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Original Article

USP11 facilitates colorectal cancer proliferation and metastasis by regulating IGF2BP3 stability

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Abstract: The abnormal expression of ubiquitin-specific protease 11 (USP11) is thought to be related to tumor development and progression; however, few studies have reported the biological function and clinical importance of USP11 in colorectal cancer (CRC). Therefore, it is necessary to further explore the role of USP11 in CRC. Immunohistochemical staining was used to explore the association between prognosis and USP11 expression in CRC. Cholecystokinin octapeptide (CCK-8), colony formation, transwell, and animal assays were used to study the abilities of proliferation, migration, and invasion in CRC cells. Co-immunoprecipitation assays, Western blotting, ubiquitination assays, and rescue experiments were performed to elucidate the interaction between USP11 and insulin-like growth factor 2 mRNA binding protein 3 (IGF2BP3). We verified that USP11 was overexpressed in CRC tissues and was associated with the depth of tumor invasion and metastasis. USP11 knockdown or overexpression could weaken or reinforce the abilities of proliferation, migration, and invasion in CRC cells *in vivo* or *in vitro*. IGF2BP3 was protected by USP11 from degradation via deubiquitination. The rescue experiments revealed that IGF2BP3 overexpression could effectively reverse the decrease in cell proliferation, migration, and invasion caused by USP11 knockdown. Therefore, USP11 might be involved in CRC tumorigenesis and development through a USP11-IGF2BP3 axis pathway, and USP11 overexpression might be a novel indicator for poor prognosis and a potential therapeutic target in CRC patients.

Keywords: Colorectal cancer, IGF2BP3, metastasis, proliferation, USP11

Introduction

In 2020, an estimated 1.81 million new cancer cases occurred and 0.61 million people died from cancer in the United States [1]. Among all cancers, the incidence and mortality of colorectal cancer (CRC) accounted for 5.8% and 8.8%, respectively [1]. In recent years, the improvement in surgical techniques and the emergence of a variety of targeted therapeutic drugs and biological agents have improved the outcomes of patients with CRC; however, the prognosis and 5-year survival rate remain unsatisfactory [2, 3].

In eukaryotic cells, the ubiquitin-proteasome system is one of the major pathways for cellular protein degradation [4]. Proteins are labeled with ubiquitin through a series of ubiquitination enzyme cascade reactions, and subsequently, ubiquitinated proteins are transported to the 26S proteasome for degradation [5]. Protein ubiquitination is a post-translational modification that can not only change the stability of intracellular proteins and regulate protein localization and activity but can also affect the function of target proteins [6]. Previous studies have reported that polyubiquitination protein modification is a reversible and dynamic process [7]. Deubiquitinating enzymes (DUBs) protect target proteins from proteasome by hydrolyzing the thioester bonds between polyubiquitination chains, or between ubiquitin and its substrates, to remove the ubiquitin. USP11 is a cysteine

protease belonging to the ubiquitin-specific proteases (USPs) family-the most important family of a variety of deubiquitinating enzymes discovered to date [8-10].

USP11 participates in multiple cell metabolism processes [11], including DNA damage repair functions [12], signaling pathway regulation [12], and cell-cycle progression. Studies have reported that USP11 can interact with the breast cancer 2 tumor suppressor (BRCA2) gene to repair DNA double-strand breaks and protect cancer cells [13, 14]. USP11 was found to serve as an upstream regulator of the IkappaB kinase alpha (IKKalpha-p53) signaling pathway [15]. Moreover, it can regulate the Hippo signaling pathway by improving the stability of vestigial-like protein 4 (VGLL4) using deubiquitination [16]. In addition, phosphatase and tensin homolog (PTEN) can maintain its stability through the PTEN-PI3K-FOXO-USP11 signaling pathway [17].

USP11 plays an important role in the occurrence and development of tumors as a prooncogenic factor. Research shows that mitoxantrone, an inhibitor of USP11, can effectively inhibit the survival of pancreatic cancer cells [13]. Pathological studies have revealed that USP11 is highly expressed in breast cancer, and it can promote the metastasis of cancer cells through transforming growth factor-beta and snail protein regulation [18, 19]. Moreover, the proliferation of cervical cancer cells was inhibited by USP11 knockdown [20]. Hongze Sun and colleagues also reported that USP11 enhances the proliferation and metastatic potential of tumor cells through the ERK/MAPK signaling pathway [21]. Furthermore, USP11 is highly expressed in hepatocellular carcinoma [7], and melanoma [22], and it is closely associated with poor survival outcomes. Considering that only a few studies have reported the biological function and clinical value of USP11 in CRC, it is necessary to further explore.

In the current study, we confirmed that USP11 was overexpressed in CRC tissues; moreover, its expression level in cancer tissues was associated with tumor invasion depth, lymph node metastasis, and distant metastasis. Finally, we verified that USP11 promoted the proliferation, migration, and invasion of CRC cells by modulating IGF2BP3 stability. Therefore, USP11 might be involved in CRC tumorigenesis through a USP11-IGF2BP3 axis pathway, and USP11 overexpression might be a novel indicator for poor prognosis in CRC patients.

Materials and methods

Clinical sample collection

A total of 138 patients with primary CRC who underwent radical surgery for CRC at The Second Affiliated Hospital of Fujian Medical University between April 2010 and June 2014 were selected, and paraffin-embedded specimens of CRC tissue and para-cancerous tissue were obtained from the pathology department. The patients had not received antineoplastic therapy before surgical resection. Complete follow-up information of the 138 patients was obtained via telephonic consultations. The data of the patients are summarized in **Table 1**. The study was approved by the Ethics Committee of The Second Affiliated Hospital of Fujian Medical University (No. 2017-32). Informed consent was obtained from all patients, and the study complied strictly with the principles outlined in the Declaration of Helsinki.

Immunohistochemical (IHC) staining

For immunohistochemical (IHC) staining, the paraffin-embedded CRC tissue and para-cancerous tissue specimens were processed into 4-μm sections. Tissue sections were soaked in xylene for deparaffinization and rehydration through a series of descending concentrations of ethanol, followed by an antigen retrieval step using a high-pressure method. Endogenous peroxidase was blocked using an endogenous peroxidase blocking solution for 10 min. Then, the sections were incubated with USP11 monoclonal antibody (Proteintech, Wuhan, China) for 3 h at room temperature. Subsequently, the sections were flushed five times with phosphate-buffered saline (PBS) and then incubated with biotinylated secondary antibody (Maixin-Bio, Fuzhou, China). Finally, diaminobenzidine was used as a chromogen to reveal antigen-antibody reactions, followed by counterstaining with hematoxylin at room temperature for 40 s.

Cell lines and cell culture

Human CRC cell lines (HT-29, DLD-1, and HCT-15) and 293T cells (ATCC, Manassas, VA, USA) were cultured in DMEM or RPMI1640 medium

Table 1. Relationship between USP11 expression and the clinicopathological characteristics of CRC

Factors	Cases	USP11 expression		χ^2	*P*
		High (n=75)	Low (n=63)		
Age (year)					
< 60	71	41 (57.7%)	30 (42.3%)	0.681	0.409
≥60	67	34 (50.7%)	33 (49.3%)		
Gender					
Male	57	29 (50.9%)	28 (49.1%)	0.471	0.492
Female	81	46 (56.8%)	35 (43.2%)		
Tumor invasion					
T1+T2	30	11 (36.7%)	19 (63.3%)	4.830	0.028*
T3+T4	108	64 (59.3%)	44 (40.7%)		
Lymph node metastasis					
No	62	26 (41.9%)	36 (58.1%)	6.991	0.008*
Yes	76	49(64.5%)	27 (35.5%)		
Distant metastasis					
No	106	52 (49.1%)	54 (50.9%)	5.158	0.023*
Yes	32	23(71.9%)	9 (28.1%)		
Clinical stage					
I-II	52	23 (44.2%)	29 (55.8%)	3.442	0.064
III-IV	86	52 (60.5%)	34 (39.5%)		
Differentiation					
Poorly	10	4 (40%)	6 (60%)	0.380	0.538
Moderately, Well	128	71 (55.5%)	57 (44.5%)		
Serum CEA level					
< 5 ng/mL	71	37 (52.1%)	34 (47.9%)	0.294	0.587
≥5 ng/mL	67	38(56.7%)	29 (43.3%)		

CRC: Colorectal cancer; CEA: Carcino-embryonic antigen. *Statistically significant, $P < 0.05$.

supplemented with 10% FBS (Bovogen, Uruguay). Cells were incubated at 37°C in a humidified atmosphere containing 5% CO_2. We used Myco-Lumi™ Luminescent Mycoplasma Detection Kit (Beyotime, Shanghai, China) to detect mycoplasma.

