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Original Research

E3 Ubiquitin Ligase RNF126 Promotes Ovarian Cancer Progression and Cisplatin Resistance Through Targeting Numb/Notch1 Axis

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Abstract

Background: RING finger protein 126 (RNF126) is an E3 ubiquitin ligase that is overexpressed in various malignancies. However, its role in ovarian cancer remains poorly understood. **Methods:** Ovarian cancer cell lines, including SKOV3, A2780, and cisplatin-resistant SKOV3/DDP, were utilized in this study. RNF126 expression was modulated via lentiviral transduction. Cell viability, colony formation, and migration were assessed by cell counting kit-8 (CCK-8), colony formation, and Transwell assays. Protein and mRNA levels were measured by Western blotting and real-time quantitative PCR (qPCR). Protein interaction was evaluated by co-immunoprecipitation and ubiquitination assays. *In vivo* tumor growth and cisplatin response were examined using a xenograft model. **Results:** In SKOV3 and A2780 cells, RNF126 overexpression promoted proliferation, colony formation, and migration, while its knockdown produced the opposite effects. Notably, elevated RNF126 expression was observed in the cisplatin-resistant SKOV3/DDP line. Silencing RNF126 in these cells increased cisplatin sensitivity while concurrently reducing proliferative capacity, clonogenicity, and migratory potential. RNF126 depletion in SKOV3/DDP cells led to decreased mRNA and protein expression of Notch1 and its downstream targets Myc, Cyclin D3, and Hes1. Meanwhile, Numb mRNA levels remained unchanged, whereas its protein levels increased, indicating post-transcriptional regulation. Furthermore, Numb silencing increased cell viability, clonality, migration, and Notch signaling activation in RNF126 knockdown SKOV3/DDP cells. *In vivo*, silencing RNF126 markedly enhanced the sensitivity of cells to cisplatin, suppressed tumor growth, and inhibited Myc, Cyclin D3, Hes1, and Notch1 protein expression. Silencing Numb can reverse the inhibitory effects of RNF126 knockdown. Mechanistic studies showed that RNF126 directly binds to the Notch signaling inhibitor Numb and mediates its ubiquitination and proteasomal degradation, thereby activating the Notch1 signaling pathway and upregulating its downstream genes. In addition, analysis of the TCGA dataset revealed that elevated RNF126 expression is a significant prognostic factor associated with increased mortality risk in ovarian cancer. **Conclusions:** These findings establish RNF126 as a critical contributor to cisplatin resistance in ovarian cancer via regulation of the Numb/Notch1 pathway and highlight its potential as a therapeutic target to counteract chemoresistance.

Keywords: E3 ubiquitin ligase; ovarian neoplasms; drug resistance; cisplatin; Notch pathway

1. Introduction

Ovarian cancer (OC) originates from ovarian tissue and represents one of the most prevalent gynecological malignancies. OC ranks as the eighth most commonly diagnosed malignant tumor among women globally and has the highest mortality rate among gynecological cancers [1]. In 2022, an estimated 324,398 new cases were reported globally, of which 206,839 deaths were attributed to this disease [2]. The clinical course of OC is characterized by an insidious onset, and early symptoms are often non-specific. The nonspecific nature of early symptoms often results in delayed diagnosis, leading most patients to be diagnosed at an advanced stage. In fact, approximately 95% of patients present with non-specific symptoms at diagnosis, including abdominal pain, bloating, urgency, and increased urinary frequency. Collectively, these features highlight the urgent need for refined early detection approaches and a more thorough elucidation of the molecular mechanisms underlying disease progression. In advanced stages, symptoms such as extra-pelvic metastases, ascites, and palpable abdominal masses are frequently observed. In the United States, around 21,000 women are diagnosed with OC annually, nearly 80% of whom have advanced disease. Currently, the standard treatment for OC is surgery combined with chemotherapy. However, due to the lack of reliable early diagnostic methods, chemoresistance, post-surgical recurrence, cumulative toxicity, and tumor heterogeneity, the overall survival rate of OC patients has not significantly improved. Among these factors, resistance to chemotherapy drugs is the leading cause of poor prognosis and high mortality [3].

Platinum agents (such as cisplatin) and taxanes represent the first-line chemotherapy regimen for OC [4]. Cisplatin, a classical platinum-based DNA-damaging agent,

exerts its anticancer effect by forming DNA adducts that induce apoptosis and shows marked efficacy against various solid tumors, including ovarian, testicular, bladder, head and neck, cervical, and non-small cell lung cancers [5]. Although most patients achieve an initial response to platinum-based therapy, approximately 80% ultimately acquire platinum resistance [6]. Once resistance occurs, median survival is limited to only 12–15 months [7]. Therefore, elucidating the molecular basis of cisplatin resistance holds substantial clinical relevance, while discovering novel therapeutic targets that can restore chemotherapy sensitivity is a core priority for improving the prognosis of OC patients. The molecular mechanisms of cisplatin resistance are highly complex and multifactorial, involving reduced intracellular drug accumulation, enhanced DNA repair capacity [8], and dysregulated apoptotic signaling pathways. Various strategies have been proposed to counteract this resistance. For example, upregulation of SHCBP1 has been shown to promote OC proliferation and cisplatin resistance, whereas its inhibition significantly enhances cisplatin sensitivity both *in vitro* and *in vivo* [9]. HJURP regulates DNA repair via the MYC/WEE1 axis, and silencing HJURP enhances cisplatin sensitivity in OC cells, showing synergistic effects with AZD1775, a WEE1 inhibitor [10]. Damnacanthal suppresses OC cell growth through the ERK/mTOR/autophagy pathway and exhibits synergistic antitumor effects when combined with cisplatin in resistant cells [11]. Additionally, studies have shown that in oral squamous cell carcinoma, miR-92b-3p activates the Notch pathway by inhibiting ATXN1 and CPEB3, thereby promoting cisplatin resistance [12]. In triple-negative breast cancer, the Notch1 intracellular domain (N1ICD) has been found to directly bind the CD73 promoter and activate its transcription, thereby contributing to cisplatin resistance. Knockdown of Notch1 or blocking this pathway with inhibitors can reduce CD73 expression, thereby enhancing chemosensitivity [13]. Although these studies have yielded valuable insights, their clinical translation remains restricted, and key regulatory factors that enhance cisplatin sensitivity require further investigation.

Ring Finger Protein 126 (RNF126) is an E3 ubiquitin ligase belonging to the RING (Really Interesting New Gene) family, exerting its ubiquitination activity through a C-terminal RING domain [14]. RNF126 regulates the ubiquitination of various substrate proteins and plays crucial roles in tumorigenesis, spermatogenesis, DNA repair, metabolism, and immune response, exhibiting broad biological functions [15,16]. It has been shown that RNF126 promotes cell proliferation, migration, and invasion by ubiquitination-mediated destabilization of PTEN. In bladder cancer, RNF126 silencing suppresses tumor formation, indicating its oncogenic role [17]. E3 ubiquitin ligases including RNF6 and RNF220 have been implicated in cisplatin resistance. Previous studies have shown that RNF6 overexpression enhances DNA repair by facilitating TCF4 binding to the proliferating cell nuclear antigen (PCNA) promoter, thereby upregulating PCNA expression and conferring cisplatin resistance in A549/DDP cells [18]. RNF220, stabilized by m6A modification, markedly enhances cisplatin resistance in bladder cancer by degrading PDE10A and promoting PD-L1 expression, thus creating an immune-evading microenvironment [19]. Although the role of RNF126 in OC remains poorly characterized, these findings suggest that RNF126, as another E3 ubiquitin ligase, may exert similar functions. To date, only three studies have addressed the role of RNF126 in OC, including promoting anoikis resistance and peritoneal colonization [20], reprogramming lipid metabolism through degradation of ACAP2 [21], and its high expression in OC as a biomarker [22]. However, none of these studies have explored the involvement of RNF126 in chemotherapy resistance-a major clinical challenge in OC. Importantly, the underlying molecular mechanisms of RNF126 on cisplatin resistance in OC remain completely unexplored. The aims of this study are to investigate the critical role of RNF126 in cisplatin resistance in OC. We found that RNF126 expression significantly affects OC cell viability and cisplatin sensitivity through the Numb/Notch1 signaling pathway, which provides new insights for improving cisplatin efficacy and developing novel molecular targeting strategies.

