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Ubiquitin-Specific Protease 4-Mediated Deubiquitination and Stabilization of PRL-3 Is Required for Potentiating Colorectal Oncogenesis

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Abstract

Ubiquitin specific protease 4 (USP4) is a deubiquitinating enzyme with key roles in the regulation of p53 and TGFβ signaling, suggesting its importance in tumorigenesis. However, the mechanisms and regulatory roles of USP4 in cancer, including colorectal cancer, remain largely elusive. Here, we present the first evidence that USP4 regulates the growth, invasion, and metastasis of colorectal cancer. USP4 expression was significantly elevated in colorectal cancer tissues and was significantly associated with tumor size, differentiation, distant metastasis, and poor survival. Knockdown of USP4 diminished colorectal cancer cell growth, colony formation, migration, and invasion *in vitro* and metastasis *in vivo*. Importantly, we found that phosphatase of regenerating liver-3 (PRL-3) is indispensable for USP4-mediated oncogenic activity in colorectal cancer. Mechanistically, we observed that USP4 interacted with and stabilized PRL-3 via deubiquitination. This resulted in activation of Akt and reduction of E-cadherin, critical regulators of cancer cell growth and metastasis. Examination of clinical samples confirmed that USP4 expression positively correlates with PRL-3 protein expression, but not mRNA transcript levels. Taken together, our results demonstrate that aberrant expression of USP4 contributes to the development and progression of colorectal cancer and reveal a critical mechanism underlying USP4-mediated oncogenic activity. These observations suggest that the potential of harnessing proteolytic degradation processes for therapeutic manipulation may offer a much-needed new approach for improving colorectal cancer treatment strategies. *Cancer Res; 76(1); 83–95. ©2015 AACR.*

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Introduction

Colorectal cancer is the third leading cause of death from cancer worldwide (1). Although some advances have been made in the treatment of colorectal cancer, including surgical techniques, chemotherapy, and radiation therapy, the overall survival rate of patients with colorectal cancer has not improved dramatically. This is due to recurrence and metastasis (2). Altered regulation of some proteins and signaling pathways in the development of colorectal cancer has been described, but identification of novel regulatory mechanisms contributing to the development and progression of colorectal cancer remains a subject of significant interest.

Ubiquitination is an essential posttranslational modification. It plays critical roles in a diverse array of cellular processes, including cell-cycle progression, transcriptional regulation, and signal transduction (3–5). However, deubiquitination, the reverse process mediated by deubiquitinating enzymes (DUB), has gained increasing attention as an important regulatory mechanism in controlling protein turnover (6, 7). Currently, approximately 100 putative DUBs have been identified (8), but most have not been functionally characterized. The ubiquitin-specific proteases (USP) represent the largest subclass of DUBs. It has more than 50 members (8). Like other USPs, USP4 also mediates the removal and processing of ubiquitin. USP4 binds TRAF2, TRAF6, and TAK1 for deubiquitination and negatively regulates TNFα-induced NF-κB activation (9, 10). USP4 binds directly to and stabilizes ARF-BP1 via deubiquitination, subsequently promoting ARF-BP1–dependent p53 ubiquitination and degradation (11). USP4 also directly interacts with and deubiquitinates TGFβ type I receptor (TβRI), regulating TGFβ signaling by controlling TβRI levels at the plasma membrane (12). USP4 is significantly overexpressed in many human cancers, such as urinary, prostate, thyroid, and liver cancer (11, 13). Unfortunately, other studies show conflicting observations. USP4 protein expression is downregulated in lung cancer cell lines (14). USP4, which is located on chromosome 3 (3p21.3), is frequently deleted in human epithelial cancers, including those of the breast, kidney, and lung (15–17). However, USP4's mechanism of regulation in cancer, including colorectal cancer, has not been well established.

Phosphatase of regenerating liver-3 (PRL-3, also called PTP4A3), a member of the protein tyrosine phosphatase family (PTPs), plays an important role in cancer progression, including

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cell proliferation, migration, invasion, metastasis, and angiogenesis (18–20). The expression of PRL-3 is frequently upregulated in human tumors, such as breast cancer (21), liver cancer (22), ovarian cancer (23), gastric cancer (24), and colorectal cancer (25). This expression is associated with cancer progression and poor survival. PRL-3 modulates multiple signaling pathways, including PI3K/AKT, SRC, Rho GTPases, MAPK/ERK, and EGFR in various cancer cells (19, 26, 27). Previous findings, including ours, clearly indicate that PRL-3 plays a critical role in the epithelial–mesenchymal transition (EMT; refs. 28, 29). Recently, a new functional role of PRL-3 in epigenetic regulation was discovered. PRL-3 probably affects the activity of JMJD1B and JMJD2B, two histone demethylases, thus participating in the regulation of histone methylation (30). Despite PRL-3's importance as a regulatory of tumorigenesis, the mechanism leading to the aberrant upregulation of PRL-3 in tumors remains unclear.

In this study, results showed USP4 expression to be elevated in tumor tissues sampled from colorectal cancer patients. A series of experiments *in vitro* and *in vivo* revealed that stable knockdown of USP4 expression in colorectal cancer cells significantly impaired the capacity for growth, migration, invasion, and metastasis. In addition, the mechanism by which USP4 promotes development and progression of colorectal cancer was investigated. USP4 activates PI3K/AKT signaling and downregulates E-cadherin expression by binding to and deubiquitinating PRL-3, thereby preventing degradation of PRL-3. Collectively, these results suggest that USP4 is a *bona fide* DUB of PRL-3 and plays a pivotal role in colorectal cancer.

Materials and Methods

Antibody and reagents

Antibodies against USP4 (#2651), p-Akt (#9271), and Akt (#9272) were purchased from Cell Signaling Technology. E-cadherin (ab53033) and PRL-3 (ab50276) were purchased from Abcam. HA (F-7) and C-myc (9E10) were purchased from Santa Cruz Biotechnology, ubiquitin (#550944) was purchased from BD Pharmingen, and β-actin (A5441) was purchased from Sigma-Aldrich. Anti-mouse IgG (HRP) (GTX221667-01) and anti-rabbit IgG (HRP) (GTX221666-01) were purchased from GeneTex. Lipofectamine 2000 and TRIzol LS were purchased from Invitrogen and WesternBright ECL reagents were purchased from Advansta.

Cell culture

HCT116, SW480, SW620, RKO, LoVo, DLD-1, LS174T, and Caco-2 human colon cancer cell lines and HEK293T human embryonic kidney cells were originally obtained from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). These cells were cultured as previously described (31). NCM460, a normal human colon mucosal epithelial cell line, was maintained in 50% DMEM and 50% RPMI-1640 medium containing 20% fetal bovine serum in a humidified atmosphere containing 5% CO_2 at 37°C. All cell lines were passaged for fewer than 4 months after resuscitation and were used at the fifth through tenth passage in culture for this study.

Tissue samples and evaluation

From 2003 to 2007, 86 human colorectal cancer tissues were collected immediately after surgical resection at the First Affiliated Hospital of Soochow University. Another 81 colorectal cancer tissues were purchased from Shanghai Outdo Biotech Co., Ltd., collected between 2006 and 2007. A total of 167 patients were involved in the study. The clinical characteristics of all patients are listed in Supplementary Table S1. The study was approved by Soochow University for Biomedical Research Ethics Committee, and all of the patients provided informed consent. Tissues were probed using the indicated antibodies.

The staining score was evaluated using two blinded individuals. The staining scoring criteria were as follows: 0, 1%–4% of the cells stained positive; 1, 5%–25% of the cells stained positive; 2, 26%–50% of the cells stained positive; 3, 51%–75% of the cells stained positive; and 4, $\geq$75% of the cells stained positive. The staining intensity of cancer cells was scored as 0 (no staining), 1 (weak staining), 2 (intermediate staining), and 3 (strong staining). An intensity score of $\geq$2 with at least 50% of USP4-positive cells was regarded as high expression, and <50% of USP4-positive cells or <2 in intensity score was considered low expression. The multiple of the intensity and extent score was used as the final score (0–12) for USP4.

