

Original Article

MLN4924 promotes the transcriptional activation of BTG2 via the ROS/EGR1-mediated signaling axis to suppress acute myeloid leukemia progression

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Abstract

Acute myeloid leukemia (AML) is a highly heterogeneous hematological malignancy characterized by a high relapse rate and a low survival rate. Although chemotherapy and allogeneic hematopoietic stem cell transplantation (allo-HSCT) have improved the prognosis for AML patients, the overall survival rate remains suboptimal. MLN4924 is a neddylation inhibitor and is considered a promising treatment approach for AML. However, the exact molecular mechanism remains elusive and requires further investigation. This study aims to investigate the molecular mechanisms of MLN4924 and novel molecular pathways in AML. RNA sequencing (RNA-seq) reveals that the transcription factor (TF) EGR1 serves as a core regulator of MLN4924 and is upregulated by MLN4924 in AML. Mechanistic studies demonstrate that MLN4924 induces apoptosis by generating reactive oxygen species (ROS) and facilitates the nuclear translocation of EGR1. This translocated EGR1 interacts with the promoter region of *BTG2*, promoting its transcription and inhibiting the progression of AML. Notably, the ROS generated by MLN4924 influences the expression of both EGR1 and *BTG2* and establishes a positive feedback loop between EGR1 and ROS. *In vivo*, we confirm that MLN4924 reduces the leukemic burden in AML cell-derived xenograft models by increasing the expression of both EGR1 and *BTG2*. In conclusion, these findings suggest that MLN4924 exerts an anti-tumor effect on AML by inducing apoptosis through the ROS-EGR1-BTG2 signaling axis. Our research provides a novel theoretical basis for the clinical potential of MLN4924 in improving the treatment of AML patients, offers novel strategies for AML treatment, and thereby advances the implementation of precision medicine.

Key words acute myeloid leukemia, MLN4924, reactive oxygen species, EGR1, BTG2

Introduction

Acute myeloid leukemia (AML) is a highly heterogeneous hematological malignancy characterized by complex genetic alterations and molecular abnormalities [1]. The median age at diagnosis of AML is 68 years, with a 5-year overall survival rate of 32% [2]. Although chemotherapy regimens, such as the “7+3” regimen and allogeneic hematopoietic stem cell transplantation (allo-HSCT), have improved survival rates for AML patients, some individuals continue to struggle to achieve remission and may experience relapse [3]. Despite significant advances in targeted therapy and immunotherapy for AML, the proportion of patients who achieve long-term remission or cure remains less than 50% [4,5]. Therefore,

there is an urgent need to explore innovative therapeutic strategies for AML patients.

In recent years, targeting protein post-translational modifications has emerged as a promising strategy for cancer treatment. Neddylation is a highly conserved and reversible post-translational modification of proteins [6]. This process involves the activation of the ubiquitin-like protein NEDD8 through a three-step enzymatic reaction, which involves the NEDD8-activating enzyme (NAE), the NEDD8-conjugating enzyme (E2), and the NEDD8-ligase (E3) [7,8]. The attachment of the ubiquitin-like protein NEDD8 facilitates the degradation of substrate proteins and plays a crucial role in regulating significant biological functions [9]. As a selective small-

molecule inhibitor of NAE, MLN4924 disrupts the ubiquitin ligase activity of Cullin-RING ligase (CRL) by inhibiting the neddylation of Cullin proteins [10]. This inhibition leads to the abnormal accumulation of tumor suppressor proteins, such as p53, CDT1, and NOXA, ultimately inducing apoptosis in cancer cells [11–13]. Phase I/II trials of MLN4924, in combination with azacitidine and venetoclax, have shown a significant improvement in the complete response (CR) rate among newly diagnosed secondary AML patients [14]. Additionally, combination therapy with azacitidine, venetoclax, and MLN4924 significantly increased the rate of minimal residual disease (MRD) negativity among patients who achieve CR in relapsed or refractory AML patients [15]. However, the precise molecular mechanism underlying the induction of apoptosis properties of MLN4924 remains to be further elucidated. There is a lack of information on the precise molecular mechanisms by which MLN4924 inhibits AML progression, and the absence of reliable biomarkers restricts the implementation of personalized treatment.

ROS play a complex dual role in tumor biology. On the one hand, ROS accumulation can induce genomic instability, which subsequently drives oncogenic mutations and tumor progression. On the other hand, excessive ROS activate oxidative stress and the mitochondria-dependent apoptotic pathway, ultimately leading to cell death [16]. Liang *et al.* [17] reported that MLN4924 inhibits NF- κ B activation, reducing catalase expression, which promotes ROS accumulation and ultimately leads to ATF3 accumulation and autophagy. Additionally, a previous study has shown that MLN4924 induces cell death by increasing ROS levels in MV4-11 cells [18]. However, the specific downstream signaling pathways and mechanisms of ROS regulation underlying its anti-leukemia effects remain to be elucidated.

Transcription factors (TFs) are proteins that specifically bind to DNA sequences and initiate the transcription of target genes under certain conditions. Dysregulation of TFs can contribute to the emergence of cancer cells and tumor progression. Identifying TFs with significant regulatory roles in tumors is critical for understanding cancer biology [19,20]. Early growth response 1 (EGR1), a zinc-finger TF, plays a pivotal role in cancer and has dual regulatory functions. Elevated levels of EGR1 are associated with carcinogenesis in prostate and gastric cancers [21,22], whereas in glioma and melanoma, EGR1 can promote apoptosis in tumor cells [23,24]. A previous study indicated that the loss of TP53 activity, combined with haploinsufficiency of *EGR1* or *APC*, accelerated the development of AML in genetically engineered mice [25]. However, the specific mechanism by which MLN4924 mediates the apoptosis of AML cells through crosstalk between ROS and EGR1 remains unclear and requires further study. Hence, our study aimed to explore the exact mechanism of MLN4924 and the role of EGR1 in AML.

In this study, we verified that EGR1 expression was significantly greater in the MLN4924 treatment group and that high expression of EGR1 could suppress AML proliferation *in vivo* and *in vitro*. Furthermore, we found that MLN4924 promoted ROS accumulation and enhanced the nuclear translocation of EGR1, establishing a positive feedback loop between EGR1 and ROS. This translocation facilitated EGR1 binding to the promoter region of *BTG2*, leading to the transcriptional activation of *BTG2* expression and consequently the inhibition of AML progression. These findings support the potential of MLN4924 as a therapeutic agent for AML and highlight

its potential for targeted therapies.

Materials and Methods

Clinical samples

Peripheral blood mononuclear cells (PBMCs) were collected from newly diagnosed patients with AML ($n = 32$) and healthy volunteers ($n = 10$) at the Hematology Department of the First Affiliated Hospital of Lanzhou Medical University. PBMCs were isolated via density gradient centrifugation. All patients signed informed consent forms, and this study was approved by the Ethics Committee of the First Hospital of Lanzhou University (Approval number: LDYYLL-2025-787).

Cell culture and reagents

The human leukemia cell line Kasumi-1 was purchased from the American Type Culture Collection (ATCC, Manassas, USA). The MOLM-13 cell line was purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). HL-60 and K562 leukemia cell lines were purchased from Servicebio (Wuhan, China). Kasumi-1 cells were cultured in RPMI-1640 medium (Gibco, Beijing, China) supplemented with 20% fetal bovine serum (FBS); MOLM-13, HL-60, and K562 cells were cultured in RPMI-1640 medium supplemented with 10% FBS. All the cell lines were combined with 1% penicillin/streptomycin (Thermo Fisher Scientific, Waltham, USA) as an additive. All the cells were cultured at 37°C and 5% CO₂ in a humidified atmosphere.

MLN4924, SC79, acetylcysteine (NAC), and SCH772984 were purchased from TargetMol (Shanghai, China). Dimethyl sulfoxide (DMSO) was purchased from Solarbio (Beijing, China).

RNA sequencing (RNA-seq)

Kasumi-1 cells were treated with different concentrations of MLN4924 (0.1 μ M, 0.3 μ M, or 3.0 μ M) for 24 h. Total RNA was extracted, and reverse transcription was performed to convert it into cDNA to construct the sequencing libraries. High-throughput sequencing was performed via the Illumina NovaSeq 6000 platform by OE Biotech Co., Ltd. (Shanghai, China) to analyze the differential gene expression between the MLN4924-treated and untreated groups. *EGR1*-overexpressing MOLM-13 and negative control cells were also analyzed via RNA-seq at OE Biotech Co., Ltd. (Shanghai, China).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR)

Total RNA extraction was performed via an M5 Universal RNA Kit (Mei5bio, Beijing, China). Reverse transcription was performed via Hifair® III 1st Strand cDNA Synthesis SuperMix (gDNA digester plus) (11141ES60; Yeasen, Shanghai, China). Hieff® qPCR SYBR Green Master Mix (11202ES08; Yeasen) was used in RT-qPCR assay on the QuantStudio 3 system (Thermo Fisher Scientific), and the relative quantification of the *EGR1*, *BTG2*, *KLF2*, *KLF6*, and *NAB2* genes was performed via the 2^{- $\Delta\Delta$ CT} method, with *GAPDH* used as a reference gene. The sequences of the qPCR primers used are listed in Supplementary Table S1.

Western blot analysis

Total protein was extracted via RIPA lysis buffer, containing PMSF and a protease inhibitor cocktail (Servicebio). The protein concentration was measured via a BCA protein assay kit (PC0020;

Solarbio). Protein lysates were subjected to SDS-PAGE and transferred onto methanol-activated PVDF membranes. The membranes were then blocked in blocking buffer (P0023B; Beyotime, Shanghai, China) and incubated with primary antibodies overnight at 4°C. The primary antibodies were subsequently removed, and the membranes were washed three times with 1× TBST before incubation with the horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. The protein bands were visualized via a high-sensitivity enhanced chemiluminescence (ECL) kit (P2200; NCM Biotech, Suzhou, China) and a chemiluminescent imaging system (Vilber, Paris, France). The following primary antibodies were used: EGR1 (4154S; Cell Signaling Technology, Danvers, USA), BTG2 (22339-1-AP; Proteintech, Wuhan, China), PI3K (4292S; Cell Signaling Technology), phospho-PI3K (4228T; Cell Signaling Technology), AKT (4691; Cell Signaling Technology), phospho-AKT (4060; Cell Signaling Technology), ERK1/2 (T40071; Abmart, Shanghai, China), phospho-ERK1/2 (T40072; Abmart), and GAPDH (60004-1-Ig; Proteintech).

Cell viability assay

Cell viability was measured via the Cell Counting Kit 8 (CCK-8) assay following the manufacturer's protocol. In brief, approximately 1×10^4 cells (100 μ L/well) were seeded into each well of a 96-well plate and cultured with 10 μ L of CCK-8 reagent (BOSTER, Wuhan, China) at 37°C for 2 h. The absorbance at 450 nm was measured via a microplate reader (BioTek Synergy Neo2, Winooski, USA) to evaluate cell activity.

Apoptosis and cell cycle analysis

Apoptotic cells were assessed via an Annexin V-FITC/PI apoptosis kit (E-CK-A218; Elabscience, Wuhan, China). Approximately 1×10^6 cells per well were seeded into a 6-well plate and treated with DMSO, MLN4924, or MLN4924 + NAC. After 24 h, the cells were harvested, washed with phosphate-buffered saline (PBS), and resuspended in binding buffer. The cells were then incubated with APC and 7AAD reagents at room temperature in the dark for 20 min. Following incubation, the samples were analyzed via flow cytometry (NovoCyte Quanteon, Singapore) to determine the proportion of cells at various stages of apoptosis. To assess apoptosis in transfected leukemia cells, an Annexin V-APC/7-AAD Apoptosis Kit (E-CK-A218; Elabscience) was used, and the same pretreatment method described above was used. For cell cycle analysis, 5×10^5 cells were collected, stained with 10 μ L of PI solution at room temperature for 30 min and detected via flow cytometry.