Plasmids, small interfering RNA treatment, and gene overexpression

For target gene knockdown, the control and two shRNA sequences against USP11 were inserted into the pLv-puro interference plasmid. Next, using TurboFect transfection reagent, the plasmids carrying the knockdown gene and the virus packaging plasmids were transfected into 293T cells. The cell culture supernatant containing the lentivirus was collected at 48 h and 72 h. Then, CRC cells were infected with the supernatant. Infected cells were cultured in puromycin for selection after infecting twice. The target sequences of USP11 were as follows: Sh1: F: 5'-3'CCGGTGCCGTGACTACAACAACTCCTACTCGAGTAGGAGTTGTTGTAGTCACGGTTTTTG R: 5'-3'AATTCAAAAACCGTGACTACAACAACTCCTACTCGAGTAGGAGTTGTTGTAGTCACGGCA; Sh2: F: 5'-3'CCGGTGCCGTGATGATATCTTCGTCTACTCGAGTAGACGAAGATATCATCACGGTTTTTG R: 5'-3' AATTCAAAAACCGTGATGATATCTTCGTCTACTCGAGTAGACGAAGATATCATCACGGA. For USP11 and IGF2BP3 overexpression, we transfected the plasmids (GENE, Shanghai, China) carrying the overexpressed sequence into cells using TurboFect transfection reagent.

Quantitative reverse transcription PCR

Total RNA was isolated from cell lines using TRIzol reagent following the manufacturer's instructions. cDNA was synthesized using the SuperRT cDNA synthesis kit. RT-qPCR was performed using the UltraSYBR One-Step RT-qPCR Kit and the ABI Prism 7000 System. The primer sequences were as follows: GAPDH, F: 5'-3' CTTTGGTATCGTGGAAGGACTC, R: 5'-3' AGTAGAGGCAGGGATGATGT; USP11, F: 5'-3'CGTTTCCGGGACCAGAATCC, R: 5'-3'CATCGCCGTCCGTTCTCTTC; IGF2BP3, F: 5'-3' CCATAGAAGTTGAGCACTCGGTCC, R: 5'-3' TCTCCACCACTCCATACTGGACTAG.

Western blotting analysis

Cells were lysed in RIPA lysis buffer containing a protease and phosphatase inhibitor cocktail for 30 min. Thirty micrograms of protein were separated using 10% SDS-PAGE and then transferred onto polyvinylidene difluoride membranes. After blocking with Beyotime blocking buffer at room temperature for 15 min, the membranes were incubated with the primary

antibodies (Proteintech, Wuhan, China) at 4°C overnight and then incubated with the appropriate secondary antibodies at room temperature for 1 h. The protein bands were visualized using the BeyoECL Moon detection system.

Cell proliferation assay

Approximately 2 × 10^3 cells/well for DLD-1, HT-29, and HCT-15 were seeded in 96-well plates with five repeats. After culturing for the appropriate time, the CCK-8 solution was injected into each well. After 1 h of incubation, the OD values of all the wells were measured at a wavelength of 450 nm.

Colony formation

Approximately 2000 cells were seeded in six-well plates with three repeats. The cells were cultured for some days, and some of them showed the growth of macroscopic cell clusters. Thereafter, cells were fixed with 4% paraformaldehyde for 20 min, stained with crystal violet for 20 min, and washed three times with PBS.

Migration and invasion assays

Invasion and migration of cells were assessed using transwell chambers with 8-μm pore filter inserts with or without Matrigel. In the cell migration assay, 5 × 10^4 cells with 200 μL serum-free medium were added to the upper chamber, and 500 μL cell culture medium with 10% fetal calf serum was added to the bottom chamber. After incubation for some time, the passed cells were fixed with 4% paraformaldehyde for 60 min and stained with crystal violet for 10 min. Finally, photographs were taken, and the numbers of cells adhered to the lower side of the filter were counted. In the cell invasion assay, 10 × 10^4 cancer cells were seeded in the top chamber and incubation was continued for a longer period.

Co-immunoprecipitation (co-IP) assay

To understand protein-protein interactions, total protein was extracted and incubated with magnetic beads and antibodies (anti-USP11 and anti-IGF2BP3) at 4°C overnight. After fixing the magnetic beads with magnets, the supernatant was removed, and the beads were washed three times with PBS. SDS-PAGE loading buffer was added to the Eppendorf tube and boiled at 100°C for 10 min. After fixing the magnetic beads with magnets again, the supernatant was collected for the Western blot assay.

Ubiquitination assay

MG132, as a proteasome inhibitor, can effectively reduce ubiquitin-conjugate protein degradation by the 26S proteasome complex. Cells were treated with MG132 (10 μg/mL) for 12 h and then collected and lysed with RIPA buffer for the immunoprecipitation assay to quantitate the ubiquitin molecules binding to the target proteins.

Cycloheximide assay

To explore the half-life of the proteins, cells were treated with cycloheximide (CHX) (10 μM) for 0, 3, 6, 12, and 24 h; then, a Western blot assay was performed.

In vivo tumor growth and peritoneum implanting metastasis model

Male BALB/c nude mice were purchased from Shanghai SLAC Laboratory Animal Center. HT-29 cells (5 × 10^6) transfected with shUSP11 or shCtrl plasmids were suspended in 100 μL basal culture medium and were subcutaneously injected into the armpits of the mice (n=8). The tumor volumes were measured and calculated every 3 days using the formula v=0.5 × length × width^2. After 15 days, the mice were euthanized, and the tumors were dissected, weighed, and photographed.

Subsequently, we selected 5 × 10^6 HT-29 cells for the peritoneal implantation metastasis model. The mice were randomly divided into two groups (n=8). Tumor formation and the metastasis of shUSP11 and shCtrl cancer cells were examined following injection into the abdominal cavity of the nude mice. After 4 weeks, all mice were euthanized, and the abdominal neoplasms and metastatic nodules of the liver were removed, counted, and weighed.

In addition, 16 nude mice were randomly allocated into two groups (n=8). Then, shUSP11 and shCtrl HT-29 cancer cells (5 × 10^6) were injected into the abdominal cavity of the nude mice. The survival time of the mice were

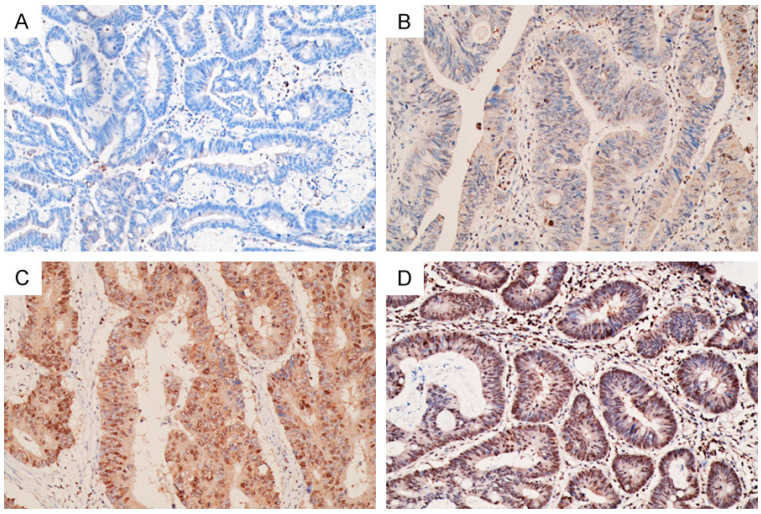

Figure 1. Representative IHC staining of USP11 in CRC tissue. Tissue samples exhibiting USP11 staining based on staining intensity scores (0-4). (A) 0: no expression; (B) 1: weak expression; (C) 2, moderate expression; and (D) 3: strong expression. (IHC, 200 ×).

observed within 60 days after injection. After 60 days of intervention, the surviving mice were euthanized. All the experiments were approved by the ethics committee of The Second Affiliated Hospital of Fujian Medical University (No. 2017-32). All animal experiments complied with the ARRIVE guidelines and were carried out in accordance with the U.K. Animals Act.