Generation of stable cell lines

HCT116 and SW480 cell lines stably expressing USP4-specific shRNA (USP4/shRNA) or scrambled shRNA control (USP4/Ctr) were constructed using a lentiviral shRNA technique. Oligonucleotides were constructed in GV248 lentiviral RNAi vector (GeneChem). SW480 and HCT116 cells were transduced with serial dilutions of lentiviral supernatant and selected for using 5 μg/mL puromycin for 3 to 4 weeks. The human USP4 shRNA target sequences are listed in Supplementary Table S2.

siRNA

Human USP4 and PRL-3 siRNA and siRNA nonspecific control were synthesized by RiboBio. Colorectal cancer cells were transiently transfected with these siRNAs using Lipofectamine 2000. The human USP4 and PRL-3 siRNA target sequences are listed in Supplementary Table S3.

Cell extraction and Western blotting

Cellular proteins were extracted using RIPA lysis buffer (50 mmol/L Tris–HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mmol/L NaCl) containing protease inhibitors (Roche). Equal amount of proteins were electrophoresed and transferred onto PVDF membranes. Protein expression was detected using primary and secondary antibodies and visualized with an enhanced chemiluminescence system.

RNA extraction and qPCR analysis

Total RNAs were purified using TRIzol LS. First-strand cDNA was generated with RevertAid First-strand cDNA Synthesis Kits (Fermentas). qPCR was carried out using SYBR Premix EX Taq (TaKaRa) on an ABI 7500 PCR system (Applied Biosystems). The primers for PCR reactions are listed in Supplementary Table S4.

Immunoprecipitation and ubiquitination assay

Cells were lysed in RIPA lysis buffer. Whole-cell lysates were immunoprecipitated with the indicated antibodies on protein A/G beads (Santa Cruz Biotechnology) overnight. The beads were then washed and boiled in SDS loading buffer. Immunoprecipitated protein complexes were assessed using Western blotting. To detect PRL-3 ubiquitination, 10 mmol/L N-ethylmaleimide was included in the lysis buffer.

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Colony formation assays

Colorectal cancer cells (1×10^3) were cultured in 6-well plates for 14 days and then subjected to Wright-Giemsa staining. The number of foci containing >50 cells was determined.

Wound-healing assays

SW480 cells were seeded into 24-well plates containing coverslips. The confluent monolayers were wounded in a line across the slides with a sterile 20 μL plastic pipette tips. All cellular debris was removed by washing the coverslips with phosphate buffer saline (PBS). The distances migrated by the cell monolayer to close the wounded area during the indicated time were measured. Cell migration, indicating wound-healing effect, is here expressed as a migration index, i.e., the distance migrated by SW480 cell at the indicated point in time relative to the initial length of the wound. Experiments were performed in at least triplicate.

Transwell migration assay

In brief, 200 μL of cell suspension (1×10^5 cells) was added to the upper compartment of migration chambers (BD Biosciences). The bottom chamber was filled with 500 μL RPMI-1640 medium with 10% FBS. Then, 24 hours later, cells were fixed with 100% methanol and stained with Wright-Giemsa. To quantify the migratory cells microscopically, cells were counted in five random fields (magnification, ×200).

Matrigel Transwell invasion assay

BD BioCoat Matrigel Invasion Chambers (catalog no. 354480) were used for the invasion assay according to the manufacturer's protocol.

Xenografts

To evaluate *in vivo* tumor growth, 100 μL cells (2×10^6) were subcutaneously injected into nude mice (BALB/c, SPF grade, 4–5 weeks old, $n = 6$ per group). Body weight and tumor sizes were measured every 3 days. Then, 3 weeks later, mice were killed and tumors were removed for assessments.

To evaluate *in vivo* tumor metastasis, SW480/USP4/control and SW480/USP4/shRNA cells (2×10^6) were injected into the lateral vein in the nude mouse tail (BALB/c, SPF grade, 4–5 weeks old, $n = 6$ per group). After 6 weeks, the mice were killed under anesthesia. Lung tissues were collected for metastatic foci evaluation and standard histopathological study.

To further evaluate *in vivo* tumor metastasis, an orthotopic metastatic mouse model of colorectal cancer was established. Briefly, 2×10^6 cells were subcutaneously injected into nude mice (BALB/c, SPF grade, 4–5 weeks old, $n = 4$ per group). The cells were allowed to grow into a tumor over the course of 2 weeks. Then, the nude mice with established subcutaneous colorectal tumors were euthanized. Subcutaneous tumors were removed and divided into 2 to 3 mm pieces in PBS, and tumor pieces were inoculated into the cecum of host nude mice (BALB/c, SPF grade, 4–5 weeks old, $n = 10$ per group) after the laparotomy. Because the common first site of metastasis of colorectal cancer is in the liver, the livers were examined after 7 weeks of implantation. The metastatic foci in the livers were evaluated using a standard histopathological study.

All manipulations involving live mice were approved by the Animal Care and Use Committee of Soochow University.

Statistical analysis

Data were expressed as mean ± SD. Each assay was performed in three independent experiments. Statistical analyses were conducted using SPSS package (version 18.0). The Student *t* test (unpaired, two-tailed) was used to compare two groups of independent samples. One-way ANOVA was used for multiple comparisons. The relationships between USP4 expression and other clinicopathological factors were determined using the Pearson χ^2 test. Kaplan–Meier survival analysis was used to illustrate the prognostic relevance of USP4 in univariate analysis. The correlations between the expression levels of USP4 and PRL-3 were calculated using the Spearman rank correlation. Values of $P < 0.05$ were considered significant.

Results

Overexpression of USP4 in colorectal cancer is significantly correlated with tumor size, distant metastasis, and poor survival

To explore the role of USP4 in colorectal cancer, USP4 expression was first analyzed in colorectal cancer tissues. Results showed that 67% of primary colorectal cancer tumors had more USP4 transcripts (Fig. 1A) and protein expression (Fig. 1B and C) than matched surrounding tissues. Immunohistochemical staining further confirmed that USP4 was overexpressed in tumor tissues ($P < 0.01$; Fig. 1D and E). Further analysis showed USP4 levels to be markedly higher in the colorectal cancer tissues with distant metastasis than in colorectal cancer tissues without distant metastasis ($P < 0.01$; Fig. 1F and G). In addition, statistical analysis revealed USP4 expression to be significantly associated with tumor size ($P = 0.041$) and differentiation ($P = 0.034$). However, there was no significant correlation between USP4 expression and other clinicopathological features, such as patient gender, age, and tumor–node–metastasis stage ($P > 0.05$; Supplementary Table S1). Kaplan–Meier analysis indicated that patients with high USP4 expression had a significantly lower survival rate than those with low USP4 expression (Fig. 1H). The median survival time of colorectal cancer patients with high USP4 expression was 34 months, which was significantly shorter than that of those with low USP4 expression (87 months). There was more USP4 protein expression in all eight colorectal cancer cell lines than in NCM460, a normal human colon mucosal epithelial cell line (Supplementary Fig. S1). Together, these results clearly show that USP4 is overexpressed in colorectal cancer tissues and colorectal cancer cells, suggesting that USP4 may play a role in colorectal cancer development and progression.

Knockdown of USP4 inhibits growth, migration, and invasion of colorectal cancer cells *in vitro*

The prevalence of USP4 upregulation raises an intriguing possibility that USP4 overexpression may be a cancer-promoting event in colorectal cancer. To test this possibility, USP4 expression was stably knocked down using a lentiviral shRNA technique in SW480 and HCT116 cells expressing high levels of USP4 protein. The knockdown efficiency was confirmed by Western blotting (Supplementary Fig. S2A). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that knocking down USP4 significantly reduced cellular proliferation (Supplementary Fig. S2B and S2C). Colony formation assays were also used to investigate the role of USP4 in cell proliferation. Figure 2A showed that the ability of SW480 and

