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OTU deubiquitinase, ubiquitin aldehyde binding 2 (OTUB2) modulates the stemness feature, chemoresistance, and epithelial-mesenchymal transition of colon cancer via regulating GINS complex subunit 1 (GINS1) expression

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Abstract

Background Colon cancer is one of the most prevalent tumors in the digestive tract, and its stemness feature significantly contribute to chemoresistance, promote the epithelial-mesenchymal transition (EMT) process, and ultimately lead to tumor metastasis. Therefore, it is imperative for researchers to elucidate the molecular mechanisms underlying the enhancement of stemness feature, chemoresistance, and EMT in colon cancer.

Methods Sphere-formation and western blotting assays were conducted to assess the stemness feature. Edu, flow cytometry, and cell viability assays were employed to evaluate the chemoresistance. Immunofluorescence and western blotting assays were utilized to detect EMT. Immunoprecipitation, ubiquitination, agarose gel electrophoresis, chromatin immunoprecipitation followed by quantitative PCR (chip-qPCR), and dual luciferase reporter gene assays were employed for mechanistic investigations.

Results We demonstrated a markedly higher expression level of OTUB2 in colon cancer tissues compared to adjacent tissues. Furthermore, elevated OTUB2 expression was closely associated with poor prognosis and distant tumor metastasis. Functional experiments revealed that knockdown of OTUB2 attenuated stemness feature of colon cancer, enhanced its sensitivity to oxaliplatin, inhibited its EMT process, ultimately reduced the ability of tumor metastasis. Conversely, overexpression of OTUB2 exerted opposite effects. Mechanistically, we identified OTUB2 as a deubiquitinase for SP1 protein which bound specifically to SP1 protein, thereby inhibiting K48 ubiquitination of SP1 protein. The SP1 protein functioned as a transcription factor for the GINS1, exerting its regulatory effect by binding to

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the 1822–1830 region of the GINS1 promoter and enhancing its transcriptional activity. Ultimately, alterations in GINS1 expression directly regulated stemness feature, chemosensitivity, and EMT progression in colon cancer.

Conclusion Collectively, the OTUB2/SP1/GINS1 axis played a pivotal role in driving stemness feature, chemoresistance, and EMT in colon cancer. These results shed new light on understanding chemoresistance and metastasis mechanisms involved in colon cancer.

Keywords OTUB2, GINS1, Stemness feature, Chemoresistance, EMT

Introduction

Colon cancer is the second leading cause of cancer-related mortality worldwide [1]. According to statistics, in 2023, the incidence of tumor recurrence and distant metastasis in patients with colon cancer is estimated to be 20%, with over 50% of cases developing distant metastasis within five years after initial diagnosis [2]. Chemotherapy resistance remains the primary factor contributing to tumor recurrence and metastasis [3]. The first-line chemotherapy regimens recommended by the 2019 National Comprehensive Cancer Network (NCCN) guidelines include FOLFOX and CapeOX regimens containing oxaliplatin. However, some patients still exhibit clinical insensitivity towards these treatments [4]. Studies have indicated a strong association between the development of chemotherapy resistance and stem-like characteristics exhibited by tumor cells [5]. Epithelial mesenchymal transition (EMT) plays a crucial role in promoting metastasis across various tumors [6, 7], characterized by reduced expression of E-cadherin alongside increased expression of N-cadherin and Vimentin as key feature during this process [8, 9]. Therefore, investigating the molecular mechanisms underlying colon cancer recurrence and metastasis caused by stem-like characteristics, chemoresistance, and EMT may provide novel insights for overcoming chemotherapy resistance in colon cancer treatment while enabling precision-based therapies.

OTU deubiquitinase, ubiquitin aldehyde binding 2 (OTUB2), a deubiquitination enzyme that has been discovered in recent years, has garnered increasing attention from researchers due to its role in malignant tumors [10]. The OTUB2 gene is located in the q32.12 region of chromosome 14 and encodes a protein consisting of 234 amino acids, including an OTU domain and Ubal binding site [11]. Overexpression of OTUB2 has been shown to promote tumor progression and metastasis in various cancers [10, 12, 13]. In colon cancer, Yu et al. [14] demonstrated that OTUB2 promotes the malignant progression of colorectal cancer by enhancing PKM2 activity and glycolysis. Xu et al. [15]. showed that inhibiting OTUB2 can suppress the growth of colorectal cancer cells by modulating the β-catenin signaling pathway. Despite extensive research on the molecular biology of OTUB2, there is currently no relative research demonstrating its regulation of stemness feature, chemoresistance, and EMT in colon cancer.

GINS complex subunit 1 (Psf1 homology, GINS1) is a component of the tetramer complex of GINS (Go-Ichi-Nii-San), which comprises SLD5, GINS2, and GINS3. It is evolutionarily conserved and plays an integral role in the replication helicase mechanism [16]. Moreover, it serves as a key player in DNA replication initiation and extension, being indispensable for accurate DNA replication and maintenance [17]. Under physiological conditions, GINS1 primarily participates in early embryogenesis [18]. In recent years, there has been increasing research on the involvement of GINS1 in malignant tumors. The expression of the GINS1 gene is upregulated across various cancers such as colon cancer, hepatocellular carcinoma, synovial sarcoma, prostate cancer, and lung cancer. Furthermore, high levels of GINS1 expression are closely associated with malignant biological behaviors including tumor proliferation and metastasis [19, 20]. Our focus has also extended to exploring the relationship between GINS1 and gastrointestinal tumors [21, 22]. Recent studies have highlighted the crucial role played by GINS1 in maintaining tumor cell stemness. Li et al. [20] demonstrated that GINS1 promoted tumor stem cell properties and induced sorafenib chemoresistance in human liver cancer cells. Therefore, we also intended to investigate whether OTUB2 enhances the stem cell properties of colon cancer cells by modulating GINS1, thereby promoting chemotherapy resistance and EMT.

SP1 can be categorized into four domains based on their respective functions: the dsDNA binding domain, the SP1 transcriptional activity domain, the Btd domain, and the SP domain [23]. The distribution of SP1, a transcription factor, has been extensively observed in the nucleus of virtually all mammalian tissue cells. Its transcriptional mechanism involves binding to GC/GT sequence-rich (GC box) regions within target gene promoters [24]. In addition, it also plays a significant role in various malignant tumors such as colon cancer, liver cancer, stomach cancer, and pancreatic cancer [25, 26]. However, few current researches reported the correlations between SP1 and stemness feature, chemoresistance, or EMT in colon cancer.

In our study, we identified SP1 as a potential target protein of the deubiquitination enzyme OTUB2 in colon

cancer cells. OTUB2 upregulated the protein expression of SP1 by inhibiting its ubiquitination degradation, thereby promoting the transcriptional activity of GINS1 and subsequently enhancing the stemness feature of colon cancer. This ultimately led to chemoresistance and EMT in colon cancer. In conclusion, our findings validated OTUB2 as a promising therapeutic target for treating colon cancer. Furthermore, combining OTUB2 targeted drug with oxaliplatin treatment may improve oxaliplatin sensitivity and reverse chemoresistance in colon cancer patients, offering a novel approach for precision treatment.

Methods

Cell culture and transfection

Normal human colon mucosal epithelial cell line (NCM460), colon cancer cell lines (HCT116, DLD1, SW620, HT29, and SW480), and human embryonic kidney cell line (HEK293T) were purchased from the Shanghai Institute of Cell Research, Chinese Academy of Sciences. NCM460, DLD1, SW620, HT29, and SW480 were cultured in DMEM medium (Solarbio Science & Technology Co., Ltd, Beijing, China) contained 10% fetal bovine serum (FBS, ExCell Bio, Suzhou, China), 100 U/ml streptomycin and 100 U/ml penicillin (Solarbio Science & Technology Co., Ltd, Beijing, China). HCT116 and HEK293T were cultured in RPMI 1640 medium (Solarbio Science & Technology Co., Ltd, Beijing, China) contained 10% FBS, 100 U/ml streptomycin and 100 U/ml penicillin. All cell lines were incubated in a constant temperature incubator set at 37℃ and with a CO2 concentration of 5%.

Lentiviral vectors for knockdown (sh-OTUB2#1, sh-OTUB2#2, sh-SP1, and sh-GINS1) and overexpression (OTUB2, SP1, and GINS1) were purchased from Hanbio (Shanghai, China). FLAG-OTUB2, HA-SP1, and MYC-ubiquitin (wild type, K6R, K11R, K27R, K29R, K33R, K48R, K63R, and K48) plasmids were purchased from Genechem (Shanghai, China). The transfection procedures for all lentiviral vectors and plasmids were conducted in strict adherence to the manufacturer's prescribed protocols. And the sequences of all shRNAs were presented in the Table S1.

Establishment of tumor stem cells and oxaliplatin-resistant cells

For the establishment of tumor stem cells, colon cancer cells (HCT-116 and DLD1) were inoculated onto ultra-low adhesion 6-well culture plates (Corning, Glendale, Arizona, USA) at a density of 5×10^3 cells per well. The cells were cultured in serum-free DMEM/F12 medium (Gibco, Grand Island, New York, USA) supplemented with B27(2%, Gibco, Grand Island, New York, USA), EGF (20 ng/ml, PEPROTECH, Rocky Hill, USA), and bFGF (20 ng/ml, EPROTECH, Rocky Hill, USA). After 7–14 days of culture under these conditions, tumor stem-like cell spheres formed uniformly and densely. These spheres were collected for further culturing to obtain a large number of tumor stem cells.

For the construction of oxaliplatin-resistant cells, the colon cancer cells were exposed to oxaliplatin at initial concentrations equivalent to 1/5 of the IC50 value in parent cells (HCT-116 and DLD1) for a duration of 48 h. Subsequently, the medium was discarded, and the cells were washed twice with PBS before being replenished with drug-free normal medium. Once the cells resumed normal growth, this process was repeated eight more times. Following stable cell growth at this particular drug concentration, the oxaliplatin dosage was gradually increased for further culturing. The drug concentration of oxaliplatin gradually increased until the resistance index (RI) exceeded 5 (RI = IC50 value in oxaliplatin-resistant cells / IC50 value in parent cells), signifying the successful establishment of oxaliplatin-resistant colon cancer cell lines.

Quantitative real-time PCR (qRT-PCR)

The extraction of total RNA from cells or tissues was performed using the Trizol method, followed by reverse transcription into cDNA (TransGen Biotech, Beijing, China). The cDNA was utilized for real-time quantitative PCR analysis (TaKaRa, Biomedical Technology Co., Ltd, Beijing, China). The $2^{-\rho\rho Ct}$ method was employed for data analysis. The primer sequence is provided in Table S2.

Western blotting

The total protein was extracted from cells or tissues using RIPA supplemented with 1% PMSF and 1% phosphatase inhibitor (Beyotime Biotechnology, Shanghai, China). The protein samples were subjected to electrophoresis on a 10% SDS-PAGE gel, followed by transfer onto a PVDF membrane (Merck Millipore Ltd, Germany). The PVDF membrane was blocked with 5% skim milk for 2 h. Subsequently, the PVDF membrane was incubated with the specific primary antibody at 4℃ for a duration of 12 h. Finally, the PVDF membrane was incubated with the secondary antibody (1:10000, Proteintech, Wuhan, China) for 1 h and then subjected to exposure using BIO-RAD gel electrophoresis imaging system. All primary antibodies employed in Western blotting were as follows: anti-OTUB2(1:1000,Affinity), anti-SP1(1:2000, Proteintech), anti-GINS1(1:10000,Abcam), anti-CD44 (1:1000, Affinity), anti-CD133 (1:1000, Affinity), anti-NANOG (1:1000, Affinity), anti-OCT4 (1:2000, Proteintech), anti-SOX2 (1:5000, Proteintech), N-cadherin (1:2000, Proteintech), E-cadherin (1:10000, Proteintech), Vimentin (1:2000,

Proteintech), anti-GAPDH (1:10000, Proteintech), anti-β-tubulin (1:5000, Proteintech).

Immunohistochemistry (IHC)

The tissue was embedded in paraffin blocks and sectioned into 4-micron-thick slices. The tissue sections were incubated with primary specific antibodies followed by incubation with an HRP-conjugated secondary antibody. The specimen was subsequently subjected to DAB staining followed by hematoxylin restaining, and ultimately captured under a microscope. All primary antibodies employed in immunohistochemistry were as follows: anti-OTUB2(1:50, Affinity), anti-CD44 (1:50, Affinity), anti-CD133 (1:50, Affinity), anti-Ki67 (1:2000, Proteintech), anti-PCNA (1:1500, Proteintech), anti-ubiquitin (1:200, Santa Cruz).