2. Materials and Methods

2.1 Cell Culture

Ovarian cancer cells (SKOV3, A2780, and the cisplatin-resistant SKOV3/DDP, A2780/DDP), obtained from KeyGEN BioTECH (Nanjing, China), were maintained in high-glucose DMEM (Gibco, USA) with 10% FBS and 1% penicillin-streptomycin. Standard incubation parameters (37 °C, 5% CO_2, and high humidity) were applied for cell growth. All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.2 Lentiviral Transfection

RNF126 silencing expression (sh-RNF126) lentiviral vectors, control scramble lentiviral vectors (sh-Scramble), RNF126 expression lentiviral vectors, and EGFP expression lentiviral vectors (OBiO scientific service, China) were transfected into SKOV3 and A2780 cells, and puromycin was added to these cells to establish stable RNF126 overexpression or silencing cells. The RNF126 shRNA target sequences were as follows: sh-RNF126#1: TATCGAGGAGCTTCCGGAAGA; sh-RNF126#2: GCCTGCCGGATTATATCTGTC; sh-RNF126#3: TGCCATCATCACACAGCTCCT; control scramble shRNA: CCTAAGGTTAAGTCGCCCTCG. The efficiency of transfection was determined by Western blot assay.

2.3 Cell Viability Assay

Ovarian cancer cells at the exponential growth phase were inoculated into 96-well plates at an initial density of 2

Table 1. Primer sequence.

Gene	Forward (5′-3′)	Reverse (5′-3′)
NUMB	CCCCAGACAGGAACTTTGATAG	GCTCTAAACAGGCTGCAAAAG
MYC	TTCGGGTAGTGGAAAACCAG	AGTAGAAATACGGCTGCACC
CCND3	AACTGTGCATCTACACCGAC	GCCAGGAAATCATGTGCAATC
HES1	AAGGCGGACATTCTGGAAAT	TCACCTCGTTCATGCACTC
NOTCH1	TGATGTTGGAGAAGGGAAGTTG	GGATGCAGCTTCTCCTAACAG
GAPDH	ACATCGCTCAGACACCATG	TGTAGTTGAGGTCAATGAAGGG

$\times 10^4$ cells. Following the designated treatment period, 10 µL of CCK-8 reagent (Solarbio Life Sciences, China) was added to each well. The plates were then incubated for an additional 2 h at 37 °C. Subsequently, the optical density (OD) at 450 nm was quantified using a microplate reader.

2.4 Colony Formation Assay

For the clonogenic potential evaluation, 5×10^2 cells per well were distributed into six-well plates and cultured in 10% FBS-supplemented DMEM for 7–14 days. Following incubation, the resulting cell clusters underwent fixation with 10% formalin (30 min), followed by a 15-min staining period using 1% crystal violet (C8470, Solarbio Life Sciences, China). Colony visualization and data acquisition were finalized utilizing the FluorChem M imaging platform (FR0145, ProteinSimple, USA).

2.5 Cell Migration Assay

To evaluate chemotactic migration, 5×10^5 cells were suspended in serum-deprived DMEM and inoculated into the upper inserts of a Transwell apparatus. The lower chambers were supplemented with 10% FBS-containing medium to establish a nutrient gradient. After a 24 h migration period, cells remaining on the initial seeding surface were mechanically removed. The membrane-traversed cells underwent fixation in 4% paraformaldehyde and subsequent labeling with 1% crystal violet. Migratory capacity was quantified by averaging the cell counts from three stochastic fields per membrane via light microscopy.

2.6 Quantitative Real-Time PCR (qPCR)

Total cellular RNA was extracted employing TRIzol reagent (1 mL), with subsequent cDNA synthesis derived from 1 µg of the RNA template. The amplification reactions were conducted using SYBR Green Master Mix (Q711, Vazyme Biotechnology, China) on a real-time PCR platform. Transcript abundance of target genes was normalized against the endogenous control (GAPDH), and fold-change analysis was performed utilizing the $2^{-\Delta\Delta Ct}$ algorithm. Specific primer sequences utilized for the quantification are detailed in Table 1.

2.7 Western Blot Assay

Total protein fractions were retrieved from cell lysates and homogenized tumor samples using protease- or phosphatase-inhibitor-fortified RIPA buffer. After centrifugation at 12,000 g, protein concentrations in the supernatants were measured via BCA assay (P0010, Beyotime, China). Equivalent protein loads were separated by SDS-PAGE and electro-transferred onto PVDF membranes. Following a 1 h blocking in 5% non-fat milk, membranes were probed overnight (4 °C) with the following primary antibodies: RNF126 (Abcam, #ab234812, dilution ratio 1:1000), Numb (Abcam, #ab220362, dilution ratio 1:1000), Notch1 (Cell signaling technology, #3608, dilution ratio 1:1000), Hes1 (Cell signaling technology, #11988, dilution ratio 1:1000), Myc (Cell signaling technology, #5605, dilution ratio 1:1000), Cyclin D3 (Cell signaling technology, #2936, dilution ratio 1:1000), Tubulin (Affinity, #DF7967, dilution ratio 1:2000), K48-linkage Polyubiquitin (Absin, abs158754, dilution ratio 1:1000), ubiquitin (Proteintech, 10201-2-AP, dilution ratio 1:2000). After washing, the membranes were incubated with the secondary antibody for 1 h at room temperature. Immunoreactive bands were visualized using the ECL detection reagent kit, which was then subjected to quantification using ImageJ 1.54 Java8 software (NIH, USA).

2.8 Co-Immunoprecipitation and Ubiquitination Assay

SKOV3 cells overexpressing RNF126 or Numb, or both, were lysed with Co-IP buffer containing protease inhibitors, followed by centrifugation at 12,000 g at 4 °C for 15 min. A portion of the supernatant was reserved as input, and the remaining supernatant was incubated with anti-Numb antibody overnight at 4 °C. Immune complexes were captured using Protein A/G agarose beads for 2 h. Centrifugation at 3000 rpm for 5 min at 4 °C was employed to precipitate the beads. Immunoprecipitation wash buffer was utilized five times to ensure purity. To liberate bound proteins for analysis, heat-induced denaturation was performed within SDS-PAGE sample buffer. Western blotting served as the analytical tool to confirm the physical association of RNF126 with Numb through the visualization of specific protein signals. For ubiquitination assays, overexpressed RNF126 and Numb SKOV3 cells were treated with 20 µM proteasome inhibitor MG132 (HY-13259, MedChemExpress, USA) for 6 h. The Numb primary antibody was used to capture complexes, and the ubiquitination level was determined by Western blot analysis.

2.9 Animal Experiments

Vital River Laboratory Animal Technology Co. (Beijing, China) provided the 6-week-old female BALB/c nude mice utilized in this study. Maintenance occurred in the standard laboratory cages (4 mice per cage) under specific pathogen-free conditions with temperature (22–25 °C), humidity (50–60%), and a 12-h light/dark cycle strictly controlled. Animals exhibiting marked behavioral disturbances, clinical signs of illness, or a body weight reduction exceeding 20% of their baseline were removed from the subsequent analysis. No individuals or data points were excluded. The sample size was chosen based on the standards established in previous ovarian cancer xenograft studies. To minimize potential bias, establishment of the animal model and biochemical analyses were performed by investigators blinded to the experimental group allocation and the identity of the biological samples. Subcutaneous inoculation of 1×10^7 SKOV3/DDP cells (0.1 mL suspension) into the right lower flank was performed to initiate tumor growth. A total of 24 mice were randomly assigned to four groups (6 mice per group): sh-Scramble, sh-RNF126, sh-Numb, and sh-RNF126+sh-Numb. One week after inoculation, cisplatin (3 mg/kg) was administered intraperitoneally (i.p.) every three days for four weeks. Tumor volume (V) was monitored weekly and estimated using the formula: $V = 0.5 \times (\text{length} \times \text{width}^2)$. On day 35, the mice were anesthetized with a single intraperitoneal injection of ketamine (100 mg/kg) and xylazine (5 mg/kg) mixture. After adequate anesthesia was confirmed by the absence of a pedal reflex, the mice were euthanized by cervical dislocation. The excised neoplasms were immediately snap-frozen and stored at –80 °C. Formal authorization for all experimental protocols was granted by the Animal Ethics Committee of The First Affiliated Hospital of Harbin Medical University (CODE:2026139). The study was carried out in accordance with the ARRIVE guidelines.

