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USP30 promotes cytarabine resistance in acute myeloid leukemia by deubiquitinating and stabilizing FOXM1

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Abstract. Acute myeloid leukemia (AML) is a hematologic malignancy. Cytarabine (Ara-C) is a key drug in its chemotherapy, yet the development of drug resistance remains a major cause of treatment failure. Ubiquitin specific peptidase 30 (USP30) and forkhead box M1 (FOXM1) have been implicated in the progression of AML, yet the mutual regulatory mechanism between them remains unclear. Western blot was employed to assess the protein expression of USP30 and FOXM1 in tissues and cells. Cell proliferation and cell cycle were evaluated by EdU and flow cytometry assays. Cell apoptosis was determined using flow cytometry and TUNEL assays. The UbiBrowser 2.0, Co-IP, and cycloheximide (CHX) assays were applied to assess the interaction between USP30 and FOXM1. Subsequently, Ara-C-resistant cell lines and animal models were established, and immunohistochemistry (IHC) was performed to analyze Ki-67 and USP30 expression in tumor tissues from mice. The results showed that USP30 and FOXM1 were highly expressed in AML. Knockdown of USP30 repressed cell proliferation and cell cycle progression and promoted cell apoptosis. Additionally, USP30 stabilized FOXM1 protein expression through its deubiquitination activity. FOXM1 overexpression reversed the impacts of USP30 silencing on proliferation, cell cycle, and apoptosis in AML cells. Furthermore, USP30 enhanced Ara-C resistance in AML cells *in vivo*. Together, USP30 promoted Ara-C resistance in AML by deubiquitinating and stabilizing FOXM1, thereby enhancing tumor growth and cell survival.

Key words: Acute myeloid leukemia — USP30 — FOXM1 — Cytarabine resistance — Deubiquitination

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Introduction

Acute myeloid leukemia (AML), a form of blood malignancy, is characterized by the excessive proliferation of abnormal myeloid progenitor cells in the bone marrow. Such proliferation disrupts the generation of normal red blood cells, white blood cells, and platelets (Khwaja et al. 2016). AML represents the predominant form of acute leukemia in adults, and its incidence increases with age (Zhou et al. 2024). Cytarabine (Ara-C) serves as the first-line therapeutic drug for AML (Kong et al. 2025). While Ara-C-based regimens achieve complete remission in most newly diagnosed AML patients, treatment efficacy is often suboptimal owing to the development of drug resistance (Wu et al. 2017). Thus, unraveling the molecular mechanisms underlying Ara-C resistance and identifying new therapeutic targets are imperative to overcome these challenges and enhance clinical outcomes for AML patients.

Deubiquitinating enzymes (DUBs) modulate protein stability, localization, and activity in diverse biological processes by removing ubiquitin chains from substrate proteins (Fang et al. 2023). In cancer, DUBs act as critical regulators of tumorigenesis by controlling the stability of oncoproteins and tumor suppressors. Aberrant DUB expression or activity, arising from genetic mutations or transcriptional/post-translational modifications, can disrupt vital cellular processes like DNA repair, apoptosis, and autophagy, thereby promoting tumor cell growth, invasion, and metastasis (Fraile et al. 2012; Christine et al. 2022). Consequently, the DUB system represents a promising therapeutic target, and efforts are underway to develop DUB inhibitors for cancer treatment (Xian et al. 2024). Accumulating studies have uncovered the oncogenic or tumor-suppressive roles of specific DUBs across various malignancies. For instance, ubiquitin-specific protease 33 (USP33) suppresses lung adenocarcinoma metastasis by downregulating the SLIT2/ROBO1 signaling pathway (Wang et al. 2018). Similarly, USP10 stabilizes YAP/TAZ in hepatocellular carcinoma *via* deubiquitination, potentiating downstream oncogenic responses (Zhu et al. 2020). In AML, USP47 drives disease progression by deubiquitinating and stabilizing the oncoprotein YBX1 (Lei et al. 2024). USP30, another member of DUB family, has been implicated in solid tumor progression. Studies have shown that downregulation of USP30 markedly inhibits the malignant behaviors of breast cancer cells *in vitro* (Sun et al. 2024). While considerable progress has been achieved, the molecular mechanisms by which USP30 influences AML remain largely undefined. Investigating USP30's function in AML could provide novel insights into disease mechanisms and identify potential therapeutic opportunities.

Forkhead box M1 (FOXM1), a well-characterized transcription factor, is pivotal in driving cell proliferation across diverse cancer types (Wang et al. 2010). FOXM1 overexpression is observed in numerous solid tumors, underscoring its involvement in tumorigenesis and progression (Borhani and Gartel 2020). Analysis of 127 AML patient samples revealed aberrant overexpression of FOXM1, which correlates with chemoresistance in this disease (Khan et al. 2017). FOXM1-targeted therapeutic strategies exhibit potential in reducing the risk of relapse in AML patients (Sheng et al. 2018). Furthermore, FOXM1 exerts a significant influence on chemoresistance in AML (Khan et al. 2018; Khan et al. 2023). Post-translational modifications, including ubiquitination, play a crucial role in regulating FOXM1 in cancers (Liao et al. 2018). Emerging evidence on USP30's role in stabilizing oncoproteins and FOXM1's involvement in AML chemoresistance led us to the hypothesis that USP30 interacts with FOXM1 to promote Ara-C resistance.

This investigation focuses on the regulation of FOXM1 by USP30, with the goal of revealing the molecular mechanisms of Ara-C resistance in AML and uncovering new therapeutic targets.

Materials and Methods

Subjects

Bone marrow (BM) samples were collected from 4 healthy donors and 4 newly diagnosed primary AML patients at Chongqing University Cancer Hospital. Collected BM aspirates were immediately anticoagulated with heparin, and mononuclear cells were subsequently isolated *via* Ficoll-Paque density gradient centrifugation.