Immunofluorescence staining assay

The 24-well culture plate was inoculated with 2×10^4 colon cancer cells that were treated differently, and then incubated at 37 °C for 24–36 h. Then, the adherent cells were washed three times with PBS solution and subsequently fixed with a 4% paraformaldehyde for a duration of 20 min. Subsequently, at room temperature, the attached cells were immersed in 0.5% Triton X-100 solution for 20 min and then incubated with 5% goat serum for 30 min to mitigate non-specific binding. Then, anti-OTUB2(1:200, Affinity), anti-CD44(1:200, Affinity), anti-CD133(1:200, Affinity), anti-N-cadherin (1:100, Proteintech), anti-E-cadherin (1:100, Proteintech), anti-Vimentin (1:100, Proteintech) were added into each well and incubated at 4°C for 12 h. The labeled antibodies of various hues were added and incubated at ambient temperature for a period of 1 h, followed by staining the nucleus with DAPI for 5 min. Ultimately, fluorescence microscopy was employed for the purpose of imaging.

Subcutaneous xenograft assay

Forty female BALB/c nude mice that were 4 weeks old were purchased from Nanjing Kris Biotechnology Co., LTD, the forty BALB/c nude mice were randomly divided into four groups (NC-OTUB2, sh-OTUB2#1, Vector-OTUB2, and OTUB2). HCT116 cells (1×10^7) stably transfected with NC-OTUB2, sh-OTUB2#1, Vector-OTUB2, and OTUB2 were resuspended in 200μL PBS and subcutaneously injected into the armpit of BALB/c nude mice. After tumors reached approximately 70 mm^3, each stable transfection group was subsequently randomly allocated into two subgroups: chemotherapy group (oxaliplatin injection) and placebo group (DMF injection). The chemotherapy group received intraperitoneal injections of oxaliplatin (5 mg/kg, every 3 days) for a duration of 3 weeks, while the placebo group received intraperitoneal injections of an equivalent volume of DMF as oxaliplatin within the same time frame. The tumor's length and width were measured using a vernier caliper at 5-day intervals, and the tumor volume (V) was calculated using the following formula: $V=0.52 \times length \times width^2$. After a period of 35 days following the inoculation of HCT116 cells, all nude mice were euthanized and the tumor mass was extracted for photographic analysis, H&E staining, and immunohistochemistry. The Institutional Animal Care and Use Committee of Nanchang Royo Biotech Co,. Ltd has granted approval for all animal experiments in our research.

Tumor lung metastasis assay

Twenty female BALB/c nude mice that were 4 weeks old were purchased from Nanjing Kris Biotechnology Co., LTD, the twenty BALB/c nude mice were randomly divided into four groups (NC-OTUB2, sh-OTUB2#1, Vector-OTUB2, and OTUB2). HCT116 cells (2×10^6) stably transfected with NC-OTUB2, sh-OTUB2#1, Vector-OTUB2, and OTUB2 were resuspended in 100μL PBS injected into the caudal vein of BALB/c nude mice. After a period of 35 days following the inoculation of HCT116 cells, all nude mice were euthanized and the lungs of mice was extracted for H&E staining. The Institutional Animal Care and Use Committee of Nanchang Royo Biotech Co,. Ltd has granted approval for all animal experiments in our research.

Sphere-formation assay

The colon cancer cells (1×10^3) were implanted in ultra-low adhesion 6-well culture plates (Corning, Glendale, Arizona, USA). The colon cancer cells were cultured in serum-free DMEM/F12 medium (Gibco, Grand Island, New York, USA) supplemented with B27(2%, Gibco, Grand Island, New York, USA), EGF (20 ng/ml, PEPROTECH, Rocky Hill, USA), and bFGF (20 ng/ml, EPROTECH, Rocky Hill, USA). After a duration of 14 days, microscopic photographs were captured and the quantity and size of the spheres were quantified.

Cell viability assay

The colon cancer cells (1×10^4) were seeded in 96-well culture plate and incubated at 37 °C for 8–12 h. Subsequently, the 96-well plates were supplemented with medium containing varying concentrations of oxaliplatin and incubated for 48 h. The medium was supplemented with 10 μL of cell counting kit 8 (UElandy, Suzhou, China) per 100 μl, followed by incubation at 37℃ for 2 h. Finally, the absorbance at 450 nm was measured using the microplate reader (Thermo Fisher Scientific, Waltham, USA). The cell viability rate (%) was determined using the formula: [(OD treatment - OD blank)/ (OD control - OD blank)] × 100%.

EdU assay

The 96-well plates were inoculated with 2×10^4 colon cancer cells and incubated at 37 °C for a duration of 8–12 h. Then, the culture was supplemented with EDU (UElandy, Suzhou, China) at a concentration of 50 μM and incubated for 2 h. The cells were subsequently fixed using a 4% paraformaldehyde solution and then neutralized with a glycine solution at a concentration of 2 mg/mL. Then, the cells were treated with a 3% BSA solution as a cell cleanser and stimulated with a 0.5% Triton X-100 solution as an osmotic enhancer. Ultimately, the cells were incubated in the dark with Click-iT working solution for 30 min and with 1×Hoechst 33,342 solution for 15 min respectively, followed by fluorescence microscopy imaging.

Flow cytometry

For apoptosis detection, the colon cancer cells were inoculated into 6-well culture plate. Once the cell density in each well exceeded 80%, the cells were gently washed with pre-cooled PBS and subsequently collected in flow cell tubes. Subsequently, the cells were treated with FITC-Annexin V and PI dyes (UElandy, Suzhou, China), followed by a 15-minute incubation in the dark. Finally, flow cytometry was employed for detection. The positive rate of FITC-Annexin V staining indicates the percentage of cells undergoing early apoptosis, while the positive rate of PI staining represents the proportion of cells in late-stage apoptosis.

For cell cycle detection, the colon cancer cells were collected, centrifuged, washed with pre-cooled PBS, fixed with pre-cooled 70% ethanol, and stored overnight in a -20℃ refrigerator. On the following day, the fixed cells were rinsed with pre-cooled PBS and subsequently treated with PI (UElandy, Shanghai, China). Finally, flow cytometry was employed for detection.

Cell counting kit-8 (CCK8) assay

The colon cancer cells (5000 cells/well) were inoculated into a 96-well plate. Subsequently, the medium containing 10% CCK-8 (Biosharp, Beijing, China) was added to each well at the specified time points (6 h, 24 h, 48 h, and 96 h). Following incubation at 37℃ for a duration of 2 h, the absorbance of each well was measured at a wavelength of 450 nm. The obtained data were then analyzed to assess cell proliferation capacity.

Colony formation assay

The colon cancer cells (1000 cells/well) were seeded into 6-well plates. Following a two-week incubation period, the cells were fixed using 4% paraformaldehyde, stained with crystal violet, and subsequently photographed to determine the number of spherical cells for assessing cellular proliferation ability.

Transwell migration assay

The transwell chamber (JET BIOFIL, Guangzhou, China) was positioned within 24-well plates. The colon cancer cells were resuspended in serum-free medium at a concentration of 10,000 cells per 100 μl. Subsequently, these cells were inoculated into the upper chambers (200 μl/upper chamber), while each lower chamber was filled with 600 μl of complete media containing 10% fetal bovine serum. After 24 to 72 h of incubation, the cells that migrated from the upper chamber to the lower chamber were fixed using a 4% paraformaldehyde solution. Subsequently, they were stained with crystal violet and ultimately visualized and quantified under a microscope.

Wound healing assay

The colon cancer cells (5×10^5) were inoculated into 6-well culture plate. After the cell monolayer was attached to the 6-well plate, we utilized a 200ul pipette tip to create a wound in the center of the adherent cells. Next, the medium in the 6-well culture plate was replaced with serum-free medium to suppress cell proliferation. Finally, the wound was documented at 0 h and 24 h through photography. The migratory capacity of cells was assessed by quantifying the extent of wound healing.

Immunoprecipitation (IP)

Colon cancer cells or 293T cell were lysed using pre-cooled RIPA lysis supplemented with 1% PMSF and 1% phosphatase inhibitor for 30 min, followed by centrifugation at 12,000 rpm for 10 min to collect the cell-containing supernatant. The supernatant was then incubated with a primary antibody (2 μg) at room temperature for 1 h and subsequently mixed with protein A/G plus-agarose (40μL) from Santa Cruz, which was incubated in a shaker at 4℃ for 8–12 h. After incubation, centrifuge at 2500 RPM for 5 min, collect the sediment, and perform a wash with pre-cooled 10% RIPA lysis. Finally, the sediment was dissolved in electrophoresis sample buffer (2×) and boiled for 10 min before Western blotting.

Mass spectrometry analysis

The technique of liquid chromatography-tandem mass spectrometry (LC-MS/MS) was employed in our study for the purpose of conducting mass spectrometry analysis. The anti-OTUB2 or anti-IgG immunoprecipitate was isolated from HCT116 cell lysates using the IP method as described above. Subsequently, the immunoprecipitated proteins were subsequently subjected to digestion using trypsin of modified sequence-grade. Finally, the fragmented peptides were subjected to LC-MS/MS analysis in order to identify the interacting proteins of OTUB2.

Ubiquitination assays

The colon cancer cells or HEK 293T cells were initially treated with 15μM MG-132 at 37 °C for a duration of 8 h. Subsequently, immunoprecipitates targeting anti-sp1, anti-GINS1, anti-ubiquitin, or anti-HA were isolated from the lysates of either colon cancer cells or 293T cells using the aforementioned IP method. Finally, Western blotting was performed to analyze the obtained immunoprecipitates.

Chromatin immunoprecipitation (ChIP-qPCR)

The digested samples of colon cancer cells were cross-linked using 1% formaldehyde, and the crosslinking reaction was terminated with glycine. Subsequently, ultrasound was employed to disrupt the DNA-protein complex formed through cross-linking. Then, anti-SP1 or anti-IgG antibodies were added to samples form an antibody-target protein-DNA complex (with a suitable number of samples reserved as Input DNA samples). Next, Protein A beads were utilized for precipitation in order to selectively enrich DNA fragments that bind to the target protein. Subsequently, the samples were subjected to multiple washes with a wash buffer to eliminate non-specifically bound chromatin and achieve purification. The washed sample was eluted using ChIP eluting buffer. Following this, the decrosslinking buffer (0.2 M NaCl) was added to samples overnight at 65℃ for reversing the crosslinking process. Subsequently, the samples were fixed with RNaseA at 37℃ for 1 h and treated with 0.5 M EDTA, 1 M Tris HCl, and protease K at 45℃ for 2 h. Finally, the sample was dissolved in ddH2O for conducting subsequent quantitative PCR (q-PCR) experiments. Primers for all regions were presented in Table S3. And the data analysis involved employing the "ΔΔCt" method.

Agarose gel electrophoresis

Weigh 2 g of dry agarose powder and dissolve it in 50 ml of TBE electrophoresis solution (Sangon Biotech, Shanghai, China). Heat the mixture in a microwave oven on medium heat. Once boiling, remove from heat and allow it to cool to 60℃ at room temperature. Repeat the heating and cooling process twice, removing it from heat and allowing it to cool to approximately 50℃ each time. Immediately add 1 μl of GoldView solution (Solarbio Science & Technology Co., Ltd, Beijing, China), thoroughly mix, and transfer the mixture into a rubber plate. Insert a comb into the plate and let it completely cool before proceeding with electrophoresis. The amplified binding region products of each decross-linked purified DNA were sequentially sampled as follows: input, IgG (negative control), Histone E3(positive control), and IP: SP1. After completing electrophoresis, perform exposure photograph analysis using a multi-functional gel analyzer.

Luciferase reporter assay

The 24-well plates were seeded with 1×10^5 colon cancer cells and incubated at 37℃ for 8–12 h. Next, plasmid co-transfection was performed using lipofectamine 2000 (Invitrogen, USA) as the transfection reagent. Finally, the relative luciferase reporter activity was measured 48 h post-transfection using the Dual-Luciferase Reporter kit (Promega, USA) in accordance with the manufacturer's instructions.

