# Zinc finger and BTB domain-containing protein 46 is essential for survival and proliferation of acute myeloid leukemia cell line but dispensable for normal hematopoiesis

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## Abstract

**Background:** Zinc finger and BTB domain-containing protein 46 (*Zbtb46*) is a transcription factor identified in classical dendritic cells, and maintains dendritic cell quiescence in a steady state. *Zbtb46* has been reported to be a negative indicator of acute myeloid leukemia (AML). We found that *Zbtb46* was expressed at a relatively higher level in hematopoietic stem and progenitor cells (HSPCs) compared to mature cells, and higher in AML cells compared to normal bone marrow (BM) cells. However, the role of *Zbtb46* in HSPCs and AML cells remains unclear. Therefore, we sought to elucidate the effect of *Zbtb46* in normal hematopoiesis and AML cells.

**Methods:** We generated *Zbtb46*$^{fl/fl}$ and *Zbtb46*$^{fl/fl}$*Mx1-Cre* mice. The deletion of *Zbtb46* in *Zbtb46*$^{fl/fl}$*Mx1-Cre* mice was induced by intraperitoneal injection of double-stranded poly (I). poly (C) (poly(I:C)), and referred as *Zbtb46* cKO. After confirming the deletion of *Zbtb46*, the frequency and numbers of HSPCs and mature blood cells were analyzed by flow cytometry. Serial intraperitoneal injection of 5-fluorouracil was administrated to determine the repopulation ability of HSCs from *Zbtb46*$^{fl/fl}$ and *Zbtb46* cKO mice. The correlation between *Zbtb46* expression and prognosis was analyzed using the data from the Cancer Genome Atlas. To investigate the role of *Zbtb46* in AML cells, we knocked down the expression of *Zbtb46* in THP-1 cells using lentiviral vectors expressing small hairpin RNAs targeting *Zbtb46*. Cell proliferation rate was determined by cell count assay. Cell apoptosis and bromodeoxyuridine incorporation were determined by flow cytometry.

**Results:** The percentages and absolute numbers of HSPCs and mature blood cells were comparable in *Zbtb46* cKO mice and its *Zbtb46*$^{fl/fl}$ littermates (*Zbtb46*$^{fl/fl}$*vs. Zbtb46* cKO, HPC: 801,310 ± 84,282 *vs.* 907,202 ± 97,403, $t = 0.82$, $P = 0.46$; LSK: 86,895 ± 7802 *vs.* 102,210 ± 5025, $t = 1.65$, $P = 0.17$; HSC: 19,753 ± 3116 *vs.* 17,608 ± 3508, $t = 0.46$, $P = 0.67$). The repopulation ability of HSCs from *Zbtb46*$^{fl/fl}$*Mx1-Cre* mice was similar to those from *Zbtb46*$^{fl/fl}$ control ($P = 0.26$). *Zbtb46* had elevated expression in AML cells compared to total BM cells from normal control. Knockdown of *Zbtb46* in THP-1 cells led to a significant increase in cell apoptosis and reduced cell growth and proliferation.

**Conclusion:** Collectively, our data indicate that *Zbtb46* is essential for survival and proliferation of AML cells, but dispensable for normal hematopoiesis.

**Keywords:** Zbtb46 transcription factor; AML; Hematopoietic stem cells

## Introduction

Zinc finger and BTB domain-containing protein 46 (*Zbtb46)* is an important member of the broad complex, tram-track, bric-a-brac, and zinc finger protein family, which plays a critical role in the transcriptional regulation of genes.[1] As a transcription factor, *Zbtb46* is selectively expressed in classical dendritic cells (cDCs) and their committed progenitors and acts as a specific marker to distinguish them from the other immune cell lineages.[2-4] In quiescent cDCs, *Zbtb46* functions as a transcriptional repressor to maintain a steady-state, although it is non-essential for the development of cDCs.[4] *Zbtb46* is highly

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expressed in quiescent endothelial cells and inhibits cell proliferation through regulating cell cycle proteins as a shear-sensitive transcription factor.[5]

Emerging evidence supports the roles of the *Zbtb46* gene in many kinds of cancers. Mutations in *Zbtb46* are significantly associated with glioblastoma and astrocytoma risk.[6] In breast cancer, *RP4-583P15.10* lncRNA, which is located downstream of the natural antisense strand of the *Zbtb46* gene, is up-regulated and controls the progression of breast cancer by influencing the immune system.[7]*The zbtb46-WFDC13* fusion gene is a novel candidate in high-grade serous ovarian carcinoma.[8] In prostate cancer, *Zbtb46* is highly expressed and induces the expression of inflammatory response genes and contributes to neuroendocrine differentiation.[9,10] In leukemia stem cells (LSCs) in acute myeloid leukemia (AML), *Zbtb46* is a member of 17-biomarkers to accurately predict the prognosis and clinical outcomes of current treatments.[11]

However, the function of *Zbtb46* in normal hematopoiesis and hematological malignant cells is unclear. In the present study, we investigated the function of *Zbtb46* in normal hematopoiesis using a *Zbtb46*$^{fl/fl}$*Mx1-Cre* conditional knockout mouse model. Further, we evaluated the role of *Zbtb46* in AML cells by knocking down the expression of *Zbtb46* in THP-1 cells.

## Methods

### Ethics statement

All the animal experiments were approved by the Animal Committee of the Third Military Medical University (No. SYXK-2017-0002).