Cell culture

Human bone marrow stromal cells HS-5 and human promyelocyte leukemia cells HL-60 were obtained from Pricella (Wuhan, China). HS-5 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, USA) containing 10% fetal bovine serum (FBS) (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomycin (P/S) (Invitrogen). HL-60 cells were maintained in Iscove's Modified Dulbecco Medium (IMDM) (Pricella) containing 20% FBS (Gibco) and 1% P/S (Invitrogen). Human AML cell line AML-193 was sourced from the National Collection of Authenticated Cell Culture (Shanghai, China) and maintained in IMDM (Pricella) supplemented with 0.005 mg/ml insulin (Yeasen, Shanghai, China), 0.005 mg/ml transferrin (Yeasen), 5 ng/ml granulocyte-macrophage colony-stimulating factor (GM-CSF) (Yeasen) and 10% FBS (Gibco). These cells were maintained in a humidified incubator at 37°C with 5% CO_2.

Western blot analysis

The cells were collected and then lysed in RIPA buffer. After centrifugation, total protein was obtained by collecting the

supernatant. Following concentration determination *via* the BCA method, the protein samples were mixed with loading buffer. Protein separation was performed *via* SDS-PAGE, followed by transferring protein bands onto a PVDF membrane (GE Healthcare, Piscataway, NJ, USA). The membrane was blocked with 5% skim milk at room temperature for 2 hours and then sequentially incubated with primary and secondary antibodies. Signal visualization was conducted using RapidStep ECL Reagent (Millipore Corp. Billerica, MA, USA). β-actin served as the internal reference protein. Primary antibodies included anti-USP30 (1:1000, Cat: 15402-1-AP, Proteintech, Wuhan, China), anti-FOXM1 (1:1000, Cat: 13147-1-AP, Proteintech), anti-BAX (1:1000, Cat: ab32503, Abcam), anti-Bcl-2 (1:1000, Cat: ab182858, Abcam), and anti-β-actin (1:1000, Cat: 66009-1-Ig, Proteintech). Secondary antibodies consisted of HRP-conjugated goat anti-rabbit (1:5000, Cat: RGAR001, Proteintech) and goat anti-mouse (1:5000, Cat: RGAM001, Proteintech).

Cell transfection

Short hairpin RNAs targeting USP30 (sh-USP30 and sh-USP30#2), with sh-NC serving as the negative control, were synthesized by GeneChem (Shanghai, China). USP30 and FOXM1 overexpression vectors (labeled as USP30 and FOXM1) were constructed by cloning the full-length USP30 and FOXM1 genes into pcDNA3.1 plasmids (Invitrogen). The empty pcDNA3.1 (vector) served as the negative control. shRNAs and overexpression vectors were transfected into AML cells using Lipofectamine 2000 (Invitrogen). Each procedure was strictly conducted in compliance with the manufacturer's protocol.

5-Ethynyl-2'-deoxyuridine (EdU) assay

Cell proliferation was monitored using the E-Click EdU Cell Proliferation Imaging Assay Kit (Red, Elab Fluor® 594) (Elabscience, Wuhan, China). HL-60 and AML-193 cells were incubated in culture medium containing EdU at 37°C for 4 hours. Next, cells were fixed with 1 ml of PBS containing 4% paraformaldehyde (Beyotime) for 15 min. Following fixation, cells were permeabilized with 0.3% Triton X-100 (Beyotime) for 20 min. After that, 500 μl of Click reaction solution was added. Nuclear staining was conducted by incubating cells with DAPI working solution at room temperature in the dark for 5 min. Lastly, EdU-positive (EdU^+) cells were observed under a fluorescence microscope.

Detection of cell cycle

AML cells were harvested *via* centrifugation, washed with PBS, and stained with Cell Cycle Staining Kit (MultiSciences, Hangzhou, China). Then, the percentages of cells in each phase were analyzed using a flow cytometer.

Cell apoptosis assay

Cell apoptosis was assessed by using the Annexin V-FITC/PI Apoptosis Detection Kit (Yeasen). HL-60 and AML-193 cells (1×10^5) were collected and resuspended in 100 μl of 1×Binding Buffer. Subsequently, 5 μl of Annexin V-FITC and 10 μl of PI Staining Solution were added to the cell suspension. The mixture was incubated at room temperature in the dark for 15 min. After adding 400 μl of 1×Binding Buffer, apoptotic cells were quantified using a flow cytometer.

TUNEL assay

TUNEL assay was carried out using the One Step TUNEL Apoptosis Assay Kit (Beyotime). Cells were collected and treated with 4% paraformaldehyde solution (Beyotime) at room temperature for 15 min. Subsequently, cells were permeabilized with 0.1% Triton X-100 (Beyotime) *via* ice-bath treatment for 10 min. TUNEL detection solution was prepared as *per* the kit instruction. Cells were resuspended in 50 μl of the solution and incubated at 37°C in the dark for 60 min. Following nuclear counterstaining of DAPI, TUNEL-positive cells were observed using a fluorescence microscope.

Detection of caspase 3 activity

Cells were harvested and lysed, followed by centrifugation at 20,000×*g* for 15 min at 4°C to collect the supernatant. A small portion of the sample was used for protein concentration determination. An equal amount of protein from each sample was combined with reaction buffer and the caspase-3 substrate Ac-DEVD-pNA, followed by incubation at 37°C for 2 hours. At last, the absorbance at 405 nm (A405) was measured using a spectrophotometer.

Bioinformatics analysis

UbiBrowser 2.0 (http://ubibrowser.ncpsb.org.cn), a multi-species ubiquitin ligase (E3)/deubiquitinating enzyme (DUB)-substrate protein interaction prediction and visualization system, was applied to predict whether deubiquitination regulation exists between USP30 and FOXM1.

Co-immunoprecipitation (Co-IP) assay

Co-IP experiments were performed to examine the interaction between FOXM1 and USP30. Cells were collected and lysed in IP lysis buffer. Following the centrifugation of cell lysates at 4°C, the supernatant (protein samples) was obtained. Part of the supernatant served as the Input control. For immunoprecipitation, the sample was incubated with IgG (negative control) (Cat: 36113ES60, Yeasen) or FOXM1 (Cat: 13147-1-AP, Proteintech) antibody at 4°C overnight.

Protein A/G beads (Beyotime) were then added to each sample for another 4 h incubation to capture the antibody-protein complexes. After repeated washing to eliminate non-specific binding proteins, the complexes were eluted by boiling with protein loading buffer. Finally, both the Input samples and the eluted IP samples were subjected to Western blot assay using anti-USP30 (Cat: 15402-1-AP, Proteintech) and anti-FOXM1 (Cat: 13147-1-AP, Proteintech).