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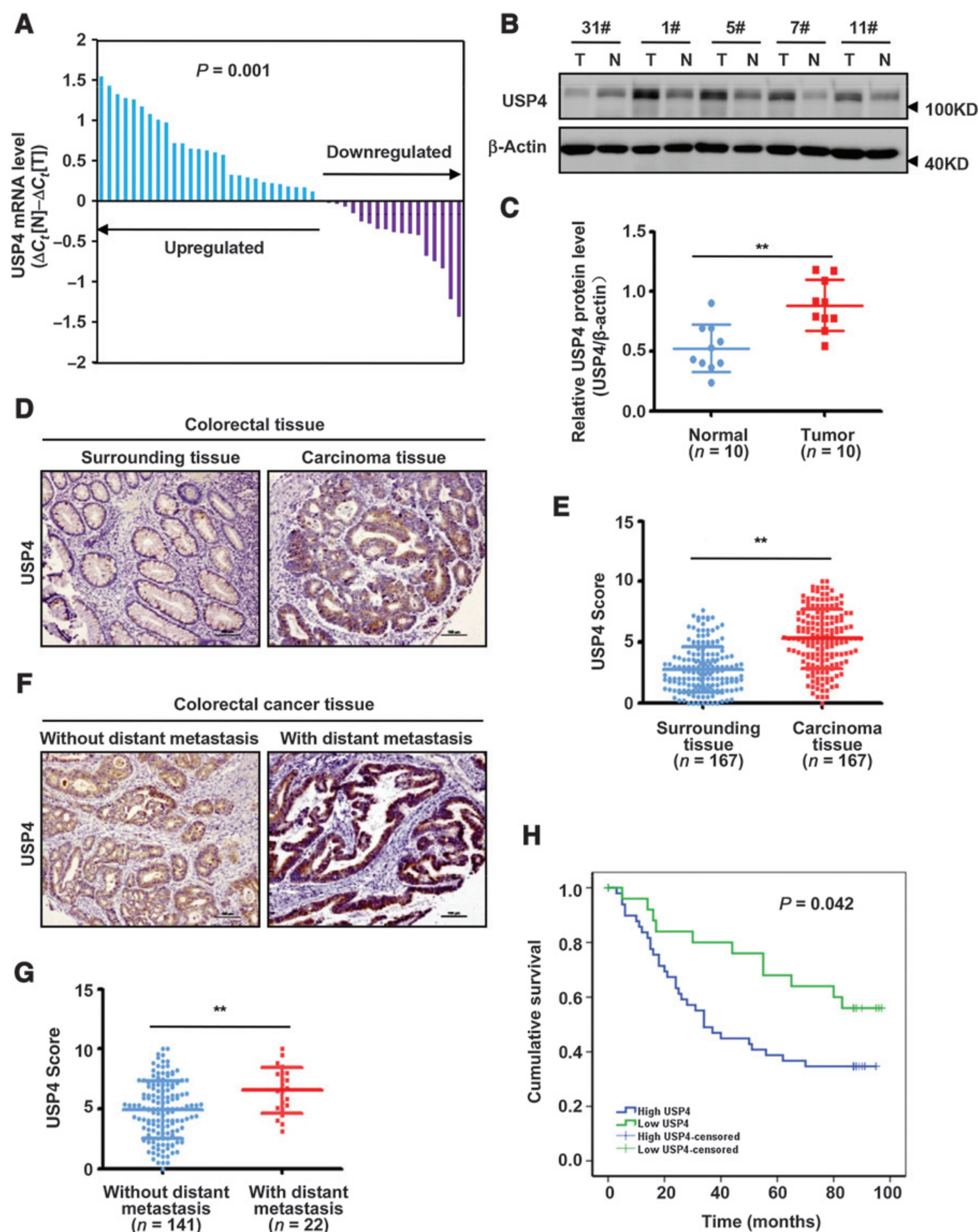

Figure 1.

Overexpression of USP4 in colorectal cancer. A, qPCR analysis of the relative USP4 mRNA expression in 45 cases of colorectal cancer tissue samples. The expression of USP4 was normalized to β-actin. Data were analyzed in triplicate. Bar, value of $\Delta C_t[N] - \Delta C_t[T]$. [T], colorectal cancer tumors; [N], matched adjacent colonic mucosa. Statistical significance was determined by one-sample *t* test; $P = 0.001$. B, Western blotting for USP4 protein in colorectal cancer tumors. Five randomly selected pairs of colorectal cancer tumors (T) and matched adjacent colonic mucosa (N) are presented. C, scatter plot analysis of the expression levels of USP4 protein. USP4 expression levels were quantified by densitometry. The expression of USP4 was normalized to β-actin. Statistical significance was determined by a two-tailed, paired Student *t* test. **, $P < 0.01$. D, immunohistochemical staining of USP4 in surrounding tissue and carcinoma tissue of colorectal cancer (magnification, ×100). E, immunohistochemical scores for USP4 expression in 167 cases of colorectal cancer tissues. Statistical significance was determined by a two-tailed, paired Student *t* test. **, $P < 0.01$. F, immunohistochemical staining of USP4 in colorectal cancer tissues with distant metastasis ($n = 22$) and without distant metastasis ($n = 141$; magnification, ×100). G, immunohistochemical scores for USP4 expression in colorectal cancer tissues with distant metastasis ($N = 22$) and without distant metastasis ($N = 141$). Statistical significance was determined using a two-tailed, unpaired Student *t* test. **, $P < 0.01$. H, a Kaplan–Meier survival curve shows significant association between high levels of USP4 and poor survival in colorectal cancer patients ($P = 0.042$). Low USP4 ($n = 29$) and high USP4 ($n = 52$).

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HCT116 cells to form colonies was significantly impaired when cells lacked USP4. Colorectal cancer cell migration and invasion were also assessed in the absence of USP4 *in vitro*. Scratch–wound-healing assays showed that knockdown of USP4 significantly reduced the ability of colorectal cancer cell migration (Fig. 2B), which is consistent with the observations made in the migration assay (Fig. 2C). Similarly, invasion assays indicated a lower invasion ability in USP4/shRNA cells than in USP4/control cells (Fig. 2D). Collectively, these data demonstrated that USP4 can promote colorectal cancer cell growth, migration, and invasion *in vitro*.

USP4 promotes the growth and metastasis of colorectal cancer cells in *in vivo* mouse models

To further investigate the role of USP4 in colorectal cancer cell growth and metastasis, the ability of USP4 knockdown to affect tumor growth and metastasis was tested in *in vivo* mice models.

First, the same number of USP4/shRNA colorectal cancer cells or control cells was injected into different flanks of nude mice. Tumor formation was monitored and tumor sizes were measured every 3 days. Twenty-five days after implantation, tumors were found to have formed, indicated by the fact that USP4/shRNA cells grew much more slowly than control cells (Fig. 3A and B), and the sizes and weight of tumors from these mice were significantly smaller than those of control mice (Fig. 3C and D). The degree of USP4 depletion in the tumors was also confirmed by immunohistochemical staining (Supplementary Fig. S3A and S3B).

The ability of USP4 knockdown to affect metastasis of colorectal cancer cells was tested further in a xenografted metastasis model. SW480/USP4/shRNA and SW480/USP4/control cells were injected into the lateral vein in the tails of the nude BALB/c mice. Five weeks after implantation, results showed that SW480/USP4/control cells formed large metastatic nodules in the lung but SW480/USP4/shRNA cells did not (Supplementary Fig. S4A). These findings are summarized in Supplementary Fig. S4B. Similarly, histological examination showed that the USP4/control group developed more pulmonary micrometastases than the USP4/shRNA group (Supplementary Fig. S4C). The degree of USP4 depletion in the lung nodules was confirmed by immunohistochemical staining (Supplementary Fig. S4D). Consistent with these findings, in an orthotopic metastatic mouse model of colorectal cancer, results further confirmed that knockdown of USP4 in tumor derived from SW480/USP4/shRNA cells produced far fewer hepatic metastases than controls (Fig. 3E and F). Hematoxylin and eosin (H&E) staining also revealed that knockdown of USP4 was associated with the development of fewer micrometastases than control group (Fig. 3G).

Collectively, these results demonstrate that high levels of USP4 expression were sufficient to promote growth and metastasis of colorectal cancer cells, strongly indicating that USP4 functions as a tumor promoter in colorectal cancer.

USP4 induces PRL-3–dependent Akt activation and E-cadherin loss

Dysregulation of PI3K/Akt signaling pathways and other key events, such as the EMT, is associated with cancer development (32, 33). To study the mechanism by which USP4 promotes the growth and metastasis of colorectal cancer, results showed that overexpression of USP4 significantly increased levels of phosphorylated Akt protein but not levels of phosphorylated JNK protein (Fig. 4A). Interestingly, these results further showed that knockdown of PRL-3 reversed the effect of USP4 on Akt phosphorylation (Fig. 4A). Inversely, inhibition of USP4 expression in colorectal cancer cells impaired Akt phosphorylation but not JNK phosphorylation (Fig. 4B), which were significantly reversed by PRL-3 overexpression (Fig. 4B). These results also showed USP4 to be involved in the regulation of EMT. As shown in Fig. 4C, USP4 knockdown significantly increased E-cadherin expression, an epithelial marker implicated in metastasis of colorectal cancer (31). Overexpression of PRL-3 profoundly impaired E-cadherin expression induced by USP4 knockdown (Fig. 4D). The effect of USP4 on Akt activation and EMT transition could also be observed *in vivo*. In SW480 xenografts, USP4 expression is positively correlated with Akt phosphorylation but negatively correlated with E-cadherin expression (Fig. 4E and F). This line of data suggests that USP4 may play a role in the development of colorectal cancer through induction of PRL-3–dependent Akt activation and E-cadherin reduction.