Immunofluorescence (IF) staining

Clean coverslips were placed at the bottom of a 24-well plate and coated with poly-L-lysine (C0313; Beyotime). Subsequently, 2×10^5 cells per well were seeded in the plate and treated with MLN4924 at 37°C for 24 h. Next, a 4% paraformaldehyde solution was used to fix the cells at room temperature for 20 min, after which three washes with PBS were performed. The cells were then permeabilized with 0.2% Triton X-100 for 15 min and washed with PBS. Then, 3% BSA was added and incubated for 1 h, after which the primary antibody was added, and the mixture was incubated overnight at 4°C. The cells were washed with PBS and incubated with a fluorescently labeled secondary antibody (RGAR004; Proteintech) at room temperature in the dark for 1 h. After the cells

were washed with PBS, DAPI staining solution was added, and the cells were incubated in the dark for 10 min. Finally, the cells were washed with PBS and mounted onto a slide using anti-fade mounting medium, and images were captured under a fluorescence microscope (ECLIPSE Ti2-E; Nikon, Tokyo, Japan).

Reactive oxygen species (ROS) detection

The level of intracellular ROS was evaluated via an ROS detection kit (CA1420; Solarbio). The cells were collected and adjusted to a concentration of 1×10^6 cells/mL in PBS. Then, 200 μ L of the cell suspension was transferred to a 96-well plate. Next, 1 μ L of dihydroethidium (DHE) red fluorescent dye was added, and the cells were incubated at 37°C for 30 min. After incubation, the staining solution was removed, and the cells were washed twice with PBS. Finally, the cells were detected via fluorescence microscopy.

Chromatin immunoprecipitation (ChIP) assay

A ChIP assay kit (P2078, Beyotime) was used to conduct chromatin immunoprecipitation assays following the manufacturer's instructions. Initially, the cells were incubated with 37% formaldehyde at a concentration of 1% at 37°C for 10 min to facilitate DNA-protein crosslinking, after which glycine solution was added to terminate the crosslinking process. Cell lysis was performed on ice for 10 min using SDS lysis buffer containing PMSF. The genomic DNA was fragmented via sonication (10 seconds on, 20 seconds off, 70 watts, 30 cycles) with a Scientz-IID sonicator (Scientz, Ningbo, China). The sonicated samples were diluted with ChIP dilution buffer containing PMSF, followed by immunoprecipitation with either an EGR1 ChIP-grade antibody (22008-1-AP; Proteintech) or a normal rabbit IgG antibody (30000-0-AP; Proteintech). The following day, the immunoprecipitation complexes were incubated with protein A+G beads at 4°C with slow rotation for 1 h. The precipitate was subsequently washed with low-salt, high-salt, LiCl, and TE wash buffers. Next, elution buffer was added, and the mixture was incubated at room temperature for 5 min with continuous rotation. The DNA-protein complexes were de-crosslinked at 65°C for 4 h. DNA was extracted via a PCR purification kit (D0033; Beyotime) and subjected to RT-qPCR analysis. The primer sequences are listed in [Supplementary Table S1](#).

Dual-luciferase reporter assay

The WT/MT sequence of the synthesized *BTG2* promoter was cloned and inserted into the pGL3-basic vector, while the coding sequence (CDS) of *EGR1* was inserted into the pcDNA3.1 vector. The cells were subsequently seeded in 24-well plates for co-transfection with the appropriate reporter plasmids. The cells were incubated at 37°C for 48 h. Finally, luciferase activity was assessed via a Double-Luciferase Reporter Assay Kit (JKR23008; Genecreate, Wuhan, China) following the manufacturer's operating guidelines.

Lentiviral transduction

The *EGR1* knockdown lentivirus, *EGR1* overexpression lentivirus, *BTG2* knockdown lentivirus, *BTG2* overexpression lentivirus, and blank vector lentivirus were purchased from Genechem Co., Ltd (Shanghai, China). HL-60 and MOLM-13 cells were seeded into a 24-well plate at 2×10^5 cells/well. The cells were transduced with the lentiviral particles at an appropriate MOI of 50, and HitransG P or HitransG A (Genechem Co., Ltd, Shanghai, China) were used to

increase the infection efficiency. After incubation at 37°C for 12 h, the medium was refreshed. The infection efficiency was observed under a fluorescence microscope after 72 h. Cells were screened using puromycin, and infection efficiency was validated with RT-qPCR and western blot analysis. Lentiviral vectors expressing shRNAs targeting EGR1 and BTG2 were designed as follows: shEGR1#2 (5'-GCCTGCGACATCTGTGAAGA-3'), shEGR1#3 (5'-GCTGTCACTCACTCTTCAGC-3'), shBTG2#2 (5'-GCATTGCGATCAACCACAAGA-3'), shBTG2#3 (5'-GCCAGATCGGACTCAGCAGC-3'), and the scramble shRNA sequence (negative control) was 5'-TTCTCCGAACGTGTCACGT-3'.

Animal models

Female NOD/SCID mice (5–6 weeks old, weighing 20 ± 2 g) used for the tumor xenograft experiment were obtained from Cavens Experimental Animal Co., Ltd. (Changzhou, China). This study received approval from the Institutional Ethics Committee at the First Hospital of Lanzhou University (approval number: LDYLL-2025-787). The experimental animals were randomly divided into four groups, with five mice in each group. Mice in Groups 1 and 2 were subcutaneously injected with 5×10^6 HL-60 cells, suspended in 200 μ L of PBS, into the right flank. Meanwhile, the remaining two groups received subcutaneous injections of 5×10^6 MOLM-13 cells infected with EGR1-overexpressing vector or an empty vector, suspended in 200 μ L of PBS, into the right flank. The mice were monitored every two days. Once tumors formed, Group 1 received an intraperitoneal injection of 200 μ L of MLN4924 at 60 mg/kg per mouse, while Group 2 was administered with the same volume of PBS. Both groups were treated once daily for a total of 10 consecutive days. The remaining two groups were solely observed, with body weight recorded and tumor size measured every other day. At the end of experiment, the mice were euthanized, and the tumor tissues were collected, weighed, photographed, and used for subsequent analyses.

Immunohistochemistry (IHC)

The tumor tissue was initially fixed in 4% paraformaldehyde, followed by embedding in paraffin and slicing into thin sections of 4 μ m. The sections were dewaxed in xylene and rehydrated in ethanol. Antigen retrieval was performed using a pressure cooker. To inhibit endogenous peroxidase activity, the sections were incubated in a 3% hydrogen peroxide solution at room temperature in the dark for 25 min. Subsequently, the sections were blocked with goat serum and incubated with the anti-EGR1 (55117-1-AP; Proteintech), anti-BTG2 (22339-1-AP; Proteintech), and anti-Ki67 (27309-1-AP; Proteintech) antibody at 4°C overnight. After washing with PBS, the HRP-conjugated secondary antibody (Servicebio) was applied and incubated at room temperature for 50 min, after which DAB chromogen was added. The nuclei were counterstained with hematoxylin for 1–2 min. Following dehydration and mounting, the sections were imaged using a digital slide scanner (3DHISTECH, Budapest, Hungary) and analyzed with analysis software for visualization.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 8. Unpaired Student's *t*-tests were used to assess significant differences between the two groups. For comparisons involving three or more groups, one-way or two-way ANOVA was applied. The

significance levels were set at $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$. The Pearson correlation coefficient was utilized to evaluate the relationship between the two variables.

Results

Transcription factor EGR1 is upregulated after MLN4924 treatment

Previous studies have demonstrated that MLN4924 inhibits the proliferation of AML cells and induces apoptosis [26,27]. To investigate the transcriptional targets in the inhibition of AML progression mediated by MLN4924, we obtained a total of 429 differentially expressed genes (DEGs) across three concentrations (IC30, IC50, and IC80) from the RNA-seq analysis. A total of 1639 human TFs were retrieved from the humanTFs database (<https://humantfs.ccb.utoronto.ca/allTFs.php>). Ultimately, 38 TFs were found to be differentially expressed, including EGR1, which was significantly upregulated by MLN4924 (Figure 1A,B). We identified four candidate genes by intersecting the DEGs from the AML dataset GSE37642 with 38 TFs: *HIVEP2*, *HLF*, *EGR1*, and *AHR* (Figure 1C). The protein-protein interaction (PPI) network was visualized using Cytoscape software, which showed that EGR1 is centrally positioned within the network (Figure 1D). As a result, we chose EGR1 as the primary focus of our research. RT-qPCR assays confirmed that *EGR1* expression increased following treatment with MLN4924 (0.3 μ M) for 24 h in MOLM-13, HL-60, Kasumi-1, and K562 cells, consistent with the RNA-seq data (Figure 1E). Furthermore, Figure 1F demonstrates that MLN4924 enhances EGR1 protein levels in a concentration-dependent manner across all four cell lines.

EGR1 is a potential novel therapeutic target for MLN4924 in AML treatment

EGR1 is a TF that has been shown to play a dual role in various cancers. However, its function in AML remains controversial. Therefore, we compared *EGR1* expression levels in AML patients and normal PBMCs using RT-qPCR. The results demonstrated that *EGR1* expression was significantly lower in AML patients (Figure 2A). Moreover, Kaplan-Meier analysis revealed that AML patients with low EGR1 expression had significantly lower overall survival compared to those with high EGR1 expression (Figure 2B). Considering that *EGR1* mRNA displayed the lowest expression level in the HL-60 cell line and the highest in the MOLM-13 cell line (Supplementary Figure S1A), we selected these two cell lines for the construction of stable transfection *EGR1* knockdown and overexpression cell lines (Figure 2C,D and Supplementary Figure S1B). EGR1 overexpression significantly inhibited the growth of both HL-60 and MOLM-13 cells, while *EGR1* knockdown notably promoted the growth of MOLM-13 cells, as demonstrated by CCK-8 assay (Figure 2E and Supplementary Figure S1C). Next, we performed apoptosis analysis by flow cytometry. As shown in Figure 2F and Supplementary Figure S1D, EGR1 overexpression in HL-60 and MOLM-13 cells resulted in a marked increase in apoptosis compared to the control group. In contrast, *EGR1* knockdown in MOLM-13 cells resulted in a decreased apoptosis rate. Considering that the inhibition of cell proliferation and promotion of apoptosis may be associated with dysregulation in cell cycle mechanisms, we examined the impact of EGR1 on cell cycle progression in AML cells. The results indicated that EGR1 overexpression arrested the cell cycle in the G0/G1 phase, whereas *EGR1* knockdown promoted the transition from G0/G1 to the S phase (Figure 2G and