### Generation of Zbtb46 conditional knockout mice

Conditional *Zbtb46*$^{fl/fl}$*Mx1-Cre* knockout mice were generated by crossing loxP-flanked *Zbtb46* (*Zbtb46*$^{fl/fl}$) mice with *Mx1-Cre* transgenic mice. In *Zbtb46*$^{fl/fl}$*Mx1-Cre* mice, *Mx1-Cre* was induced by intraperitoneal (IP) injection of 10 μg/g of body weight of the interferon-α inducer, double-stranded poly (I).poly (C) (poly(I:C); GE Healthcare Life Sciences, Lithuania) every second day for a total of three injections. All data were obtained from mice at 4 to 8 weeks of age following poly(I:C) induction.

### Flow cytometry

Single-cell suspensions were prepared from bone marrow (BM; femurs and tibiae), spleen, and thymus. Red cells were lysed with ammonium-chloride-potassium buffer. Cells were incubated for 30 min on ice with the antibodies. The following biotin-conjugated mouse antibodies were used (all from BioLegend, San Diego, CA, USA): anti-Gr-1 (Category number, #108404), anti-Ter119 (#116204), anti-B220 (#103204), anti-CD19 (#115504), anti-IgM (#408903), anti-IL-7R (#135006), and anti-CD3 (#100304), for lineage markers in mice. The fluorochrome-conjugated antibodies used were as follows (all from Biolegend except for the ones specifically mentioned): streptavidin-PerCP-Cy5.5 (#405214), PE-anti-Sca-1 (#108108), allophycocyanin (APC)-Cy7-anti-c-Kit (#105826), PE-Cy7-anti-CD48 (#103424), and APC-anti-CD150 (#115910) for analysis of hematopoietic progenitor cells (HPCs), LSK cells (Lin$^-$Sca-1$^+$c-Kit$^+$), and hematopoietic stem cells (HSCs); streptavidin-APC-Cy7 (#405208), PE-anti-Sca-1, APC-anti-c-Kit (#105812), and PE-Cy5-anti-Flt3/CD135 (eBioscience, San Diego, CA, USA, #46-1351-82), and BV421-anti-CD34 (#152208) for analysis of lymphoid-primed multipotent progenitors (LMPPs), long term HSCs (LT-HSCs), short term HSCs (ST-HSCs), and multipotent progenitors (MPPs); streptavidin-APC-Cy7, PE-anti-Sca-1, APC-anti-c-Kit, PE-Cy7-anti-CD16/32 (#101318), and BV421-anti-CD34 for analysis of common myeloid progenitors (CMPs), granulocyte-monocyte progenitors (GMPs), megakaryocyte-erythroid progenitors (MEPs), and CD34$^-$LSK (CD34$^-$Lin$^-$Sca-1$^+$c-Kit$^+$). The antibodies used for testing the mature cell populations were as follows (all from Biolegend): PE-anti-Gr-1 (#108408) and APC-anti-MAC (#101211) for analyzing myeloid cells, APC-anti-Ter119 (#116212) and PE-anti-CD71 (#113808) for red cells, PE-anti-B220 (#103208) and APC-anti-IgM (#406509) for B cells, PE-anti-CD4 (#100408) and APC-anti-CD8a (#100712) for T cells. While to analyze apoptosis in the cell lines, a simplified staining protocol based on APC-annexin V and 4′,6-diamidino-2-phenylindole (DAPI) were used. The cells were analyzed using a BD FACSCanto (BD Bioscience, San Jose, CA, USA). All fluorescence-activated cell sorting (FACS) data were analyzed by FlowJo software, version 10 (Tree Star, Inc., San Carlos, CA, USA).

### Cell isolation

Lineage negative (Lin$^-$; Gr-1$^-$Ter119$^-$B220$^-$CD19$^-$IgM$^-$IL-7R$^-$CD3$^-$), HPC (Lin$^-$Sca-1$^-$c-Kit$^+$), LSK (Lin$^-$Sca-1$^+$c-Kit$^+$), MPP (Lin$^-$Sca-1$^+$c-Kit$^+$CD48$^+$), HSC (Lin$^-$Sca-1$^+$c-Kit$^+$CD48$^-$CD150$^+$), and myeloid (Gr-1$^+$MAC$^+$) cells were sorted from the BM of C57/B6 mice through FACS (BD FACS AriaII). B cells (B220$^+$IgM$^+$) and red cells (CD71$^-$Ter119$^+$) were sorted from the spleen of C57/B6 mice. CD8$^+$ T (CD4$^-$CD8a$^+$) and CD4$^+$ T (CD4$^+$CD8a$^-$) were sorted from the thymus of C57/B6 mice to detect T cells.

### Treatment with 5-fluorouracil (5-FU)

The cell cycle-dependent myelotoxic reagent, 5-FU, kills proliferating cells by acting as a DNA synthesis inhibitor. Administration of 5-FU (Sigma-Aldrich, Saint Louis, MO, USA) was carried out via IP injection at a dose of 150 mg/kg. The 5-FU treatments were repeated once a week for a total of 3 weeks. The survival of individuals was monitored daily and analyzed with Graphpad Prism software 6.0 (GraphPad Software, San Diego, CA, USA).

### Cell culture

Five human AML cell lines THP-1, mv4-11, Kasumi, U937, and HL-60 were obtained from the American Type Culture Collection (https://www.atcc.org/). Total BM cells were used as normal control. The AML cell lines were cultured in Roswell Park Memorial Institute-1640 medium (RPMI) containing 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin. The human embryonic kidney

cell line, 293T, was cultured in high-glucose Dulbecco modified Eagle medium (DMEM) containing 10% FBS. All the cells were maintained in a 37°C incubator with 5% $CO_2$.

