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Biology of Human Tumors

Clinical Cancer Research

RING-Finger Protein 6 Amplification Activates JAK/STAT3 Pathway by Modifying SHP-1 Ubiquitylation and Associates with Poor Outcome in Colorectal Cancer

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Abstract

Objective: The E3 ubiquitin ligase RNF6 (RING-finger protein 6) plays a crucial role in carcinogenesis. However, the copy number and expression of RNF6 were rarely reported in colorectal cancer. We aimed to explore the mechanical, biological, and clinical role of RNF6 in colorectal cancer initiation and progression.

Design: The copy number and expression of RNF6 were analyzed from Tumorscape and The Cancer Genome Atlas (TCGA) datasets. Gene expressions were examined by real-time PCR, Western blot, and immunohistochemical staining. Gene expression profiling studies were performed to identify pivotal genes regulated by RNF6. Biological function of RNF6 on tumor growth and metastasis was detected *in vivo* and *in vitro*. Role of RNF6 in modulating SHP-1 expression was examined by coimmunoprecipitation and confocal microscopy, respectively.

Results: The copy number of RNF6 was significantly amplified in colorectal cancer, and the amplification was associated with RNF6 expression level. Amplification and overexpression of RNF6 positively correlated with patients with colorectal cancer with poor prognosis. The gene set enrichment analysis (GSEA) revealed cell proliferation, and invasion-related genes were enriched in RNF6 high-expressed colorectal cancer cells as well as in patients from TCGA dataset. Downregulation of RNF6 impaired the colorectal cancer cell proliferation and invasion *in vitro* and *in vivo*. RNF6 may activate the JAK/STAT3 pathway and increase pSTAT3 levels by inducing the ubiquitination and degradation of SHP-1.

Conclusions: Genomic amplification drives RNF6 overexpression in colorectal cancer. RNF6 may be a novel biomarker in colorectal carcinogenesis, and RNF6 may increase pSTAT3 level via promoting SHP-1 ubiquitylation and degradation. Targeting the RNF6/SHP-1/STAT3 axis provides a potential therapeutic option for RNF6-amplified tumors. *Clin Cancer Res; 24(6); 1473–85. ©2017 AACR.*

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Transcript Profiling: The RNA sequence data have been deposited in NCBIs Gene Expression Omnibus and are accessible through GEO Series accession number GSE107980.

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Introduction

Colorectal cancer is the second most commonly diagnosed cancer in females and the third most common in males (1). Although the increased uptake of screening and removal of precancerous adenomas decrease colorectal cancer incidence in the United States (2), the incidence is still increasing in several Asian and European countries owing to a prevalence of risk factors for colorectal cancer, such as unhealthy diet, obesity, and smoking (3). Moreover, colorectal cancer mortality rates are still high in countries that have increasing incidence and limited resources. With the rapid development of genetic knowledge and technologies, some biomarkers concerning with the initiation, progression, and metastasis of colorectal cancer have been utilized to predict clinical parameters including survival (4, 5). Novel diagnostic, prognostic, and treatment biomarkers still need to be explored and be applied to improve tumor behaviors and patient survival.

Ubiquitylation is one of the posttranslational modifications, which has a crucial role in the degradation of short-lived regulatory proteins including many oncogene products and tumor suppressors (6). This modification regulates degradation of cellular proteins by the ubiquitin–proteasome system (UPS), and

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Translational Relevance

Colorectal cancer is one of the most common cancers in the world. The E3 ubiquitin ligases play an important role in gene regulatory pathways for various human diseases, including cancer. DNA copy-number variation (CNV) is a large kind of genome variations in the human genome. CNVs of the E3 ubiquitin ligases have been reported to be associated with colorectal cancer occurrence and development. In this study, we found an E3 ubiquitin ligase containing a RING-finger domain, RNF6, which is amplified and overexpressed in colorectal cancer cells as well as in patients. RNF6 is a functional and clinical marker for colorectal cancer progression and may be a potential target for therapeutic intervention. RNF6 may act as a novel E3 ubiquitin ligase to facilitate the ubiquitination and elimination of SHP-1, and then further increases the phosphorylation level of STAT3, ultimately causing colorectal cancer malignance. Therefore, our work is highly innovative and scientifically interesting to the biomedical field in general.

this process involves the sequential action of ubiquitin-activating enzymes (E1s), ubiquitin-conjugating enzymes (E2s), and ubiquitin ligases (E3s; ref. 7). Of the UPS components, the E3 ubiquitin ligase, which can recognize substrates with the most specific, has been regarded as the potential diagnosis and therapeutic target in cancer (8). As the major components of E3s, RING finger and RING finger–related E3s are involved in either the suppression or the progression of cancer (9). The RING-finger protein 6 (RNF6) was mapped to chromosome 13q12 (10), containing a coiled-coil domain at the N-terminus and a RING-finger domain at the C-terminus which contributes to its E3 ubiquitin ligase activity (11). RNF6 has been found as an oncogene in several cancers, such as breast cancer (12), leukemia (13), prostate cancer (14), lung adenocarcinoma (15), and esophageal squamous cell carcinoma (16). However, the pathologic and clinical role of RNF6 has not been revealed in colorectal cancer.

DNA copy-number variation (CNV) is a large kind of genome variations in the human genome (17, 18). Accumulating reports illuminate the hypothesis that CNV could be used as a molecular biomarker for cancer diagnosis and prognosis (19), e.g., amplification of oncogenes or deletion of tumor suppressors can lead to tumorigenesis (20). A large proportion of tumors contain copy-number gains and amplifications of several oncogenes, such as BRAF, which is known to be amplified in lung squamous cell carcinoma (21) and in ovarian carcinoma (22); FGFR2, which is amplified in gastric cancer (23). CNVs of the E3 ubiquitin ligases have been reported to be associated with colorectal cancer occurrence and development (24, 25). As whole-genome sequencing (WGS) becomes more accessible, there are opportunities to characterize the CNVs of E3 ubiquitin ligases with more accurate and efficient algorithms than before.

In this study, we first reported that RNF6 CNV is common and associated with poor prognosis in patients with colorectal cancer. Functionally, RNF6 promotes the proliferation and metastasis of colorectal cancer cell *in vivo* and *in vitro*. Mechanistically, RNF6 facilitates the ubiquitination and elimination of SHP-1 by interacting with SHP-1, and further increases the phosphorylation level of signal transducer and activator of transcription 3 (STAT3), ultimately causing colorectal cancer malignance.

Materials and Methods

Patient samples

We have studied three cohorts of patients with colorectal cancer from Renji Hospital affiliated to Shanghai Jiaotong University School of Medicine (Shanghai, China) between 2012 and 2016. These cohorts comprise randomly selected cohort 1 from the West campus of Renji Hospital with 62 fresh tissues, cohort 2 from the East campus of Renji Hospital, and cohort 3 from the South campus of Renji Hospital with 78 and 97 formalin-fixed paraffin-embedded tissues, respectively. Patients were pathologically and clinically diagnosed as colorectal cancer. Tumor–node–metastasis staging was based on pathology reports and histologic slices. All the research was carried out in accordance with the provisions of the Declaration of Helsinki of 1975. Cases that received preoperative radiochemotherapy before surgical resection were excluded. And after surgical resection, patients received adjuvant treatments according to physicians' advices. Dates of death were retrieved from medical writings or telephone follow-up. This study was approved by the ethics committee of Shanghai Jiao Tong University School of Medicine, Renji Hospital.

Bioinformatics analysis and high-throughput sequencing

The DNA copy-number data were analyzed from The Cancer Genome Atlas (TCGA) dataset (http://www.cbioportal.org/) and Tumorscape (http://portals.broadinstitute.org/tumorscape). The effect of RNF6 CNV on expression was evaluated with one-sided Jonckheere–Terpstra test. The Kaplan–Meier curve comparing survival of patients with colorectal cancer with or without copy-number alterations of RNF6 was estimated using the log-rank test. The detailed RNA sequencing and bioinformatics analysis were described in the Supplementary Materials and Methods.