Transcriptome sequencing

Large-scale expression profiling was conducted by performing transcriptomic analysis on stable HCT116 cells (NC and sh-OTUB2#1). Total RNA was extracted from HCT116 cells using the Trizol method, followed by DNase I treatment (TaKaRa, Biomedical Technology Co., Ltd, Beijing, China) to remove DNA. The Illumina TruSeqTM RNA Sample Preparation Kit (San Diego, CA) was utilized to prepare an RNA-SEQ transcriptome library with 1 μg of total RNA, following the provided instructions. After quantification using TBS380, the RNA-seq sequencing library was sequenced on the Illumina HiSeq xten sequencer. The original reads were subjected to SeqPrep (https://github.com/jstjohn/SeqPrep) and Sickle (https://github.com/najoshi/sickle) for trimming and quality control purposes using default parameters. Data analysis was performed online on the Majorbio Cloud Platform (www.majorbio.com).

Clinical samples collection

We collected a total of 124 pairs of tumor and adjacent tissue samples from the Gastrointestinal Surgery Department of the Second Affiliated Hospital of Nanchang University, all pathologically diagnosed with colon cancer. The clinical and pathological characteristics of the patients are summarized in Table 1. The study has received approval from the Ethics Committee of the Second Affiliated Hospital of Nanchang University.

Bioinformatics analysis

The TCGA-COAD dataset (https://portal.gdc.cancer.gov/) was utilized for the analysis of mRNA expression levels of OTUB2, CD44, and CD133 in colon cancer and adjacent normal colon tissues. The Human Protein Atlas (HPA) database was employed to investigate the protein expression level of OTUB2 in colon cancer tissues compared to normal colon tissues. AlphaFold (https://alphafold.ebi.ac.uk/), a structural prediction database, was used to predict the protein structure of OTUB2. Additionally, the UCSC database (https://genome.ucsc.edu/) and PROMO usage database (https://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3) were utilized to predict SP1 as a potential transcription factor for GINS1. GSE10950, GSE39582, GSE41258,

Table 1 The relationships between OTUB2 expression and clinicopathological characteristics in colon cancer patients

Characteristics	Low expression of OTUB2	High expression of OTUB2	*p* value
n	62	62	
Age, n (%)			0.7196
≤ 50	30(24.19%)	33 (26.61%)	
>50	32(25.81%)	29(23.39%)	
Gender, n (%)			0.5831
Male	35 (28.23%)	39 (31.45%)	
Female	27 (21.77%)	23 (18.55%)	
Diameter of tumor(cm), n (%)			***0.0116**
≤ 5	39 (31.45%)	24 (19.35%)	
>5	23 (18.55%)	38 (30.65%)	
TNM stage, n (%)			0.1054
I/II	35 (28.23%)	25 (20.16%)	
III/IV	27 (21.77%)	37 (29.84%)	
Lymphatic metastasis, n (%)			***0.0289**
Negative	42 (33.87%)	29 (23.39%)	
Positive	20 (16.13%)	33 (26.61%)	
Distant metastasis, n (%)			***0.0439**
Negative	59 (47.58%)	51 (41.13%)	
Positive	3 (2.42%)	11 (8.87%)	

and GSE44861 datasets from Gene Expression Omnibus (https://www.ncbi.nlm.nih.gov/geo/) database were used to validate the correlation between GINS1 and transcription factors. Furthermore, the UbiBrowser database (http://ubibrowser.ncpsb.org/) predicted OTUB2 was the deubiquitinating enzymes of SP1. Lastly, the JASPAR database (https://jaspar.elixir.no/) was employed to identify potential SP1 binding sites within the promoter region of GINS1.

Statistical analysis

All data were analyzed using GraphPad Prism 9.0 software (GraphPad, La Jolla, USA) or R software (version:4.3.2). Student t-test was employed to compare the mean differences between two groups. One-way ANOVA test was employed to assess the mean differences among multiple groups. Kaplan-Meier survival curves were assessed using the Log-rank test. Nonlinear regression was utilized to fit the inhibition curve, and the IC50 value of oxaliplatin was calculated using GraphPad Prism 9.0 software. Spearman correlation analysis was employed to examine the relationship between two variables. All experiments were independently repeated three times. Statistical significance was considered at $p<0.05$.

Results

OTUB2 was highly expressed in colon cancer tissues and cells and was associated with poor prognosis in patients

OTUB2 is a member of the ovarian tumor (OTU) deubiquitinase family, which belongs to the group of deubiquitinase enzymes. It has been observed that OTUB2 is highly expressed in various types of tumors and is closely associated with poor prognosis in cancer patients [12, 13, 27, 28]. However, the role of OTUB2 played in colon cancer remains unclear. Therefore, we conducted a series of studies on OTUB2 in colon cancer. Initially, we utilized data from the TCGA-COAD database to demonstrate higher mRNA expression levels of OTUB2 in 44 colon cancer samples compared to their corresponding para-cancer samples (Fig. 1A). Subsequently, through analysis of IHC results from the HPA database, we further confirmed elevated protein expression levels of OTUB2 in colon cancer tissue samples as compared to normal tissue samples (Fig. 1B). These findings were subsequently validated through qRT-PCR and Western blotting experiments where both mRNA and protein levels were found to be higher in colon cancer cells than normal colon epithelial cell (Fig. 1C, D). Additionally, we collected a total of 124 pairs of clinical samples from colon cancer patients at the Second Affiliated Hospital of Nanchang University and performed qRT-PCR along with IHC and Western blotting experiments which consistently demonstrated increased mRNA and protein levels for OTUB2 within these colon cancer tissue samples when compared to adjacent normal tissues (Fig. 1E-G). Finally, we also conducted an analysis on the overall survival (OS) rate of these 124 patients. The 124 patients were stratified into low and high expression groups for Kaplan-Meier survival analysis based on the median OTUB2 expression level. The result revealed that the high expression group exhibited a significantly lower OS compared to the low expression group, indicating a negative correlation between OTUB2 expression level and patient prognosis ($p=0.0173$, HR=1.951) (Fig. 1H). Finally, we also investigated the correlation between OTUB2 expression levels and clinicopathological characteristics in patients with colon cancer. The results revealed that high OTUB2 expression was significantly associated with increased tumor diameter ($p=0.0116$), lymphatic metastasis ($p=0.0289$), and distant metastasis ($p=0.0439$) (Table 1). In conclusion, our findings demonstrated a significant upregulation of OTUB2 expression in colon cancer samples compared to normal colon samples. And the high expression of OTUB2 was strongly associated with unfavorable prognosis of colon cancer patients.

OTUB2 regulated stemness feature, chemosensitivity, and EMT of colon cancer cells in vitro

Recent studies have demonstrated that OTUB2 promotes the progression of colorectal cancer by facilitating glycolysis [14]. However, the involvement of OTUB2 in regulating colon cancer cell stemness feature remains unclear and require further investigation. CD44 and

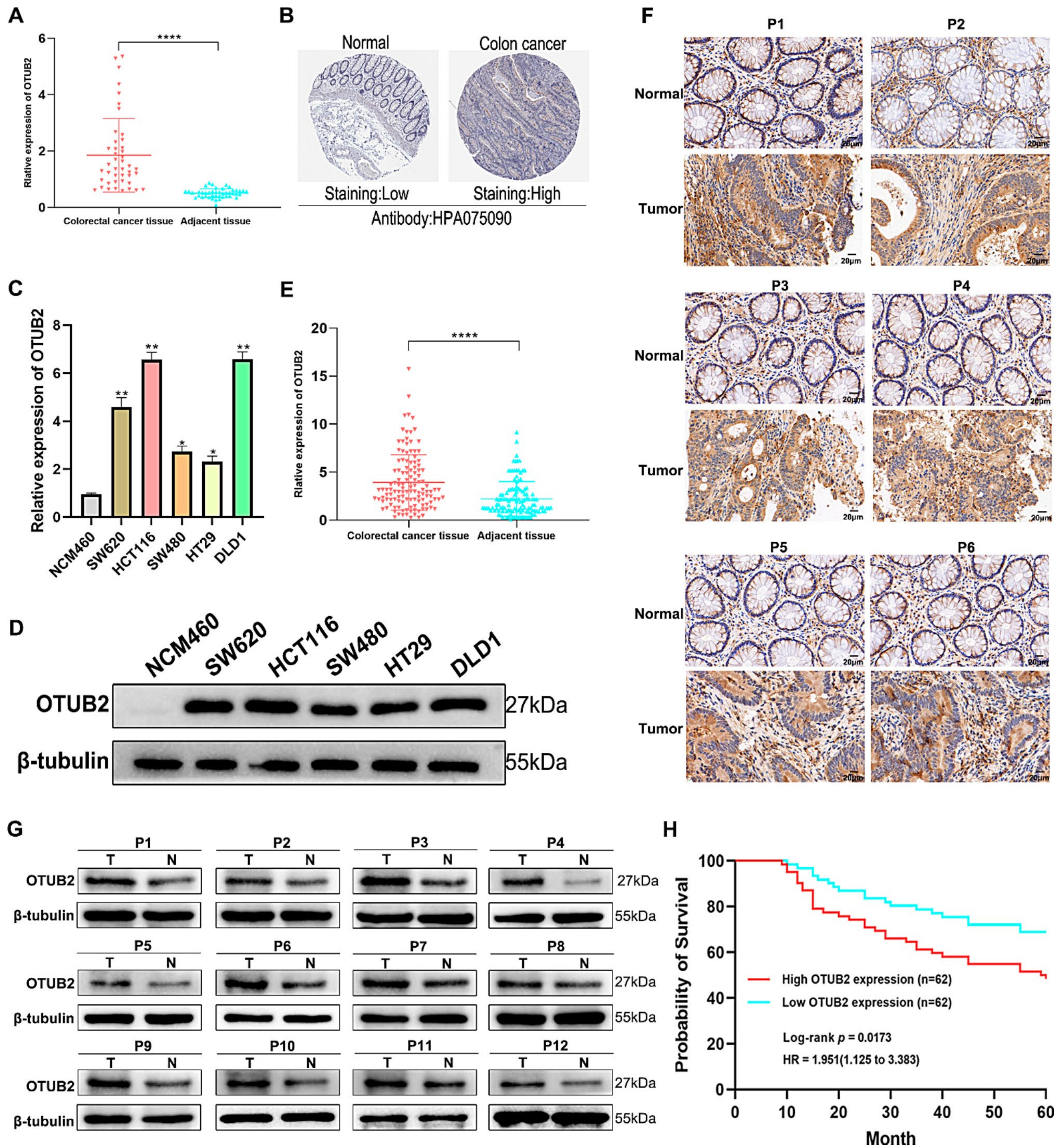


Fig. 1 OTUB2 was highly expressed in colon cancer tissues and cells. **A** The mRNA expression of OTUB2 in colon cancer specimens from TCGA-COAD database ($n = 44$; Student's t-test). **B** The IHC results of OTUB2 in normal colon tissue and colon cancer tissue from HPA database. **C** The mRNA expression of OTUB2 among in colon epithelial cell and colon cancer cells were analyzed by qRT-PCR (One-way ANOVA test). **D** The protein expression of OTUB2 among normal colon epithelial cell and colon cancer cells were analyzed by western blotting. **E** The mRNA expression of OTUB2 in our collected colon cancer samples ($n = 124$; Student's t-test). **F** The representative IHC results of OTUB2 in our collected colon cancer samples. **G** The representative western blotting results of OTUB2 in our collected colon cancer samples. **H** The OTUB2 related Kaplan–Meier survival analysis on our collected clinical data ($n = 124$; Log-rank test). All experiments were repeated three times and the data were represented as mean ± SD (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

CD133 are crucial markers for predicting the stemness of colon cancer [29, 30]. Interestingly, analysis of TCGA-COAD database revealed a positive correlation between the expression levels of OTUB2 and CD44/CD133 (Fig. 2A), suggesting that OTUB2 may regulate colon cancer cell stemness to influence the progression of colon cancer. To verify this hypothesis, we conducted a series of experiments. HCT116 and DLD1 were selected for in vitro validation due to their relatively high expression levels among five colon cancer cell lines (Fig. 1C, D) Knockdown and overexpression efficiency of OTUB2 in HCT116 and DLD1 were confirmed through qRT-PCR and Western blotting experiments (Fig. 2B, C), demonstrating effective modulation of OTUB2 expression levels. The spheroid formation assay is an established method for assessing tumor cell stemness feature; thus, we initially performed this experiment to evaluate the impact of OTUB2 on colon cancer cell stemness feature. Results showed that downregulation or upregulation of OTUB2 significantly inhibited or enhanced spheroid-forming ability respectively in colon cancer cells (Fig. 2D). Similarly, Western blotting experiments further validated that downregulation or upregulation of OTUB2 led to concurrent downregulation or upregulation respectively in tumor stemness markers such as CD44, CD133, Nanog, OCT4, and SOX2.This indirectly suggested that alteration in OTUB2 expression could affect the stemness feature of colon cancer cell (Fig. 2E). Additionally, our immunofluorescence experiments also revealed that the expression levels of tumor stemness markers CD44 and CD133 were modulated in accordance with the knockdown or overexpression of OTUB2, providing further evidence to support the hypothesis that OTUB2 exerts an influence on the stemness feature of colon cancer cell (Fig. 2F). In summary, the aforementioned experiments collectively provided direct or indirect evidence supporting the role of OTUB2 in regulating the stemness feature of colon cancer cells.