Immunostaining evaluation and statistical analysis

IHC evaluation of USP11 expression was independently performed by two experienced pathologists blinded to the clinical data. Positive expression of USP11 located in the nucleus and cytoplasm. IHC staining intensity scores included 0 (no expression), 1 (weak expression), 2 (moderate expression), and 3 (strong expression) (**Figure 1**), and the percentage score of the positively stained area included 0 (no stain), 1 (< 25% stain), 2 (26-50% stain), 3 (51-75% stain), and 4 (76-100% stain). The two scores were multiplied to obtain a total IHC score ranging from 0 to 12. The cases were divided into two groups: low (total score ≤5) and high (total score >5) USP11 expression.

IBM SPSS Statistics version 20.0 software, GraphPad Prism version 6.0 software, and Image J software were used as statistical tools. The Pearson's chi-squared test and Wilcoxon matched-pairs test were used to describe the relationship between USP11 expression and clinicopathological features, as well as the differences in total IHC scores between cancerous and non-cancerous tissues. Student's t-test was used to analyze the differences between groups. Two-tailed *P*-values < 0.05 were considered statistically significant.

Results

USP11 expression status

IHC/hematoxylin-eosin (HE) staining results of 138 pathological specimens were reanalyzed. USP11 expression in non-cancerous tissues was low/zero (**Figure 2F**) compared to high expression in the CRC tissues (**Figure 2E**). The expression level of USP11 was significantly higher in cancerous tissues than in non-cancerous tissues (**Figure 2G**, $P < 0.001$). The percentage of high USP11 expression was higher in CRC tissues than in non-CRC tissues (**Figure 2H**, 54.3% vs. 16.7%, $P < 0.001$).

High expression of USP11 is associated with clinicopathological characteristics of CRC

To further evaluate the clinical implication of USP11 expression in the occurrence and development of CRC, the clinicopathological information of 138 patients was summarized (**Table 1**). The Pearson's chi-squared test was used to analyze the association of USP11 expression with clinical features. The expression of USP11 in CRC tissues was related to the depth of tumor invasion (P=0.028), lymph node metastasis (P=0.008), and distant metastasis (P= 0.023).

USP11 knockdown weakened the abilities of proliferation, migration, and invasion of CRC cells in vitro

The expression of USP11 was significantly reduced in DLD-1 and HT-29 cell lines in terms of protein levels (**Figure 3A**) and RNA levels (**Figure 3B**); therefore, USP11 knockdown models (shCtrl, sh1, and sh2) were established.

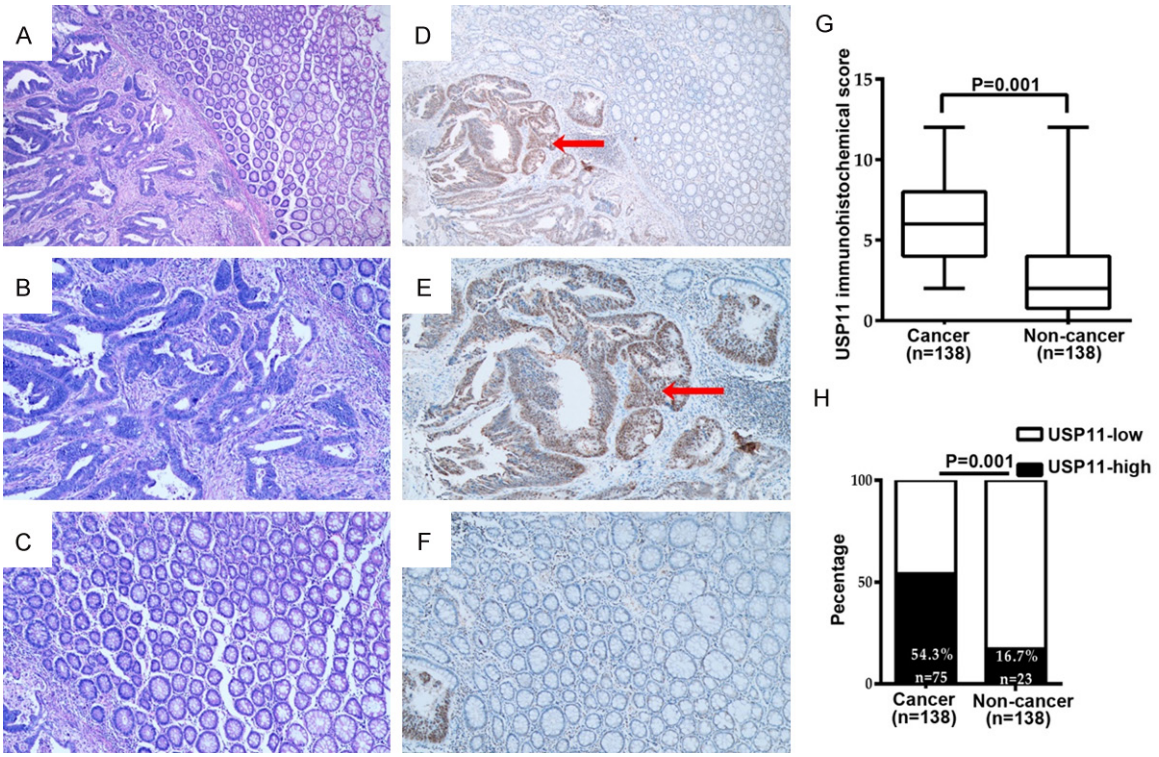

Figure 2. USP11 expression in paired cancerous and para-cancerous tissue. The representative expression levels of USP11 in one paired cancerous and para-cancerous tissue (A-C: HE; D-F: IHC). USP11 is strongly expressed in the cancerous tissue (E), while it is weakly expressed in the para-cancerous tissue (F). (HE and IHC: A, D 100 ×; B, C, E, F 200 ×). (G) The expression level of USP11 is significantly higher in cancerous tissues (P=0.001). (H) The percentage of high USP11 expression is higher in cancerous tissues (54.3% vs. 16.7%, P=0.001). The red arrows represent USP11 high expression.

Next, CCK-8 and clone formation assays were used to evaluate the role of USP11 in cellular proliferation and clonogenicity. We found that USP11 knockdown significantly inhibited proliferation in CRC cell lines (**Figure 3C**). The clone formation assay indicated that USP11 knockdown significantly decreased the number of cell clusters (**Figure 3D**).

Migration and invasion assays revealed that USP11 knockdown cells displayed weak migration and invasion behaviors (**Figure 3E**, **3F**). Collectively, these data strongly suggest that USP11 knockdown weakened the abilities of proliferation, migration, and invasion in CRC cells *in vitro.*

USP11 knockdown weakened the proliferative and metastatic abilities of CRC cells in vivo

To further clarify the role of USP11 in CRC tumorigenicity *in vivo*, a xenograft mouse model was established via subcutaneous injection of shUSP11 and shCtrl HT-29 cancer cells into nude mice. The effect of USP11 knockdown on CRC tumorigenesis *in vivo* is shown in **Figure 4A**. IHC and HE were performed to verify the expression level of USP11 in subcutaneous tumor tissues (**Figure 4B**). USP11-knockdown CRC cells showed slower cell growth and lower tumor volume and weight than the shCtrl cells (P < 0.05, **Figure 4A**, **4C**, **4D**).

