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Original Article

FBXL18 is required for ovarian cancer cell proliferation and migration through activating AKT signaling

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Abstract: Background: F-box and leucine-rich repeat protein 18 (FBXL18) is an F-box protein that functions as an E3-ubiquitin ligase, and it plays pivotal roles in multiple disease processes. However, its role and underlying mechanism in ovarian cancer (OC) are still unknown. We investigated the impact and mechanism of FBXL18 in OC cell growth and tumorigenesis. Methods: Silent interfering RNAs and overexpression plasmids were employed to knock down and overexpress *FBXL18* in OC cells (A2780 and OVCAR3). CCK-8, colony formation, cell migration, and nude mouse xenograft assays were used to assess the effect of FBXL18 on OC cell proliferation and migration. Western blotting and co-immunoprecipitation followed by ubiquitination assays were performed to detect the mechanism of the FBXL18/AKT axis in OC. Results: *FBXL18* knockdown inhibited OC cell proliferation and migration, whereas FBXL18 overexpression showed the opposite results. Phosphorylated-AKT (S473) protein expression was increased by FBXL18 overexpression and markedly decreased after phosphorylated-AKT inhibitor (MK-2206) treatment. Co-immunoprecipitation assays demonstrated that FBXL18 strongly interacted with AKT in OC cells. Ubiquitination assays revealed that FBXL18 promoted K63-linked AKT ubiquitination to activate AKT. MK-2206 treatment reversed the increase in proliferation and migration of OC cells induced by FBXL18 overexpression. Conclusions: FBXL18 promoted OC cell proliferation and migration and facilitated OC tumorigenesis. Mechanically, FBXL18 interacted with AKT and promoted K63-linked ubiquitination of AKT to activate AKT in OC cells. Our study revealed that the FBXL18/AKT axis plays a crucial role in the OC process, indicating that FBXL18 may be a valuable target for OC diagnosis and treatment.

Keywords: FBXL18, ovarian cancer, cell proliferation, migration, AKT

Introduction

Ovarian cancer (OC) refers to malignant tumor growth in the ovaries. Typically, OC is often not diagnosed early and since it has highly aggressive characteristics, coupled with a 5-year survival rate of less than 40%, it has one of the highest mortality rates of gynecological cancers [1]. The current treatment strategies include cytoreductive surgery and platinum-based chemotherapy, but the treatment effect is still suboptimal [2]. Therefore, in-depth exploration of the mechanism of OC occurrence and development and the identification of effective targets are currently urgently needed.

F-box and leucine-rich repeat protein 18 (FBXL18) is a member of the F-box protein (FBP) family. As subunits of the S-phase kinase-associated protein 1 (Skp1)-Cullin-F-box protein (SCF) E3 ligase complex, FBPs contain two domains: an N-terminal F-box motif and a C-terminal leucine-rich repeat (LRR) motif or tryptophan-aspartic acid (WD) repeat motif. Accordingly, the FBP family is divided into three classes, including F-box and WD repeat domain-containing proteins, F-box and LRR domain-containing proteins (FBXL), and F-box-only proteins (FBXO) [3]. The F-box motif recruits Skp1, whereas the LRR/WD motif is engaged in recognizing diverse substrates for ubiquitin ligation

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and subsequent degradation [4]. The increasing characterization of FBPs and the discovery of their substrates have increased the understanding of how FBPs play various roles in cell cycle progression, signal transduction, and tumorigenesis [5-7]. FBXL18 has been shown to play pivotal roles in multiple cellular processes [8, 9]. However, the detailed functions of FBXL18 in OC development and cell growth have not been elucidated.

In this study, evidence is presented for the first time to show that FBXL18 functions as a key factor for OC cell growth maintenance. Specifically, deletion of *FBXL18* significantly inhibited OC cell proliferation and migration, while FXBL18 overexpression showed the opposite result. Further study showed that FBXL18 increased phosphorylated-AKT (p-AKT) protein expression by directly interacting with AKT and promoting K63-linked ubiquitination to active AKT. However, application of a p-AKT inhibitor (MK-2206) abolished the increase in OC cell proliferation and migration induced by FBXL18 overexpression, confirming that FBXL18 mediates OC cell proliferation and tumorigenesis and that these processes are closely associated with activation of AKT signaling. These results provide an increased understanding of the role of the FBXL18/AKT axis in OC tumorigenesis and propose a laboratory basis for the diagnosis and treatment targets of OC.

Materials and methods

Cell culture and treatments

Human OC cell lines (A2780, OVCAR3) were obtained from the Chinese Academy of Cell Collection (Shanghai, China) and then cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (FBS) (Gibco, Carlsbad, CA, USA) and RPMI-1640 (Gibco, USA) containing 20% FBS at 37°C in a humidified chamber with 5% CO_2, respectively.

Silent interfering RNAs (siRNAs) (GenePharma, Suzhou, China) targeting *FBXL18* and the negative control were transfected into cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). Overexpression plasmids including pcDNA3.1-Flag-labeled FBXL18, pEGFP-labeled AKT, pRK5-HA-labeled ubiquitin WT/K48/K63/K0, or an empty vector (Sangon Biotech, Shanghai, China) were transfected into cells using X-tremeGENE HP DNA Transfection Reagent (Roche, Mannheim, BW, Germany). Functional assays were conducted after transfection for 48 h. The target sequences recognized by the siRNAs included the following: si-*FBXL18*$^{1\#}$ 5'-3': GGACAUAUCCAAUGAUGAUTT; si-*FBXL18*$^{2\#}$ 5'-3': GCCUUGACAAGAGCCUCAUTT; negative control siRNA (si-NC) 5'-3': ACGUGACACGUUCGGAGAA.

MK-2206 2HCL (MK-2206, 10 μM/L, Selleck, USA) is a specific phospho-AKT inhibitor and was applied to block AKT phosphorylation when cells were transfected with plasmids.

Cell proliferation assays

For the CCK-8 assay, post-treatment cells at a density of $3*10^3$ were plated on 96-well plates and inoculated with CCK-8 solution (Beyotime Institute of Biotechnology, Shanghai, China). Then, the optical density was assessed every 24 h at 450 nm using a microplate reader (Bio-Rad Model 680, Hercules, CA, USA).

For the colony formation assay, 1500 cells were added to six-well plates and cultured for approximately 2 weeks before fixing with methanol. Then, the cells were stained with 0.1% crystal violet (Beyotime, Shanghai, China) and counted for analysis.

Cell migration assay

After treatment, cells were incubated with 300 μL of serum-free medium and deposited in the upper chamber (Corning, NY 14831, USA), whereas the lower chamber was filled with 700 μL of complete medium. After approximately 48 h, the cells crossed the chamber membrane and were harvested outside the chamber. Then, the cells were fixed with 4% paraformaldehyde, stained with 0.1% crystal violet, and observed under a bright-field microscope.

Mouse xenografts

Four-week-old female athymic BALB/c nude mice were housed in suitable barrier facilities under specific pathogen-free conditions with individually ventilated cages, sterilized food, and water at the Animal Center of Nanjing Medical University and taken care of by members of professor Mingxi Liu's lab. Animal use was approved by the Animal Ethical and Welfare Committee of Nanjing Medical University (Permit Number: IACUC-2004020).

Human OC cells (OVCAR3) were treated with sh-*FBXL18* and the control group (sh-NC) for 48 h. Then, the cells were harvested, washed, and re-suspended in 100 μL of phosphate-buffered saline. The flanks of nude mice were subcutaneously injected with sh-*FBXL18*-treated cells, and another flank was injected with control cells (n = 6). The tumor volumes were calculated every 3 days ($V = 0.5 \times D \times d^2$ (V, volume; D, longitudinal diameter; d, latitudinal diameter)). After approximately 2 weeks, the mice were sacrificed, and subcutaneous tumors were removed, measured, and fixed in formalin.

