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ORIGINAL RESEARCH

# Crosstalk Between FOXN3 and E2F5 Reveals a Novel Tumor Suppressive Pathway in Acute Myeloid Leukemia via MAPK Signaling: Implications for Potential Future Targeted Therapy

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**Objective:** To investigate the mechanism of FOXN3 in acute leukemia.

**Methods:** ChIP-seq experiments were performed using FOXN3-specific antibodies to identify FOXN3 target genes in acute myeloid leukemia (AML). Bioinformatics analyses were conducted to determine the enrichment of biological processes and pathways related to cell cycle regulation and apoptosis among the target genes. The transcriptional regulation of the gene of interest was confirmed through RT-qPCR, Western blotting, and luciferase reporter assays. Additionally, we examined the significance of FOXN3 on the prognosis of AML patients. Functional studies were performed following the knockdown and overexpression of the target gene in AML cells. Furthermore, we investigated the interaction between FOXN3 and the target gene by co-transfecting AML cells with lentiviruses overexpressing the target gene, followed by examinations of downstream signaling pathways through RNA-seq and pathway enrichment analyses.

**Results:** FOXN3 regulates E2F5 expression, leading to decreased mRNA and protein levels of E2F5 upon FOXN3 overexpression, though additional factors may contribute to this repression. Notably, E2F5 expression was elevated in AML patients and cell lines, correlating with unfavorable clinical outcomes. Functional investigations revealed that E2F5 functions as an oncogene in AML, promoting cell proliferation, inhibiting apoptosis, and influencing cell cycle progression. Co-transfection experiments demonstrated that E2F5 could counteract the proliferation-inhibitory effect of FOXN3. Additionally, FOXN3 was found to modulate the MAPK signaling pathway and its downstream target, EZH2.

**Conclusion:** This study reveals a novel regulatory axis involving FOXN3 and E2F5 in AML. FOXN3 acts as a tumor suppressor by regulating E2F5 and modulating downstream MAPK signaling pathways.

**Keywords:** forkhead box N3, E2F5, acute myeloid leukemia, mechanism, MAPK, sequencing, survival

## Introduction

Acute myeloid leukemia (AML) is the most common type of adult leukemia and is characterized by chromosomal abnormalities, recurrent gene mutations, and epigenetic alterations. These factors result in unchecked proliferation, impaired differentiation, and reduced apoptosis of leukemic cells.[1,2] Despite significant advancements in targeted therapies, includingBcl-2, FLT3, and IDH1/2 inhibitors,[3,4] challenges such as resistance and relapse continue to hinder successful treatment outcomes.

In previous studies,[5–7] we identified Forkhead box N3 (FOXN3) as a novel tumor suppressor in AML. Low expression of FOXN3 is associated with advanced age, elevated white blood cell count, lower complete remission rates, and shorter overall survival. Functionally, overexpression of FOXN3 inhibits AML cell proliferation and induces apoptosis.[6,7] However, the precise molecular mechanisms underlying FOXN3's role in leukemogenesis remain largely unclear. As a forkhead

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transcription factor, FOXN3 can recognize two distinct motifs, as demonstrated by the unique co-crystal structures within its DNA-binding domain.[8] Interestingly, in chronic lymphocytic leukemia, truncated forms of FOXN3, which lack essential tumor suppressor regions, have been shown to increase in expression due to intron polyadenylation, thereby suppressing its tumor suppressor activity,[9] thereby suggesting that FOXN3 may contribute to leukemogenesis by binding to specific target genes, likely oncogenes implicated in AML.

Furthermore, FOXN3 has been observed to be downregulated in various tumors, including Hodgkin lymphoma, ovarian cancer,[10] pancreatic cancer,[11] thyroid cancer,[12] and others, indicating its broader role as a tumor suppressor.[13] The FOX gene family, which includes FOXN3, is implicated in carcinogenesis through mechanisms such as gene amplification and chromosomal translocation.[14] Deregulation of FOX genes can lead to congenital disorders, diabetes mellitus, and various cancers. Thus, current research highlights FOXN3 as an essential tumor suppressor gene, with its downregulation in AML and other malignancies suggesting its potential as a therapeutic target. Further investigation into FOXN3 could yield valuable insights into novel therapeutic strategies for multiple diseases.

This study aimed to elucidate the regulatory role of FOXN3 in AML by identifying its key transcriptional targets and investigating their functional impact on leukemogenesis.

# Materials and Methods

## Samples and Cell Culture

The study protocol was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (CMU). Bone marrow samples were obtained from 66 newly diagnosed AML patients and 18 healthy donors who received treatment or underwent physical examinations at CMU. AML cell lines, comprising THP1, Kasumi1, K562, and 293T, were purchased from the Haematology Institute of the First Affiliated Hospital of CMU. And the Ethics Committee of hospital approval this experimental.

The THP1 and K562 cell lines were cultured in RPMI 1640 medium (BI, Israel) supplemented with 10% fetal bovine serum (FBS; Clark, USA) and 1% penicillin/streptomycin (HyClone, Logan, USA). Kasumi1 cells were cultured in RPMI 1640 medium with 20% FBS and 1% penicillin/streptomycin. The 293T cells were cultured in DMEM (BI, Israel) with 10% FBS and 1% penicillin/streptomycin. All cell lines were maintained at 37°C in a humidified atmosphere with 5% $CO_2$.

## Transfection

Lentiviruses carrying E2F5 shRNA and negative scrambled shRNA, as well as the E2F5 and FOXN3 overexpression plasmids, along with empty plasmids, were synthesized and provided by Wableibio (Shenyang, China). Detailed sequences are provided in Supplementary Table S1. AML cells THP1 and Kasumi1 were seeded in 6-well plates at a density of $2\times10^5$ cells/well and subsequently subjected to lentivirus infection. The transfection efficiencies were then validated through quantitative real-time polymerase chain reaction (RT-qPCR) and Western blot analysis.

## RNA Isolation and RT-qPCR Analysis

Based on the top 100 enriched peaks with the smallest p-values from the ChIP-seq data, we selected candidate genes for validation through an integrative approach that incorporated publicly available datasets, pathway analyses, UCSC Genome Browser (https://genome.ucsc.edu/) searches to examine FOXN3-binding regions in cell cycle and apoptosis pathways, and gene annotations from the Atlas of Genetics and Cytogenetics in Oncology and Haematology (https://atlasgeneticsoncology.org/).Based on these approaches, we identified ISG15, FLOT1, INPP5F, CCNT2, AKT3, SHC4, CDC42, YWHAZ, CDK1, NMNAT2, HDAC1, PML, BMP4, HDAC9, PIM2, E2F5, FTL, SHC3, MAPK8, B3GALT4, RASGRF2, PD4ED and EFNA4 as the most promising candidate genes, which were subsequently validated through RT-qPCR following FOXN3 overexpression in AML cells.

The bone marrow mononuclear cells were obtained from samples using Ficoll-Paque™ PLUS (GE Healthcare, Uppsala, Sweden). Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA, USA), and 1 μg of RNA was reverse-transcribed into cDNA using the PrimeScript™ RT Reagent Kit with gDNA Eraser (TaKaRa, Dalian, China). RT-

qPCR was performed using SYBR Green technology on an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA), following previously described protocols.[6,7] The amplification conditions were as follows: initial denaturation at 95°C for 15 minutes, followed by 40 cycles of denaturation at 95°C for 10 seconds, annealing at 58°C for 25 seconds, and extension at 72°C for 15 seconds. Relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, with β-actin as the reference control. The primer sequences are provided in Supplementary Table S2.

## Protein Extraction and Western Blot

Cellular proteins were extracted using radioimmunoprecipitation assay (RIPA) buffer containing phosphatase inhibitors. Protein concentrations were determined using a bicinchoninic acid (BCA) protein assay kit (Beyotime, Shanghai, China). Then, 40 μg of proteins were separated on 10%-12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, MA, USA). The membranes were blocked with 5% non-fat milk for 1 hour at room temperature, followed by overnight incubation at 4°C with primary antibodies. Afterward, the blots were washed with Tris-buffered saline with Tween-20 (TBST) and incubated with secondary antibodies (1:10,000; Jackson, USA) for 1 hour at room temperature. Protein bands were visualized using an enhanced chemiluminescence system (Millipore) and captured with the Image Quant LAS-4000 image acquisition system (FujiFilm, Wuhan, China).

