells were referred to as *Twist1*$^{-/-}$ and control (Ctrl), respectively, in the figures hereafter. Colony formation and serial replating assays revealed that *Twist1* deficiency led to a significant reduction in colony-forming activity (*Online Supplementary Figure S4A*). This result was corroborated by BM transplantation (BMT). Equal numbers of MLL-AF9-transduced c-kit$^+$ cells from *Twist1* knockout or wild-type mice were transplanted into lethally irradiated recipients. Deletion of *Twist1* was verified by quantitative reverse transcription PCR (qRT-PCR) in *Twist1*$^{-/-}$ BM cells from the transplantation recipients (*Online Supplementary Figure S4B*). The expression of *Twist2* was not altered in *Twist1*-deficient BM cells (*Online Supplementary Figure S4B*). Mice transplanted with MLL-AF9-transduced wild-type cells developed AML and died within 41 days after transplantation (Figure 2B). In contrast, recipients transplanted with MLL-AF9-transduced cells from *Twist1*$^{-/-}$ mice showed a significantly prolonged survival (Figure 2B), suggesting that loss of *Twist1* delays the onset of leukemia. Moreover, these recipients showed remarkable reductions in splenomegaly, white blood counts (WBC), and GFP$^+$ AML cells in the BM and spleen compared with the control (*Online Supplementary Figure S4C-F*). The histological analysis also revealed prominently reduced leukemic infiltration of BM, spleen, and liver (Figure 2C), suggesting a decrease in disease burden upon *Twist1* loss. Meanwhile, *Twist1* deletion led to significantly enhanced apoptosis (Figure 2D), as well as differentiation of leukemia cells (Figure 2E; *Online Supplementary Figure S4G*). No obvious difference in proliferation was observed between Ctrl and *Twist1*-deleted leukemia cells (*Online Supplementary Figure S4H*). These results suggest that TWIST1 promotes the initiation of MLL-AF9-mediated AML by enhancing differentiation blockage and inhibiting apoptosis of leukemia cells.

We next assess the effect of Twist1 deficiency on the maintenance of established leukemia. We transplanted primary MLL-AF9 (*ER-Cre*$^{+/-}$;*Twist1*$^{flox/flox}$ or *ER-Cre*$^{+/-}$;*Twist1*$^{wt/wt}$) AML cells into irradiated wild-type mice, followed by injection of tamoxifen (TAM) to ablate *Twist1* (Figure 2F). As expected, the deletion of *Twist1* significantly prolonged the survival of recipient mice and retarded the progression of leukemia (Figure 2G; *Online Supplementary Figure S5A*). Significantly decreased disease burden in the peripheral blood (PB), BM, and spleen (*Online Supplementary Figure S5B-E*), and increased myeloid differentiation of AML cells were observed upon *Twist1* deletion (*Online Supplementary Figure S5F, G*). In order to further validate the role of TWIST1, we transplanted murine AML cells transduced with retroviral vectors containing *Twist1* or control into irradiated recipient mice (*Online Supplementary Figure S6A*). Enforced expression of *Twist1* significantly accelerated MLL-AF9-induced AML development (*Online Supplementary Figure S6B-D*), and arrested myeloid differentiation (*Online Supplementary Figure S6E*). Together, our findings indicate that TWIST1 plays a critical role in the initiation and progression of MLL-AF9-induced AML.