CNV detection

DNA samples were extracted from 62 (cohort 1), 78 (cohort 2), and 97 (cohort 3) colorectal cancer samples and the adjacent nontumor tissues using Tguide S32 Magnetic Tissue Genomic DNA Kit (TIANGEN). The RNF6 gene copy numbers were detected by using the QX200 Droplet Digital PCR Assays. A 20 μL ddPCR reaction mix was created to generate droplets by a QX200 Droplet Generator (BIO-RAD). The RNF6 reaction volume contained 30 ng DNA, 10 μL Probe SuperMix, 1.8 μL RNF6 F&R primer, 0.5 μL target probe (sequence: 5′-CAGAGA-CAGAGTGGCAC-3′), and 4.9 μL water. The reference reaction system contained 10 μL Probe SuperMix, 1 μL copy-number reference, 1 μL DNA, and 8 μL water. The quantitative assays were performed using the T100 Polymerase Chain Reaction machine (BIO-RAD) after the droplets were transferred into a 96-well plate. The reaction was implemented using the following cycling conditions: 95°C for 10 minutes, 94°C for 30 seconds, and 60°C for 1 minute for 40 cycles, 98°C for 10 minutes, and 4°C holding. After PCR completing, we loaded the 96-well plate into a QX200 Droplet Reader (BIO-RAD) to read positive and negative droplets. Positive droplets containing at least one copy of RNF6 presented stronger fluorescence than negative droplets. We analyzed concentrations with QuantaSoft software v1.4 to estimate RNF6 copy numbers in every sample.

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Cell culture and treatment

Human colorectal cancer cell lines RKO, SW1116, SW480, Caco2, LoVo, HT29, and HCT116 were purchased from the American Type Culture Collection. All cell lines were genotyped for identity by Beijing Microread Genetics Co., Ltd and tested routinely for Mycoplasma contamination (last date of testing: January 3, 2017). Cells were cultured and treated as described in detail in the Supplementary Materials and Methods section.

Fluorescence in situ hybridization assay

The FISH assay was performed in colorectal cancer tissue slides to detect the *RNF6* gene CNV. The detailed FISH assay was described in the Supplementary Materials and Methods.

In vivo experiments

To investigate the effect of RNF6 on tumor growth *in vivo*, 4-week-old male BALB/c nude mice were purchased from Slac Laboratory Animal. Mouse experiments were conducted in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. The study procedures were approved by the Institutional Animal Care and Use Committee of Renji Hospital, School of Medicine, Shanghai Jiaotong University. The detailed information of *In vivo* Experiments was described in the Supplementary Materials and Methods.

Statistical analysis

The detailed statistical analysis was described in the Supplementary Materials and Methods.

Results

RNF6 genomic amplification is prevalent in colorectal cancer and correlated with shortened patient survival

There are about 700 different RING E3 ligases, most of which are not well studied (26). As estimated by Sabscience, approximately 300 ubiquitin ligase genes could be regarded as potential drug targets. To evaluate the function of E3 ubiquitin ligases in colorectal cancer, 246 RNA microarrays of colorectal cancer patients from TCGA database were analyzed for 332 ubiquitin ligases genes. Differential expression analysis showed that 55 candidate ubiquitin ligases genes were increased (fold change > 1.25, $P < 0.005$) and 83 candidate ubiquitin ligases genes were decreased (fold change < 0.8, $P < 0.005$) in colorectal cancer tissues compared with adjacent tissues (Fig. 1A, Supplementary Fig. S1A, and Supplementary Table S1). Further CNV analysis revealed that 10 of 55 genes showed copy-number amplification in 608 patients with colorectal cancer from TCGA dataset (with amplification frequency $\geq$ 1%), and RNF6 showed the most significant gains (Fig. 1B). To explore more beneficial prognosis biomarkers for patients with colorectal cancer, we analyzed the correlation between the CNV and mRNA levels of the 10 genes with the clinical outcome in TCGA dataset. The Kaplan–Meier analyses showed that ASB9, BRCA2, CDC16, CUL4A, FBXL20, RNF24, and TCEB1 expressions have no predictive value for the clinical outcome of patients with colorectal cancer, whereas high expressions of UHRF2, RNF6, and SKP2 were significantly associated with a poor prognosis in these patients (Fig. 1C and Supplementary Fig. S1B). Further survival analysis showed that only CNV amplification of RNF6 was significantly associated with a poor prognosis in TCGA dataset, but not the other nine genes (Fig. 1D and Supplementary Fig. S1C). Because the mRNA expression and CNV of RNF6 are both significant as a prognostic marker in patients with colorectal cancer of TCGA dataset (Supplementary Fig. S1D), we focused our study on RNF6.

Next, the CNV of RNF6 was further analyzed in colorectal cancer tissues using data from TCGA colorectal cancer cohort and the Tumorscape cohort (http://portals.broadinstitute.org/tumorscape/pages/PortalHome.jsf). The RNF6 gene is located in a recurrently amplified region in chromosome 13 (Supplementary Fig. S1E), with 13 of 161 patients (8.1%) showing focal amplification and 83 of 161 patients (51.6%) showing broad amplification (FDR < 0.001) in Tumorscape dataset (Fig. 1E). This result was confirmed by TCGA colorectal cancer patient cohort, which reported 36 in 608 patients with colorectal cancer (6%) with focal amplification and 377 in 608 patients with colorectal cancer (62%) with broad colorectal cancer amplification of RNF6 gene (Fig. 1E). Further combined analysis on the mRNA and CNV data in the TCGA dataset revealed that gained CNV of RNF6 associated with significantly higher mRNA level in colorectal cancer (Fig. 1F). To validate the CNV data from Tumorscape and TCGA dataset, the expression level of RNF6 was first examined using qRT-PCR in 62 cases of patients with colorectal cancer of Renji hospital (cohort 1 fresh tissues, Supplementary Table S2). Real-time PCR revealed that RNF6 expression was remarkably increased in 35 of the 62 (56.5%) colorectal cancer tissues when compared with the paired adjacent normal samples ($P < 0.05$; Fig. 1G). Next, to confirm the bioinformatics data that upregulation of RNF6 in colorectal cancer tissues was partly caused by CNV of its coding sequence, we analyzed the copy number of RNF6 by real-time PCR in cohort 1, and cohort 2 to 3 paraffin-embedded tissues (Supplementary Tables S3 and S4), which were from different campus of Renji hospital. Real-time PCR showed that RNF6 amplification was detected in six of the 62 (9.7%) of cohort 1, six of the 78 (7.7%) of cohort 2, and eight of the 97 (8.2%) of cohort 3, respectively (Fig. 1H), and FISH analysis using probe targeting RNF6 also suggested that RNF6 is amplified in tumors from patients with colorectal cancer, relative to a centromere 13 probe (Supplementary Fig. S1F). The data indicate that RNF6 CNV was remarkably common in colorectal cancer. In addition, the prognostic value of RNF6 CNV was analyzed in cohorts 2 and 3 as well. Colorectal cancer cases with RNF6 copy-number amplifications exhibited significant association with poorer prognosis than those without alterations (Fig. 1I) in cohorts 2 and 3, which further supported TCGA data (Fig. 1D), suggesting that high expression level of RNF6 and shortened patient survival outcome in patients with colorectal cancer are copy-number driven.

RNF6 is clinically relevant in colorectal cancer

To evaluate the pathologic and clinical value of RNF6 with different clinicopathologic features, we next analyzed RNF6 mRNA expression in cohort 1. We found that RNF6 expression positively correlated with AJCC stage ($P < 0.05$), histologic differentiation ($P < 0.05$), and tumor size ($P < 0.05$; Fig. 2A), whereas no significant correlation was found with gender, age, tumor location, invasive depth, and vascular invasion.

To further validate the pathologic and clinical significance of RNF6 in colorectal cancer, we detected and compared RNF6 expression by immunohistochemical (IHC) staining assay in cohort 1 and two additional 78 and 97 paraffin-embedded colorectal cancer and adjacent tissues (cohort 2 and cohort 3).