The primary antibodies used were as follows: FOXN3 (ab129453, 1:2500, Abcam, UK), E2F5 (A16042, 1:500, ABclonal, China), CDC42 (2466T, 1:1000, Cell Signaling Technology, MA), HDAC1 (34589T, 1:1000, Cell Signaling Technology, MA), PCNA (WL03213, 1:500, Wanleibio, China), CyclinE1 (WL01072, 1:1000, Wanleibio, China), CDK2 (WL01543, 1:500, Wanleibio, China), CyclinD1 (WL01435a, 1:400, Wanleibio, China), CDK6 (WL03460, 1:500, Wanleibio, China), Bcl-2 (WL01556, 1:1000, Wanleibio, China), EZH2 (WL00795, 1:1000, Wanleibio, China), p-ERK (WLP1512, 1:1000, Wanleibio, China), ERK (WL01864, 1:1000, Wanleibio, China), p-38 (WL00764, 1:1000, Wanleibio, China), and GAPDH (5174, 1:1000, Cell Signaling Technology, MA).

## Cell Proliferation Assays

Cell proliferation was assessed using Cell Counting Kit-8 (CCK-8, WLA074b) assays. Briefly, the cells were plated in 96-well plates at a density of 3000 cells per well in 100 μL of culture medium. Following incubation for 24, 48, 72, and 96 hours, 10 μL of CCK-8 reagent was added to each well and incubated for an additional hour. Absorbance was then measured at 450 nm using a microplate reader (Biotek, Winooski, VT).

## Flow Cytometry

The transfected cells were harvested and washed twice with phosphate-buffered saline (PBS). For cell apoptosis analysis, the collected cells were suspended in 500 μL of binding buffer and incubated with 5 μL of Annexin V-PE and 10 μL of 7-AAD at room temperature in the dark for 15 minutes. For cell cycle analysis, the collected cells were fixed in 70% cold ethanol at 4°C for 2 hours or overnight. Subsequently, they were resuspended in 500 μL of staining buffer and incubated with 25 μL of propidium iodide (PI) and 10 μL of RNase A at 37°C in the dark for 30 minutes. Flow cytometry (BD Biosciences, Franklin Lakes, NJ) was performed to assess cell cycle and cell apoptosis following the manufacturer's instructions.

## Chromatin Immunoprecipitation-Sequencing (ChIP-Seq) and ChIP-PCR

Approximately $2\times10^7$ THP1 and Kasumi1 cells were cross-linked with 1% formaldehyde for 15 minutes at room temperature and quenched with 125mM glycine. The ChIP assay was conducted using the One-Day Chromatin Immunoprecipitation Kit (Millipore, USA) following the manufacturer's protocol and 5μg of a specific antibody against FOXN3 (711585, Invitrogen, USA). ChIP-seq analysis was performed using the Illumina Sequencing Platform by KangChen Bio-tech Inc. Corporations (Shanghai, China). To confirm FOXN3 binding to the E2F5 promoter, the precipitated DNA samples were subjected to RT-qPCR amplification using primers designed for the E2F5 promoter region, with SIRT6 as a positive control. The ChIP-PCR primer sequences were as follows: E2F5 forward, 5′-AGGCAGCATAAGTTGAGTGTT-3′; E2F5 reverse, 5′-CTTACCTATGAATG ATAACCCTG-3′; SIRT6 forward, 5′-ATGAGCCGAGGGAGGAGACA-3′; SIRT6 reverse, 5′-ATGATAGGGAA GATCGTGGGTG-3′; and GAPDH forward, 5′-CCACAGTCCAGTCCTGGGAACC-3′, and reverse, 5′-GAGCTA CGTGCGCCCGTAAAA-3′.

## Dual-Luciferase Reporter Gene Assay

The E2F5 promoter construct was generated by amplifying the 2000 bp upstream region from the transcription start site of E2F5, based on the NCBI reference sequence (NC_000008.11:85,175,154–85177153, Homo sapiens chromosome 8, GRCh38.p13 Primary Assembly). The amplified sequence was cloned into the pGL3-Basic luciferase reporter vector using the NheI/HindIII restriction sites. Human 293T cells were seeded into 12-well plates and transfected with 1 μg of either the empty vector or pcDNA3.1-FOXN3, along with 1 μg of either the pGL3-basic plasmid or the pGL3-E2F5-promoter plasmid. Transfection was performed using Lipofectamine 3000 Reagent (Invitrogen, USA) in accordance with the manufacturer's instructions. Forty-eight hours post-transfection, reporter gene assays were conducted using the Dual-Luciferase Reporter Gene Assay Kit (KeyGEN Bio TECH, China), following the manufacturer's guidelines.

## RNA-Sequencing

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA) and reverse-transcribed to create the cDNA by SuperScript™ II Reverse Transcriptase (Invitrogen, cat. 1896649, USA) following the manufacturer's protocol. The average insert size for the final cDNA library was 300 ± 50 bp. We performed the 2×150 bp paired-end sequencing (PE150) on an Illumina Novaseq™ 6000 following the vendor's recommended protocol, and data analysis was performed by LC-Bio Technology Co., Ltd. (Hangzhou, China). Differentially expressed mRNAs were identified based on a fold change of >2 or <0.5, coupled with a p-value < 0.05. Subsequently, Gene Ontology (GO) enrichment and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were conducted on the differentially expressed mRNAs.

## GO Enrichment Analyses and KEGG Pathway Analysis

GO enrichment analyses for FOXN3 were conducted, along with Gene Set Enrichment Analysis (GSEA), to identify associated phenotypes and signaling pathways. The analyses utilized Hallmark v7.2, GO c5 v7.2, and KEGG c2 v7.2 gene sets to identify relevant mechanistic pathways. These analyses were performed using the clusterProfiler R package (version 3.14.3; https://bioconductor.org/packages/release/bioc/html/clusterProfiler.html). To ensure robustness, only terms with a P-value< 0.05 were deemed significant in the pathway enrichment analysis. Additionally, the fold change cut-off criterion was set to $|\log2FoldChange| \geq 1$ to enhance the identification of differentially expressed genes.

## Statistical Analysis

Statistical analyses were performed using GraphPad Prism 7.0a software, with all data expressed as mean ± standard deviation (SD). Differences between groups were evaluated using the Mann–Whitney test for two-group comparisons or one-way analysis of variance (ANOVA), followed by Tukey's post hoc test for multiple group comparisons. For cell proliferation assays, a two-way ANOVA followed by Bonferroni's post hoc test was applied to compare data across multiple time points. A *P*-value of less than 0.05 was considered statistically significant. Significance levels are denoted as *$P < 0.05$,** $P < 0.01$, *** $P < 0.001$, and **** $P < 0.0001$.

# Results

## Identification of E2F5 as the Transcriptional Target Gene of FOXN3

The analysis revealed a total of 205,115 FOXN3 binding peaks in THP1 cells, distributed as follows: 2.49% in promoters, 1.47% in exons, 33.09% in introns, and 11.09% in regions upstream of promoters (region 2 kilobases (kb) upstream of the transcription start site) (Figure 1A). In Kasumi1 cells, FOXN3 binding peaks were distributed as follows: 3.46% in promoters, 1.79% in exons, 30.34% in introns, and 11.93% in upstream regions (Figure 1A). Furthermore, by mapping each binding peak to the nearest gene within 5 kb upstream and 5 kb downstream of the transcription start site, 5719 potential FOXN3 target genes (including both protein-coding and non-coding genes) were identified. The genomic occupancy profile of FOXN3 binding sites showed a strong enrichment around transcription start sites (TSS) (Figure 1B), highlighting the potential role of FOXN3 in transcriptional regulation.