2.10 Statistics

GraphPad Prism 8.0 (GraphPad software, San Diego, CA, USA) served as the computational platform for all data processing tasks. All experimental data were expressed as mean ± standard deviation (SD) from at least three independent experiments. Prior to the statistical analyses, the assumptions of normality and homogeneity of variance were assessed. For comparisons between two groups, unpaired two-tailed Student's *t*-test was applied. For comparisons among multiple groups, one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test. For data involving two independent variables, two-way ANOVA followed by Tukey's post hoc test was performed. The detailed statistical tests are also indicated in the corresponding figure legends. A p value < 0.05 was required to determine statistical significance. Significance levels are denoted as: $^*p < 0.05$, $^{**}p < 0.01$.

3. Results

3.1 RNF126 Promotes the Cell Viability, Colony Formation and Migration in SKOV3 and A2780 Cells

Lentiviral transduction was employed to establish stable SKOV3 and A2780 cell lines exhibiting either RNF126 overexpression (Lv-RNF126) or knockdown (sh-RNF126) (Fig. 1A,B). In cell viability assays, significantly elevated optical density (OD) values were observed in the Lv-RNF126 group compared with the Lv-EGFP control group over the 24–72 h period in both cell lines, whereas RNF126 knockdown markedly suppressed proliferation (Fig. 1C). Consistently, colony formation was significantly enhanced by RNF126 overexpression and substantially diminished by its silencing in both SKOV3 and A2780 cells (Fig. 1D,E). Evidence from functional assays demonstrates that the invasive potential of ovarian cancer cells is significantly enhanced by RNF126 overexpression, whereas its suppression results in a marked decline in motility (Fig. 1F,G). Collectively, these results establish that RNF126 serves as a fundamental driver of cell multiplication, colony-forming capacity, and migratory behavior within this specific malignancy.

3.2 Silencing RNF126 Increases the Sensitivity of SKOV3/DDP Cells to Cisplatin

In comparison to the parental SKOV3 line, SKOV3/DDP cells exhibited a marked elevation in RNF126 protein expression as determined by Western blotting (Fig. 2A). Following 72 hours of cisplatin treatment across a 0–40 μM dosage range, this variant also demonstrated superior survival rates (Fig. 2B), a result consistent with its established chemoresistant characteristics. Silencing RNF126 in SKOV3/DDP cells markedly reduced cell viability under cisplatin treatment (Fig. 2C). Evidence obtained from SKOV3/DDP models indicates that silencing RNF126 effectively suppresses colony initiation and migratory potential during cisplatin exposure (Fig. 2D,E). Such results suggest that the depletion of RNF126 serves to reinstate therapeutic vulnerability in ovarian cancer cells previously characterized by cisplatin insensitivity.

3.3 Silencing RNF126 Inhibits the Activation of the Notch Pathway in SKOV3/DDP Cells

To explore the involvement of RNF126 in platinum-based chemoresistance, Notch signaling dynamics were assessed within SKOV3/DDP models deficient in this protein. Quantitative PCR results indicated that RNF126 depletion significantly attenuated Notch1 mRNA transcription levels, along with its downstream effectors HES1, MYC, and CCND3. This reduction in NOTCH1 mRNA is consistent with the well-established positive feedback regulation of the Notch pathway [23]. The inhibition of Notch pathway activation regulated by RNF126-mediated Numb upregulation disrupted this auto-regulatory loop, leading to

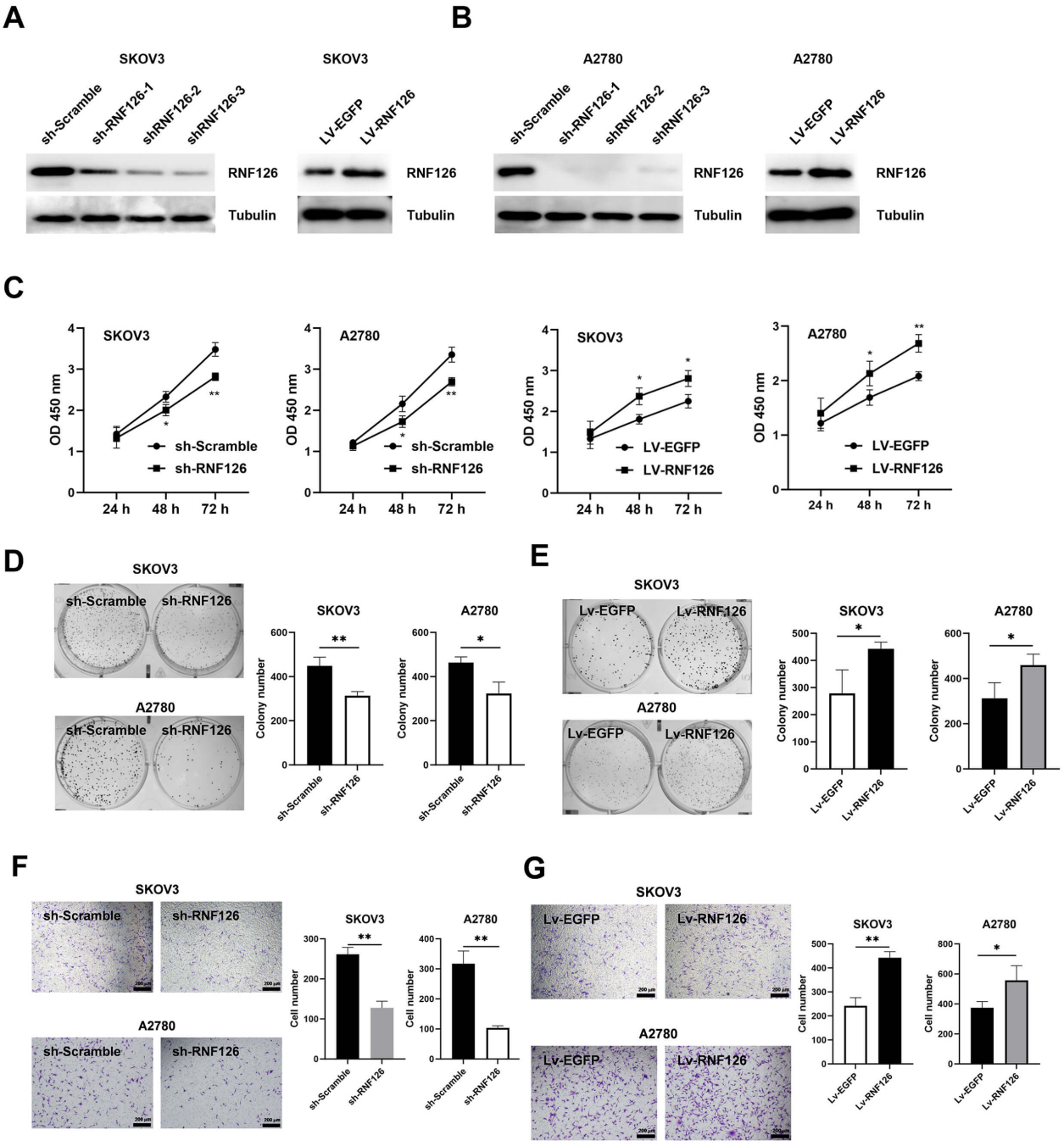

Fig. 1. RNF126 promotes the cell viability, clonality and migration in SKOV3 and A2780 cells. RNF126 silencing expression (sh-RNF126) lentiviral vectors, control lentiviral vectors (sh-Scramble), RNF126 expression lentiviral vectors, and EGFP expression lentiviral vectors were transfected into SKOV3 and A2780 cells, respectively. (A,B) Western blot verifies the successful construction of RNF126 stable silencing/overexpression cell lines in SKOV3 and A2780 cells. (C) The cell proliferation was determined by CCK8 assay. (D,E) Colony formation assay. Cells (5×10^2) were cultured in 6-well plate and colony numbers were counted after 14 (D) or 7 (E) days. (F,G) The cell migration was determined by transwell migration assay. Cells (5×10^5) were cultured into the transwell chamber and cell numbers were counted after 24 h (scale bar: 200 μm). Data are presented as the mean ± SD from three independent experiments. Comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. $*p < 0.05$, $**p < 0.01$, (n = 3).

decreased Notch1 transcript levels. By contrast, no significant alteration in Numb mRNA levels, despite its established role as a Notch pathway suppressor, was observed (Fig. 3A). In SKOV3/DDP models, RNF126 serves to potentially accelerate the turnover of Numb while influencing the cellular response to cisplatin by altering Notch sig-