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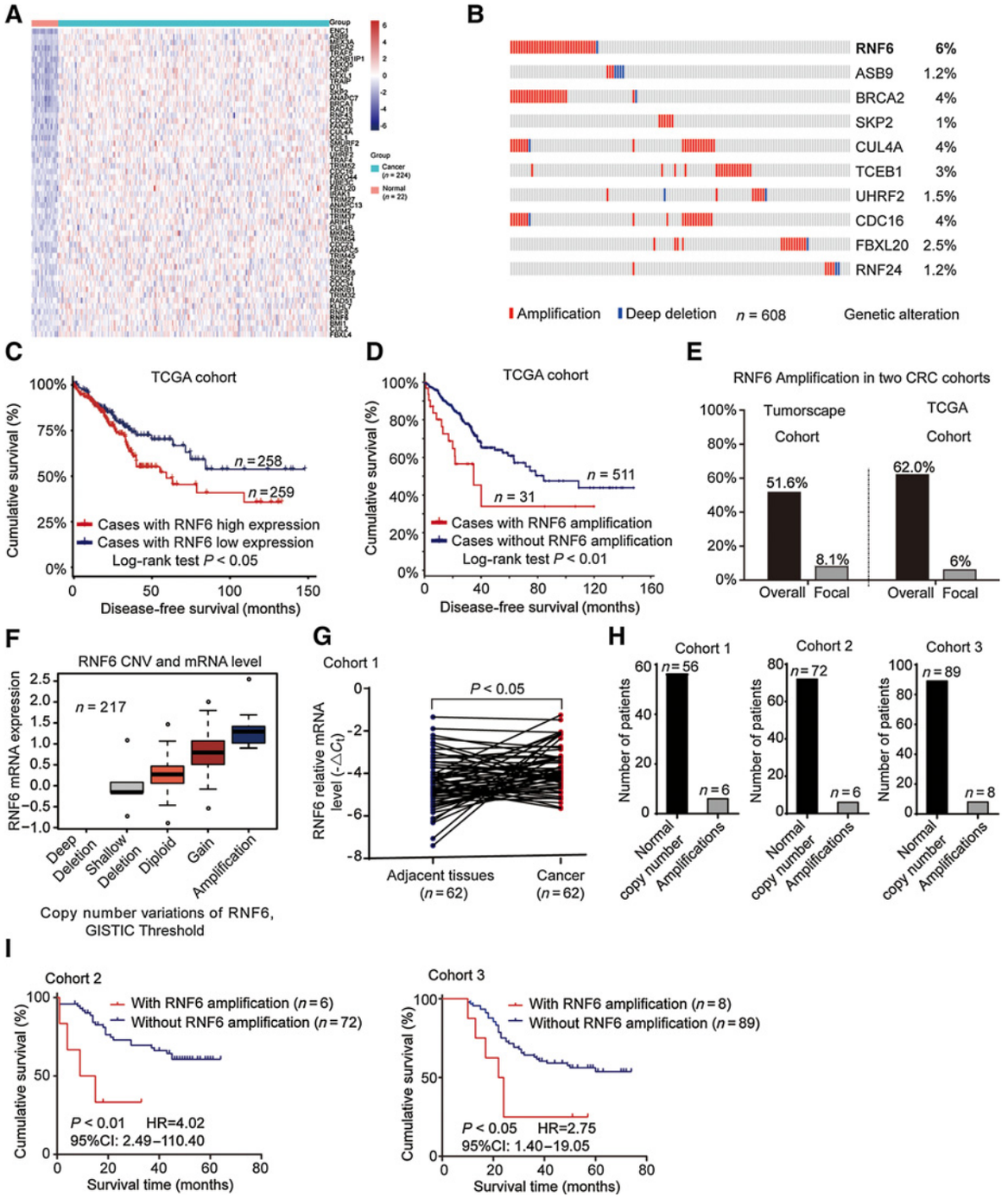

Figure 1.

RNF6 genomic amplification is prevalent in colorectal cancer and correlated with shortened patient survival. **A,** Analysis of 332 human E3 ubiquitin ligases gene expression of colorectal cancer in TCGA database [$n = 246$ (224 vs. 22), fold change > 1.25, limma package, $P < 0.005$]. **B,** Representative data of CNV analysis of 55 E3 ubiquitin ligases genes that upregulated in colorectal cancer tissues in TCGA database ($n = 608$, red bar indicates amplification, gray bar indicates normal copy number, and blue bar indicates deep deletion). **C,** High expression of RNF6 mRNA associated with poor disease-free survival in TCGA cohort ($n = 517$, log-rank test, $P < 0.05$). TCGA performed CNV analysis in 542 samples with DFS information (517 out of these samples had mRNA-Sequencing information). **D,** Amplification of RNF6 gene copy number associated with poor disease-free survival in TCGA cohort ($n = 542$, log-rank test, $P < 0.01$). **E,** The frequency of RNF6 amplification in Tumorscape and TCGA cohorts as indicated. **F,** RNF6 mRNA levels were significantly higher in samples with RNF6-gained CNA compared with the samples without CNV in the TCGA dataset ($n = 217$, one-sided Jonckheere–Terpstra test, $P < 0.01$). **G,** Statistical analysis of RNF6 mRNA expression in colorectal cancer and the paired adjacent normal tissues of Renji cohort 1 ($n = 62$, nonparametric Mann–Whitney test, $P < 0.05$). **H,** Statistical analysis of RNF6 copy number by real-time PCR in three independent Renji datasets (cohort 1, $n = 62$; cohort 2, $n = 78$; cohort 3, $n = 97$). **I,** Survival analysis was performed between patients with or without RNF6 amplification in two independent Renji datasets (cohort 2, $n = 78$, $P < 0.01$; cohort 3, $n = 97$, $P < 0.05$; log-rank test).

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RNF6 expression was higher in colorectal cancer tissues than adjacent tissues both in Renji cohorts 1 ($n = 62$, $P < 0.05$), 2 ($n = 78$, $P < 0.05$), and 3 ($n = 97$, $P < 0.05$; Fig. 2B–D; Supplementary Fig. S2A–S2C). The Kaplan–Meier analysis revealed that the high expression level of RNF6 was markedly associated with a poor prognosis of patients with colorectal cancer

