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Article

TRIM28 protects CARM1 from proteasome-mediated degradation to prevent colorectal cancer metastasis

Jinyuan Cui [a,b,1], Jia Hu [b,1], Zhilan Ye [b], Yongli Fan [b], Yuqin Li [b], Guobin Wang [a,b,*], Lin Wang [b,*], Zheng Wang [a,b,*]

[a] Department of Gastrointestinal Surgery, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China
[b] Research Center for Tissue Engineering and Regenerative Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan 430022, China

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ABSTRACT

TRIM28 (Tripartite motif-containing protein 28), a member of TRIM family, is aberrantly expressed and reportedly has different functions in many types of human cancer. However, the biological roles of TRIM28 and related mechanism in colorectal cancer (CRC) remain unclear. Here, we showed that TRIM28 was downregulated in colorectal cancer compared with normal mucosa, especially at advanced stages, and acted as an independent prognostic factor of favorable outcome. Functional studies demonstrated that TRIM28 restrained CRC migration and invasion in vitro and in vivo. Mechanistically, we reported that CARM1 (co-activator-associated arginine methyltransferase1) was a critical player downstream of TRIM28. TRIM28 interacted with CARM1, and protected CARM1 from proteasome-mediated degradation through physical protein-protein interaction to suppress CRC metastasis. Further, TRIM28 suppressed the migration and invasion of CRC cells through inhibiting WNT/β-catenin signaling in a CARM1-dependent manner, but independent of CARM1′s methyltransferase activity. The protein expression of CARM1 was positively correlated with TRIM28 in CRC tissues. Patients with high levels of TRIM28 and CARM1 had improved prognosis, whereas patients with low TRIM28 and CARM1 expression had the poor outcomes. Thus, our study reveals an inhibitory role of TRIM28 in CRC metastasis, which was achieved through a TRIM28-CARM1-WNT/β-catenin axis. This work provides potential prognostic and therapeutic targets for CRC treatment.

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1. Introduction

Colorectal cancer (CRC) is the fourth most common cancer and the third leading cause of cancer-related death in the world [1]. The global burden of CRC is expected to increase by 60% to over 2.2 million new cases and 1.1 million deaths by 2030 [2]. Approximately 40%–50% CRC patients suffer from metastasis either at the time of diagnosis or amid treatment [3]. Most of CRC patients with distant metastasis are not suitable for conventional therapy and exhibit a poor 5-year survival rate, even lower than 10% [4]. Thus, it is necessary to advance the understanding of the molecular mechanisms underlying CRC metastasis and to identify effective therapeutic targets for blocking CRC metastasis.

Tripartite motif family (TRIMs) is a large family of proteins usually containing four typical motifs: a RING domain in N-terminal, a B-box domain, a coiled-coil domain, and a variable C-terminal domain. TRIMs play important roles in various cellular processes, such as innate immunity, autophagy, protein quality control, and carcinogenesis [5,6]. Among these, TRIM28 protein, also known as KAP1 (Krüppel-Associated Box (KRAB)-Associated Protein1), is a widely studied molecule in cancer [7–9]. Whether TRIM28 acts as a pro-oncogene or tumor-inhibiting factor appears to depend on types of cancers. For example, TRIM28 protected TRIM24 from ubiquitination and degradation to promote tumor growth in prostate cancer [10]. Similarly, in breast cancer, TRIM28-mediated stimulation of KRAB-ZNF contributes to cancer progression in vitro and in vivo [11,12]. On the contrary, TRIM28 had anti-proliferative activity via modulating HDAC1-E2F3/E2F4 interactions in lung cancer, and positively associated with improved overall survival in early-stage lung cancer [13]. However, the functional roles of TRIM28 in CRC remain elusive.

CARM1 (co-activator-associated arginine methyltransferase1), a member of protein arginine methyltransferase (PRMT) family, plays a critical role in cancer by methylating histone or nonhistone

* Corresponding authors.
E-mail addresses: wgb@hust.edu.cn (G. Wang), lin_wang@hust.edu.cn (L. Wang), zhengwang@hust.edu.cn (Z. Wang).
[1] These authors contributed equally to this work.