A considerable body of research has demonstrated that increasing tumor stemness level is associated with reduced sensitivity of tumors to chemotherapy drugs, leading to the development of chemoresistance [31–33]. Oxaliplatin is the primary chemotherapy drug used in clinical treatment for colon cancer patients; however, many patients still exhibit insensitivity to oxaliplatin [4]. Therefore, we began to investigate whether alterations in OTUB2 expression levels also influence colon cancer sensitivity towards oxaliplatin. Initially, we employed the cell viability assays and observed that colon cancer cell lines with higher relative expression levels of OTUB2 exhibited higher IC50 values for oxaliplatin compared to cell lines with lower expression levels (Fig. 2G). This finding demonstrated that the expression level of OTUB2 could indeed influence the sensitivity of colon cancer cells towards oxaliplatin. Furthermore, when OTUB2 was either knocked down or overexpressed in HCT116 and DLD1 cell lines, there was a corresponding decrease or increase in the IC50 value for oxaliplatin, further confirming that OTUB2 expression level could impact the sensitivity of colon cancer cells towards oxaliplatin (Fig. 2H). Additionally, we validated our findings using EdU cell proliferation assay and flow cytometry apoptosis assay. The results revealed that knockdown or overexpression of OTUB2 significantly enhanced or weakened the inhibitory effect of oxaliplatin on colon cancer cell proliferation (Fig. 2I), while simultaneously augmenting or diminishing its promoting effect on apoptosis induction in colon cancer cells (Fig. 2J). In summary, these results collectively provided direct or indirect evidence of the impact of OTUB2 on the chemosensitivity of colon cancer cells towards oxaliplatin.

A plethora of studies have demonstrated that cellular overgrowth often contributes to the development of chemoresistance in cells [34–36]. Therefore, we hypothesize that modulation of OTUB2 expression through knockdown or overexpression may also impact the growth of colon cancer cells, consequently influencing cellular chemoresistance. We conducted CCK8 assay (Fig. S1A), colony formation assay (Fig. S1B), and flow cell cycle assay (Fig. S1C), collectively revealing that manipulation of OTUB2 expression either suppressed or enhanced cell proliferation. Collectively, these findings establish a link between OTUB2 modulation and tumor cell growth as well as its indirect influence on cellular chemoresistance.

The previous studies have demonstrated that alterations in the stemness characteristics and chemoresistance of tumors can impact the process of EMT in tumor cells [37, 38]. Simultaneously, we conducted Western blotting experiments to validate the differential expression of EMT biomarkers between tumor cells and tumor stem cells. The results demonstrated that N-cadherin and Vimentin exhibited higher expression levels in tumor stem cells compared to tumor cells, whereas E-cadherin displayed lower expression levels in tumor stem cells relative to tumor cells (Fig. S2A). Furthermore, we also confirmed the disparity in EMT biomarker expression between tumor cell lines and oxaliplatin-resistant tumor cell lines through Western blotting experiments. The findings revealed elevated levels of N-cadherin and Vimentin in oxaliplatin-resistant tumor cell lines as opposed to tumor cell lines, while the expression level of E-cadherin was reduced in oxaliplatin-resistant tumor cell lines compared to tumor cell lines (Fig. S2B). Consequently, we further investigated whether OTUB2 could influence the EMT process in colon cancer cells. Western blotting and immunofluorescence experiments showed that as OTUB2 was knocked down or overexpressed, the biomarkers of EMT, N-cadherin and Vimentin,

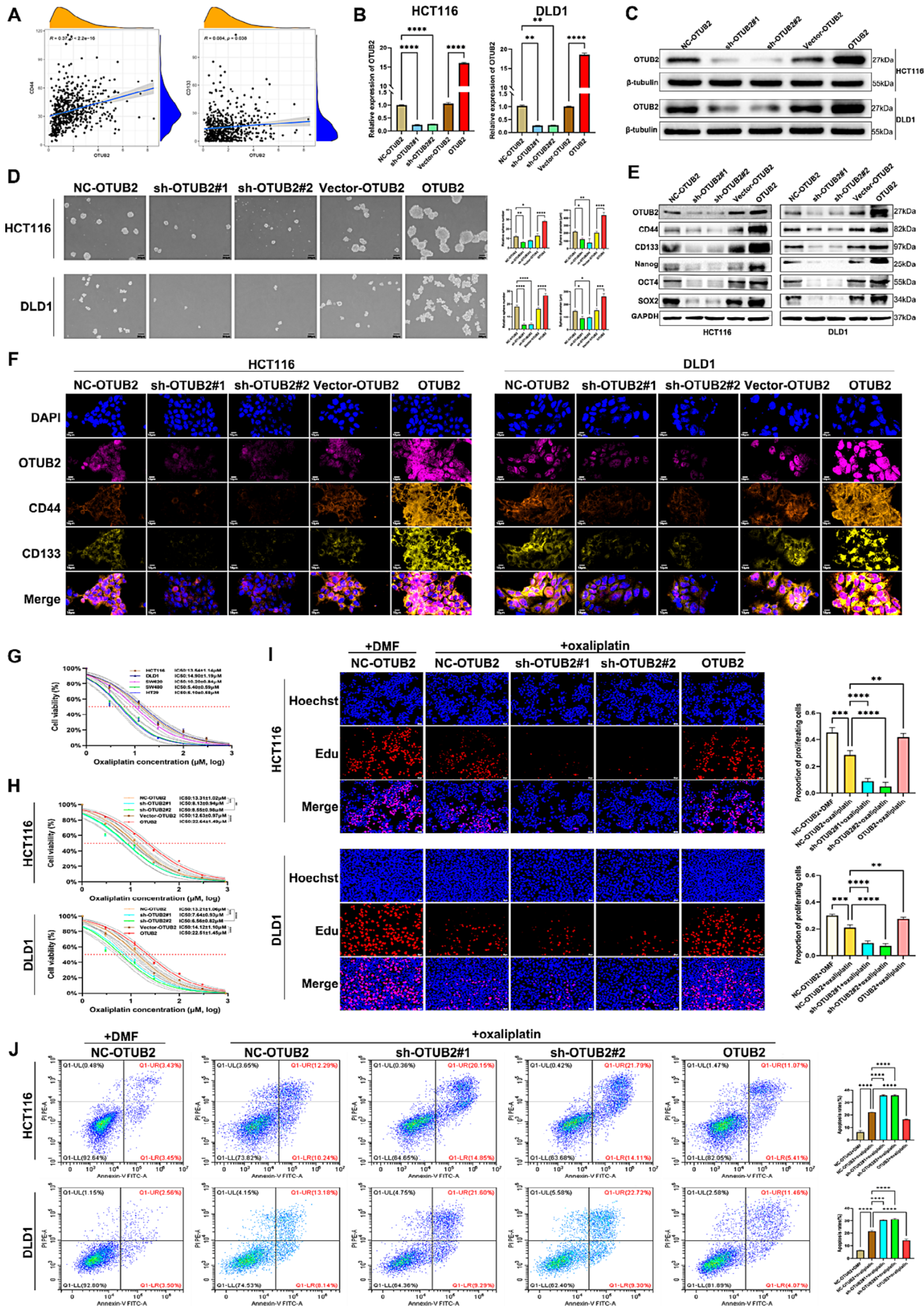


Fig. 2 OTUB2 regulated stemness feature and chemosensitivity of colon cancer in vitro. **A** The correlation between the expression of OTUB2 and CD44/CD133 in colon cancer specimens from TCGA-COAD database ($n=568$; Spearman correlation analysis). **B**, **C** The lentiviral transfection efficiency was validated through qRT-PCR and western blotting (One-way ANOVA test). **D** The spheroid formation assay of colon cancer cells transfected with indicated lentivirus. **E** The western blotting assay showed the alteration of stemness markers in colon cancer cells transfected with indicated lentivirus. **F** The immunofluorescence experiments revealed the alteration of CD44 and CD133 in colon cancer cells transfected with indicated lentivirus. **G** The IC50 values for oxaliplatin in five colon cancer cell lines (Nonlinear regression analysis). **H** The IC50 values for oxaliplatin in colon cancer cells transfected with indicated lentivirus (Nonlinear regression analysis). **I** The EdU assay in colon cancer cells transfected with indicated lentivirus and treated with oxaliplatin (One-way ANOVA test). **J** The flow cytometry assay in colon cancer cells transfected with indicated lentivirus and treated with oxaliplatin (One-way ANOVA test). All experiments were repeated three times and the data were represented as mean ± SD (*$p<0.05$, ** $p<0.01$, ***$p<0.001$, ****$p<0.0001$)

were downregulated or upregulated, while E-cadherin was upregulated or downregulated, which proved that OTUB2 could regulate the EMT process in colon cancer cells (Fig. 3A, B). However, upon addition of the EMT activator (TGF-β), we observed a reversal of changes in the expression levels of these EMT biomarkers caused by OTUB2 knockdown (Fig. 3C). Interestingly, our immunofluorescence experiments also unveiled that although OTUB2 knockdown resulted in decreased expression levels of cancer stemness markers CD44 and CD133, this effect was abrogated upon increasing the presence of TGF-β (Fig. 3D). Additionally, the cell viability assays confirmed that despite enhancing chemosensitivity to oxaliplatin in colon cancer through OTUB2 knockdown initially, this effect was nullified when TGF-β was introduced (Fig. 3E). Henceforth, these findings validated that OTUB2 exerted its regulatory influence on the EMT process within colon cancer cells by modulating their stemness feature and chemosensitivity.

Furthermore, previous studies have demonstrated that the process of EMT could induce a phenotypic shift in tumor cells towards mesenchymal characteristics, thereby augmenting their migratory capacity [39]. Consequently, we also examined whether alterations in OTUB2 expression could impact the migratory capacity of colon cancer cells. In line with our expectations, we observed a significant decrease or increase in the migratory capacity of colon cancer cells when OTUB2 was silenced or overexpressed, as evidenced by the Transwell migration assay (Fig. 3F) and wound healing assay (Fig. 3G).

In summary, the aforementioned experimental results collectively demonstrated that OTUB2 exerted regulatory control over the stemness feature, chemosensitivity, and EMT, thereby influencing the migratory capacity of colon cancer cells.