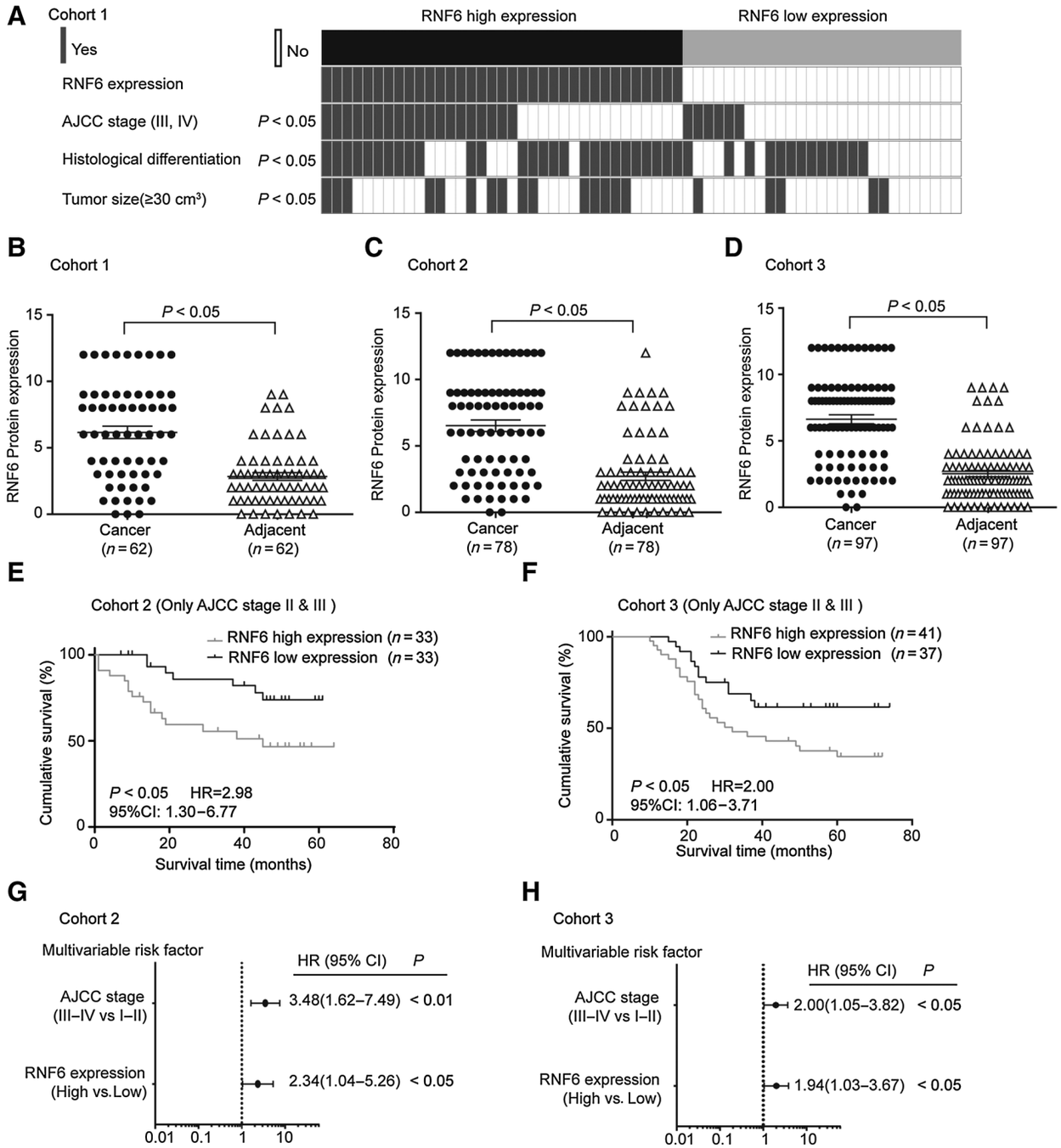

Figure 2.

The clinical relevance of RNF6 in colorectal cancer. **A,** Comparing AJCC stage, histologic differentiation, and tumor size between RNF6 high- and low-expression tumors in the Renji dataset. The heat map illustrates the association of different clinicopathologic features with RNF6 high- and low-expression (cohort 1, $n = 62$, χ^2 test, $P < 0.05$). **B–D,** Statistical analysis of RNF6 protein expression in colorectal cancer tissues and paired adjacent tissues using IHC staining in three independent Renji datasets (cohort 1, $n = 62$; cohort 2, $n = 78$; cohort 3, $n = 97$; nonparametric Mann–Whitney test, $P < 0.05$). **E** and **F,** Survival analysis of patients with colorectal cancer (stages II and III) stratified by expression of RNF6 in cohort 2 and cohort 3 (cohort 2, $n = 66$; cohort 3, $n = 78$; log-rank test, $P < 0.05$; all the bars correspond to 95% confidence intervals). **G** and **H,** Multivariate regression analysis was performed in cohort 2 and cohort 3 (all the bars correspond to 95% confidence intervals).

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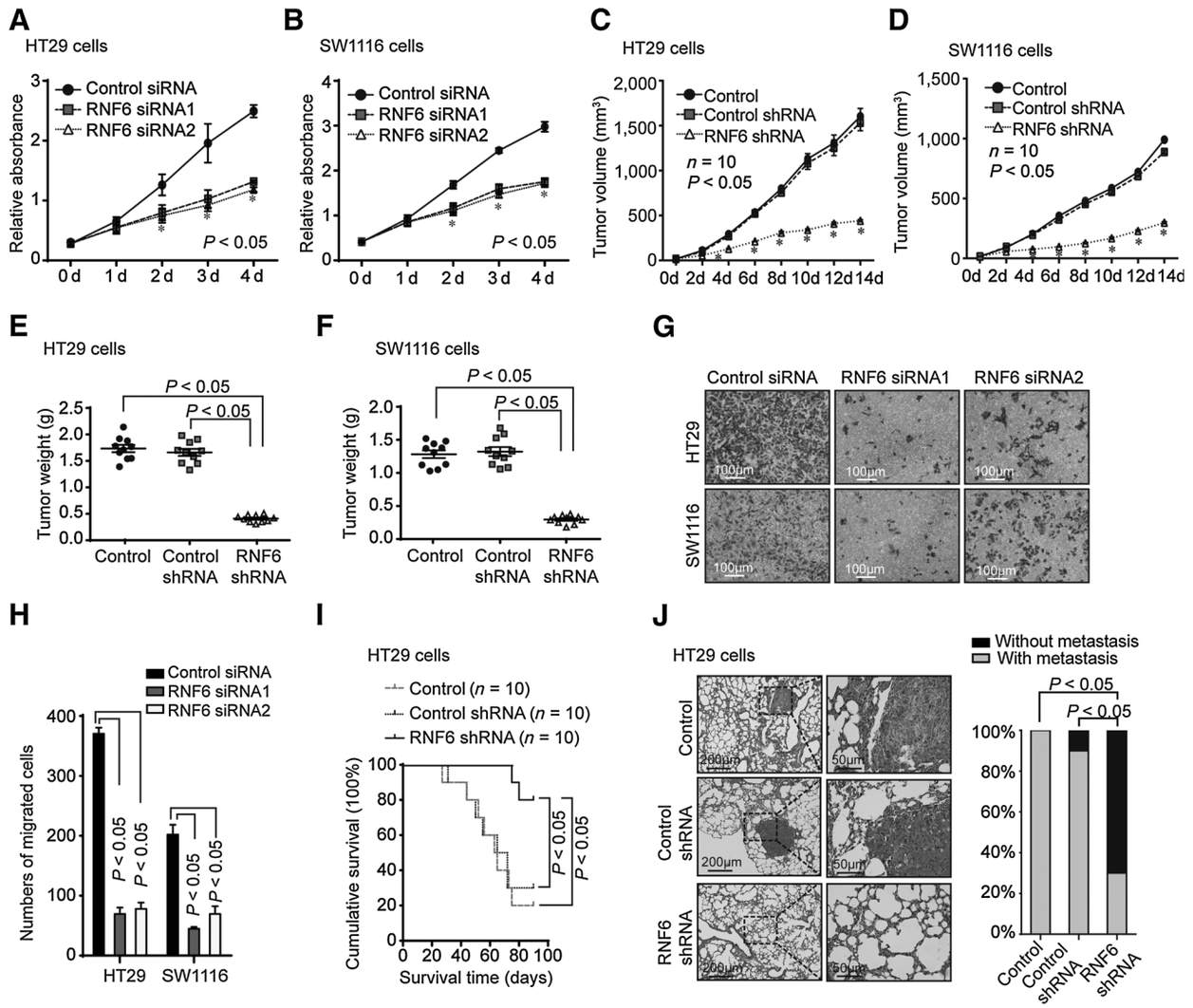

Figure 3.

RNF6 is an oncogenic gene in colorectal cancer. **A** and **B,** Cell proliferation assay was performed in HT29 and SW1116 cells transfected with control siRNA and RNF6 siRNAs ($n = 3$, nonparametric Mann–Whitney test, $P < 0.05$). **C** and **D,** Statistical analysis of xenograft tumors volumes in nude mice bearing HT29 or SW1116 cells treated with PBS, control shRNA adenovirus, and RNF6 shRNA adenovirus ($n = 10$, nonparametric Mann–Whitney test, $P < 0.05$). **E** and **F,** Statistical analysis of xenograft tumor weights of HT29 or SW1116 cells in nude mice after different treatments ($n = 10$, nonparametric Mann–Whitney test, $P < 0.05$). **G** and **H,** Transwell invasion assay was performed in HT29 and SW1116 cells transfected with control siRNA and RNF6 siRNAs ($n = 3$, nonparametric Mann–Whitney test, $P < 0.05$). **I,** Survival analysis in nude mice bearing colorectal cancer cells treated with PBS, control shRNA adenovirus, and RNF6 shRNA adenovirus ($n = 10$, log-rank test, $P < 0.05$). **J,** Representative hematoxylin–eosin staining and summarized data on tumor lung foci in nude mice at 13 weeks after subcutaneously injecting with PBS (control), control shRNA adenovirus, and RNF6 shRNA adenovirus into the right flank of nude mice, respectively ($n = 10$, nonparametric Mann–Whitney test, $P < 0.05$).

in the AJCC stage II and III patients of cohort 2 ($n = 66$, $P < 0.05$) and cohort 3 ($n = 78$, $P < 0.05$; Fig. 2E and F), as well as in the whole patients of two cohorts (Supplementary Fig. S2D and S2E). In addition, univariate and multivariate regression analyses of cohorts 2 and 3 demonstrated that RNF6 expression was an independent predictor of colorectal cancer aggressiveness with significant HRs for predicting clinical outcome. Its predictive value was comparable with that of the AJCC stage (Fig. 2G and H; Supplementary Fig. S2F and S2G). Collectively, RNF6 is upregulated and significantly associated with clinicopathologic characters as well as poor prognosis in human colorectal cancer.