Ubiquitination assay

HL-60 and AML-193 cells were collected and lysed in RIPA buffer. Supernatant proteins were obtained through centrifugation. A portion of the supernatant served as the Input control, while the remaining samples underwent IP overnight with FOXM1 antibody (Cat: 13147-1-AP, Proteintech) or IgG conjugated to Protein A/G beads (Beyotime). Subsequently, precipitated proteins were eluted by boiling in SDS loading buffer and then subjected to Western blot using primary anti-ubiquitin (Ub) antibody (Cat: ab140601, Abcam, Cambridge, UK). After secondary antibody incubation and chemiluminescent development, FOXM1 ubiquitination levels in each group were analyzed.

Cycloheximide (CHX) assay

CHX (MCE, Monmouth Junction, NJ, USA), a protein synthesis inhibitor, was added to the transfected HL-60 cells. Cells were harvested at 0, 2, 4, and 8 hours, and total proteins were extracted. Subsequently, the regulatory effect of USP30 on FOXM1 protein stability was analyzed by Western blot.

Establishment of Ara-C-resistant AML cell line

To establish the Ara-C-resistant subline HL-60/C, cells were seeded at a density of 3×10^5 cells/ml in T25 flasks. During the logarithmic growth phase, varying concentrations (0, 1, 2, 5, and 10 μM) of Ara-C were added to the culture. The medium containing the corresponding drug was replaced every 3–4 days, and the cell density was maintained between $(2–10)\times10^5$ cells/ml for 3–4 weeks. The IC50 of Ara-C in parental HL-60 cells was determined to be 5 μM. The resistant cell line HL-60/C was subsequently established and maintained under continuous selection pressure with 5 μM Ara-C.

Animal experiment

6–8-week-old male BALB/c nude mice were purchased from Sibeifu Biotechnology Co. Ltd. (Beijing, China). The animals were of specific pathogen-free status, genetically modified as nude mice with genotype nu/nu (exhibiting T lymphocyte deficiency due to thymic aplasia), and had no previous experimental procedures prior to this study. Ten nude mice were randomly divided into two groups: sh-NC group and sh-USP30 group ($n = 5$). Sample size of 5 BALB/c mice per group aligned with previous exploratory studies in this field using the same strain (Zhou et al. 2011). Mice were housed in standard cages (5 *per* cage) under SPF conditions: temperature 22 ± 2°C, humidity 50 ± 10%, 12 hours light/dark cycle, with sterilized bedding changed twice weekly. To reduce distress, injections were performed using 27G needles; mice were monitored daily for activity and tumor growth. Humane endpoints included tumor size >20 mm, >20% weight loss, or inability to access food/water. No adverse events or endpoint breaches occurred. HL-60/C cells (2×10^6) stably transfected with sh-NC or sh-USP30 were resuspended in 100 μl Matrigel and injected into the right dorsal midline of nude mice. On day 7, the mice were *intraperitoneally* injected with Ara-C (20 mg/kg, administered three times a week) for a total of 2 weeks. Tumor growth was continuously observed for 21 days. Subsequently, the mice were euthanized *via* cervical dislocation to collect tumor tissue samples.

Immunohistochemistry (IHC)

Tumor tissues were collected, fixed, and embedded in paraffin to prepare 4-μm paraffin sections. These sections sequentially underwent dewaxing, hydration, and antigen retrieval to expose target antigens. Thereafter, the sections were incubated with goat serum (Beyotime), followed by incubation with anti-Ki-67 (Cat: ab15580, Abcam) or anti-USP30 (Cat: 15402-1-AP, Proteintech). After washing, HRP-labeled goat anti-rabbit (Cat: RGAR001, Proteintech) was added, and DAB (Yeasen) was utilized for color development. Finally, cell nuclei were counterstained with hematoxylin, and the sections were dehydrated, mounted, and observed under a microscope.

Statistical analysis

Statistical analysis and data visualization were conducted using GraphPad Prism 8.0.1. Data were presented as mean ± standard deviation. Student's *t*-test was employed for two-group comparisons, while One-way ANOVA or Two-way ANOVA was applied for multi-group analyses. Statistical significance was defined as $p < 0.05$.

Results

USP30 and FOXM1 expression levels were elevated in primary AML samples

Initially, USP30 and FOXM1 expression levels in BM cells from healthy donors ($n = 4$) and primary AML patients ($n = 4$) were detected. Clinical characteristics of these patients are presented in Table 1. As displayed in Figure 1A and B,

USP30 and FOXM1 levels in BM cells from AML patients were significantly higher than those from healthy controls. Therefore, USP30 and FOXM1 were upregulated in AML.

Silencing USP30 inhibited cell proliferation and cell cycle progression, while triggering apoptosis in AML

Compared with normal HS-5 cells, USP30 protein levels in HL-60 and AML-193 cells were significantly upregulated (Fig. 2A). Subsequently, to elucidate the function of USP30 in AML progression, AML cells were transfected with sh-NC, sh-USP30, and sh-USP30#2. USP30 protein expression in the sh-USP30 and sh-USP30#2 groups was substantially downregulated compared to control cells (Fig. 2B). Proliferation assay revealed a marked reduction of EdU-positive cells in the sh-USP30 group (Fig. 2C). Cell cycle analysis showed that USP30 knockdown significantly increased the proportion of cells in G0/G1 phase, while decreasing the proportion of cells in S phase compared to the sh-NC group (Fig. 2D). Following the silencing of USP30, both the apoptotic cells and TUNEL$^+$ cells were observed to substantially increase (Fig. 2E,F). Additionally, after USP30 was knocked down, caspase 3 activity in AML cells was markedly augmented (Fig. 2G). Taken together, USP30 knockdown constrained the proliferation of HL-60 and AML-193 cells, induced G0/G1 cell cycle arrest, and facilitated apoptosis.