Immunofluorescence assay

Paraffin-embedded tissues were dewaxed and hydrated. Then, antigen retrieval was performed by boiling samples in 10 mM sodium citrate buffer (pH 6.0). After blocking with 1% bovine serum albumin for 2 h, slides containing target tissues were incubated with the primary antibody (anti-Ki67 mouse antibody, Santa Cruz Biotechnology, Dallas, TX, USA) and then with Alexa-Fluor secondary antibodies (Thermo Scientific, Wilmington, DE, USA). Finally, cell nuclei were stained with 5 μg/mL 4',6-diamidino-2-phenylindole (DAPI, Beyotime) for 10 min. All samples were viewed using a Zeiss laser confocal microscope (LSM810, Carl Zeiss, Oberkochen, Germany) and quantified with Zeiss software.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR) assays

Total RNA in cells was extracted using the TRIzol reagent (Vazyme, Nanjing, China) according to the manufacturer's protocol and reverse transcribed into cDNA using Hiscript III RT Super Mix from the qPCR kit (Vazyme). RT-qPCR was quantified using SYBR Green Master Mix (Vazyme) with an Applied Biosystems 7500 Real-Time PCR System. Finally, the relative expression levels of genes were normalized to that of *18s rRNA* using the $2^{-\Delta\Delta CT}$ method. The *FBXL18* gene primers were 5'-AGTCGCTTCTCCGCAGGTG-3' and 5'-ACCGCAAGGACAACAGACTACC-3'. The *18s rRNA* gene primers were 5'-AAACGGCTACCACATCCAAG-3' and 5'-CCTCCAATGGATCCTCGTTA-3'.

Western blotting assay

A2780 and OVCAR3 cells were lysed using lysis buffer (RIPA, Beyotime), total proteins were extracted, and the protein concentration was measured using a BCA protein assay kit (Beyotime). Subsequently, the proteins were denatured at 100°C for 10 min, separated by 10% sodium dodecyl-sulfate polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes (Millipore, Billerica, MA, USA), followed by blocking with 5% nonfat milk at room temperature for 2 h. The membranes were incubated overnight with corresponding primary antibodies at 4°C. The next day, after washing with TBST, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 1 h at room temperature. Finally, protein bands were visualized using enhanced chemiluminescence (Beyotime) and a digital gel image analysis system. Protein expression levels were quantified using ImageJ software. The following primary antibodies were used: anti-FBXL18 mouse antibody (1:1000; Santa Cruz Biotechnology), anti-AKT rabbit antibody (1:1000; Proteintech, Wuhan, China), anti-p-AKT (Ser-473) mouse antibody (1:1000; Proteintech), anti-GAPDH mouse antibody (1:1000; Proteintech), anti-GFP mouse antibody (1:1000; Proteintech), anti-HA mouse antibody (1:1000; Santa Cruz Biotechnology), and anti-Flag mouse antibody (1:1000; Sigma, St. Louis, MO, USA).

Co-immunoprecipitation (Co-IP) and ubiquitination assays

Cells were transfected with overexpression plasmids including Flag-labeled FBXL18, GFP-labeled AKT, and HA-labeled ubiquitin WT/K48/K63/K0. After 48 h of transfection, the cell lysates were incubated with anti-GFP or anti-Flag-labeled antibodies overnight at 4°C, followed by co-incubation with washing protein A beads (Invitrogen) for 3 h at 4°C. Finally, the antigen-antibody-bead complex was denatured by heating at 100°C for 10 min, and the supernatant was detected with specific primary antibodies by western blotting.

Statistical analysis

Data are presented as the mean ± standard deviation of at least three replicates. SPSS 17.0 (IBM Corporation, Armonk, NY, USA) and GraphPad Prism 9.0 (GraphPad Software, La Jolla, CA, USA) software were used for the statistical analyses. Differences between the two groups were analyzed using Student's t-test,

and differences among multiple groups were analyzed using a one-way analysis of variance. A value of $P < 0.05$ was considered a significant difference.

Results

FBXL18 is a risk factor that affects the prognosis of OC

We identified the E3 ubiquitin protein ligase genes associated with overall survival (OS) of OC through univariate Cox regression analysis. In total, 45 and 36 genes associated with OC prognosis were identified in The Cancer Genome Atlas (TCGA) (**Figure 1A**) and the GSE63885 database (**Figure 1B**), respectively. By intersecting the above two sets of gene data and plotting the Kaplan-Meier curve (suggesting the relationship between the expression level and OS time), we obtained a total of four meaningful genes, namely DDB1 and CUL4 associated factor 15 (*DCAF15*), PTEN-induced putative kinase 1 (*PINK1*), Lysine-specific demethylase 2A (*KDM2A*), and *FBXL18*. High expression of *DCAF15* and *PINK1* was associated with a better prognosis of OC (**Figure 1C**, **1D**), but high expression of *KDM2A* and *FBXL18* was associated with a worse prognosis (**Figure 1E**, **1F**).

We selected the OC prognostic risk factor genes *KDM2A* and *FBXL18* and mapped their expression levels in OC tissue and normal ovarian tissue using the Gene Expression Profiling Interactive Analysis (GEPIA) database. We found that the expression level of *FBXL18* in OC was significantly higher than that in normal ovarian tissue, and the difference in expression of *KDM2A* in the two groups was not statistically significant (**Figure 1G**, **1H**). These results indicate that *FBXL*18 is a risk factor that affects the prognosis of OC and has potential research value for the occurrence and development of OC. Therefore, we selected *FBXL18* for follow-up studies.

FBXL18 knockdown inhibits OC cell proliferation and migration in vitro

To clarify the biological role of FBXL18 in OC cells in vitro, we first transfected A2780 and OVCAR3 cells with two independent siRNAs that both dramatically decreased FBXL18 mRNA and protein expression, as measured by RT-qPCR and western blotting (**Figure 2A-C**). CCK-8 and in vitro colony formation assays showed that repression of *FBXL18* significantly decreased the proliferative ability of OC cells (**Figure 2D-G**). Given that cancer cell migration also plays an important part in tumor malignant progression, the effect of *FBXL18* knockdown on cell migration capacity was determined using a Transwell assay. The results showed a significant reduction in the migration capacity of *FBXL18*-siRNA-treated OC cells compared with that of the si-NC group (**Figure 2H**, **2I**). The above results strongly suggested that FBXL18 could promote the proliferation and migration of OC cells in vitro.

Repression of FBXL18 decreases OC cell proliferation in vivo

To further assess the effects of FBXL18 on tumorigenesis in vivo, we subcutaneously injected *FBXL18* knockdown (sh-*FBXL18*) and NC group (sh-NC) OC cells (OVCAR3) into the armpits of nude mice to establish a subcutaneous xenograft tumor model. The nude mice were sacrificed after approximately 2 weeks. The tumor volumes and weights in the sh-*FBXL18* group were significantly lower than those in the sh-NC group (**Figure 3A-C**). Furthermore, immunofluorescence analysis showed that the proportion of Ki67-positive cells was significantly lower in the sh-*FBXL18* group than in the sh-NC group (**Figure 3D**, **3E**). Collectively, these results indicated that FBXL18 could facilitate ovarian tumor progression in vivo.

Overexpression of FBXL18 promotes OC cell proliferation and migration

Similarly, we transfected A2780 and OVCAR3 cells with pcDNA3.1-Flag-FBXL18 to overexpress FBXL18, as confirmed by western blotting (**Figure 4A**). As expected, FBXL18 overexpression significantly increased OC cell proliferation (**Figure 4B-E**) and migration (**Figure 4F**, **4G**) in vitro. Taken together, these results suggested that FBXL18 is required for the proliferative and migratory abilities of OC cells.