A peritoneal implantation metastasis model was applied through intraperitoneal injection of shUSP11 and shCtrl HT-29 cancer cells into nude mice. Four weeks after injection, the mice were euthanized, and the abdominal neoplasms were stripped (**Figure 4E**). IHC and HE were performed to further verify the expression level of USP11 in abdominal neoplasms and hepatic metastatic neoplasms (**Figure 4F**, **4G**). The mice injected with shCtrl HT-29 cancer cells developed more and heavier tumors (P < 0.05, **Figure 4H**, **4I**), and exhibited more hepatic metastatic lesions as indicated by HE and IHC staining (P < 0.05, **Figure 4J**). The USP11 knockdown group in the peritoneal implantation model showed a better survival rate (P <

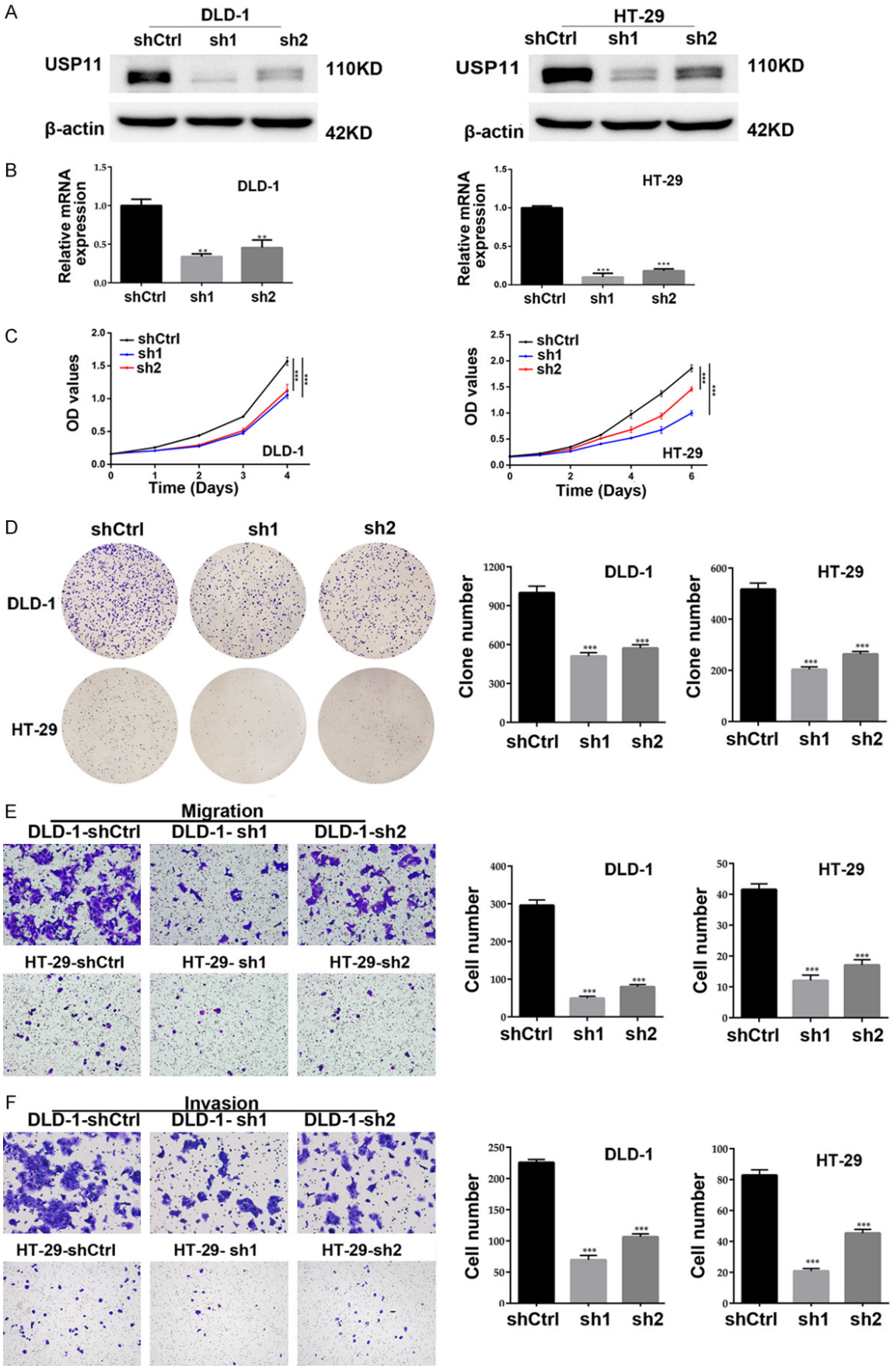
A
DLD-1
shCtrl
sh1
sh2
USP11
110KD
β-actin
42KD
HT-29
B
Relative mRNA expression
C
OD values
Time (Days)
D
DLD-1
HT-29
Clone number
E
Migration
DLD-1-shCtrl
DLD-1- sh1
DLD-1-sh2
HT-29-shCtrl
HT-29- sh1
HT-29-sh2
Cell number
F
Invasion

Figure 3. USP11 knockdown weakened the abilities of proliferation, migration, and invasion of CRC cells *in vitro*. (A, B) The protein and RNA levels of USP11 are significantly reduced following transfection with shRNA lentiviruses compared with those after transfection with an empty vector. USP11 knockdown significantly inhibits proliferation (C), clone formation (D), migration (E) and invasion (F) behavior in DLD-1 and HT-29 cell lines. Data are presented as means ± SEM. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