### BrdU-incorporation assay

Cultured THP-1 cells were treated with the final concentration of 10 μmol/L bromodeoxyuridine (BrdU) (BD Biosciences) for 30 min before analysis. Cells incubated with BrdU were harvested and staining with fluorescein isothiocyanate (FITC)-anti-BrdU antibody according to the manufacturer's instructions provided in the FITC BrdU flow kit (BD Biosciences) and the treated cells were analyzed by flow cytometry (BD FACSCanto).

### Lentiviral constructs and packaging

To generate lentiviral vectors expressing *Zbtb46*-specific small hairpin RNAs (shRNAs), we cloned shZbtb46-1, 2, and 3 [Table 1] into a pLKO.1 vector followed by puromycin screening. The correct cloning of the shRNAs was confirmed by Sanger DNA sequencing. The 293T cells were transfected with shZbtb46-1, 2, 3, and pLKO.1-scrambled control along with the helper plasmids, psPAX2 and pMD.2G, using polyetherimide (PEI) transfection reagent (Sigma-Aldrich) for lentivirus packaging. Following co-culturing for 12 h, the medium containing PEI and vectors was replaced with fresh complete DMEM. The viral supernatants were collected at 48 and 72 h following transfection and stored at −80°C until use.

### Lentiviral infection of cells

THP-1 cells were seeded onto six-well plates and cultured with RPMI containing 10% FBS. Lentiviral stocks of shZbtb46-1, 2, 3, and pLKO.1-scrambled were added to the medium. After overnight incubation at 37°C with 5% $CO_2$, the supernatants were removed by centrifugation, and the cell pellets were washed once with cold phosphate-buffered saline and then re-suspended with fresh complete RPMI. Positive clones were selected in 3 μg/mL final concentration of puromycin for 5 days. The proliferation of shZbtb46-3 and pLKO.1-scrambled infected THP-1 cells was analyzed daily for 7 days. The cellular density was determined by manual cell counting using Counting Slides with a TC20 Automated Cell Counter (Bio-Rad, Hercules, CA, USA).

### RNA extraction and quantitative reverse transcription polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted from cells using RNAiso Plus reagent (TaKaRa, Dalian, China) and quantified by NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). The complementary DNA (cDNA) was reverse transcribed using a PrimeScript™ reverse transcription (RT) reagent Kit with genomic DNA (gDNA) Eraser (Perfect Real Time; TaKaRa). *Zbtb46* expression was determined by qRT-PCR with TB Green® Premix Ex Taq™ II (Tli RNaseH Plus; TaKaRa) using a CFX96 Touch Real-Time PCR system (Bio-Rad). All primers used for the qRT-PCR were listed in Table 1. All procedures in this study were performed according to standard molecular biology protocols or as the manufacturer's instructions.

### Statistical analysis

Two-tailed Student's *t* test was used to calculate the statistical significance of LMPP, *Zbtb46* knockdown efficiency, and cell apoptosis. Differences between two or more independent groups were analyzed using multiple *t* tests for HSPC populations. $P < 0.05$ was considered to be significantly different.

## Results

### Generation of Zbtb46 conditional knockout mice

The function of *Zbtb46* in HSCs and HPCs has not been reported previously. Therefore, to determine its role we first analyzed the expression of *Zbtb46* in hematopoietic stem and progenitor cells (HSPCs) and mature hematopoietic populations. We found that *Zbtb46* had relatively higher expression in HSPCs compared to the mature populations, including myeloid cells (Mac-1$^+$Gr-1$^+$), B cells (B220$^+$IgM$^+$), red cells (CD71$^-$Ter119$^+$), and mature T

**Table 1: Primer list used in RNA interference, genotyping, and qRT-PCR.**

| Primers | Sequences | |
|---|---|---|
| *shZbtb46-1* | CCGGGCTACTTCAAGACGCTCTACTCTCGAGAGTAGAGCGTCTTGAAGTAGCTTTTTTG | |
| *shZbtb46-2* | CCGGGATGTTTCTTCACAGCCTCTACTCGAGTAGAGGCTGTGAAGAAACATCTTTTTTG | |
| *shZbtb46-3* | CCGGGCTCATGAGTAAGAACAGCCTCTCGAGAGGCTGTTCTTACTCATGAGCTTTTTTG | |
| *pLKO.1-scrambled* | CCGGTCCTAAGGTTAAGTCGCCCTCGCTCGAGCGAGGGCGACTTAACCTTAGGATTTTTTG | |
| >q*Zbtb46* | ATCACTTCTCACTACCGGCAT | ACGTTCTTATGTGCCTTGAAGAC |
| >q*Actin* | ACCTTCTACAATGAGCTGCG | CTGGATGGCTACGTACATGG |
| >q*Zbtb46* for human | CGAGACTCAAATGCGGACCT | GTCCTTCTCTGGGGTGAGGG |
| >q*GAPDH* | ATTGACCTCAACTACATGGTTTACATG | TTGGAGGGATCTCGCTCCTGGAAG |
| >*Zbtb46*-Genotyping | TCGGTTGAGCAAGCTCGTCCAT | CATTCCTCCCTCAGTGTAAAGCTGT |
| >*Zbtb46*-deletion | TGGGAACAAAGCAACAGGGAT | CATTCCTCCCTCAGTGTAAAGCTGT |
| >*Mx1-Cre* | TTCCCAACCTCAGTACCAAGC | AACCTCATCACTCGTTGCATCGAC |

qRT-PCR: Quantitative reverse transcription-polymerase chain reaction; *Zbtb46*: Zinc finger and BTB domain-containing protein 46; *GAPDH*: Glyceraldehyde-3-phosphate dehydrogenase.