Gene Ontology (GO) and KEGG pathway enrichment analyses revealed that FOXN3 regulates biological processes including cell cycle arrest, apoptosis, development, and stress response (Table 1), as well as pathways such as cell cycle,

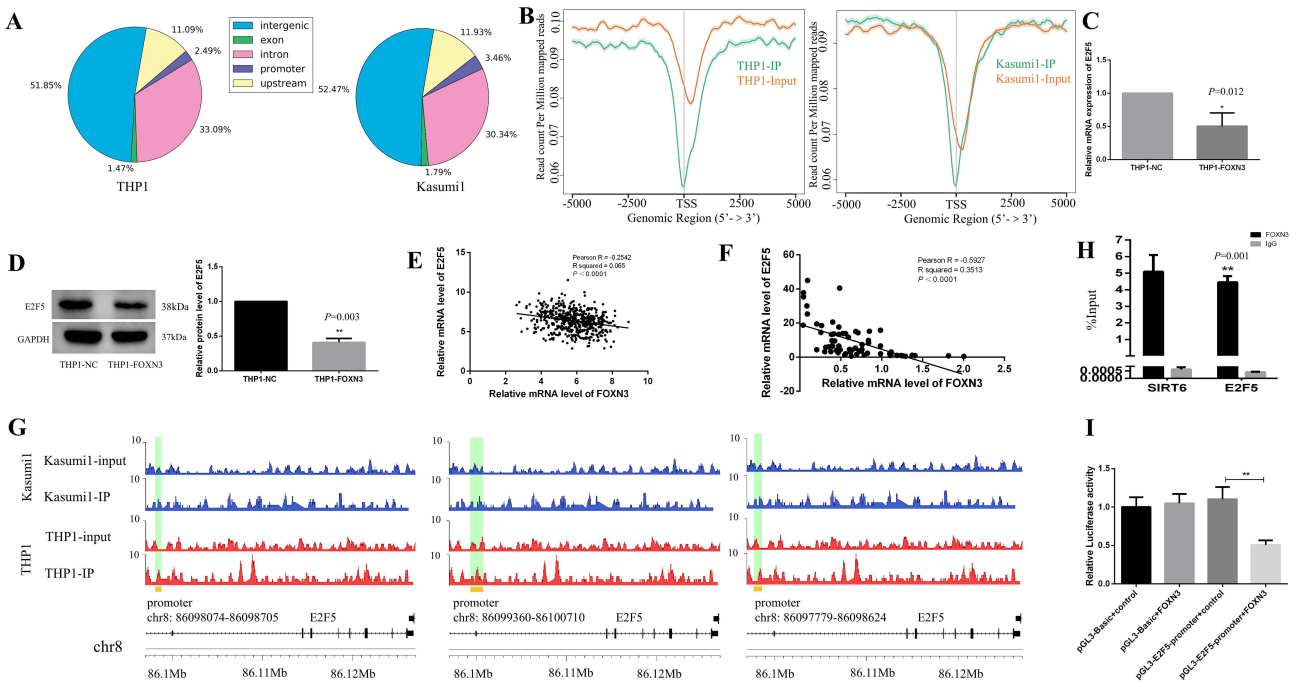


**Figure 1** Identification of E2F5 as a transcriptional target of FOXN3. (**A**) Genomic distribution of FOXN3 binding sites, represented through pie charts. (**B**) Distribution of binding sites relative to transcription initiation peaks within 5 kb upstream and downstream of the transcription start site (TSS). (**C** and **D**) RT-qPCR (**C**) and Western blotting (**D**) analyses of E2F5 expression in THP1 cells following FOXN3 overexpression. (**E**) Scatter plot illustrating the correlation between FOXN3 and E2F5 expression levels in AML patient samples from the GSE13159 dataset, indicating a negative correlation between the two genes. (**F**) Scatter plot confirming the negative correlation between FOXN3 and E2F5 expression in AML patient samples collected from our institution. (**G**) Visualization of representative FOXN3 binding peaks in the genome browser using karyoploteR, specifically in the E2F5 promoter region. The tracks display both input and immunoprecipitation (IP) data for the Kasumi1 and THP1 cell lines. The highlighted region denotes the E2F5 promoter area, where significant binding peaks were detected in the ChIP-seq experiment. (**H**) ChIP-PCR analysis demonstrating FOXN3 binding to the E2F5 promoter. Significant enrichment of FOXN3 binding is observed at the E2F5 promoter compared to the IgG control, with the SIRT6 promoter serving as a positive control ($P = 0.001$). (**I**) Results from the luciferase reporter assay illustrating the repressive effect of FOXN3 on E2F5 promoter activity. The relative luciferase activity is significantly lower in the presence of FOXN3 ($P < 0.01$). Data are presented as mean ± SD of three independent experiments. Statistical significance is indicated as follows: $^{*}P < 0.05$, $^{**}P < 0.01$.

adhesion junction, and galactose metabolism (Table 2). Based on these findings, we focused on cell cycle-related signaling and identified 23 candidate target genes of FOXN3—including ISG15, FLOT1, CDC42, CDK1, HDAC1, and MAPK8—through systematic screening using the UCSC Genome Browser and the Atlas of Genetics and Cytogenetics in Oncology and Haematology.

FOXN3 overexpression in THP1 cells was validated at both mRNA and protein levels (Supplementary Figure S1A and B). Among 23 candidate target genes screened, RT-qPCR analysis revealed that FOXN3 overexpression significantly down-regulated the mRNA expression of E2F5, CDC42, and HDAC1, while the remaining 20 candidates showed no significant change (Figure 1C, Supplementary Figure S1C and D). Western blotting further confirmed reduced protein expression of E2F5 and CDC42, though HDAC1 protein levels remained unchanged (Figure 1D, Supplementary Figure S1E and F).

Analysis of public AML datasets and clinical samples consistently demonstrated a significant negative correlation between FOXN3 and E2F5 expression (Figure 1E and F and Table S3). ChIP-seq data from Kasumi1 and THP1 cells identified FOXN3-binding peaks in the promoter region of E2F5, which were validated by ChIP-qPCR (Figure 1G and H). Moreover, dual-luciferase assays showed that FOXN3 overexpression significantly repressed E2F5 promoter activity (Figure 1I).

Collectively, these results indicate that FOXN3 directly binds to the E2F5 promoter and suppresses its transcriptional activity, supporting E2F5 as a functional downstream target of FOXN3 in AML.

## E2F5 Expression is Upregulated and Associated with Poor Outcomes in AML

To evaluate the expression profile of E2F5 in newly diagnosed AML patients, we performed RT-qPCR using bone marrow samples obtained from 66 AML patients (Supplementary Table S4) and 18 healthy donors. Our analysis revealed a significant upregulation of E2F5 mRNA expression in AML patients, with a median of 4.199 (range: 0.403–65.345), compared to


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**Table 1** The Top 20 Major Gene Ontology Terms-Biological Processes Enriched for the Potential Target Genes of FOXN3

| Term | *P* value |
|---|---|
| Protein import into nucleus | 0.0011 |
| Regulation of apoptotic signaling pathway | 0.0013 |
| Regulation of acrosome reaction | 0.0014 |
| Endoderm development | 0.0019 |
| Cell cycle arrest | 0.0020 |
| Protein localization to nucleus | 0.0020 |
| Import into nucleus | 0.0025 |
| Regulation of interferon-gamma production | 0.0035 |
| Regulation of fertilization | 0.0037 |
| Positive regulation of apoptotic signaling pathway | 0.0037 |
| Regulation of protein insertion into mitochondrial membrane involved in Apoptotic signaling pathway | 0.0045 |
| Positive regulation of protein insertion into mitochondrial membrane Involved in apoptotic signaling pathway | 0.0045 |
| Antigen processing and presentation via MHC class Ib | 0.0053 |
| Receptor guanylyl cyclase signaling pathway | 0.0053 |
| Epithelial cell proliferation involved in lung morphogenesis | 0.0053 |
| Protein insertion into mitochondrial membrane involved in apoptotic Signaling pathway | 0.0054 |
| Interferon-gamma production | 0.0057 |
| Muscle hypertrophy in response to stress | 0.0058 |
| Cardiac muscle adaptation | 0.0058 |
| Cardiac muscle hypertrophy in response to stress | 0.0058 |

