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ORIGINAL ARTICLE

YBX1 regulates the survival of chronic myeloid leukemia stem cells by modulating m^{6}A-mediated *YWHAZ* stability

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Abstract

Purpose Chronic myeloid leukemia (CML) is a myeloproliferative disease derived from hematopoietic stem cells (HSCs) that harbor Philadelphia chromosome (Ph chromosome). In clinic, leukemia stem cells (LSCs) in CML are insensitive to the treatment with tyrosine kinase inhibitors, and are responsible for disease relapse. However, the molecular mechanisms for maintaining LSCs survival remain elusive.

Methods CML patient-derived cell lines and BCR-ABL-induced CML mouse model were used to explore the role of YBX1 in regulating the survival of CML LSCs. Bone marrow transduction and transplantation, and colony-forming unit assay were used to investigate LSC function. The underlying mechanism of how YBX1 regulates LSCs survival were assessed using flow cytometry, RNA sequencing, western blot, RNA decay assay, co-immunoprecipitation and RNA immunoprecipitation.

Results Here we show that RNA-binding protein YBX1 plays an important role in regulating survival of CML LSCs. We find that YBX1 expression is significantly increased in CML cells, and confirm that YBX1 is required for maintaining survival of LSCs. Deletion of YBX1 impairs the propagation of CML through blocking cell proliferation and inducing apoptosis of LSCs. Mechanistically, we find that YBX1 regulates expression of apoptotic associated genes. YBX1 cooperates with RNA m^{6}A reader IGF2BPs to stabilize *YWHAZ* transcript in an m^{6}A-dependent manner, and loss of YBX1 decreases *YWHAZ* expression by accelerating mRNA decay. Restoration of *YWHAZ* efficiently rescues the defects of YBX1-deficient CML cells.

Conclusion Our findings reveal a critical role of YBX1 in maintaining survival of CML LSCs, which provides a rationale for targeting YBX1 in CML treatment.

Keywords Chronic myeloid leukemia · Leukemia stem cells · YBX1 · RNA m^{6}A · YWHAZ

1 Introduction

Chronic myeloid leukemia (CML) is a clonal hematopoietic stem cell (HSC) disorder caused by the Philadelphia chromosome due to a reciprocal translocation between chromosomes 9 and 22 [1, 2]. This chromosomal translocation results in the formation of BCR-ABL oncoprotein, which has a constitutive tyrosine kinase activity and promotes the proliferation of leukemia cells via various signaling pathways (e.g. JAK-STAT, MAPK/ERK, and PI3K/Akt/mTOR) [2]. Tyrosine kinase inhibitors (TKIs) can efficiently inhibit BCR-ABL kinase activity, and have become very successful in treating patients with CML in clinic [3]. However, increasing evidence indicates that leukemia stem cells (LSCs) in CML are insensitive to TKIs treatment [4, 5]. Thus, it is generally accepted that eradication of LSCs is required for curing CML [6]. Although previous studies have uncovered some key regulators for CML LSCs maintenance [7–12], identifying the underlying mechanisms for regulating LSCs survival remains a challenge in this field.

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RNA-binding proteins (RBPs) play important roles in both transcriptional and post-transcriptional processing of RNA, such as fine-tuning gene expression, RNA splicing, polyadenylation, localization, stability, and translation [13, 14]. Dysregulation of some RBPs have been implicated in various human diseases including cancers. Recently studies also reveal the essential roles of RBPs in normal hematopoiesis and hematopoietic malignancies [15–17]. For instance, frequent mutations in genes encoding RNA splicing factors have been identified in myeloid dysplasia [18, 19]. N^6-methyladenosine (m^6A) is the most prevalent mammalian mRNA modification, and plays critical role in determining RNA fates [20, 21]. The writers, erasers, and readers of RNA m^6A have emerged as critical players in normal and malignant hematopoiesis [22–28]. Our recent