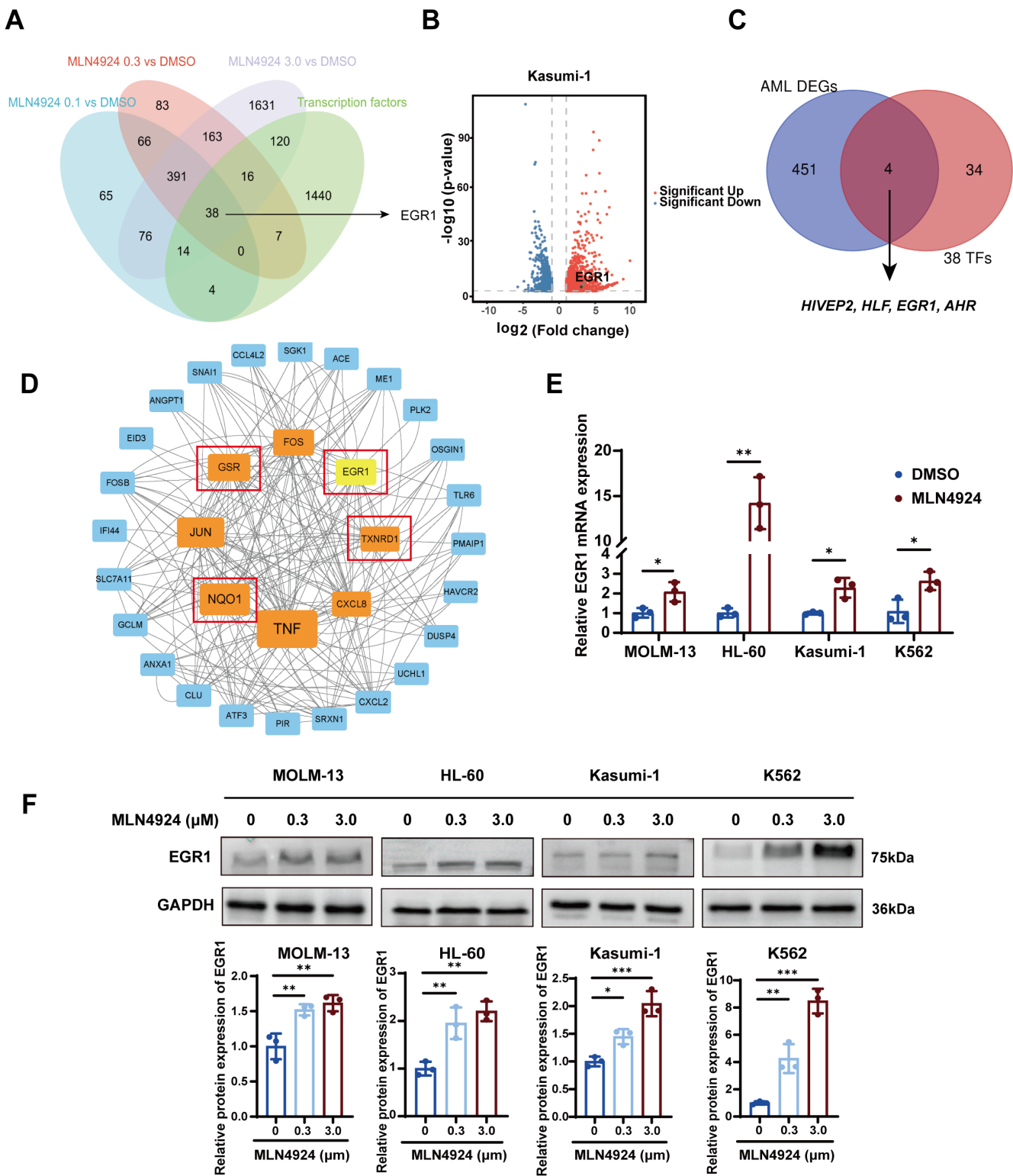


Figure 1. EGR1 is upregulated after MLN4924 treatment (A) Venn diagram of the intersection among DEGs in Kasumi-1 cells treated with three different concentrations of MLN4924 for 24 h and in human TFs database. (B) Volcano plot of RNA-seq analysis showed 429 DEGs following MLN4924 treatment. (C) The Venn diagram of the overlap between 455 DEGs in AML patients from the GEO database and 38 screened TFs. (D) Cytoscape was used to rank the top 8 hub genes of the PPI network (the top 8 genes are displayed in the inner circle). (E) RT-qPCR results of four AML cells treated with MLN4924 (0.3 μM) for 24 h. (F) Western blot results of four AML cell lines treated with MLN4924 for 24 h. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

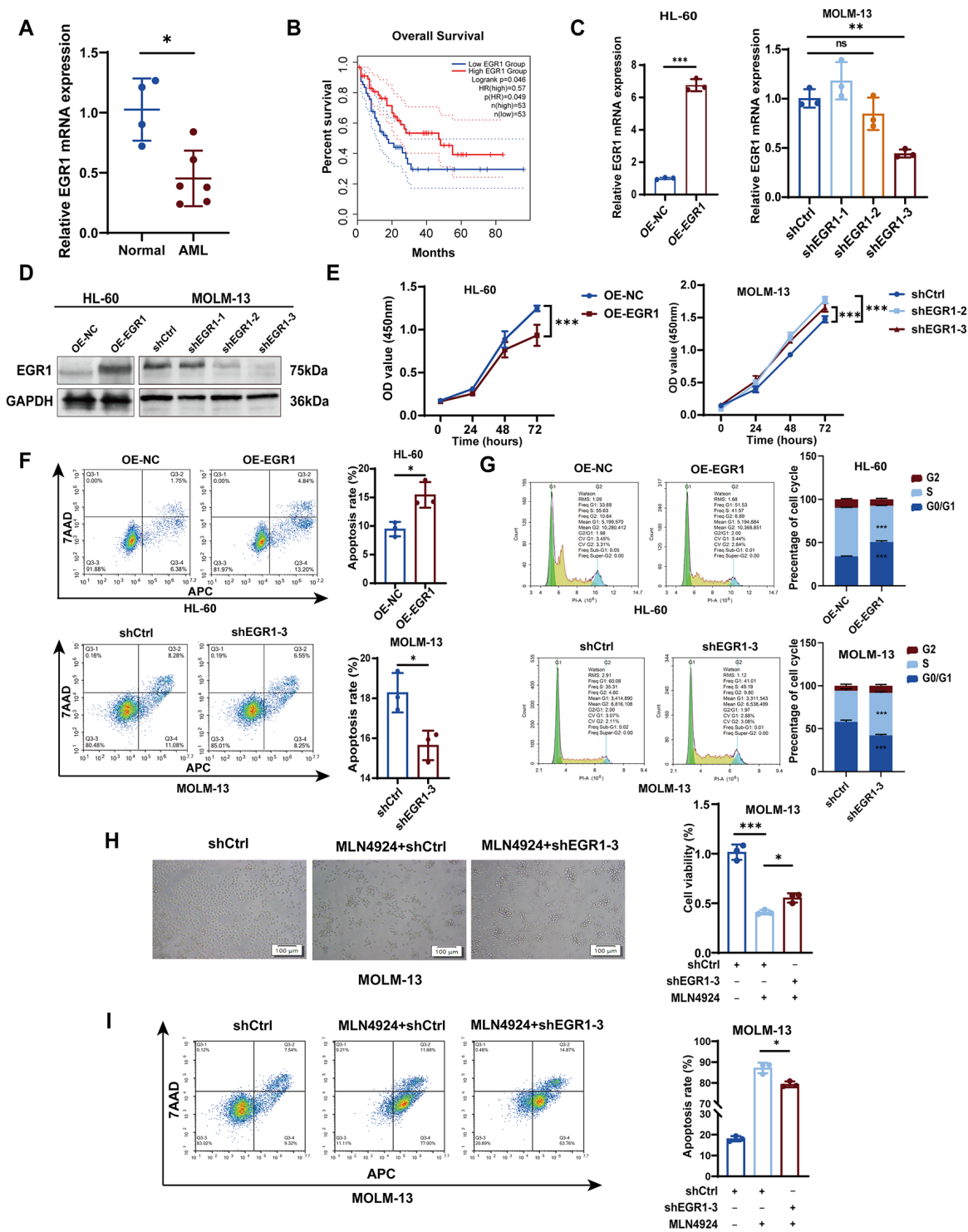


Figure 2. MLN4924 inhibits AML cell growth by upregulating EGR1 (A) The *EGR1* mRNA expression in normal hematopoietic cells and AML patients. (B) Overall survival analyses between AML patients with high and low EGR1 expression in the GEPIA database. (C,D) RT-qPCR and western blot analyses showing EGR1 expression in HL-60 and MOLM-13 cells after lentivirus transfection. (E) CCK-8 assay was used to evaluate the cell viability of HL-60 and MOLM-13 cells with EGR1 overexpression or *EGR1* silencing. (F,G) The effects of EGR1 overexpression and knockdown on the apoptosis and cell cycle distribution of HL-60 and MOLM-13 cells were evaluated by flow cytometry. (H) Cell morphology of MOLM-13 cells transduced with shNC or shEGR1-3 was examined under a microscope after 24 h of exposure to MLN4924 (0.3 μ M). CCK-8 assay was used to detect the cell viability of MOLM-13 cells transduced with shNC or shEGR1-3 and treated with MLN4924 (0.3 μ M) for 24 h. Scale bar: 100 μ m. (I) Flow cytometry was used to detect the apoptosis rate of MOLM-13 cells transduced with shNC or shEGR1-3 and treated with MLN4924 (0.3 μ M) for 24 h. * P <0.05, ** P <0.01, and *** P <0.001.

Supplementary Figure S1E). To confirm that EGR1 is the primary target of MLN4924 in inhibiting AML, we conducted rescue experiments using MOLM-13 cells with *EGR1* knockdown to evaluate cell proliferation and apoptosis, both with and without MLN4924 treatment. Notably, MOLM-13 cells with *EGR1* knockdown, treated with MLN4924, exhibited improved morphology and increased cell counts. The CCK-8 assay revealed that *EGR1* knockdown diminished the antiproliferative effects of MLN4924 on MOLM-13 cells (Figure 2H). Additionally, flow cytometry results showed that *EGR1* knockdown reversed the pro-apoptotic effects of MLN4924 (Figure 2I). These results suggested that EGR1 may serve as a novel therapeutic target for MLN4924 to suppress AML progression.

Identification of *BTG2* as a novel downstream target gene of *EGR1* in AML cells

To elucidate the molecular mechanism by which EGR1 inhibits AML cell growth, we performed RNA-seq experiments on MOLM-13 cells with EGR1 overexpression. Our analysis revealed that EGR1 regulated a total of 490 genes, with 427 upregulated and 63 downregulated (Fold change > 1.5, $P < 0.05$; Figure 3A,B). KEGG pathway analysis identified DEGs enriched in the PI3K/AKT pathway. Gene Set Enrichment Analysis (GSEA) revealed that EGR1 overexpression led to the negative regulation of the PI3K/AKT pathway (Figure 3C). Western blot analysis confirmed that EGR1 overexpression reduced the phosphorylation levels of both PI3K and AKT, while the total levels of PI3K and AKT remained unchanged (Figure 3D). Importantly, *EGR1* knockdown reversed the inhibitory effect of MLN4924 on the PI3K/AKT pathway (Figure 3E). Furthermore, MLN4924 significantly inhibited the phosphorylation of PI3K/AKT, while treatment with SC79 notably increased the level of AKT phosphorylation (Figure 3F), indicating that AKT activity had been restored. Treatment with SC79 effectively reversed the inhibitory effects of MLN4924 on cell proliferation and significantly alleviated the pro-apoptotic effects of MLN4924 on AML cells (Figure 3G,H). Additionally, ChIP-seq data for *EGR1* from K562 and HL-60 cells were retrieved from the Gene Expression Omnibus (GEO) database (GSE105316 and GSE106359). These ChIP-seq datasets were intersected with predicted target genes from the TF database GTRD, as well as genes exhibiting significant correlation predicted by GEPIA ($PCC > 0.25$). This comprehensive analysis identified 42 target genes strongly correlated with *EGR1* (Figure 3I). We then intersected these 42 target genes with RNA-seq data from *EGR1*-overexpressing cells, leading to the identification of four candidate genes (*BTG2*, *KLF2*, *KLF6*, and *NAB2*) for further investigation (Figure 3J). RT-qPCR was performed to verify the expression levels of these genes in MOLM-13 and HL-60 cells with stable *EGR1* overexpression, as well as in their corresponding negative control cells. The results showed that *BTG2* and *KLF2* were significantly upregulated in both MOLM-13 and HL-60 cells with *EGR1* overexpression (Figure 3K). PPI network analysis of DEGs revealed a significant regulatory relationship between EGR1 and BTG2 (Supplementary Figure S2A). Therefore, *BTG2* was selected for further investigation as a candidate target gene of *EGR1*. Western blot analysis showed that EGR1 overexpression in MOLM-13 and HL-60 cells resulted in increased BTG2 protein levels (Figure 3L). In contrast, *EGR1* knockdown in MOLM-13 cells led to decreased *BTG2* mRNA and protein expression levels (Figure 3M, N). These findings suggest a positive regulatory relationship

between EGR1 and BTG2 at the protein level, showing that BTG2 functions as a downstream target of EGR1 in AML cells. In addition, RT-qPCR analysis of *EGR1* and *BTG2* mRNA expression was performed in 32 clinical AML patients at diagnosis. The Pearson correlation coefficient between the two genes was $r = 0.91$, indicating a strong correlation (Figure 3O), consistent with results from the GEO and starBase databases (Supplementary Figure S2B). Collectively, these results suggest that *BTG2* is a novel downstream target gene of *EGR1* in AML cells.