FBXL18 is required for OC cell proliferation and migration via activation of AKT signaling

Activation of AKT is the most common signaling mechanism involved in OC cell survival, growth, and proliferation [10-12]. Previous studies have demonstrated that FBXL18 promotes glioblas-

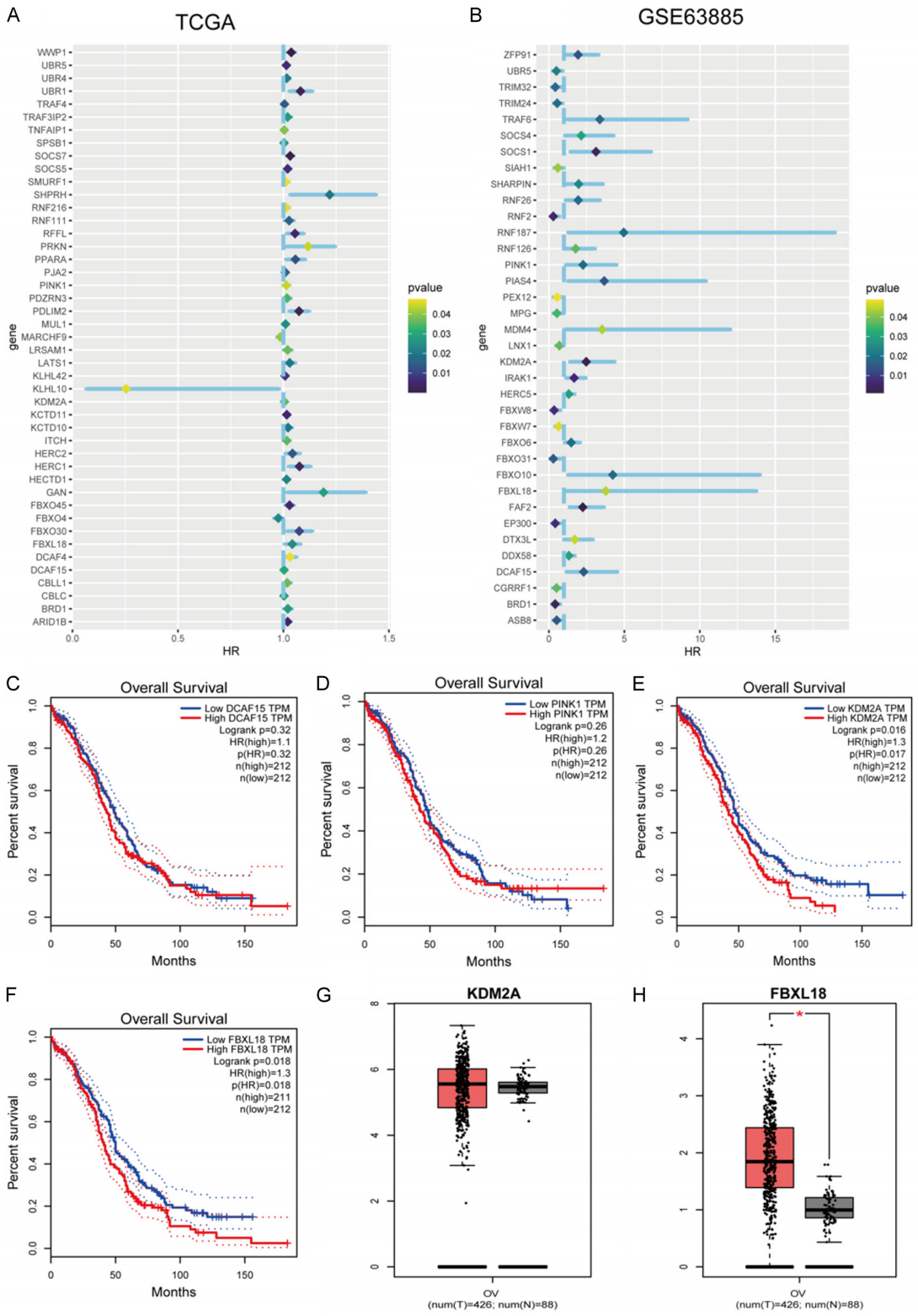


Figure 1. F-box and leucine-rich repeat protein 18 (FBXL18) is a risk factor for and is negatively associated with the prognosis of ovarian cancer (OC). A. E3 ubiquitin protein ligase genes associated with overall survival (OS) of OC in The Cancer Genome Atlas database (TCGA). B. E3 ubiquitin protein ligase genes associated with OS of OC in the GSE63885 database. C-F. The Kaplan-Meier curves of four meaningful genes from the intersecting portion of the two gene data sets. G, H. The expression levels of Lysine-specific demethylase 2A *(KDM2A)* and *FBXL18* in OC tissue (n = 426) and normal ovarian tissue (n = 88) from the Gene Expression Profiling Interactive Analysis database (t-test). *$P < 0.05$.