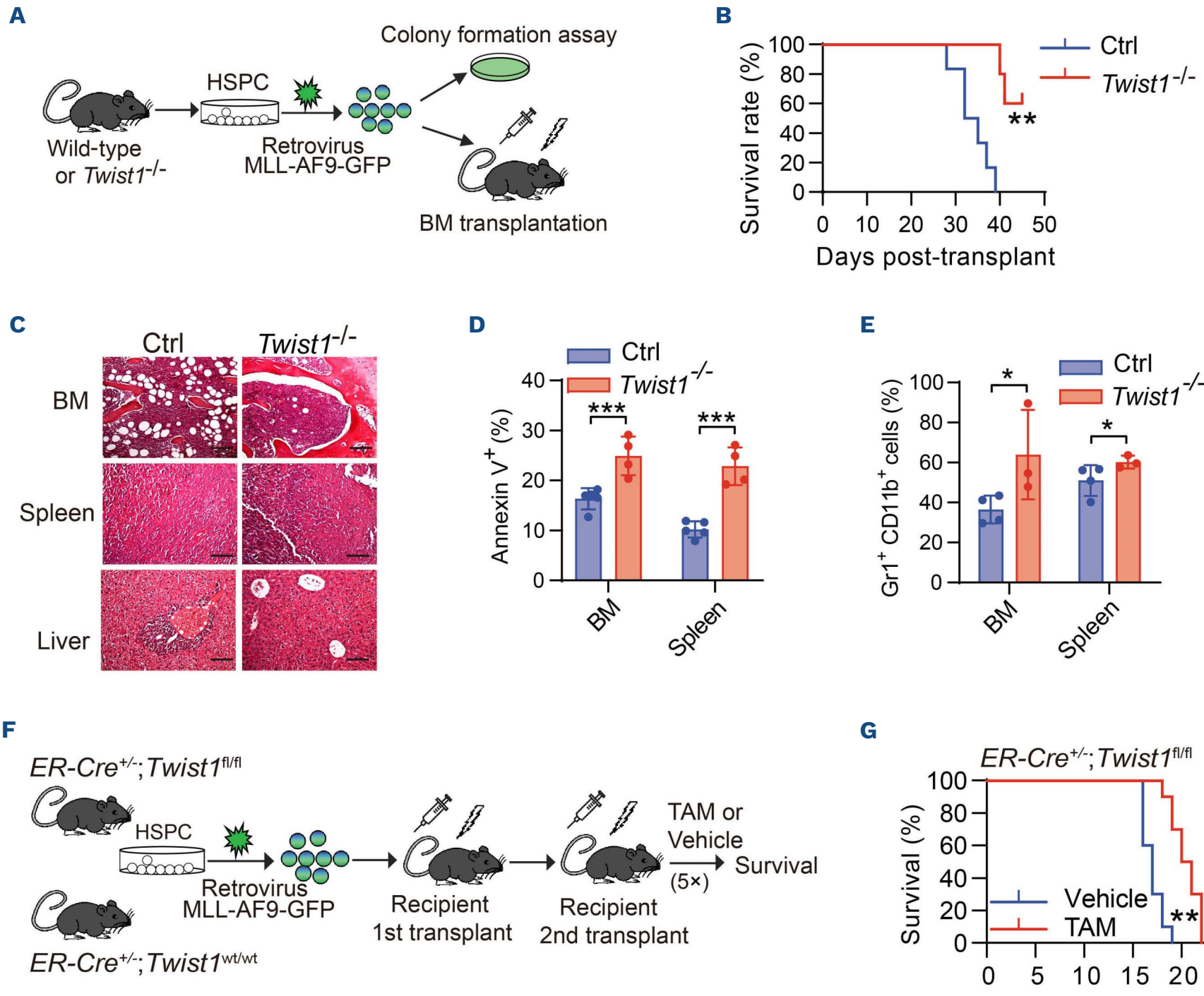



**Figure 2. TWIST1 is important for the initiation and maintenance of MLL-AF9-mediated acute myeloid leukemia.** (A) Schematic of the colony formation and leukemic transplant experiments. (B) Kaplan-Meier survival curves of mice injected with control or *Twist1*$^{-/-}$ cells transformed with MLL-AF9 (N=5-6). (C) One hundred thousand MLL-AF9-transduced c-kit$^{+}$ cells from wild-type or *Twist1*-knockout mice were transplanted into lethally irradiated recipients. The leukemia burden was examined 1 month after transplantation. Representative hematoxylin and eosin-stained histology sections of bone marrow (BM), spleen and liver. The length of the bars represents 100 μm. (D-E) Leukemic BM cells from sick primary mice were transplanted into the secondary recipients. The biological characteristics of leukemia cells were examined when the recipient mice became moribund. Apoptosis in GFP$^{+}$ BM and GFP$^{+}$ spleen cells (D, N=4-5). Frequency of leukemic myeloid Gr1$^{+}$ CD11b$^{+}$ cells in GFP$^{+}$ BM and GFP$^{+}$ spleen cells (E, N=3-4). (F) Schematic of the experimental procedure for evaluation of the role of TWIST1 in acute myeloid leukemia (AML) maintenance. (G) Kaplan-Meier survival curves of mice in AML maintenance experiment (N=10). Data are represented as mean ± standard deviation. *$P$<0.05; **$P$<0.01; ***$P$<0.001, by Student's $t$ test (D and E) or log-rank test (B and G).

### TWIST1 is essential for the maintenance of leukemia stem cell

In order to elucidate the role of TWIST1 in LSC, we evaluated the number and apoptotic status of the LSC from *Twist1*$^{-/-}$ AML recipients. Flow cytometric analysis revealed that the percentages of L-GMP and c-kit$^{+}$Gr1$^{-}$ cells were markedly reduced in the BM and spleen of recipients of *Twist1*$^{-/-}$ AML cells (Figure 3A, B), probably due to the increase in apoptotic rate (Figure 3C). In addition, cell cycle analysis showed a significant decrease in the proportion of these cells in the G0 phase (Figure 3D).

In order to assess the impact of *Twist1* deletion on LSC function, we performed secondary transplantation into irradiated mice using leukemic whole BM cells from sick primary mice. Similar to primary transplantation, secondary recipient mice receiving *Twist1*$^{-/-}$ leukemia cells exhibited significantly longer leukemia-free survival (*Online Supplementary Figure S7A*). No significant difference in homing capacity was observed between *Twist1*$^{-/-}$ and control AML cells (*Online Supplementary Figure S7B*). In order to quantitate the functional LSC, we performed a limiting dilution assay (LDA). The frequency of LSC in *Twist1*$^{-/-}$ AML mice was dramatically reduced by approximately 69-fold compared to the control (1:21,472 *vs*. 1:310 cells) (Figure 3E). In addition, transplantation of *Twist1*$^{-/-}$ LSC also led to markedly delayed onset of AML compared to that of control LSC (*Online Supplementary Figure S7C*). Overall, these data suggest that TWIST1 is necessary for LSC survival and function in MLL-AF9$^{+}$ AML.

### TWIST1 promotes leukemogenesis of human MLL-AF9$^{+}$ acute myeloid leukemia

In order to verify the function of TWIST1 in human MLL-AF9 leukemia, we performed small haipin RNA (shRNA)-mediated *TWIST1* knockdown (KD) in THP-1 and MOLM-13 cell lines (*Online Supplementary Figure S8A*). TWIST1 insufficiency significantly attenuated cell growth and colony formation, and promoted differentiation, while its induction of apoptosis was mild but statistically significant compared with the controls (Figure 4A, B; *Online Supplementary Figure S8B, C*). In order to ascertain if *TWIST1* KD inhibits leukemia development *in vivo*, we developed a xenograft model of human AML. MOLM-13-luc2 cells transduced with *TWIST1* shRNA or control scramble shRNA were injected into NOD/SCID mice. *TWIST1* KD led to remarkably reduced tumor burden (Figure 4C, D), and significantly prolonged survival in comparison with the control (Figure 4E). Collectively, our results demonstrate a critical role of TWIST1 in the propagation of human MLL-AF9$^{+}$ AML cells.