USP30 stabilized FOXM1 expression via deubiquitination

Prediction using the UbiBrowser 2.0 software revealed that USP30 has the potential to regulate deubiquitination of FOXM1 (Fig. 3A). After IP using the FOXM1 antibody, the USP30 protein could be detected, whereas no USP30 signal was observed in the IgG negative control (Fig. 3B). This confirmed the direct protein interaction between FOXM1 and USP30 in HL-60 and AML-193 cells. Additionally, FOXM1 protein levels were decreased upon USP30 knockdown, and this reduction was reversed by the proteasome inhibitor MG132. In contrast, USP30 overexpression increased FOXM1 protein abundance (Fig. 3C). Collectively, these findings demonstrated that USP30 maintained FOXM1 protein expression by inhibiting proteasome-mediated degradation of FOXM1. Moreover, in comparison with the sh-NC group, FOXM1 ubiquitination signals were notably augmented in the sh-USP30 group, indicating that USP30 knockdown promoted the ubiquitination modification of FOXM1 and thus mediated its degradation (Fig. 3D). Consistently, CHX chase assay showed that FOXM1 protein degraded more rapidly in USP30-knockdown HL-60 cells compared to the sh-NC group (Fig. S1A see in Supplementary material). In conclusion, USP30 could bind to FOXM1 and stabilize its expression via deubiquitination.

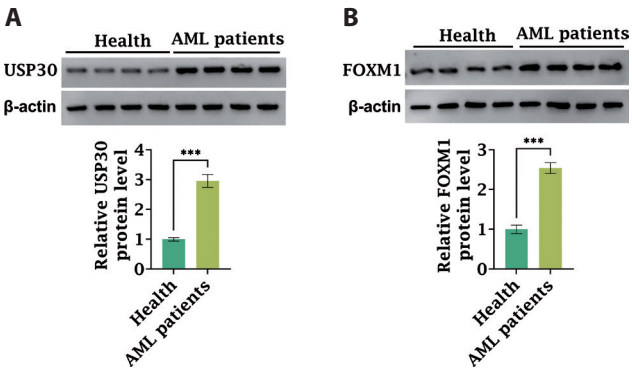


Figure 1. USP30 and FOXM1 expressions are upregulated in AML samples. USP30 (**A**) and FOXM1 (**B**) protein expression levels were detected by Western blot in bone marrow cells from healthy individuals ($n = 4$) and AML patients ($n = 4$). *** $p < 0.001$.

FOXM1 overexpression counteracted the effects of USP30 knockdown on proliferation, cell cycle progression, and apoptosis in AML cells

Subsequently, we analyzed whether the roles of USP30 in AML were associated with FOXM1. FOXM1 expression in HS-5, HL-60, and AML-193 cells was tested. Obviously, FOXM1 protein expression was upregulated in AML cells compared with normal HS-5 cells (Fig. 4A). Then, Western blot verified that FOXM1 protein level was elevated after cells were transfected with FOXM1 overexpression vector (Fig. 4B). Besides, FOXM1 expression was decreased with the downregulation of USP30, while FOXM1 overexpression vector rescued its expression (Fig. 4C). In addition, USP30 knockdown suppressed cell proliferation and induced G0/G1 phase cell cycle arrest, while FOXM1 upregulation rescued the inhibitory effects of USP30 depletion on cell proliferation and cell cycle progression (Fig. 4D,E). Moreover, USP30 knockdown markedly elevated the cell apoptosis rate, the

Table 1. Clinical characteristics of acute myeloid leukemia (AML) patients

Parameter	AMI ($n = 4$)
Age (years)	53.24±24.68
Sex	2/2
White blood cell ($\times 10^9$ l)	22.33±8.65
Hemoglobin (g/l)	85.47±27.89
Platelet count (10^9/l)	44.12±29.34
FAB classification	
M2	1
M3	1
M4	1
M5	1