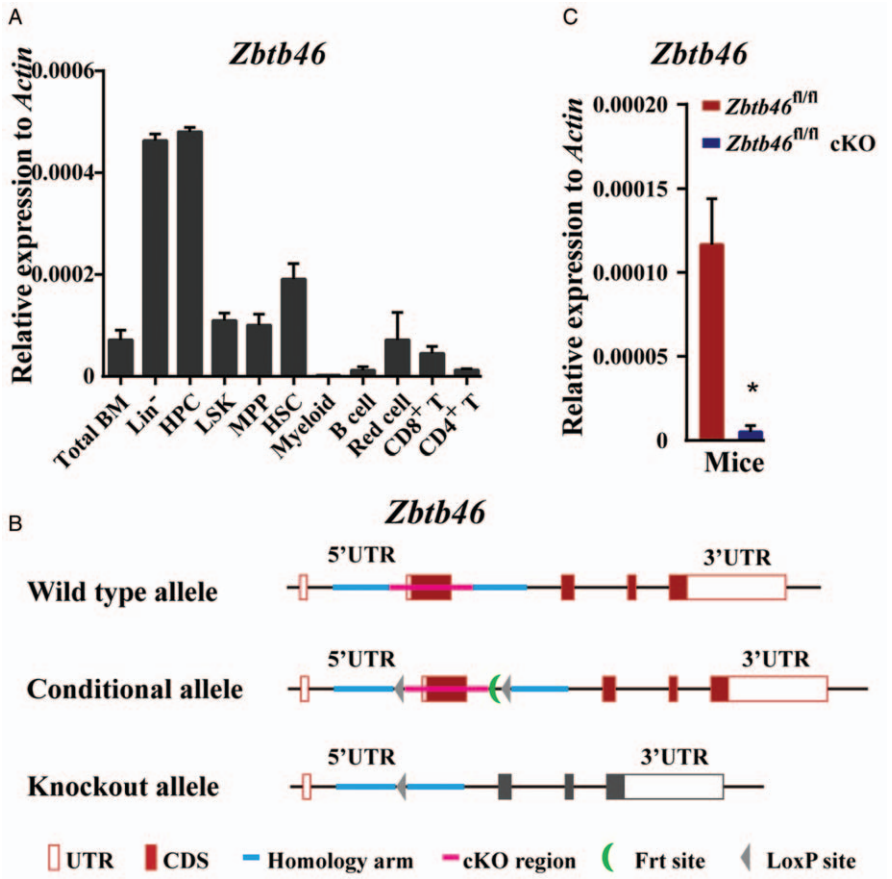


**Figure 1:** Generation of hematopoietic-specific *Zbtb46* knockout mice. (A) mRNA expression profile of *Zbtb46* in hematopoietic stem and progenitor cells (HSPCs) and mature hematopoietic populations. mRNA was isolated from the sorted cell population and analyzed by qRT-PCR. $n = 3$. (B) Schema-construction strategy of *Zbtb46* conditional knockout mice. The partial 5′-UTR and flanked exon were deleted for disrupting *Zbtb46* transcription. (C) qRT-PCR assay for the mRNA expression level of *Zbtb46* in total bone marrow cells from $Zbtb46^{fl/fl}$ and $Zbtb46^{fl/fl}$*Mx1-Cre* mice. $^{*}P < 0.001$ compared with $Zbtb46^{fl/fl}$ group. CDS: CoDing sequence; HPC: Hematopoietic progenitor cell; HSC: Hematopoietic stem cell; $Lin^-$: Lineage negative; LSK: $Lin^-c\text{-}Kit^+Sca\text{-}1^+$; MPP: Multipotent progenitors; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; UTR: Untranslated region; *Zbtb46*: Zinc finger and BTB domain containing protein 46.

cells ($CD4^+$ T and $CD8^+$ T) [Figure 1A]. This suggests that Zbtb46 has an important role in HSPCs. To evaluate the function of *Zbtb46* in the normal hematopoietic system of mice, we generated the $Zbtb46^{fl/fl}$*floxed mice* [Figure 1B], then crossed these mice with $Zbtb46^{fl/fl}$ alleles with *Mx1-Cre* mice to generate $Zbtb46^{fl/fl}$ mice and $Zbtb46^{fl/fl}$*Mx1-Cre* mice. The deletion of *Zbtb46* was induced by three IP injections of double-stranded poly (I). poly (C) (poly(I:C); 10 mg/g of body weight) every second day for a total of three injections. The high efficiency of *Zbtb46* deletion was confirmed by semiquantitative PCR analysis using genomic DNA from the BM cells of the $Zbtb46^{fl/fl}$*Mx1-Cre* mice (hereafter referred to as *Zbtb46* cKO mice) [Supplementary Figure 1A, http://links.lww.com/CM9/A240]. Accordingly, loss of *Zbtb46* mRNA was confirmed by qRT-PCR of BM cells from *Zbtb46* cKO mice [Figure 1C].