OTUB2 regulated chemosensitivity, stemness feature, and metastasis of colon cancer in vivo

Following this, we proceeded to further validate the effects of OTUB2 on colon cancer stemness, chemotherapy, and metastasis through in vivo experiments. The specific procedures for constructing subcutaneous tumor and pulmonary metastasis nude mouse models are illustrated in Fig. 4A, B. HCT116 cells stably transfected with the expression vector were injected into the nude mice used in our mouse model. The results of the subcutaneous tumor experiment in nude mice demonstrated that compared to the NC-OTUB2 group, both tumor volume and weight were reduced in the sh-OTUB2#1 group; similarly, when treated with oxaliplatin, both tumor volume and weight decreased in both the NC-OTUB2+oxaliplatin group and sh-OTUB2#1+oxaliplatin group compared to the group without oxaliplatin treatment. Notably, the reduction in tumor volume and weight was more significant in the sh-OTUB2#1+oxaliplatin group than that observed in the NC-OTUB2+oxaliplatin group (Fig. 4C-E). Moreover, compared to Vector-OTUB2 group, both tumor volume and weight increased significantly in OTUB2 groups; however, when treated with oxaliplatin, there was a decrease observed in both Vector-OTUB2+oxaliplatin and OTUB2+oxaliplatin groups regarding their respective tumor volumes and weights. Among them, the reduction in tumor volume and weight in the OTUB2+oxaliplatin group was comparatively lower than that observed in the Vector-OTUB2+oxaliplatin group, which was worth noting (Fig. 4F-H). The body weight changes of the mice in each group were also recorded simultaneously, and the findings indicated a slight decrease in body weight among mice in the oxaliplatin treatment group compared to those in the placebo control group after completion of the treatment (Fig. S3A-B). After observing weight loss in the oxaliplatin treatment group, we questioned whether our drug dosage was appropriate; however, a substantial body of experimental research has consistently demonstrated that 5 mg/kg is the standard dose of oxaliplatin in mice [40–42]. Therefore, we have ruled out inappropriate dosage as the cause. Upon reviewing relevant literature, it has been found that oxaliplatin can indeed induce side effects such as anorexia, nausea, and vomiting during clinical treatment [43, 44]. Consequently, weight loss is a prevalent adverse effect of oxaliplatin in clinical practice. Henceforth, we hypothesize that weight loss is one of the common side effects associated with oxaliplatin treatment in mice. Simultaneously, this study serves as a cautionary reminder to be vigilant regarding its side effects during clinical administration and to promptly employ preventive and therapeutic measures to enhance the efficacy of oxaliplatin for patients. Furthermore, the results of IHC also demonstrated a significant reduction in the protein expression of Ki67 and PCNA in the sh-OTUB2#1 group compared to the NC-OTUB2 group. Additionally, treatment with oxaliplatin resulted in a decreased protein expression of Ki67 and PCNA when compared to the control group without oxaliplatin, observed in both the NC-OTUB2+oxaliplatin group and the sh-OTUB2#1+oxaliplatin group. Notably, there was a more pronounced reduction in the protein expression of Ki67 and PCNA within the sh-OTUB2#1+oxaliplatin group as compared to the NC-OTUB2+oxaliplatin group (Fig. 4I). Meanwhile, in comparison to the Vector-OTUB2 group, the protein expression of Ki67 and PCNA was found to be upregulated in the OTUB2 group. Additionally, treatment with oxaliplatin resulted in a decrease in the protein expression of Ki67 and PCNA in both the Vector-OTUB2+oxaliplatin and OTUB2+oxaliplatin groups when compared to the group without oxaliplatin. Notably, there was a less pronounced reduction

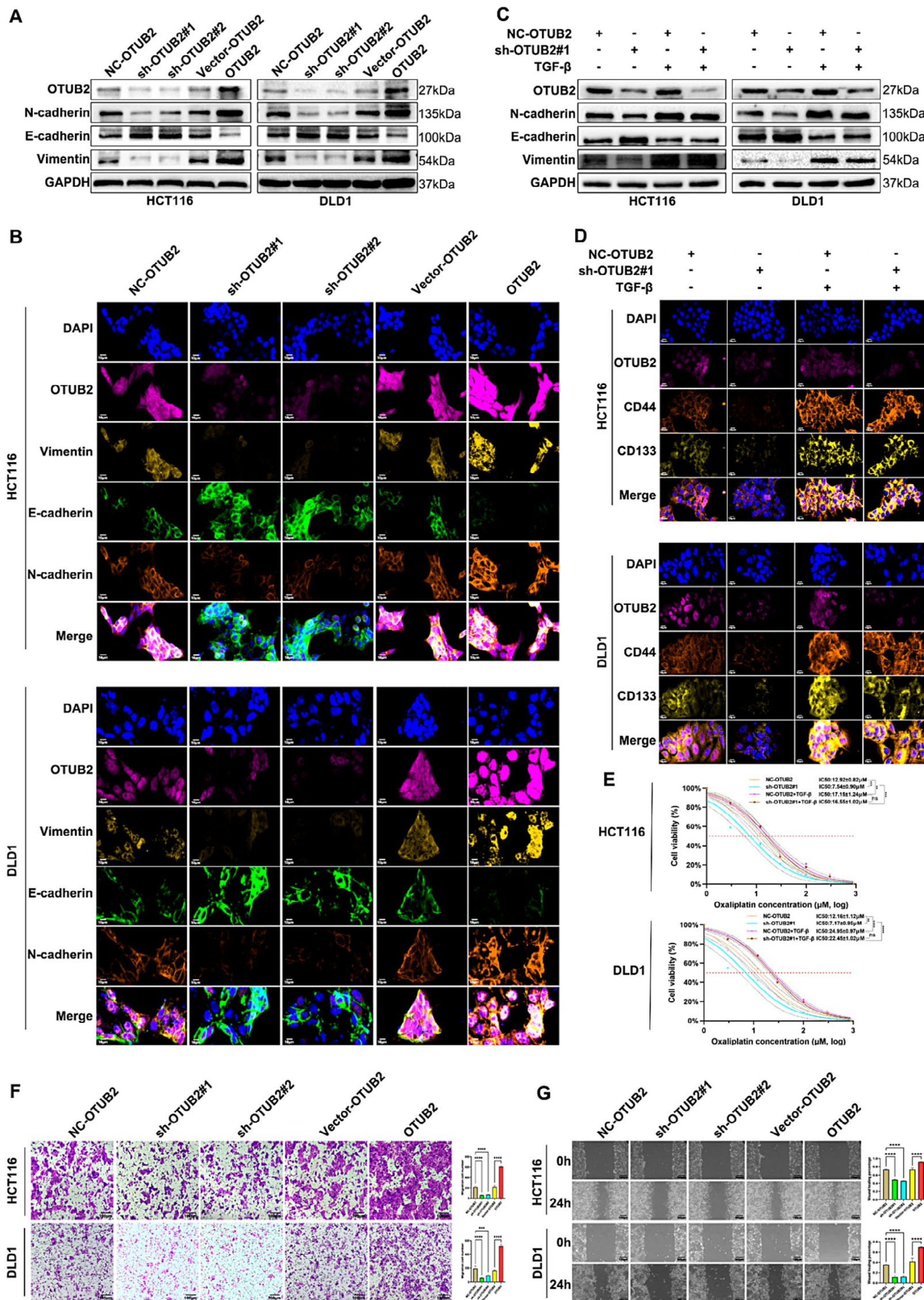


Fig. 3 OTUB2 promoted EMT and migration of colon cancer in vitro. **A** The western blotting assay revealed the alteration of EMT markers in colon cancer cells transfected with indicated lentivirus. **B** The immunofluorescence experiments revealed the alteration of EMT markers in colon cancer cells transfected with indicated lentivirus. **C** The western blotting assay revealed the alteration of EMT markers in colon cancer cells transfected with indicated lentivirus and EMT activator (TGF-β, 10ng/mL). **D** The immunofluorescence experiments revealed the alteration of EMT markers in colon cancer cells transfected with indicated lentivirus and TGF-β. **E** The IC50 values for oxaliplatin in colon cancer cells transfected with indicated lentivirus and TGF-β (Nonlinear regression analysis). **F**, **G** The Transwell migration assay and wound healing assay revealed the alteration of migration capability in colon cancer cells transfected with indicated lentivirus (One-way ANOVA test). All experiments were repeated three times and the data were represented as mean ± SD (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

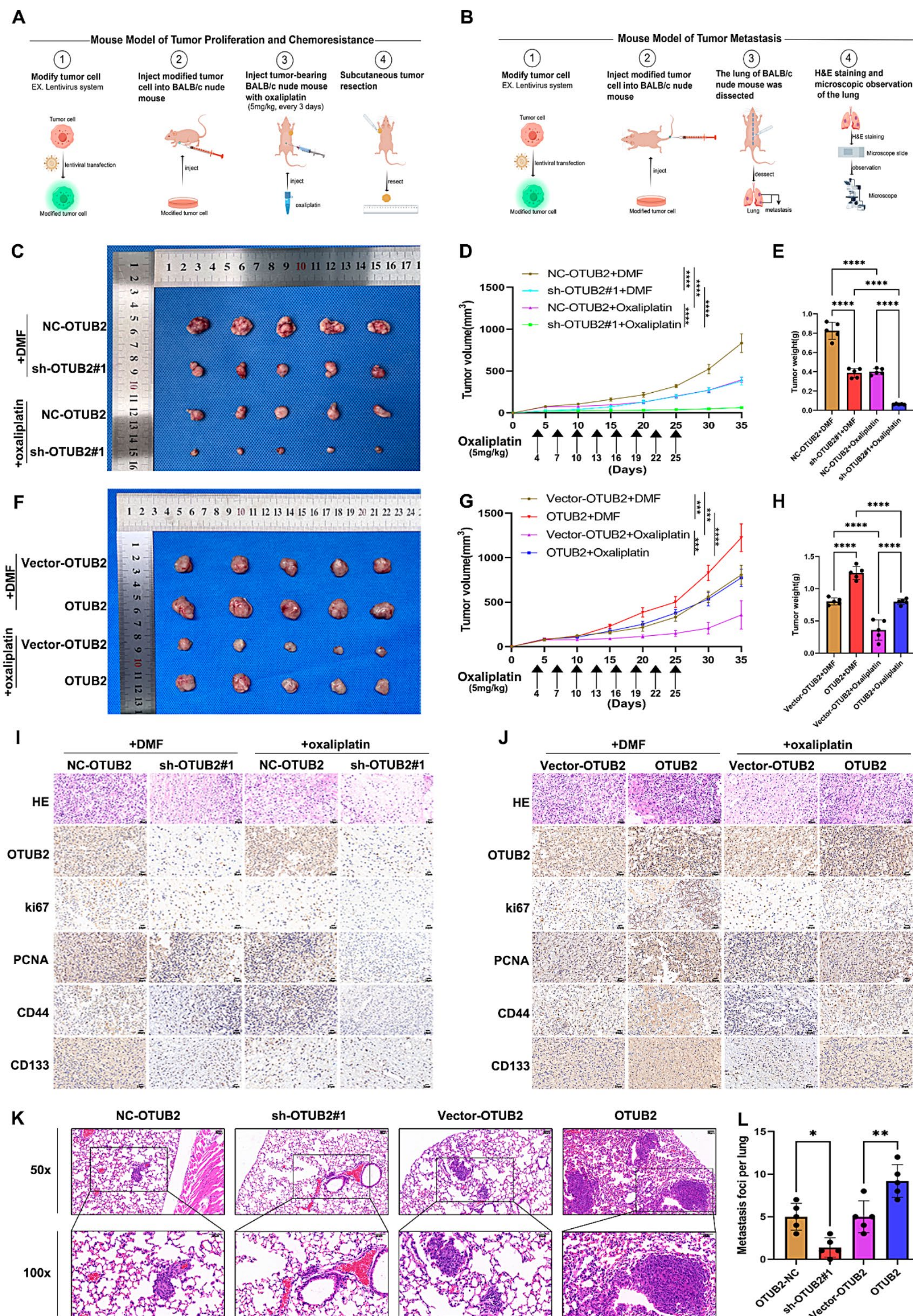


Fig. 4 OTUB2 regulated chemosensitivity, stemness feature and metastasis of colon cancer in vivo. **A**, **B** The specific procedures for constructing subcutaneous tumor and pulmonary metastasis nude mouse models. **C-H** The subcutaneous tumor experiment revealed the subcutaneous tumor volume and weight in colon cancer cells transfected with indicated lentivirus and treated with oxaliplatin (One-way ANOVA test). **I-J** The representative HE and IHC results of indicated proteins in subcutaneous tumors. **K** The representative HE results of pulmonary metastasis (One-way ANOVA test). All experiments were repeated three times and the data were represented as mean ± SD (**p* < 0.05, ** *p* < 0.01, ****p* < 0.001, *****p* < 0.0001)

in the protein expression of Ki67 and PCNA within the OTUB2+oxaliplatin group as compared to the Vector-OTUB2+oxaliplatin group (Fig. 4J). Therefore, the above findings collectively demonstrated that the expression of OTUB2 significantly impacted the chemosensitivity of colon cancer to oxaliplatin in vivo. Furthermore, it was noteworthy that the results of IHC also revealed that the downregulation or upregulation of OTUB2 also leads to a concomitant downregulation of CD44 and CD133 (Fig. 4I-J), which indirectly demonstrated that alterations in the expression of OTUB2 could impact the stemness feature of colon cancer in vivo. Additionally, we also established the nude mouse model for pulmonary metastasis of tumors. Histopathological examination (HE) results showed that there were fewer lung tumor foci observed in the sh-OTUB2#1 group compared to the NC-OTUB2 group, while more lung metastatic foci were observed in the OTUB2 group compared to the Vector-OTUB2 group (Fig. 4K). The obtained results provided direct evidence that the expression of OTUB2 exerted a significant impact on the metastasis of colon cancer in vivo. In conclusion, the aforementioned in vivo experimental findings collectively demonstrated that modulation of OTUB2 expression levels exerted a significant influence on the stemness feature, chemosensitivity, and metastatic potential in colon cancer.