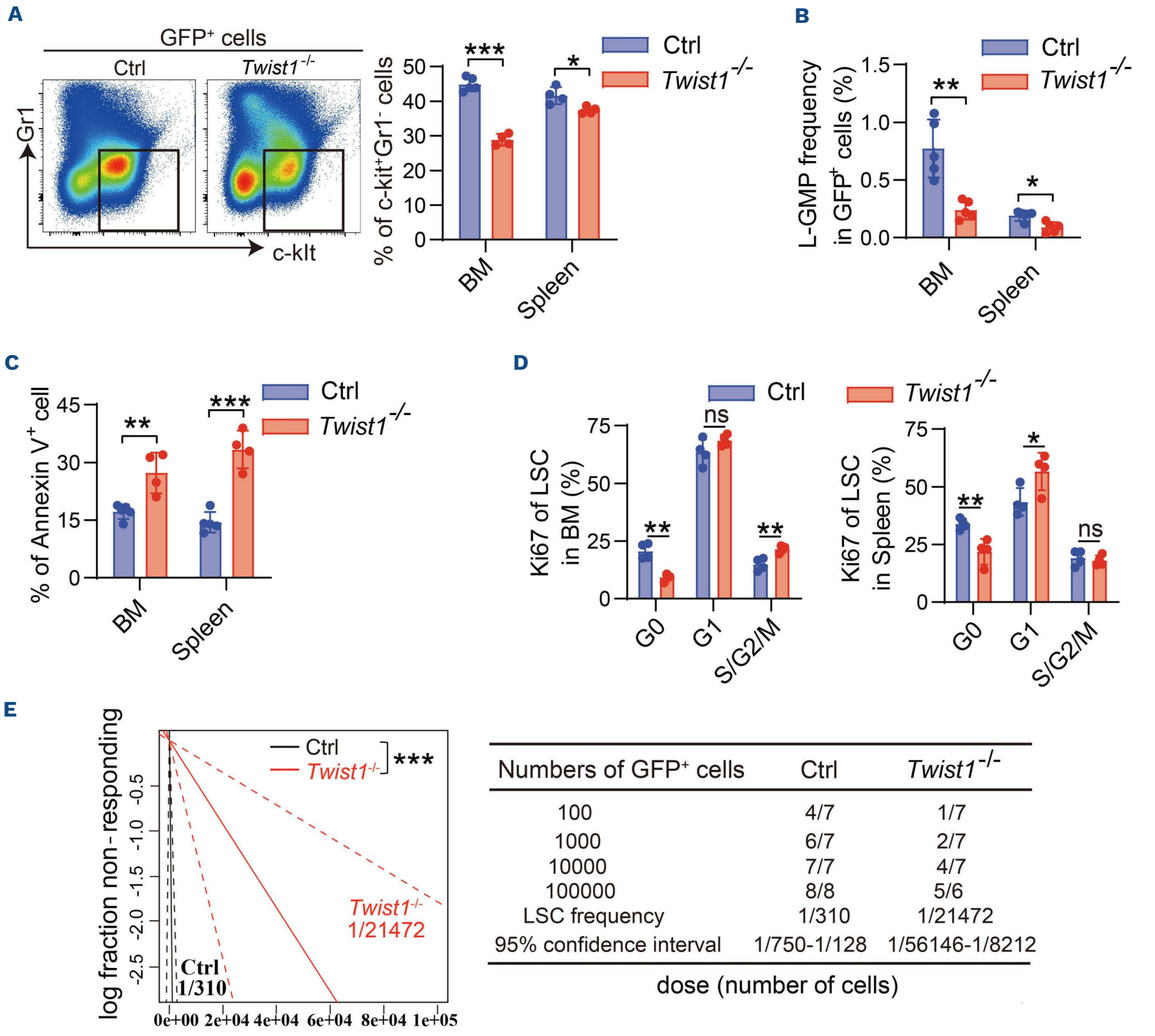
### TWIST1 promotes the development of MLL-AF9-induced acute myeloid leukemia via activation of the WNT5a/RAC1 axis

In order to identify the downstream targets of TWIST1, we performed RNA sequencing on LSC from *Twist1*$^{-/-}$ and *Twist*$^{+/+}$ MLL-AF9 AML mice. A total of 1,033 genes were differentially expressed, of which 329 were downregulated and 704 were upregulated (log2 fold change >1, and $P$<0.05; Figure 5A). Gene ontology (GO) analysis revealed that regulation of Rho protein signal transduction was among the top enriched terms (Figure 5B). Enrichment of the Rho protein signal pathway was confirmed through gene set enrichment analysis (GSEA) (Figure 5C). RAC1, RHOA, and CDC42 are the three best-characterized members of the Rho GTPase family,[32] and RAC1 plays an important role in MLL-AF9-induced leukemogenesis.[33,34] In order to determine whether RAC1 is involved in TWIST1-mediated LSC maintenance, we first examined RAC1 activity using a Rac1-GTP pull-down assay in *Twist1*-deficient LSC and control cells. The results demonstrated that the depletion of *Twist1* led to a significant decrease in RAC1 activity (Figure 5D), while RAC2 activity was unchanged (Figure 5D). We next examined whether RAC1 mediated the contribution of TWIST1 to MLL-AF9-driven AML development. A constitutively active form of RAC1 (Q61L), a dominant-negative RAC1 mutant (T17N), or an empty vector was overexpressed in *Twist1*-deleted LSC, and the transduced cells were then injected into recipient mice (Figure 5E). As expected, overexpression of Q61L Rac1 markedly abrogated the impact of *Twist1* ablation on murine survival (Figure 5F), spleen size, LSC frequency, and myeloid differentiation (*Online Supplementary Figure S9A-C*). In contrast, overexpression of T17N Rac1 or control vector showed no similar effects (Figure 5F; *Online Supplementary Figure S9A-C*). In summary, these data suggest that RAC1 activation mediates, at least partially, the pathogenic role of TWIST1 in MLL-AF9-driven AML.

GSEA analysis also revealed significant negative enrichment of the non-canonical WNT signaling pathway in *Twist1*-deficient LSC (*Online Supplementary Table S1*). Validation of the expression of genes associated with the Wnt pathway revealed that the expression levels of Wnt5a were most dramatically reduced (Figure 6A, B; primer sequences and antibodies are listed in *Online Supplementary Tables S2* and *S3*). In addition, in a cohort of MLL-r AML, *WNT5a* expression correlates significantly with *TWIST1* (*Online Supplementary Figure S10*). We then reintroduced TWIST1 expression in *Twist1*-deleted LSC, and found that the reintroduction of TWIST1 completely restored Wnt5a expression (Figure 6C). In order to investigate whether TWIST1 played a direct role in the transcription regulation of Wnt5a, we first searched the JASPAR database and found E-box consensus sequences in the promoter region of *Wnt5a*. We then generated luciferase constructs to examine the promoter activity of various lengths of *Wnt5a* 5' flanking sequences (0.5, 1, 1.5, and 1.8 kb). Only the 1.8 kb fragment responded to TWIST1 overexpression by enhancing the luciferase activity (Figure 6D), indicating that TWIST1 bind to the *Wnta5a* promoter region from -1.8 kb to -1.5 kb. ChIP assay further confirmed a direct binding of endogenous TWIST1 to the *Wnt5a* promoter in LSC isolated from MLL-AF9$^{+}$ AML mice (Figure

6E). Taken together, these results indicate that TWIST1 is a transcriptional activator of WNT5a.