**Table 2** The Top ten Significantly Enriched Pathways for the Potential Target Genes of FOXN3

| Pathway | *P* value |
|---|---|
| Olfactory transduction | 0.0013 |
| Arrhythmogenic right ventricular cardiomyopathy (ARVC) | 0.0027 |
| Non-homologous end-joining | 0.0100 |
| Homologous recombination | 0.0179 |
| Proteasome | 0.0196 |
| Pathogenic Escherichia coli infection | 0.0326 |
| Cell cycle | 0.0405 |
| Adhesion junction | 0.0418 |
| Autoimmune thyroid disease | 0.0447 |
| Galactose metabolism | 0.0494 |

controls, which had a median of 1.289 (range: 0.344–3.914) (Figure 2A, $P < 0.0001$). This elevated expression of E2F5 was further confirmed using the online Bloodpool dataset (https://www.bloodspot.eu), which similarly demonstrated significantly higher E2F5 expression in common AML subtypes with varying prognostic risks compared to other control cell types (Figure 2B). At the cellular level, additional experiments indicated that the mRNA expression levels of E2F5 in AML cell lines were significantly elevated compared to the control 293T cell line (Figure 2C). Furthermore, analysis based on the online GEPIA dataset (https://gepia.cancer-pku.cn) revealed that AML patients with higher E2F5 expression had shorter overall survival compared to those with lower E2F5 expression (Figure 2D, $P = 0.0052$), similarly as previously reported.[15] These findings suggest that E2F5 is abnormally upregulated and associated with poor outcomes in AML, indicating a potential oncogenic role for E2F5 in this malignancy.

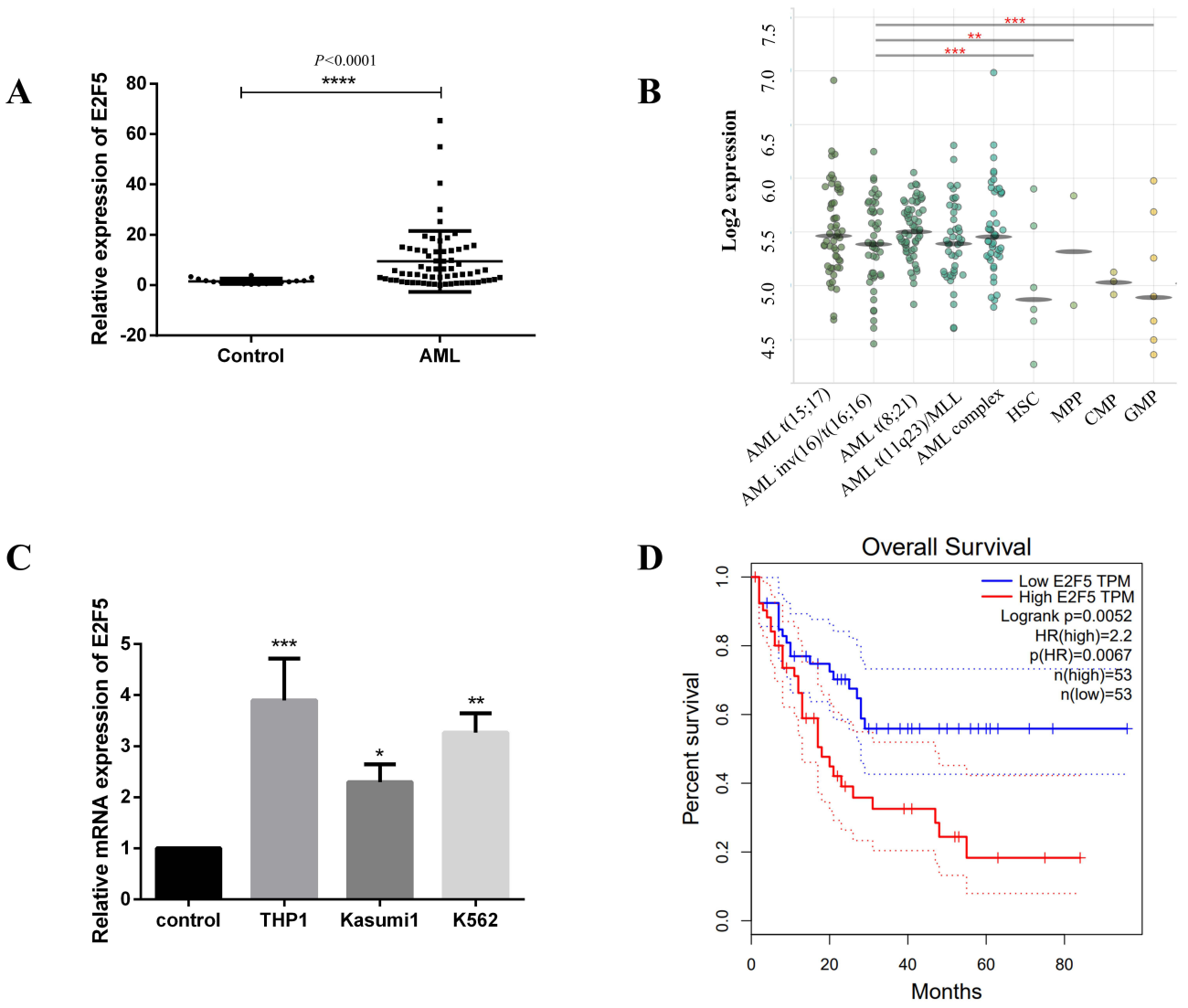


**Figure 2** Aberrant upregulation of E2F5 expression and its association with poor outcomes in AML. (**A**) RT-qPCR analysis showing elevated E2F5 mRNA expression in AML patients compared to healthy controls. (**B**) E2F5 mRNA levels are significantly higher in AML compared to hematopoietic stem cells (HSCs), as demonstrated using the online Bloodpool dataset. (**C**) RT-qPCR analysis revealing increased E2F5 mRNA expression in AML cell lines compared to control cell lines. (**D**) Correlation between higher E2F5 expression and shorter overall survival (OS) in AML, determined using the median value as the cutoff to distinguish between high and low E2F5 expression levels from the online GEPIA dataset. Data are presented as mean ± SD of three independent experiments. Statistical significance is indicated as follows:*$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001, and ****$P$ < 0.0001.

**Abbreviations**: MPP, multipotential progenitors; CMP, Common myeloid progenitor cell; GMP, Granulocyte monocyte progenitors.

## Functional Impact of E2F5 as an Oncogene in AML

To investigate the potential functions of E2F5 in AML cells, we transfected Kasumi1 and THP1 cells with E2F5 shRNA lentivirus to induce knockdown of E2F5 expression (designated as shE2F5), with cells transfected with negative scrambled shRNA (NC) serving as the negative control. The significant downregulation of E2F5 expression was confirmed through RT-qPCR (Supplementary Figure S2A and B) and Western blot analysis (Supplementary Figure S2C and D). Then, we assessed the proliferation capacity of the cells using a CCK-8 assay, which revealed a significant inhibition of cell proliferation in the shE2F5 group compared to the control group (Figure 3A). Western blot analysis further demonstrated that E2F5 knockdown resulted in decreased protein levels of PCNA compared to NC cells (Supplementary Figure S3A). Additionally, flow cytometry analysis indicated an increase in cell apoptosis following E2F5 knockdown (Figure 3B), accompanied by a reduction in Bcl-2 protein levels after 48 hours compared to NC cells (Supplementary Figure S3B). Cell cycle analysis