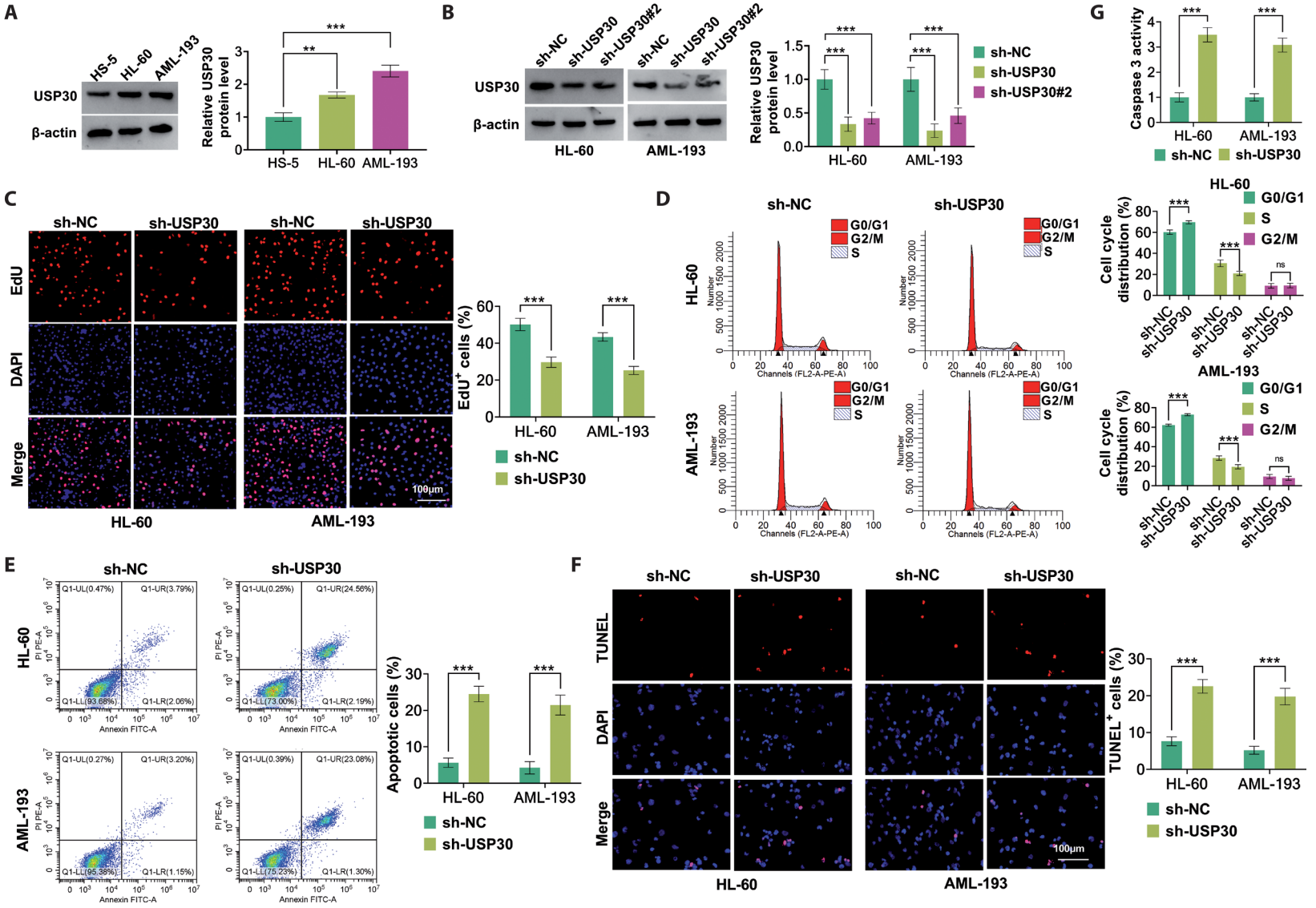


Figure 2. Silencing USP30 inhibits cell proliferation and cell cycle progression, while promoting cell apoptosis. **A.** Western blot analysis of USP30 levels in HS-5, HL-60, and AML-193 cells. **B.** USP30 protein levels in HL-60 and AML-193 cells transfected with sh-NC, sh-USP30, and sh-USP30#2 were detected by Western blot. HL-60 and AML-193 cells were transfected with sh-NC and sh-USP30. **C.** Cell proliferation was examined by EdU assay. **D.** Cell cycle was evaluated by using the flow cytometry. Cell apoptosis was detected by flow cytometry (**E**) and TUNEL assays (**F**). **G.** Caspase 3 activity was detected by using caspase 3 activity detection kit. ** $p < 0.01$, *** $p < 0.001$, *ns,* not significant.

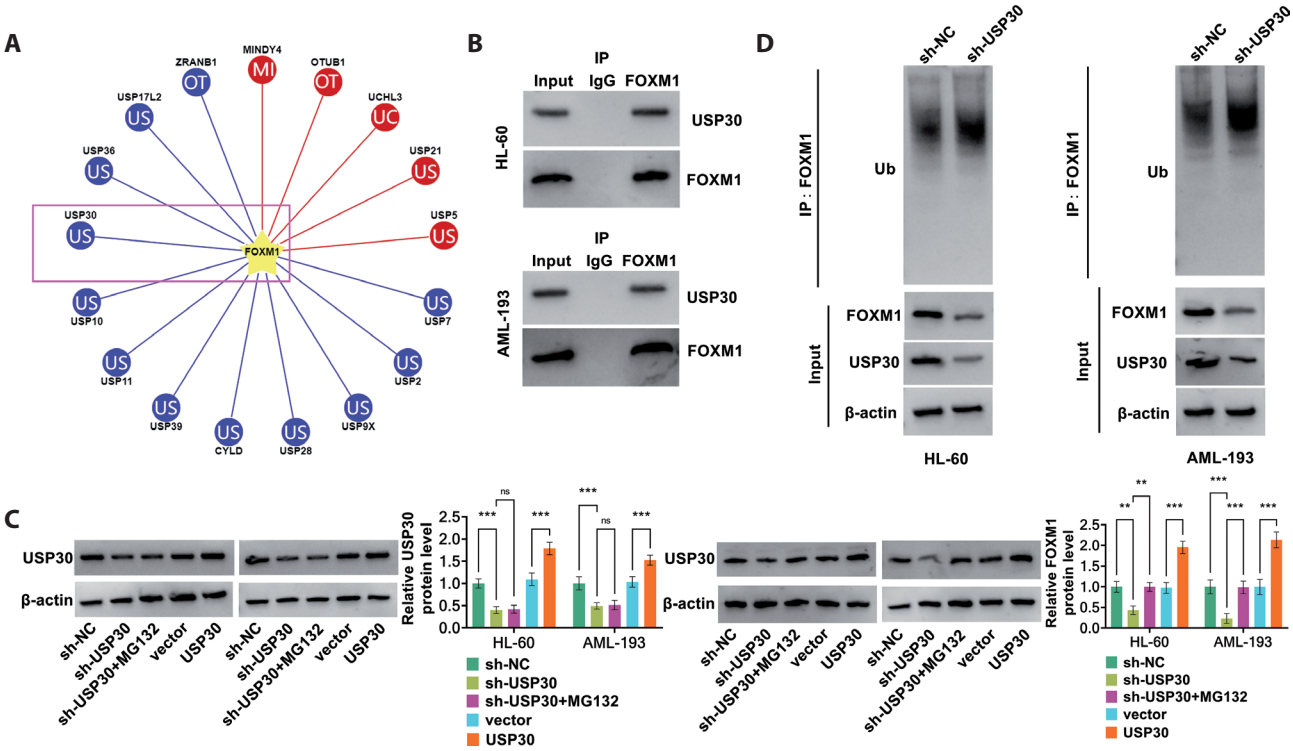


Figure 3. USP30 stabilizes FOXM1 expression *via* deubiquitination. **A.**The possible deubiquitination modification of USP30 on FOXM1 was predicted by UbiBrowser. **B.** Co-IP assay was used to detect the interaction between FOXM1 and USP30. **C.** HL-60 and AML-193 cells were treated with sh-NC, sh-USP30, sh-USP30+MG132, vector, and USP30. The protein levels of USP30 and FOXM1 were determined by Western blot. **D.** IP and Western blot were performed to analyze the ubiquitination level of FOXM1 in AML cells transfected with sh-NC and sh-USP30. ** $p < 0.01$, *** $p < 0.001$, *ns,* not significant.

number of TUNEL$^+$ cells, and the caspase 3 activity. By contrast, FOXM1 overexpression counteracted these effects (Fig. 4F–H), demonstrating that FOXM1 overexpression alleviated the increase in apoptosis triggered by USP30 knockdown. Together, USP30 knockdown exerted a suppressive effect on AML by inhibiting FOXM1 expression.