Previous studies have reported that WNT5a promotes the self-renewal of tumor stem cells,[35] and it induces RAC1 activation in a β-catenin-independent pathway.[36,37] Therefore, we speculated that TWIST1 may activate RAC1 by upregulating WNT5a. In order to test this, we ectopically expressed *Wnt5a* in *Twist1*-wild-type and *Twist1*-deficient LSC and analyzed RAC1 activity. The results revealed that enforced expression of Wnt5a in *Twist1*-wild-type LSC led to significantly enhanced activities of RAC1 (Figure 6F). In addition, WNT5a overexpression substantially diminished




| Numbers of GFP+ cells | Ctrl | *Twist1*$^{-/-}$ |
|---|---|---|
| 100 | 4/7 | 1/7 |
| 1000 | 6/7 | 2/7 |
| 10000 | 7/7 | 4/7 |
| 100000 | 8/8 | 5/6 |
| LSC frequency | 1/310 | 1/21472 |
| 95% confidence interval | 1/750-1/128 | 1/56146-1/8212 |

**Figure 3. *Twist1* deletion reduces the frequency and function of leukemic stem cell in MLL-AF9+ acute myeloid leukemia.** (A-D) The frequency and biological characteristics of leukemic stem cells (LSC) were examined when the secondary recipient mice became moribund. Frequencies of c-kit+Gr1- subpopulations in the bone marrow (BM) and spleen (right) and representative fluorescence-activated cell sorting plots (left) (A, N=4-5). Frequencies of leukemic granulocyte/macrophage progenitor (L-GMP, Lin-c-kit+Sca1-CD127-CD34+CD16/32+) in the BM and spleen (B, N=4-5). Apoptosis analysis of c-kit+Gr1- LSC in the BM and spleen (C, N=4-5). Cell-cycle analysis of c-kit+Gr1- LSC in the BM and spleen (D, N=3-4). (E) Limiting dilution assay using BM cells from primary *Twist1*-deleted or control MLL-AF9+ acute myeloid leukemia mice. Logarithmic plot (left) showing the percentage of non-responding recipients transplanted with different cell doses of GFP+ cells. Table (right) showing the number of mice used as BM transplantation recipients in the assay and the number of recipients that developed leukemia. Data are represented as mean ± standard deviation. *$P$<0.05; **$P$<0.01; ***$P$<0.001, by Student's $t$ test (A-D) or $\chi^2$ test (E).

the inhibitory effect of *Twist1* deletion on RAC1 activity (Figure 6F). We further transplanted *Twist1*$^{-/-}$ AML cells overexpressing *Wnt5a* or empty vector into recipient mice, and found that enforced expression of *Wnt5a* completely abrogated the changes in murine survival, leukemia cell infiltration, LSC frequency, and myeloid differentiation caused by *Twist1* deletion (Figure 6G; *Online Supplementary Figure S11A-D*). These data suggest that the oncogenic role of TWIST1 in MLL-AF9-induced AML is mediated via activation of WNT5a-RAC1 signaling pathway.

### TWIST1 directly binds to HOXA9 and the co-expression of *Twist1* and *Hoxa9* induces fatal acute myeloid leukemia in mice

Transcription factors often interact with other factors, in complexes, to alter DNA binding specificity or enhance