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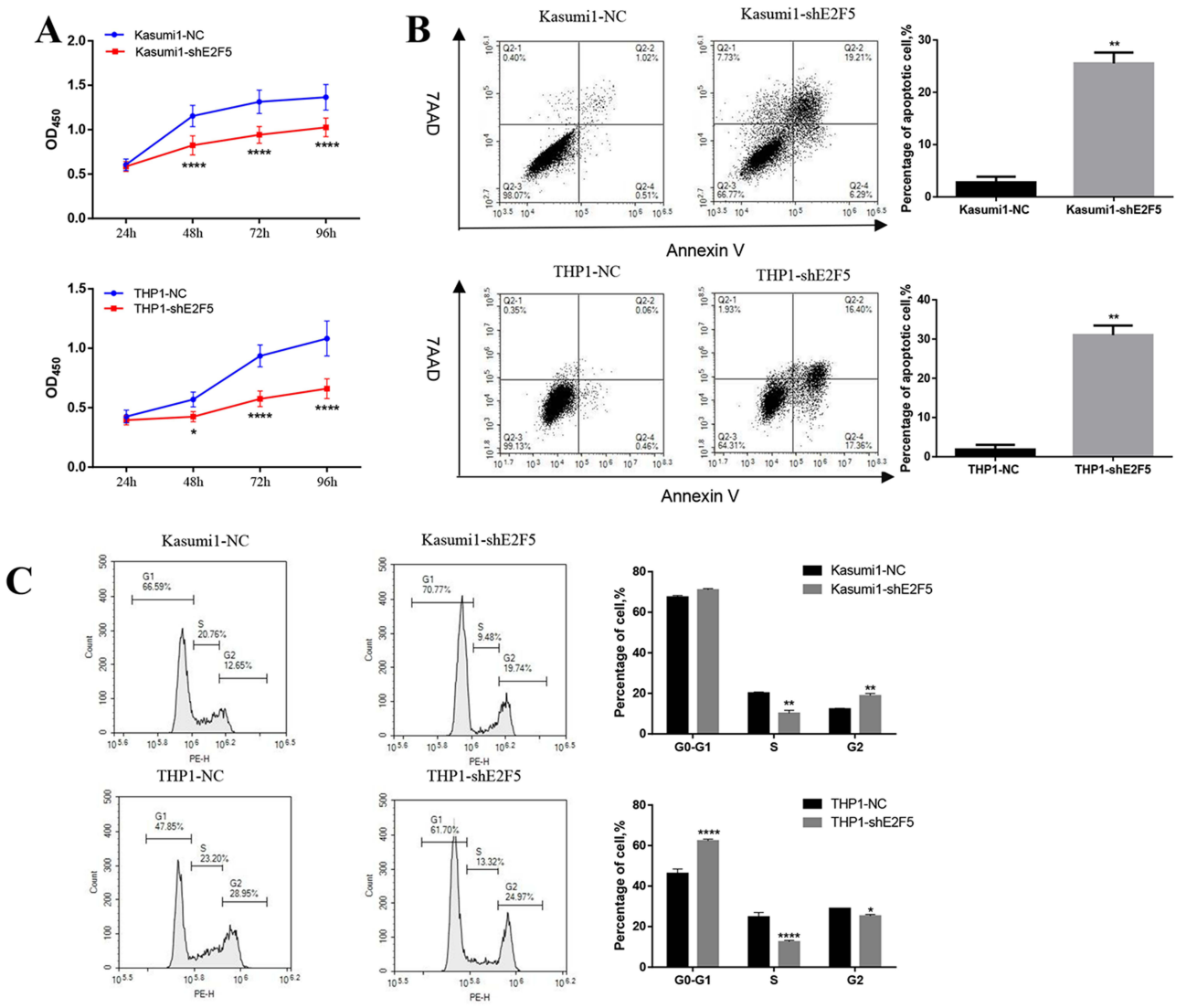


**Figure 3** Inhibition of cell proliferation, induction of apoptosis, and cell cycle arrest following E2F5 knockdown in AML cells. (**A**) CCK-8 assay was conducted to assess the proliferation of Kasumi1 and THP1 cells following E2F5 knockdown. (**B**) Flow cytometry analysis evaluated the induction of apoptosis in Kasumi1 and THP1 cells after E2F5 knockdown. (**C**) Cell cycle analysis was performed on Kasumi1 and THP1 cells with E2F5 knockdown using flow cytometry. Data are presented as mean ± SD of three independent experiments. Statistical significance is indicated as follows: **P* < 0.05, ***P* < 0.01, and *****P* < 0.0001.

revealed a blockade of the cell cycle in the G0-G1 phase, characterized by an increase in G0-G1 phase cells and a decrease in S phase cells following E2F5 knockdown after 48 hours (Figure 3C). Furthermore, we observed downregulation of the protein levels of CyclinD1, CDK6, CDK2, and CyclinE1 upon E2F5 knockdown compared to NC cells (Supplementary Figure S3C). Collectively, these findings indicate that E2F5 knockdown can induce cell cycle arrest, increase cell apoptosis, and inhibit the proliferation of AML cells, suggesting that E2F5 functions as a novel oncogene in AML.

## FOXN3 Counteracts Leukemia Cell Proliferation via Transcriptional Regulation of E2F5

Western blot and quantitative RT-PCR experiments confirmed successful overexpression of E2F5 in both Kasumi1 and THP1 cell lines (Supplementary Figure S4A–D). To assess the functional effects of E2F5 overexpression in Kasumi1 and THP1 cells, we performed CCK-8 and cell cycle assays, and the results demonstrated that E2F5 overexpression significantly increased cell proliferation, as indicated by higher OD450 values (Figure 4A).