PRL-3 is required for USP4 to stimulate growth, migration, and invasion of colorectal cancer cells

Because PRL-3 is involved in USP4-mediated Akt activation and E-cadherin loss, it is possible that PRL-3 may affect the oncogenic effect of USP4 in colorectal cancer. To test this, the role of PRL-3 in USP4-mediated colorectal cancer cell proliferation and growth was investigated. The results of the present MTT assays (Supplementary Fig. S5A) and colony formation assays (Supplementary Fig. S5B) showed that knockdown of USP4 significantly impaired colorectal cancer cell proliferation and growth, which could be reversed by overexpression of PRL-3, suggesting that PRL-3 is required for the pro-proliferative function of USP4. Wound-healing assays (Supplementary Fig. S5C) and migration assays (Supplementary Fig. S5D) indicated that knockdown of USP4 markedly reduced colorectal cancer cell migration. However, silencing of USP4 showed only a limited effect on colorectal cancer cell migration when PRL-3 was overexpressed. Similar results were also obtained in invasion assays (Supplementary Fig. S5E), and overexpression of PRL-3 reversed the inhibitory effect of USP4 knockdown on colorectal cancer cell invasion. Together, these data demonstrate that PRL-3 is indispensable to USP4-mediated oncogenic activity in colorectal cancer.

USP4 stabilizes PRL-3 protein expression

It was here observed that PRL-3 is required for USP4-mediated oncogenic activity, which prompted examination of whether USP4 could regulate PRL-3 expression. Colorectal cancer cells, including RKO and LS174T, were transfected with increasing amounts of USP4 plasmid, and Western blotting indicated that USP4 could largely increase the endogenous PRL-3 protein level in a dose-dependent manner (Fig. 5A). Similarly, overexpression of USP4 markedly increased exogenous PRL-3 expression (Fig. 5B). Inversely, inhibition of USP4 expression by siRNA significantly decreased endogenous (Fig. 5C) and exogenous (Fig. 5D) PRL-3 protein expression, suggesting that USP4 could positively regulate PRL-3 expression. The next task was to determine whether USP4 regulates PRL-3 expression at the transcriptional level. SW480 cells were transfected with USP4-expressing vector, and then PRL-3 transcription was examined by qPCR. No significant change in the PRL-3 mRNA level was detected (Supplementary Fig. S6A). Knockdown of USP4 by shRNA in colorectal cancer cells also did not affect the PRL-3 mRNA level

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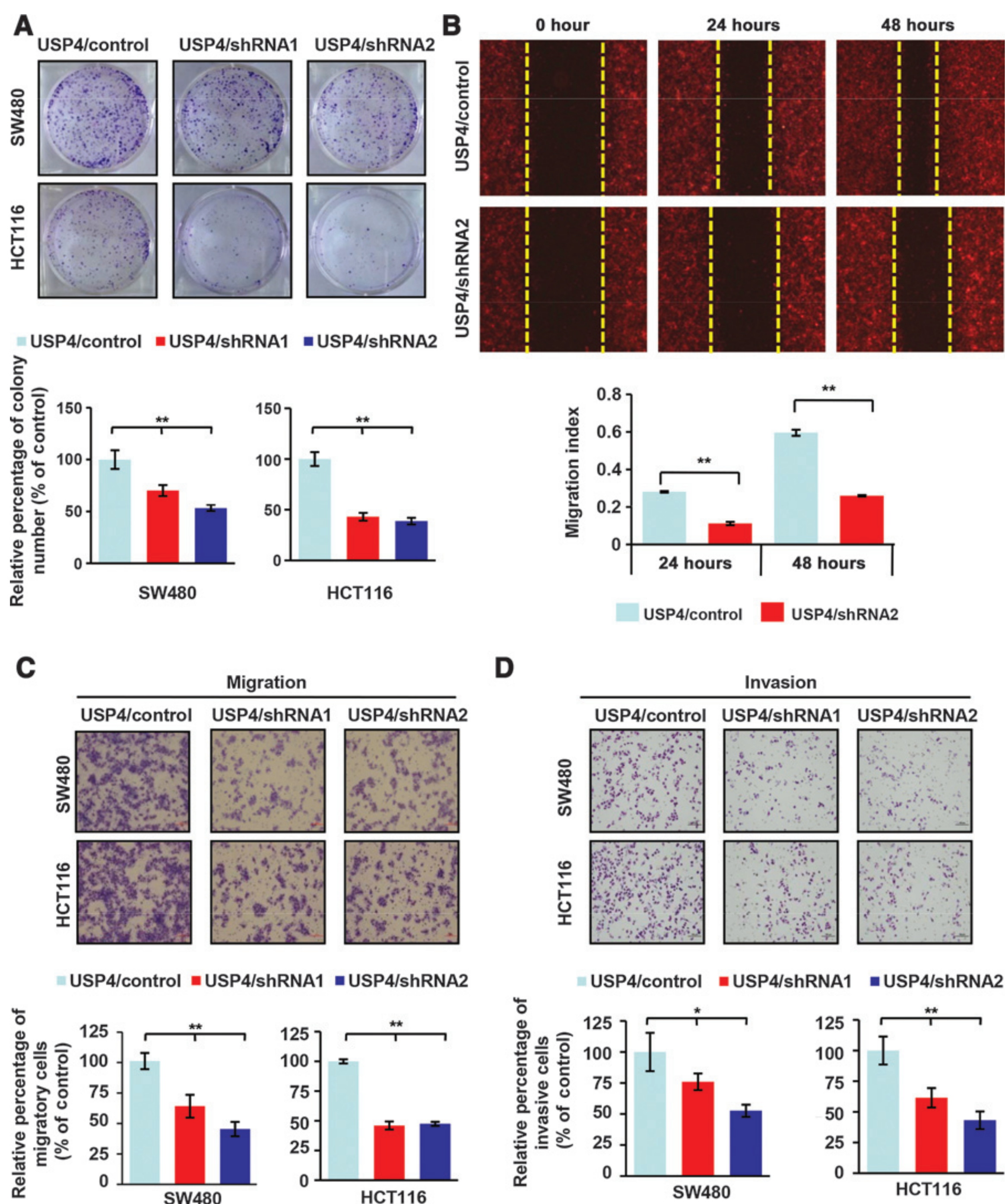

Figure 2.
Knockdown of USP4 suppresses the growth, migration, and invasion of colorectal cancer cells *in vitro*. A, colony formation assays were performed in wild-type cells (USP4/control) and in cells with stable knockdown of USP4 (USP4/shRNA; top), the relative number of colonies was quantified (bottom). Error bars, mean ± SD from three biological replicates. Statistical significance was determined by one-way ANOVA. **, $P < 0.01$. B, USP4 is required for colorectal cancer cell migration. SW480 wild-type cells (USP4/control) and SW480 cells USP4 knockdown (USP4/shRNA) were wounded by a 20 μL plastic pipette tip and cultured in serum-free medium for the indicated time, and cell migration into the wounded area was evaluated (top). Migration index was calculated (bottom). Error bars, mean ± SD. Statistical significance was determined using a two-tailed, unpaired Student *t* test. **, $P < 0.01$. C, migration assays were performed in SW480 and HCT116 cells (top), and the relative percentage of migratory cells was calculated (bottom). Error bars, mean ± SD from three biological replicates. Statistical significance was determined using one-way ANOVA. **, $P < 0.01$. D, a Matrigel invasion assay was performed in SW480 and HCT116 cells (top), and the relative percentage of invasive cells was calculated (bottom). Error bars, mean ± SD from three biological replicates. Statistical significance was determined by one-way ANOVA. *, $P < 0.05$; **, $P < 0.01$.