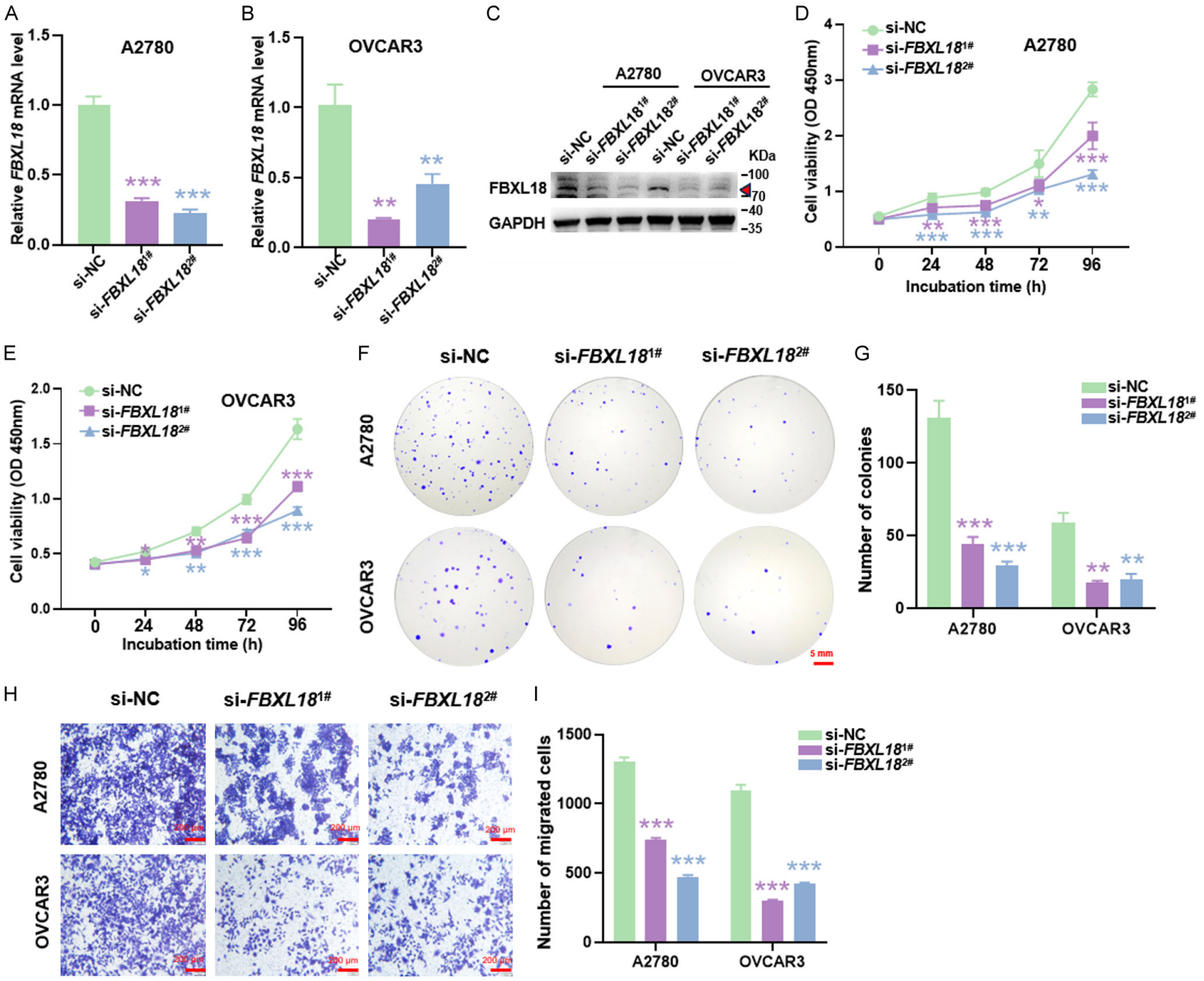

A
A2780
Relative FBXL18 mRNA level
si-NC
si-FBXL18¹#
si-FBXL18²#
B
OVCAR3
C
A2780
OVCAR3
KDa
FBXL18
GAPDH
D
si-NC
si-FBXL18¹#
si-FBXL18²#
Cell viability (OD 450nm)
Incubation time (h)
E
F
si-NC
si-FBXL18¹#
si-FBXL18²#
5 mm
G
Number of colonies
H
200 μm
I
Number of migrated cells

Figure 2. Knockdown of *FBXL18* suppresses OC cell (A2780 and OVCAR3) proliferation and migration in vitro. (A, B) Real-time quantitative PCR was used to detect the relative expression of *FBXL18* mRNA in A2780 and OVCAR3 cells after *FBXL18* knockdown (analysis of variance, ANOVA). (C) Western blotting was employed to verify the efficiency of *FBXL18* knockdown. (D, E) A CCK-8 assay (n = 4 for each group) was conducted to determine the viability of A2780 and OVCAR3 cells (ANOVA). (F) A colony formation assay (n = 3) was performed to examine the proliferation of A2780 and OVCAR3 cells. Scale bar: 5 mm. (G) Quantification of the results from (F) (ANOVA). (H) A Transwell assay (n = 3) was conducted to assess the migratory abilities of A2780 and OVCAR3 cells. Scale bar: 200 μm. (I) Quantification of (H) (ANOVA). *P < 0.05, **P < 0.01, ***P < 0.001, compared with the si-NC condition.

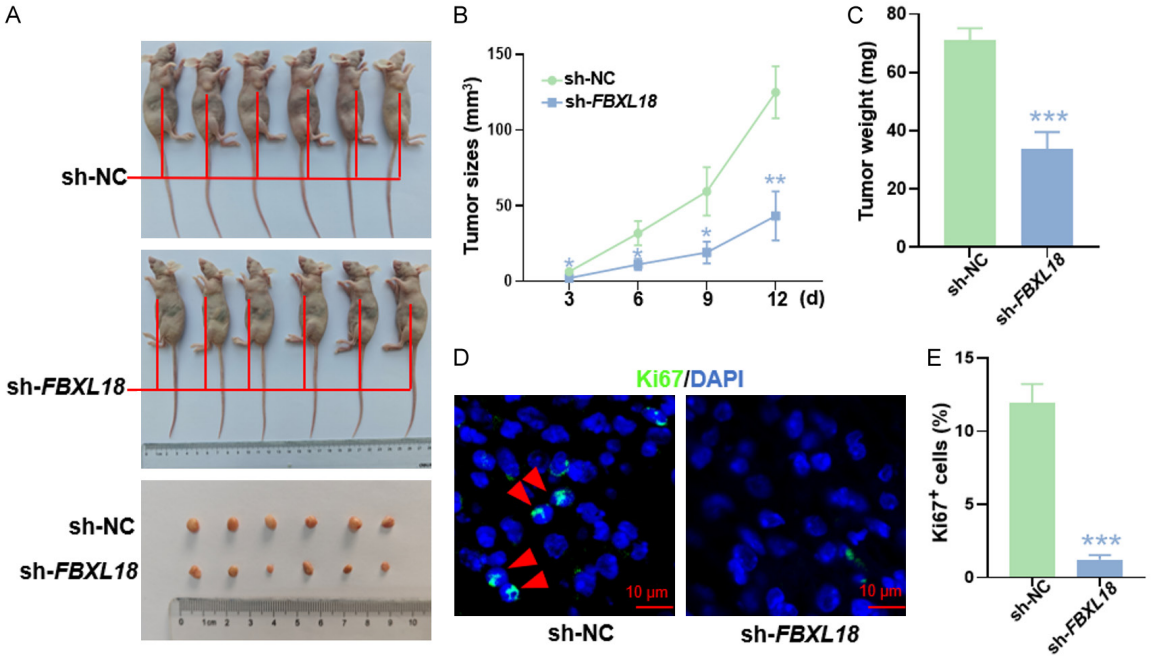


Figure 3. Decreasing *FBXL18* attenuates OC cell proliferation in vivo. (A) OVCAR3 cells were treated with sh-*FBXL18* and sh-NC and then subcutaneously injected into the armpits of nude mice (n = 6). The macroscopic appearance of tumors is shown. (B) Tumor sizes were calculated every 3 days and are represented as the mean tumor size ± standard deviation (n = 6) (t-test). (C) The weights of subcutaneous tumors were measured (n = 6) (t-test). (D) Ki67 expression in tumors was examined using immunofluorescence. n = 3. Scale bar: 10 μm. (E) Quantification of Ki67-positive cells in (D) (t-test). *P < 0.05, **P < 0.01, ***P < 0.001, compared with the sh-NC condition.

toma cell proliferation via promoting K63-linked ubiquitination to activate AKT signaling in human glioma cancer [13]. Therefore, we next examined whether the effect of FBXL18 on the promotion of proliferation in OC cells was associated with AKT activation.

To address this question, we knocked down and overexpressed *FBXL18* in A2780 and OVCRA3 cells to assess AKT activation. Western blotting results revealed that decreased *FBXL18* expression prominently reduced the level of p-AKT (S473) (**Figure 5A-D**), while FBXL18 overexpression enhanced the level of p-AKT (S473) (**Figure 5E-H**). However, treatment with MK-2206, a specific p-AKT inhibitor, could rescue the upregulation of p-AKT (S473) induced by FBXL18 overexpression. Together, these findings indicated that AKT activation is highly likely to participate in the OC cell proliferation caused by FBXL18 overexpression.

To further verify that AKT activation contributes to the OC cell proliferation and migration induced by FBXL18, MK-2206 was applied to inhibit p-AKT activation after FBXL18 overexpression. Consistently, CCK-8 (**Figure 5I**, **5J**), colony formation (**Figure 5K**, **5L**), and migration assays (**Figure 5M**, **5N**) validated that FBXL18 overexpression noticeably enhanced the proliferative and migratory abilities of OC cells, whereas MK-2206 treatment could dramatically rescue the altered phenotypes in FBXL18-overexpressed OC cells. The above findings