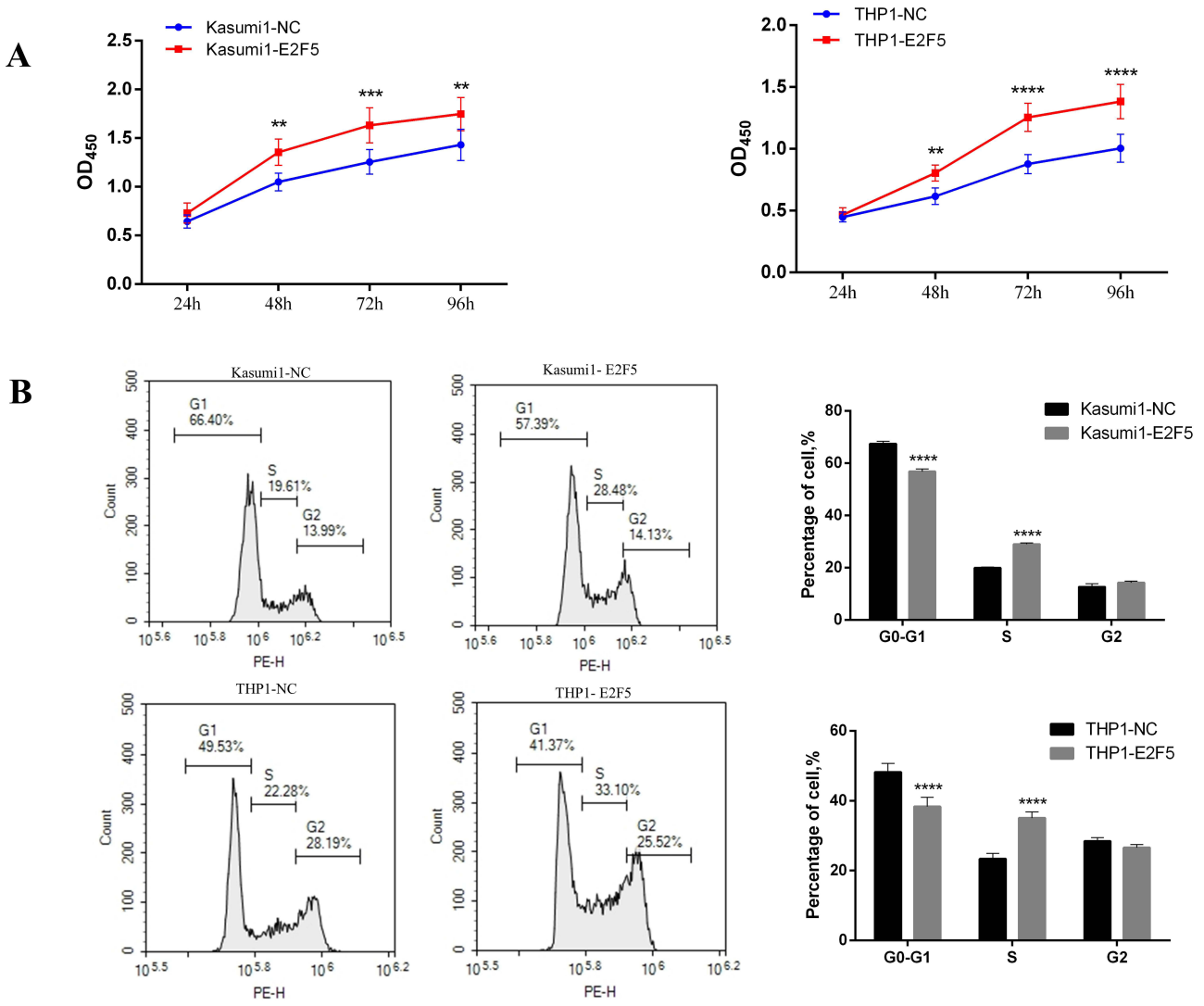


**Figure 4** Enhancement of cell proliferation and cell cycle following E2F5 overexpression in AML cells. (**A**) CCK-8 assay evaluated the proliferation of Kasumi1 and THP1 cells following E2F5 overexpression. (**B**) Cell cycle analysis was conducted on Kasumi1 and THP1 cells with E2F5 overexpression using flow cytometry. Data are presented as mean ± SD of three independent experiments. Statistical significance is indicated as follows: $^{**}P < 0.05$, $^{***}P < 0.01$, and $^{***}P < 0.0001$.

Additionally, the impact of E2F5 overexpression on PCNA protein levels was assessed, revealing increased expression of PCNA, a marker for cell proliferation, in both Kasumi1 and THP1 cells overexpressing E2F5 (Supplementary Figure S5A). Cell cycle assays indicated a significant decrease in the G0-G1 phase ($P < 0.0001$) and a significant increase in the S phase ($P < 0.0001$) for the E2F5 group compared to the control in both cell lines (Figure 4B), which suggests that E2F5 overexpression facilitates cell cycle progression by reducing the percentage of cells in the G1 phase while increasing those in the S phase, indicating enhanced cell cycle progression and proliferation. Confirmatory experiments via Western blot analysis showed that E2F5 overexpression increased the levels of key cell cycle regulatory proteins, including CyclinD1, CDK6, CDK2, and CyclinE1, in both Kasumi1 and THP1 cells (Supplementary Figure S5B). These results confirm that E2F5 promotes cell cycle progression, likely by facilitating the transition from the G1 to the S phase, thus supporting its role as a novel oncogene in AML.

Subsequently, we assessed the expression levels of FOXN3 and E2F5 in each group (Kasumi1-NC, Kasumi1-FOXN3, Kasumi1-FOXN3+E2F5, and THP1-NC, THP1-FOXN3, THP1-FOXN3+E2F5) through RT-qPCR (Supplementary Figure S6) and Western blotting (Supplementary Figure S7). As shown in Figure 5A, CCK-8 assays


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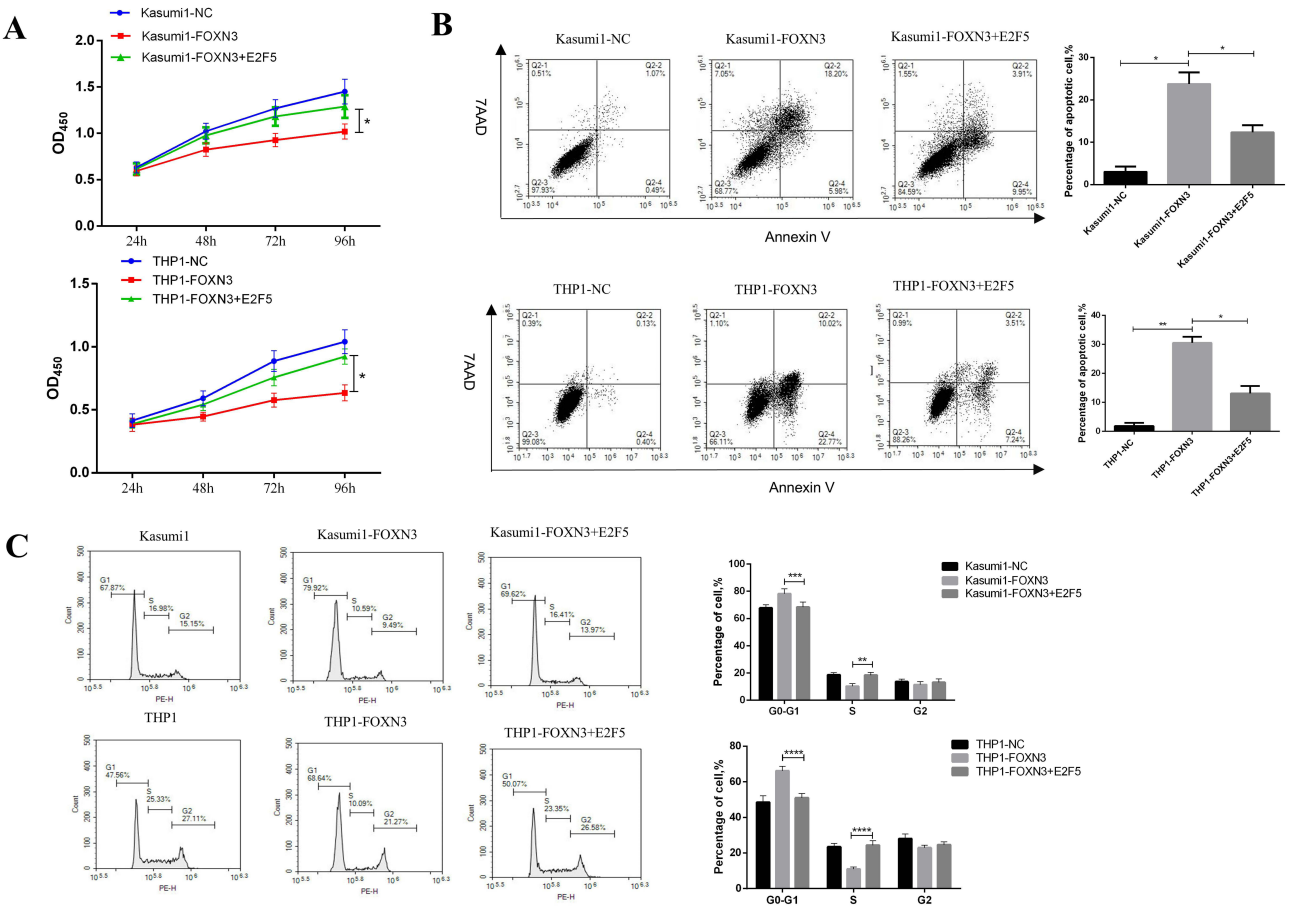


**Figure 5** Partial rescue of proliferation inhibition and induction of apoptosis by E2F5 upregulation in AML cells overexpressing FOXN3. (**A**) Assessment of cell proliferation in leukemic cells co-transfected with lentiviruses for FOXN3 overexpression and E2F5 overexpression using the CCK-8 assay. (**B**) Flow cytometry analysis evaluated apoptosis in leukemic cells co-transfected with FOXN3 and E2F5 overexpressing lentiviruses. (**C**) Cell cycle analysis was performed on leukemic cells co-transfected with FOXN3 and E2F5 overexpressing lentiviruses using flow cytometry. Data are presented as mean ± SD of three independent experiments. Statistical significance is indicated as follows:*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, and ****$P < 0.0001$.

revealed that E2F5 overexpression could counteract the inhibition of cell proliferation induced by FOXN3 overexpression. Flow cytometric analysis indicated that E2F5 overexpression significantly reduced the increased cell apoptosis caused by FOXN3 overexpression in Kasumi1 and THP1 cells (Figure 5B). Furthermore, E2F5 overexpression notably reversed the cell cycle arrest effect in the G0-G1 phase induced by FOXN3 overexpression in both cell lines (Figure 5C). Consistent with these findings, we observed that the protein levels of PCNA, Bcl-2, CyclinD1, CDK6, CDK2, and CyclinE1, which were downregulated in FOXN3-overexpressing cells, were significantly restored upon E2F5 overexpression (Supplementary Figure S8A–C).