RNF6 is an oncogenic gene in colorectal cancer

The consistently high expression of RNF6 in colorectal cancer suggests it contributes to tumorigenesis. RNF6 expression was examined in eight different colorectal cancer cells and normal colorectal cell FHC by real-time PCR and Western blotting. The data showed that SW1116 and HT29 expressed higher levels of RNF6, whereas LoVo and SW480 expressed lower levels of RNF6 (Supplementary Fig. S3A and S3B). Real-time PCR and Western blotting analysis showed that RNF6 siRNAs significantly decreased RNF6 expression (Supplementary Fig. S3C and S3D). To elucidate the functional significance of RNF6,

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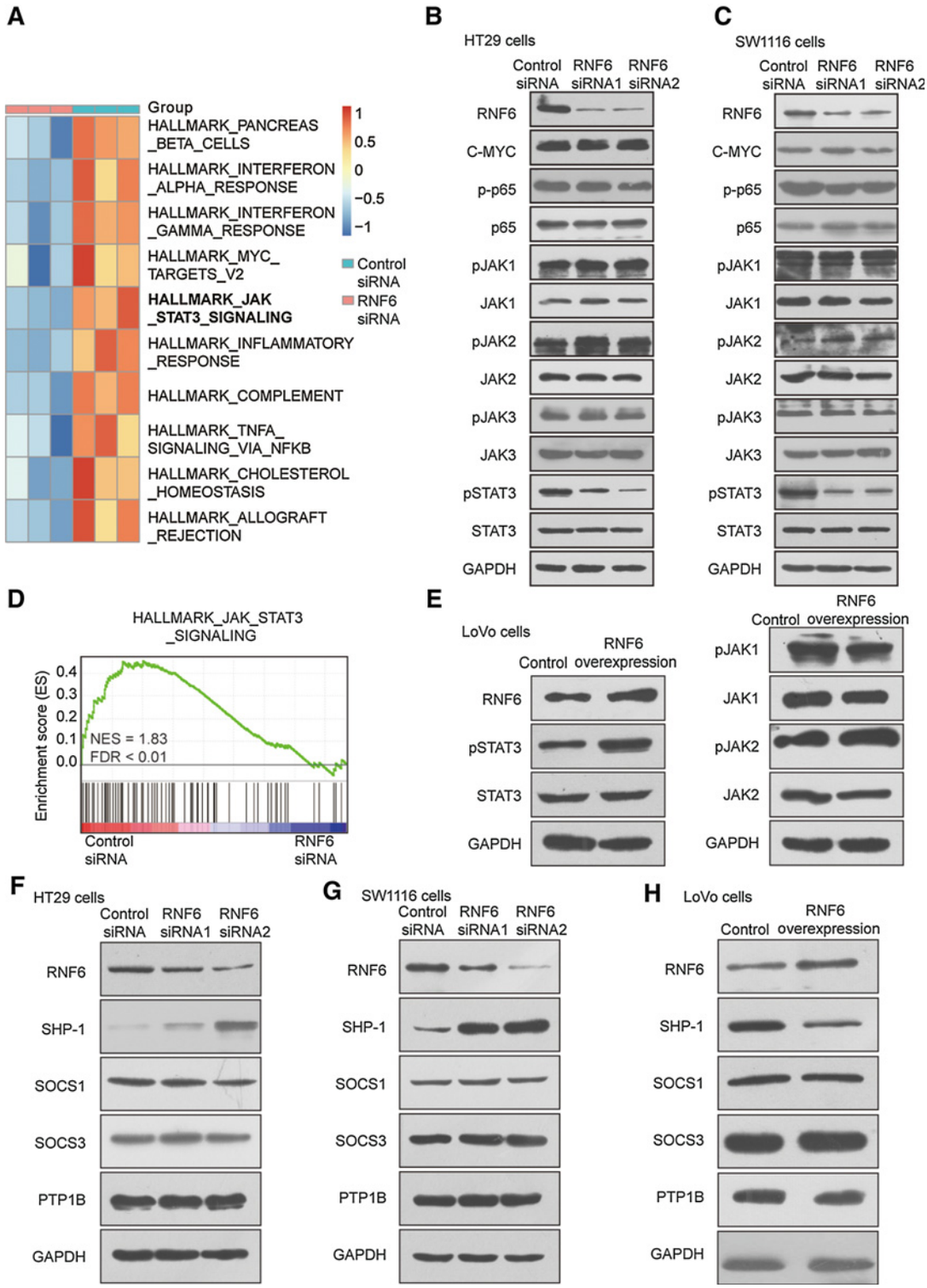

Figure 4.

RNF6 upregulates pSTAT3 via downregulating SHP-1 expression. **A,** SsGSEA analysis was conducted to show the pathways closely correlated with RNF6 expression levels in colorectal cancer cells. **B** and **C,** Western blot was performed in HT29 (**B**) and SW1116 cells (**C**) transfected with control siRNA and RNF6 siRNAs ($n = 3$). **D,** GSEA analysis was conducted to show a set of activated genes related to JAK-STAT3 signaling pathway (NES = 1.83, FDR < 0.01). **E,** Western blot was performed in LoVo cells transfected with RNF6 overexpression plasmids ($n = 3$). **F** and **G,** Western blot was performed in HT29 (**F**) and SW1116 (**G**) cells transfected with control siRNA and RNF6 siRNAs. ($n = 3$). **H,** Western blot was performed in LoVo cells transfected with RNF6 overexpression plasmids ($n = 3$).

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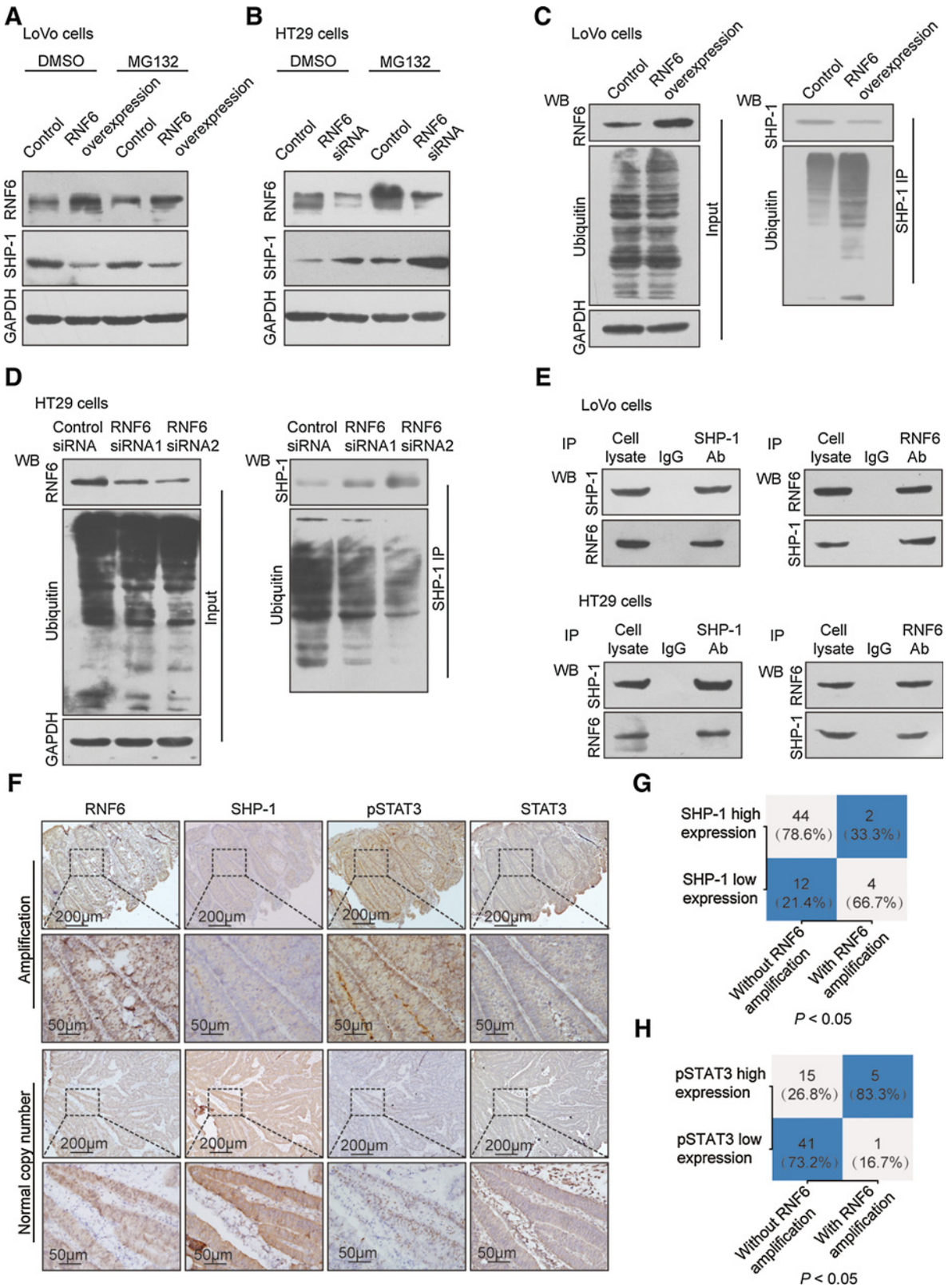