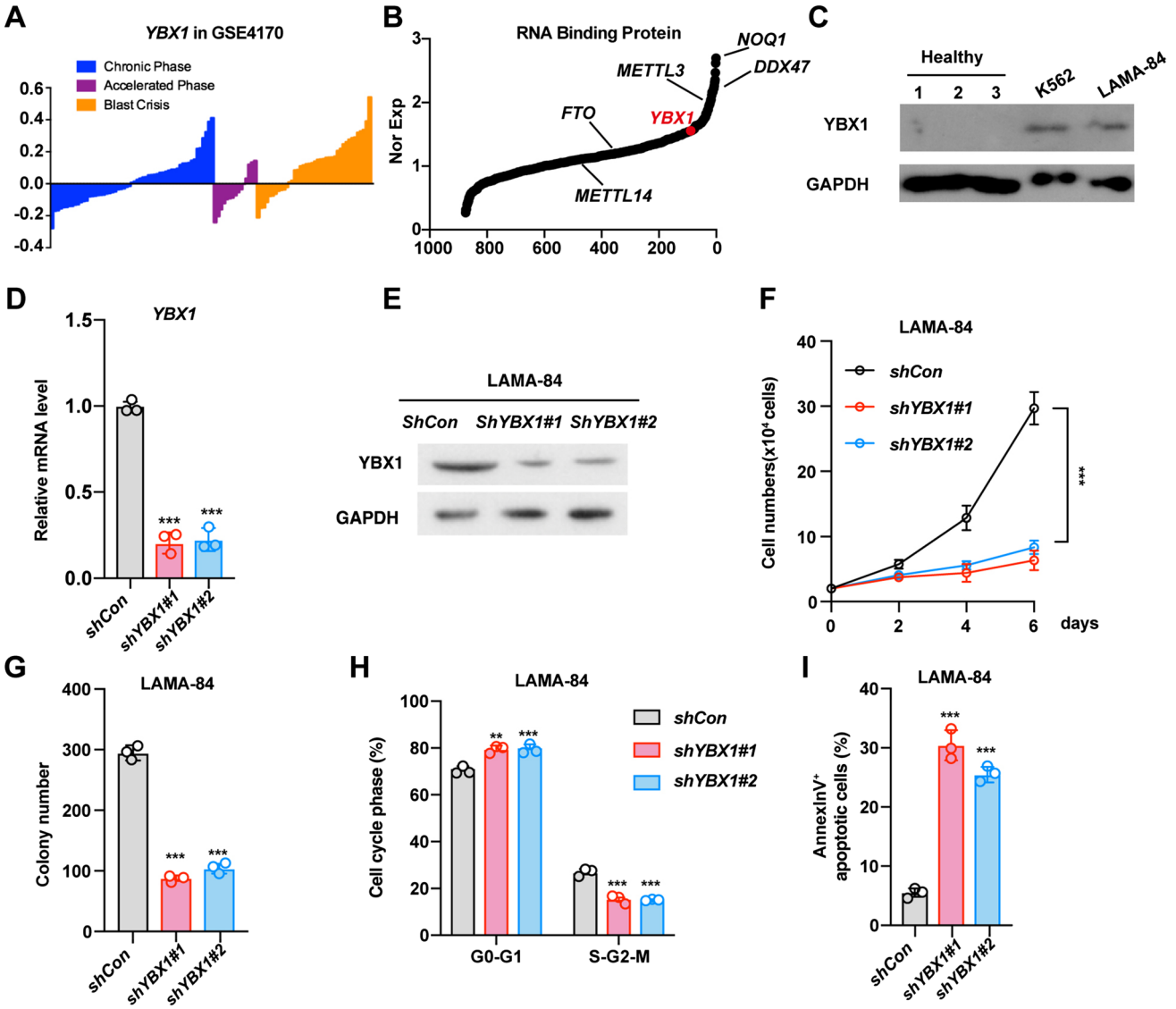


Fig. 1 Elevated expression of YBX1 is required for survival of human myeloid leukemia cells. (**A**) *YBX1* expression in human CML samples. Microarray analysis (GSE4170) showed expression of *YBX1* in $CD34^+$ the bone marrow and peripheral blood (PB) cells from 42 chronic-phase (blue), 17 accelerated (purple), and 32 blast-crisis-phase (brown) CML patients compared to normal human $CD34^+$ cells. (**B**) Expression profiling of 876 RNA-binding protein-encoding genes in CML patients. The public microarray database GSE4170 was used, and 876 RBPs were detected in this database. (**C**) Western blot analysis of YBX1 expression in various patient-derived myeloid leukemia cell lines K562 and LAMA-84; mononuclear cells derived from cord blood were used as a healthy control, and GAPDH served as the loading control. (**D**) qPCR analysis of *YBX1* expression in LAMA-84 leukemia cells after transduction with shRNA lentiviruses targeting *YBX1*. (**E**) Immunoblot showing YBX1-KD efficiency in LAMA-84 leukemia cells. (**F**) Growth curves of LAMA-84 leukemia cells after transduction with lentiviruses for *shYBX1#1*, *shYBX1#2*, or *shControl*. (**G**) Colony formation assay of LAMA-84 leukemia cells after transduction with indicated lentiviruses. (**H**) Cell cycle distribution of leukemia cells after transduction with the indicated lentiviruses. Cells were labeled with Hoechst 33342 and assessed by flow cytometry. (**I**) Percentages of apoptotic leukemia cells at day 4 after YBX1 knockdown. Error bars denote mean ± SD. Two-tailed Student's *t* test: $^*P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

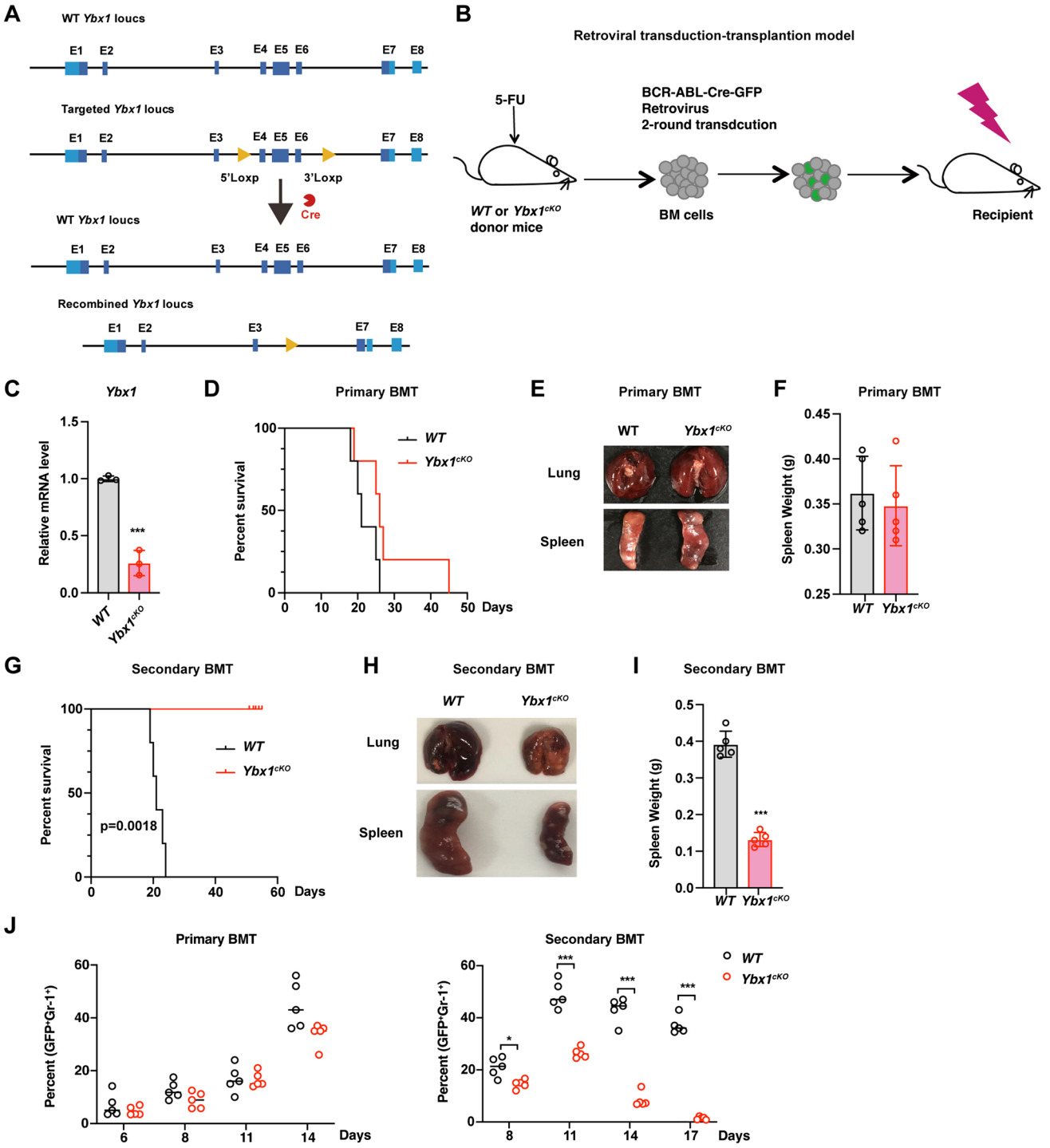
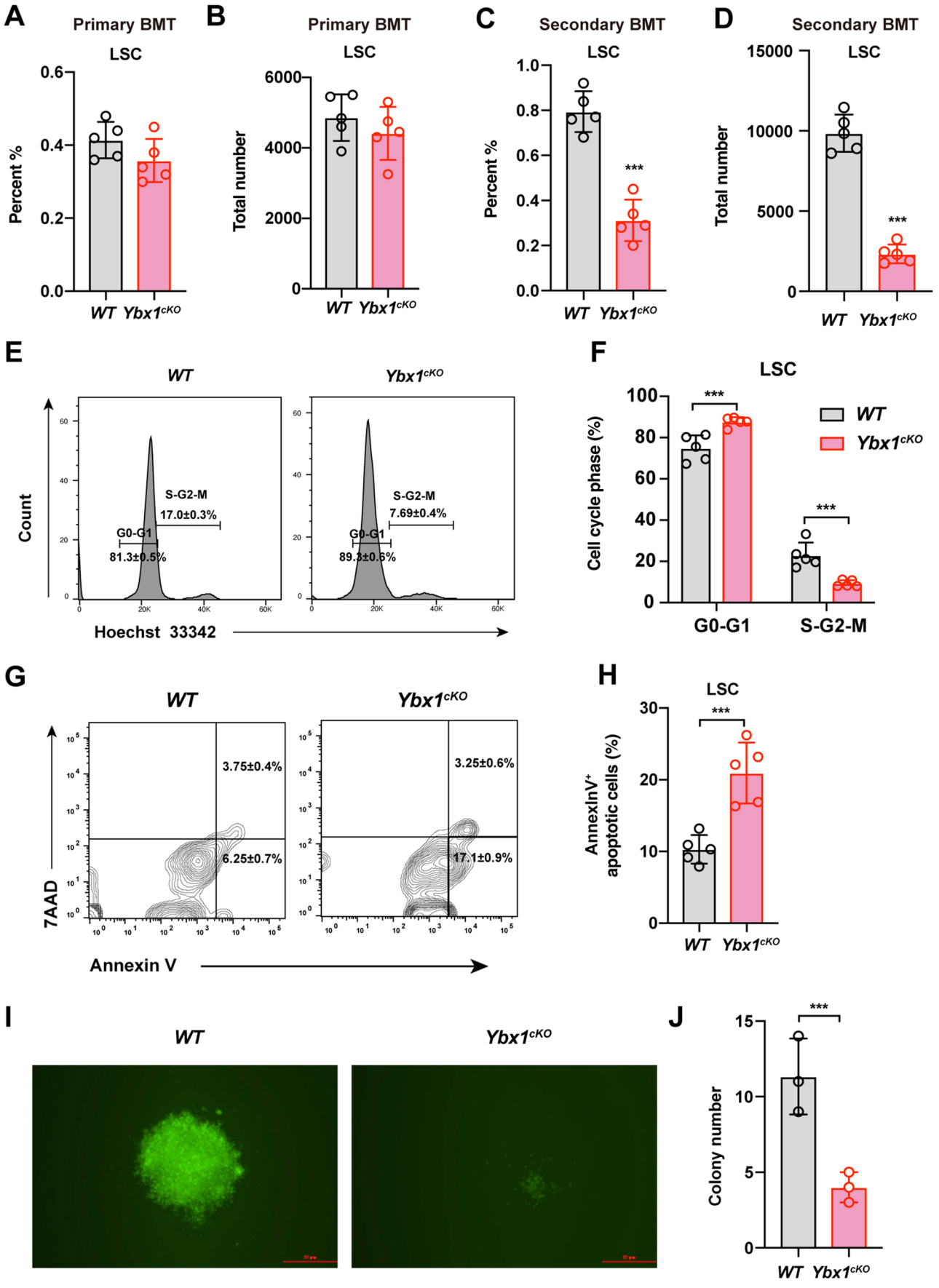