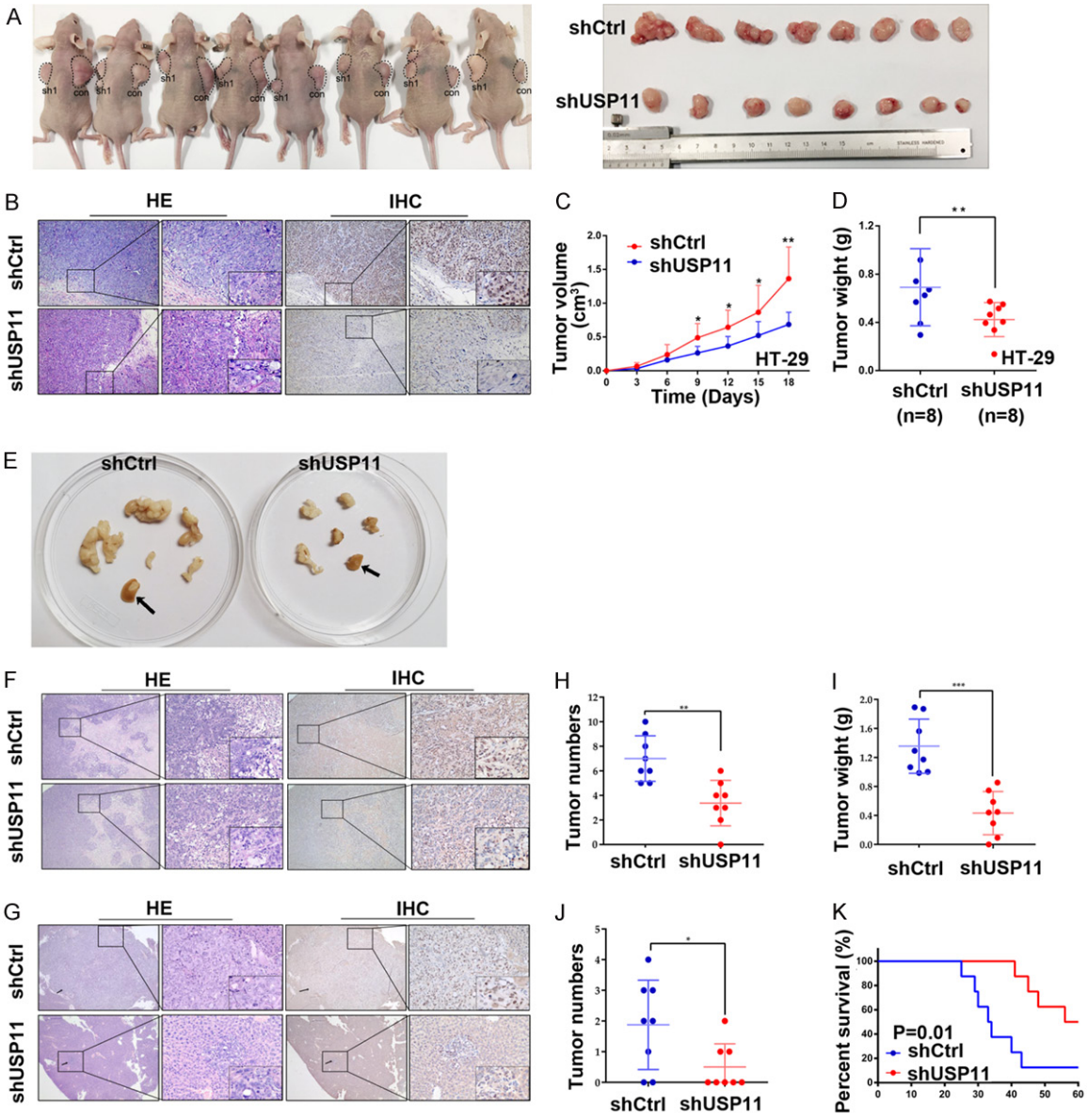

Figure 4. USP11 knockdown weakened the abilities of cell proliferation and metastasis of CRC cells *in vivo*. (A) Effect of USP11 knockdown on CRC tumorigenesis in subcutaneous tissue. (B) HE and IHC used to verify the expression level of USP11 in subcutaneous tumor tissues (100 ×, 200 ×, and 400 ×). (C) The measured and calculate tumor volumes. (D) Tumors weighed. (E) Effect of USP11 knockdown on CRC tumorigenesis and metastasis in the abdominal cavity. Black arrows indicate hepatic metastases (F, G). USP11 expression in abdominal neoplasms and hepatic metastatic neoplasms (Representative HE and IHC images, 50 ×, 200 ×, and 400 ×) (H, I). The abdominal neoplasms were counted and weighed. (J) Metastatic nodules of the liver were counted. (K) Kaplan-Meier survival analysis shows that the USP11 knockdown group in the peritoneal implantation model had a better survival rate. **P* < 0.05, ***P* < 0.01, ****P* < 0.001.

0.05, **Figure 4K**). Collectively, the data demonstrate that USP11 knockdown weakened the proliferative and metastatic abilities of CRC cells *in vivo*.

USP11 and IGF2BP3 could interact with each other

To explore the possible mechanism through which USP11 promoted the proliferation, migration, and invasion of CRC cells, we reviewed numerous relevant articles in the literature and selected several potentially relevant downstream target genes. Of all the possible USP11-associated targets, IGF2BP3 was chosen for follow-up research based on studies that showed that the upregulation of IGF2BP3 in CRC cells could promote tumor cell proliferation and metastasis through the adjustment of mRNA stability [23-25]. An endogenous co-IP assay primitively validated that USP11 and IGF2BP3 interact with each other in CRC cell lines (**Figure 5A**). GFP-USP11 and Myc-IGF2BP3 were co-transfected into 293T cells for exogenous reciprocal co-IP assays to further prove the interaction of USP11 and IGF2BP3, and the result was consistent with that of endogenous co-IP assay (**Figure 5B**). Knockdown of USP11 did not lead to significant changes in IGF2BP3 mRNA levels, as revealed by qRT-PCR (**Figure 5C**). Moreover, we also observed that the expression of IGF2BP3 decreased in shUSP11 cancer cells (**Figure 5D**). Besides, we detected the protein levels of USP11 and IGF2BP3 *in vivo* using Western blotting. We observed that the expression levels of both proteins decreased in the subcutaneous neoplasms which formed from shUSP11 HT-29 cells (**Figure 5E**). To improve the accuracy of our experiment, HCT-15 CRC cells were transfected with a wild-type USP11 (HCT-15-USP11^{WT}) or USP11-mutant (HCT-15-USP11^{MUT}) overexpression plasmid. The USP11^{MUT} had no catalytic activity. As shown in **Figure 6A**, USP11 overexpression in HCT-15-USP11^{WT} cells increased IGF2BP3 expression; however, USP11^{MUT} overexpression in HCT-15-USP11^{MUT} cells did not influence IGF2BP3 protein levels. Moreover, the abilities of cell proliferation, migration, and invasion were reinforced in HCT-15-USP11^{WT} but not in HCT-15-USP11^{MUT} cells compared with those in HCT-15 control cells (**Figure 6B-E**). In conclusion, USP11 and IGF2BP3 interact with each other.

USP11 protects IGF2BP3 protein from degradation by deubiquitination

USP11, belonging to the DUB family, protects downstream target proteins from proteasome hydrolysis. Therefore, we hypothesized that USP11 could stabilize IGF2BP3 expression via deubiquitination. To test our hypothesis, a ubiquitination assay was performed to reveal the number of ubiquitins bound to IGF2BP3. The results showed that the knockdown of USP11 led to an increase in the polyubiquitination level of IGF2BP3 (**Figure 7A**). We further treated DLD-1 and HT-29 cells with 10 μg/mL MG132 for 12 h to investigate whether it can effectively reduce ubiquitin-conjugate protein degradation. Expectedly, when treated with MG132, IGF2BP3 was protected from USP family-mediated downregulation (**Figure 7B**). For USP11 to affect protein levels through post-translational modifications, we used 10 μM cycloheximide (CHX) to determine the half-life of USP11 and IGF2BP3 proteins. The results showed that USP11 knockdown led to a decrease in the half-life of IGF2BP3 (**Figure 7C**). Therefore, USP11 protects the IGF2BP3 protein from degradation. Collectively, these findings suggest that USP11 protects the IGF2BP3 protein from degradation via deubiquitination in CRC cells.

USP11 promotes proliferation, migration, and invasion of CRC cells by modulating IGF2BP3 stability

The biological significance of IGF2BP3 in the USP11-mediated poor prognosis of CRC remained unclear. To further investigate whether the function of USP11 is dependent on IGF2BP3, we reintroduced IGF2BP3 into shUSP11 and shCtrl DLD-1 and HT-29 CRC cells for rescue experiments (**Figure 8A**). CCK-8, and colony formation, and transwell assays revealed that IGF2BP3 overexpression could effectively reverse the decrease in cells proliferation, tumor formation, migration and invasion caused by USP11 knockdown (**Figure 8B-D**). In summary, USP11 promotes the proliferation, migration, and invasion of CRC cells by modulating IGF2BP3 stability.

Discussion

Protein ubiquitination is a dynamic and multilevel process of post-transcriptional modification that is involved in almost all biological behaviors of eukaryotes. Previous studies have reported that polyubiquitination protein modification is a reversible process [26]. USPs, belonging to the DUB family, protect down-