BTG2 suppresses AML progression and mediates the pro-apoptotic effect of MLN4924

To investigate the role of *BTG2* in AML, we assessed its expression in 21 AML patients and 10 normal samples. RT-qPCR analysis revealed that *BTG2* expression was significantly downregulated in AML patients compared to normal samples (Figure 4A). Kaplan-Meier survival analysis showed that high BTG2 expression was associated with improved survival in AML patients (Figure 4B). To further explore the biological functions of BTG2 in AML, we established stable MOLM-13 and HL60 cell lines with *BTG2* knockdown or overexpression using lentiviral vectors (Figure 4C, D and Supplementary Figures S3A,B). CCK-8 assays demonstrated that upregulation of BTG2 inhibited AML cell proliferation, while downregulation of BTG2 promoted proliferation (Figure 4E and Supplementary Figure S3C). The apoptosis rate in the BTG2 overexpression group was significantly higher than that in the control group, whereas the apoptosis rate in the *BTG2* knockdown group was lower (Figure 4F and Supplementary Figure S3D). Additionally, BTG2 overexpression notably increased the proportion of cells in the G0/G1 phase, whereas *BTG2* knockdown decreased the proportion of cells in the G0/G1 phase, accompanied by an increase in cells in the S phase (Figure 4G and Supplementary Figure S3E). To investigate whether MLN4924 could inhibit AML progression through BTG2 regulation, RT-qPCR and western blot analyses were performed on MOLM-13 and HL-60 cells treated with MLN4924. The results showed a dose-dependent increase in BTG2 expression (Figure 4H,I). Furthermore, the knockdown of *BTG2* inhibited the MLN4924-induced upregulation of *BTG2* at both the mRNA and protein levels (Figure 4J,K). CCK-8 and flow cytometry analyses revealed that MLN4924 effectively inhibited AML cell proliferation and promoted apoptosis. Importantly, *BTG2* knockdown significantly reversed the antiproliferative and pro-apoptotic effects of MLN4924 on AML cells (Figure 4L,M).

MLN4924 induces AML cells death through ROS-mediated EGR1/BTG2 expression

Previous studies have demonstrated that MLN4924 is involved in regulating ROS [28]. In this study, we found that MLN4924 mediates the accumulation of ROS in AML cells. The results demonstrated a significant increase in ROS levels within 24 h of MLN4924 treatment, and the addition of NAC, a ROS scavenger, partially reversed the effects of MLN4924 on MOLM-13 and HL-60 cells (Figure 5A). To verify whether ROS accumulation plays a pivotal role in MLN4924-induced apoptosis, we compared microscopy images of MOLM-13 and HL-60 cells treated with MLN4924, both with and without NAC, over 24 h. The results indicated that the combination group (MLN4924 + NAC) exhibited intact cell morphology and increased cell counts compared to treatment with MLN4924 alone. Furthermore, NAC mitigated the inhibition of cell

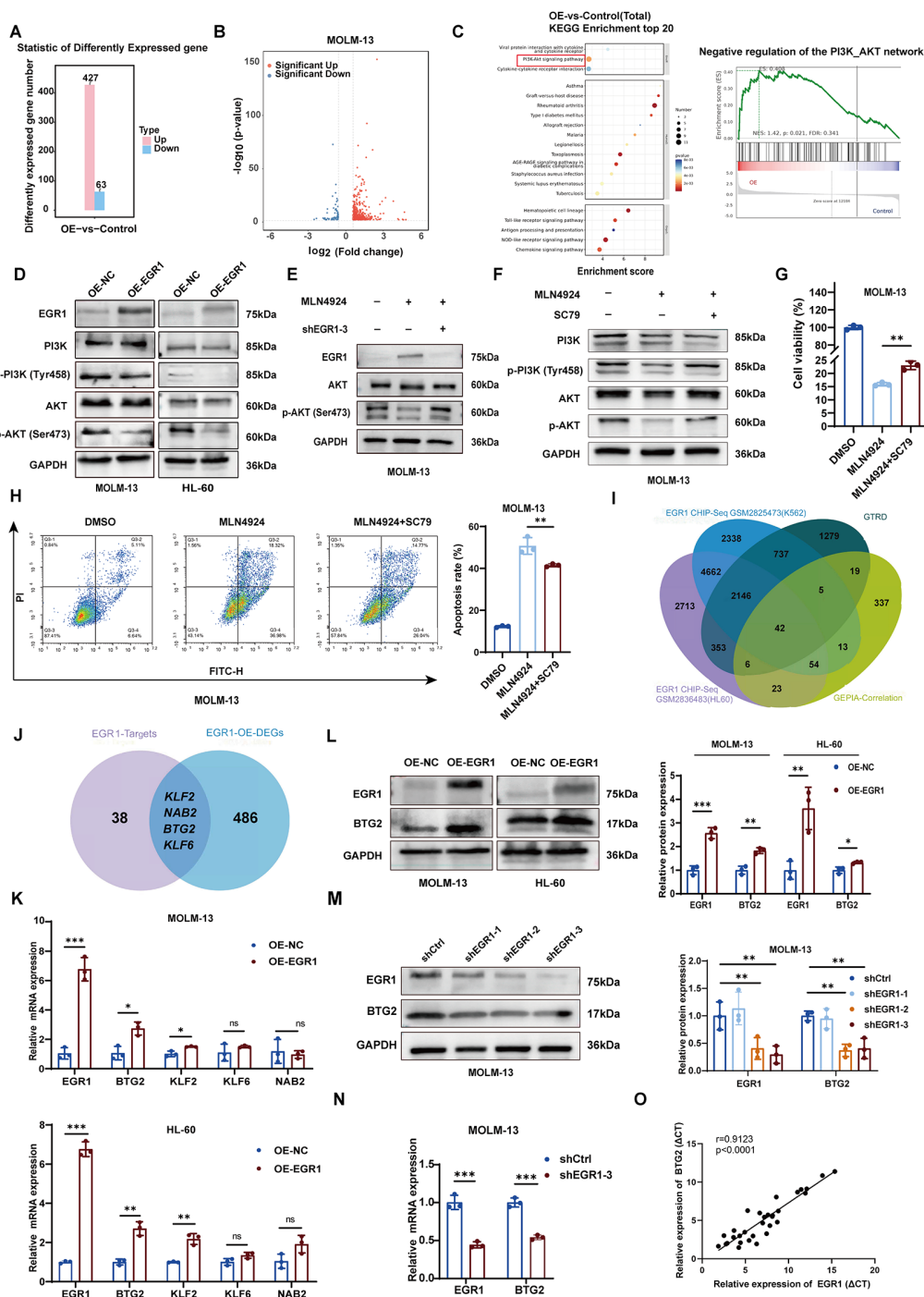


Figure 3. BTG2 is a novel downstream target of EGR1 in AML (A,B) The bar chart and volcano plot displaying 490 DEGs between the EGR1 overexpression group and the control group. (C) Enrichment pathways of DEGs in MOLM-13 cells overexpressing EGR1 and control cells were analyzed using KEGG enrichment analysis and GSEA. (D) Western blot analysis of the effect of EGR1 overexpression on the PI3K/AKT signaling pathway in MOLM-13 and HL-60 cells. (E) Western blot results showing that the knockdown of *EGR1* reversed the inhibition of the PI3K/AKT signaling pathway by MLN4924 (0.3 μ M) in MOLM-13 cells. (F) Western blot analysis showing that SC79 (5 μ M) effectively restored the AKT activity that had been inhibited by MLN4924 (0.3 μ M) in MOLM-13 cell lines. (G) The CCK-8 assay indicated that SC79 (5 μ M) treatment for 48 h restored the inhibitory effect of MLN4924 (0.3 μ M) on cell proliferation. (H) Flow cytometry results showed that SC79 (5 μ M) rescued MLN4924 (0.3 μ M)-induced apoptosis in MOLM-13 cells. (I,J) The Venn diagram illustrating the intersection of EGR1 ChIP-seq gene sets (GSE105316, GSE106359), *EGR1* AML correlation analysis gene sets, GTRD datasets, and EGR1 overexpression differential genes. (K) RT-qPCR was performed to analyze the expression of four potential target genes following the overexpression of EGR1 in MOLM-13 and HL-60 cell lines. (L,M) Western blot analysis of BTG2 protein expression levels following EGR1 overexpression and knockdown in AML cells. (N) RT-qPCR was performed to analyze the expression of *BTG2* following the *EGR1* knockdown. (O) Correlation analysis was performed to evaluate the association between *EGR1* and *BTG2* in 32 clinical AML patients. * P < 0.05, ** P < 0.01, *** P < 0.001, and ns (not significant).