### *Zbtb46 is dispensable for normal hematopoiesis in mice*

To evaluate the *in vivo* function of *Zbtb46* in the hematopoietic system in mice, we analyzed the hematopoietic cells in the BM of $Zbtb46^{fl/fl}$ and $Zbtb46^{fl/fl}$*Mx1-Cre* mice six weeks following the poly(I:C)-induction. The total number of BM cells are comparable in the *Zbtb46* cKO mice and their $Zbtb46^{fl/fl}$ control littermates [Supplementary Figure 1B, http://links.lww.com/CM9/A240]. Flow cytometry analysis also revealed that the frequency of mature myeloid, B cells, red cells, and T cells was comparable in the BM cells, spleen and Thymus (except mature B cells in BM) from *Zbtb46* cKO and the control mice [Supplementary Figure 1C–E, http://links.lww.com/CM9/A240]. This suggests that the loss of *Zbtb46* did not affect the differentiation of mature blood cells.

In normal hematopoiesis, HSCs not only have self-renewal capacity to give rise to more identical HSCs but also possess multi-potency to differentiate into all mature blood cells.[12] Given that *Zbtb46* had relatively higher expression in HSPCs compared with mature populations, we examined these stem and progenitor cell compartments by flow cytometry. We observed that *Zbtb46* cKO mice showed similar frequencies and absolute numbers of LT-HSCs ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD48^-CD150^+$ or $Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^-Flt3^-$), $CD34^-$LSKs ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$ $CD34^-$), LSKs ($Lin^-Sca\text{-}1^+c\text{-}Kit^+$), MPPs ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^+Flt3^+$), and HPC ($Lin^-Sca\text{-}1^-c\text{-}Kit^+$) but a slight increase in ST-HSCs ($Lin^-Sca\text{-}1^+c\text{-}Kit^+CD34^+Flt3^-$) in comparison with their wild-type littermates [Figure 2A and 2B]. The frequencies