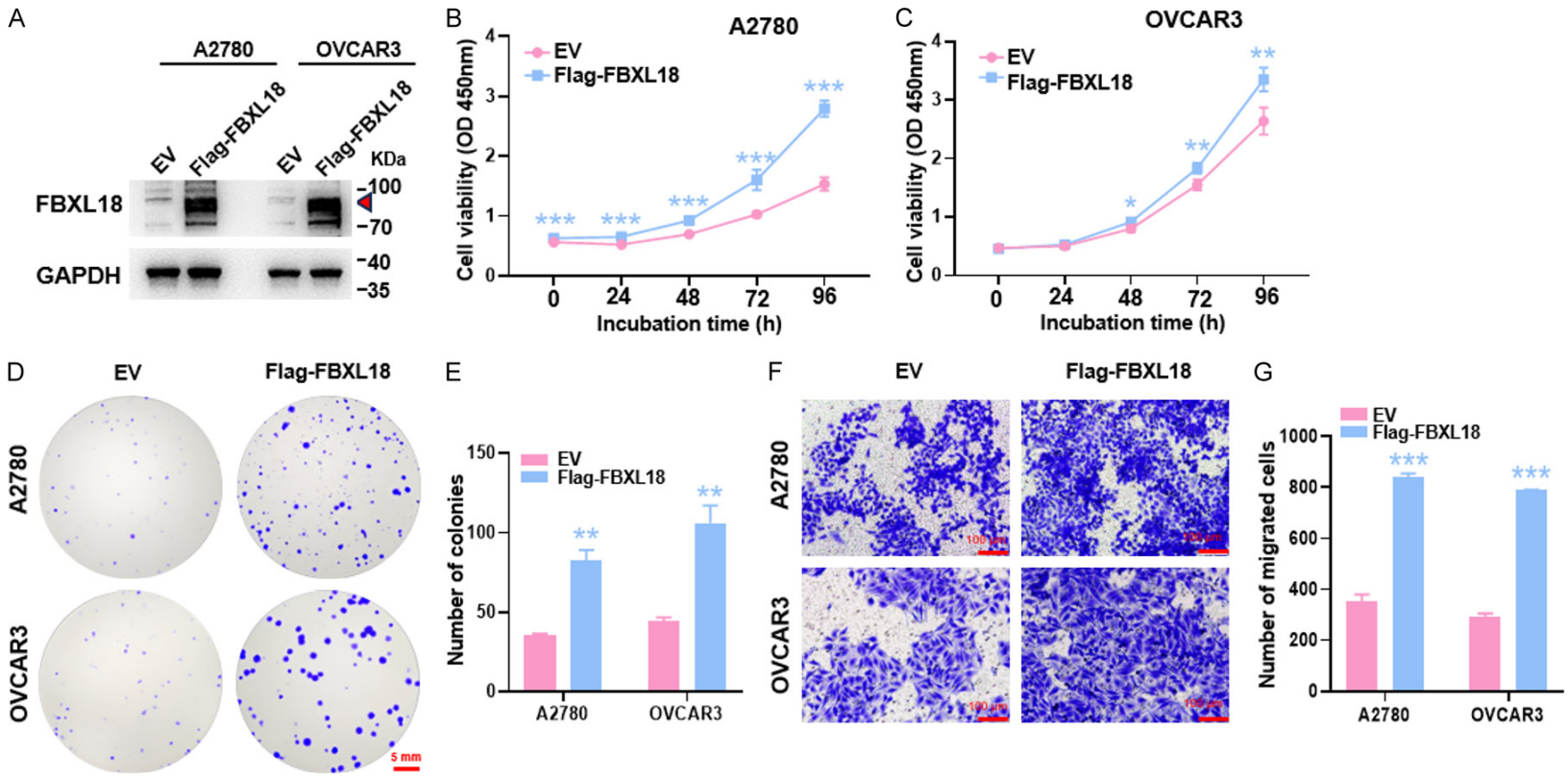
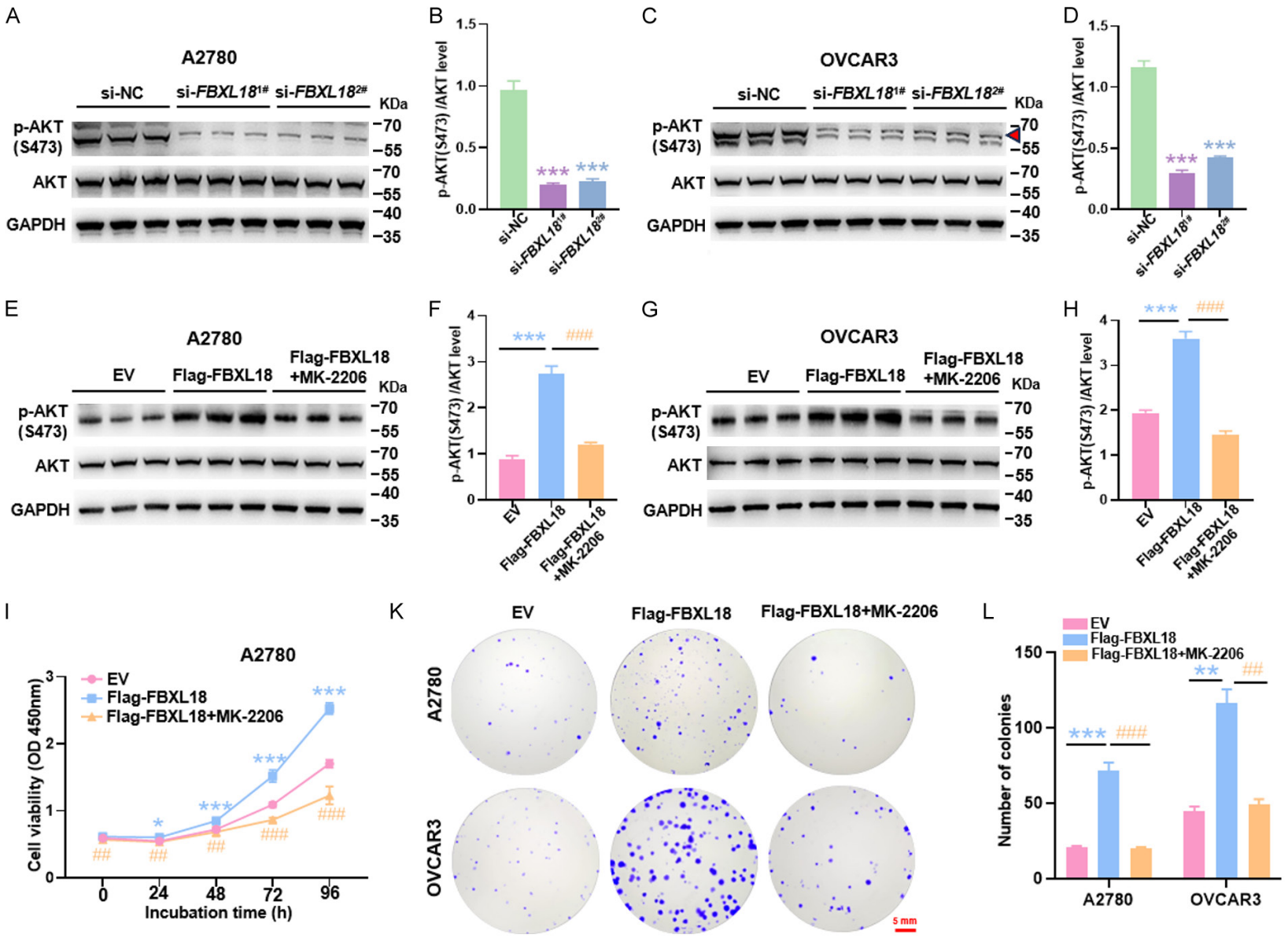


Figure 4. Overexpression of FBXL18 promotes OC cell proliferation and migration in vitro. (A) Western blotting of FBXL18 protein expression in A2780 and OVCAR3 cells transfected with pcDNA3.1-Flag-FBXL18 and pcDNA3.1-NC (EV). (B, C) The proliferative viability of OC cells was detected using a CCK-8 assay after OC cells were treated with the FBXL18 and EV overexpression plasmids (n = 4) (t-test). A colony formation assay (D, E) (n = 3) (t-test, scale bar: 5 mm) was conducted, and the migratory ability was assessed using a cell migration assay (F, G) (n = 3) (t-test). Scale bar: 100 μm. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the EV condition.