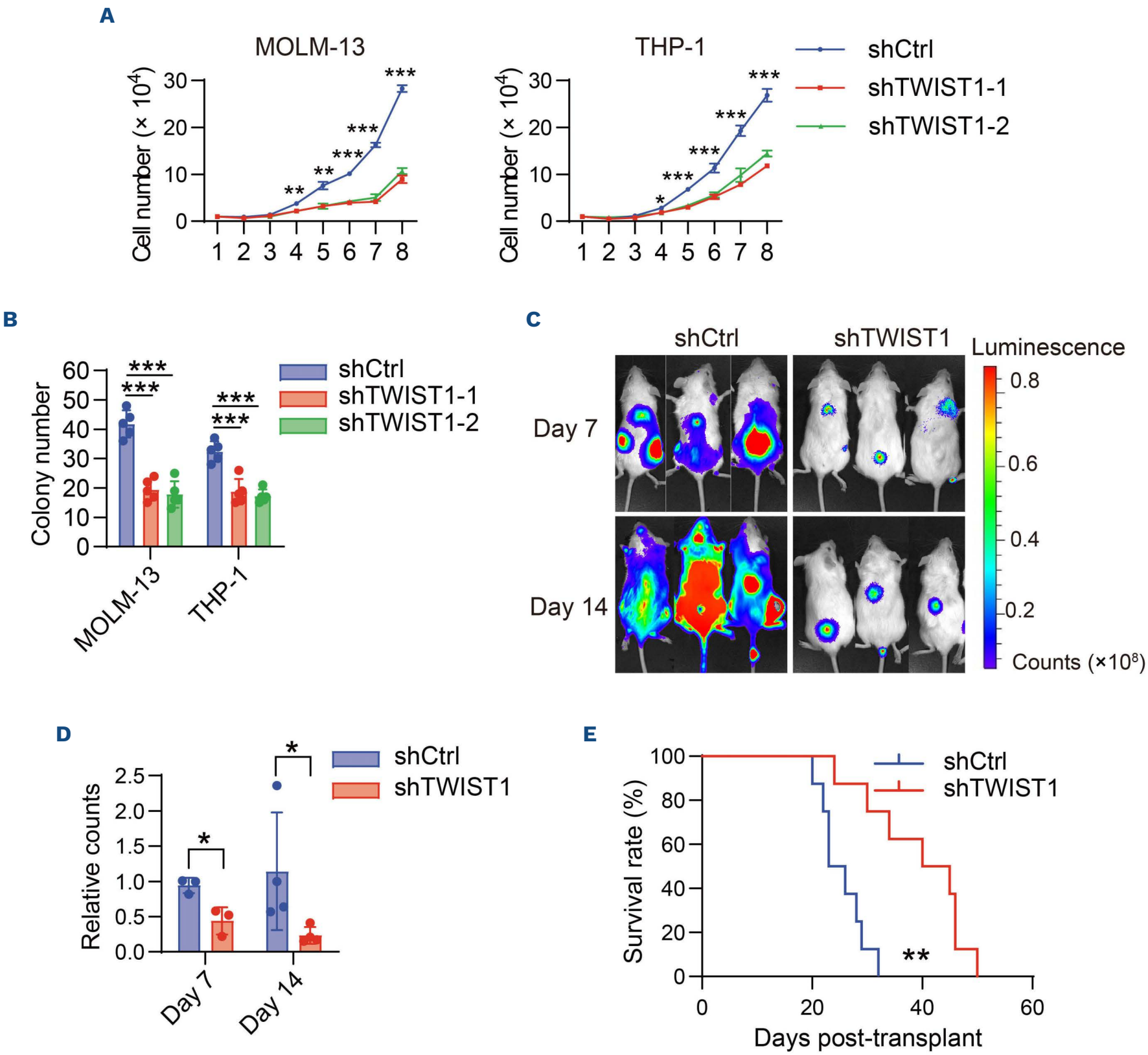



**Figure 4. Human MLL-AF9$^{+}$ acute myeloid leukemia cells are sensitive to *TWIST1* knockdown.** (A, B) Effects of *TWIST1* knockdown on cell proliferation (A) and colony formation (B) in MOLM-13 and THP-1 cells, as determined by cell counting and colony-forming assay, respectively (N=3-5). (C-E) MOLM-13-luc2 cells were transfected with *TWIST1* small hairpin RNA (shRNA) or control scramble shRNA and engrafted into NOD/SCID mice (8×10$^{5}$ cells per mouse). Bioluminescence imaging of representative mice from each group was taken on day 7 and day 14 post-transplantation (C). Quantification of the bioluminescence imaging at the indicated time points (D, N=3-4). Kaplan-Meier survival curves of MOLM13-transplanted mice (E, N=8). Data are represented as mean ± standard deviation. *$P$<0.05; **$P$<0.01; ***$P$<0.001, by Student's $t$ test (A, B and D) or log-rank test (E).

transcription.[38] in order to investigate whether TWTST1 binds to any factors, we performed a liquid chromatography-tandem mass spectrometry analysis of endogenous TWIST1 immunoprecipitates in the LSC isolated from MLL-AF9-driven AML mice, and identified HOXA9, the only protein reported to be essential for MLL-dependent leukemogenesis and LSC maintenance,[9,10] as a potential TWIST1-interacting protein (dataset available via ProteomeXchange with identifier PXD037217). The interaction of TWIST1 with HOXA9 in the LSC was further confirmed by reciprocal co-immunoprecipitation using antibodies against either TWIST1 or HOXA9 (Figure 7A; *Online Supplementary Figure S12A*). Similar interactions were also observed in MOLM-13 and THP-1 cells (Figure 7A; *Online Supplementary Figure S12A*). In order to further test whether this interaction is direct, GST pull-down assay was performed using purified