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RNA sequencing analysis was performed to compare the gene expression profiles of RNF6 siRNA and control siRNA groups. A total of 1,511 downregulated genes and 1333 upregulated genes (adjusted $P < 0.01$) were detected (raw data accessible via GEO number: 96074) after knockdown of RNF6 in HT29 colorectal cancer cells (Supplementary Table S5). Gene ontology (GO) analysis revealed changes in gene sets related to colorectal cancer, cell proliferation and metastasis (Supplementary Fig. S3E–S3H). To gain further insight into the biological pathways involved in colorectal cancer pathogenesis stratified by the median of RNF6 expression level, GSEA analysis was performed in TCGA datasets. Enrichment plots of GSEA showed that the gene signatures of COLON_AND_RECTAL_CANCER_UP (colorectal cancer), REGULATION_OF_CELL_PROLIFERATION (cell proliferation), and RICKMAN_METASTASIS_UP (metastasis) were more correlated with patients with RNF6-higher expression versus patients with RNF6-lower expression in TCGA datasets (Supplementary Fig. S3I–S3K). The top-scoring genes recurring in the three gene sets included key cancer genes, MYC, EGFR, and EZR. Real-time PCR confirmed that alteration of RNF6 expression dramatically affected the key tumorigenesis gene signatures (Supplementary Fig. S3L–S3N), suggesting that RNF6 may be an important modulator in colorectal tumorigenesis.

To functionally validate the pathway findings, functional assays were performed after RNF6 siRNA transfection in colorectal cancer cells. We found that knockdown of RNF6 significantly impaired cell proliferation both in HT29 (Fig. 3A) and SW1116 cells (Fig. 3B). Downregulation of RNF6 dramatically reduced colorectal cancer tumor growth (Fig. 3C and D, Supplementary Fig. S3O and S3P) and tumor weight (Fig. 3E and F) in xenograft mouse tumor models. In support of the protumor role of RNF6, Ki67 staining revealed that downregulation of RNF6 decreased tumor cell proliferation *in vivo* (Supplementary Fig. S3Q and S3R). The data suggest that RNF6 may be an oncogenic gene in colorectal cancer and control colorectal cancer cell proliferation.

Next, we examined the effects of RNF6 on colorectal cancer cell invasion and metastasis. In the invasion assay, we showed that downregulation of RNF6 significantly reduced the invasion ability in colorectal cancer cells (Fig. 3G and H). In a colorectal cancer metastatic model, the mice inoculated with RNF6 shRNA–expressing tumor cells had a longer overall survival time than the mice that received control shRNA–expressing tumor cells or phosphate buffered solutions (PBS, control; Fig. 3I). There were fewer metastatic foci in the lungs of nude mice at 13 weeks after injection of RNF6 shRNA adenovirus, when compared with control groups (Fig. 3J). In the gain-of-function assays, overexpression of RNF6 increased cell proliferation (Supplementary Fig. S3S) and invasion (Supplementary Fig. S3T) ability of LoVo cells. The data strongly suggest that RNF6 may promote colorectal cancer progression by regulating colorectal cancer cell proliferation and metastasis, which is consistent with the clinicopathologic parameters in patients.

RNF6 upregulates the phosphorylation of STAT3 via dowregulating SHP-1 expression

To investigate the mechanisms by which RNF6 induced cell proliferation and invasion of colorectal cancer, we projected our RNA-sequence profiling data of cell line samples from these two treatment groups into the space of the 50 hallmarks by means of single-sample GSEA (ssGSEA; refs. 27, 28). SsGSEA revealed that the gene sets including Hallmark_Myc_Targets_V2, Hallmark_IL6_Jak_STAT3_signal, and Hallmark_TNFA_Signaling_Via_NFkB closely correlated with RNF6 alteration in colorectal cancer cells (Fig. 4A). We next investigated the typical genes including C-MYC, NF-κB (p65), p-p65, pJAK1, JAK1, pJAK2, JAK2, pJAK3, JAK3, pSTAT3, and STAT3, which were involved in the major pathways of ssGSEA analysis by real-time PCR and Western blot. The mRNA and protein levels of these genes were rarely changed, whereas the expression of pSTAT3 was decreased in HT29 (Fig. 4B; Supplementary Fig. S4A) and SW1116 (Fig. 4C; Supplementary Fig. S4B) cells, indicating that RNF6 may regulate the JAK-STAT3 signaling pathway. This result was further confirmed by GSEA analysis (Fig. 4D). In addition, overexpression of RNF6 significantly increased pSTAT3 level, but not pJAK1, JAK1, pJAK2, JAK2, and STAT3 levels in LoVo cells (Fig. 4E; Supplementary Fig. S4C). Furthermore, knockdown of RNF6 effectively altered the target genes of the JAK/STAT3 pathway (Supplementary Fig. S4D and S4E; refs. 29–31). These data reveal that RNF6 may modulate the JAK/STAT3 pathway by regulating the phosphorylation of STAT3, but not other components of this pathway.

As an E3 ubiquitin ligase, RNF6 is responsible for protein degradation and recycling (11, 32), and we next hypothesized that RNF6 may upregulate pSTAT3 expression level via degrading the expression of the negative regulator of pSTAT3. Several phosphatases may act as negative regulators, which modulate the phosphorylation of STAT3, such as protein tyrosine phosphatase-1B (PTP1B; ref. 33), suppressor of cytokine signaling 1 (SOCS1), SOCS3 (34), and SH2-containing protein tyrosine phosphatase 1 (SHP-1; ref. 35). To verify our hypothesis, Western blot was performed to investigate which phosphatase may be regulated by RNF6. The protein expression of SHP-1 was increased, whereas other phosphatases have no significant change in response to RNF6 knockdown in HT29 and SW1116 cells (Fig. 4F and G). These data are also confirmed in gain-of-function assays (Fig. 4H). Further real-time PCR showed that there was no significant change of SHP-1 mRNA level after alteration RNF6 expression (Supplementary Fig. S4F–S4H),

Figure 5.