Fig. 2 Ybx1 is essential for CML development. (**A**) Scheme for generating *Ybx1* conditional knockout mouse. (**B**) Experimental scheme for establishing CML mouse model. Bone marrow cells from 5-FU (200 mg/kg) pretreated WT or *Ybx1*^{cKO} donor mice are transduced with *BCR-ABL-iCre-GFP* retrovirus twice and transplanted into lethally irradiated recipients for induction of CML. (**C**) qRT-PCR analysis of *Ybx1* expression in sorted LSCs (GFP$^+$Lin$^-$Sca-1$^+$c-Kit$^+$) from CML mice. (**D**) Kaplan–Meier survival curves for primary recipients of *BCR-ABL-iCre-GFP*-transduced BM cells from *WT* or *Ybx1*^{cKO} donor mice ($n=5$). BMT, bone marrow transplantation. (**E**) Gross appearance of the lung and spleen from primary recipients 14 days after BMT ($n=5$). (**F**) The spleen weight of primary recipients 14 days after BMT ($n=5$). (**G**) Kaplan–Meier survival curves for secondary recipients of *BCR-ABL-iCre-GFP*-transduced BM cells from *WT* or *Ybx1*$^{fl/fl}$ donor mice ($n=5$). (**H**) Gross appearance of the lung and spleen of secondary recipients 15 days after BMT ($n=5$). (**I**) The spleen weight of secondary recipients 15 days after BMT ($n=5$). (**J**) FACS analysis of GFP$^+$Gr-1$^+$ cells in PB of primary and secondary recipients of *BCR-ABL-iCre-GFP*-transduced BM cells from *WT* or *Ybx1*$^{fl/fl}$ donor mice ($n=5$). (**C-J**) Shown is 1 representative of 3 independent experiments. Error bars denote mean ± SD. Two-tailed Student's *t* test: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$


A
Primary BMT
LSC
Percent %
WT Ybx1cKO
B
Primary BMT
LSC
Total number
WT Ybx1cKO
C
Secondary BMT
LSC
Percent %
***
WT Ybx1cKO
D
Secondary BMT
LSC
Total number
***
WT Ybx1cKO
E
WT
Ybx1cKO
Count
S-G2-M
17.0±0.3%
G0-G1
81.3±0.5%
S-G2-M
7.69±0.4%
G0-G1
89.3±0.6%
Hoechst 33342
F
LSC
Cell cycle phase (%)
***
***
WT
Ybx1cKO
G0-G1
S-G2-M
G
WT
Ybx1cKO
3.75±0.4%
6.25±0.7%
3.25±0.6%
17.1±0.9%
7AAD
Annexin V
H
LSC
***
AnnexinV+ apoptotic cells (%)
WT Ybx1cKO
I
WT
Ybx1cKO
J
***
Colony number
WT Ybx1cKO

◂**Fig. 3** Ybx1 is required for maintaining LSC function. (**A-B**) The percentages (**A**) and total numbers (**B**) of LSCs (GFP$^+$ Lin-Sca-1$^+$c-Kit$^+$ cells) in BM of primary CML recipients ($n=5$). (**C-D**) The percentages (C) and total numbers (D) of LSCs (GFP$^+$ Lin-Sca-1$^+$c-Kit$^+$ cells) in BM of secondary CML recipient mice ($n=5$). (**E–F**) The cell-cycle analysis of LSCs from BM of CML mice ($n=5$). (**G-H**) The apoptosis analysis of LSCs from BM of CML mice ($n=5$). (**I-J**) Colony formation assay. LSCs were sorted and cultured in MethoCult M3434 for 7 days. Data show results from two representative experiments. Two-tailed Student's *t* test: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

findings uncover the key role of RNA m^{6}A reader, IGF2BP2, in sustaining HSCs function [29]. We also reveal that the RNA m^{6}A demethylase ALKBH5 is selectively required for maintaining the function of acute myeloid leukemia (AML) stem cells but not normal hematopoietic stem cells [30]. Despite of these recent advances, it is still necessary to explore the roles of different RBPs under various physiological and pathological hematological conditions.

YBX1 belongs to the RBP family and is a multifunctional protein containing the evolutionarily conserved cold-shock domain, which could serve as an oncoprotein functioning in cell proliferation, survival, drug resistance, and chromatin instability in human cancers. YBX1 has been implicated in various biological processes including cell proliferation, survival, drug resistance, and chromatin destabilization [31–33]. Interestingly, our recent study demonstrates that YBX1 is selectively required for maintaining AML cell survival, and deletion of YBX1 does not obviously affect normal hematopoiesis [34]. YWHAZ (also called 14–3-3ζ), an isoform of the 14–3-3 family, is a tyrosine 3-monooxygenase/tryptophan 5-monooxygenase, and involves in many oncogenic processes. YWHAZ is a molecular target and prognostic marker for many cancers [35, 36]. YWHAZ interacts with target proteins via phosphorylation motif [37] or directly regulating phosphorylation [38], and plays a key role in signaling transduction. Previous studies have discovered overexpression of YWHAZ in many cancers, including AML [39, 40], in which it promotes cancer initiation and progression by activating PI3K/AKT/mTOR signaling pathway [41]. In here, we uncover that YBX1 regulates YWHAZ expression via an m^{6}A-dependent manner and is required for maintaining the survival of LSCs in CML.

2 Material and methods

2.1 Mice

C57BL/6 J (CD45.2) background *Ybx1$^{f/f}$* mice were generated by Biocytogen. Mx1-cre mice and B6.SJL (CD45.1) were obtained from The Jackson Laboratory.

2.2 Cell line culture

Human CML cell lines (K562 and LAMA-84) were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS), 1% penicillin–streptomycin (Sigma). HEK293T were maintained in DMEM (Hyclone) with 10% FBS and 1% penicillin/streptomycin.

2.3 Bone marrow transduction and transplantation

The retroviral construct BCR-ABL-iCre-GFP carrying the BCR-ABL cDNA were used for inducing CML, and expression of iCre was used to induce deletion of Ybx1 in cells from *Ybx1$^{f/f}$* mice. Briefly, donor mice (Eight-week-old, Animal Center of Medical Research Institute at Wuhan University) were primed with 5-fluorouracil (5-FU; 200 mg/kg). The bone marrow cells from donor mice were seeded at a density of 2×10^6 /mL in DMEM with 10 ng/mL IL-3 (PeproTech), 10 ng/mL IL-6 (PeproTech), and 100 ng/mL SCF (PeproTech). After incubating at 37 °C for 24 h, the cells were transduced twice with retroviral stocks. Recipient mice were irradiated with 10 Gy and bone marrow cells were transplanted by intravenous injection. For secondary transplantation, recipient mice were irradiated with lethal dose, and then 2×10^5 bone marrow cells from primary transplantation mice were transplanted.