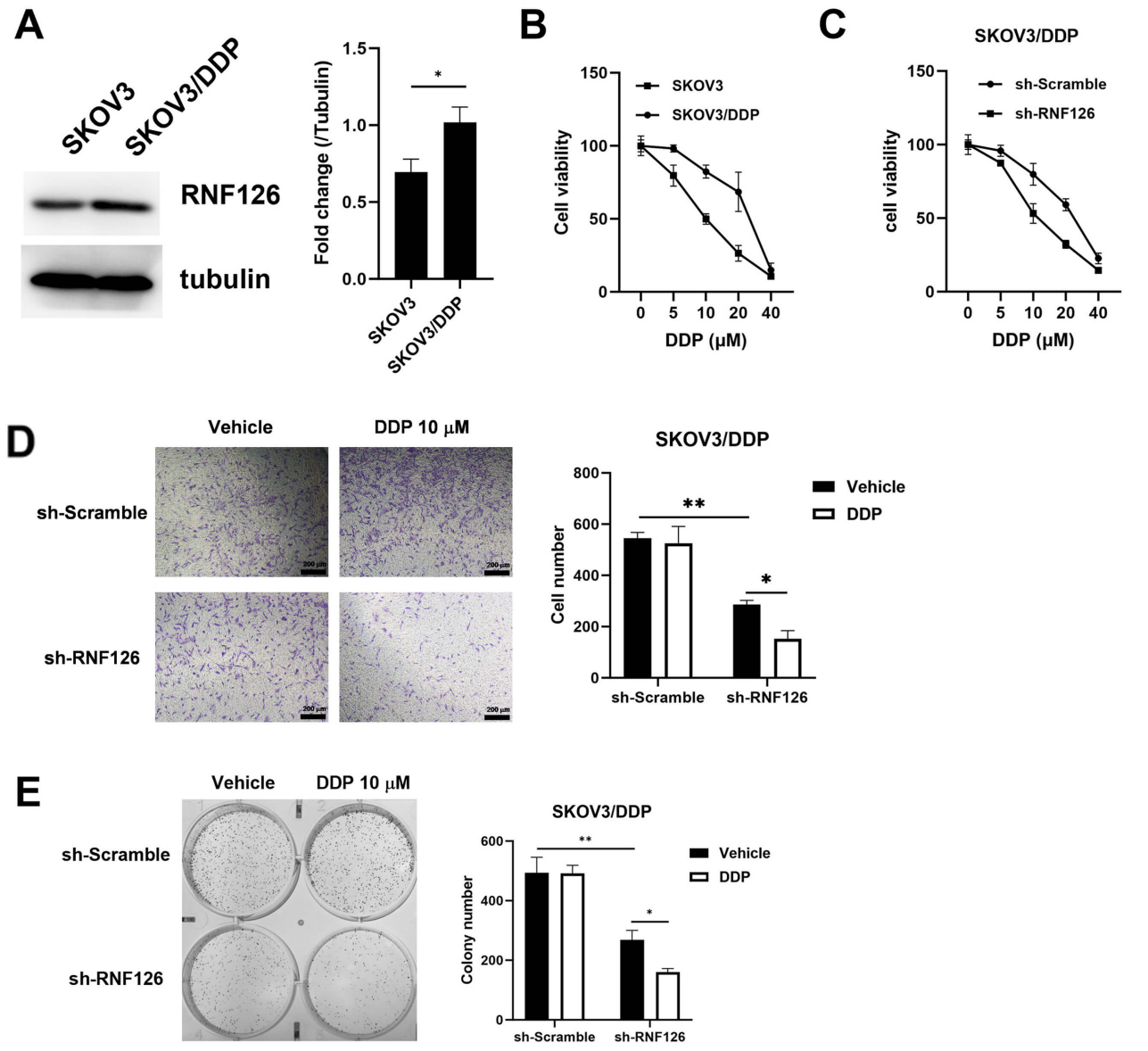

Fig. 2. Silencing RNF126 increases the sensitivity of SKOV3/DDP cisplatin-resistant cells to cisplatin. (A) The RNF126 protein expression were determined by Western blot assay in SKOV3 and SKOV3/DDP cells. Comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. (B,C) The cell viabilities of SKOV3 and SKOV3/DDP cells were determined by CCK8 assay. Cells (2×10^4) were cultured in 96-well plate and treated with indicated concentrations of cisplatin for 72 h. (D) The cell migration was determined by transwell migration assay. Cells (5×10^5) cultured into the transwell chamber were treated with 10 μM cisplatin and cell numbers were counted after 24 h (scale bar: 200 μm). (E) Colony formation assay. Cells (5×10^2) cultured in 6-well plate were treated with 10 μM cisplatin and colony number were counted after 14 days. Data are presented as the mean ± SD from three independent experiments. Comparisons among four groups were performed using two-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$, (n = 3). DDP, cis-Diamminedichloroplatinum.

naling flux. Protein quantification through Western blotting demonstrated that Numb concentrations was increased substantially following RNF126 depletion, whereas levels of Notch1 and its associated downstream targets, including Hes1, Cyclin D3, and Myc, were effectively suppressed (Fig. 3B).

3.4 Silencing RNF126 Inhibits the Viability, Colony Formation, and Migration of SKOV3/DDP Cells Through the Numb/Notch1 Axis

To further verify that RNF126 promotes the progression of OC and cisplatin resistance by regulating the Numb/Notch1 axis, SKOV3/DDP cells were divided into four different treatment groups: sh-Scramble, sh-