USP30 enhanced Ara-C resistance in AML

Given that both FOXM1 and USP30 have been implicated in cancer progression and chemoresistance, it was hypothesized that they might play crucial roles in Ara-C resistance in AML. Understanding their functions could provide new insights into overcoming drug resistance. An Ara-C-resistant AML cell line was established. Western blot results showed that USP30 and FOXM1 expression in these Ara-C-resistant (HL-60/C) cells was higher than that in the control cells (Fig. 5A and Fig. S1B, see in Supplementary material). When HL-60/C cells were transfected with sh-USP30, USP30 protein expression was decreased (Fig. 5B). Furthermore, USP30 knockdown curbed proliferation and promoted apoptosis of HL-60/C cells (Fig. 5C,D). Subsequently, animal experiments were carried out. Animal treatment protocol was depicted in Figure 5E. Compared with the control group, the tumor weight in the sh-USP30 group was significantly reduced (Fig. 5F). The proliferation marker Ki-67 was notably diminished in tumor tissues of the sh-USP30 group, as demonstrated by IHC. Additionally, the expression level of USP30 was notably downregulated in tumor tissues from sh-USP30 group (Fig. 5G). Also, the expression of apoptotic markers (Bax and Bcl-2) in tumor tissues was detected via Western blot. As shown in Fig. S1C, USP30 knockdown significantly elevated the pro-apoptotic protein Bax levels and reduced the anti-apoptotic protein Bcl-2 levels. In summary, USP30 knockdown restrained the proliferation of Ara-C-resistant cells, promoted their apoptosis, thereby attenuating drug resistance and suppressing tumor growth *in vivo*.

Discussion

AML is a highly aggressive hematologic malignancy characterized by clonal expansion of immature myeloid cells, often

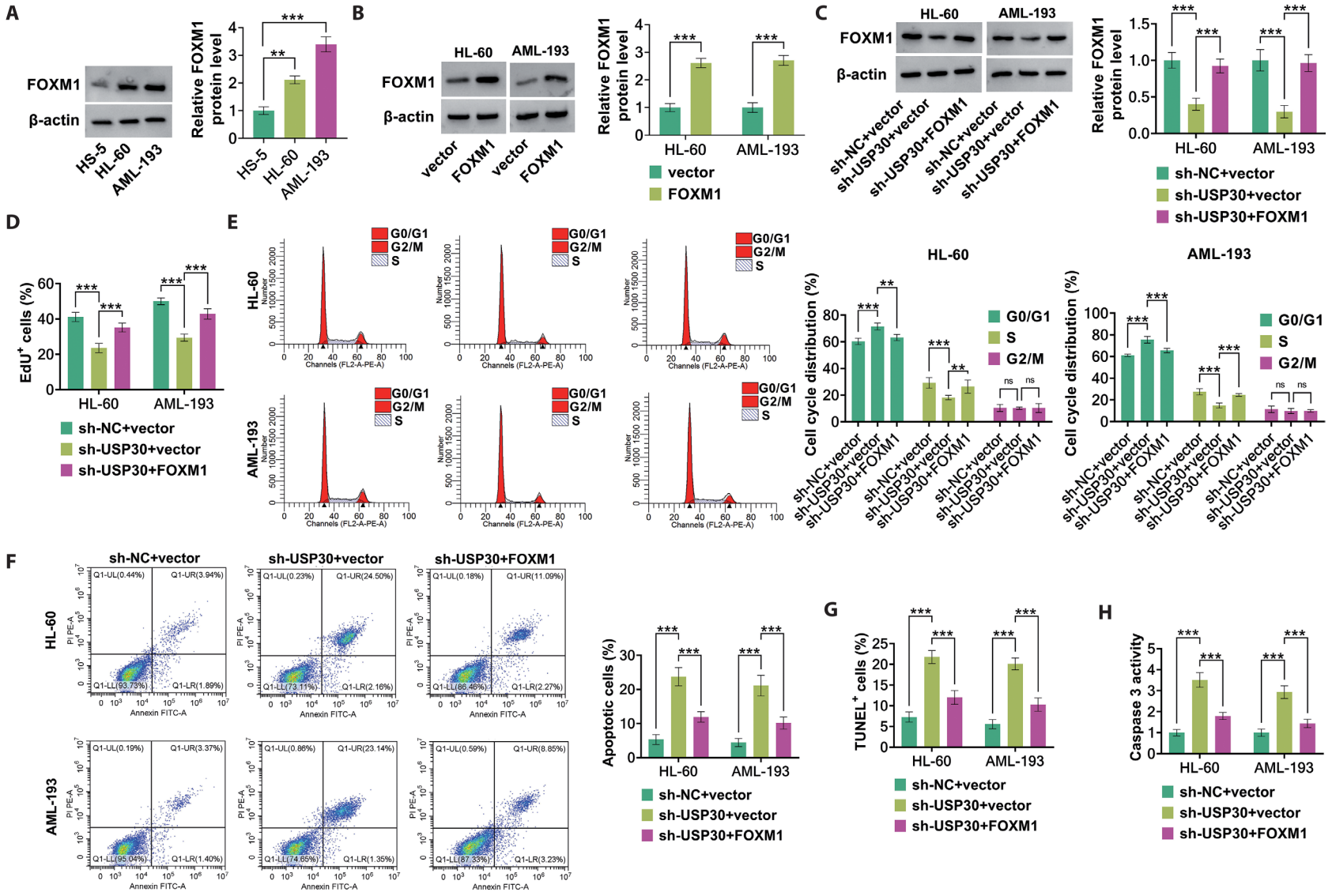


Figure 4. FOXM1 overexpression reverses the effects of USP30 knockdown on proliferation, cell cycle, and apoptosis in AML cells. **A.** FOXM1 protein expression in HS-5, HL-60, and AML-193 cells was determined by Western blot. **B.** FOXM1 protein levels in HL-60 and AML-193 cells transfected with vector and FOXM1 were tested by Western blot. **C–G.** HL-60 and AML-193 cells were divided into 3 groups: sh-NC+vector, sh-USP30+vector, and sh-USP30+FOXM1. **C.** FOXM1 protein levels detected by Western blot. **D.** Cell proliferation was detected using EdU assay. **E.** Cell cycle was examined using the flow cytometry. Cell apoptosis was analyzed by flow cytometry (**F**) and TUNEL assays (**G**). **H.** Caspase 3 activity was evaluated using caspase 3 activity detection kit. ** $p < 0.01$, *** $p < 0.001$, *ns,* not significant.

demonstrating resistance to conventional chemotherapy due to intrinsic or acquired drug resistance (Pollyea et al. 2023). In this work, we elucidated a new molecular mechanism driving AML progression and Ara-C resistance, in which the deubiquitinating enzyme USP30 stabilized the transcription factor FOXM1.