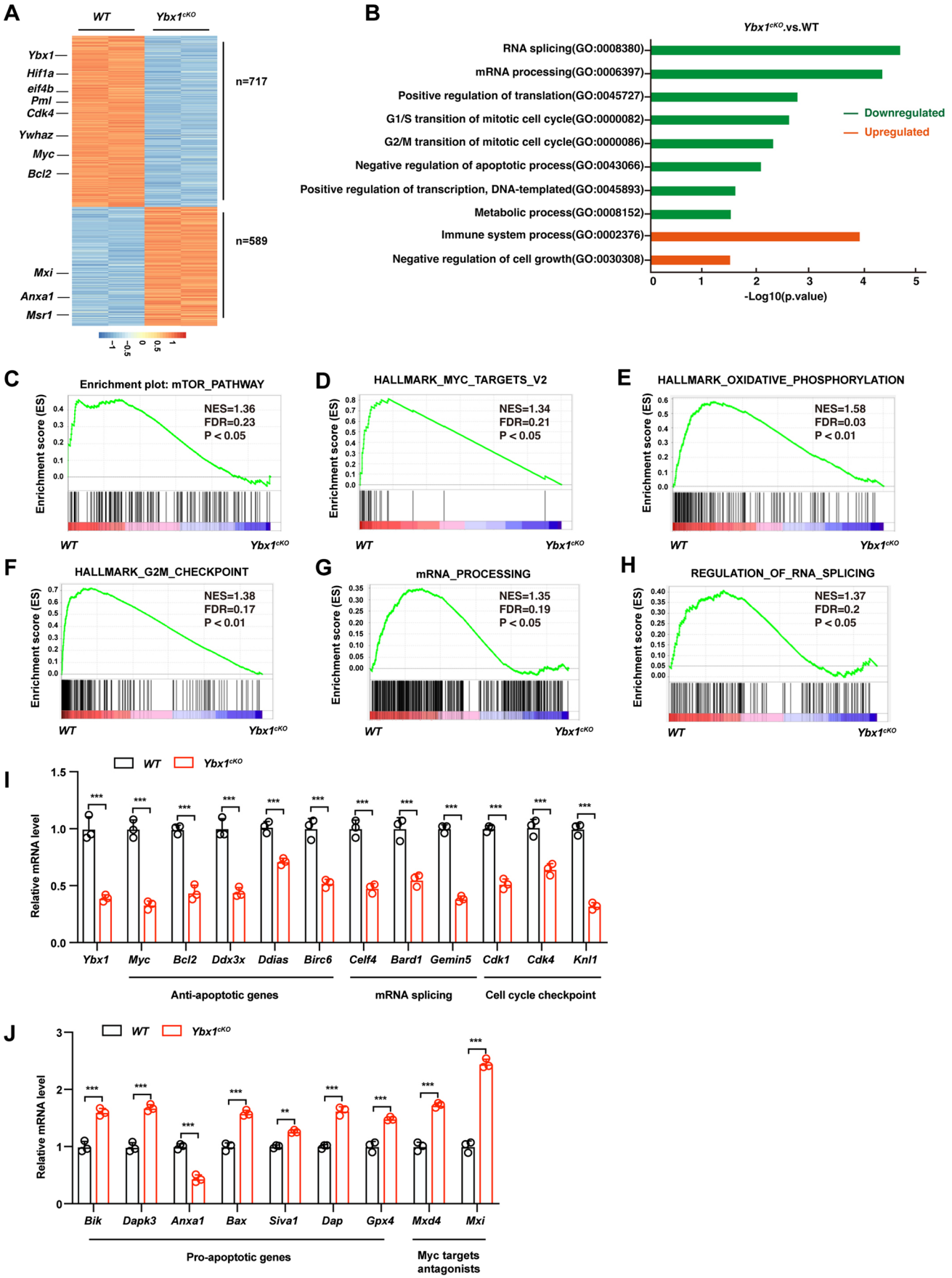
2.4 Flow cytometry analysis and cell sorting

Bone marrow (BM) and peripheral blood (PB) of CML mice were collected for FACS analysis. Cells were stained in PBS containing 2% FBS with primary antibodies (CD3, CD4, CD8, B220, Gr-1, Mac-1 and Ter119) at 4 °C for 15 min. Following PBS washing, the secondary antibody (APC-Cy7-conjugated streptavidin for recognizing biotin and PE-conjugated c-Kit and APC-conjugated Sca-1) were added at 4 °C for 15 min in the dark. The CML stem cell population (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) was analyzed by FACS. All of these antibodies were purchased from eBioscience.

2.5 Plasmid constructions and Lentiviruses transduction

shRNAs targeting human *YBX1* and *IGF2BP1* were designed and inserted into pLKO.1 according to instructions. For the overexpression of YWHAZ, BCL2 and MYC, pCDH-CMV-Blast was used by standard molecular cloning methods. The sequences were listed in Supplemental Table 1.

Lentiviruses were produced in HEK293T cells after transfecting with viral packaging constructs pMD2.G and pSPAX2. Viral supernatants were collected at 48 and 72 h after transfection. Target cells were infected with virus and 8 μg/mL polybrene, and the medium was changed 12 h after infection, and added antibiotics (2 μg/mL puromycin) 48 h after infection.


A
WT
Ybx1cKO
Ybx1
Hif1a
eif4b
Pml
Cdk4
Ywhaz
Myc
Bcl2
Mxi
Anxa1
Msr1
n=717
n=589
B
Ybx1cKO.vs.WT
RNA splicing(GO:0008380)
mRNA processing(GO:0006397)
Positive regulation of translation(GO:0045727)
G1/S transition of mitotic cell cycle(GO:0000082)
G2/M transition of mitotic cell cycle(GO:0000086)
Negative regulation of apoptotic process(GO:0043066)
Positive regulation of transcription, DNA-templated(GO:0045893)
Metabolic process(GO:0008152)
Immune system process(GO:0002376)
Negative regulation of cell growth(GO:0030308)
Downregulated
Upregulated
-Log10(p.value)
C
Enrichment plot: mTOR_PATHWAY
NES=1.36
FDR=0.23
P < 0.05
Enrichment score (ES)
D
HALLMARK_MYC_TARGETS_V2
NES=1.34
FDR=0.21
P < 0.05
E
HALLMARK_OXIDATIVE_PHOSPHORYLATION
NES=1.58
FDR=0.03
P < 0.01
F
HALLMARK_G2M_CHECKPOINT
NES=1.38
FDR=0.17
P < 0.01
G
mRNA_PROCESSING
NES=1.35
FDR=0.19
P < 0.05
H
REGULATION_OF_RNA_SPLICING
NES=1.37
FDR=0.2
P < 0.05
I
Relative mRNA level
Ybx1
Myc
Bcl2
Ddx3x
Ddias
Birc6
Celf4
Bard1
Gemin5
Cdk1
Cdk4
Knl1
Anti-apoptotic genes
mRNA splicing
Cell cycle checkpoint
J
Relative mRNA level
Bik
Dapk3
Anxa1
Bax
Siva1
Dap
Gpx4
Mxd4
Mxi
Pro-apoptotic genes
Myc targets antagonists

◂**Fig. 4** Loss of Ybx1 changes the expression of cell cycle and apoptotic associated genes in CML LSCs. (**A**) Heatmap showing differential expression of Ybx1 targets in LSCs from WT and *Ybx1*^{cKO} CML mice. (**B**) GO enrichment analysis showing the enriched terms for significantly downregulated and upregulated genes in *Ybx1*^{cKO} LSCs. (**C-H**) Gene set enrichment analysis (GSEA) plots showing enrichment of mTOR signaling pathway, MYC targets, genes for oxidative phosphorylation, G2/M checkpoint, mRNA processing, and RNA splicing in WT LSCs comparing to *Ybx1*^{cKO} LSCs. FDR, false discovery rate; NES, normalized enrichment score. **(I)** qRT-PCR analysis validating the change of *Myc*, *Ddx3x*, *Bcl2, Ddias*, *Birc6, Cefl4*, *Bard1*, *Gemin5*, *Cdk1*, *Cdk4*, and *Knl1* in *Ybx1*^{cKO} LSCs comparing to WT LSCs (**J**) qRT-PCR analysis showing the expression levels of pro-apoptotic genes (*Bik*, *Dapk3*, *Anxa1*, *Bax*, *Siva1*, *Dap*, and *Gpx4)*, Myc antagonist genes (*Mxd4* and *Mxi)* in WT and *Ybx1*^{cKO} LSCs. Two-tailed Student's *t* test: $*P<0.05$; $**P<0.01$; $***P<0.001$

2.6 Cell proliferation and colony-forming unit assay

For proliferation assay, human leukemia cells (K562 and LAMA-84) were transduced with lentivirus followed by puromycin selection. 20,000 cells were seeded into 24-well plates, counting cells every other day. For colony-forming unit assay, transduced human leukemia cells were cultured in 1.2% methylcellulose medium (100 IU/mL penicillin and 100 µg/mL streptomycin, 10% FBS) at 37 °C. Mouse LSCs ($GFP^+Lin^-Sca\text{-}1^+c\text{-}Kit^+$) cells were sorted by FACS, and plated in methylcellulose medium (Methocult GF M3434; StemCell Technologies). Colonies were counted at day 7.