RNF6 interacts with SHP-1 and enhances ubiquitylation and degradation of SHP-1. **A,** Western blot was performed in LoVo cells transfected with RNF6 overexpression plasmids and treated with DMSO or MG132 ($n = 3$). **B,** Western blot was performed in HT29 cells transfected with RNF6 siRNAs and treated with DMSO or MG132 ($n = 3$). **C,** The amount of ubiquitin that coimmunoprecipitated with SHP-1 in LoVo cells transfected with RNF6 overexpression plasmids. Western blot data of RNF6, ubiquitin, and GAPDH from 20% input (left). Antiubiquitin and anti–SHP-1 antibody were used for Western blot to determine the ubiquitination level of SHP-1 (right; $n = 3$). **D,** The amount of ubiquitin that coimmunoprecipitated with SHP-1 in HT29 cells transfected with RNF6 siRNAs. Western blot data of RNF6, ubiquitin, and GAPDH from 20% input (left). Antiubiquitin and anti–SHP-1 antibody were used for Western blot to determine the ubiquitination level of SHP-1 (right; $n = 3$). **E,** Co-IP detected the interaction of RNF6 and SHP-1 in LoVo and HT29 cells. The 20% of cell lysate and RNF6 or SHP-1 immunoprecipitates were separated by SDS-PAGE. The specific immunoprecipitation of RNF6 and SHP-1 was confirmed by Western blot ($n = 3$). **F,** Immunohistochemical staining of RNF6, SHP-1, pSTAT3, and STAT3. **G** and **H,** Statistical analysis of colorectal cancer tissues under different staining conditions in cohort 1 ($n = 62$, χ^2 test, $P < 0.05$).

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suggesting that RNF6 may regulate SHP-1 expression via posttranscription.

RNF6 Interacts with SHP-1 and enhances ubiquitylation and degradation of SHP-1

Next, we explored the molecular mechanisms by which RNF6 regulated SHP-1 protein expression level. SHP-1 could be polyubiquitinated and degraded by the UPS (36, 37). We next found that MG132 (a proteasome inhibitor) treatment significantly rescued RNF6-induced downregulation of SHP-1 in LoVo cells (Fig. 5A). These data were further confirmed in loss-of-function assays in HT29 cells (Fig. 5B). Given the fact that RNF6 contains a RING-finger domain which plays the E3 ubiquitin ligase role (10), we hypothesized that RNF6 may downregulate SHP-1 expression via promoting SHP-1 protein ubiquitination and degradation. As shown in Fig. 5C, the amount of ubiquitin that coimmunoprecipitated with SHP-1 was significantly enhanced in LoVo cells with RNF6 overexpression. Consistently, knockdown of RNF6 impaired SHP-1–ubiquitin association (Fig. 5D). In addition, we observed that RNF6 and SHP-1 interacted with each other in LoVo and HT29 cells (Fig. 5E). Subsequently, confocal microscopy revealed that SHP-1 and RNF6 colocalized in LoVo cells (Supplementary Fig. S5A). Together, these data indicate that RNF6 serves as E3 ubiquitin ligase for SHP-1–ubiquitin association.

We next performed IHC staining in colorectal cancer patients' tissues of cohort 1. Interestingly, the samples with RNF6 amplification displayed strongly staining for pSTAT3, whereas the SHP-1 staining was weak. On the contrary, samples with nonamplification of RNF6 appeared low levels of pSTAT3 while displaying high levels of SHP-1 (Fig. 5F). The data are statistically significant (Fig. 5G and H).

RNF6 promotes colorectal cancer progression via modulating SHP-1 levels

We next hypothesized that RNF6 may act as an oncogene by ubiquitylating and degrading SHP-1, and then eventually elevating pSTAT3 levels in colorectal cancer. To test this hypothesis, we transfected SHP-1 overexpression plasmid into colorectal cancer cell and examined its effects on cancer cell biological function. Overexpression of SHP-1 significantly reduced colorectal cancer cell proliferation and invasion induced by RNF6 in LoVo (Supplementary Fig. S6A and S6B) and SW480 cells (Supplementary Fig. S6C and S6D). Furthermore, upregulation of SHP-1 significantly blocked RNF6-induced pSTAT3 in LoVo (Supplementary Fig. S6E) and SW480 cells (Supplementary Fig. S6F). Thus, SHP-1 may mediate the regulatory function of RNF6 in colorectal cancer cells.

Effectiveness of pSTAT3 inhibitors in treating RNF6-amplified tumors

Knockdown of RNF6 in colorectal cancer cells causes a decrease in proliferation. However, treatment with siRNAs still requires further development before it can be used in clinical practice (38, 39). Hence, we sought to explore whether any small molecules and drugs could recapitulate the effects of siRNA knockdown in RNF6-amplified colorectal cancer cells. RNF6 is amplified in HT29 and SW1116 cells (data downloaded from http://www.cbioportal.org/). In addition, HT29 and SW1116 cells display higher RNF6 and pSTAT3 levels, and lower SHP-1 expression, compared with that in LoVo cells (Fig. 6A). Because we have proved that RNF6 may function as an oncogene by upregulating pSTAT3 levels in colorectal cancer cells, and several pSTAT3 inhibitors have been developed as antitumor drug candidates, we next hypothesized that those patients with colorectal cancer who harbor RNF-amplified colorectal cancer cells will be more sensitive to the antitumor drug candidates, which are major targets of the JAK/STAT3 pathway or pSTAT3 activation.

To test this prediction, we treated the amplified colorectal cancer cell lines HT29 and SW1116 with cryptotanshinone (an inhibitor of pSTAT3). Cryptotanshinone treatment resulted in 50% and 38.7% inhibition of cell proliferation in HT29 and SW1116 cells, respectively. Conversely, treatment of LoVo, which has normal copy number of RNF6, with the same dose of cryptotanshinone resulted in only 6.2% inhibition of cellular proliferation (Fig. 6B). These inhibition results could also be recapitulated *in vivo*. Cryptotanshinone treatment of HT29 and SW1116 xenografts resulted in a decrease in xenograft size and tumor growth ($n = 8$, $P < 0.05$; Fig. 6C and D). Treatment of Lovo xenografts with the same dose of cryptotanshinone had no significant effect ($n = 8$; Fig. 6E). In addition, the immunostaining data showed that cryptotanshinone could more effectively inhibit the phosphorylation of STAT3 in HT29 and SW1116 tumor tissues, than that in LoVo tumor tissues (Fig. 6F). However, cryptotanshinone has no effect on the expression of RNF6, SHP-1, and STAT3 in the all xenografts tumors (Fig. 6F; Supplementary Fig. S6G–S6I). Taken together, these data suggest that targeting the pSTAT3 may be more effective in RNF6-amplified colorectal cancer cells than in non–RNF6-amplified colorectal cancer cells.

Discussion

Overexpression of ubiquitin E3 ligase with CNV may contribute to various cancer tumorigenesis (5, 25); however, the potential involvement of ubiquitin E3 ligase with CNV is poorly defined in human colorectal cancer. Through a combination of genomic, biochemical, and cell biological analyses, we have demonstrated that RNF6 is amplified in colorectal cancer and may function as an oncogene in colorectal carcinogenesis. Patients with overexpression or copy-number amplification of RNF6 had a statistically significantly poorer prognosis compared with patients with wild-type levels of RNF6, and genomic amplification may be a basis for RNF6 overexpression in colorectal cancer. GSEA analyses have demonstrated that cell proliferation, metastasis, and colorectal cancer–related pathways are significantly enriched in response to RNF6 alteration in the datasets of patients with colorectal cancer. The bioinformatic analyses have been functionally validated in several *in vitro* and *in vivo* experimental models. In cultured colorectal cancer cells and xenograft mouse models, downregulation of RNF6 markedly suppresses colorectal cancer cell growth and metastasis. The data consistently point to the notion that high RNF6 expression and RNF6 copy-number amplification may be a decisive factor of controlling human colorectal cancer aggressiveness.