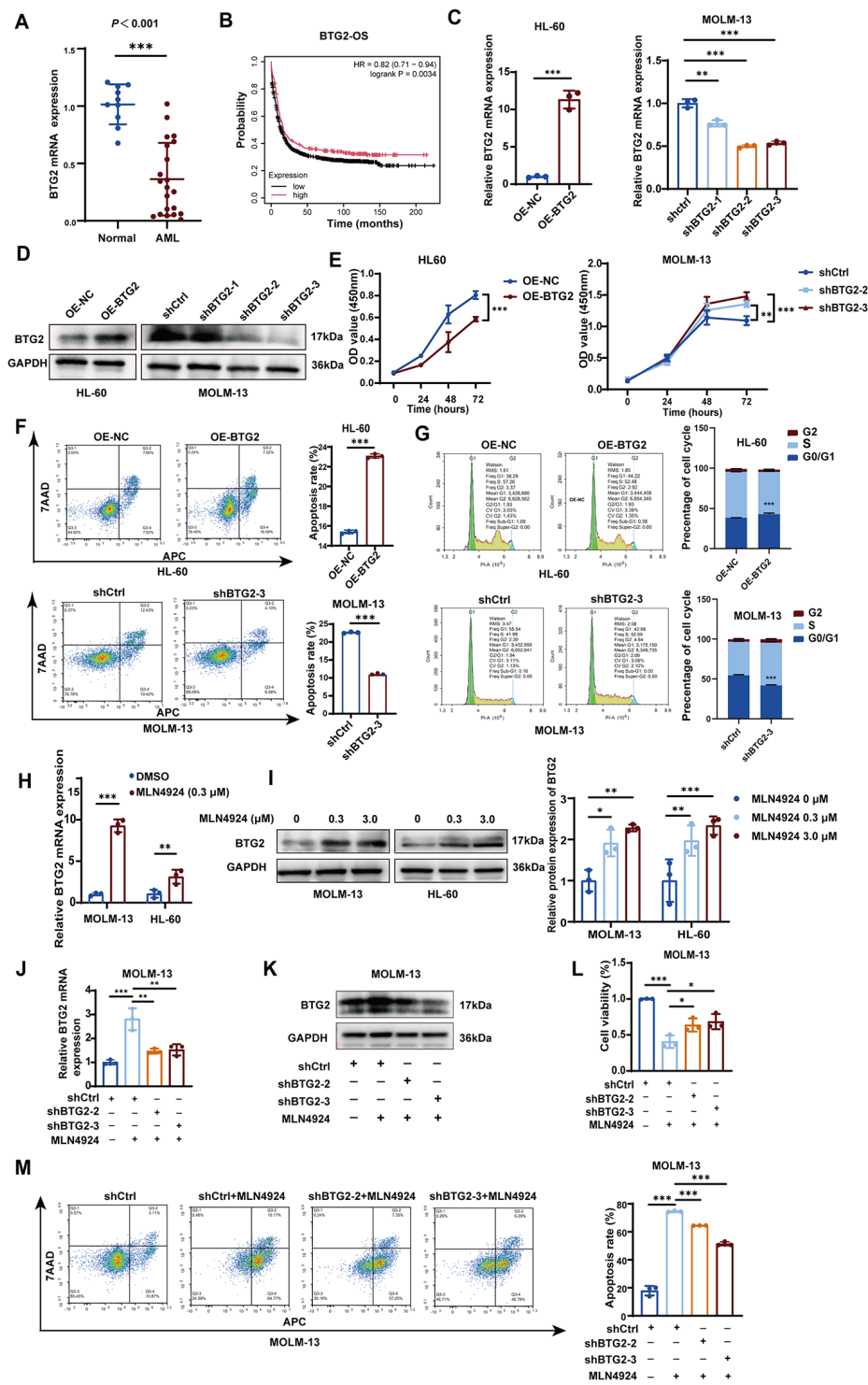


Figure 4. MLN4924 induces apoptosis in AML cells by upregulating BTG2 expression (A) RT-qPCR showed that *BTG2* was downregulated in AML patients. (B) The Kaplan-Meier Plotter was used to analyze the overall survival between AML patients with high or low *BTG2* expression. (C,D) RT-qPCR and western blot analyses showing *BTG2* expression in HL-60 and MOLM-13 cells after lentivirus transfection. (E) CCK-8 assay was used to evaluate the cell viabilities of HL-60 and MOLM-13 cells with *EGR1* overexpression or silencing. (F,G) The effects of *BTG2* overexpression and knockdown on the apoptosis and cell cycle distribution of HL-60 and MOLM-13 cells were evaluated by flow cytometry. (H,I) RT-qPCR and western blot analyses were performed to analyze the *BTG2* expression following MLN4924 treatment. (J,K) RT-qPCR and western blot analyses were used to detect the mRNA and protein levels in MOLM-13 cells with *BTG2* knockdown following treatment with MLN4924 (0.3 μ M). (L,M) Flow cytometry and CCK-8 assay were used to detect the apoptosis rate and cell viability of MOLM-13 cells transduced with shNC or shBTG2 and treated with MLN4924 (0.3 μ M) for 24 h. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$.

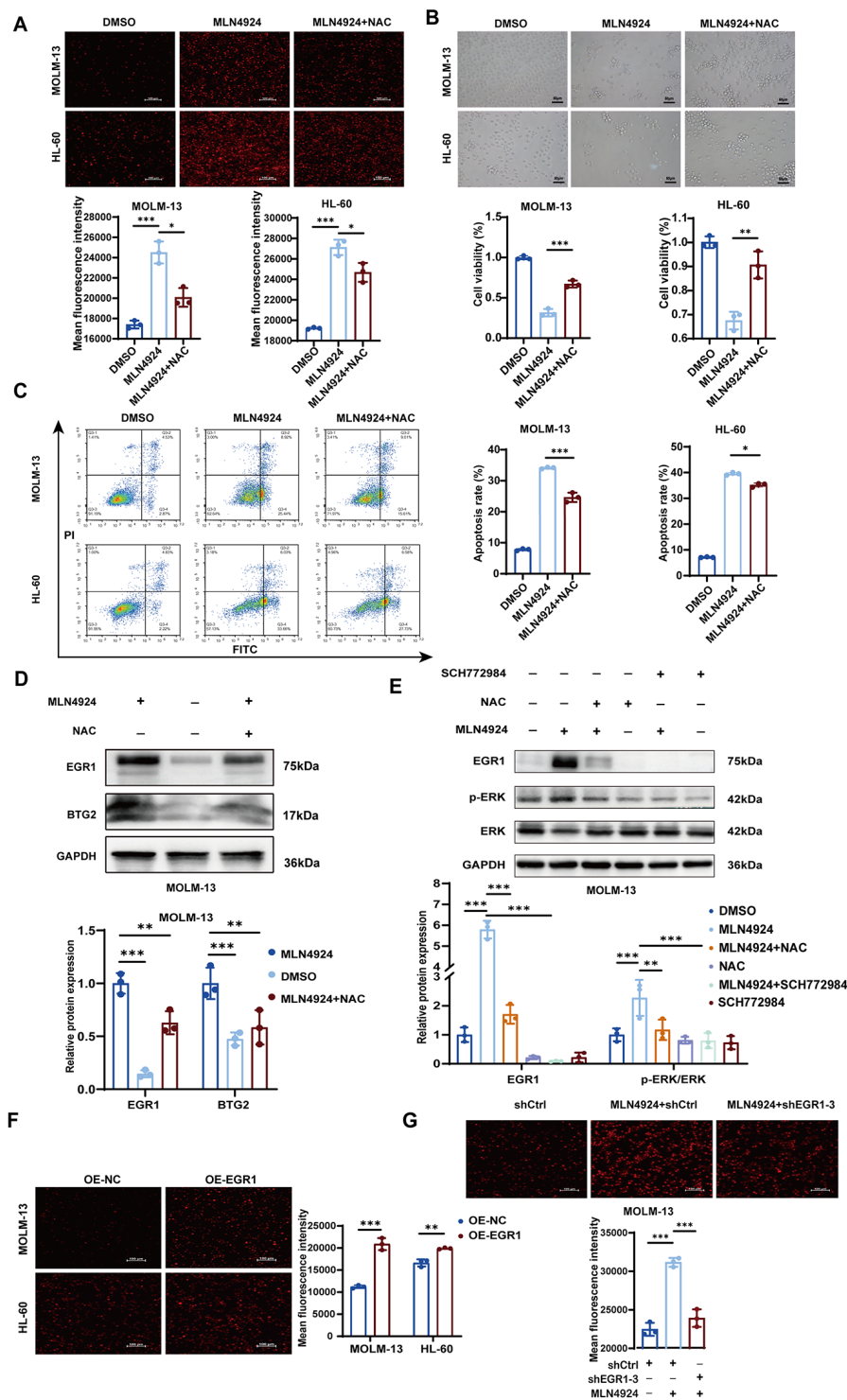


Figure 5. MLN4924 promotes cell death through the regulation of ROS-induced EGR1 and BTG2 (A) Detection of the accumulation of ROS in cells treated with MLN4924 (0.3 μ M), as well as in cells treated with MLN4924 (0.3 μ M) in combination with NAC (5 mM) for 24 h. Scale bar: 100 μ m. (B,C) The cell morphology of AML cells was observed under a microscope following exposure to MLN4924 (0.3 μ M), both alone and in combination with NAC (5 mM) for 24 h, cell viability and apoptosis rates were assessed by CCK-8 assay and flow cytometry, respectively. Scale bar: 50 μ m. (D) Western blot results of MOLM-13 cells treated with MLN4924 (0.3 μ M), as well as MLN4924 (0.3 μ M) in combination with NAC (5 mM) for 24 h. (E) Western blot results of MOLM-13 cells treated with MLN4924 (0.3 μ M), MLN4924 in combination with NAC (5 mM), as well as MLN4924 in combination with SCH772984 (5 μ M) for 24 h. (F) The accumulation of ROS in control MOLM-13 cells and MOLM-13 cells overexpressing EGR1. Scale bar: 100 μ m. (G) The accumulation of ROS in MOLM-13 cells transduced with shNC or shEGR1 and treated with MLN4924 (0.3 μ M) for 24 h. Scale bar: 100 μ m. * P < 0.05, ** P < 0.01, and *** P < 0.001.

viability induced by MLN4924 in MOLM-13 and HL-60 cells (Figure 5B). After co-incubation with NAC, the apoptosis rate in the combination group was lower than that observed in the MLN4924-only group (Figure 5C). These results suggest that the accumulation of ROS induced by MLN4924 triggers apoptosis in AML cells. Additionally, western blot analysis showed a significant increase in EGR1 and BTG2 protein expression in the presence of ROS. However, when NAC neutralized ROS, the expression levels of EGR1 and BTG2 decreased markedly (Figure 5D). To further explore the precise molecular mechanism, we found that inhibiting ROS reduced the expression of p-ERK1/2. The expression level of EGR1 was significantly decreased after treatment with the ERK inhibitor SCH772984 (Figure 5E). These results indicated that ROS facilitated the phosphorylation of ERK1/2, thereby activating EGR1. Interestingly, the fluorescence intensity of the ROS probe in the EGR1 overexpression group was significantly higher than that in the control group (Figure 5F). Furthermore, *EGR1* knockdown reduced the impact of MLN4924 on ROS generation (Figure 5G). MLN4924 inhibited AML cell growth by upregulating EGR1 and BTG2, as validated in the aforementioned results. Combining the results above, we demonstrated that MLN4924 induces apoptosis in AML cells via the ROS-mediated EGR1/BTG2 axis, highlighting a positive feedback loop between ROS and EGR1 in AML cells.

MLN4924 transcriptionally activates *BTG2* by upregulating *EGR1*

EGR1 is a TF that regulates metastasis, differentiation, and apoptosis in tumor cells. Upon exposure to stimuli such as tumor necrosis factors, inflammatory factors, ionizing radiation, and ROS, EGR1 can be activated through the MAPK signaling pathway [29]. However, it remains unclear whether EGR1 can be activated under MLN4924 treatment. To determine if MLN4924 activates EGR1, we performed immunofluorescence microscopy on MOLM-13 cells treated with MLN4924 for 24 h. Immunofluorescence staining revealed that MLN4924 stimulated the translocation of EGR1 to the nucleus and increased BTG2 expression (Figure 6A). We then used the Integrative Genomics Viewer (IGV) browser to analyze the binding of EGR1 to the *BTG2* promoter region, based on ChIP-seq data from the Cistrome DB. This analysis revealed a distinct binding peak of EGR1 in the *BTG2* promoter region (Figure 6B), and two potential binding motifs (P1 and P2) were identified using the JASPAR database (Figure 6C). ChIP assays indicated that MLN4924 (0.3 μ M) treatment for 24 h significantly increased EGR1 enrichment at the P1 site within the *BTG2* promoter region, while the P2 site had no significant difference (not shown). Similarly, in EGR1-overexpressing MOLM-13 cells, an enrichment of EGR1 was observed at the P1 region of the *BTG2* promoter (Figure 6D). Subsequently, dual-luciferase assays showed that *EGR1* significantly increased luciferase activity of the wild-type *BTG2* promoter but had no effect on the activity of *BTG2* promoter mutants at the P1 and P2 sites (Figure 6E). The RT-qPCR results indicated that when *EGR1* was knocked down, MLN4924 significantly reduced the transcription level of *BTG2* (Figure 6F). Furthermore, we generated a stable MOLM-13 cell line that overexpresses EGR1 while simultaneously knocking down *BTG2* (Figure 6G). To further investigate whether BTG2 influences EGR1-mediated proliferation and apoptosis in AML cells. We demonstrated that silencing of *BTG2* inhibited the antiproliferative effect of EGR1 by the CCK-8 assay (Figure 6H). Flow cytometry results showed that silencing of

BTG2 reversed the pro-apoptotic effect of EGR1 in MOLM-13 cells (Figure 6I). These results suggest that MLN4924 upregulates EGR1 expression, which subsequently increases BTG2 expression through transcriptional regulation.