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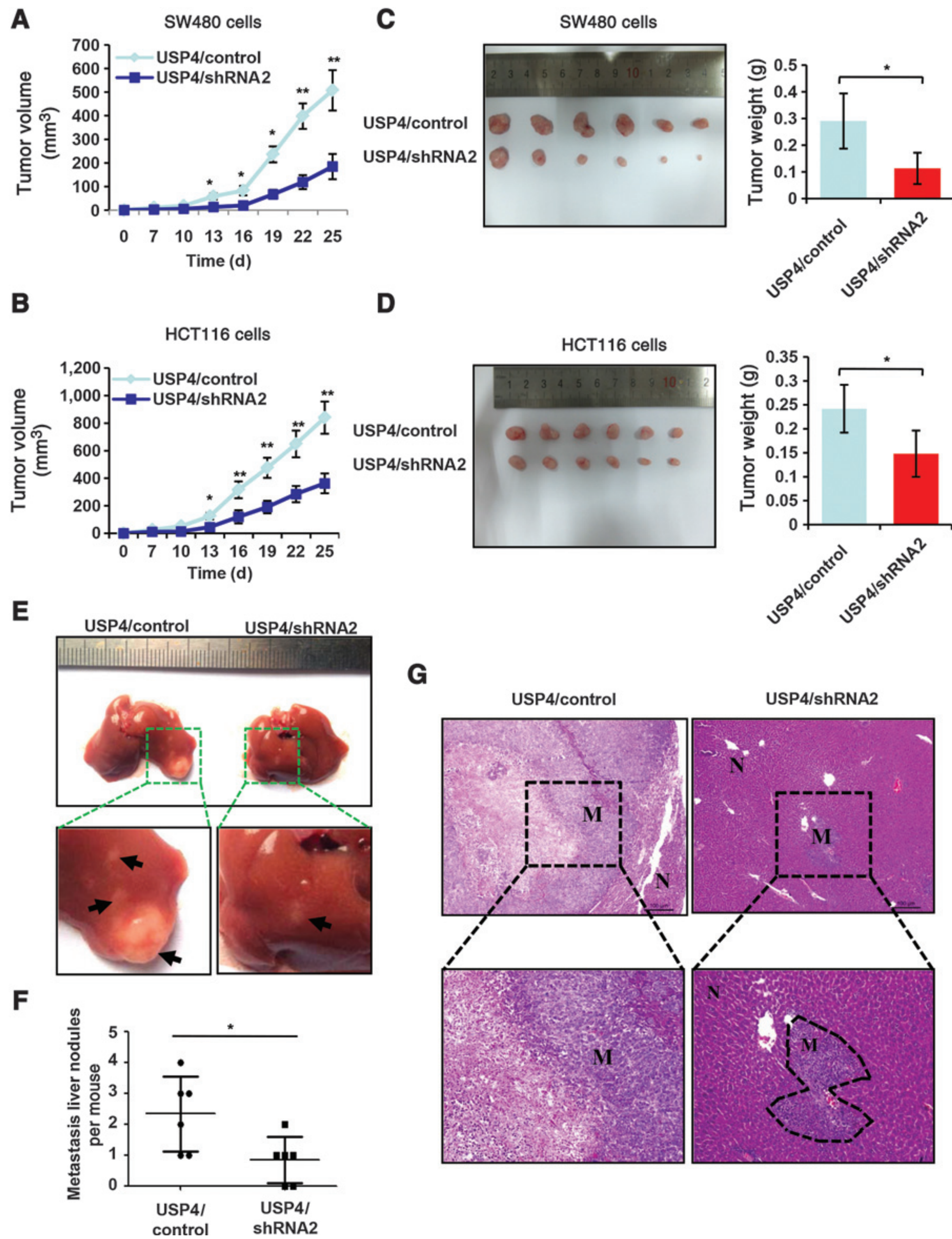

Figure 3.
Oncogenic activity of USP4 *in vivo*. A–D, knockdown of USP4 suppresses colorectal cancer tumorigenicity *in vivo*. Subcutaneous tumor regeneration with SW480 and HCT116 cells was assessed in nude mice ($n = 6$ per group). A and B, the volume of the generated tumors was measured during the indicated period. C (left) and D (left), photographs of tumors at autopsy are presented. C (right) and D (right), the tumor weight was noted. E and F, USP4 depletion suppresses hepatic metastasis in an orthotopic metastatic mouse model of colorectal cancer. E, representative photographs of liver with metastatic nodules are shown (arrowheads). F, metastatic nodules in the livers of mice were counted ($n = 6$ per group). G, representative micrographs of liver tissues with metastatic cells are shown using H&E staining at a magnification of ×40 (top) and ×100 (bottom). M, metastatic lesion; N, adjacent normal liver tissue. Error bars, mean ± SD. Statistical significance was determined by a two-tailed, unpaired Student *t* test. *, $P < 0.05$; **, $P < 0.01$.

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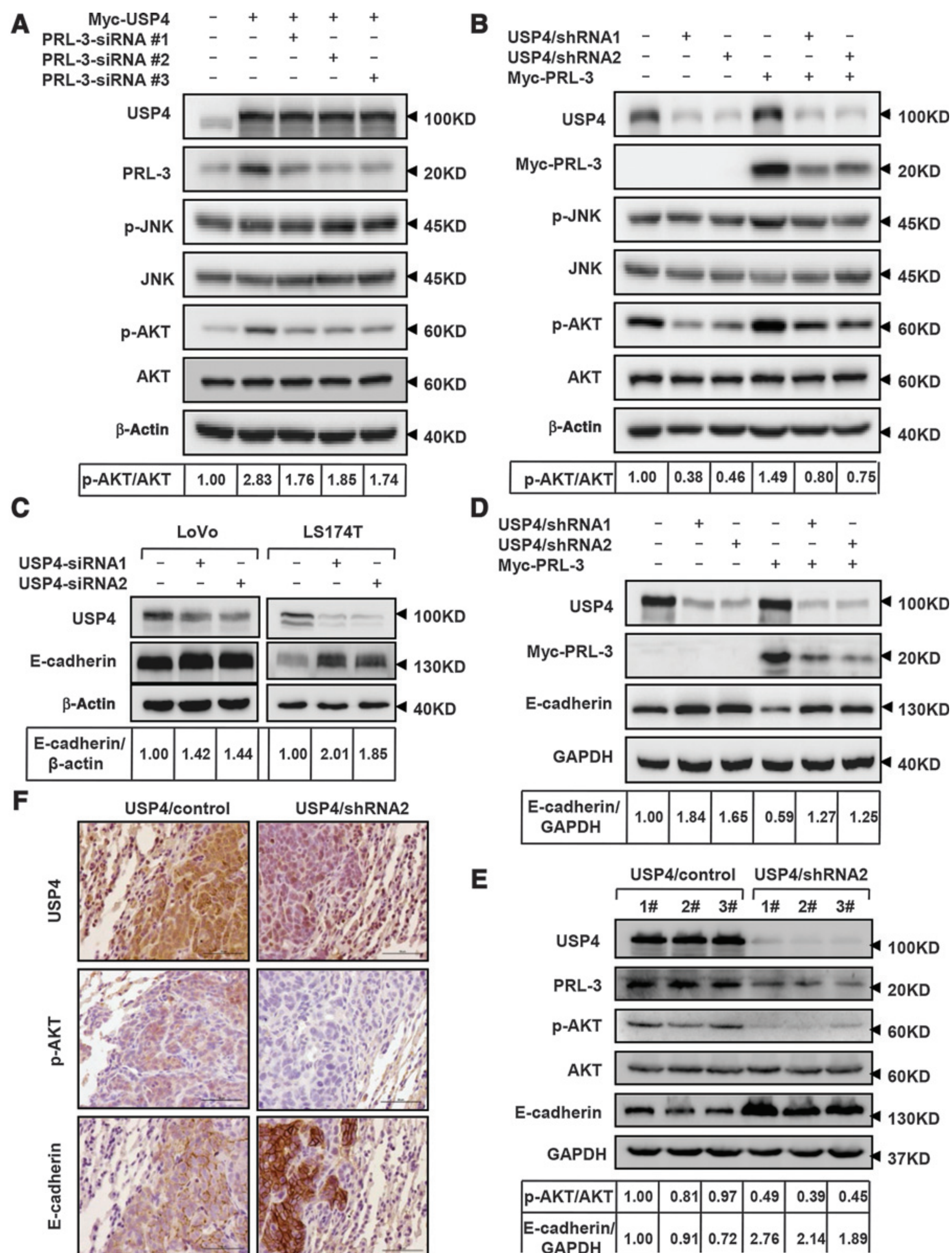

Figure 4.