E3 ligases usually participate in carcinogenesis by degrading or stabilizing target proteins (40); however, the underlying molecular mechanisms of RNF6 in colorectal cancer remain unknown. Our GSVA pathway analysis data demonstrated that JAK/STAT3 pathway–related genes were enriched in RNF6 high-expression colorectal cancer cells. STAT3 plays a crucial role in a wide variety of biological processes such as cell proliferation, invasion,

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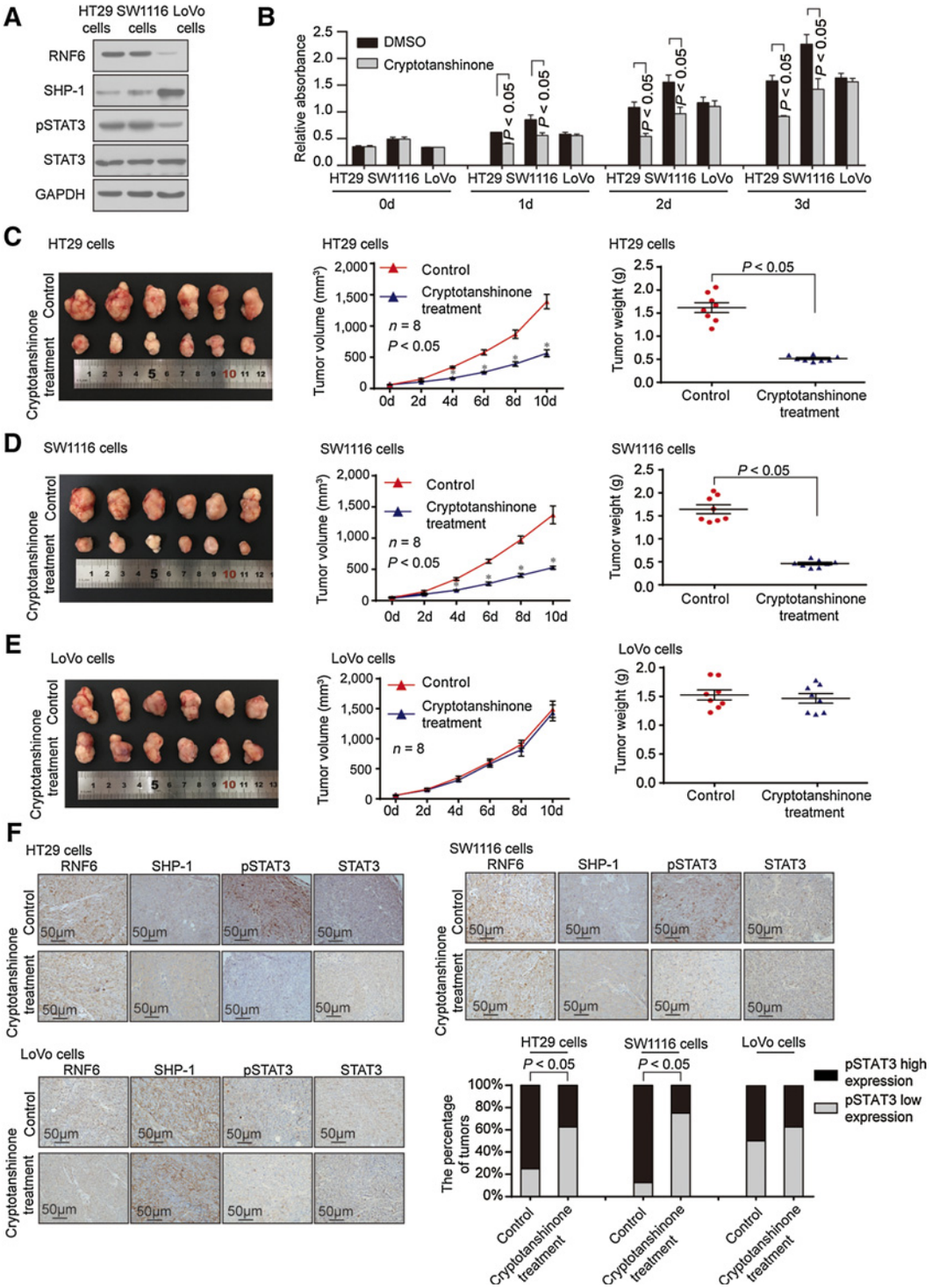

Figure 6.

Effectiveness of pSTAT3 inhibitors in treating RNF6-amplified tumors. **A,** Western blot was performed to detect different protein expressions in HT29, SW1116, and LoVo cells ($n = 3$). **B,** Cell proliferation assays were performed in HT29, SW1116, and LoVo cells with different treatment ($n = 3$, nonparametric Mann–Whitney test, $P < 0.05$;). **C,** Representative images of tumors, statistical analysis of tumor volume, and weights in nude mice bearing HT29 cells treated with DMSO or cryptotanshinone at 10 mg/kg ($n = 8$). **D,** Representative images of tumors, statistical analysis of tumor volume, and weights in nude mice bearing SW1116 cells treated with DMSO or cryptotanshinone at 10 mg/kg ($n = 8$). **E,** Representative images of tumors, statistical analysis of tumor volume, and weights in nude mice bearing LoVo cells treated with DMSO or cryptotanshinone at 10 mg/kg ($n = 8$). **F,** Representative immunohistochemical staining and statistical analysis of RNF6, SHP-1, pSTAT3, and STAT3 in different tumor tissues from xenograft mouse model.

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apoptosis, and immunity (41). It has been reported that STAT3 activation was associated with a poor prognosis of diverse cancers including colorectal cancer (42), gastric cancer (43), and breast cancer (44). In our study, we have dissected the mechanisms by which RNF6 mediates the JAK/STAT3 pathway activation. RNF6 may stabilize STAT3 phosphorylation by ubiquitylating and degrading SHP-1, but not other negative regulators of STAT3. This notion is supported by four lines of experimental evidence. (i) Genetic deficiency of RNF6 decreased the phosphorylation level of STAT3, and the data were verified in gain function assay. (ii) Knockdown of RNF6 restored the expression of SHP-1 in colorectal cancer cells, but not other negative regulator of pSTAT3, and the data were verified in gain function assay as well. (iii) MG132 treatment, the inhibitor of proteasome, disrupted RNF6-induced SHP-1 downregulation in colorectal cancer cells. (iv) The association between SHP-1 and ubiquitin was reduced by RNF6 downregulation in coimmunoprecipitation (co-IP) data, and RNF6 overexpression leads to a significant increase of the ubiquitin that co-IP with SHP-1. Our findings are supported by two additional studies in different research models (11, 32). Moreover, because RNF6 may have other client proteins, such as estrogen receptor alpha and androgen receptor (11, 12), we need to explore more RNF6-regulated signal pathways and molecules in colorectal carcinogenesis in the future study. In short, RNF6 is the oncogene capable of modulating the ubiquitination of SHP-1 and further stabilizing the phosphorylation of STAT3 in colorectal cancer cells (Supplementary Fig. S6J).

In addition to its biological importance, our work may be relevant in clinical management of patients with colorectal cancer. Survival analyses illustrate that both RNF6 amplification and overexpression can predict poor clinical outcome in patients with colorectal cancer from TCGA and Renji datasets, which indicate that the measurement of RNF6 CNV after surgery may be an effective approach to predict patient outcome, and RNF6 may be a promising therapeutic or treatment target in patients with colorectal cancer. Accordingly, bioinformatic analysis, *in vitro* experiments, and *in vivo* experiments provided us with a link between RNF6 overexpression and high JAK/STAT3 pathway activity. We further found that cryptotanshinone (a chemical inhibitor of pSTAT3) is more effective to inhibit pSTAT3 activity in colorectal cancer cells with RNF6 amplification than those cells without RNF6 amplification. Because cryptotanshinone inhibits other signal pathways (45, 46) and other cancer (47), and contains multiple biological activities (48), more specific inhibitors of pSTAT3 need to be developed for patients with colorectal cancer, especially for those with RNF6 amplification treatment. Besides, to more really and effectively develop chemicals or other molecules for the treatment of patients with colorectal cancer with RNF6 amplification, an orthotopic murine model of human colorectal cancer needs to be employed in our future research. Thus, given the significance of RNF6 in the clinical, bioinformatic, genetic, and functional aspect, we regard RNF6 as a biomarker to guide early diagnosis and therapy in colorectal cancer, and it is important to detect RNF6 CNV and differentially manage patients with different CNV levels of RNF6.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

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Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Q. Liang, X. Zhu, H. Chen, J. Hong
Writing, review, and/or revision of the manuscript: D. Ma, J.-Y. Fang, H. Chen, J. Hong
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): Q. Liang, F. Guo, Z. Wang, J. Hong
Study supervision: J.-Y. Fang, H. Chen, J. Hong

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RING-Finger Protein 6 Amplification Activates JAK/STAT3 Pathway by Modifying SHP-1 Ubiquitylation and Associates with Poor Outcome in Colorectal Cancer

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