Taken together, these results indicate that FOXN3 functions as a tumor suppressor through transcriptional regulation of E2F5 in AML.

## Transcriptional Regulation of E2F5 by FOXN3 via MAPK Signaling Pathways in AML

GO and KEGG pathway analyses revealed significant alterations in gene expression profiles. Specifically, a total of 181 genes exhibited differential expression, with 97 genes upregulated and 84 genes downregulated upon FOXN3 overexpression (Figure 6A). GO analysis demonstrated associations with various biological processes, including transcriptional regulation, cell cycle progression, and apoptosis, while KEGG pathway analysis revealed enrichment in signaling pathways such as MAPK, ErbB, and NF-kappa B (Figure 6B).

Western blotting findings showed that FOXN3 overexpression resulted in a significant decrease in the levels of EZH2 and p-ERK1/2. Importantly, when E2F5 was overexpressed in cells with stable FOXN3 overexpression, it restored the

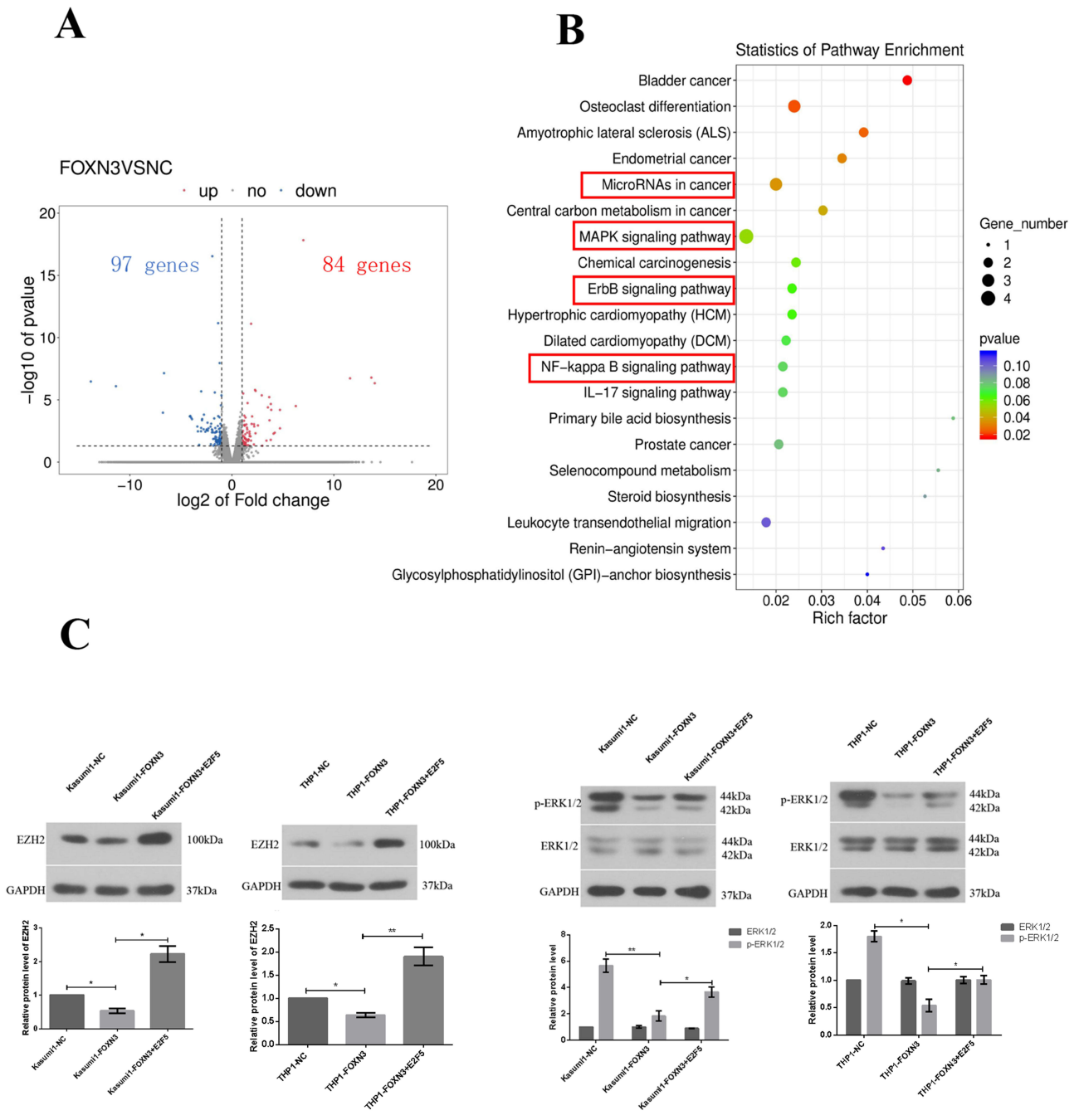


**Figure 6** Regulation of EZH2 and MAPK pathways by the FOXN3-E2F5 axis in AML. (**A**) Volcano plots illustrating differentially expressed genes in THP1 cells with FOXN3 overexpression compared to control THP1 cells. (**B**) The top twenty significant KEGG pathways related to differentially expressed genes in THP1 cells with FOXN3 overexpression compared to control THP1 cells. The red box highlights the signal pathway that are in agreement with the functional results. (**C**) Western blot analysis showing the protein levels of EZH2, ERK1/2, and phosphorylated ERK1/2 (p-ERK1/2) in cells co-transfected with lentiviruses for FOXN3 and E2F5 overexpression. The experiments were conducted using three independent replicates and the data are presented as mean ± SD. Statistical significance is indicated as follows: $^{*}P < 0.05$, and $^{**}P < 0.01$.

expression levels of EZH2 and p-ERK1/2, indicating their involvement in the FOXN3-mediated regulatory mechanism (Figure 6C). Overall, these findings suggest that FOXN3 functions as a tumor suppressor in AML, potentially through the transcriptional targeting of E2F5 via the MAPK signaling pathway.


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## Discussion

In this study, we identified E2F5 as a transcriptional target gene of FOXN3 in AML through ChIP-seq analysis. Subsequent GO and KEGG pathway analyses confirmed FOXN3's role in regulating genes associated with cell cycle arrest, apoptotic signaling, and other essential biological processes. We established the direct regulation of E2F5 by FOXN3, which was further validated through ChIP-PCR and dual-luciferase reporter gene assays. Notably, we observed a decrease in both mRNA and protein levels of E2F5 upon FOXN3 overexpression. Furthermore, E2F5 was found to be overexpressed in AML patients and cell lines, correlating with poor overall survival outcomes. Functional investigations identified E2F5 as an oncogene that promotes cell proliferation, inhibits apoptosis, and influences cell cycle dynamics. Co-transfection experiments indicated that E2F5 could counteract the proliferation-inhibitory effects of FOXN3, potentially through the modulation of the MAPK signaling pathway and its downstream target, EZH2 (Figure 7). Overall, these findings elucidate the regulatory axis between FOXN3 and E2F5, providing valuable insights into potential therapeutic targets for the treatment of AML.