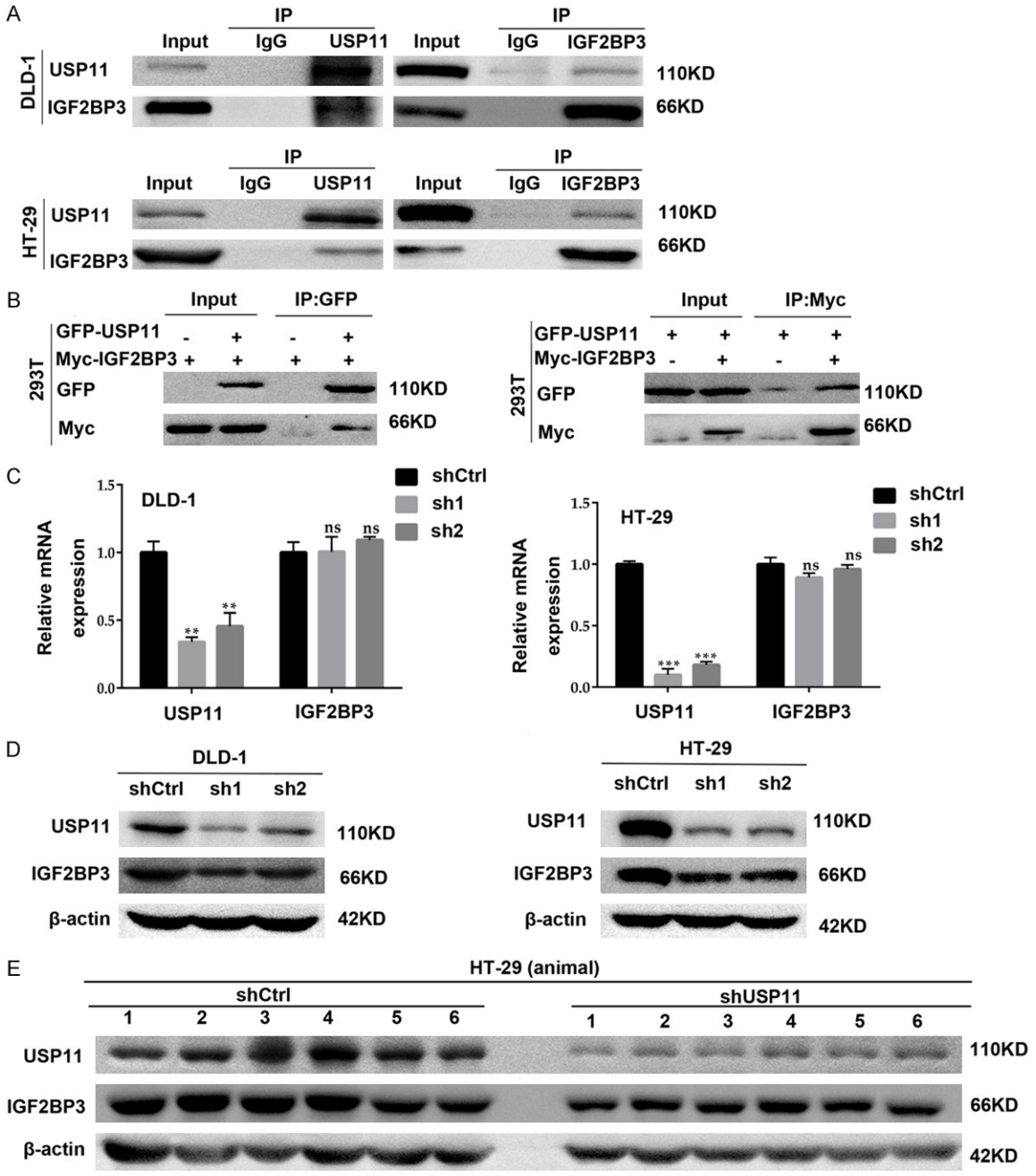

Figure 5. USP11 and IGF2BP3 could interact with each other. Endogenous co-IP (A) and exogenous reciprocal co-IP assay (B) showing interaction of USP11 and IGF2BP3 with each other. (C) Knockdown of the USP11 did not lead to significant changes in IGF2BP3 mRNA levels. (D) Decreased IGF2BP3 expression in shUSP11 cancer cells. (E) Decreased IGF2BP3 expression in subcutaneous neoplasms formed from shUSP11 HT-29 cells.

stream target proteins from proteasome hydrolysis, and they have also been found to be associated with a variety of cell-life activities, including tumor formation [27]. Studies have shown that an aberrant expression of USP22 is associated with liver metastasis and poor prognosis of CRC [28], that USP22 can drive CRC invasion and metastasis via EMT by the adjustment of AP4 expression [29], and that the deubiquitinase USP54 can promote intestinal tumorigenesis [30]. USP11, belonging to USPs, has similar capabilities. Typically, abnormal USP11 expression has been linked to tumors in the brain [31], breast [18], pancreas [32], and liver [7]. In

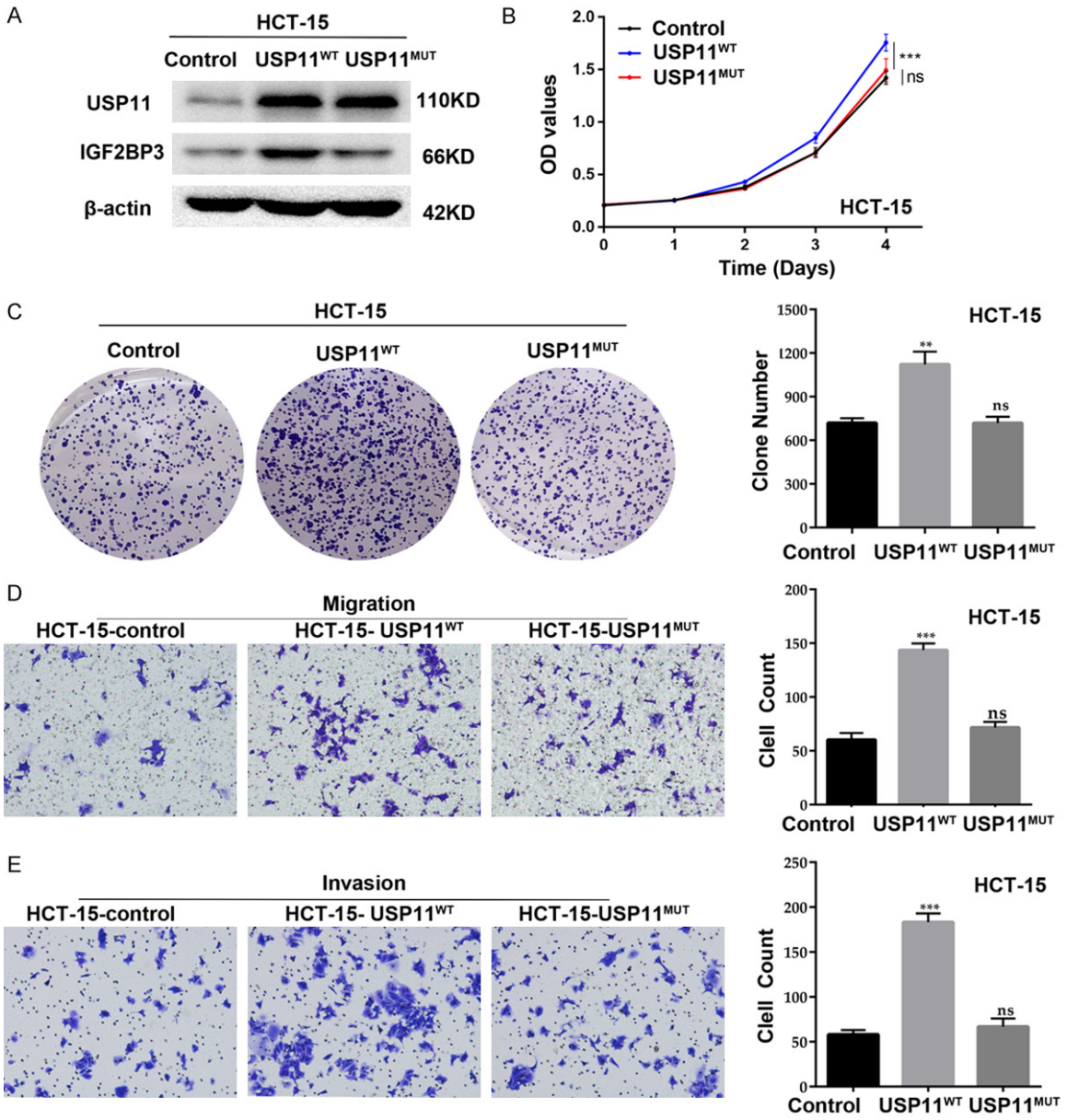

Figure 6. USP11 overexpressed reinforced the abilities of proliferation, migration, and invasion of CRC cells *in vitro*. (A) USP11 overexpression in HCT-15-USP11^{WT} cells leads to an increased in IGF2BP3 expression. USP11 overexpression significantly reinforces proliferation (B), clone formation (C), migration (D) and invasion (E) behaviors in HCT-15-USP11^{WT} but not in HCT-15-USP11^{MUT} cells. Data are presented as means ± SEM. *P < 0.05, **P < 0.01, ***P < 0.001.

the current study, we further investigated the role of USP11 in the occurrence and development of CRC.