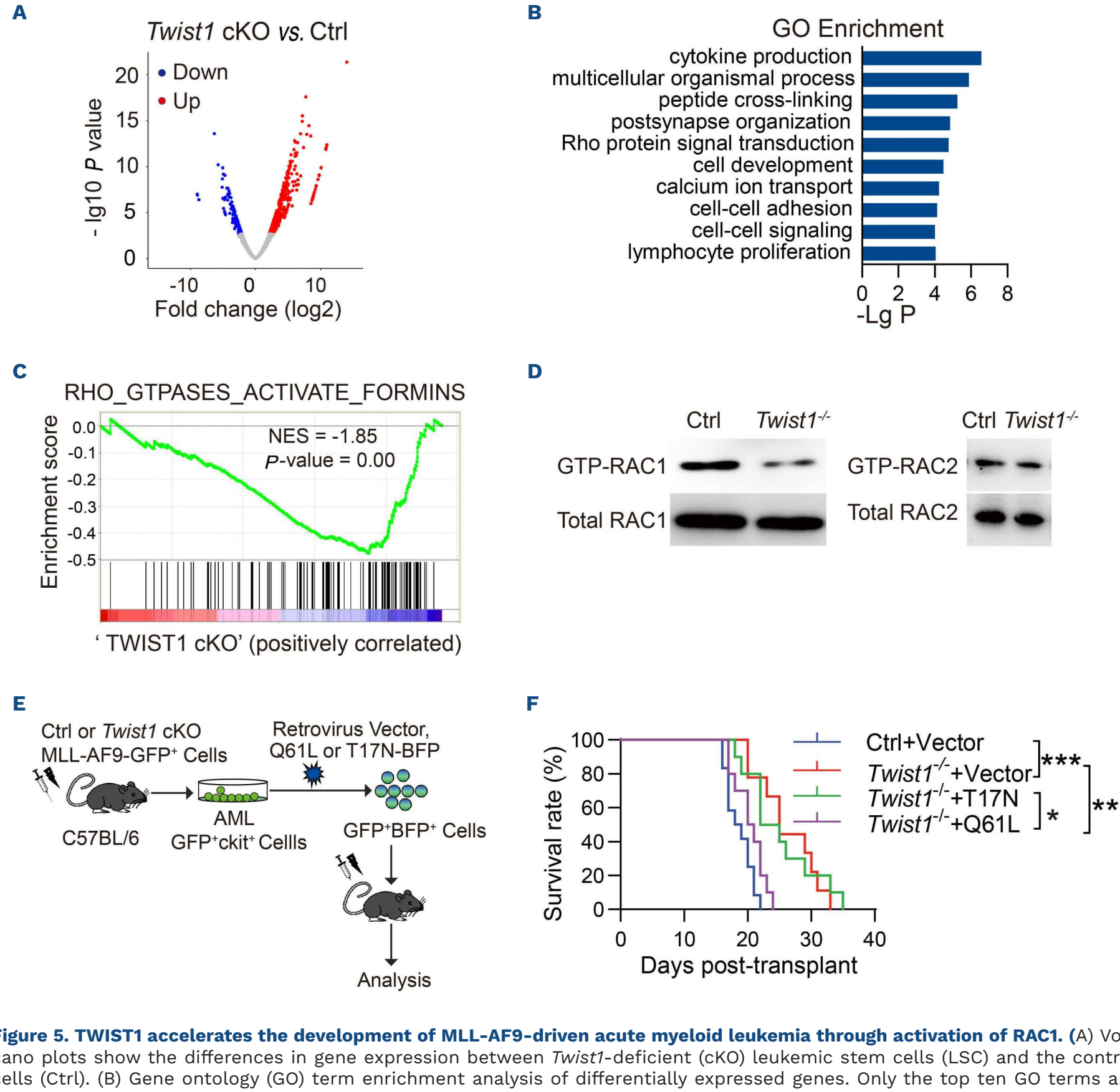



**Figure 5. TWIST1 accelerates the development of MLL-AF9-driven acute myeloid leukemia through activation of RAC1. (**A) Volcano plots show the differences in gene expression between *Twist1*-deficient (cKO) leukemic stem cells (LSC) and the control cells (Ctrl). (B) Gene ontology (GO) term enrichment analysis of differentially expressed genes. Only the top ten GO terms are listed. (C) Gene set enrichment analysis showing reduced Rho protein signal pathway in *Twist1*-deficient LSC. The normalized enrichment score (NES) and *P* value are shown. (D) Western blot analysis of activated RAC1 (GTP-RAC1), total RAC1, activated RAC2 (GTP-RAC2), and total RAC2 in *Twist1*-deficient LSC and the control cells. (E) Schematic of the experimental procedure for evaluation of the role of RAC1 in TWIST1-mediated development of acute myeloid leukemia (AML). (F) Kaplan-Meier survival curves of mice transplanted with the indicated transduced cells (N=9-12). *$P$<0.05; **$P$<0.01; ***$P$<0.001, by log-rank test.

proteins of GST-tagged TWIST1 and MBP-tagged HOXA9. The GST-TWIST1, but not GST alone, could pull down MBP-tagged HOXA9 (Figure 7B), suggesting that TWIST1 directly interacts with HOXA9.

Our previous study revealed that the enforced expression of *Twist1* promotes HSC self-renewal and myeloid skewing, but it is not sufficient to induce hematopoietic malignancies.[21] Overexpression of individual *Hoxa9* leads to myeloproliferative neoplasm in mice.[15] Given that TWIST1 and HOXA9 are both highly expressed in patients with MLL-r AML and TWIST1 interacts with HOXA9, we next sought to determine whether co-expression of *Twist1* and *Hoxa9* could initiate AML. c-kit$^+$ BM cells from wild-type mice co-transduced with *Twist1* and *Hoxa9* retroviruses were subjected to trans-

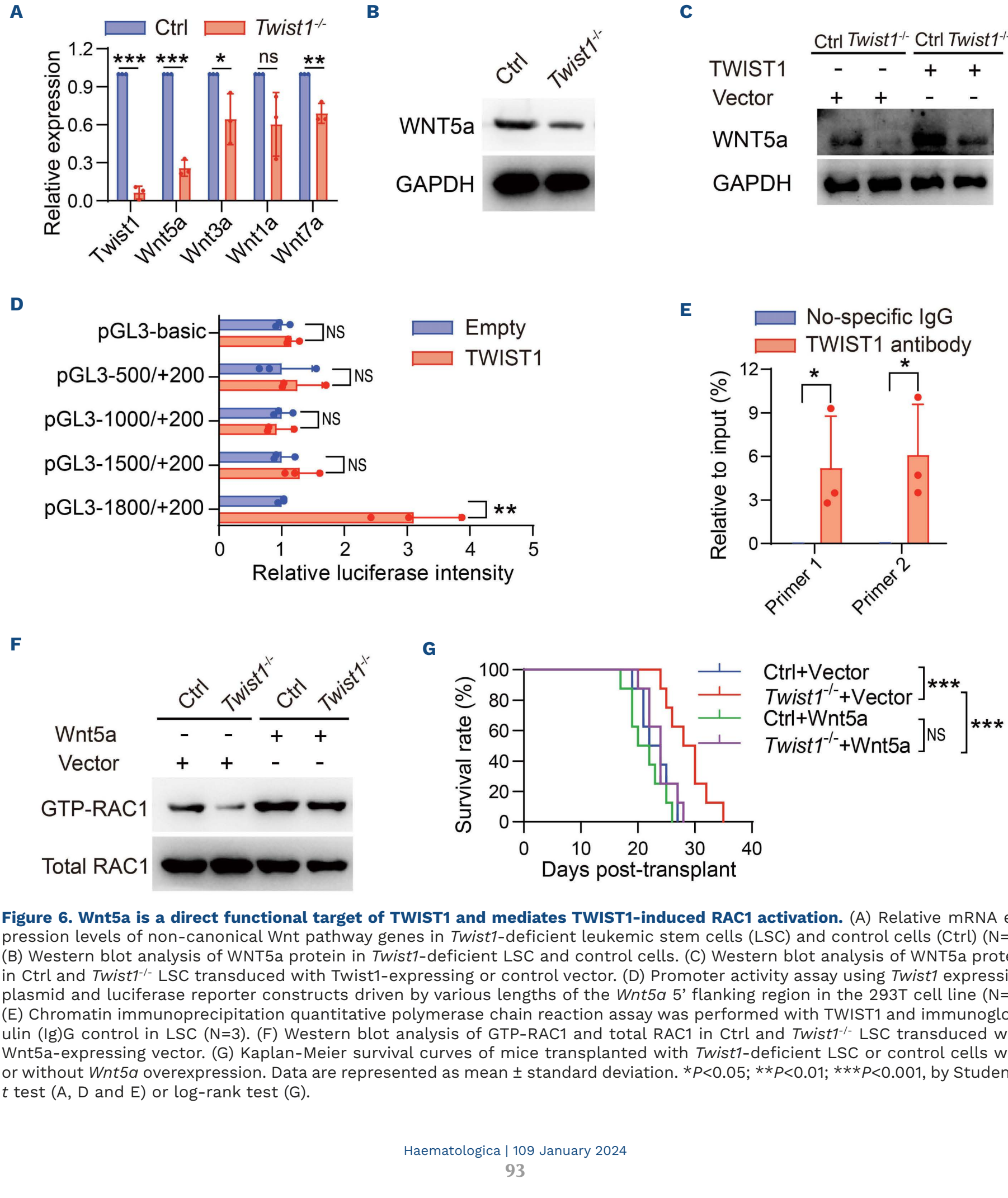



**Figure 6. Wnt5a is a direct functional target of TWIST1 and mediates TWIST1-induced RAC1 activation.** (A) Relative mRNA expression levels of non-canonical Wnt pathway genes in *Twist1*-deficient leukemic stem cells (LSC) and control cells (Ctrl) (N=3). (B) Western blot analysis of WNT5a protein in *Twist1*-deficient LSC and control cells. (C) Western blot analysis of WNT5a protein in Ctrl and *Twist1*$^{-/-}$ LSC transduced with Twist1-expressing or control vector. (D) Promoter activity assay using *Twist1* expression plasmid and luciferase reporter constructs driven by various lengths of the *Wnt5a* 5' flanking region in the 293T cell line (N=3). (E) Chromatin immunoprecipitation quantitative polymerase chain reaction assay was performed with TWIST1 and immunoglobulin (Ig)G control in LSC (N=3). (F) Western blot analysis of GTP-RAC1 and total RAC1 in Ctrl and *Twist1*$^{-/-}$ LSC transduced with Wnt5a-expressing vector. (G) Kaplan-Meier survival curves of mice transplanted with *Twist1*-deficient LSC or control cells with or without *Wnt5a* overexpression. Data are represented as mean ± standard deviation. **P*<0.05; ***P*<0.01; ****P*<0.001, by Student's *t* test (A, D and E) or log-rank test (G).