Prior literature highlights the significant roles of USP30 in cancer progression (Doherty and Mills, 2022). Notably, other members of the USP family, such as USP7, have also been reported to contribute to AML proliferation and drug resistance by regulating key oncoproteins (Cartel et al. 2021; Zangooie et al. 2024), underscoring the broader involvement of deubiquitinating enzymes in AML pathogenesis. Analysis of clinical samples revealed elevated USP30 expression in AML. Core pathological features of AML include uncontrolled cell proliferation, dysregulated cell cycle control, and apoptotic imbalance (Wachter and Pikman 2024). Here, USP30 knockdown reduced the proportion of EdU-positive cells, indicating suppressed DNA synthesis and impaired proliferative capacity in AML cells. This suggests USP30 drives disease progression by maintaining the hyperproliferative activity of AML cells, aligning with the leukemic cell infiltration and excessive proliferation hallmark of AML. Dysregulated cell cycle is critical for AML cell survival (Bouligny et al. 2023). Our findings show that USP30 silencing induced G0/G1 arrest and reduced S phase entry, indicating USP30 promotes cell cycle progression by overcoming the G0/G1 restriction point. This regulatory role in the cell cycle provides a critical foundation for the continuous proliferation of AML cells (Schnerch et al. 2012). Additionally, AML cells evade immune elimination by suppressing apoptosis, and caspase-3 serves as a key executioner of the apoptotic pathway (Man

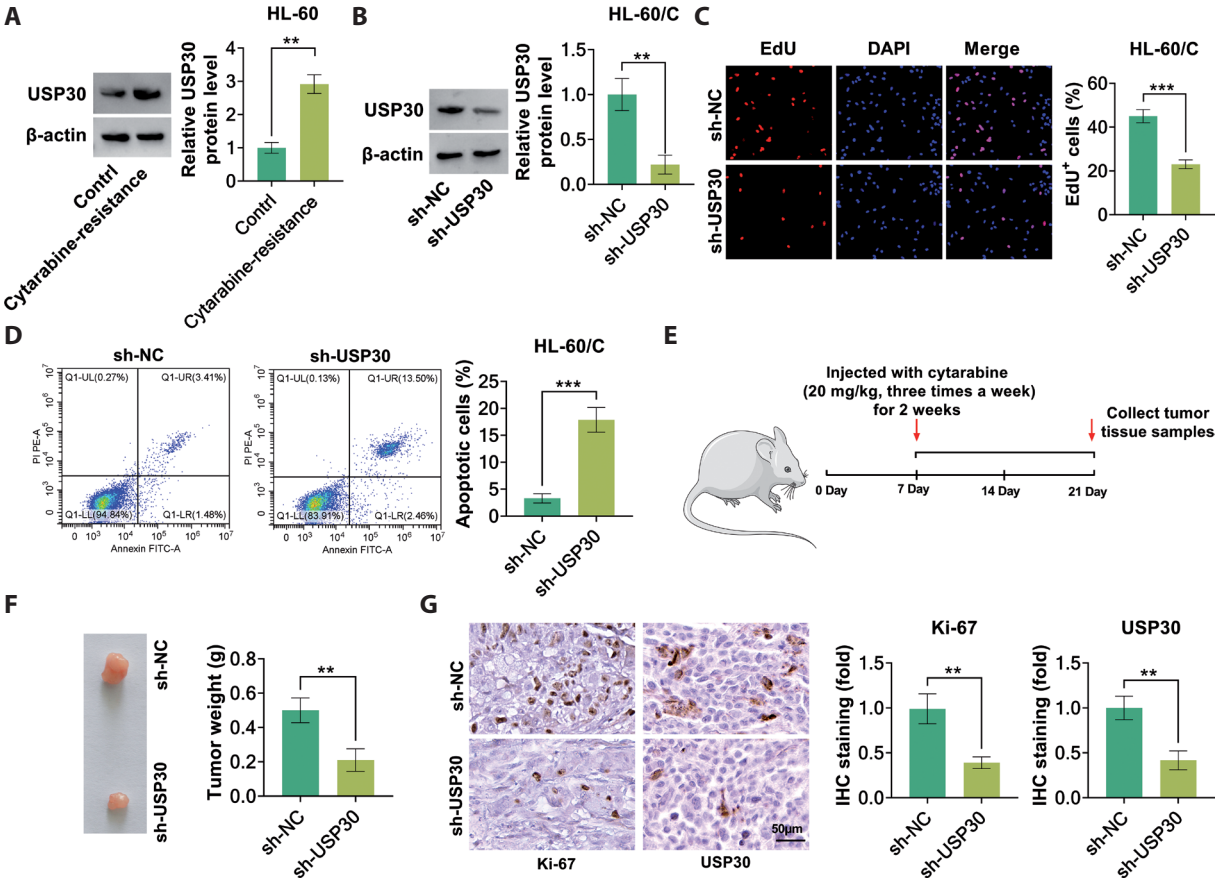


Figure 5. USP30 promotes Ara-C resistance in AML. **A.** USP30 expression in Ara-C-resistant HL-60 cells was determined by Western blot. **B.** Western blot analysis of USP30 protein level in HL-60/C cells transfected with sh-NC and sh-USP30. **C.** EdU assay was employed to assess cell proliferation of HL-60/C cells transfected with sh-NC and sh-USP30. **D.** Apoptosis of HL-60/C cells transfected with sh-NC and sh-USP30 was evaluated by flow cytometry. **E-G.** HL-60/C cells stably transfected with sh-NC and sh-USP30 were injected into BALB/c nude mice. From the 7th day, these mice were injected with Ara-C (20 mg/kg) three times a week for 2 weeks. **E.** Animal treatment protocol schematic diagram. **F.** Tumor tissues were excised, photographed, and weighed. **G.** IHC was performed to assess the protein levels of Ki-67 and USP30 in tumor tissues. ** $p < 0.01$, *** $p < 0.001$.

et al. 2017). Downregulation of USP30 resulted in enhanced caspase-3 activity and a marked increase in apoptotic cell death, indicating that USP30 inhibited the apoptotic pathway in AML cells. Under normal conditions, caspase-3 activation triggers apoptosis (Jiang et al. 2020), but in AML, USP30 maintains cell survival by suppressing caspase-3 activity, thereby promoting disease progression.