Association of USP4 with PRL-3–dependent Akt activation and E-cadherin reduction. A, immunoblotting of the indicated protein in USP4-overexpressed HCT116 cells with and without PRL-3 siRNA. B, immunoblotting of the indicated protein in USP4 shRNA-transduced SW480 cells in the presence and absence of PRL-3. C and D, immunoblotting of USP4 and E-cadherin in LoVo and LS174T cells (C) and in SW480 cells (D) overexpressing PRL-3 in the presence and absence of USP4 siRNA or shRNA. E, immunoblotting of the indicated protein in tumors. Six USP4/control and six USP4/shRNA xenografts were randomly selected for determination of the indicated protein. F, immunohistochemical staining of USP4, p-Akt, and E-cadherin in the metastatic tumors derived from SW480/USP4/control and SW480/USP4/shRNA cells. Magnified (×400) images of representative staining are shown. The experiments were performed three times, and the representative images of blots are shown. Western blot analyses were quantified via densitometry, and the mean ratios of the indicated protein from three independent experiments are shown at the bottom of the figure.

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(Supplementary Fig. S6B). These data indicate that the regulation of PRL-3 expression by USP4 must occur at the protein level.

To address whether and how USP4 stabilizes PRL-3, RKO cells were treated with cycloheximide (CHX), an inhibitor of protein synthesis, and the stability of endogenous PRL-3 was monitored. As shown in Fig. 5E, the half-life of PRL-3 was increased from 4.5 hours to about 8 hours upon overexpression of USP4. However, knockdown of USP4 in SW480 cells significantly reduced the half-life of PRL-3 (Fig. 5F). These results suggest that USP4 enhances PRL-3 stability by blocking protein degradation.

USP4 interacts with and deubiquitinates PRL-3

Next, the ability of USP4 to interact with PRL-3 was investigated. Myc-USP4 and GFP-PRL-3 were coexpressed in HEK293T cells. Coimmunoprecipitation analysis showed GFP-PRL-3 to be coimmunoprecipitated with Myc-USP4 by anti-Myc antibody (Fig. 6A, top). Similarly, immunoprecipitation of GFP-PRL-3 by anti-GFP antibody led to coimmunoprecipitation of Myc-USP4 (Fig. 6A, bottom). Endogenous USP4 and PRL-3 were immunoprecipitated from SW480 cells and the presence of endogenous PRL-3 and USP4 was detected (Fig. 6B). Together, these results showed that USP4 can interact with PRL-3.

Because USP4 mediates removal and processing of ubiquitin, it was here speculated that USP4 directly regulates PRL-3 protein stability through interaction with and deubiquitination of PRL-3. To assess this possibility, GFP-PRL-3 and HA-ubiquitin were coexpressed with and without Myc-USP4 in HEK293T cells. Immunoblotting showed the ubiquitination of PRL-3 to be strongly inhibited by USP4 expression in the presence or absence of MG132, a potent inhibitor of the 26S proteasome (Fig. 6C). Knockdown of USP4 expression did the opposite (Fig. 6D). Collectively, these results indicate that USP4 is a DUB that controls the level of PRL-3 protein through interaction with and deubiquitination of PRL-3.

USP4 protein expression is positively correlated with PRL-3 protein expression in colorectal cancer cell lines and colorectal cancer tissues

To further examine the relationship between USP4 and PRL-3, the expression of USP4 and PRL-3 was analyzed in colorectal cancer tissues. Unfortunately, no correlation was observed in the expression of USP4 and PRL-3 at the mRNA level in these tissues (Supplementary Fig. S7A). Consistent with these results, there was no correlation in the mRNA expression of USP4 and PRL-3 in eight colorectal cancer cell lines (Supplementary Fig. S7B). However, results showed that the protein levels of USP4 and PRL-3 were positively correlated in the majority of these colorectal cancer cell lines (Fig. 7A and B). Similar results were obtained in clinical colorectal cancer tissues. Immunohistochemical staining showed that the high levels of USP4 expression were associated with high PRL-3 expression in Case 1. Inversely, low USP4 expression was associated with low PRL-3 expression in Case 2 (Fig. 7C). Spearman rank correlation analysis also further confirmed that USP4 protein expression was positively associated with PRL-3 protein expression (Fig. 7D, left) but not PRL-3 mRNA expression (Fig. 7D, right). In agreement with these observations, a significant positive correlation was also noticed between USP4 protein and PRL-3 protein in xenografts (Supplementary Fig. S8). Together, these results positively correlated expression between USP4 and PRL-3 protein expression but not mRNA transcript levels, strengthening our observation that USP4 serves as a deubiquitinating enzyme to control the protein level of PRL-3.

Discussion

Recent evidence confirmed a critical role for USP4 in regulating p53 and TGFβ signaling, implicating dysregulation of USP4 expression in the development of cancer (11, 12). However, the expression profile and the role of USP4 in colorectal cancer remain unclear. In this study, results showed USP4 overexpression in 67% of the human colorectal cancer tissues and all colorectal cancer cell lines examined, suggesting that USP4 may have a role in colorectal cancer progression. Results showed that downregulation of USP4 significantly impaired colorectal cancer cell proliferation, colony formation, and invasion *in vitro* and markedly inhibited tumor growth and metastasis *in vivo*, indicating that USP4 plays a critical role in colorectal cancer progression. The current results are consistent with previous reports indicating that USP4 overexpression that occurs in many human cancers, including prostate cancer, liver cancer, urinary cancer, thyroid cancer, and neck squamous cell carcinoma, contributes to their progression (11, 13, 34). In this way, this study, and those of others, point to the potential oncogenic function for USP4 in regulating tumorigenesis. Paradoxically, USP4 is frequently decreased in human epithelial tumors such as carcinomas of the breast, lung, kidney, and ovary, and USP4 antagonizes the migration of lung cancer cells (11, 14). Furthermore, USP4 has been shown to be a negative regulator of the canonical Wnt signal pathway. It is well known to have tumorigenesis activity (35). Thus, these data indicated that USP4 may act as a tumor suppressor. Collectively, these data suggest that USP4 is involved in cancer and its potential functions in cancer progression depend on cancer type.

It is concluded that USP4 acts as a tumor-promoting protein in colorectal cancer, and the USP4/PRL-3 axis is critical to controlling the progression of colorectal cancer. Modulation of USP4 expression by overexpression or siRNA methods caused a marked change in Akt phosphorylation and E-cadherin expression, critical regulators of cancer cell growth, and metastasis. These data indicate that USP4 initiates colorectal cancer growth and metastasis via regulation of Akt and E-cadherin. These events could be reversed by PRL-3, suggesting that the regulation of Akt phosphorylation and E-cadherin loss by USP4 is dependent on PRL-3. As a recent study reported a critical role of Akt in regulation of USP4 localization and activity (12). These data together with the current results indicate that there is a regulatory loop among USP4, PRL-3, and Akt, in which PRL-3 may be involved in the regulation of the Akt/USP4 signaling axis through its protein tyrosine phosphatase activity. However, the potential regulatory role of PRL-3 in Akt-mediated USP4 signaling remains to be established in the future.