plant experiments (Figure 7C). The results revealed that the recipient mice transplanted with cells co-expressing *Twist1* and *Hoxa9* have significantly shorter survival than those with *Hoxa9* or *Twist1* alone (Figure 7D). Histological staining revealed that these mice developed fatal AML, characterized by expansion of myeloid blast cells and leukemic infiltration of the BM, spleen, liver, and kidney (Figure 7E; *Online Supplementary Figure S12B*), whereas *Hoxa9* or *Twist1*

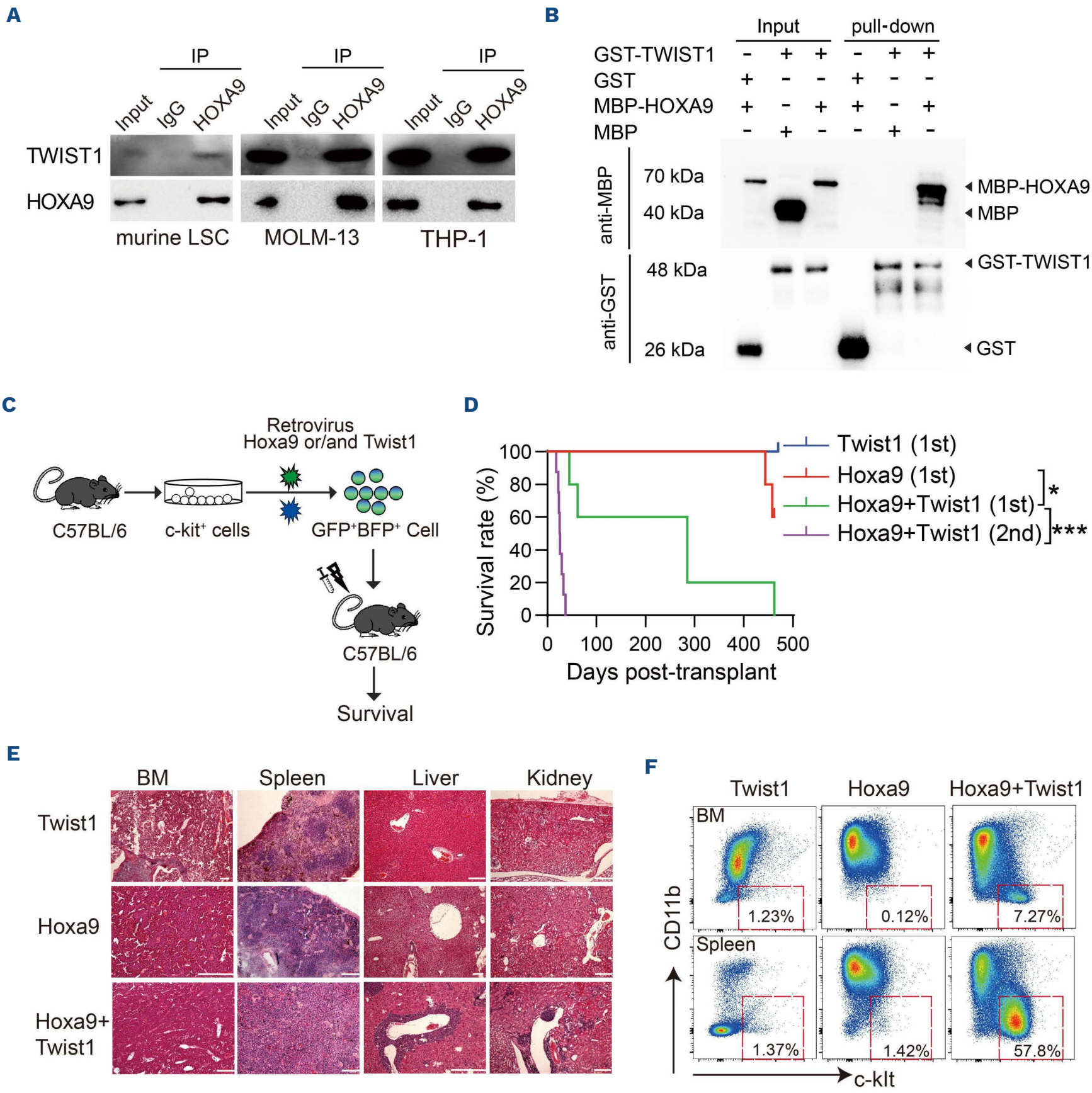



**Figure 7. TWIST1 directly interacts and collaborates with HOXA9 to induce acute myeloid leukemia in transplanted mice.** (A) Co-immunoprecipitation (Co-IP) and immunoblot (IB) analyses in murine leukemic stem cells (LSC), MOLM-13, and THP-1 cells using anti-HOXA9 or anti-rabbit immunoglobulin (Ig)G for IP and anti-HOXA9 or anti-TWIST1 for IB. (B) GST pull-down assay was performed by using bacterially purified GST-TWIST1 and MBP-HOXA9, followed by immunoblotting. GST and MBP alone were used as negative controls, respectively. (C) Schematic of the experimental procedure for evaluation of the role of co-expression of *Twist1* and *Hoxa9* in inducing acute myeloid leukemia. (D) Kaplan-Meier curves of mice transplanted with c-kit$^+$ cells transduced with the indicated genes, and secondary recipient mice transplanted with primary leukemic bone marrow (BM) cells of the Hoxa9/Twist1 group (N=5-8). (E) Representative images of tissue morphologies. The BM, spleen, liver, and kidney tissues were stained with hematoxylin and eosin. The length of bars represents 100 μm. (F) Flow cytometric analysis of c-kit$^+$CD11b$^-$ cells of BM and spleen from the recipient mice. *$P$<0.05; ***$P$<0.001, by log-rank test (D).

overexpression alone did not initiate leukemia, consistent with the previous reports.[15,21] Fluorescence-activated cell sorting analysis of BM and spleen cells from the recipient mice showed that co-expression of *Twist1* and *Hoxa9* led to a higher proportion of c-kit$^+$CD11b$^-$ blast cells than those expressing *Hoxa9* alone (Figure 7F). In order to determine whether Twist1/Hoxa9-induced AML is transplantable, we performed secondary transplantation with leukemic whole BM cells from diseased primary mice. The secondary recipients transplanted with leukemic cells co-expressing *Twist1* and *Hoxa9* exhibited significantly shortened survival (median 27 days) than the primary ones (median 227 days) (Figure 7D), indicating a more aggressive disease. In sum, TWIST1 cooperates with HOXA9 to induce AML.