FOXM1 is known to exhibit elevated expression in diverse cancer cell types (Yu et al. 2017; Chai et al. 2025). Extensive research indicates that FOXM1 is crucial for the malignant progression of tumor cells. Notably, abnormally elevated FOXM1 expression is significantly correlated with poor clinical tumor classification, high proliferation rates, and unfavorable prognosis (Kim et al. 2019; Kalathil et al. 2020). In previous studies, FOXM1 has been widely reported to be significantly correlated with AML progression and treatment efficacy (Sheng et al. 2020). Using bioinformatics tools, we identified a regulatory interaction between USP30 and FOXM1. Further experimental validation confirmed this prediction, revealing that USP30 maintained FOXM1 stability through deubiquitination. Functional validation experiments demonstrated that USP30 promoted FOXM1 expression, thereby driving malignant biological behaviors of AML cells.

Ara-C, a deoxycytidine analog and antimetabolite chemotherapy drug, primarily acts on the S phase of the tumor cell cycle. By inhibiting DNA synthesis, it disrupts tumor cell proliferation, serving as a cornerstone in AML chemotherapy (Sun et al. 2019; Sadeghi et al. 2024). The pro-resistance role of FOXM1 in AML has been confirmed (Raghuwanshi et al. 2025). Also, a prior report shows that FOXM1 enhances chemoresistance in AML, leading to treatment failure (Khan et al. 2018). Notably, the contribution of USP30 to Ara-C resistance has not been thoroughly clarified.

Therefore, an Ara-C-resistant cell line was established. *In vitro* experimental results showed that upon USP30 knockdown, the proliferative capacity of HL-60/C cells significantly declined, demonstrating that USP30 is a key factor sustaining the proliferative activity of HL-60/C cells. This suggests USP30 helps AML cells resist the chemotherapeutic inhibitory effects of Ara-C by promoting DNA synthesis and proliferation in drug-resistant cells. Meanwhile, USP30 knockdown resulted in a notable elevation in the apoptosis rate of HL-60/C cells, confirming that USP30 suppressed the apoptotic pathway in HL-60/C cells. This indicated USP30 maintained drug-resistant cell survival through dual mechanisms promoting proliferation and inhibiting apoptosis and acts as a crucial safeguard for cell survival in the Ara-C-resistant microenvironment. At the *in vivo* level, USP30 was found to enhance the tumorigenicity of Ara-C-resistant AML cells, further demonstrating that USP30 plays a critical role not only in regulating drug-resistant cell phenotypes *in vitro* but also in tumor growth *in vivo*.

Overall, this study unveiled the molecular mechanism by which USP30 stabilized FOXM1 through deubiquitination, thereby promoting AML cell proliferation, inhibiting apoptosis, and enhancing Ara-C resistance, providing a novel therapeutic target for AML drug resistance. However, several limitations, such as limited experimental models, incomplete understanding of the mechanistic network, and insufficient evidence for clinical translation, merit consideration. Moving forward, future studies should expand the diversity of cell and animal models, delve into the interactions between the USP30-FOXM1 axis and other resistance pathways, validate its prognostic value through large-scale clinical cohorts, and develop USP30-targeted combination therapeutic strategies to bridge the gap between basic discoveries and clinical applications.

Ethics approval. The study was approved by the Institutional Ethics Committee of Chongqing University Cancer Hospital (IRB No. CZLS2023335-A), with every participant having provided the written informed consent. The procedures performed on all animals complied with the standards formulated by the Experimental Animal Ethics Committee of Chongqing University Cancer Hospital (No. CQCH-LAE-20231020023).

Data Availability Statement. The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' contributions. YJ and ZZ designed and performed the research. HS, RL, and ZZ collected and analyzed the data. BM and YW plotted the figures. YJ and ZZ wrote the manuscript. All authors read and approved the final manuscript.

Conflict of interest. The authors declare that they have no financial conflicts of interest.

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Supplementary Material

USP30 promotes cytarabine resistance in acute myeloid leukemia by deubiquitinating and stabilizing FOXM1

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Supplementary Figure

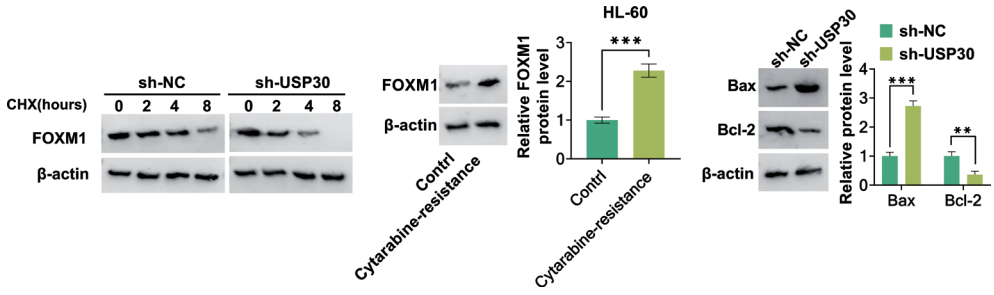


Figure S1. A. CHX chase assay was conducted to evaluate the protein stability of FOXM1 in HL-60 cells transfected with sh-NC or sh-USP30 at 0, 2, 4, and 8 hours. **B.** Western blot was used to detect FOXM1 protein expression in HL-60 parental cells (Control) and Ara-C-resistant HL-60 cells. **C.** Western blot analysis of Bax and Bcl-2 protein levels in tumor tissues from mice transfected with sh-NC or sh-USP30. ** $p < 0.01$, *** $p < 0.001$.