2.7 Cell cycle and apoptosis analysis

To analyze cell cycle, cells were cultured with Hoechst 33342 at 37 °C for 90 min, followed by flow cytometric analysis. To analyze apoptosis, cells and Annexin V (eBioscience, 88–8005-72) were incubated at 37 °C for 30 min, and 7AAD was added before flow cytometric analysis. FlowJo software was used to analyze the results.

2.8 Quantitative real-time PCR (qRT-PCR)

Total RNA was isolated using TRIzol reagent (Takara). After DNase I treatment, reverse transcription reactions were performed using ReverTra Ace qPCR RT Kit (TOYOBO) according to the manufacturer's instructions. Relative gene expression was measured using Fast SybrGreen PCR Master Mix with a Bio-Rad real-time PCR System. All the primer sequences were showed in Supplemental Table 2.

2.9 MeRIP-qPCR

Cells were crosslinked by UV (stratalinker 1800, 400 mJ/cm^2). The nucleus was fragmented and pre-cleaned, and then incubated with protein A beads-antibody at 4 °C overnight. After washing with high salt wash buffer and RIP buffer for three times respectively, beads (Thermo Fisher Scientific) were resuspended in PBS, followed by DNA digestion at 37 °C for 15 min and proteinase K (20 mg/mL, Thermo Fisher) digestion at 55 °C for 30 min. RNAs were isolated by TRIzol, extraction and analysed by quantitative PCR. All the primer sequences were showed in Supplemental Table 2.

2.10 m^{6}A modification site prediction

The potential m^{6}A modification sites of YWHAZ were predicted by SRAMP (http://www.cuilab.cn/sramp).

2.11 Co-immunoprecipitation

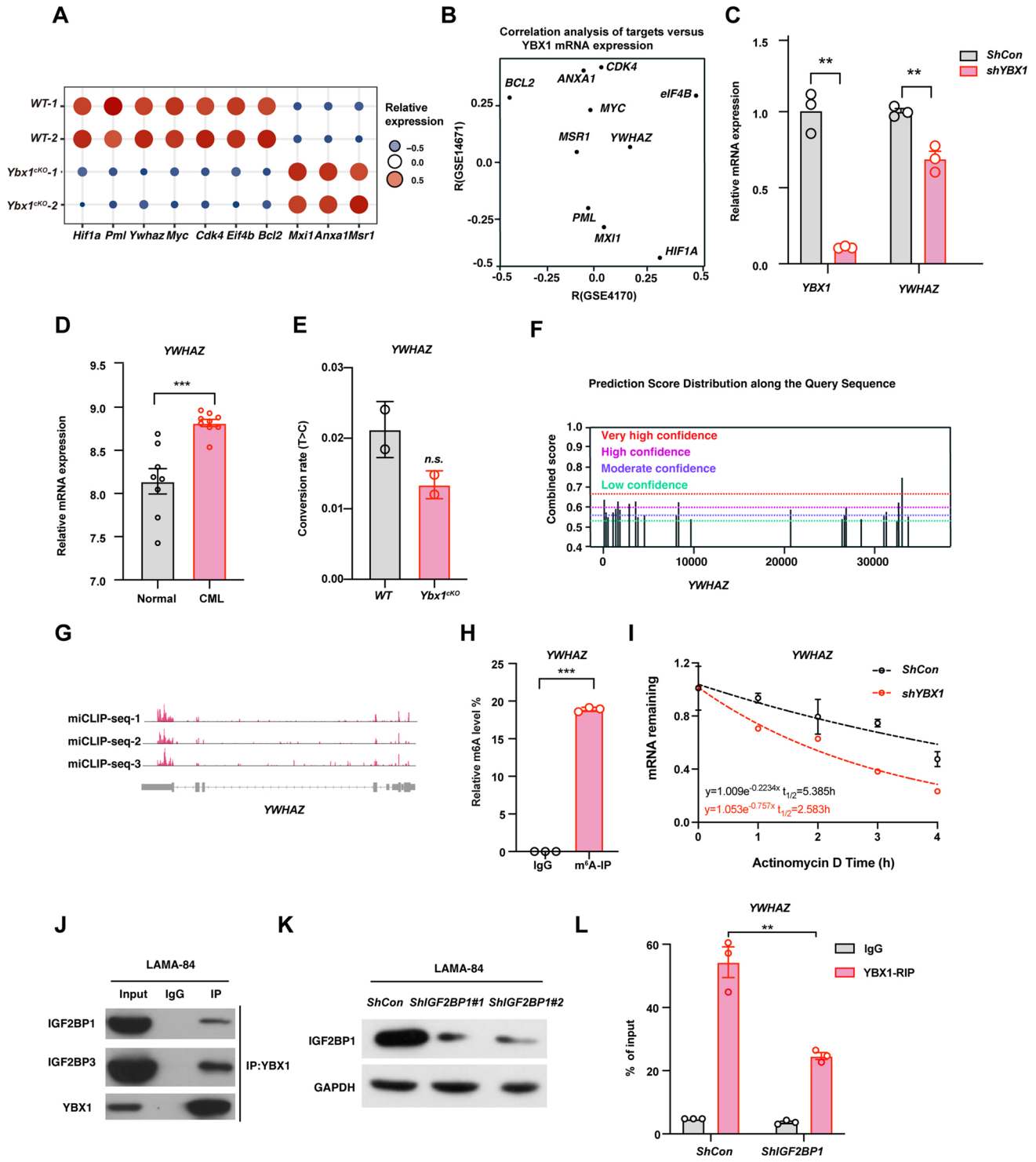
Cells transfected with plasmids were lysed with lysis buffer (20 mM Tris–HCl pH 7.4, 150 mM NaCl, 2 mM EDTA pH 8.0, 1% NP40, 1 × Protease inhibitor) on ice for 30 min and then fragmented. After centrifugation, the supernatant was incubated with anti-FLAG beads or HA antibody for 4 h at 4 °C. The immune complexes were washed three times and then boiled in 2 × SDS loading buffer for western blot detection.

2.12 Western blot analysis

Cells were lysed by use of RIPA with protease inhibitor cocktail (Roche), the total cell lysates were separated by SDS- PAGE gels. Membranes were blocked and incubated overnight at 4 °C with primary antibody, after which HRP-linked secondary antibodies were incubated for 1 h at room temperature. Antibodies used were as follows: YBX1 (Cat#ab12184, Abcam), IGF2BP1 (IMP1, clone D33A2, Cat#8482, CST), IGF2BP3 (IMP3, Cat#ab177477, Abcam), MYC (Cat#18583, CST), BCL2 (Cat#ab182858, Abcam), and GAPDH (Cat#60004–1, Proteintech) were used as loading control.