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proteins [14–19]. CARM1 methylates chromatin remodeling factor BAF155 to enhance metastasis in breast cancer [17]. It can also methylate GAPDH to repress the glycolysis and proliferation in liver cancer cells [18]. Arginine methylation of MDH1 by CARM1 suppresses the pancreatic cancer [19]. Although multiple substrates of CARM1 were identified, the molecular regulation on CARM1 is rarely studied.

Here, we found that TRIM28 was downregulated and acted as an independent prognostic factor of favor outcomes in CRC patients. In vitro and in vivo results demonstrated that TRIM28 suppressed the migration and invasion of CRC. Mechanistically, TRIM28 protected CARM1 from proteasome-mediated degradation through physical protein-protein interaction. TRIM28 inhibited WNT/β-catenin signaling in a CARM1-dependent manner. There was a positive expressional correlation between CARM1 and TRIM28 in CRC patients. This work may provide new targets for designing therapeutic strategies for CRC.

2. Materials and methods

2.1. Antibodies and reagents

Antibodies used in this study were as follows: Antibodies against TRIM28, CARM1, β-catenin, β-actin, GAPDH and Ubiquitin were purchased from Proteintech (USA). Mono-methyl arginine antibody and asymmetric di-methyl arginine antibody were purchased from Cell Signaling Technology (CST, USA). Anti-Myc and anti-Flag were obtained from Abbkine (USA). Other reagents used in this study were: the eukaryote protein synthesis inhibitor (cycloheximide), the specific CARM1 inhibitor (CARM1-IN-1) and WNT/β-catenin pathway inhibitor (XAV939) were purchased from MedchemExpress (MCE, USA). Proteasome inhibitor (MG132) was purchased from Selleck chemicals (USA).

2.2. Human colorectal cancer samples

Fifteen fresh samples of CRC tissues and paired normal tissues were collected with patients' informed consent from Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, China. The study was approved by the Human Ethics Committee of Union Hospital and in accordance with the Declaration of Helsinki II.

2.3. Cell lines and transfection

HEK293T cell, human normal mucosa cell NCM460 and human CRC cell lines (LoVo, HCT116, SW48, DLD-1 and HT-29) were obtained from American Type Culture Collection (ATCC). Cells were cultured in DMEM medium (Hyclone, Logan, UT) containing 10% fetal bovine serum (ScienCell, Carlsbad, CA). Transfection of siRNAs and plasmids into cells was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's protocols.

2.4. Plasmids and small interfering RNAs

Human TRIM28 cDNA was amplified from cDNA library of HEK293T cells and cloned into the p3 × FLAG-CMV-10 vector (sigma aldrich, USA) at the restriction sites of *Eco*R1 and *Bam*H1. Human CARM1 cDNA was cloned into pcDNA.3.1 vector (Invitrogen) at the restriction sites of *Xho*1 and *Bam*H1. The following plasmids expressing full length or truncated TRIM28/CARM1 were also constructed: Flag-TRIM28 (C), Flag-TRIM28 (N), Flag-TRIM28 (M), Flag-TRIM28 (ΔN), Myc-CARM1 (FL), Myc-CARM1 (ΔN). C65/68A RING mutant of TRIM28 was obtained from TsingKe biological technology. Oligonucleotides specific shRNAs against TRIM28 or CARM1 were inserted into PLKO.1 vector (Addgene Plasmid #10878). All plasmids were confirmed by DNA sequencing. The primers used in this study were shown in Table S1 (online). Small interfering RNAs (siRNAs) targeting TRIM28, CARM1 were purchased from Ribobio (Guangzhou, China). The siRNA sequence was shown in Table S1 (online).

2.5. Lentiviral transduction

HEK293T cells were transfected with indicated lentiviral plasmids. Virus-containing supernatant was collected at 48 h or 72 h post-transfection. To generate the stably transfected cell lines, LoVo, HCT116 and SW48 cells were infected with lentivirus in medium in the presence of polybrene (8 μg/mL) and then selected by puromycin (1 μg/mL) for one week. The overexpression or knockdown efficiency was identified using Western blot.