MLN4924 triggers EGR1-dependent BTG2 upregulation *in vivo*

To further investigate the anti-tumor effect of MLN4924 *in vivo*, we established a subcutaneous xenograft model. After 10 days of treatment, the mice were euthanized, and the tumors were collected, weighed, and photographed (Figure 7A). Tumor weight in the MLN4924-treated group was significantly reduced compared to that in the control group. Tumor volume was measured every two days using calipers, and the tumor growth curve was plotted. MLN4924 treatment significantly reduced tumor growth rates (Figure 7B,C). To determine whether MLN4924 induces cell death in AML cells through the upregulation of EGR1 and BTG2 *in vivo*, we analyzed the expression levels of EGR1 and BTG2 by western blot analysis and immunohistochemistry. The expression levels of EGR1 and BTG2 were higher in MLN4924-treated tumor tissues compared to controls, consistent with the results from *in vitro* experiments (Figure 7D,E). To verify that EGR1 suppresses AML progression through upregulation of BTG2 *in vivo*, NOD-SCID mice were injected with MOLM-13 cells overexpressing EGR1. Tumor size was measured every three days, and after 27 days, all mice were euthanized (Figure 7F). The results showed that tumor weight in the EGR1 overexpression group was significantly lower than that in the control group, and tumor volume was also smaller in the *EGR1*-overexpressing mice (Figure 7G,H). Western blot analysis confirmed that BTG2 protein levels were upregulated in tissues from mice overexpressing EGR1 (Figure 7I). Ki67 staining revealed that EGR1 significantly inhibited tumor growth, with a marked increase in BTG2 expression (Figure 7J). These findings suggest that MLN4924 effectively inhibits tumor growth *in vivo* by regulating the EGR1/BTG2 axis.

Discussion

As the sole activating enzyme of neddylation, NAE has emerged as a promising target for treating hematological and solid tumors [30]. Numerous studies have confirmed that MLN4924, a novel NAE inhibitor, exhibits significant antitumor effects across various cancer types, particularly in hematological malignancies such as leukemia [15], myelodysplastic syndromes (MDS) [31], multiple myeloma (MM) [32], and lymphoma [33]. Despite ongoing research in AML, our understanding of the molecular mechanisms underlying MLN4924's effects in AML treatment remains limited. Increasing evidence suggests that targeting TFs may provide an effective strategy for cancer therapy. A significantly altered TF-EGR1 was identified by our study, through RNA-seq and the humanTFs database, addressing the gap in research exploring the relationship between MLN4924 and transcriptional regulation. Validation experiments demonstrated that MLN4924 significantly enhanced both mRNA and protein expression levels of EGR1, promoting its translocation to the nucleus. Further investigations revealed that EGR1 binds to the promoter region of the tumor suppressor gene *BTG2*, facilitating its transcriptional activation and inhibiting AML progression. Additionally, our findings indicated that MLN4924 stimulates the production of ROS and that a positive feedback loop exists between ROS and EGR1. In this study, we

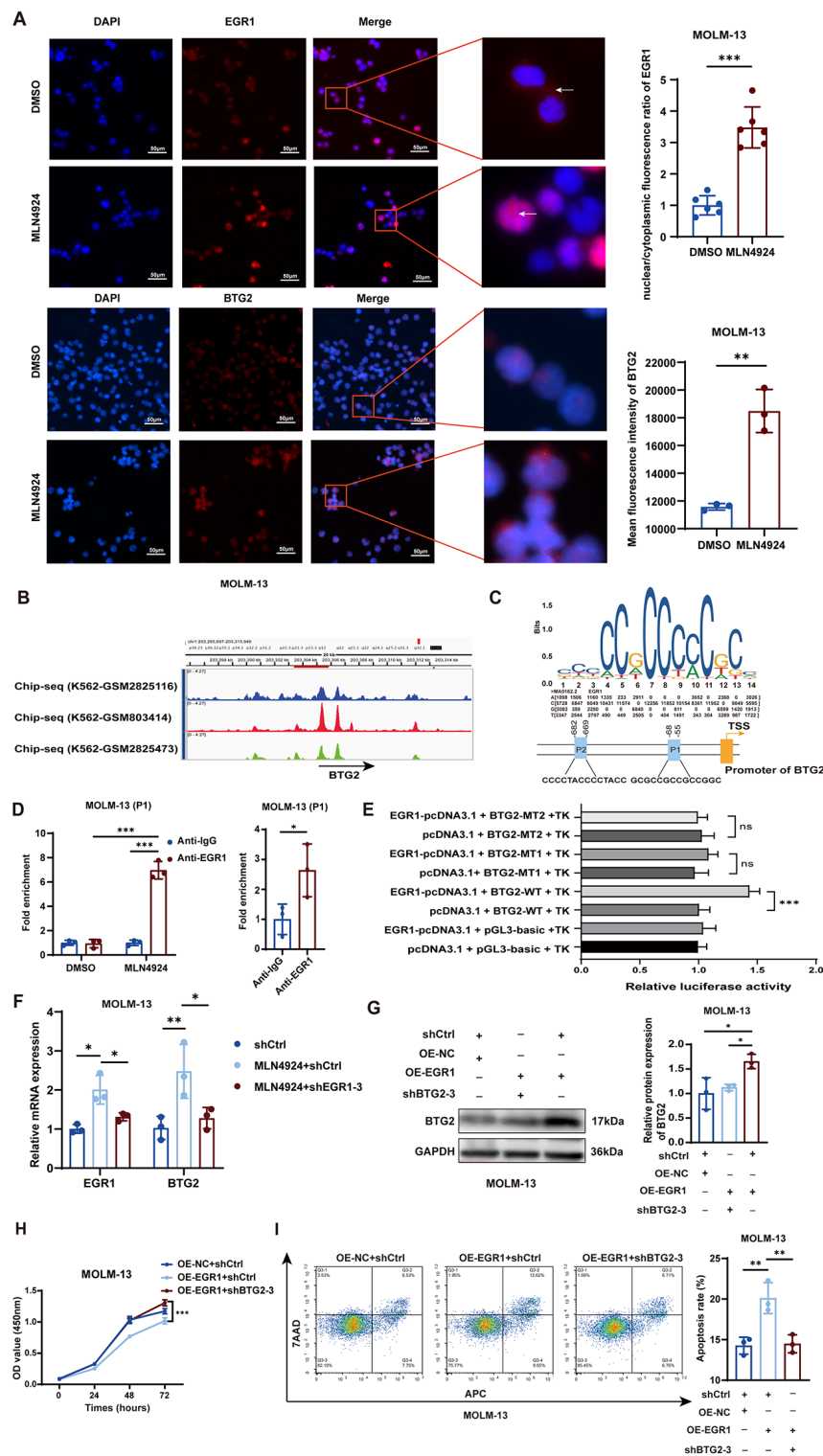


Figure 6. MLN4924 transcriptionally activates BTG2 by upregulating EGR1 (A) Immunofluorescence staining was used to assess the localization of EGR1 and expression of BTG2 in MOLM-13 cells treated with MLN4924 (0.3 μ M). Scale bar: 50 μ m. (B) The IGV genome browser displayed the binding peak of EGR1 in the *BTG2* promoter region from the CHIP-seq data. (C) JASPAR website predicted potential EGR1-binding site sequences and the binding sites in the *BTG2* promoter region. (D) CHIP assays were conducted in MOLM-13 cells. (E) The dual-luciferase reporter assay provided evidence for the binding of *EGR1* to the P1 and P2 sites of *BTG2* in 293T cells. (F) RT-qPCR analysis of *BTG2* transcriptional levels in MLN4924-treated MOLM-13 cells with *EGR1* knockdown. (G) Western blot analysis of the protein expression levels in the MOLM-13 cell line with *EGR1* overexpression and *BTG2* knockdown. (H,I) Flow cytometry and CCK-8 assays were conducted to investigate the pro-apoptotic and anti-proliferative effects of EGR1 in MOLM-13 cells with *BTG2* knockdown. * P < 0.05, ** P < 0.01, *** P < 0.001, and ns (not significant).

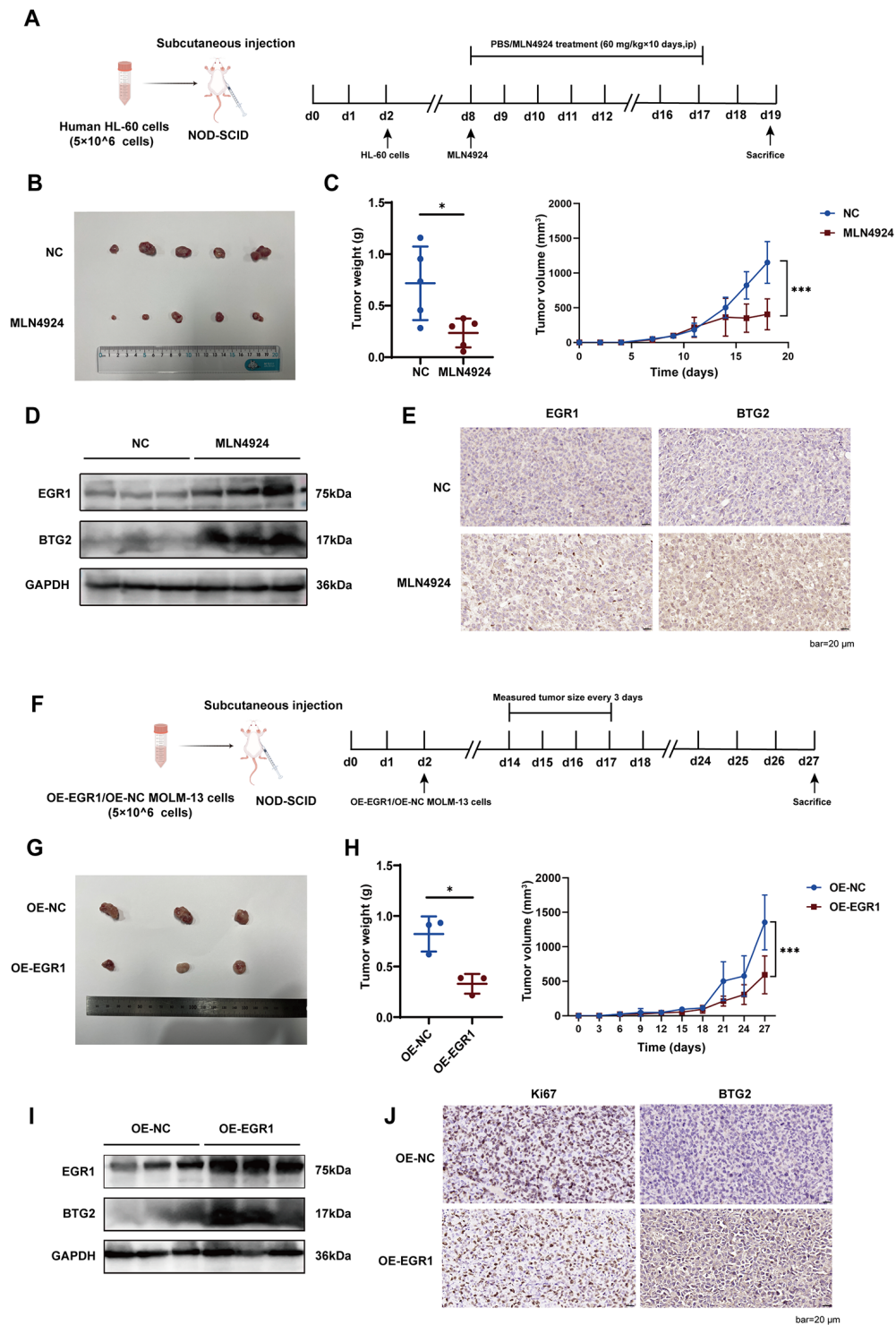


Figure 7. MLN4924 exerts anti-acute myeloid leukemia effects by upregulating EGR1 and BTG2 in a xenograft *in vivo* model (A) *In vivo* subcutaneous implantation of NOD-SCID mouse models induced by HL-60 cells with PBS or MLN4924 treatment. (B,C) HL-60 cells were utilized to investigate the effect of MLN4924 on the growth of xenograft tumors. Tumor volume was measured every two days. (D) Western blot analysis was used to detect the protein expression of EGR1 and BTG2 in xenograft tumors of mice treated with MLN4924. (E) Immunohistochemistry verified the expression levels of EGR1 and BTG2 in xenograft tumors of mice treated with MLN4924. Scale bar: 20 μ m. (F) *In vivo* subcutaneous implantation of NOD-SCID mouse models induced by OE-EGR1 or OE-NC MOLM-13 cells. (G,H) MOLM-13 cells were utilized to investigate the effect of EGR1 on the growth of xenograft tumors. Tumor volume was measured every three days. (I) Western blot analysis of the protein expression of EGR1 and BTG2 in EGR1 overexpression xenograft tumors. (J) Immunohistochemistry verified the expression levels of BTG2 and Ki67 in EGR1 overexpression xenograft tumors. Scale bar: 20 μ m. * P < 0.05 and *** P < 0.001.

demonstrated that MLN4924 induces apoptosis in AML cells via the ROS-EGR1-BTG2 axis. These findings suggest new therapeutic strategies and targets for AML, providing a theoretical foundation for precise treatments involving MLN4924.