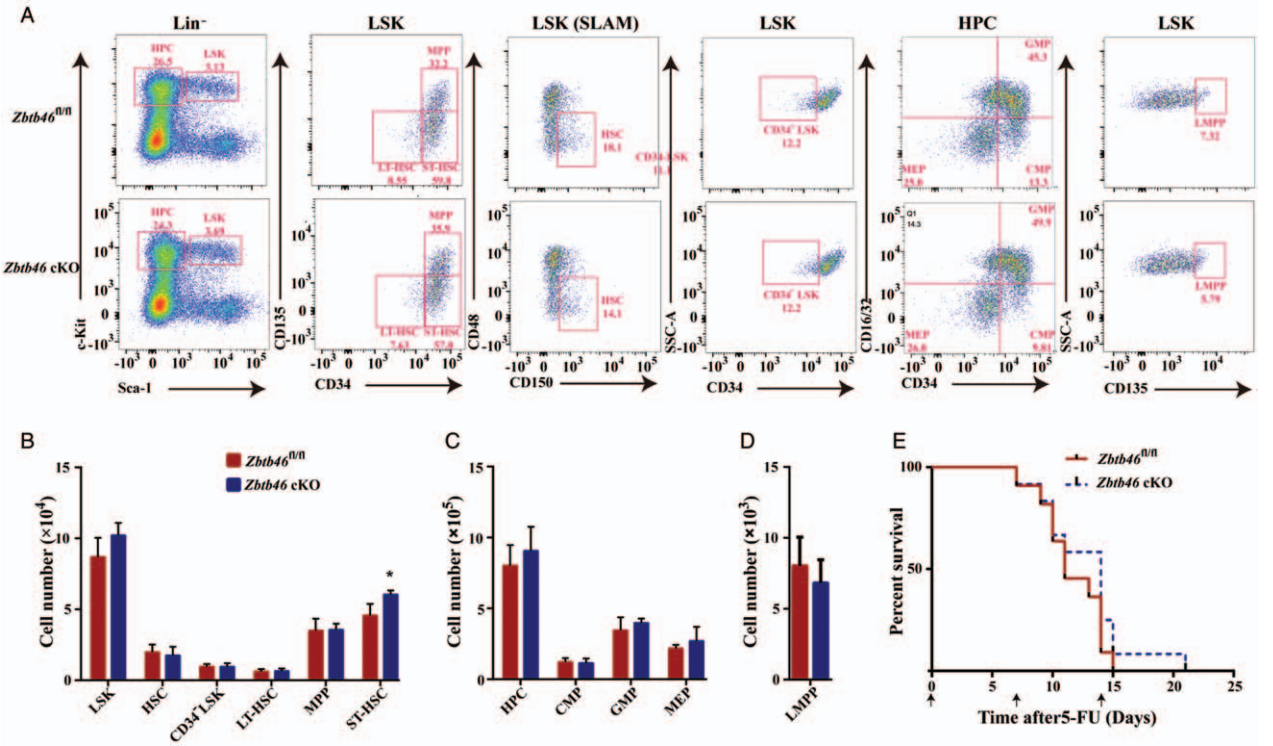


**Figure 2:** *Zbtb46* is dispensable for normal hematopoiesis determined by primary *Zbtb46*$^{fl/fl}$ and *Zbtb46*$^{fl/fl}$*Mx1-Cre* mice (referred to as *Zbtb46* cKO mice). (A) The representative flow cytometry of BM cells from *Zbtb46*$^{fl/fl}$ and *Zbtb46* cKO mice. BM cells were stained with SLAM family markers to distinguish HPC, LSK, and HSC, and Flt3/CD135 and CD34 to distinguish LT-HSC, MPP, ST-HSC, and LMPP, while CD34 and CD16/32 were used to distinguish CMP, GMP, and MEP cell populations, respectively. The percentage of each population is shown next to the outlined areas in the flow charts. All mice were induced with poly (I:C) for 1 month ($n = 3$). (B, C, and D) Total LSKs, HSCs, CD34$^-$LSKs, LT-HSCs, MPPs, ST-HSCs, HPCs, CMPs, GMPs, MEPs, and LMPPs in BM calculated by flow cytometric analysis ($n = 3$, $^*P < 0.05$). (E) Survival curve following 5-FU injection of *Zbtb46*$^{fl/fl}$ ($n = 11$) and *Zbtb46* cKO mice ($n = 12$). 5-FU: 5-Fluorouracil; CMP: Common myeloid progenitor; CD34LSK: Lin$^-$c-Kit$^+$Sca-1$^+$CD34$^-$; GMP: Granulocyte-monocyte progenitor; HPC: Hematopoietic progenitor cell; HSC: Hematopoietic stem cell; Lin$^-$: Lineage negative; LMPP: Lymphoid-primed multipotent progenitors; LSK: Lin$^-$c-Kit$^+$Sca-1$^+$; LT-HSC: Long term hematopoietic stem cell; MEP: Megakaryocyte-erythroid progenitor; MPP: Multipotent progenitors; ST-HSC: Short term hematopoietic stem cell; *Zbtb46*: Zinc finger and BTB domain containing protein 46.

and absolute numbers of the myeloid lineages including CMP, GMP, and MEP, were unaffected [Figure 2A and 2C]. LMPPs are the main source for the generation of B cells, T cells, and monocytes.[13] Our result indicated that the number of LMPP (Lin$^-$Sca-1$^+$c-Kit$^+$Flt3$^+$) declined slightly in the *Zbtb46* cKO mice although the difference was not significant [Figure 2A and 2D]. In summary, Zbtb46 conditional knockout in primary mice had no obvious phenotype in either progenitor or lineage cells.

In the hematopoietic system, 5-FU injection kills proliferating HSPCs and stimulates quiescent HSC entering the cell cycle for hematopoietic reconstitution.[14] 5-FU injection assay was performed to further explore whether the loss of *Zbtb46* affected hematopoietic reconstitution in primary *Zbtb46* cKO and *Zbtb46*$^{fl/fl}$ mice. The survival of the treated animals was monitored daily for 3 weeks. The survival curve of the 5-FU injection assay showed comparable self-renewal capacity between *Zbtb46* cKO and *Zbtb46*$^{fl/fl}$ WT mice [Figure 2E]. Together these data suggest that *Zbtb46* is dispensable for normal hematopoiesis in mice.

### High Zbtb46 expression is associated with a poor prognosis in AML

It has been reported that *Zbtb46* is a negative indicator of LSC.[11] However, the role of *Zbtb46* in AML remains unclear. To further evaluate the role of *Zbtb46* in human AML, we evaluated the expression of *Zbtb46* in AML by analyzing public databases.[15,16] We found that the *Zbtb46* gene was highly expressed in peripheral blood or BM from AML patients (542 individuals) compared to peripheral blood mononuclear cells from normal samples [74 individuals; Figure 3A]. Simultaneously, by analyzing the correlation between *Zbtb46* expression and prognosis in the AML cohort in the Cancer Genome Atlas (TCGA),[17] we found that patients with low *Zbtb46* expression had significantly better overall survival outcomes than those with high *Zbtb46* expression [$P = 0.028$; Figure 3B]. Further, we analyzed *Zbtb46* expression in human total BM cells of normal control and AML cell lines including THP-1, U937, HL-60, mv4-11, and Kasumi-1. The results showed that *Zbtb46* was highly expressed in most AML cell lines compared to the normal BM cells [Figure 3C]. Collectively, the results indicate that *Zbtb46* may play important roles in AML cells.

### Zbtb46 is essential for survival and proliferation of AML cells

To investigate the role of *Zbtb46* in the AML cells, we knock down the expression of *Zbtb46* in THP-1 cells using lentiviral vectors expressing shRNAs targeting *Zbtb46*. Three Zbtb46-specific shRNA (shZbtb46-1, 2, and 3) were cloned into the vector pLKO.1, and shZbtb46-3 was