In this study, results further indicated that USP4 was the key regulatory factor responsible for PRL-3 stabilization and that knockdown of USP4 decreased the protein level of PRL-3 and suppressed colorectal cancer cell growth and invasion *in vitro* and tumorigenicity *in vivo*. These observations are compatible with the idea that PRL-3 acts as an oncogenic regulator to promote cancer development. Elevated PRL-3 expression is associated with the growth, metastatic potential, and poor prognosis of multiple cancer types, including colorectal cancer, breast cancer, gastric cancer, and lung cancer. It has been shown to increase the activity of multiple progrowth and

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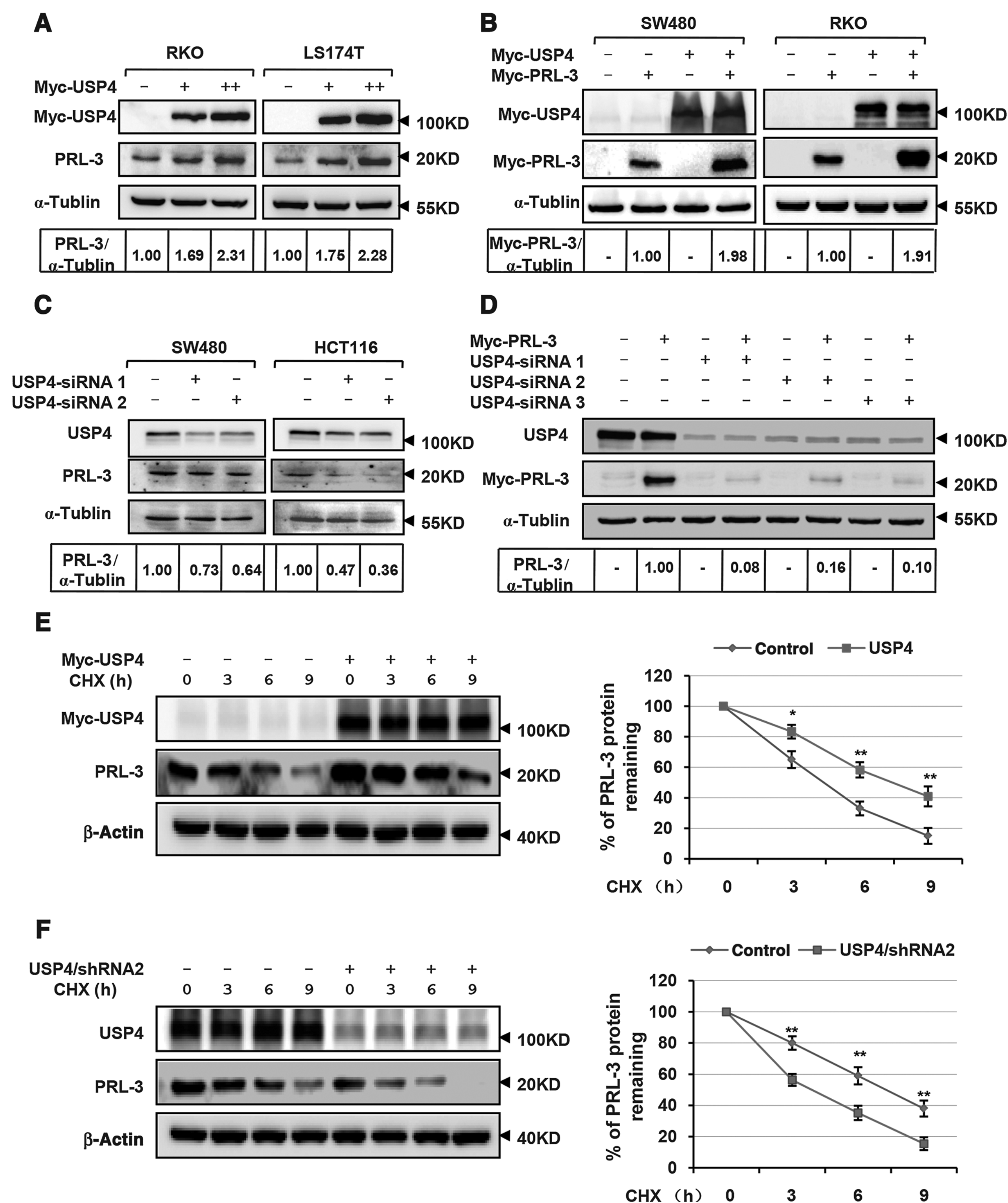

Figure 5.

USP4 Stabilizes PRL-3. A and B, overexpressed USP4 increases endogenous and exogenous PRL-3 expression. A, immunoblotting of endogenous PRL-3 in RKO and LS174T cells expressing increasing amounts of USP4. B, immunoblotting of exogenous PRL-3 in SW480 and RKO cells coexpressing PRL-3 and the increasing amounts of USP4. C and D, knockdown of USP4 decreases endogenous and exogenous PRL-3 expression. C, SW480 and HCT116 cells were transfected with control or USP4 siRNA, and expression of endogenous USP4 and PRL-3 was analyzed by immunoblotting. D, immunoblotting of exogenous PRL-3 in WT and USP4-silenced SW480 cells with or without overexpressed PRL-3. E and F, USP4 enhances the stability of endogenous PRL-3 protein. E, Myc-USP4 was transfected into RKO cells. (*Continued on the following page.*)

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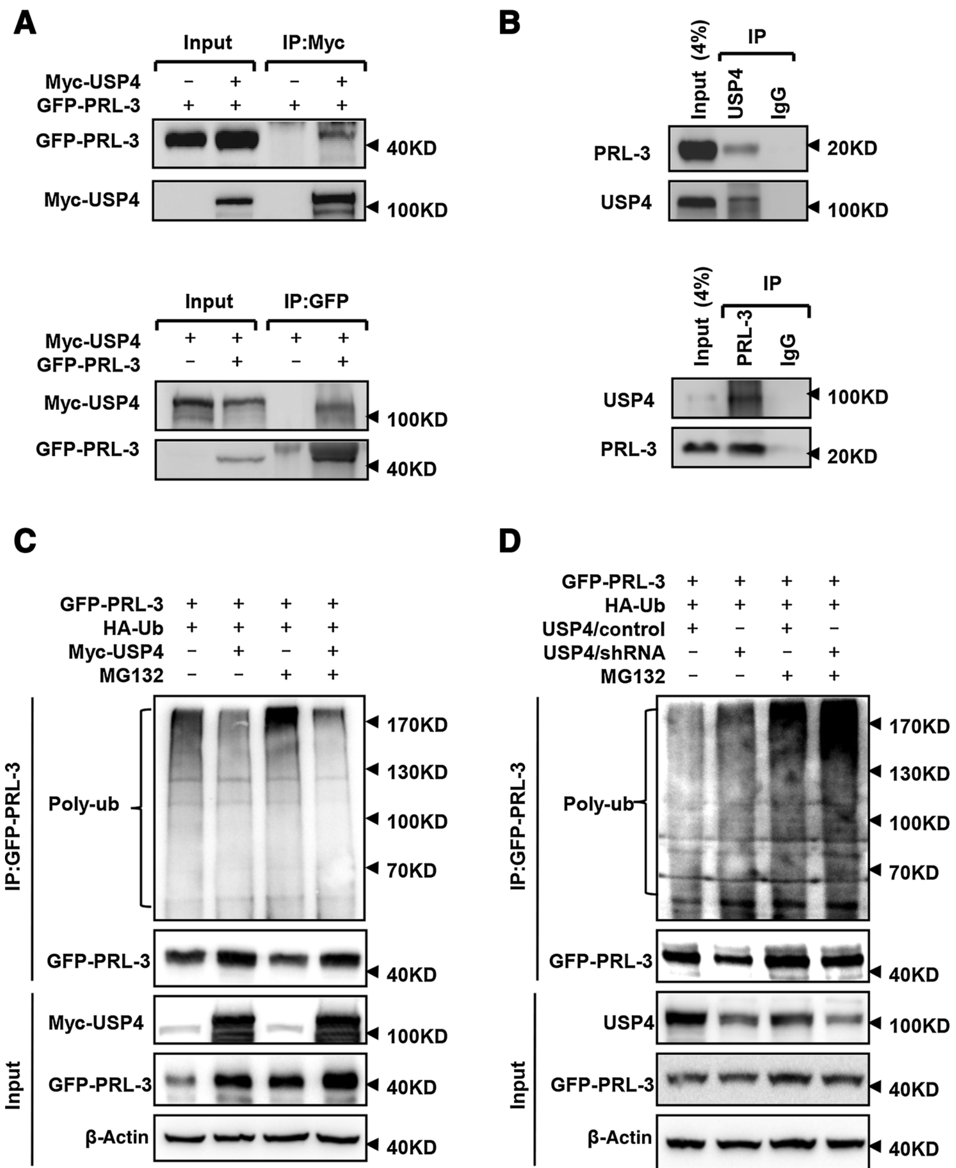

Figure 6.
USP4 interacts with and deubiquitinates PRL-3. A, USP4 interacts with PRL-3 at exogenous levels. Immunoblotting analysis of lysates after immunoprecipitation from HEK293T cells transfected with Myc-USP4 and GFP-PRL-3. B, USP4 interacts with PRL-3 at endogenous levels. Cell lysates from SW480 cells were immunoprecipitated with anti-USP4 or anti-PRL-3 antibody, followed by immunoblotting with anti-PRL-3 or anti-USP4 antibody, respectively. IgG was used as a control. C, GFP-PRL-3 and HA ubiquitin were coexpressed with Myc-USP4 in HEK293T cells. After cells were treated with and without 10 mmol/L MG132 for 6 hours, PRL-3 was immunoprecipitated with anti-GFP antibody, and the polyubiquitination of PRL-3 was detected by immunoblotting. D, GFP-PRL-3 and HA ubiquitin were coexpressed in SW480 cells stably transfected with control or USP4 shRNA for 24 hours, and then cells were treated with and without 10 mmol/L MG132 for 6 hours. Extracts were immunoprecipitated with anti-GFP antibody, and the polyubiquitination of PRL-3 was examined by immunoblotting using anti-HA antibody. The experiments were repeated three times, and representative images of blots are shown.