We confirmed that USP11 expression is higher in CRC tissue specimens than in para-cancerous specimens. Notably, in cancerous tissues, the upregulation of USP11 was associated with the depth of tumor invasion, lymph node metastasis, and distant metastasis.

We then tested this result in CRC cells. By CCK-8, clone formation, and transwell assays, we found that the proliferation, migration, and invasion capacity of CRC cells were down-regulated after USP11 knockdown. In contrast, these abilities were reinforced in HCT-15 CRC cells transfected with wild-type USP11 overexpression plasmid. Using animal experiments, we also discovered that tumors grew more slowly, hepatic metastatic lesions were fewer, and the

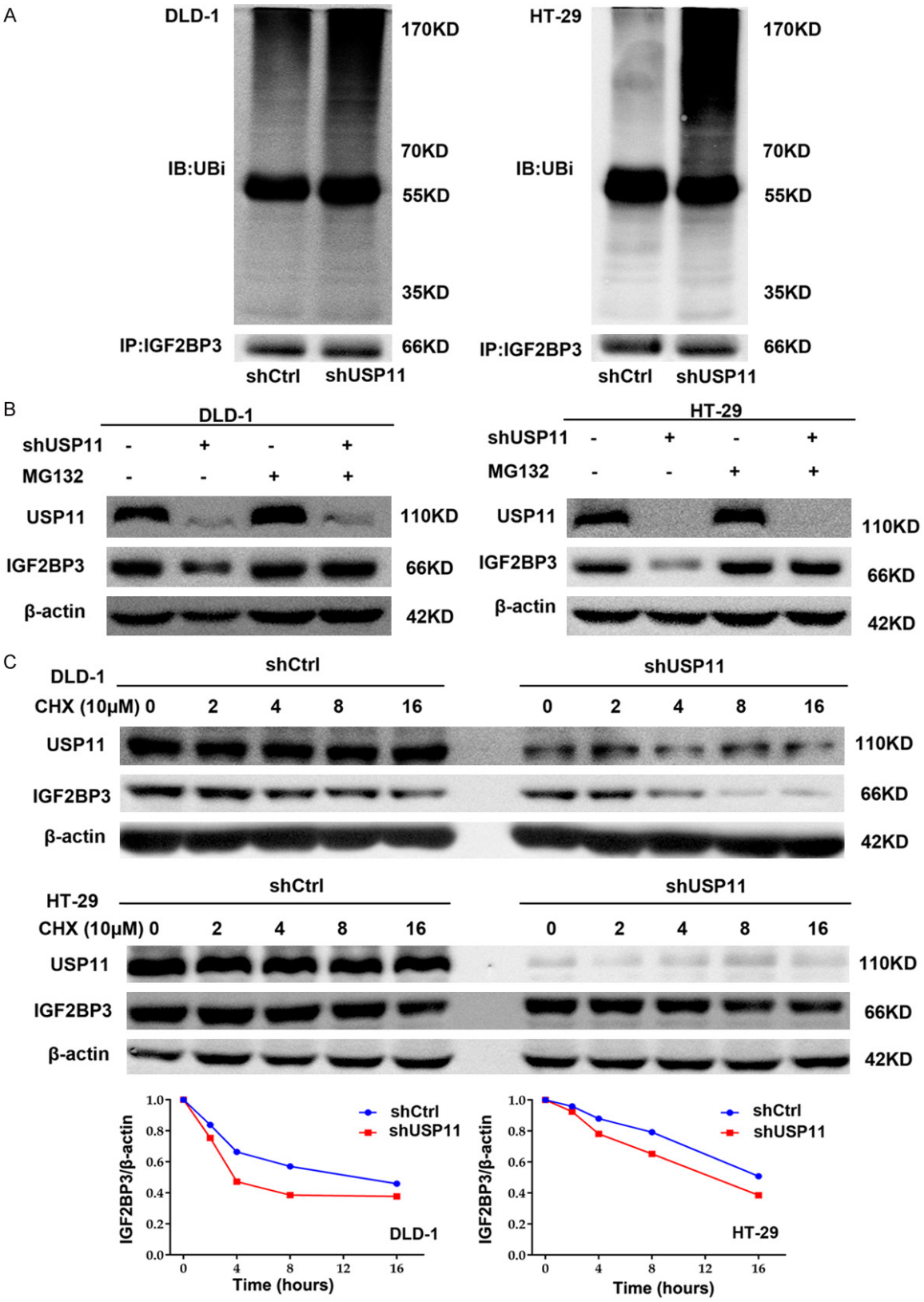

Figure 7. USP11 protects IGF2BP3 protein from degradation by deubiquitination. A. Western blotting analysis shows knockdown of USP11 leads to an increase in the polyubiquitination level of IGF2BP3. B. Cells (treated with 10 μg/mL MG132 for 12 h), show that IGF2BP3 is protected from degradation. C. We used 10 μM CHX, a protein synthesis inhibitor, to explore the half-life of USP11 and IGF2BP3 proteins. The results showed that USP11 knockdown led to a decrease in the half-life of IGF2BP3 in both DLD-1 and HT-29 cells.

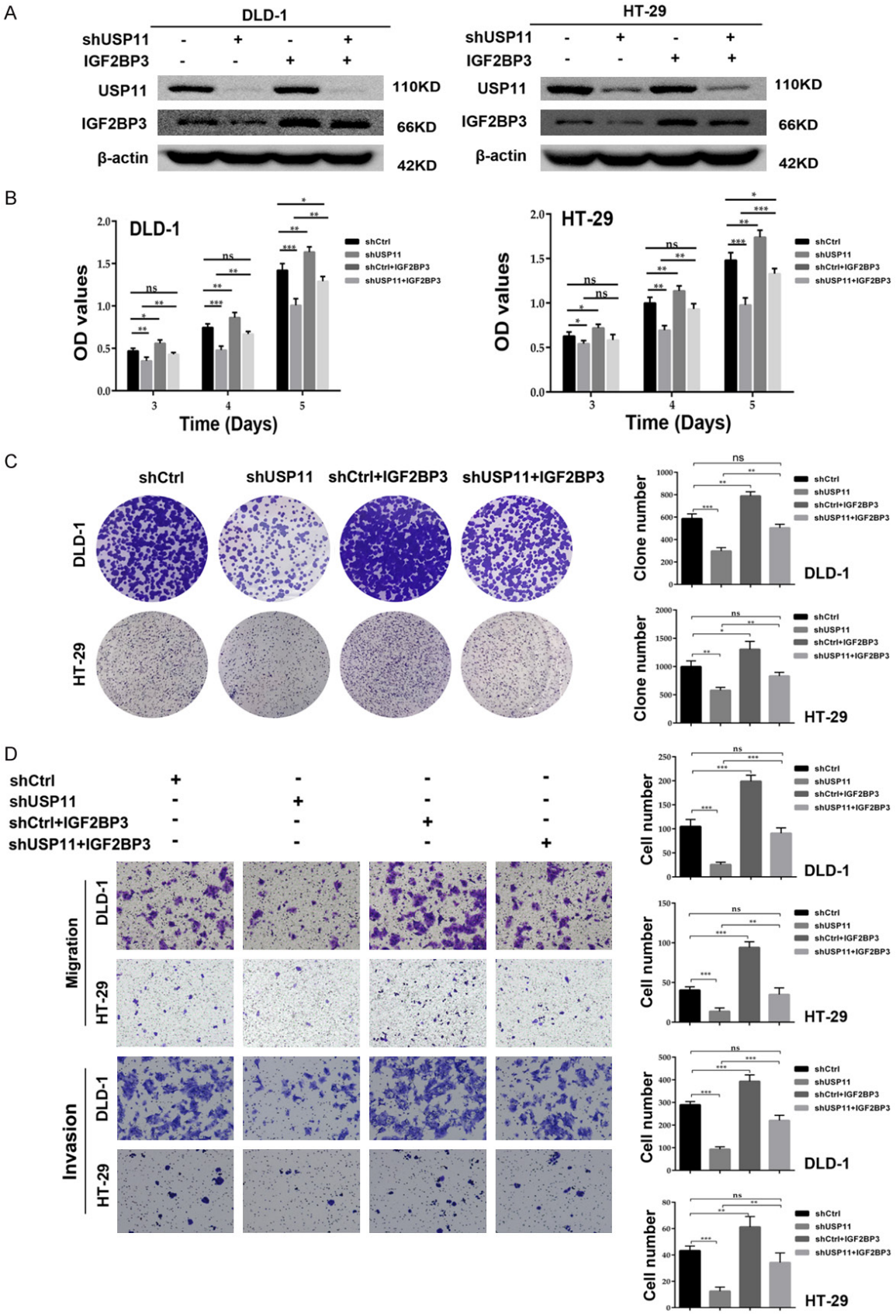
A
DLD-1
HT-29
shUSP11
IGF2BP3
USP11
IGF2BP3
β-actin
110KD
66KD
42KD
B
DLD-1
HT-29
OD values
Time (Days)
shCtrl
shUSP11
shCtrl+IGF2BP3
shUSP11+IGF2BP3
C
shCtrl
shUSP11
shCtrl+IGF2BP3
shUSP11+IGF2BP3
DLD-1
HT-29
Clone number
D
Migration
Invasion
Cell number