A
A2780
si-NC
si-FBXL18¹#
si-FBXL18²#
KDa
p-AKT (S473)
AKT
GAPDH
70
55
40
35
B
p-AKT(S473) /AKT level
C
OVCAR3
D
E
EV
Flag-FBXL18
Flag-FBXL18 +MK-2206
F
G
H
I
Cell viability (OD 450nm)
Incubation time (h)
K
5 mm
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Number of colonies

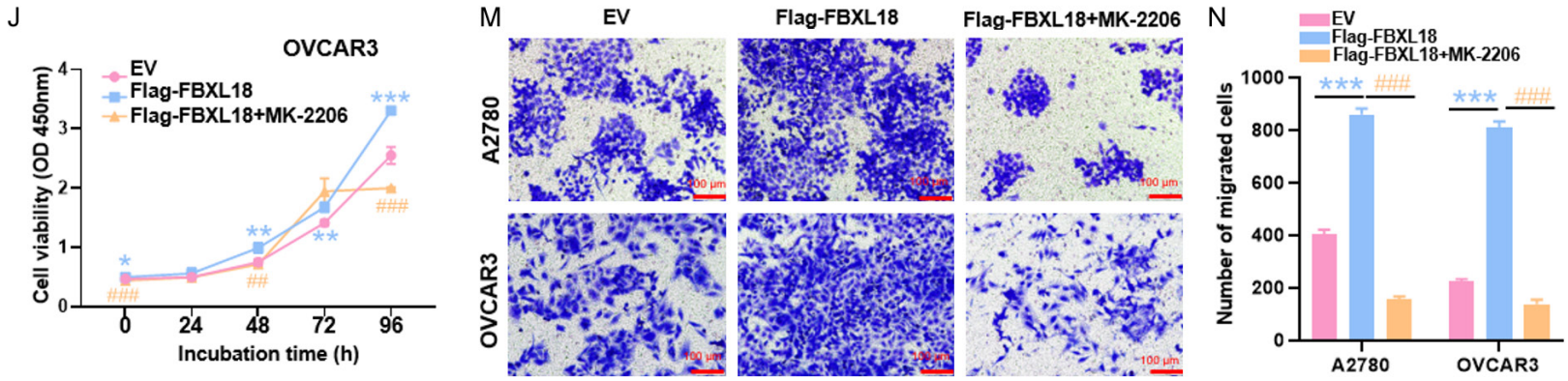

Figure 5. AKT activation is implicated in OC cell proliferation and migration induced by FBXL18 overexpression. (A-D) A2780 and OVCAR3 cells were treated with si-*FBXL18*s and si-NC for 48 h, followed by western blotting analysis of phosphorylated-AKT (p-AKT) (Ser473) protein expression (n = 3). (B) and (D) show the quantification of (A) and (C) (ANOVA). (E-H) OC cells were transfected with FBXL18 overexpression plasmids with or without MK-2206 treatment (p-AKT inhibitor, 10 μM), and the levels of AKT phosphorylation at Ser473 were determined by western blot analysis in A2780 (E, F) (t-test) and OVCAR3 (G, H) cells (t-test, n = 3). CCK-8 (I, J) (n = 4) (t-test) and colony formation (K, L) (n = 3) (t-test, scale bar: 5 mm) assays were used to assess the effect of p-AKT inhibition on the proliferative viability of OC cells induced by FBXL18. (M, N) A cell migration assay (n = 3) (t-test, scale bar: 100 μm) was performed to reveal the relationship between AKT activation and the migratory ability of OC cells induced by FBXL18. **P* < 0.05, ***P* < 0.01, ****P* < 0.001, compared with the si-NC or EV condition. $^{\#}P$ < 0.05, $^{\#\#}P$ < 0.01, $^{\#\#\#}P$ < 0.001, compared with the Flag-FBXL18 condition.

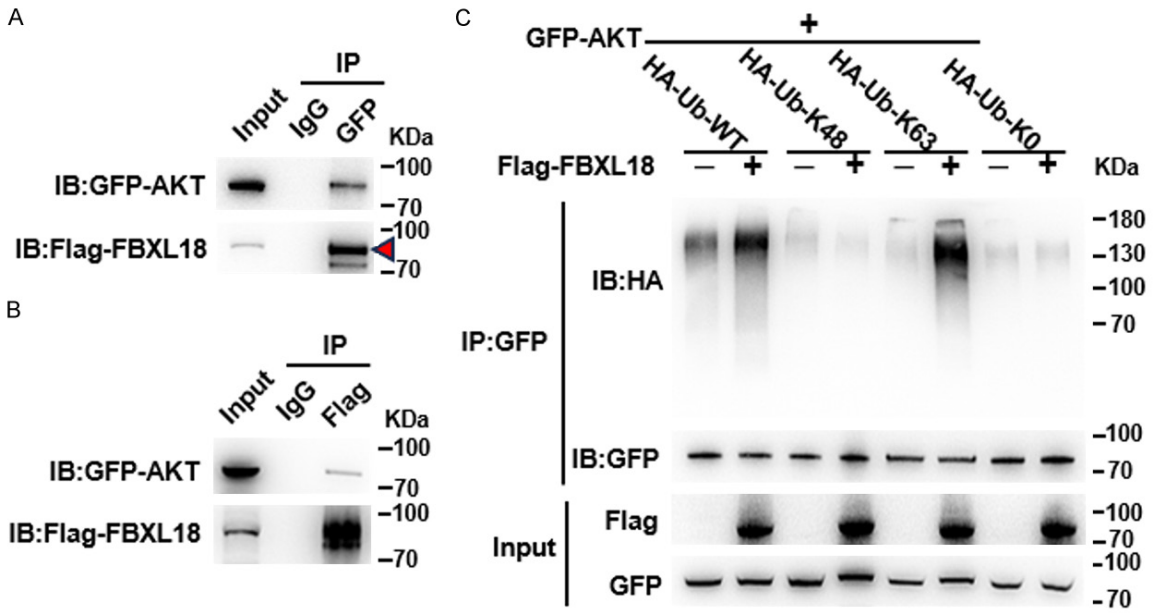

Figure 6. FBXL18 interacts with AKT and promotes AKT K63-linked ubiquitination. A, B. GFP-labeled AKT was co-transfected with Flag-labeled FBXL18 in OVCAR3 cells followed by a co-immunoprecipitation assay. C. The OVCAR3 cells were transiently transfected with Flag-labeled FBXL18, GFP-labeled AKT, and HA-labeled-Ub-WT/K48/K63/K0 plasmids, followed by a ubiquitin assay.

confirmed that FBXL18 promotes proliferation of OC cells and this effect is closely related to activation of AKT signaling.

FBXL18 interacts with AKT and promotes K63-linked ubiquitination of AKT in OC cells

To identify the working relationship between FBXL18 and AKT, we co-expressed Flag-tagged FBXL18 with GFP-tagged AKT in OVCAR3 cells and then detected their interaction. Co-IP assays demonstrated that FBXL18 exhibited a strong interaction with AKT (**Figure 6A**, **6B**). Because FBPs play crucial roles in diverse cellular processes through ubiquitination and subsequent function of target proteins, we next explored the relationship between FBXL18 and AKT ubiquitination in OC cells. K63-linked ubiquitination usually induces protein activation or strengthens protein stability, whereas K48-linked ubiquitination usually promotes protein degradation. Therefore, we overexpressed HA-tagged wild-type ubiquitin (containing all seven lysine residues), ubiquitin mutants such as K48 and K63 (only K48 or K63 were retained and the other six lysine residues were replaced with arginine), and K0 (none of the seven lysine residues were retained) together with Flag-tagged FBXL18 and GFP-tagged AKT in OVCAR3 cells. Ubiquitination assays revealed that only K63-linked polyubiquitination of AKT was excessively induced by FBXL18 overexpression, while K48-linked polyubiquitination was not affected (**Figure 6C**). Overall, these results indicated that FBXL18 may promote K63-linked ubiquitination of AKT to activate AKT signaling in OC cells.