OTUB2 modulated stemness feature, chemosensitivity, and EMT of colon cancer cells through regulating GINS1 expression

Next, we initiated an investigation into the specific molecular mechanisms through which OTUB2 regulates stemness feature, chemosensitivity, and EMT in colon cancer. We initially conducted transcriptome sequencing analysis using stably transfected HCT116 cells (NC and sh-OTUB2#1). The results of the transcriptome sequencing analysis are presented in Table S4. Subsequently, we performed differential gene expression analysis on the transcriptome sequencing data (NC vs. sh-OTUB2#1) and observed a significant down-regulation in the expression levels of numerous genes following OTUB2 knockdown (Table S5; Fig. S4). Among the top 50 genes exhibiting the most pronounced decrease, GINS1 was the sole regulatory gene associated with tumor stemness [45, 46]. Furthermore, it has been implicated in modulating chemotherapy resistance and EMT process in various malignancies [20, 47, 48]. Therefore, we boldly hypothesized that OTUB2 might regulate the expression level of GINS1 to influence stemness feature, chemosensitivity, and EMT in colon cancer. The initial validation of our hypothesis was achieved by conducting Western blotting experiments, which demonstrated that the expression level of GINS1 decreases or increases correspondingly as OTUB2 was knocked down or overexpressed (Fig. S5A). Subsequently, we employed qRT-PCR experiments to confirm the co-transfection efficiency of OTUB2 and GINS1 knockdown or overexpression (Fig. S5B), followed by a series of functional recovery experiments. The spheroid formation experiments and Western blotting assays revealed that knockdown of OTUB2 weakened the stemness feature of colon cancer cells, which could be restored by overexpressing GINS1; conversely, overexpression of OTUB2 enhanced the stemness feature, but this effect could be reversed by knocking down GINS1 (Fig. S5C, D). Furthermore, the cell viability assays demonstrated that the decreased IC50 value in colon cancer cells induced by OTUB2 knockdown could be restored through GINS1 overexpression, while the increased IC50 value in colon cancer cells induced by OTUB2 overexpression could be restored through GINS1 knockdown (Fig. S5E). The EdU cell proliferation assay revealed that the inhibitory effect of oxaliplatin on colon cancer cell proliferation was potentiated by OTUB2 knockdown, while this effect could be attenuated by GINS1 overexpression. Conversely, the inhibitory effect of oxaliplatin on colon cancer cell proliferation was weakened by OTUB2 overexpression, which could be enhanced by GINS1 knockdown (Fig. S5F). The flow cytometry-based apoptosis assays demonstrated that the enhancement of oxaliplatin-induced apoptosis in colon cancer cells through OTUB2 knockdown was mitigated by GINS1 overexpression, whereas the promotion of oxaliplatin-induced apoptosis in colon cancer cells via OTUB2 overexpression was augmented by GINS1 knockdown (Fig. S5G). Finally, Western blotting experiments, Transwell migration assays, and wound healing assays indicated that OTUB2 knockdown reduced EMT and migration ability of colon cancer cells, which could be restored by GINS1 overexpression. Conversely, OTUB2 overexpression weakened EMT and migration ability of colon cancer cells but could be restored by GINS1 knockdown (Fig. S6A-C). In summary, these experiments collectively demonstrated that modulation of GINS1 expression level allowed OTUB2 to regulated the stemness feature, chemosensitivity, and EMT in colon cancer.

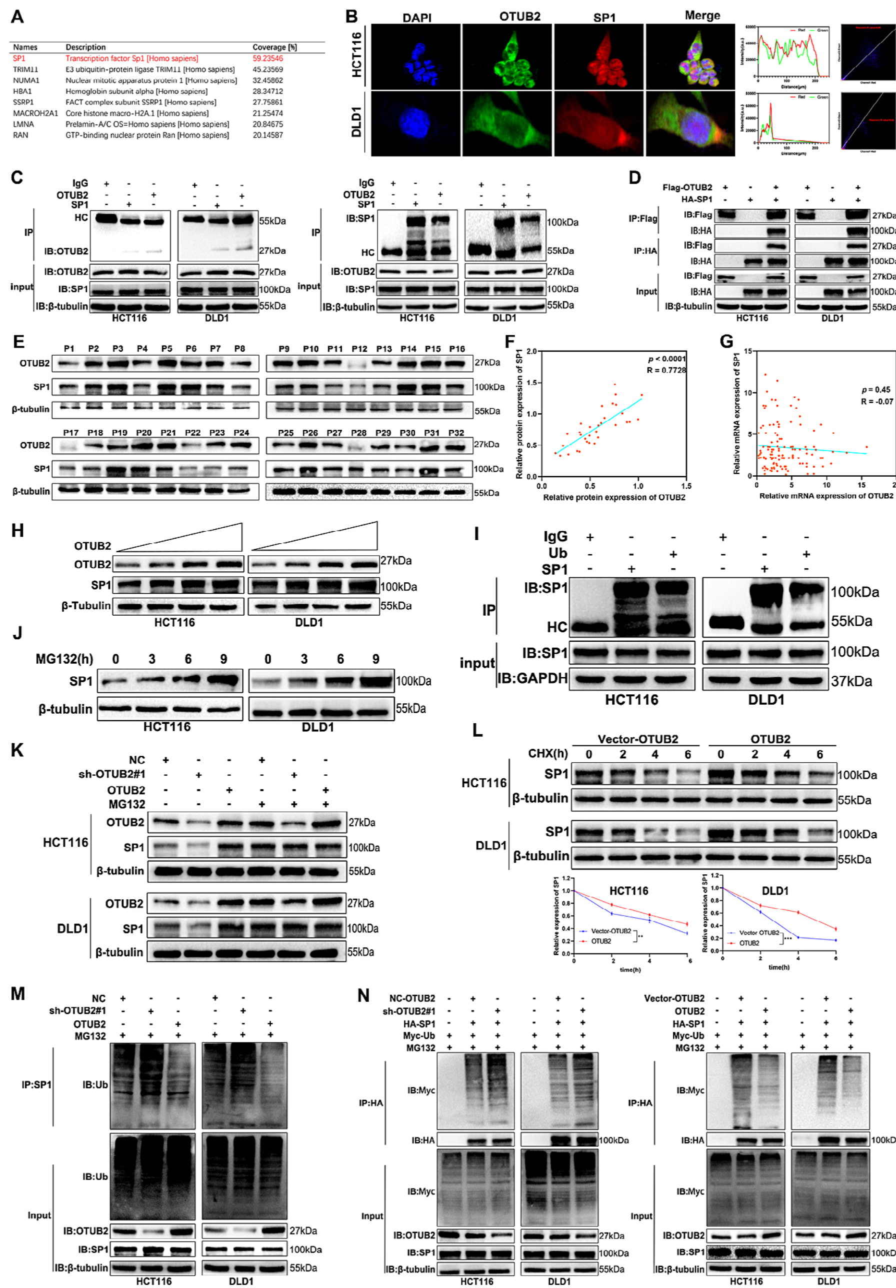
OTUB2 regulated stemness feature, chemosensitivity, and EMT of colon cancer cells by stabilizing the protein expression of SP1

Although we have demonstrated the ability of OTUB2 to regulate GINS1 expression and subsequently modulate stemness feature, chemosensitivity, and EMT in colon cancer, the specific regulatory mechanisms remain elusive. Considering that OTUB2 belongs to the deubiquitinase family, we hypothesized that OTUB2 might regulate GINS1 by inhibiting its deubiquitination and stabilizing its expression. Therefore, we conducted ubiquitination experiments; however, the results showed no

effect on the level of GINS1 ubiquitination upon downregulation or upregulation of OTUB2 (Fig. S7A). Hence, we postulated that there might be involvement of other key molecules in the regulation between OTUB2 and GINS1. The initial thought that came to mind was that OTUB2 might exert its influence on the expression level of GINS1's transcription factor by deubiquitinating it, thereby indirectly regulating the transcriptional activity of GINS1. To validate our hypothesis, we utilized both the UCSC and PROMO databases to predict a substantial number of transcription factors for GINS1(Fig. S8A). Subsequently, we conducted a comprehensive analysis of these factors by randomly selecting four colon cancer datasets (GSE10950, GSE39582, GSE41258, and GSE44861) to examine their correlation with GINS1(Fig. S9A-D). Interestingly, only SP1 exhibited a significant positive correlation with GINS1 across all four datasets (Fig. S9E-I); thus leading us to identify SP1 as the regulatory factor for GINS1. Furthermore, through examination in the UbiBrowser database, we confirmed OTUB2 as one of SP1's deubiquitinating enzymes (Fig. S8B). Based on these findings, we boldly proposed that OTUB2 could enhance the stability of SP1 protein expression by deubiquitination, consequently promoting GINS1 transcription and governing critical feature such as stemness feature, chemosensitivity, and EMT in colon cancer cells. To validate our hypothesis, we initially conducted Western blotting and qRT-PCR assays. The results of Western blotting assays demonstrated that the downregulation or upregulation of OTUB2 led to corresponding decreases or increases in the protein expression levels of SP1 and GINS1 (Fig. S7B). The results of qRT-PCR assays revealed that the mRNA expression level of GINS1 was also downregulated or upregulated upon OTUB2 knockdown or overexpression, while the mRNA level of SP1 remained unchanged (Fig. S7C), thereby confirming the involvement of OTUB2 in post-transcriptional regulation of SP1. Hence, the combined results of Western blotting and qRT-PCR aasys provided preliminary validation for our hypothesis. Subsequently, we employed the qRT-PCR experiments to verify co-transfection efficiency between OTUB2 and SP1 knockdown or overexpression (Fig. S7D), followed by a series of functional recovery experiments. Spheroid formation and Western blotting assays confirmed that the stemness feature of colon cancer cells were attenuated by OTUB2 knockdown but could be restored through SP1 overexpression, and vice versa (Fig. S7E, F). Cell viability assays, EdU cell proliferation assays, and flow cytometry apoptosis assays demonstrated that sensitivity to oxaliplatin in colon cancer cells decreased due to OTUB2 knockdown but could be regained via SP1 overexpression, and vice versa (Fig. S7G-I). Additionally, Western blotting assays along with Transwell migration assays and wound healing assays verified that EMT process as well as migration ability in colon cancer cells were suppressed by OTUB2 knockdown but could be rescued through SP1 overexpression, and vice versa (Fig. S8C-E). In summary, these experiments provided evidence supporting the role of OTUB2 in regulating stemness feature, chemosensitivity, and EMT in colon cancer by stabilizing the protein expression level of SP1.