Figure 8. USP11 promotes proliferation, migration, and invasion of CRC cells by modulating IGF2BP3 stability. (A) IGF2BP3 overexpression plasmid reintroduced into shUSP11 and shCtrl DLD-1 and HT-29 CRC cells. IGF2BP3 overexpression could effectively reverse the decrease in cell proliferation (B), clone formation (C), migration, (D) and invasion (D) caused by USP11 knockdown.

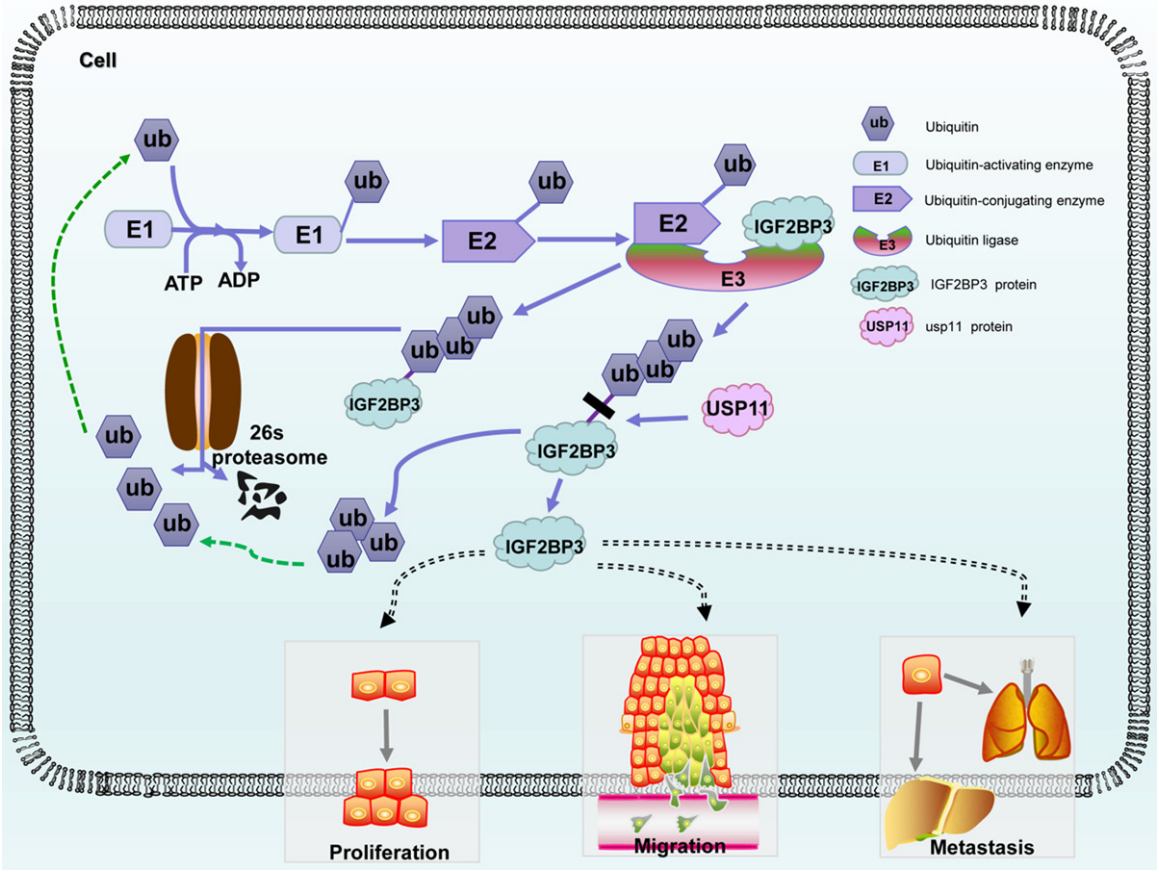

Figure 9. The USP11-IGF2BP3 pathway. USP11 can protect IGF2BP3 protein from degradation by deubiquitination to promote CRC proliferation, migration, and metastasis.

survival rate was better in the shUSP11 group than in the shCtrl group.

Previous research has shown that IGF2BP3, as a carcinoma embryo protein, is upregulated in lung carcinoma [33], ovarian carcinoma [34], hepatocellular carcinoma [35], and pancreatic carcinoma [36]. XiaoPing H [37], Gong Y [25] and Paul Lochhead [38] reported that IGF2BP3, as a tumor initiator, was closely related to the prognosis of CRC patients and could also promote tumor cell proliferation and metastasis. WeiMin Xu [39] found that IGF2BP3 expression was obviously upregulated in CRC tissues and was associated with poor prognosis and that IGF2BP3 can be deubiquitinated by USP10 [33]. We demonstrated that USP11 and IGF-2BP3 interacted with each other and that IGF2BP3 expression decreased in shUSP11 cells, increased in HCT-15-USP11^{WT} cells, and was not influenced in HCT-15-USP11^{MUT} cells compared with that in shCtrl and HCT-15-controls. The protein levels of USP11 and IGF2BP3 in subcutaneous tumor tissues of mice were examined, and the findings confirmed the above results.

Researchers have found that USP11 can deubiquitinate both TGFBR1 and TGFBR2 leading to breast cancer cell metastasis [19, 40, 41]. Encheng Zhang [16] also verified that USP11 controls VGLL4 protein stability by deubiquitination. Therefore, we hypothesized that USP11 stabilizes IGF2BP3 expression via deubiquitination. We established that USP11 knockdown led to an increase in the polyubiquitination

level of IGF2BP3 in DLD-1 and HT-29 cells. Moreover, USP11 knockdown led to a decrease in the half-life of IGF2BP3.

Given that the biological significance of IGF-2BP3 in USP11-mediated poor prognosis of CRC is unclear, we reintroduced IGF2BP3 into shUSP11 and shCtrl DLD-1 and HT-29 CRC cells. Rescue experiments revealed that IGF-2BP3 overexpression could effectively reverse a decrease in cell proliferation, tumor formation, migration and invasion caused by USP11 knockdown. Therefore, USP11 promotes the proliferation, migration, and invasion of CRC cell by modulating IGF2BP3 stability.

To our knowledge, this is the first report that describes the role of the USP11-IGF2BP3 pathway in tumor proliferation and metastasis (**Figure 9**). These findings provide a novel molecular biological mechanism for the role of USP11 in CRC, which will aid us in further understanding of the molecular basis of the occurrence and development of CRC. Furthermore, the proposed USP11-IGF2BP3 pathway is a potential new therapeutic target for CRC.

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Disclosure of conflict of interest

None.

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