2.13 RNA decay assay

LAMA-84 cells were treated with actinomycin D (5 µg/mL) for indicated time and collected. Total RNA was extracted and analyzed by RT-PCR. GAPDH was used as endogenous control. The rate of disappearance of mRNA concentration at a given time (dC/dt) is proportional to both the rate constant for decay (Kdecay) and the cytoplasmic concentration of the mRNA (C). This relation is described by the following equation: $dC/dt = -KdecayC$. The mRNA degradation rate Kdecay was estimated by: $\ln(C/C0) = -Kdecay\ t$. To determine the half-life ($t_{1/2}$), this means 50% of the mRNA is decayed ($C/C0 = 1/2$). Substituted to the above equation got the following equation: $\ln(1/2) = -Kdecay\ t_{1/2}$. from where: $t_{1/2} = \ln 2/Kdecay$.



2.14 RNA-seq and data analysis

For RNA-seq, LSCs were sorted from *WT* and *Ybx1*^{cKO} CML mice. Total RNA was isolated. Poly(A) mRNA was subsequently purified from 1 μg total RNA using NEBNext Poly (A) mRNA Magnetic Isolation Module. The RNA-seq library was prepared with a TruSeq Sample Prep Kit v2 (Illumina), according to the manufacturer's protocol. RNA libraries were sequenced on an Illumina Hiseq X Ten platform with paired-end reads (150-bp read length). For RNA-seq analysis, reads were mapped to mouse genome version 38 (GRCm38) with Hisat2 (v 2.1.0), FeatureCounts (v 1.6.4) was used to calculate counts from bam files. DESeq2 (v 1.26.0) was employed for data normalization and differential expression analysis of RNA-seq counts.

◂**Fig. 5** YBX1 regulate YWHAZ expression in a m^{6}A-dependent manner in CML leukemia cells. (**A**) Dot plot showing the relative expression of differential genes in *WT* and *Ybx1*^{cKO} LSCs. (**B**) Expression correlation analysis of potential downstream targets versus *YBX1* in CML patients. The correlation values (R) for YBX1 vs. individual candidate were shown (y axis for GSE14671 and x axis for GSE4170). Each dot represents the R value for each paired analysis. (**C**) qRT-PCR analysis showing the downregulation of *YWHAZ* in CML leukemia cells upon Ybx1 knockdown. (**D**) The expression level of *YWHAZ* in CML patients. The public database GSE5550 including 9 CML paitents and 8 healthy controls were used. (**E**) The transcription rates of *YWHAZ* mRNA in *WT* or *Ybx1*^{cKO} leukemia cells. The public database GSE159152 was used. (**F**) The potential m^{6}A modification sites of YWHAZ were predicted by SRAMP. (**G**) IGV tracks showing the distribution of m^{6}A peaks in *YWHAZ* transcripts by analyzing the public database GSE98623. (**H**) MeRIP-qPCR analysis of m^{6}A enrichment of *YWHAZ* in LAMA-84 cells. (**I**) The mRNA half-life of *YWHAZ* in control and YBX1-KD LAMA-84 cells. (**J**) Co-IP and western blot detection of the interaction of YBX1 with IGF2BP1 and IGF2BP3. (**K**) Immunoblot for IGF2BP1 expression in LAMA-84 cells 4 days after transduction with the indicated lentiviruses (GAPDH was used as a loading control). (**L**) YBX1 RIP-qPCR analysis showing YBX1 binding to *YWHAZ* mRNA in shCon or shIGF2BP1 leukemia cells. Two-tailed Student's *t* test: $^*P<0.05$; $^{**}P<0.01$; $^{***}P<0.001$

2.15 Statistical analysis

The log-rank test was used to compare survival curves, and all the other experiments were analyzed using two-way Student's *t* test. For the comparison of different specimens, the unpaired *t* test was used. For the comparison of different treatments within the same specimen, the paired *t* test was used. *P* values of less than 0.05 were considered statistically significant. In the figures, asterisks indicate $^*p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$.

3 Results

3.1 High expression of YBX1 in human chronic myeloid leukemia cells

To investigate the role of YBX1 in CML, we first analyzed its expression level by surveying the publicly available gene expression profiling database for human bulk CD34$^+$ cells from CML patients [42]. Interestingly, higher expression of *YBX1* was observed in the majority patients with CML, especially in patients in blast crisis phase (Fig. 1A). Using the same database, we also comprehensively assessed the expression levels of the detected 876 RBP mRNAs, and found that *YBX1* mRNA level ranked among the top candidates in these RBPs (Fig. 1B). And higher expression of YBX1 was also detected at the protein level in patient-derived CML cell lines (K652 and LAMA-84), compared with healthy control cells (Fig. 1C). These data promoted us to assess YBX1 function in chronic myeloid leukemia.

3.2 YBX1 is essential for maintaining human chronic myeloid leukemia cell survival

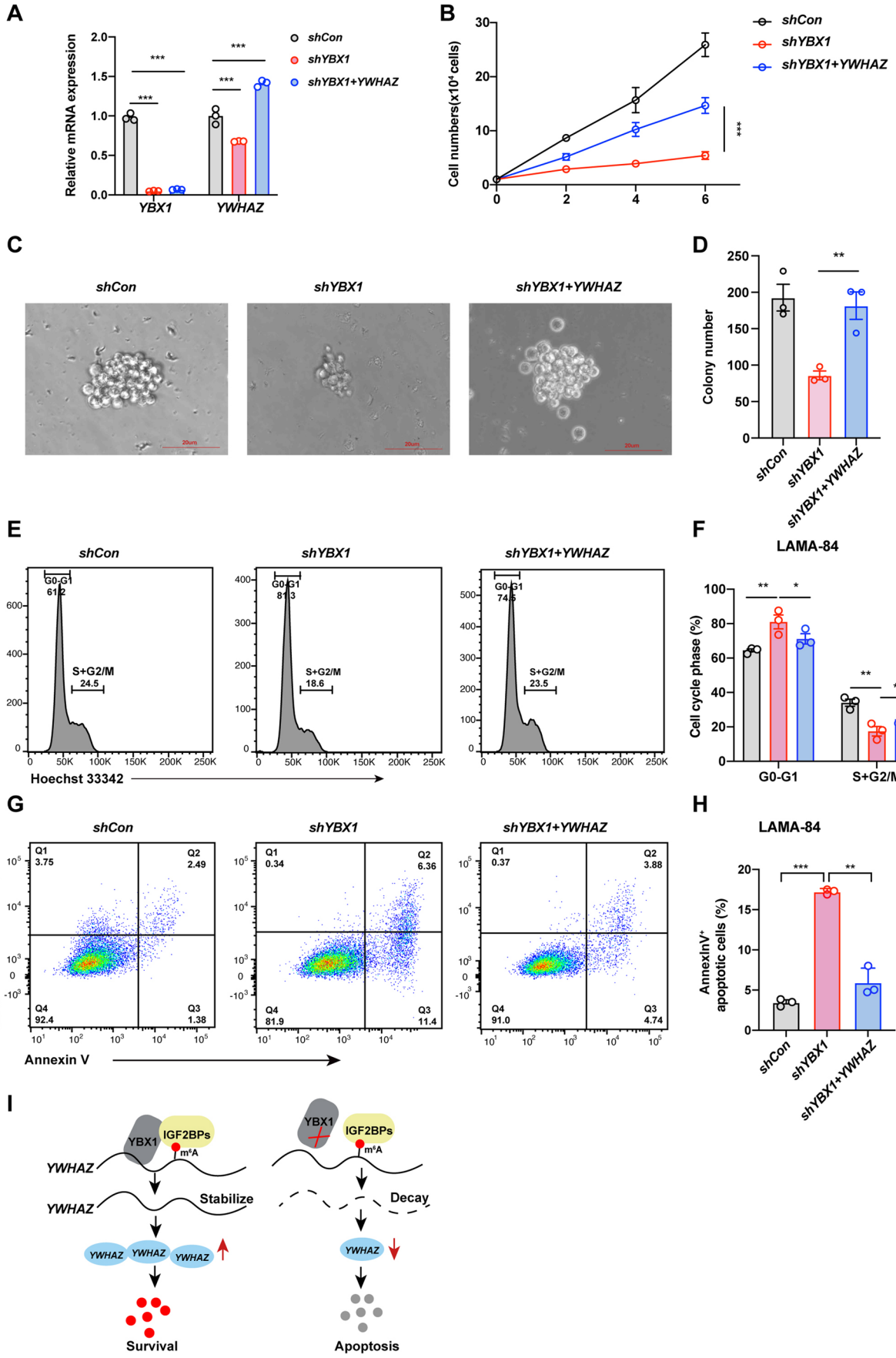
To explore the role of YBX1 in CML cells, we knocked down YBX1 using short hairpin RNAs (shRNA). Both shRNAs markedly deleted YBX1 expression (Fig. 1D, E, Fig. S1A-B). We found that knockdown of YBX1 obviously inhibited proliferation and clonogenicity of both LAMA-84 and K562 CML cells (Fig. 1F-G, Fig. S1C-E). In addition, deletion of YBX1 moderately blocked cell cycle, and significantly promoted apoptosis of CML cells (Fig. 1H-I). Together, these results indicate that YBX1 is required to maintain human CML cell survival.