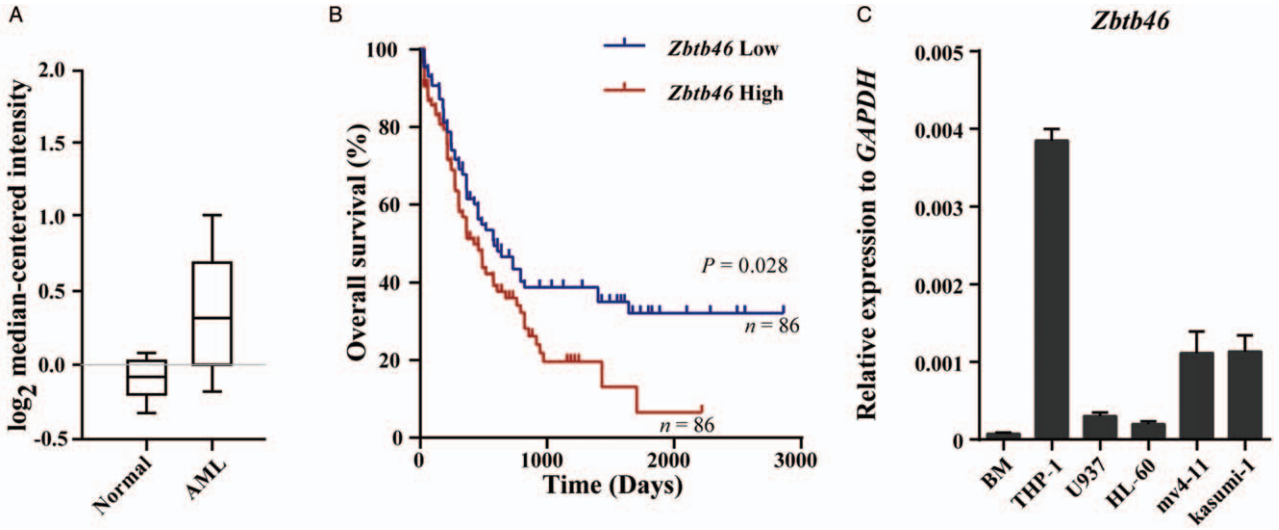


**Figure 3:** High expression of *Zbtb46* was associated with a poor prognosis in AML. (A) The expression profile of *Zbtb46* in normal (74 individuals) and AML patients (542 individuals). (B) Kaplan-Meier overall survival analysis based on *Zbtb46* mRNA levels (227329_at) from the TCGA AML cohort. $N = 172$, log-rank $P = 0.028$. (C) Expression of *Zbtb46* in classic AML cell lines and total BM-derived normal control cells determined by qRT-PCR. AML: Acute myeloid leukemia; BM: Bone marrow; *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; TCGA: The Cancer Genome Atlas; *Zbtb46*: Zinc finger and BTB domain containing protein 46.

selected for the subsequent experiments for its high-knockdown efficiency [around 70%; Figure 4A]. First, we evaluated the proliferation rate of THP-1 cells with shZbtb46-3-mediated silencing of *Zbtb46* and found that the growth rate of *Zbtb46*-silenced THP-1 was significantly reduced following the silencing of *Zbtb46* compared to pLKO.1-scrambled control [Figure 4B]. We further assessed the incorporation of the thymidine analog, BrdU, to investigate the cell cycle kinetics of *Zbtb46*-silenced THP-1 cells and corresponding control cells (THP-1 cells infected with pLKO.1-scrambled). While 32% of THP-1 cells incorporated BrdU when infected with pLKO.1-scrambled, 21% of $BrdU^+$ cells were detected in the *Zbtb46*-silenced THP-1 cells [Figure 4C and 4D], which indicated that the silencing of *Zbtb46* reduced the proliferation of THP-1 cells. We next analyzed apoptosis in *Zbtb46*-silenced THP-1 cells and its control by staining with Annexin V and DAPI. The percentage of early (Annexin $V^+$/$DAPI^-$) and late (Annexin $V^+$/$DAPI^+$) apoptotic cells were remarkably increased in the shZbtb46-3 knockdown group [Figure 4E and 4F]. These results suggest that *Zbtb46* is essential for the survival and proliferation of AML cells.

## Discussion

In this study, using a mouse model with conditional deletion of *Zbtb46*, we identified that *Zbtb46* is dispensable for the maintenance of HSPCs in a steady state. Further, we also identified that *Zbtb46* acts as an important factor in maintaining the survival and proliferation of AML cells.

Emerging evidence supports the important role of the *Zbtb46* gene in cancers.[6-8] In malignant prostate cancer, Zbtb46 has been reported as a novel tumor promoter and is essential for metastasis.[18] However, in AML, *Zbtb46* has been identified as a negative indicator of LSC, and the expression of *Zbtb46* in AML LSCs negatively correlated with the clinical prognosis. [11] In this study, we demonstrated that *Zbtb46* was highly expressed in most AML cell lines. By analyzing the correlation between *Zbtb46* expression and prognosis in the AML cohort from TCGA,[17] we found that patients with low *Zbtb46* expression had significantly better survival outcomes than those with high *Zbtb46* expression. Human AML is organized as a hierarchy initiated by LSC that give rise to progenitors, and eventually to terminally differentiated blasts. [19] Hence, *Zbtb46* may function in a context-dependent manner. In LSC, high expression of *Zbtb46* may impair their function, while in the more differentiated blasts, like in the AML cell lines, *Zbtb46* is essential for survival and proliferation of the cells. Further studies using *in vivo* mouse models of AML would enable us to develop a clearer understanding of the underlying mechanisms.

HSCs give rise to all lineages of blood cells, and the balance between their proliferation and quiescence is carefully regulated by a complex network under homeostatic or stress conditions. Many genes may have limited effects on hematopoiesis under homeostatic conditions; however, they could play important functions under stress conditions.[20] In this study, we have only identified the role of *Zbtb46* under the homeostatic condition and found that *Zbtb46* is dispensable for normal hematopoiesis in mice. Further studies are needed to clarify the role of *Zbtb46* under stress conditions.

The limitations of the study are as follows: First, we evaluated the role of *Zbtb46* only under homeostatic conditions. Further studies regarding the function of *Zbtb46* under stress conditions would make this study more complete. Secondly, when we studied the function of *Zbtb46* in THP-1 cells, we found that the silencing of *Zbtb46* impaired the survival and proliferation of THP-1 cells. However, how *Zbtb46* overexpression might affect