The regulatory mechanism of MLN4924 on EGR1 involves the synergistic effects of multiple signaling pathways. A previous study has indicated that MLN4924 enhances the accumulation of EGR1 by inactivating the SCF^{FBXW7} (CRL1) complex, suggesting that EGR1 is a substrate protein of MLN4924 [34]. Our study demonstrated that *EGR1*, as an early stress response gene, can be activated by the ROS/ERK pathway, thereby exerting its transcriptional regulatory functions. However, the role of EGR1 in inhibiting AML progression via MLN4924 remains unclear. Several studies have suggested that EGR1 plays a dual role in malignant tumors. Liu and colleagues [35] reported that EGR1 recruits Septin 2 and Septin 9 to the promoters of *E2F1-3*, enhancing the transcription of these genes and promoting the progression of triple-negative breast cancer. Another study found that EGR1 is a crucial regulatory factor in myeloid differentiation [36]. Joslin and colleagues [37] discovered that haploinsufficiency of EGR1 contributes to the development of leukemia and the progression of AML/MDS, particularly the abnormalities in chromosome 5. Consistent with previous findings in myeloid neoplasms, we observed that EGR1 was expressed at low levels in AML patients and positively correlated with the overall survival of patients. Overexpression of EGR1 induced cell cycle arrest at the G0/G1 phase in AML cells, while promoting apoptosis and inhibiting proliferation. These studies suggest that the role of EGR1 in malignant tumors is highly dependent on tumor-type. In addition, we further discovered that knockdown of *EGR1* inhibited the antiproliferative and pro-apoptotic effects of MLN4924 on AML cells. Although this effect may be weak, it still holds statistical significance ($P < 0.05$). This weaker trend could be attributed to the broad target spectrum of MLN4924, which can lead to the simultaneous accumulation of both pro-apoptotic and pro-proliferative proteins [38]. The signaling pathway mediated by EGR1 may represent a significant branch activated by MLN4924; however, it is not the only determinant. When *EGR1* is knocked down, alternative targets or signaling pathways are activated, which partially compensate for the effects resulting from *EGR1* deficiency. These results suggest that EGR1 is a novel target for inducing apoptosis in AML cells via MLN4924. In addition, studies have demonstrated that EGR1 acts as a TF that primarily exerts its tumor-suppressive effects by enhancing the transcriptional activation of *PTEN* [39, 40]. This increase in *PTEN* expression subsequently inhibits the phosphorylation levels of PI3K and AKT [41]. Consistent with previous studies, our results demonstrated that overexpression of EGR1 reduced the phosphorylation levels of both PI3K and AKT. Furthermore, we discovered that the knockdown of *EGR1* can restore the inhibitory effect of MLN4924 on the PI3K/AKT pathway. The inhibition of the PI3K/AKT signaling pathway by MLN4924 is closely related to both cell proliferation and apoptosis. These discoveries provided a novel therapeutic strategy for MLN4924 in treating AML.

To further screen downstream regulatory molecules of EGR1 in AML, we conducted RNA-seq to identify DEGs in EGR1-over-expressing MOLM-13 and corresponding control cells. We identified *BTG2* as a downstream target gene regulated by *EGR1* through RNA-seq of MOLM-13 cells with EGR1 overexpression and GEO ChIP-seq data. The *BTG2* gene is a key member of the BTG/TOB

gene family and plays an essential role in biological processes such as cell cycle regulation, DNA damage repair, and tumor suppression. A study demonstrated that the combination therapy of T1-44 and Vactosertib exerts a synergistic effect by upregulating BTG2, thereby inhibiting pancreatic tumor growth [42]. However, the role of BTG2 in AML remains unclear. In this study, we found that BTG2 expression was low in AML patients, and its expression level was positively correlated with survival time. Overexpression of BTG2 led to cell cycle arrest in the G0/G1 phase, inhibiting cell proliferation and inducing apoptosis. In contrast, the knockdown of *BTG2* reduced the number of cells in the G0/G1 phase, increased the proportion of cells in the S phase, promoted cell proliferation, and inhibited apoptosis. Additionally, in MOLM-13 and HL-60 cell lines, MLN4924 significantly upregulated *BTG2* expression, consistent with RNA-seq data. Notably, the knockdown of *BTG2* diminished the anti-proliferative and pro-apoptotic effects of MLN4924 on AML cells, indicating that BTG2 is a crucial biomarker for MLN4924 in inhibiting AML progression.

Swords and colleagues [18] demonstrated that MLN4924 induces apoptosis in MV4-11 cells through ROS generation. MLN4924 generates ROS primarily through the inhibition of CRL activity, leading to the abnormal accumulation of pro-apoptotic and pro-stress substrate proteins [13,43]. This accumulation can induce mitochondrial dysfunction, disrupt the normal function of the electron transport chain, significantly increase electron leakage, and convert it to superoxide anion ($O_2^{\cdot-}$) [44]. Additionally, the accumulated stress signals may activate intracellular ROS-generating enzymes, such as NADPH oxidase (NOX), ultimately resulting in increased ROS production [45]. Our study suggested that inhibiting ROS generation not only reversed MLN4924-induced apoptosis but also mitigated its antiproliferative effects. A previous study showed that ROS stimulate the expression of EGR1, which in turn promotes the expression of the apoptosis-inducing factor Bax, ultimately leading to osteosarcoma cell apoptosis [46]. The MAPK signaling pathway is activated by ROS and plays a crucial role in regulating cell death [47]. Numerous studies indicated that increased levels of ROS trigger cell death through the activation of the MAPK pathway [48,49]. Chuang *et al.* [50] demonstrated that ROS enhance EGR1 expression via the ERK signaling pathway. In this study, MLN4924-induced ROS generation significantly increased EGR1 expression, while the removal of ROS markedly reduced EGR1 and BTG2 protein levels. A previous study has demonstrated that p-ERK enters the nucleus, where it phosphorylates Elk-1 to stimulate *EGR1* transcription upon cellular stimulation [51]. Subsequently, EGR1 quickly translocates to the nucleus. In our study, we found that inhibiting p-ERK reduced EGR1 expression levels, a relationship that was influenced by ROS. We also found that overexpression of EGR1 increased ROS production, while knockdown of *EGR1* reversed the ROS-inducing effects of MLN4924. These studies suggest that the activation of EGR1 is closely linked to ROS and ERK phosphorylation, and a positive feedback loop exists between ROS and EGR1.

Previous studies have shown that MLN4924 inhibits the ubiquitination and degradation of IKBA by inactivating CRL through the suppression of NAE [17,33]. The accumulation of IKBA inhibits the NF- κ B signaling pathway, leading to the downregulation of BCL2 family members, which ultimately suppresses the progression of AML. The inhibition of NF- κ B by MLN4924 serves as a key molecular mechanism underlying its significant anti-AML activity

[18]. Our research findings indicated a novel mechanism that differs from the previous studies. We further validated the hypothesis that MLN4924 inhibits AML progression through the ROS/EGR1-mediated transcriptional activation of *BTG2*. Rescue experiments revealed that knockdown of *BTG2* diminished the anti-proliferative and pro-apoptotic effects of EGR1. The role of MLN4924 as an activator of *BTG2* through the ROS/EGR1 axis is closely associated with its function as an inhibitor of NAE. MLN4924 induces the accumulation of ROS by inhibiting NAE activity [52]. Our study further demonstrated that ROS enhanced the expression of EGR1, which in turn transcriptionally activated *BTG2*, indicating that the ROS/EGR1/*BTG2* axis is a significant downstream pathway of NAE. A previous study reported that EGR1 promoted apoptosis in rat neuronal cells by upregulating *BTG2* [53], which corroborates our findings. This study presented the ROS-EGR1-*BTG2* signaling pathway and demonstrated that MLN4924 inhibited AML progression through the regulation of this pathway.

However, this study has certain limitations. In our research, we used a subcutaneous tumor formation model for the *in vivo* experiments. While this method was effective in establishing the leukemia model, it did not replicate the authentic leukemia microenvironment. In contrast, utilizing tail vein injection promotes the homing of cells to the bone marrow, providing a more accurate representation of the true state of leukemia. Moving forward, this approach will be the focus of our further studies.

In summary, our study demonstrated that MLN4924 inhibits AML by promoting ROS generation and stimulating the nuclear translocation of the EGR1, which subsequently activates *BTG2* transcriptionally. Our findings indicate that MLN4924 suppresses AML progression through the ROS-EGR1-*BTG2* signaling pathway. Both EGR1 and *BTG2* may serve as potential biomarkers for MLN4924 treatment in AML, aiding in the identification of patients who are more likely to benefit from this therapy. These conclusions provide a theoretical foundation for precision medicine and individualized treatment, thereby optimizing stratified treatment strategies for AML patients.

Supplementary Data

Supplementary data are available at *Acta Biochimica et Biophysica Sinica* online.

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Conflict of Interest

The authors declare that they have no conflict of interest.