OTUB2 deubiquitinated SP1 protein by removing K48 ubiquitin chain

Although the aforementioned experiments have demonstrated that OTUB2 is capable of regulating stemness feature, chemosensitivity, and EMT in colon cancer by stabilizing the protein expression level of SP1, it remains unknown whether OTUB2 achieves this stabilization through deubiquitination. Therefore, we aimed to verify this hypothesis in this section. We confirmed through mass spectrum analysis that OTUB2 was indeed one of the target proteins of SP1 in HEK293T cell (Fig. 5A). Confocal microscopy results also revealed the binding between OTUB2 and SP1 in colon cancer cells (Fig. 5B). Additionally, the results from endogenous and exogenous co-immunoprecipitation (CO-IP) experiments further supported the interaction between OTUB2 protein and SP1 protein (Fig. 5C, D). Hence, the above results collectively provided compelling evidence that OTUB2 served as the specific target protein of SP1.Subsequently, we also performed Western blotting assays to reveal a significant positive correlation between the protein expression levels of OTUB2 and SP1 in colon cancer patients ($p<0.0001$, $R=0.7728$) (Fig. 5E, F), while qRT-PCR experiments showed no significant correlation was observed between their mRNA expression levels ($p=0.45$, R = -0.07) (Fig. 5G). Moreover, as the concentration of the OTUB2 overexpression plasmid increased, both the protein expression level of OTUB2 and SP1 also increased accordingly (Fig. 5H). Collectively, these findings provided initial evidence supporting that OTUB2 was a target protein regulated by SP1 and involved in its post-transcriptional modification processes. Next, the subsequent experiments were conducted to explore whether the post-transcriptional modification of SP1 by OTUB2 involves deubiquitination. Firstly, we conducted the endogenous CO-IP experiments to demonstrate the interaction between SP1 protein and Ub protein in colon cancer cells (Fig. 5I). Additionally, treatment with the proteasome inhibitor (MG132) also led to the accumulation of SP1 protein in colon cancer cells (Fig. 5J). Hence, these findings collectively supported the involvement of the ubiquitin-proteasome system in mediating the degradation of SP1 protein in colon cancer. Subsequently, we added the MG132 to both the sh-OTUB2#1 group and the OTUB2 group of colon cancer cells. The results demonstrated the addition of MG132 abolished



Names	Description	Coverage [%]
SP1	Transcription factor Sp1 [Homo sapiens]	59.23546
TRIM11	E3 ubiquitin-protein ligase TRIM11 [Homo sapiens]	45.23569
NUMA1	Nuclear mitotic apparatus protein 1 [Homo sapiens]	32.45862
HBA1	Hemoglobin subunit alpha [Homo sapiens]	28.34712
SSRP1	FACT complex subunit SSRP1 [Homo sapiens]	27.75861
MACROH2A1	Core histone macro-H2A.1 [Homo sapiens]	21.25474
LMNA	Prelamin-A/C OS=Homo sapiens [Homo sapiens]	20.84675
RAN	GTP-binding nuclear protein Ran [Homo sapiens]	20.14587

Fig. 5 OTUB2 bound to SP1 protein and deubiquitinated it. **A** The LC–MS/MS results of OTUB2 binging proteins. **B** The colocalization between OTUB2 and SP1 visualized by confocal laser scanning microscope. **C** The endogenous CO-IP assay revealed the interaction between OTUB2 and SP1 in colon cancer cells. **D** The exogenous endogenous CO-IP assay showed the interaction between OTUB2 and SP1 in colon cancer cells. **E** The representative western blotting results showed the protein expression of OTUB2 in our collected colon cancer samples. **F** The correlation between the protein expression of OTUB2 and SP1 in our collected colon cancer samples (Spearman correlation analysis). **G** The correlation between the mRNA expression of OTUB2 and SP1 in our collected colon cancer samples (Spearman correlation analysis). **H** The alterations in SP1 protein expression were examined in colon cancer cells following transfection with OTUB2 overexpression plasmids at varying concentrations. **I** The endogenous CO-IP assay revealed the interaction between SP1 and Ub in colon cancer cells. **J** With proteasome inhibitor (MG132, 15μM) added to colon cancer cells, western blotting was employed to assess the protein expression of SP1 at different time points (0 h, 3 h, 6 h, and 9 h). **K** With MG132 added to colon cancer cells transfected with OTUB2 knockdown or overexpression lentivirus, western blotting was performed to assess the alteration of protein expression of SP1. **L** With protein synthesis inhibitor (CHX, 15μM) added to colon cancer cells transfected with empty or OTUB2 overexpression lentivirus, western blotting was utilized to test the protein expression of SP1 at different time points (0 h, 2 h, 4 h, and 6 h). **M** Endogenous ubiquitination experiment revealed the alteration of ubiquitination level of SP1 in colon cancer cells transfected with OTUB2 knockdown or overexpression lentivirus. **N** Exogenous ubiquitination experiment showed the alteration of ubiquitination level of SP1 in colon cancer cells transfected with OTUB2 knockdown or overexpression lentivirus. All experiments were repeated three times and the data were represented as mean ± SD (*$p < 0.05$, ** $p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$)

the regulatory effects of either downregulating or upregulating OTUB2 on the protein expression level of SP1 (Fig. 5K). Additionally, cycloheximide (CHX) was added to the Vector-OTUB2 group and the OTUB2 group to impede protein synthesis in colon cancer cells. The results revealed that the half-life of the SP1 protein was significantly prolonged in the OTUB2 group compared to the Vector-OTUB2 group (Fig. 5L). Hence, these results collectively demonstrated that OTUB2 inhibited the ubiquitin-proteasome system-mediated degradation process of SP1 protein, thereby contributing to the stabilization of SP1 protein expression levels. Finally, to further validate the specific role of OTUB2 in the degradation process of SP1 protein, we performed endogenous and exogenous ubiquitination experiments in colon cancer cells. The results of endogenous ubiquitination experiments demonstrated that knockdown or overexpression of OTUB2 led to an upregulation or downregulation in the ubiquitination level of SP1 protein (Fig. 5M). Consistently, the findings from the exogenous ubiquitination experiments aligned with those obtained from the endogenous study (Fig. 5N). Hence, these ubiquitination experiments substantiated that OTUB2 can impede SP1 protein ubiquitination via deubiquitination to maintain a stable expression level. In conclusion, all the aforementioned results collectively proved that OTUB2 exerted an inhibitory effect on the ubiquitination process of SP1 through its deubiquitinating action, thereby leading to the stabilization of SP1 protein expression levels.

The current evidence adequately substantiated our hypothesis that OTUB2 could stabilize the protein expression level of SP1 through its deubiquitinating action. However, the ubiquitin chain system in the proteasome degradation pathway primarily consists of seven major types of ubiquitin chains: K6, K11, K27, K29, K33, K48, and K63 [49]. The specific ubiquitin chains that OTUB2 could inhibit to exert its deubiquitinating effect on the SP1 protein remain unknown. Therefore, we proceeded to conduct a series of experiments to resolve this mystery. We transfected HEK293T cells with different types of ubiquitin mutants (K6R, K11R, K27R, K29R, K33R, K48R, and K63R) and overexpressed OTUB2 in these cells to assess the exogenous ubiquitination levels of SP1 protein by ubiquitination assays. The results revealed that the overexpression of OTUB2 attenuated the ubiquitination level of SP1 protein in K6R, K11R, K27R, K29R, K33R, and K63R groups, however, no significant alteration of ubiquitination level was observed in K48R group (Fig. 6A). Hence, the preliminary results demonstrated that OTUB2 inhibited the ubiquitination process of SP1 protein mediated by the K48 ubiquitin chain. Subsequently, to further substantiate this finding, we transfected HEK293T cells with the K48-only Ub plasmid (K48) and manipulated OTUB2 expression through knockdown or overexpression in order to evaluate the exogenous ubiquitination level of SP1 protein using ubiquitination assays. The results indicated that the ubiquitination level of SP1 protein increased or decreased accordingly with the downregulation or overexpression of OTUB2 in the K48 group (Fig. 6B). Therefore, the results verified that OTUB2 inhibited the ubiquitination process of SP1 protein mediated by the K48 ubiquitin chain again.

In conclusion, the results presented above collectively provided compelling evidence that OTUB2 exerted regulatory control over the deubiquitination process of SP1 by suppressing K48 ubiquitin-mediated ubiquitination of SP1.

SP1 regulated stemness feature, chemosensitivity, and EMT of colon cancer cells by enhancing transcriptional activity of GINS1

The transcription factor role of SP1 for GINS1 has been previously confirmed based on data from the UCSC and PROMO databases (Fig. S8A). However, further validation through in vitro experiments was not conducted. Hence, in this section, we aimed to prove that the SP1 protein is one transcription factor of GINS1 and investigate its specific binding regions for transcription in vitro. Initially, by analyzing data from the JASPAR database, we identified potential binding regions of SP1 on the GINS1 promoter and obtained five putative binding sites (Fig. 6C, D). The five putative binding sites were named Region 1, Region 2, Region 3, Region 4, and Region 5, respectively. Noteworthily, three binding sites (Region 3, Region 4, and Region 5) on the GINS1 promoter have been consolidated into a unified region termed as Region 3/4/5 due to their close proximity. Subsequently, we conducted a series of assays in vitro. The initial step of our investigation involved conducting the agarose gel electrophoresis experiments, which yielded results indicating that SP1 exhibited a chromatin-level binding affinity towards Region 2 (1822–1830) of GINS1 promoter (Fig. 6E). Subsequently, ChIP-qPCR and dual luciferase reporter assays were conducted, which further validated that SP1 indeed bound to Region 2 (1822–1830) (Fig. 6F, G). In summary, these findings presented herein provided compelling evidence that SP1 functioned as a transcription factor for GINS1, exerting regulatory control over GINS1 transcription through specific binding to the 1822–1830 region of the GINS1 promoter.

Additionally, we also conducted rescue experiments to demonstrate that the knockdown or overexpression of GINS1 can restore the stemness feature, chemosensitivity, and EMT induced by SP1 overexpression or knockdown. The results depicted in Fig. S10A validate the efficacy of co-transfection involving SP1 knockdown or overexpression and GINS1 overexpression or

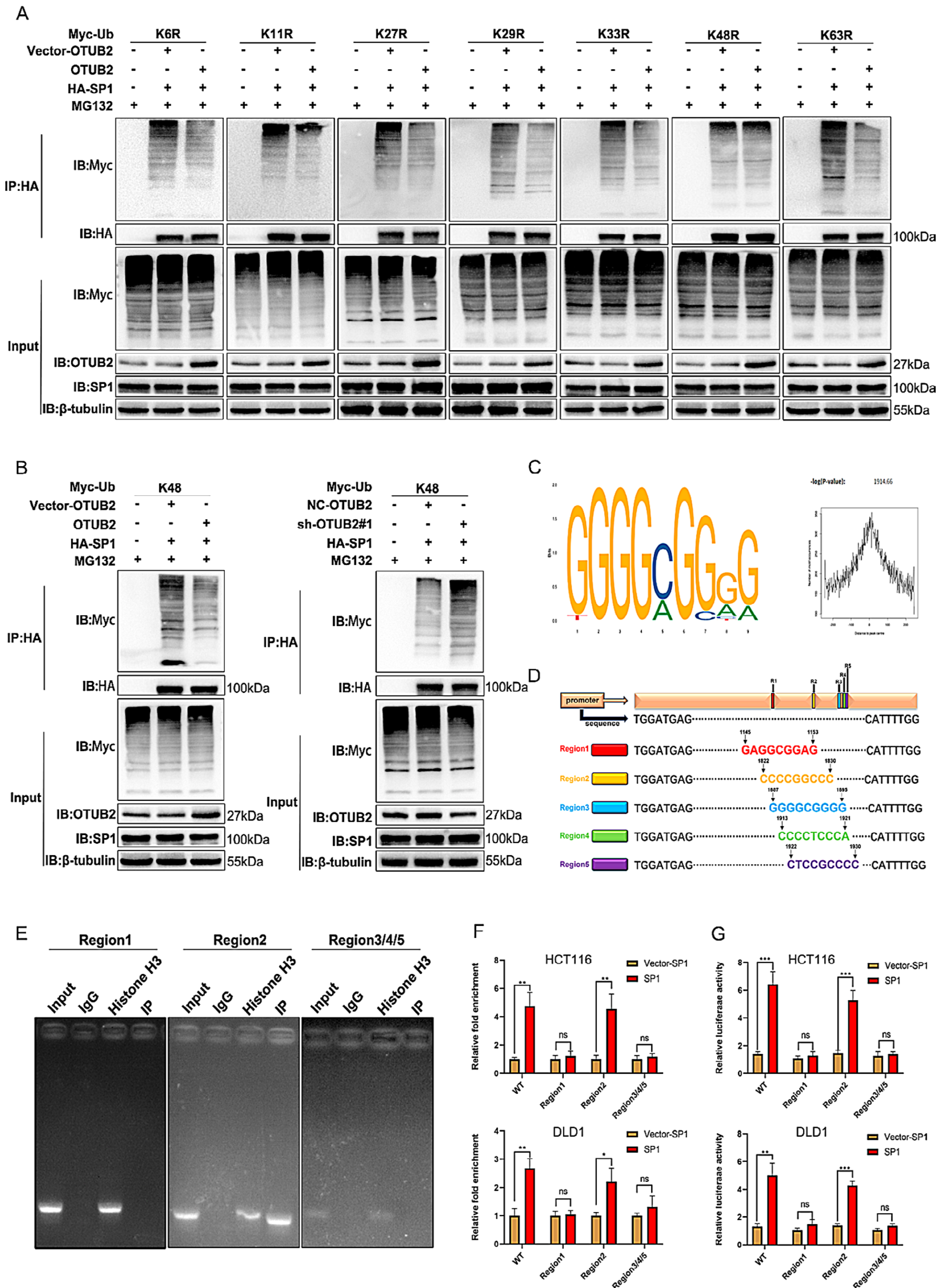


Fig. 6 The K48 ubiquitination of SP1 protein was abrogated by OTUB2, and SP1 interacted with the region 1822–1830 of GINS1 promoter to augment the transcriptional activity of GINS1. **A** The exogenous ubiquitination experiments revealed the alteration of ubiquitination level of SP1 protein in HEK293T cells transfected with OTUB2 overexpression lentivirus and Ub mutants (K6R, K11R, K27R, K29R, K33R, K48R, or K63R). **B** The exogenous ubiquitination experiments revealed the alteration of ubiquitination level of SP1 protein in HEK293T cells transfected with OTUB2 overexpression or knockdown lentivirus and K48-only Ub plasmid (K48). **C** The predicted motif of SP1 from JASPAR database. **D** The potential five binding regions of SP1 on the GINS1 promoter predicted by JASPAR database. **E** The result of agarose gel electrophoresis experiment revealed SP1 exhibited a chromatin-level binding affinity towards region 2 of GINS1 promoter in HEK293T cell. **F** The result of ChIP-qPCR assay showed a chromatin-level binding between SP1 and region 2 of GINS1 promoter in colon cancer cells (One-way ANOVA test). **G** The result of dual luciferase reporter assay further revealed the binding between SP1 and region 2 of GINS1 promoter in colon cancer cells (One-way ANOVA test). All experiments were repeated three times and the data were represented as mean ± SD (**p* < 0.05, ** *p* < 0.01, ****p* < 0.001, *****p* < 0.0001)