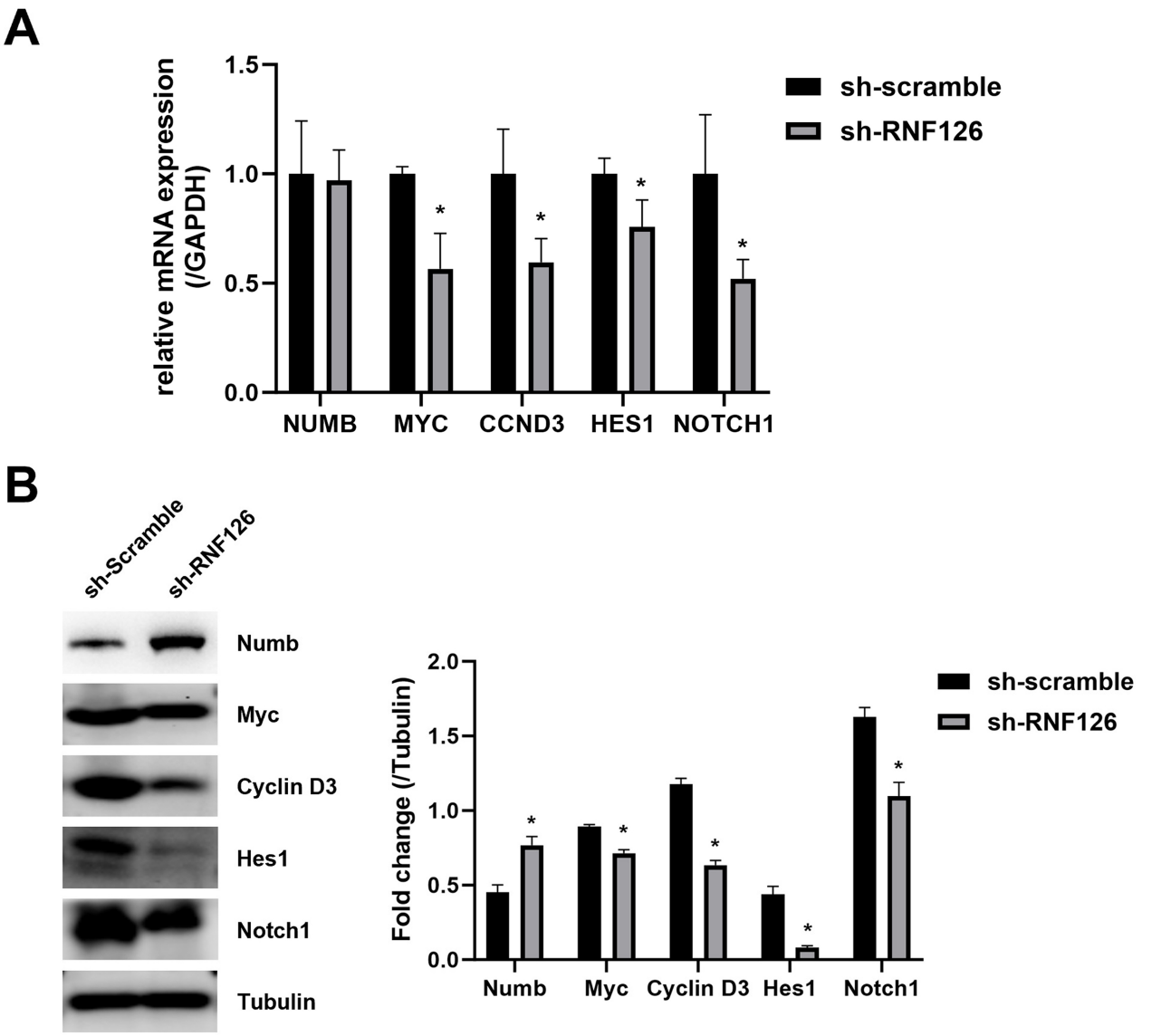

Fig. 3. Silencing RNF126 inhibits the activation of the Notch signaling pathway in SKOV3/DDP cells. (A) The expression levels of Notch pathway-related genes mRNA were determined in RNF126-silenced SKOV3/DDP cells by qPCR. (B) The expression levels of Notch pathway-related proteins were determined in RNF126-silenced SKOV3/DDP cells by Western blot assay. Data are presented as the mean ± SD from three independent experiments. Comparisons between two groups were performed using an unpaired two-tailed Student's *t*-test. *$p < 0.05$, (n = 3).

RNF126, sh-Numb, and the double knockdown group sh-RNF126+sh-Numb. After exposure to cisplatin at concentrations ranging from 0–40 μM (0, 5, 10, 20, 40 μM) for 72 hours, cell viability was assessed using CCK-8 assay. RNF126 silencing led to a marked reduction in cell viability compared to the sh-Scramble control group. However, concurrent knockdown of Numb in the sh-RNF126 background significantly counteracted this suppressive effect (Fig. 4A). Consistently, Western blot analysis showed that compared with sh-Scramble, protein levels of Notch1, Hes1, Myc, and Cyclin D3 were significantly downregulated in the sh-RNF126 group, but these proteins were significantly upregulated in the sh-RNF126+sh-Numb group relative to sh-RNF126 alone (Fig. 4B). Similarly, clone formation was significantly reduced after RNF126 knockdown, but was partially restored by additional silencing of Numb (Fig. 4C). A comparable phenotype was observed in migration assays, wherein RNF126 silencing markedly attenuated migratory capacity, and this effect was partially rescued by concomitant Numb depletion (Fig. 4D). These results indicate that RNF126 modulates the viability, colony formation, and migration in SKOV3/DDP cells through the Numb/Notch1 axis.

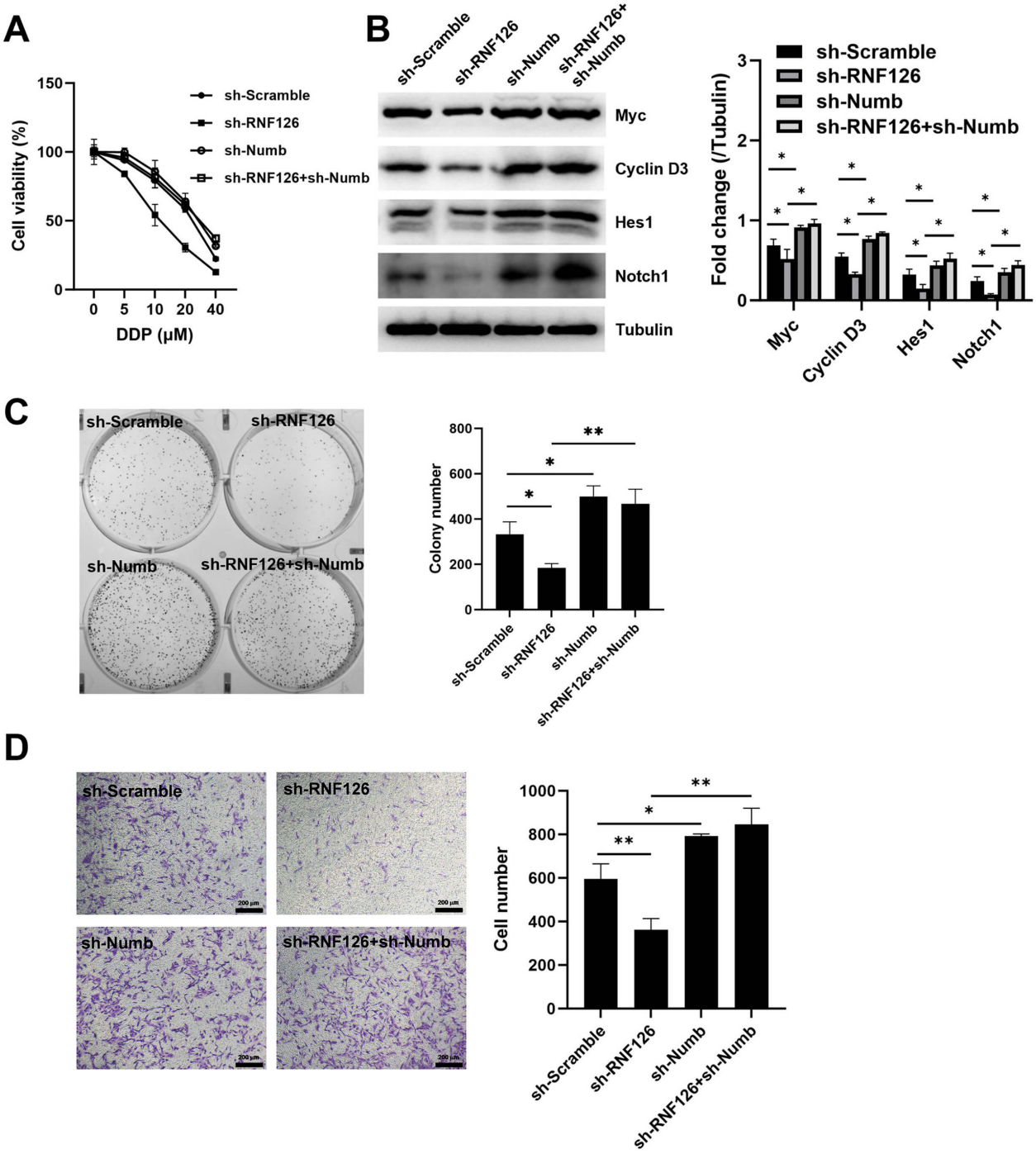

Fig. 4. Silencing RNF126 inhibits the viability, colony formation, and migration of SKOV3/DDP cells through the Numb/Notch1 axis. RNF126 silencing expression (sh-RNF126) lentiviral vectors, Numb silencing expression (sh-Numb) lentiviral vectors and control lentiviral vectors (sh-Scramble) were transfected into SKOV3/DDP cells, respectively. (A) The cell viabilities of SKOV3/DDP cells were determined by CCK8 assay. Cells (2×10^4) cultured in 96-well plate were treated with the different concentrations of cisplatin for 72 h. (B) The expression levels of Notch pathway-related protein were determined in SKOV3/DDP cells by Western blot assay. (C) Colony formation assay. Cells (5×10^2) cultured in 6-well plate were treated with 10 μM cisplatin and colony numbers were counted after 14 days. (D) The cell migration was determined by transwell migration assay. Cells (5×10^5) cultured into the transwell chamber were treated with 10 μM cisplatin and cell numbers were counted after 24 h (scale bar: 200 μm). Data are presented as the mean ± SD from three independent experiments. Comparisons among four groups were performed using two-way ANOVA followed by Tukey's post hoc test. $^*p < 0.05$, $^{**}p < 0.01$, (n = 3).