Discussion

Because of its highly lethal characteristics and lack of suitable biomarkers for diagnosis and treatment, OC has become a major malignancy that threatens women's health and quality of life [14]. FBXL18 has been identified as an E3 ubiquitin ligase and is widely involved in the tumorigenesis of many tumors, such as hepatocellular carcinoma (HCC) [15], glioma [13], and bladder cancer [8]. However, research on FBXL18 has not been reported in the biological process of OC. Our study is the first to address the function and molecular mechanism of FBXL18 in OC tumorigenesis. In this study, we demonstrated that FBXL18 acts as an oncogene in OC tumorigenesis because of its effect on promoting proliferation of OC cells in vitro and in vivo. Additionally, the proliferation and migration of OC cells induced by FBXL18 over-

expression were suppressed by a p-AKT inhibitor. Furthermore, we found that FBXL18 directly interacted with AKT and promoted K63-linked polyubiquitination of AKT, thus activating AKT signaling in OC cells.

Protein ubiquitination is meticulously regulated by the coordinated actions of an E1 ubiquitin-activating enzyme, an E2-conjugating enzyme, and an E3-ubiquitin ligase and is indispensable for a wide range of cellular regulatory processes [16-18]. Among the large number of E3 ligases, the SCF family has been identified as a class of E3 proteins that regulates diverse biological processes [19-21]. In the SCF complex, FBPs recognize diverse substrates for ubiquitin ligation and subsequent degradation. Aberrations in the expression or activity of FBPs could contribute to the abnormal accumulation or degradation of their substrate proteins, and this process is usually involved in tumorigenesis [22-24]. FBXL8 loss excessively supported oncogene-induced transformation in vitro and lymphoma progression in vivo, which was mediated by polyubiquitylation of cyclin D3 and regulation of cyclin D3 protein stability [25]. FBXL10 has been reported to act as a crucial tumor accelerator in gastric cancer [26], colon cancer [27], and breast cancer [28]. Additionally, studies have indicated that upregulation of the FBXL20 protein level is associated with malignant histological features of OC and that the depletion of FBXL20 activity may improve OC clinical outcomes [29, 30].

In general, polyubiquitylation via different linkages is abundant in cells and often results in distinct signaling activation and trafficking. FBXL18 can promote the K63-linked polyubiquitination of Ribosomal protein S15A (RPS15A) and facilitates its stability during HCC progression [15]. FBXL18 regulated cell apoptosis by mediating the proteasomal degradation of FFBXL7 in HeLa cells [31]. FBXL18 attenuated lung epithelial cell death by mediating the degradation of mortality factor 4-like protein 1 (Morf4l1) [32]. Regarding its other functions, FBXL18 targets Leucine-rich repeat protein kinase 2 (LRRK2) for degradation by ubiquitination and abrogates neuron toxicity, and some mutations of FBXL18 are the most common genetic causes of Parkinson's disease [33]. Another study reported that FBXL18 could enhance K63-linked polyubiquitination of AKT and activate AKT signaling in glioma [13]. Congruously, our data showed that FBXL18 interacted with AKT and promoted its stability via K63-linked polyubiquitination, followed by increasing the expression level of p-AKT (S473), indicating activation of AKT signaling in OC cells.

AKT, a major kinase in the Phosphoinositide 3-kinase (PI3K)-AKT pathway, is often activated in various biological processes and usually functions as a beneficial factor for cellular survival and proliferation [34, 35]. Many studies have shown that AKT activation primarily occurs through PI3K-dependent phosphorylation mediated by Mammalian target of rapamycin complex 2 (mTORC2) components and 3-phosphoinositide-dependent protein kinase 1 (PDK1) [36, 37]. However, aberrant activation pathways of AKT have also been identified, such as ubiquitination [38], SUMOylation [39], and acetylation [40]. Moreover, studies have revealed that the K63-linked ubiquitination of AKT plays a crucial role in AKT membrane recruitment, phosphorylation, and activation [41].

Studies have reported that other FBPs also play a crucial role in OC progression. FBXO6 promoted K48-dependent ubiquitination of Ribonuclease T2 (RNASET2), thereby contributing to the development of OC [42]. FBXO16 interacted with Heterogeneous nuclear ribonucleoprotein L (hnRNPL) and triggered its proteasomal degradation, exerting a tumor suppressor role in OC tumorigenesis [43]. Based on the pivotal characteristics of FBPs in the biological processes of OC through ubiquitination of substrates, these studies might provide a perspective to design small-molecule FBP agonists to slow the course of OC-related diseases and enhance treatment in the future.

In summary, we identified FBXL18 as an oncogenic factor both in vitro and in vivo in OC tumorigenesis and demonstrated that the FBXL18/AKT axis is critical for OC cell proliferation and migration. These findings will provide potential new directions for diagnostic and therapeutic strategies for OC.

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Disclosure of conflict of interest

None.

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References

[1] Tossetta G and Inversetti A. Ovarian cancer: advances in pathophysiology and therapies. Int J Mol Sci 2023; 24: 8930.

[2] Wu Y, Li P, Zhu Y, Zhang F, Su L, Ye Y, Huang Y and Zhang M. Effectiveness and safety of mono-anlotinib mono therapy or in combination with chemotherapy in platinum-resistant recurrent ovarian cancer: a single-center retrospective study. Am J Transl Res 2023; 15: 1973-1981.

[3] Horn-Ghetko D, Krist DT, Prabu JR, Baek K, Mulder MPC, Klügel M, Scott DC, Ovaa H, Kleiger G and Schulman BA. Ubiquitin ligation to F-box protein targets by SCF-RBR E3-E3 superassembly. Nature 2021; 590: 671-676.

[4] Nguyen KM and Busino L. The biology of F-box proteins: the SCF family of E3 ubiquitin ligases. Adv Exp Med Biol 2020; 1217: 111-122.

[5] Uddin S, Bhat AA, Krishnankutty R, Mir F, Kulinski M and Mohammad RM. Involvement of F-BOX proteins in progression and development of human malignancies. Semin Cancer Biol 2016; 36: 18-32.

[6] Yumimoto K, Yamauchi Y and Nakayama KI. F-box proteins and cancer. Cancers (Basel) 2020; 12: 1249.

[7] Dang F, Nie L and Wei W. Ubiquitin signaling in cell cycle control and tumorigenesis. Cell Death Differ 2021; 28: 427-438.

[8] Huang W, Lu Y, Wang F, Huang X and Yu Z. Circular RNA circRNA_103809 accelerates bladder cancer progression and enhances chemoresistance by activation of miR-516a-5p/FBXL18 axis. Cancer Manag Res 2020; 12: 7561-7568.

[9] Ueda M, Matsuura K, Kawai H, Wakasugi M and Matsunaga T. Spironolactone-induced XPB degradation depends on CDK7 kinase and SCF^{FBXL18} E3 ligase. Genes Cells 2019; 24: 284-296.

[10] Dong J, Ru Y, Zhai L, Gao Y, Guo X, Chen B and Lv X. LMNB1 deletion in ovarian cancer inhibits the proliferation and metastasis of tumor cells through PI3K/Akt pathway. Exp Cell Res 2023; 426: 113573.

[11] Chen H, Zhang L, Zuo M, Lou X, Liu B and Fu T. Inhibition of apoptosis through AKT-mTOR pathway in ovarian cancer and renal cancer. Aging (Albany NY) 2023; 15: 1210-1227.

[12] Nie X, Liu D, Zheng M, Li X, Liu O, Guo Q, Zhu L and Lin B. HERPUD1 promotes ovarian cancer cell survival by sustaining autophagy and inhibit apoptosis via PI3K/AKT/mTOR and p38 MAPK signaling pathways. BMC Cancer 2022; 22: 1338.