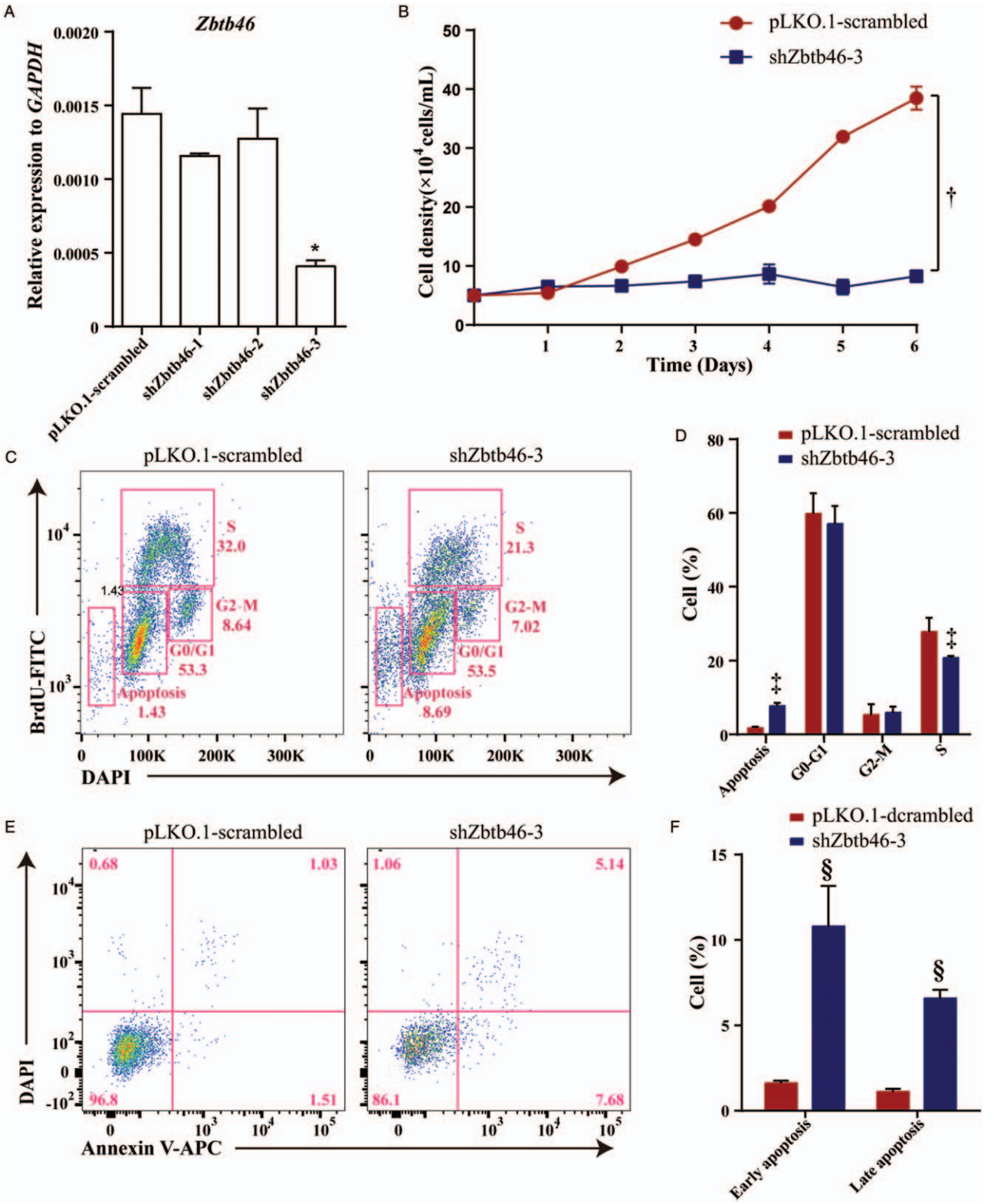


**Figure 4:** Knockdown of *Zbtb46* significantly affected the survival and proliferation of AML cells. (A) Knockdown efficiency of the *Zbtb46* gene mediated by three lentiviral vectors (shZbtb46-1, 2, 3) in THP-1 was determined by qRT-PCR and compared to pLKO.1-scrambled control. $^{*}P < 0.05$ compared to pLKO.1-scrambled. (B) The proliferation rate of the THP-1 cell line was significantly inhibited after the silencing of *Zbtb46*. $^{\dagger}P < 0.01$ compared with pLKO.1-scrambled control. (C) Representative flow cytometry of pLKO.1-scrambled and shZbtb46-3 lentiviral silenced THP-1 stained with BrdU and DAPI. (D) The histograms revealed apoptosis frequency (sub-G0/G1), G0/G1, S, and G2-M phase in pLKO.1-scrambled and shZbtb46-3 interfering THP-1. $^{\ddagger}P < 0.05$ compared with pLKO.1-scrambled control. (E and F) The percentage of apoptotic cells in the shZbtb46-3 knockdown group and control cells (THP-1 cells infected with pLKO.1-scrambled) was analyzed by flow cytometry. $^{\S}P < 0.01$ compared with pLKO.1-scrambled control. AML: Acute myeloid leukemia; APC: Allophycocyanin; BrdU: bromodeoxyuridine; DAPI: 4′,6-Diamidino-2-phenylindole; FITC: Fluorescein isothiocyanate; *GAPDH:* Glyceraldehyde-3-phosphate dehydrogenase; qRT-PCR: Quantitative reverse transcription polymerase chain reaction; *Zbtb46*: Zinc finger and BTB domain containing protein 46.

AML proliferation and apoptosis remains unclear. Further studies that explore the effect of *Zbtb46* overexpression in THP-1 cells will make our results more significant and relevant.

In this study, we demonstrated that *Zbtb46* is dispensable for normal hematopoiesis but indispensable for survival and proliferation of AML cells. This may provide us a potential target in the clinical treatment of AML.

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## Conflicts of interest

None.

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