3.5 Silencing RNF126 Inhibits the Growth of Xenografts Through the Numb/Notch1 Axis In Vivo

For *in vivo* experiments, cells from four groups (sh-Scramble, sh-RNF126, sh-Numb, sh-RNF126+sh-Numb) were subcutaneously injected into the right ventral side of nude mice, followed by cisplatin administration. The tumor volume and body weight were recorded weekly (Fig. 5A,B). *In vivo* xenograft experiments demonstrated that silencing RNF126 significantly suppressed tumor growth, as evidenced by a marked reduction in tumor volume in the sh-RNF126 group relative to the sh-Scramble control. This inhibitory effect was partially abrogated upon concurrent depletion of Numb, with the sh-RNF126+sh-Numb group exhibiting significantly larger tumors compared to the sh-RNF126 group alone. Importantly, Systemic toxicity was ruled out as a contributing factor to the recorded outcomes, as evidenced by the consistent maintenance of mouse body mass observed throughout each of the four study cohorts. These findings indicate that, *in vivo*, silencing RNF126 can significantly inhibit the growth of xenografts under cisplatin treatment, and silencing Numb can reverse this inhibitory effect. In the sh-RNF126 group, Western blot analysis further revealed markedly reduced protein levels of Notch1, Hes1, Myc, and Cyclin D3 compared to the sh-Scramble group. Relative to cells solely deficient in RNF126, the sh-RNF126+sh-Numb cohort demonstrated a substantial recovery of the aforementioned protein expression concentrations following concurrent depletion (Fig. 5C). These *in vivo* findings further support that RNF126 modulates the proliferation of cisplatin-resistant ovarian cancer cells via the Numb/Notch1 signaling axis.

3.6 RNF126 Mediates the Ubiquitination and Degradation of Numb

To investigate the interaction between RNF126 and Numb, RNF126-overexpressing SKOV3 and A2780 cells were treated with 10 μM cycloheximide (CHX), a protein synthesis inhibitor, for different times to evaluate the influence of RNF126 on the protein stability of Numb. The Western blot assay showed that RNF126 overexpression shortened the half-life of endogenous Numb (Fig. 6A,B). In particular, at the 0 h time point of the CHX chase, Numb protein levels were lower in RNF126-overexpressing SKOV3 cells compared to controls, reflecting the sustained degradation of Numb by RNF126 under steady-state conditions prior to CHX addition. Furthermore, SKOV3 cells stably overexpressing or depleted of RNF126 were treated with the proteasome inhibitor MG132 (20 μM) for 6 hours. Relative to the Lv-EGFP control cohort, the Lv-RNF126 assembly exhibited a substantial decline in Numb protein concentrations when no MG132 was administered, whereas RNF126 knockdown resulted in a significant elevation of Numb compared to sh-Scramble. Proteasome inhibition with MG132 substantially restored Numb expression in Lv-RNF126 cells, eliminating the difference observed between this group and MG132-treated Lv-EGFP controls. Although Numb levels in the sh-RNF126 group remained elevated relative to the sh-Scramble group following MG132 treatment, the magnitude of this increase was considerably attenuated. Collectively, these findings demonstrate that RNF126 mediates proteasome-dependent degradation of Numb (Fig. 6C). Furthermore, RNF126 and NUMB were overexpressed in SKOV3 cells, respectively, and a Co-IP assay confirmed the interaction between RNF126 and Numb (Fig. 6D). The ubiquitination assay showed that RNF126 overexpression can significantly enhance the ubiquitination level of Numb. Furthermore, immunoblotting using a K48-linked ubiquitin-specific antibody revealed that RNF126 overexpression markedly enhanced the K48-linked polyubiquitination of Numb (Fig. 6E). These results indicate that RNF126 downregulates Numb expression by mediating the ubiquitin-proteasome degradation of Numb, thereby activating the Notch signaling pathway.

3.7 External Validation of RNF126 Prognostic Value in TCGA Dataset

Finally, the prognostic significance of RNF126 in ovarian cancer was evaluated using the TCGA dataset through GEPIA analysis. The Kaplan–Meier survival analysis revealed that patients with high RNF126 expression exhibited significantly poorer overall survival compared to those with low expression (log-rank $p = 0.034$; HR = 1.4, $p = 0.036$) (Fig. 7A). The survival curves demonstrated a clear and sustained separation between the two groups, with the high-expression cohort (n = 323) showing consistently lower survival probabilities than the low-expression group (n = 102) (Fig. 7A). These findings suggest that elevated RNF126 expression is a significant prognostic factor associated with increased mortality risk in ovarian cancer. Fig. 7B presents a graphic abstract summarizing the mechanism by which RNF126 drives ovarian cancer progression and cisplatin resistance, highlighting RNF126 as a critical oncogenic driver and a potential therapeutic target.

4. Discussion

The high mortality associated with OC is largely driven by frequent late-stage diagnosis and the emergence of chemotherapy-resistant disease [20]. Although aberrant Notch signaling has been consistently implicated in ovarian tumorigenesis, the upstream mechanisms governing its activity remain incompletely understood. By modulating the Numb/Notch1 pathway, RNF126 functions as a key oncogenic driver that accelerates ovarian tumor growth while simultaneously fostering insensitivity to cisplatin-based chemotherapy. Enhanced cellular viability, clonogenic potential, and migratory capacity were observed in two ovarian cancer cell lines upon RNF126 expression, accompanied by accelerated xenograft tumor growth *in vivo*. Functioning as an E3 ubiquitin ligase, RNF126 directly binds to Numb and catalyzes its ubiquitination, thereby target-

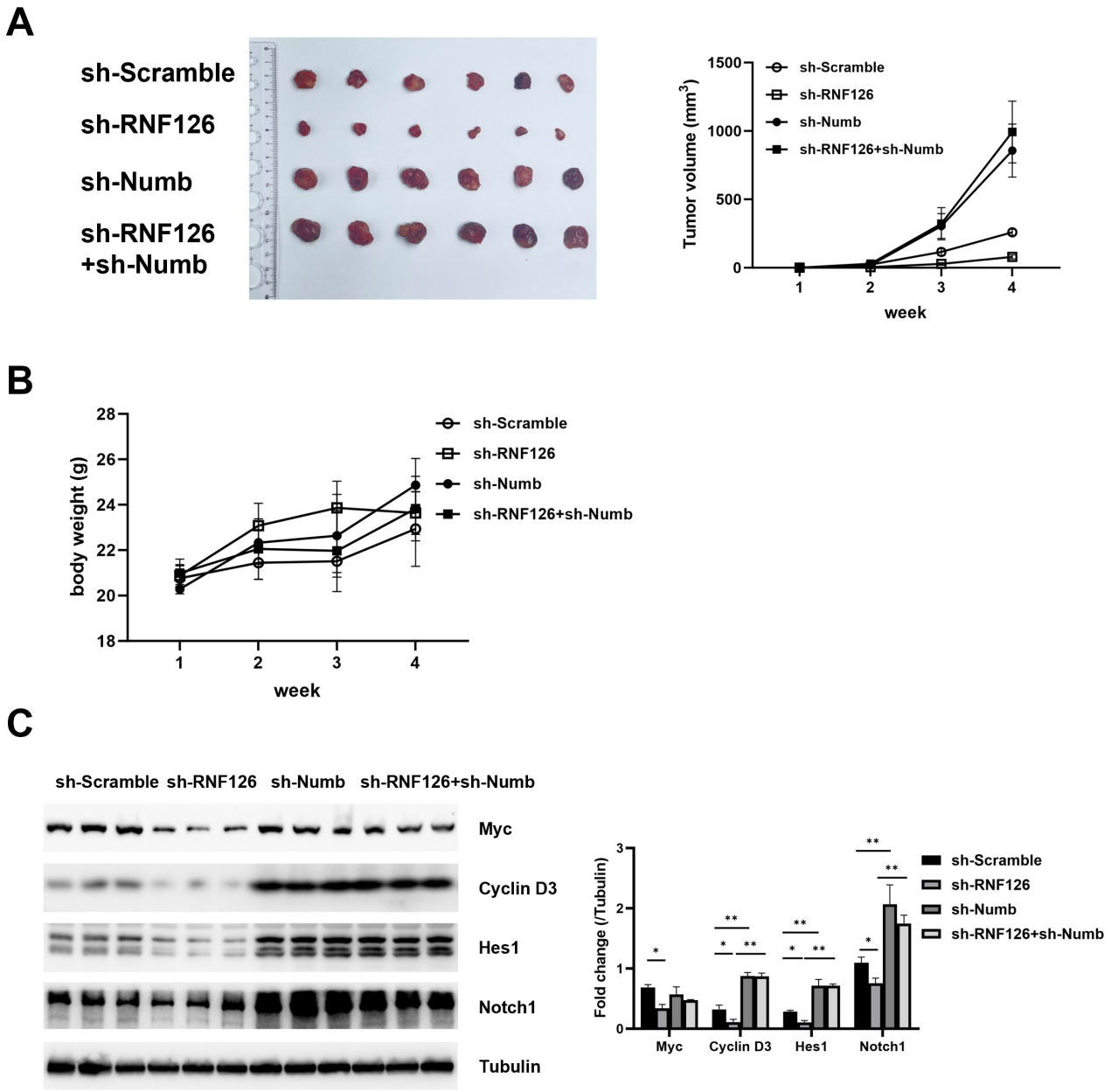

Fig. 5. Silencing RNF126 inhibits the growth of xenografts through the Numb/Notch1 axis *in vivo*. (A) The growth curve of xenograft tumors in nude mice (n = 6). (B) The growth curve of body weight (n = 6). (C) The expression levels of Notch pathway-related proteins were determined in tumor tissues by Western blot assay (n = 3). Data are presented as the mean ± SD. Comparisons among four groups were performed using two-way ANOVA followed by Tukey's post hoc test. *$p < 0.05$, **$p < 0.01$.