[13] Zhang J, Yang Z, Ou J, Xia X, Zhi F and Cui J. The F-box protein FBXL18 promotes glioma progression by promoting K63-linked ubiquitination of Akt. FEBS Lett 2017; 591: 145-154.

[14] Mahima M, Mahmood T, Ved A, Siddiqui MH, Ahsan F, Shamim A, Ansari VA, Ahmad A and Kashyap MK. An in-depth analysis of ovarian cancer: pathogenesis and clinical manifestation. Drug Res (Stuttg) 2022; 72: 424-434.

[15] Yu HQ, Li F, Xiong H, Fang L, Zhang J, Bie P and Xie CM. Elevated FBXL18 promotes RPS15A ubiquitination and SMAD3 activation to drive HCC. Hepatol Commun 2023; 7: e00198.

[16] Gao L, Zhang W, Shi XH, Chang X, Han Y, Liu C, Jiang Z and Yang X. The mechanism of linear ubiquitination in regulating cell death and correlative diseases. Cell Death Dis 2023; 14: 659.

[17] Cen X, Li Z and Chen X. Ubiquitination in the regulation of autophagy. Acta Biochim Biophys Sin (Shanghai) 2023; 55: 1348-1357.

[18] Xiao J, Wang L, Zhuang Y, Zhu Q, Li W, Liao H, Chen X and Liu Z. The deubiquitinase OTUB2 promotes cervical cancer growth through stabilizing FOXM1. Am J Transl Res 2024; 16: 75-84.

[19] Thompson LL, Rutherford KA, Lepage CC and McManus KJ. The SCF complex is essential to maintain genome and chromosome stability. Int J Mol Sci 2021; 22: 8544.

[20] Liu J, Peng Y, Zhang J, Long J, Liu J and Wei W. Targeting SCF E3 ligases for cancer therapies. Adv Exp Med Biol 2020; 1217: 123-146.

[21] Guan Y, Lin H, Leu NA, Ruthel G, Fuchs SY, Busino L, Luo M and Wang PJ. SCF ubiquitin E3 ligase regulates DNA double-strand breaks in early meiotic recombination. Nucleic Acids Res 2022; 50: 5129-5144.
[22] Hussain S, Dong J, Ma X, Li J, Chen S, Clement A and Liu H. F-box only protein 9 and its role in cancer. Mol Biol Rep 2022; 49: 1537-1544.
[23] Lin M, Xu Y, Gao Y, Pan C, Zhu X and Wang ZW. Regulation of F-box proteins by noncoding RNAs in human cancers. Cancer Lett 2019; 466: 61-70.
[24] Xia L, Chen J, Huang M, Mei J and Lin M. The functions of long noncoding RNAs on regulation of F-box proteins in tumorigenesis and progression. Front Oncol 2022; 12: 963617.
[25] Yoshida A, Choi J, Jin HR, Li Y, Bajpai S, Qie S and Diehl JA. Fbxl8 suppresses lymphoma growth and hematopoietic transformation through degradation of cyclin D3. Oncogene 2021; 40: 292-306.
[26] Li N, Song H, Chen Z, Chen C and Quan M. Novel KDM2B/SAV1 signaling pathway promotes the progression of gastric cancer. Genet Res (Camb) 2023; 2023: 1230182.
[27] Moon SW, Son HJ, Mo HY, Choi EJ, Yoo NJ and Lee SH. Mutation and expression alterations of histone methylation-related NSD2, KDM2B and SETMAR genes in colon cancers. Pathol Res Pract 2021; 219: 153354.
[28] Yang Y, Li S, Li B, Li Y, Xia K, Aman S, Yang Y, Ahmad B, Zhao B and Wu H. FBXL10 promotes ERRα protein stability and proliferation of breast cancer cells by enhancing the monoubiquitylation of ERRα. Cancer Lett 2021; 502: 108-119.
[29] Wu D, Liu C and Hong L. F-box and leucine-rich repeat protein 20 (FBXL20), negatively regulated by microRNA (miR)-195-5p, accelerates the malignant progression of ovarian cancer. Mol Biotechnol 2021; 63: 1235-1243.
[30] Zheng S and Fu Y. Age-related copy number variations and expression levels of F-box protein FBXL20 predict ovarian cancer prognosis. Transl Oncol 2020; 13: 100863.
[31] Liu Y, Lear T, Zhao Y, Zhao J, Zou C, Chen BB and Mallampalli RK. F-box protein Fbxl18 mediates polyubiquitylation and proteasomal degradation of the pro-apoptotic SCF subunit Fbxl7. Cell Death Dis 2015; 6: e1630.
[32] Zou C, Li J, Xiong S, Chen Y, Wu Q, Li X, Weathington NM, Han S, Snavely C, Chen BB and Mallampalli RK. Mortality factor 4 like 1 protein mediates epithelial cell death in a mouse model of pneumonia. Sci Transl Med 2015; 7: 311ra171.
[33] Ding X, Barodia SK, Ma L and Goldberg MS. Fbxl18 targets LRRK2 for proteasomal degradation and attenuates cell toxicity. Neurobiol Dis 2017; 98: 122-136.
[34] Gao HT, Yang Z, Sun H, Zhang Y, Wang Z, Liu WY, Wen HZ, Qu CB and Wang XL. TREM2 as an independent predictor of poor prognosis promotes the migration via the PI3K/AKT axis in prostate cancer. Am J Transl Res 2023; 15: 779-798.
[35] Chen S, Tao Y, Wang Q, Ren J, Jing Y, Huang J, Zhang L and Li R. Glucose induced-AKT/mTOR activation accelerates glycolysis and promotes cell survival in acute myeloid leukemia. Leuk Res 2023; 128: 107059.
[36] Leroux AE and Biondi RM. The choreography of protein kinase PDK1 and its diverse substrate dance partners. Biochem J 2023; 480: 1503-1532.
[37] Zheng N, Wei J, Wu D, Xu Y and Guo J. Master kinase PDK1 in tumorigenesis. Biochim Biophys Acta Rev Cancer 2023; 1878: 188971.
[38] Chan CH, Jo U, Kohrman A, Rezaeian AH, Chou PC, Logothetis C and Lin HK. Posttranslational regulation of Akt in human cancer. Cell Biosci 2014; 4: 59.
[39] Francia MG, Verneri P, Oses C, Vazquez Echegaray C, Garcia MR, Toro A, Levi V and Guberman AS. AKT1 induces Nanog promoter in a SUMOylation-dependent manner in different pluripotent contexts. BMC Res Notes 2023; 16: 309.
[40] Sundaresan NR, Pillai VB, Wolfgeher D, Samant S, Vasudevan P, Parekh V, Raghuraman H, Cunningham JM, Gupta M and Gupta MP. The deacetylase SIRT1 promotes membrane localization and activation of Akt and PDK1 during tumorigenesis and cardiac hypertrophy. Sci Signal 2011; 4: ra46.
[41] Paccosi E, Balzerano A and Proietti-De-Santis L. Interfering with the ubiquitin-mediated regulation of Akt as a strategy for cancer treatment. Int J Mol Sci 2023; 24: 2809.
[42] Ji M, Zhao Z, Li Y, Xu P, Shi J, Li Z, Wang K, Huang X and Liu B. FBXO6-mediated RNASET2 ubiquitination and degradation governs the development of ovarian cancer. Cell Death Dis 2021; 12: 317.
[43] Ji M, Zhao Z, Li Y, Xu P, Shi J, Li Z, Wang K, Huang X, Ji J, Liu W and Liu B. FBXO16-mediated hnRNPL ubiquitination and degradation plays a tumor suppressor role in ovarian cancer. Cell Death Dis 2021; 12: 758.