3.3 Ybx1 is essential for murine CML development

To determine the role of YBX1 in CML development *in vivo*, we used conditional Ybx1 knockout mice we generated previously [34]. To simultaneously express BCR-ABL and delete Ybx1 in the same cell, we used BCR-ABL-iCre-GFP retroviral construct that carry BCR-ABL and iCre (improved Cre). BM cells from 5-FU-treated *WT* and *Ybx1*$^{fl/fl}$ mice were transduced with BCR-ABL-iCre-GFP retrovirus, and transplanted into lethally irradiated recipient mice to induce CML (Fig. 2A-B). Then we sorted CML stem cell population (GFP$^+$Lin$^-$c-Kit$^+$Sca-1$^+$) from BM and confirmed *Ybx1* deletion in LSCs (Fig. 2C). In primary transplantation, recipients of BCR-ABL-transduced *WT* or *Ybx1*^{cKO} BM cells developed and succumbed to CML within 4 weeks after BMT, and no significant difference in survival was observed between *WT* and *Ybx-1*^{cKO} CML mice (Fig. 2D). In addition, there was no significant difference in the degree of splenomegaly or lung hemorrhage between these two groups of CML mice (Fig. 2E-F). We next performed secondary transplantation assay to assess the effect of *Ybx1* on LSCs. As expected, LSCs from WT CML mice efficiently induced CML and all secondary recipient mice died within 4 weeks post-BMT, however, *Ybx1*^{cKO} LSCs failed to induce CML in the secondary recipient mice (Fig. 2G). In addition, recipients of *Ybx1*^{cKO} LSCs displayed much less severe splenomegaly and lung hemorrhage than recipients of *WT* LSCs (Fig. 2H-I). The defective CML phenotype in the absence of Ybx1 was consistent with a gradual decrease of the percentages of leukemia cells in peripheral blood (Fig. 2J). Thus, these results indicate that Ybx1 is required for CML development.

3.4 Ybx1 is essential for survival maintenance of LSCs

Given that CML is a stem cell disease, we focused on LSCs and hypothesized that the defect of CML development due to Ybx1 deficiency is caused by its inhibitory effect of on LSCs. To verify this idea, we first examined LSCs (BCR-ABL-expressing LSK cells) in primary CML mice, and did not observe significant


A
Relative mRNA expression
shCon
shYBX1
shYBX1+YWHAZ
YBX1
YWHAZ
B
Cell numbers(x10⁴ cells)
C
shCon
shYBX1
shYBX1+YWHAZ
D
Colony number
E
Count
Hoechst 33342
G0-G1
S+G2/M
F
LAMA-84
Cell cycle phase (%)
G0-G1
S+G2/M
G
7AAD
Annexin V
Q1
Q2
Q3
Q4
H
LAMA-84
AnnexinV⁺ apoptotic cells (%)
I
YBX1
IGF2BPs
m⁶A
YWHAZ
Stabilize
Decay
Survival
Apoptosis

◂**Fig. 6** YWHAZ mediates the function of YBX1 in CML cells. (**A**) RT-PCR showing the expression of *YWHAZ* in control and YBX1-KD LAMA-84 leukemia cells with or without restoration of YWHAZ. (**B**) Growth curve of LAMA-84 leukemia cells after transduction with indicated lentiviruses. (**C**) Representative images from colony formation assay for YBX1-KD LAMA-84 leukemia cells with or without YWHAZ restoration. (**D**) Colony formation assay of LAMA-84 leukemia cells after transduction with indicated lentiviruses. (**E–F**) Cell cycle distribution of leukemia cells after knocking down YBX1 with or without YWHAZ restoration. (**G-H**) Percentages of apoptotic leukemia cells after knocking down YBX1 with or without tYWHAZ restoration at day 4. (**I**) Working model. Two-tailed Student's *t* test: $*P<0.05$; $**P<0.01$; $***P<0.001$

differences in the percentages and total numbers of LSCs between *WT* and *Ybx1*^{cKO} CML mice (Fig. 3A-B), consistent with the similar survival of WT and *Ybx1*^{cKO} primary CML mice. We further analyzed LSCs in the secondary recipient mice receiving *WT* or *Ybx1*^{cKO} LSCs from primary CML mice. Two weeks after the secondary BMT, the percentages and numbers of LSCs in the BM were dramatically lower in the absence of Ybx1 (Fig. 3C-D), indicating that Ybx1 is required for maintenance of the LSC function. To understand the mechanism by which Ybx1 maintains the function of LSCs, we analyzed the effect of Ybx1 on cell cycle and apoptosis of LSCs. We found that deletion of Ybx1 resulted in an accumulation of LSCs in the G0-G1 phase, accompanied with obvious reduction in the S-G2-M phase of cell cycle (Fig. 3E-F), suggesting that Ybx1 deficiency impaired cell cycle progression of LSCs. Furthermore, we found that *Ybx1*^{cKO} LSCs showed marked higher apoptosis than *WT* LSCs (Fig. 3G-H). To investigate whether Ybx1 affects self-renewal of LSCs, we carried out an in vitro colony-forming unit assay. Sorted LSCs from mice receiving *BCR-ABL*-transduced *WT* and *Ybx1*^{cKO} BM cells were plated *in vitro*, and as expected, *Ybx1*^{cKO} LSCs gave rise to less colonies than did *WT* LSCs, indicating that loss of Ybx1 impairs self-renewal ability of LSCs (Fig. 3I-J). Together, these results suggest that Ybx1 is required for regulating the function of CML LSCs.

3.5 Ybx1 regulates expression of apoptosis related genes in CML LSCs

To comprehensively understand the underlying mechanism of Ybx1 in leukemogenesis, we performed RNA-seq assay and profiled gene expression alteration of LSCs sorted from *WT* and *Ybx1*^{cKO} CML mice. A total of 1306 genes (717 downregulated and 589 upregulated) were significantly altered upon Ybx1 deletion (Fig. 4A). In line with previous studies [34, 43], GO analysis showed that the downregulated genes in *Ybx1*^{cKO} LSCs were enriched in mRNA processing, RNA splicing regulation, positive regulation of translation, cell cycle regulation, and regulation of the intrinsic apoptotic signaling pathway (Fig. 4B). GSEA also showed also similar findings (Fig. 4C-H). Consistent with increased apoptosis in *Ybx1*^{cKO} CML cells, the expression of prosurvival genes including *Myc*, *Ddx3x*, *Bcl2, Ddias*, *Birc6, Celf4*, *Bard1*, *Gemin5*, *Cdk1*, *Cdk4*, and *Knl1* were substantially decreased in *Ybx1*^{cKO} CML cells (Fig. 4I). In contrast, deletion of Ybx1 upregulated the expression of proapoptotic genes, such as *Bik*, *Dapk3*, *Anxa1*, *Bax*, *Siva1*, *Dap*, *Gpx4*, *Mxd4* and *Mxi* (Fig. 4J). Together, our data indicate that Ybx1 is essential for regulating the survival of CML LSCs.