ing it for proteasomal degradation. The RNF126-mediated depletion of Numb consequently relieves its inhibitory effect on Notch1, leading to pathway activation. Collectively, these data identify RNF126 as a previously underappreciated molecular node linking ubiquitin-dependent protein turnover to Notch-driven oncogenesis in OC.

The biological function of RNF126, a member of the RING finger E3 ligase family, has attracted considerable interest in recent research. Previous studies have shown that RNF126 is overexpressed in several cancers and participates in the promotion of malignant tumors, including breast cancer [16], gastric cancer [24], nasopharyngeal cancer [25], bladder cancer [17], cerebral cancer [26], and lung cancer [27]. However, only three studies to date have investigated its role in OC. One study reported that the clinical outlook for individuals diagnosed with OC is significantly influenced by the status of RNF126 as a distinct indicator of disease outcome. Our analysis of the TCGA dataset further confirmed that elevated RNF126 expression is significantly associated with reduced overall survival (Fig. 7A), rein-

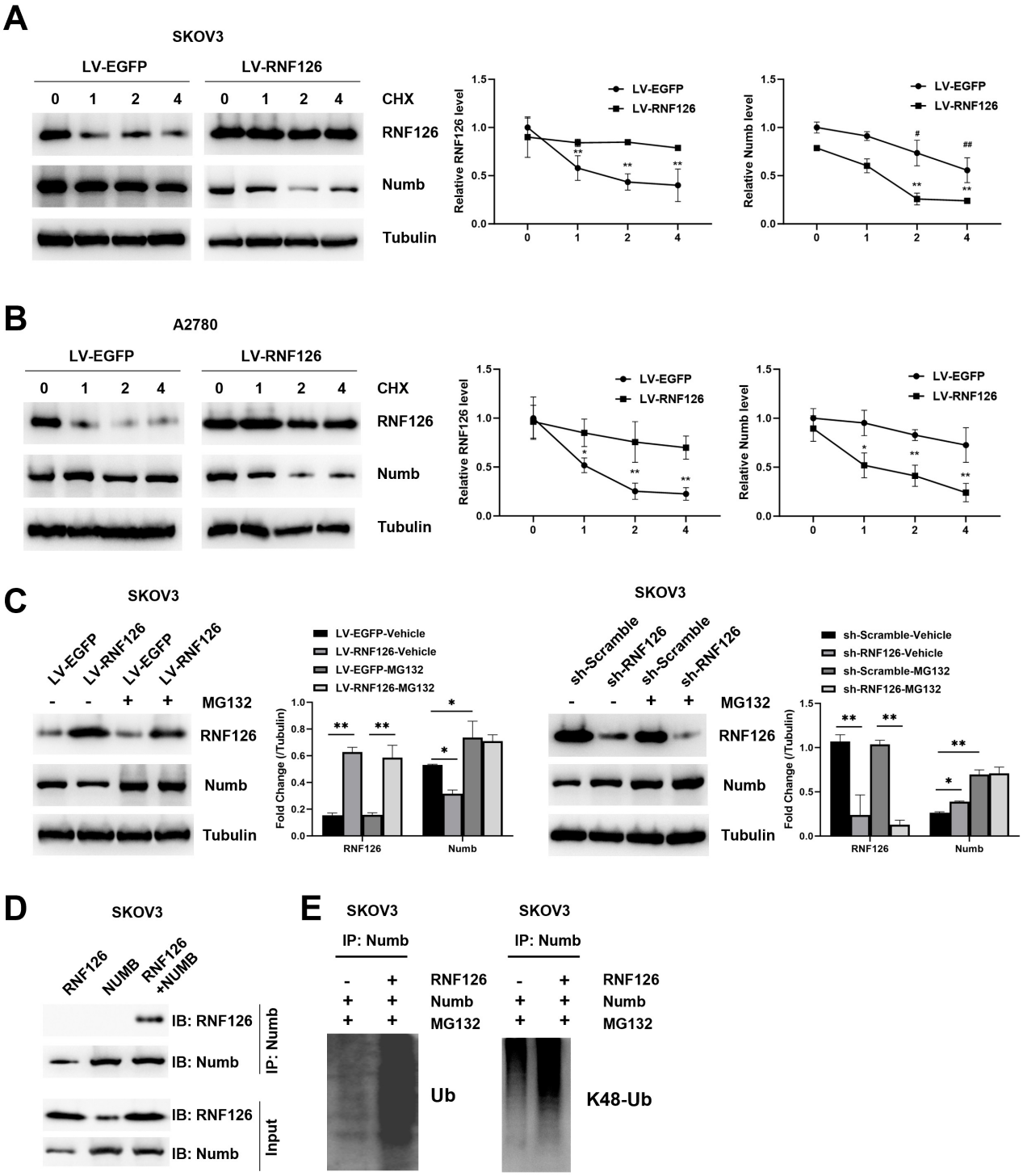

Fig. 6. RNF126 mediates the ubiquitin-proteasome degradation of Numb. (A,B) RNF126-overexpressing SKOV3 and A2780 cells were treated with 10 μM CHX for 0, 1, 2, and 4 h. The influence of RNF126 on the protein stability of Numb was evaluated by Western blot assay (n = 3). (C) RNF126-overexpressing or silenced SKOV3 cells were treated with 20 μM MG132 for 6 h. The influence of RNF126 on the proteasome degradation of Numb was evaluated by Western blot assay (n = 3). (D) RNF126 or Numb was overexpressed in SKOV3 cells. The interaction between RNF126 and Numb was determined by Co-IP assay. (E) RNF126- or Numb-overexpressing SKOV3 cells were treated with MG132. The Numb primary antibody was used to capture complexes, and the ubiquitination level of Numb was determined by Western blot assay. Data are presented as the mean ± SD from three independent experiments. Comparisons among four groups were performed using two-way ANOVA followed by Tukey's post hoc test. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ vs LV-EGFP; $^{*}p < 0.05$, $^{**}p < 0.01$ vs LV-RNF126, (n = 3).