References

- De Kouchkovsky I, Abdul-Hay M. Acute myeloid leukemia: a comprehensive review and 2016 update. *Blood Cancer J* 2016, 6: e441
- Shimony S, Stahl M, Stone RM. Acute myeloid leukemia: 2025 update on

- diagnosis, risk-stratification, and management. *Am J Hematol* 2025, 100: 860–891
- Liu H. Emerging agents and regimens for AML. *J Hematol Oncol* 2021, 14: 49
- Soleimani Samarkhazan H, Zehtabcheh S, Seraji HR, Beqaj SH, Tayefeh S, Mohammadi MH, Aghaei M. Unveiling the potential of CLL-1: a promising target for AML therapy. *Biomark Res* 2025, 13: 28
- Fathi AT. Pevonedistat, a new partner for 5-azacitidine. *Blood* 2018, 131: 1391–1392
- Ai TJ, Sun JY, Du LJ, Shi C, Li C, Sun XN, Liu Y, *et al.* Inhibition of neddylation by MLN4924 improves neointimal hyperplasia and promotes apoptosis of vascular smooth muscle cells through p53 and p62. *Cell Death Differ* 2018, 25: 319–329
- Fu DJ, Wang T. Discovery of dual tubulin-NEDDylation inhibitors with antiproliferative activity. *J Enzyme Inhibition Med Chem* 2023, 38: 166–175
- Li Y, Wang C, Xu T, Pan P, Yu Q, Xu L, Xiong X, *et al.* Discovery of a small molecule inhibitor of cullin neddylation that triggers ER stress to induce autophagy. *Acta Pharm Sin B* 2021, 11: 3567–3584
- Matondo M, Marcellin M, Chaoui K, Bousquet-Dubouch M-, Gonzalez-de-Peredo A, Monsarrat B, Burlet-Schiltz O. Determination of differentially regulated proteins upon proteasome inhibition in AML cell lines by the combination of large-scale and targeted quantitative proteomics. *Proteomics* 2017, 17: 1600089
- Yu Q, Jiang Y, Sun Y. Anticancer drug discovery by targeting cullin neddylation. *Acta Pharm Sin B* 2020, 10: 746–765
- Lin JJ, Milhollen MA, Smith PG, Narayanan U, Dutta A. NEDD8-targeting drug MLN4924 elicits DNA rereplication by stabilizing Cdt1 in S phase, triggering checkpoint activation, apoptosis, and senescence in cancer cells. *Cancer Res* 2010, 70: 10310–10320
- Vanderdys V, Allak A, Guessous F, Benamar M, Read PW, Jameson MJ, Abbas T. The neddylation inhibitor pevonedistat (MLN4924) suppresses and radiosensitizes head and neck squamous carcinoma cells and tumors. *Mol Cancer Ther* 2018, 17: 368–380
- Cojocari D, Smith BN, Purkal JJ, Arrate MP, Huska JD, Xiao Y, Gorska A, *et al.* Pevonedistat and azacitidine upregulate NOXA (PMAIP1) to increase sensitivity to venetoclax in preclinical models of acute myeloid leukemia. *Haematologica* 2022, 107: 825–835
- Short NJ, Muftuoglu M, Ong F, Nasr L, Macaron W, Montalban-Bravo G, Alvarado Y, *et al.* A phase 1/2 study of azacitidine, venetoclax and pevonedistat in newly diagnosed secondary AML and in MDS or CMML after failure of hypomethylating agents. *J Hematol Oncol* 2023, 16: 73
- Murthy GSG, Saliba AN, Szabo A, Harrington A, Abedin S, Carlson K, Michaelis L, *et al.* A phase I study of pevonedistat, azacitidine, and venetoclax in patients with relapsed/refractory acute myeloid leukemia. *Haematologica* 2020
- Cheung EC, Vousden KH. The role of ROS in tumour development and progression. *Nat Rev Cancer* 2022, 22: 280–297
- Liang Y, Jiang Y, Jin X, Chen P, Heng Y, Cai L, Zhang W, *et al.* Neddylation inhibition activates the protective autophagy through NF- κ B-catalase-ATF3 axis in human esophageal cancer cells. *Cell Commun Signal* 2020, 18: 72
- Swords RT, Kelly KR, Smith PG, Garnsey JJ, Mahalingam D, Medina E, Oberheu K, *et al.* Inhibition of NEDD8-activating enzyme: a novel approach for the treatment of acute myeloid leukemia. *Blood* 2010, 115: 3796–3800
- Bushweller JH. Targeting transcription factors in cancer - from undruggable to reality. *Nat Rev Cancer* 2019, 19: 611–624
- Vervoot SJ, Devlin JR, Kwiatkowski N, Teng M, Gray NS, Johnstone RW.

- Targeting transcription cycles in cancer. *Nat Rev Cancer* 2022, 22: 5–24
21. Li L, Ameri AH, Wang S, Jansson KH, Casey OM, Yang Q, Beshiri ML, *et al.* EGR1 regulates angiogenic and osteoclastogenic factors in prostate cancer and promotes metastasis. *Oncogene* 2019, 38: 6241–6255
 22. Ma Z, Gao X, Shuai Y, Wu X, Yan Y, Xing X, Ji J. EGR1-mediated linc01503 promotes cell cycle progression and tumorigenesis in gastric cancer. *Cell Prolif* 2021, 54: e12922
 23. Paulsson B. The multiplicative model for cancer risk assessment: applicability to acrylamide. *Carcinogenesis* 2001, 22: 1577
 24. Schmidt K, Carroll JS, Yee E, Thomas DD, Wert-Lamas L, Neier SC, Sheynkman G, *et al.* The lncRNA SLNCR recruits the androgen receptor to EGR1-bound genes in melanoma and inhibits expression of tumor suppressor p21. *Cell Rep* 2019, 27: 2493–2507.e4
 25. Stoddart A, Fernald AA, Wang J, Davis EM, Karrison T, Anastasi J, Le Beau MM. Haploinsufficiency of del(5q) genes, Egr1 and Apc, cooperate with Tp53 loss to induce acute myeloid leukemia in mice. *Blood* 2014, 123: 1069–1078
 26. Chen Y, Sun L. Inhibition of NEDD8 NEDDylation induced apoptosis in acute myeloid leukemia cells via p53 signaling pathway. *Biosci Rep* 2022, 42: BSR20220994
 27. Guo N, Azadniv M, Coppage M, Nemer M, Mendler J, Becker M, Liesveld J. Effects of neddylation and mTOR inhibition in acute myelogenous leukemia. *Transl Oncol* 2019, 12: 602–613
 28. Chen Y, Liu C, Tong J, He C, Ling X, Xiang J, Xue C, *et al.* Inhibition of cullin3 neddylation alleviates diabetic retinopathy by activating Nrf2 signaling to combat ROS-induced oxidative stress and inflammation. *Inflammation* 2025, 48: 3194–3205
 29. Wang B, Guo H, Yu H, Chen Y, Xu H, Zhao G. The role of the transcription factor EGR1 in cancer. *Front Oncol* 2021, 11: 642547
 30. Fu DJ, Wang T. Targeting NEDD8-activating enzyme for cancer therapy: developments, clinical trials, challenges and future research directions. *J Hematol Oncol* 2023, 16: 87
 31. Adès L, Girshova L, Doronin VA, Díez-Campelo M, Valcárcel D, Kambhampati S, Viniou NA, *et al.* Pevonedistat plus azacitidine vs azacitidine alone in higher-risk MDS/chronic myelomonocytic leukemia or low-blast-percentage AML. *Blood Adv* 2022, 6: 5132–5145
 32. El-Mesery M, Rosenthal T, Rauert-Wunderlich H, Schreder M, Stühmer T, Leich E, Schlosser A, *et al.* The NEDD8-activating enzyme inhibitor MLN4924 sensitizes a TNFR1+ subgroup of multiple myeloma cells for TNF-induced cell death. *Cell Death Dis* 2019, 10: 611
 33. Milhollen MA, Traore T, Adams-Duffy J, Thomas MP, Berger AJ, Dang L, Dick LR, *et al.* MLN4924, a NEDD8-activating enzyme inhibitor, is active in diffuse large B-cell lymphoma models: rationale for treatment of NF- κ B-dependent lymphoma. *Blood* 2010, 116: 1515–1523
 34. Yin L, Zhang J, Sun Y. Early growth response-1 is a new substrate of the GSK3 β -FBXW7 axis. *Neoplasia* 2022, 34: 100839
 35. Liu N, Wang S, Li M, Zhao N, Wang D, Zhang R, Yu M, *et al.* BET degrader exhibits lower antiproliferative activity than its inhibitor via EGR1 recruiting septins to promote E2F1-3 transcription in triple-negative breast cancer. *Pharmacol Res* 2024, 208: 107377
 36. Garcia-Cuellar MP, Akan S, Slany RK. A C/ebp α isoform specific differentiation program in immortalized myelocytes. *Leukemia* 2023, 37: 1850–1859
 37. Joslin JM, Fernald AA, Tennant TR, Davis EM, Kogan SC, Anastasi J, Crispino JD, *et al.* Haploinsufficiency of EGR1, a candidate gene in the del(5q), leads to the development of myeloid disorders. *Blood* 2007, 110: 719–726
 38. Tang H, Pang X, Li S, Tang L. The double-edged effects of MLN4924: rethinking anti-cancer drugs targeting the neddylation pathway. *Biomolecules* 2024, 14: 738
 39. Wang B, Wang Y, Wang W, Wang Z, Zhang Y, Pan X, Wen X, *et al.* WTAP/IGF2BP3 mediated m6A modification of the EGR1/PTEN axis regulates the malignant phenotypes of endometrial cancer stem cells. *J Exp Clin Cancer Res* 2024, 43: 204
 40. Baron V, Adamson ED, Calogero A, Ragona G, Mercola D. The transcription factor Egr1 is a direct regulator of multiple tumor suppressors including TGF β 1, PTEN, p53, and fibronectin. *Cancer Gene Ther* 2006, 13: 115–124
 41. Li Y, He X, Li S, Chen S, Zhao Z, Mu Y, Zhao AZ, *et al.* The phosphodiesterase-4 inhibitor Zl-n-91 suppresses glioblastoma growth via EGR1/PTEN/AKT pathway. *Eur J Pharmacol* 2025, 988: 177230
 42. Hong E, Barczak W, Park S, Heo JS, Ooshima A, Munro S, Hong CP, *et al.* Combination treatment of T1-44, a PRMT5 inhibitor with Vactosertib, an inhibitor of TGF- β signaling, inhibits invasion and prolongs survival in a mouse model of pancreatic tumors. *Cell Death Dis* 2023, 14: 93
 43. Li H, Tan M, Jia L, Wei D, Zhao Y, Chen G, Xu J, *et al.* Inactivation of SAG/RBX2 E3 ubiquitin ligase suppresses KrasG12D-driven lung tumorigenesis. *J Clin Invest* 2014, 124: 835–846
 44. Nigam M, Punia B, Dimri DB, Mishra AP, Radu AF, Bungau G. Reactive oxygen species: a double-edged sword in the modulation of cancer signaling pathway dynamics. *Cells* 2025, 14: 1207
 45. Lee SS, Yoo YC. NOX-NOS crosstalk in the liver-brain axis: novel insights for redox regulation and neurodegenerative diseases. *Redox Biol* 2025, 86: 103807
 46. Chen Z, Wu F, Li J, Dong J, He H, Li X, Lu Q, *et al.* Investigating the synergy of Shikonin and Valproic acid in inducing apoptosis of osteosarcoma cells via ROS-mediated EGR1 expression. *Phytomedicine* 2024, 126: 155459
 47. Liu Y, Fan D. Ginsenoside Rg5 induces G2/M phase arrest, apoptosis and autophagy via regulating ROS-mediated MAPK pathways against human gastric cancer. *Biochem Pharmacol* 2019, 168: 285–304
 48. Fan J, Ren D, Wang J, Liu X, Zhang H, Wu M, Yang G. Bruceine D induces lung cancer cell apoptosis and autophagy via the ROS/MAPK signaling pathway *in vitro* and *in vivo*. *Cell Death Dis* 2020, 11: 126
 49. Wang LJ, Lee YC, Huang CH, Shi YJ, Chen YJ, Pei SN, Chou YW, *et al.* Non-mitotic effect of albendazole triggers apoptosis of human leukemia cells via SIRT3/ROS/p38 MAPK/TTP axis-mediated TNF- α upregulation. *Biochem Pharmacol* 2019, 162: 154–168
 50. Chuang KC, Chen FW, Tsai MH, Shieh JJ. EGR-1 plays a protective role in AMPK inhibitor compound C-induced apoptosis through ROS-induced ERK activation in skin cancer cells. *Oncol Lett* 2021, 21: 304
 51. Yang J, Sun L, Han J, Zheng W, Peng W. DUSP1/MKP-1 regulates proliferation and apoptosis in keratinocytes through the ERK/Elk-1/Egr-1 signaling pathway. *Life Sci* 2019, 223: 47–53
 52. Luo Z, Pan Y, Jeong LS, Liu J, Jia L. Inactivation of the Cullin (CUL)-RING E3 ligase by the NEDD8-activating enzyme inhibitor MLN4924 triggers protective autophagy in cancer cells. *Autophagy* 2012, 8: 1677–1679
 53. Wu F, Zhang P, Zhou G. The involvement of EGR1 in neuron apoptosis in the *in vitro* model of spinal cord injury via BTG2 up-regulation. *Neurol Res* 2023, 45: 646–654