E2F5, a member of the E2F transcription factor family, is essential in various biological processes, including cell proliferation, cell cycle regulation, DNA damage repair, and developmental pathways.[16,17] Numerous investigations have highlighted the oncogenic functions of E2F5 across various malignancies,[18–22] linking its elevated expression to enhanced cell proliferation, increased migration, and inhibition of apoptosis. Furthermore, E2F5's influence extends beyond its role as an oncogene; it has been associated with chemotherapy sensitivity in several cancers.[23,24] While prior studies have examined the regulatory relationship between E2F5 and FOXN3 in hepatocellular carcinoma cells,[25] the precise significance of E2F5 in leukemogenesis has remained unclear. In this study, we demonstrate that overexpression of FOXN3 leads to decreased phosphorylation levels of ERK1/2 and reduced EZH2 protein levels. These findings suggest that FOXN3 negatively regulates the MAPK signaling pathway and its downstream targets, potentially contributing to its tumor-suppressive effects in AML. The restoration of these protein levels upon E2F5 overexpression further supports the notion of E2F5 as an important mediator in this regulatory axis. Moreover, the knockdown of E2F5 in Kasumi1 and THP1 cells significantly altered both cell cycle progression and proliferation, indicating its oncogenic role in AML, aligning with previous research suggesting that dysregulation of cell cycle control is a hallmark of many cancers, including AML.[26,27] Thus, our study represents one of

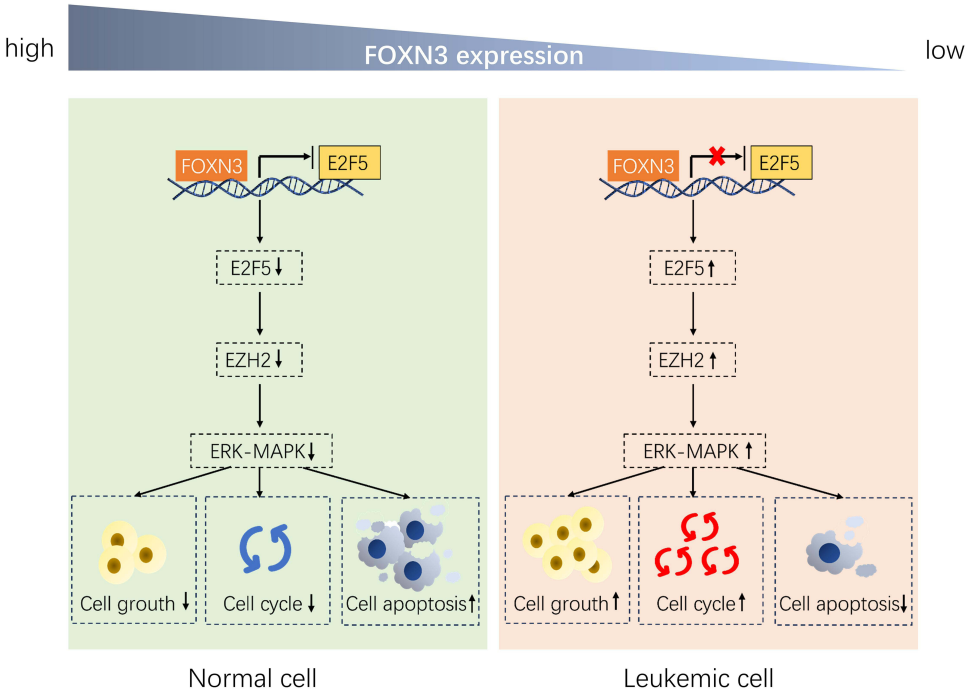


**Figure 7** The mechanism of FOXN3-E2F5-EZH2-MAPK regulatory axis in AML.

the few reports identifying elevated E2F5 expression in AML, with prior evidence limited to a single study employing cDNA microarray analysis.[28] However, the functional implications of E2F5 in AML have remained unclear.

The increased expression of E2F5 in AML patients, as detected by RT-qPCR and validated by independent datasets, along with its association with shorter survival outcomes indicated by GEPIA analysis, suggests its potential as a diagnostic and prognostic biomarker in AML. Elevated levels of E2F5 may signal a higher-risk disease, thereby guiding clinical decision-making. E2F5 has been implicated as a target of several microRNAs in various human cancers and has been shown to regulate the transcription of key genes, including UBE2T, TFP2, MMP-2, and MMP-9, promoting cell proliferation and invasion.[19,29–32] Functional assays conducted in this study confirmed E2F5's oncogenic role in leukemia. Furthermore, we investigated the regulatory axis involving FOXN3 and E2F5, along with the downstream signaling pathways implicated in leukemogenesis. Our clinical data demonstrated reduced FOXN3 expression and elevated E2F5 levels in AML patients, establishing their prognostic significance.[7] Future studies could focus on validating these findings in larger patient cohorts to confirm E2F5's functional role in AML pathogenesis and explore therapeutic interventions targeting E2F5 and its associated pathways. Additionally, the development of multiplex biomarker panels incorporating E2F5 could enhance diagnostic accuracy and prognostic predictions in AML, ultimately improving patient management.

Subsequent RNA-seq analysis identified the MAPK signaling pathway as a prominent downstream cascade associated with FOXN3. Given its well-established role in AML pathogenesis[33–36] and in the development of drug resistance among AML cells,[37] we prioritized ERK1/2 for further validation as a key effector within this pathway. The aberrant activation of MAPK signaling has been extensively implicated in AML progression, influencing cell survival, proliferation, and chemoresistance. Prior studies have demonstrated that EZH2, a histone-lysine N-methyltransferase, is regulated by the E2F family and interacts with the MAPK pathway to modulate transcriptional programs in AML.[38] As a key downstream target of the pRB-E2F pathway,[39–42] EZH2 plays a critical role in histone H3K27 methylation and epigenetic regulation. Suppression of EZH2 has been shown to inhibit tumor growth by de-repressing tumor suppressor genes,[42,43] and EZH2 knockout has been linked to apoptosis induction in AML cells, largely through thioredoxin inhibition and increased ROS accumulation.[44] Notably, interactions between EZH2 and p-ERK signaling have been reported across multiple malignancies, including lymphoma, lung cancer, and liver cancer,[45,46] further supporting its relevance in AML. Given that EZH2 inhibitors, such as Valemetostat (Ezharmia), Tazverik, SHR2554 and HH2853, are currently under clinical investigation for relapsed or refractory T-cell leukemia/lymphoma, follicular lymphoma, and classical Hodgkin lymphoma, their potential application in AML warrants further exploration. By elucidating the FOXN3-E2F5-EZH2-MAPK axis, our study provides insights into how FOXN3 suppresses oncogenic signaling in AML, reinforcing its potential role as a tumor suppressor. Thus, targeting this regulatory axis could offer a novel therapeutic strategy to enhance treatment efficacy and potentially overcome drug resistance in AML.

This study has several limitations. First, while we have established a regulatory axis involving FOXN3 and E2F5 in AML, our investigation primarily assessed correlative data between FOXN3 and E2F5 expression. As such, the precise mechanistic details of how FOXN3 represses E2F5 remain to be fully elucidated. Second, while our data support FOXN3 binding at the E2F5 promoter, it is not yet demonstrated whether this binding is necessary for E2F5 repression. Thus, we acknowledge the possibility that FOXN3 may regulate other factors that indirectly contribute to the repression of E2F5 expression. Future studies employing FOXN3 motif mutations are necessary to confirm the direct role of FOXN3 in E2F5 repression by including more functional assays, such as knockdown or rescue experiments, to thoroughly investigate the downstream signaling cascades and other possible molecular mediators beyond the MAPK pathway. Third, the potential therapeutic implications of targeting the FOXN3-E2F5 axis in preclinical AML models remain to be explored. Therefore, expanding the research to include in vivo validation in animal models and clinical correlations could help provide further insights into the clinical relevance of our findings.

## Conclusions

In this study, we identified FOXN3 as a tumor suppressor that negatively regulates the expression of E2F5 and modulates key signaling pathways involved in AML. However, further mechanistic exploration is required to fully elucidate the scope of FOXN3's regulation of the MAPK pathway. Given that reduced FOXN3 expression is associated with AML relapse and poor survival outcomes, future studies should focus on models that better reflect the physiological levels of FOXN3 observed in AML patients. This approach would enhance our understanding of how FOXN3 downregulation

contributes to disease progression and therapeutic resistance. Further investigation into additional components of the MAPK pathway is also necessary to substantiate the conclusion that FOXN3 broadly regulates this signaling network.

## Data Sharing Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## Ethics Approval and Consent to Participate

The present study was approved by the Ethics Committee of the First Affiliated Hospital of China Medical University (CMU). All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards. All patients gave their written informed consent.

## Patient Consent for Publication

Written informed consent was obtained from the study participants for the anonymous use of their samples for research purposes.

## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

The authors declare that they have no conflicts of interest in this work.

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