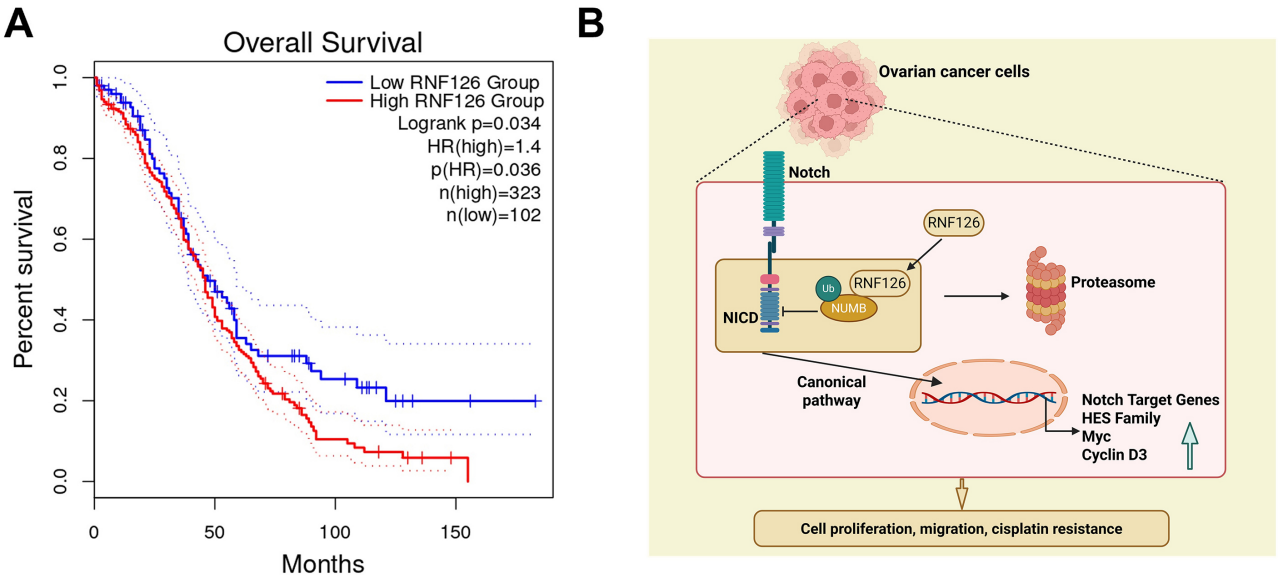

Fig. 7. High RNF126 expression correlates with poor overall survival in ovarian cancer patients. (A) Kaplan-Meier survival analysis of ovarian cancer patients stratified by RNF126 expression level based on the TCGA dataset using the GEPIA database. Patients were divided into high-expression (n = 323) and low-expression (n = 102) groups according to the custom grouping parameter function of the GEPIA platform. The log-rank test was used to compare overall survival between the two groups. High RNF126 expression was significantly associated with shorter overall survival (Log-rank p = 0.034, HR = 1.4, p(HR) = 0.036). (B) Schematic diagram of the molecular mechanism of RNF126 by targeting the Numb/Notch1 axis in ovarian cancer cells. Created in BioRender. Liu, H. (2026) https://BioRender.com/b9ep0oy.

forcing its utility as a prognostic biomarker. Importantly, our functional experiments reveal that RNF126 drives cisplatin resistance, suggesting that its expression may also possess predictive value for chemotherapy responsiveness, although this possibility requires prospective validation in patient cohorts. From a therapeutic standpoint, targeting RNF126 represents an appealing strategy for overcoming cisplatin resistance. Supporting the present observations, RNF126 overexpression was shown to markedly enhance SKOV3 cell migration and invasion [22]. However, the underlying mechanism by which RNF126 functions is completely unexplored. OC progression was shown in another study to be promoted by RNF126 through the ubiquitin-dependent degradation of ACAP2, thereby enhancing lipid synthesis [21]. Multiple proteins have been identified as targets of RNF126. PTEN was demonstrated to bind the C-terminal RING domain of RNF126 in 293T cells, leading to RNF126-mediated polyubiquitination that modulates PTEN stability, as confirmed by an *in vivo* ubiquitination assay [17]. Ubiquitination of FSP1, an anti-ferroptotic gene, was promoted by RNF126, which also governed its subcellular localization [26]. In this study, we found that enhanced cell viability, clonality, and migration were observed in SKOV3 and A2780 cells upon RNF126 overexpression. Inhibition of cell viability, clonality, and migration resulted from RNF126 silencing. Through direct interaction, RNF126 drives the ubiquitination of Numb, a well-established negative regulator of Notch signaling. This RNF126-mediated ubiquitination of Numb implies a potential mechanism by which RNF126 contributes to OC progression.

Notch, a single-pass transmembrane receptor, transduces signals via proteolytic cleavage of its intracellular domain upon ligand activation [28]. In the nucleus, cleaved Notch drives the expression of oncogenic targets such as Myc, Cyclin D3, and HES. Notch signaling contributes to cisplatin resistance by promoting cancer stem cell expansion, epithelial-mesenchymal transition (EMT), angiogenesis, and an enhanced DNA damage response [29]. In this study, the Notch signaling pathway was investigated to explore the role of RNF126 in cisplatin resistance. We found that silencing RNF126 inhibited Notch pathway activation in SKOV3/DDP cells, as evidenced by reduced mRNA and protein expression of Notch target genes. Interestingly, it was found that RNF126 silencing not only increased Numb protein levels but also reduced Notch1 mRNA transcription. The decreased Notch1 mRNA can be explained by the canonical positive feedback loop that operates in the Notch signaling pathway. Conversely, Notch signaling activation promotes the transcription of its own gene, reinforcing pathway activation [23]. Importantly, it was found that silencing of RNF126 did not alter the level of Numb mRNA expression. However, silencing RNF126 in SKOV3/DDP cells markedly increased the Numb protein level, whereas overexpressing RNF126 dramatically reduced it, as determined by Western blot analysis. Based on these results,

we hypothesized that RNF126 regulates Numb by ubiquitination and degradation, thereby activating the Notch pathway. It has been reported that Numb overexpression confers cisplatin sensitivity and suppresses tumor growth in epithelioid malignant pleural mesothelioma [30]. Notably, an increase in tumor growth was observed upon Numb silencing in the tumor xenograft model. Importantly, statistical analysis revealed that tumor volumes in the sh-RNF126+sh-Numb group were not significantly different from those in the sh-Numb group, indicating that Numb depletion completely abrogated the tumor-suppressive effect of RNF126 silencing. These results suggested that RNF126-mediated tumor promotion are predominantly mediated through the negative regulation of Numb and the subsequent activation of the Notch1 signaling pathway.

Although the RNF126-regulated Numb/Notch1 axis represents a critical mechanism driving cisplatin resistance in OC, RNF126 may exert its oncogenic functions through additional pathways beyond Numb degradation. Previous studies have identified several other substrates of RNF126, including PTEN [17], G3BP1/2 [31], MBNL1 [32], ACAP2 [21] and ILF3 [33]. Beyond Numb, RNF126 may also promote chemoresistance by ubiquitinating these substrates, a possibility that warrants further investigation. Additionally, given the established role of Notch signaling in cancer stem cell maintenance, it is conceivable that RNF126-mediated Numb degradation promotes cancer stem cell expansion through the Notch pathway and thereby contributes to chemoresistance.

5. Limitations

Several limitations of this study should be acknowledged. First, the *in vivo* experiments were conducted using immunodeficient mice, limiting the assessment of RNF126 function within the tumor immune microenvironment. Second, although we identified Numb as a critical substrate of RNF126, further work is required to precisely map the interaction domain between RNF126 and Numb to establish the structural basis of their binding. Finally, whether additional RNF126 substrates cooperatively contribute to Notch activation and cisplatin resistance warrants further investigation. Addressing these questions will help to establish a theoretical foundation for the development of novel anti-OC strategies that target RNF126.

6. Conclusion

In summary, the RNF126-mediated induction of the Numb–Notch signaling cascade facilitates heightened tumor cell reproduction and insensitivity to cisplatin treatment, as evidenced by data from cellular and animal models. Mechanistically, RNF126 functions as a novel ubiquitin E3 ligase targeting Numb ubiquitination, leading to Notch signaling activation and its target genes expression increasing. By establishing RNF126 as a critical oncogenic driver, this study provides significant mechanistic insights into the complex regulation of the Notch pathway within ovarian carcinomas. Accordingly, the therapeutic targeting of RNF126 may represent a novel and promising strategy to inhibit metastasis and circumvent chemotherapy resistance in patients with OC.

Availability of Data and Materials

The datasets utilized and analyzed in study are available from the corresponding authors upon reasonable request.

Author Contributions

TZ: Methodology, Investigation, Writing-original draft, Formal analysis and Software. HW: Methodology, Investigation. LN: Methodology, Investigation. XW: Conceptualization, Writing-review. HL: Conceptualization, Writing-review & editing, XJ: Conceptualization, Writing-review & editing, Supervision. All authors contributed to editorial changes in the manuscript. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

Ethical approval was granted by the Animal Ethics Committee of The First Affiliated Hospital of Harbin Medical University (Approval No.2026139). All animal experiments in this study were followed by the ARRIVE guidelines and approved by The First Affiliated Hospital of Harbin Medical University. All procedures involving animals were performed strictly in accordance with the National Standard of China for Institutional Animal Care and Use (GB/T 35892-2018) and relevant international welfare regulations to minimize animal suffering.

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Conflicts of Interest

The authors declare no conflicts of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at https://doi.org/10.31083/FBL54223.

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