knockdown. Spheroid formation experiments and Western blotting assays confirmed that SP1 knockdown weakened the stemness level of colon cancer cells, which could be restored by GINS1 overexpression, and vice versa (Fig. S10B, C). Cell viability assay experiments, EdU cell proliferation experiments, and flow cytometry apoptosis experiments demonstrated that SP1 knockdown reduced the sensitivity of colon cancer cells to oxaliplatin, which could be restored by GINS1 overexpression, and vice versa (Fig. S10D-F). Western blotting assays along with Transwell migration experiments and wound healing assays proved that SP1 knockdown decreased the EMT and migration ability of colon cancer cells, which could be restored by GINS1 overexpression, and vice versa (Fig. S11A-C). In summary, these results collectively demonstrated that SP1 regulated the transcriptional activity of GINS1 to influence the stemness feature, chemosensitivity, and EMT in colon cancer cells.

In conclusion, our study indicated that the deubiquitinase OTUB2 served as the target protein of SP1 protein and exerted inhibitory effects on K48 ubiquitination of SP1 protein, thereby upregulating the protein expression level of SP1. As a transcription factor for GINS1, SP1 enhanced the transcriptional activity of GINS1 by specifically binding to its promoter region at 1822–1830, ultimately elevating colon cancer stemness feature, reducing chemotherapy sensitivity in colon cancer, and promoting EMT process in colon cancer cells. Figure 7 presented a schematic representation illustrating this specific mechanism, which was drawn by Figdraw.

Discussion

Colorectal cancer is a prevalent malignant tumor in the digestive tract, posing a significant threat to human health [50]. In recent years, there has been a continuous increase in its mortality rate [51]. This can primarily be attributed to tumor recurrence and metastasis, which are predominantly caused by chemotherapy resistance [3]. The development of chemotherapy resistance is closely associated with the stemness feature of tumors [52, 53]. One notable feature of tumor cells exhibiting high resistance to chemotherapy is their heightened EMT characteristics [54, 55]. And the EMT process frequently contributes to the development of tumor metastasis [56, 57]. Therefore, investigating the molecular mechanisms underlying the stemness feature, chemosensitivity, and EMT in colon cancer that contribute to metastasis can offer novel insights for overcoming chemoresistance and achieving precision therapy in clinical practice.

Proteins are the ultimate product of gene expression and serve as the primary executors of vital biological processes. Alterations in their expression levels are closely linked to tumorigenesis and progression [58]. Ubiquitination modification is a prevalent form of post-translational protein modification [59] that plays a crucial role in various aspects of tumor development, including transcriptional regulation, DNA damage repair, cell

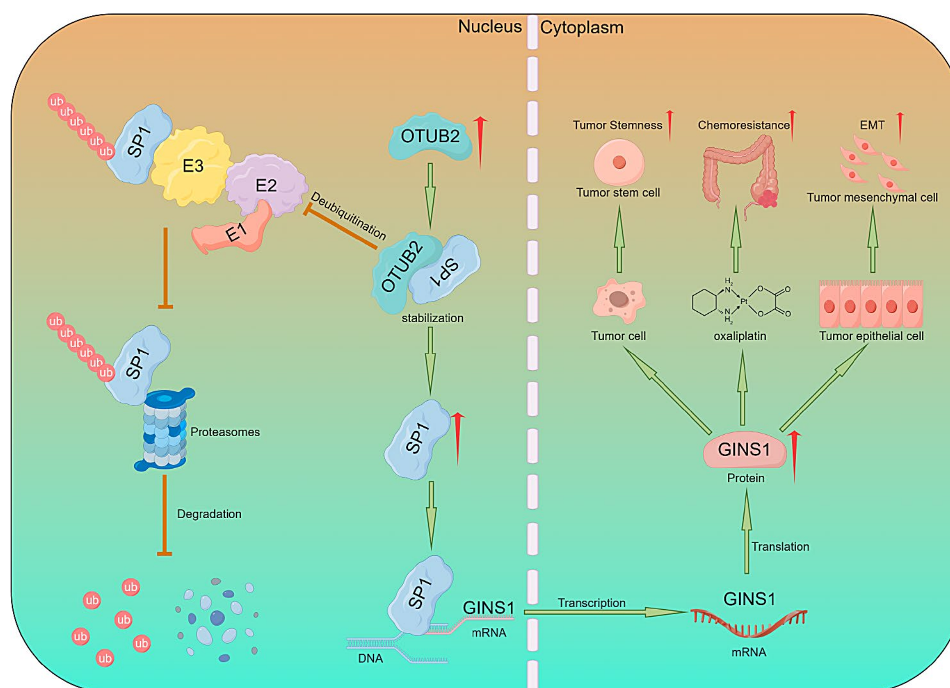


Fig. 7 The schematic diagram illustrating the regulatory mechanism through which OTUB2 modulated tumor stemness, chemosenstivity, and EMT in colon cancer

cycle control, apoptosis induction, vesicle transport, and other fundamental cellular processes [60]. In contrast to ubiquitination, deubiquitination refers to the removal of ubiquitin molecules from proteins. Deubiquitinating enzymes (DUBs) catalyze this process by hydrolyzing the isopeptide bond between ubiquitin and substrate proteins specifically removing ubiquitin molecules from them and reversing the effects of ubiquitination [61, 62]. The dynamic nature of protein ubiquitination/deubiquitination balance forms the basis for eukaryotic protein stability and function [63]. However, most studies have focused on investigating how ubiquitination contributes to tumorigenesis and progression in recent years [64–67], with less attention paid to deubiquitination. Therefore, our team aimed at exploring how deubiquitinases contribute to colon cancer development.

OTUB2, a deubiquitinase belonging to the OTU family, has been increasingly studied for its impact on malignant tumor progression. Zhang et al. [12] demonstrated that OTUB2 promoted tumor metastasis by independently activating YAP and TAZ. Ren et al. [27]. showed that drugs targeting OTUB2 could degrade PD-L1 and enhance tumor sensitivity to cytotoxic T cells. Ouyang et al. [28] found that OTUB2 promoted gastric cancer cell proliferation by stabilizing KRT80 expression through deubiquitination. Li et al. [13] revealed that in non-small cell lung cancer, OTUB2 stabilized U2AF2 expression through deubiquitination, promoting Wnt/β-catenin signaling pathway activation and tumorigenesis. Despite these findings linking OTUB2 to various malignancies, its relationships with colon cancer stemness feature, chemosensitivity, and EMT remains unknown; thus, we aimed to investigate its relationships in our current study. Our data demonstrated that OTUB2 exhibited significantly higher expression levels in colon cancer cells and tissues compared to normal colon epithelial cell or adjacent tissues. The upregulation of OTUB2 was inversely associated with the prognosis of colon cancer patients. Through functional experiments, we discovered that OTUB2 played crucial roles in regulating the stemness feature, chemosensitivity, and EMT of colon cancer both in vitro and in vivo. Further investigations revealed that OTUB2 mediated the malignant progression of colon cancer by regulating GINS1. While GINS1 primarily participates in early embryonic development under normal physiological conditions [68], it also contributes to the regulation of stemness feature, chemosensitivity, and EMT in various malignant tumors [20, 47, 69]. However, its involvement has not been explored specifically in colon cancer. Therefore, for the first time, we investigated the relationships between GINS1 and the stemness feature as well as chemosensitivity and EMT processes in colon cancer.

Next, we aimed to investigate whether OTUB2 stabilized the protein expression of GINS1 by deubiquitinating it and subsequently regulated the stemness feature, chemosensitivity, and EMT of colon cancer. Unfortunately, our ubiquitination experiments revealed that OTUB2 did not regulate the ubiquitination of GINS1. Therefore, we postulated that there might be other key molecules involved in the interaction between OTUB2 and GINS1. Through database prediction analysis, we discovered that SP1 acted as a transcription factor for GINS1 while OTUB2 functioned as a deubiquitinase for SP1. Consequently, we hypothesized that OTUB2 enhanced the stability of SP1 protein expression by deubiquitinating it, thereby promoting the transcriptional activity of GINS1. To validate our hypothesis, we performed mass spectrometry analysis, immunofluorescence co-localization experiments, CO-IP, and ubiquitination assays. The experimental results demonstrated that deubiquitinase OTUB2 was the specific target protein of SP1 in colon cancer. OTUB2 interacted with SP1 to suppress its K48 ubiquitination, thereby upregulating its protein expression level. Subsequently, we confirmed the binding of SP1 to the 1822–1830 region of the GINS1 promoter through agarose gel electrophoresis, chip-qPCR, and dual luciferase gene reporter assays, thereby augmenting the transcriptional activity of GINS1. Finally, through the integration of results from a series of functional restoration experiments, we successfully demonstrated that OTUB2 exerted its influence on SP1 protein expression levels by means of a deubiquitination process, thereby resulting in an upregulation of GINS1 transcriptional activity. This upregulation of the GINS1 gene could further enhance colon cancer's stemness feature, diminish its susceptibility to oxaliplatin, facilitate the EMT process, and ultimately promote tumor metastasis.

Although our study has demonstrated that OTUB2 can stabilize the protein expression level of SP1 through deubiquitination and promote the transcriptional activity of GINS1 to regulate the stemness feature, chemosensitivity, and EMT of colon cancer, there are still several limitations. First, although we have shown that SP1 is a target protein of OTUB2, we have not further identified the specific structural domain or binding site where OTUB2 interacts with SP1. Second, while we have demonstrated that OTUB2 can inhibit K48 ubiquitination-mediated degradation of SP1 and upregulate its expression, it remains unknown whether OTUB2 competitively binds to the ubiquitin or E3 ubiquitin ligase binding site on SP1. Third, although we have validated that OTUB2 can impact stemness feature and chemosensitivity in colon cancer cells, we have not confirmed its effects on stemness feature and chemosensitivity in tumor stem cells or resistant cell lines. Naturally, our team will continue conducting further research to address these limitations in future studies.

In conclusion, our study suggested that targeting OTUB2 could be a promising strategy for drug therapy in colon cancer. By suppressing the expression of OTUB2, we could promote the ubiquitination of SP1 protein and subsequently decrease the expression level of GINS1. Ultimately, this approach might enhance the sensitivity of colon cancer to oxaliplatin, attenuate its stemness feature, and reduce the likelihood of tumor metastasis.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12964-024-01789-2.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7
Supplementary Material 8
Supplementary Material 9
Supplementary Material 10
Supplementary Material 11
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Author contributions
Wenjie zhu: Conceptualization, investigation, formal analysis, and writing editing; Changlei Wu: Investigation and editing; Zitao Liu: Investigation; Shimin Zhao: Investigation; Jun Huang: Conceptualization, resources, supervision, and funding acquisition.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was performed in accordance with the Declaration of Helsinki. The inclusion of human participants, and the use of human data and human tissue in this study were approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. The use of animals in this study was approved by the Institutional Animal Care and Use Committee of Nanchang Royo Biotech Co,. Ltd.

Consent for publication
All the authors have declared approval of publication.

Competing interests
The authors declare no competing interests.

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