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prometastatic signaling pathways and induce the conversion of key events such as PI3K/Akt and EMT in cancer development (19, 27, 28, 36–38).

One important finding reported here is the mechanism of PRL-3 regulation by USP4 in colorectal cancer. The present and previous studies have shown PRL-3 to be transcriptionally regulated by some proteins, including STAT3, p53, and Snail (39–41). However, regulation of PRL-3 protein stability and turnover remains unknown. In the current study, results show that overexpression of USP4 stabilizes PRL-3 protein by binding to and deubiquitinating PRL-3 in colorectal cancer. These findings are consistent with the hypothesis that regulation of PRL-3 by USP4 is a posttranslational event. Examination of colorectal cancer cell lines and clinical colorectal cancer samples confirmed that USP4 and PRL-3 protein expression were closely correlated but mRNA transcription levels were not. The human genome encodes limited numbers of DUBs (~100), indicating that each DUB may have several substrates. USP4 has

(*Continued.*) After treating cells with cyclohexamide (CHX; 100 μg/mL) for indicated time intervals, expression of PRL-3 and USP4 was examined by immunoblotting (left) using the indicated antibodies. The intensity of endogenous PRL-3 expression for each time point was quantified by densitometry (right). F, shRNA-transduced SW480 and control cells were treated with CHX (100 μg/mL) for the indicated time intervals. The endogenous PRL-3 expression levels were assessed by immunoblotting (left) and quantified by densitometry (right). The experiments were repeated three times, and the representative images of blots are shown. Western blot analyses were quantified via densitometry, and the mean ratios of the indicated protein from three independent experiments are shown at the bottom of the figure. Error bars, mean ± SD. Statistical significance was determined by a two-tailed, unpaired Student *t* test. *, $P < 0.05$; **, $P < 0.01$.

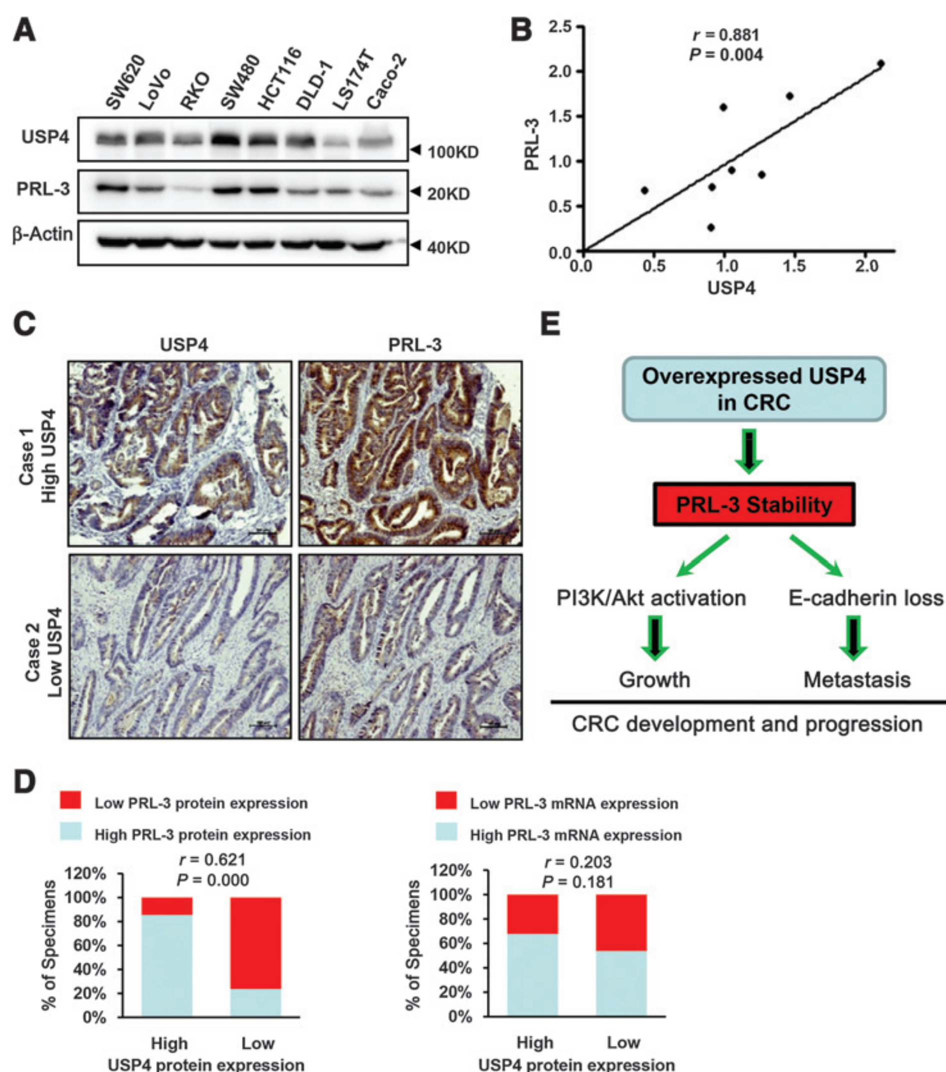

Figure 7.
USP4 and PRL-3 protein expression positively correlate in colorectal cancer (CRC) cells and clinical colorectal cancer tissues. A, immunoblotting analysis was performed to detect the expression of USP4 and PRL-3 in colorectal cancer cell lines. B, Spearman correlation analysis between USP4 and PRL-3 protein levels in colorectal cancer cell lines. C, immunohistochemical staining of USP4 and PRL-3 in human colorectal cancer tissues. Representative images of immunohistochemical staining from the same tumor samples are shown. D, Spearman correlation analysis between USP4 protein levels and PRL-3 protein levels (left) or PRL-3 mRNA levels (right) in 45 cases of colorectal cancer tissues. E, schematic representation of the function and potential mechanism of USP4 in colorectal cancer.

been shown to interact with and deubiquitinate several proteins, such as TRAF2, TRAF6, TAK1, TβRI, ARF-BP1, and RIP1 (9–12, 34). However, continuing to identify novel USP4 substrates remains a significant subject of interest. Here, PRL-3 was identified as a novel substrate for USP4. Overexpression of USP4 significantly impaired PRL-3 ubiquitination, and knockdown of USP4 markedly enhanced PRL-3 ubiquitination. Like the interaction of USP4 with ARF-BP1 (11), USP4 interacted with and deubiquitinated PRL-3, leading to the stabilization of PRL-3 and subsequently promoted colorectal cancer progression. Unsurprisingly, in its role as the substrate of USP4, PRL-3 expression was positively correlated with USP4 expression in colorectal cancer cell lines and clinical colorectal cancer samples. For this reason, it is hypothesized that USP4 drives colorectal cancer growth, invasion, and metastasis through a mechanism wherein USP4 stabilizes PRL-3 via binding with and deubiquitinating PRL-3.

In summary, the current study indicates that USP4 is a key modulator in facilitating colorectal cancer development and progression. A critical mechanism for USP4 in the regulation of colorectal cancer development and progression through interaction with and deubiquitination of PRL-3 was found here. This may highlight a new therapeutic opportunity for preventing and treating colorectal cancer by targeting the USP4/PRL-3 signaling axis.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): C. Xing, H. Wu, J.-M. Li
Writing, review, and/or revision of the manuscript: H. Wu, J.-M. Li
Study supervision: H. Wu, J.-M. Li

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