3.6 YWHAZ mediates the function of YBX1 in CML cells

Next, we investigated the molecular mechanism of how YBX1 regulates the survival of CML cells. Given that CML cell line LAMA-84 was established from the blood of a patient with CML in acute phase, and has morphological features of undifferentiated blast cells, we mainly used LAMA-84 to explore the mechanisms in the following experiments. Our previous study indicates that c-MYC and BCL2 are the functional downstream targets of YBX1 in AML, we also found the significant enrichment of G2/M checkpoint, MYC targets in *YBX1*^{high} LSCs (Fig. S2A-C). Interestingly, we found that knockdown of YBX1 in LAMA-84 cells obviously downregulated the expression of MYC and BCL2 at both mRNA and protein levels (Fig. S2D-F). Consistent with our previous findings in AML cells, YBX1 deletion did not significantly change global m^{6}A level in LAMA-84 cells (Fig. S2G), and found that YBX1 regulated the expression of BCL2 and MYC by modulating their mRNA stability in CML cells in an m^{6}A-dependent manner (Fig. S2H-L). We restored c-MYC and BCL2 and found that ectopic expression of c-MYC and BCL2 partially rescued the clonogenic and proliferative defects of YBX1-deficient CML cells (Fig. S3A-D). In addition, restoration of c-MYC and BCL2 also prevented the induction of apoptosis and rescued cell cycle in YBX1-deficient CML cells (Fig. S3E-G). Overall, these data indicate that c-MYC and BCL2 partially mediates the function of YBX1 in maintaining LSCs survival in CML.

We further sought to identify potential key regulators which expression is affected by YBX1. We integrated RNA-seq data for Ybx1-knockdown CML cells. Interestingly, we identified 10 differentially expressed genes upon Ybx1 deficiency (Fig. 5A). By interrogating publicly available gene expression datasets of CML patients and performing correlation analysis, we found that, among these 10 potential candidates, expression of *YWHAZ* and *EIF4B* consistently showed significant correlation with *YBX1* level in two datasets for CML patients (Fig. 5B). As the role of YWHAZ in CML remains unclear, we focused on YWHAZ for further investigation. Knockdown of YBX1 obviously downregulated *YWHAZ* expression in human CML cells (Fig. 5C). Furthermore, we observed a higher expression of *YWHAZ* in CML patients (Fig. 5D). Thus, these data suggest that YWHAZ is one of downstream targets of YBX1 in CML.

Next, we explored how YBX1 regulates the expression of YWHAZ. Interestingly, we did not detect obvious difference in the transcriptional rates of YWHAZ between *WT* and *Ybx1*^{cKO} leukemia cells utilizing a previously published SLAM-seq dataset (Fig. 5E), suggesting that YBX1 does not affect the transcription of YWHAZ. Our previous study demonstrates that YBX1 plays a key role in regulating gene expression in an m^{6}A-dependent manner[34], thus we investigated whether YBX1 regulates m^{6}A-dependent mRNA stability of YWHAZ. SRAMP predictive analysis result exhibited that m^{6}A modification sites were abundant in *YWHAZ*, indicating that *YWHAZ* was highly possible to be modified via m^{6}A methylation (Fig. 5F). The integrative genomics viewer (IGV) displayed high enrichment of m^{6}A peaks in *YWHAZ* (Fig. 5G). MeRIP-qPCR also showed higher m^{6}A enrichment in *YWHAZ* transcripts (Fig. 5H). As expected, we found that loss of YBX1 caused an obvious decrease in the half-life of *YWHAZ* mRNA in LAMA-84 cells (Fig. 5I). These findings suggest that YBX1 regulated m^{6}A-mediated YWHAZ mRNA stability. We observed an interaction of YBX1 with IGF2BP1/3 in LAMA84 cells (Fig. 5J). YBX1 RIP-PCR showed that YBX1 directly binds *YWHAZ* transcripts in LAMA-84 cells, which was impaired with knockdown of IGF2BP1 (Fig. 5K-L). Taken together, our data indicate that YBX1 stabilizes m^{6}A-tagged *YWHAZ* mRNA by cooperating with IGF2BPs in CML cells. Next, we assessed whether YWHAZ mediates the function of YBX1 in CML. As expected, YWHAZ restoration in YBX1-deficient CML cells significantly rescued the clonogenic and proliferative defect (Fig. 6A-D). Moreover, reintroduction of YWHAZ also partially rescued the cell cycle and apoptotic defects of YBX1-deficient CML cells (Fig. 6E-H). Overall, these data indicate that YWHAZ is a functional downstream target of YBX1 in CML cells.

4 Discussion

Studies on CML have brought great achievements in clinic, as it could serve as a paradigm for cancer research and therapy [1–3]. One of the major issues in current CML biology remains understanding the biology of LSCs. In here, our study reveals that RNA-binding protein YBX1 is critical for maintaining LSCs survival in CML by cooperating with IGF2BPs and regulating *YWHAZ* stability in m^{6}A-dependent manner (Fig. 6I). This study extend our knowledge about the role of YBX1 in hematologic malignancies.

Recent findings from both our and another group have shown that YBX1 is required for the development and maintenance of human and murine AML *in vitro* and *in vivo* [34, 43]. Meanwhile, genetic deletion of YBX1 had no obvious deleterious effects on normal hematopoiesis, which make it a potentially therapeutic target for myeloid leukemia therapy. However, it remains unknown whether YBX1 plays a role in CML. In this work, firstly provide the clear evidence for the role of YBX1 in CML LSCs. We demonstrated that YBX1 is upregulated in CML cells and is required for CML development through specifically regulating LSCs survival. Thus, the present study strengthens the feasibility of specifically targeting YBX1 in CML treatment.

As an RNA-binding protein, YBX1 plays essential roles in determining RNA fates, such as pre-mRNA splicing, mRNA packaging, translational regulation. In line with our previous study[34], our data indicate that YBX1 also cooperates with m^{6}A readers IGF2BPs in stabilizing m^{6}A-tagged RNA. In addition, many studies have revealed the role of RNA m^{6}A modifiers in AML, rather than CML. It should be further investigated the role of m^{6}A-tagged RNA in maintaining LSCs. Our study reveals the cross-talk between YBX1 and YWHAZ. YWHAZ overexpression has been found in multiple cancers, including AML and CML, associating with high risk of tumorigenesis and worse prognosis overall [39, 40, 44, 45]. It is reported that YWHAZ could regulate the activation of the mTOR signaling axes via interacting with TSC2 [46]. Interestingly, our findings show that Ybx1 deletion leads to the alteration of the mTOR signaling pathway in LSCs (Fig. 4C), suggesting that Ybx1 might regulated YWHAZ and then altered the activation of mTOR signaling axes in the survival of CML LSCs. Our study found that m^{6}A modification is essential in maintaining YWHAZ mRNA stability in CML cells. The exact mechanism of how YBX1 works in regulating m^{6}A-tagged RNA may be context dependent. In conclusion, this work uncovers a conserved mechanism of YBX1 in regulating survival of CML stem cells.

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Authors' contributions Contribution: J.C., Q.W., and H.Z. designed the experiments; J.C. and Q.W. contributed to the experimental plan and data interpretation; G.H. performed bioinformatic analyses with conceptual input from H.Z.; Q.W. and J.C. performed mouse experiments with help from Q.Q.; Q.W. performed growth curve and colony formation assays, qPCR, and western blotting on primary samples or cell lines with help from Y.C. and W.L.; J.C. and Y.C. performed plasmid constructions; Q.W. constructed the RNA-sequencing library; J.C. and Q.W. performed statistical analysis and wrote the manuscript. H.Z. wrote the manuscript and supervised this study.

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Data availability The datasets generated, RNA-seq is being deposited in GSE199304. This paper does not report original code. Any additional information required to access and analyze the data reported in this paper is available from the lead contact upon request.

Declarations

Ethical approval All mice were bred and maintained in Animal Center of Medical Research Institute at Wuhan University. All animal experiments were performed according to protocols approved by the Animal Care and Use Committee of Medical Research Institute, Wuhan University. This study did not use primary human subjects, thus ethical approval for human subjects is not applicable.

Competing interests The authors declare no competing financial interests.

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