We next carried out RNA sequencing to characterize transcriptional changes upon *Hoxa9* knockdown in LSC from MLL-AF9$^+$ AML mice. A total of 324 genes were downregulated and 367 upregulated (log2 fold change >1, and $P$<0.05; *Online Supplementary Figure S13A*). GO and Reactome pathway analyses revealed that, consistent with *Twist1* deletion, the non-canonical WNT signaling and Rho GTPase signaling were also significantly negatively enriched (*Online Supplementary Figure S13B, C*), indicating that the WNT5a-RAC1 signaling pathway may be the common downstream mediator for the pathogenic effects of HOXA9 and TWIST1. These data provide further support for the collaboration between TWIST1 and HOXA9 in leukemogenesis.

# Discussion

High relapse rate leads to high mortality in patients with MLL-r AML, indicating the importance of targeted therapy for LSC. Here, our data demonstrate that TWIST1 is highly expressed in BMMNC from patients with MLL-AF9 AML, and its level in murine LSC is strikingly higher than that in normal HSC. In addition, TWIST1 plays essential roles in maintaining the survival and function of LSC, and promotes the leukemogenesis of MLL-AF9$^+$ AML. Although our previous study showed that TWIST1 preserves dormancy and self-renewal capacities of HSC,[20] *Twist1* knockout does not induce apoptosis of HSC, inconsistent with its role in LSC, suggesting that TWIST1 is much more important for LSC than HSC.

More and more modulators of EMT, a crucial process for the invasion and metastasis of epithelial tumors, have been identified as tumor promoting factors in AML.[39,40] However, because of the lack of relevance in non-EMT contexts, the specific roles of these EMT regulators in AML remain enigmas. Here, we demonstrate that TWIST1, a key EMT-inducing transcription factor, plays crucial roles in LSC maintenance, consistent with what has been observed for SNAI1, ZEB2 and AXL, three other factors regulating EMT.[39,41,42] In addition, as a TWIST1 downstream mediator in the present study, Rac1 GTPase has also been implicated in EMT trigger,[43] as well as LSC function.[44,45] It is documented that activation of the EMT program in epithelial cells induces the acquisition of stem cell properties.[19] Therefore, it appears that the relevance of EMT with stemness in solid tumors extends to hematologic malignancies, in which the EMT inducing factors promote LSC maintenance. Recently, several compounds specifically targeting EMT-associated AXL kinase in AML LSC have advanced into clinical trials.[42] Albeit caution needs to be exercised in developing therapeutic drugs targeting TWIST1, as our previous study demonstrates that, opposite to the tumor-promoting role in LSC, TWIST1 expressed in niche cells exerts anti-leukemia activities.[27] It appears that the roles of TWIST1 in hematological malignancies are cell-type dependent.

Despite the extraordinary diversity of fusion partners of MLL-r AML, the presence of common core regulators implies that targeting such factors will be broadly applicable to MLL-r leukemia. High expression of HOXA9 is an important step toward MLL-fusion-mediated leukemic transformation.[10] The ability for HOXA9 to drive leukemia development is dependent on interaction with different co-factors.[46] The most important co-factors are MEIS1 and PBX3, which mainly play supportive roles in promoting HOXA9 to activate or repress the transcription of downstream targets.[12,15] Co-expression of HOXA9 with either MEIS1 or PBX3 promotes leukemogenesis, while MEIS1 or PBX3 expression alone is insufficient to initiate HSPC transformation.[12,15] Our present study demonstrates for the first time that TWIST1 not only directly interacts with HOXA9, but also cooperates with HOXA9 to induce AML. These findings indicate that TWIST1 acts as a novel co-factor of HOXA9.

Growing evidence shows that decreased H3K9me3 accelerates AML disease progression,[47-50] but the underlying gene loci have not been well defined. Our findings demonstrate that TWIST1 is one of the related loci marked by a low level of H3K9me3 in LSC. Furthermore, we demonstrate that lysine demethylase KDM4C is required for TWIST1 expression in LSC of MLL-AF9$^+$ AML by removing H3K9me3 silencing marks on the *TWIST1* locus. KDM4C has been implicated in various malignancies, acting as an oncoprotein.[47,51] Recent reports have identified the roles of KDM4C in maintaining AML LSC,[48] and in the leukemogenesis of AML.[47] We observed that ectopic expression of *TWIST1* partially rescued the reduction in colony formation and enhancement in apoptosis of human MLL-AF9$^+$ AML cells caused by *KDM4C* knockdown (*Online Supplementary Figure S14A, B*), suggesting that the tumor-promoting activities of KDM4C are partly mediated by TWIST1. In preclinical models of MLL-r AML, KDM4C specific inhibitor SD70 can suppress the transcription and transformation ability of MLL fusions in AML cells.[47] Our findings provide new insights into the molecular mechanisms underlying its anti-leukemia activity.

In conclusion, our studies demonstrate for the first time that TWIST1 plays a critical role in LSC maintenance, and it directly interacts and collaborates with HOXA9 in induc-

ing AML in mice. Our results provide new insights into the leukemogenesis and suggest potential therapeutic target for treatment of MLL-AF9$^{+}$ AML.

**Disclosures**

*No conflicts of interest to disclose.*

**Contributions**

*XTM designed the project. XTM and NW wrote the manuscript. NW organized the experiments and performed statistical analysis of the data. NW, JY, NY, WQZ, NNG, XYL, PWZ, WLH and YQX performed experiments and analyzed data. QR provided plasmid constructs. All authors approved the final version of the manuscript.*

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**Data-sharing statement**

*All data are available upon request sent to the corresponding author.*

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