2.6. TOP-flash/FOP-flash reporter assay

TRIM28 stably knockdown LoVo and HCT116 cells were seeded into 24-well plates and transfected with TOP-flash or FOP-flash reporter plasmid for 48 h. The relative luciferase activities (firefly luciferase activity divided by Renilla activity) were measured by a dual-luciferase reporter assay system (Promega, Madison, WI). The TOP/FOP ratio reflected the activity of the WNT/β-catenin pathway. All results were repeated at least three times.

2.7. Cell migration and invasion assay

The cell migration and invasion assay were performed using 24-well Boyden chambers (Corning Inc, Acton, MA). 1×10^5 cells were seeded in the upper chamber. The lower chamber was added with 700 μL DMEM medium containing 10% FBS. Cells on the upper side of the filter were removed after 12 h for LoVo cells, 48 h for HCT116 cells. The migrated cells were stained with 0.1% crystal violet and counted under microscopy.

Cellular invasion ability was measured with Matrigel matrix (BD Science, Sparks, MD). 1×10^5 cells were added to the matrigel-coated upper chamber and allowed to invade 24 h for LoVo cells, 72 h for HCT116 cells. Invaded cells were stained with 0.1% crystal violet and counted under the microscope.

2.8. Animal experiments

2×10^6 SW48 cells (shNC, shTRIM28#1 and shTRIM28#2) were injected into the tail vein of male Balb/c nude mice ($n = 5$/each group, 4–6 weeks). Five weeks later, mice were sacrificed, and lungs were fixed and performed with hematoxylin and eosin staining. The incidence and number of metastatic lesions were photographed and quantified. The mice in this study were maintained under specific pathogen-free (SPF) conditions. All animal experiments were approved by the Animal Ethics Committee of Union Hospital, Tongji Medical College, Huazhong University of Science and Technology, Wuhan.

2.9. Immunohistochemistry

Immunohistochemical staining was performed by the streptavidin-biotin-peroxidase detection method. Briefly, CRC tissues and adjacent normal tissues were fixed with 4% formaldehyde and embedded with paraffin. After rehydration and microwave antigen retrieval, the sections were labeled with anti-TRIM28 antibody (proteintech, 1:100) or anti-CARM1 antibody (proteintech, 1:100) overnight and followed with the specific HRP-conjugate

secondary antibody for 30 min. Staining was performed with DAB and counterstained with Mayer's hematoxylin.

2.10. Real time PCR and Quantitative Real Time PCR

Total RNA was extracted with the Trizol reagent (Invitrogen) and reverse transcribed into cDNA using the M−MLV reverse transcriptase (Thermo Scientific, Hudson, NH). Quantitative Real Time PCR was performed on StepOnePlusTM Real-time PCR system (Applied Biosystems7500, Foster City, CA) using a standard SYBR Green PCR kit (Takara Shuzo Co. Ltd, Kyoto, Japan). Quantitation of the relative expression levels of each gene was performed in triplicate and calculated using the $2^{-\Delta\Delta}$CT method. GAPDH was used as an endogenous control. The primers were shown in Table S1 (online).

2.11. Western blot

Total protein was extracted by lysing the cells with RIPA buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors. The protein concentration was measured by the BCA kit (Thermo Scientific). The cell lysate was incubated at 98 for 10 min, separated by SDS PAGE and then transferred onto nitrocellulose (NC) membranes (Bio-Rad, Richmond, CA). After blocking with 5% skimmed milk, membranes were incubated with indicated antibodies. The signals were detected by chemiluminescence system (UVP, San Gabriel, CA).

2.12. Immunoprecipitation and protein ubiquitination assay

For Immunoprecipitation and protein ubiquitination assay, cells were collected and lysed in immunoprecipitation lysis buffer (Beyotime Institute of Biotechnology) supplemented with protease inhibitors. Cell extracts were incubated with indicated antibodies overnight at 4 °C. Then protein A/G plus-agarose beads (Santa Cruz, CA, USA) were added and incubated for another 2 h at 4 °C. The beads were washed three times using immunoprecipitation lysis buffer, boiled at 98 °C for 5 min in protein loading buffer and then subjected to western blot analysis. Antibodies used in the co-immunoprecipitation and protein ubiquitination assay were as follows: anti-Flag, anti-Myc, and anti-ubiquitin antibodies.

2.13. Statistical analysis

Kaplan-Meier curves were used for survival analysis and the significance of differences was calculated with log-rank test. The Pearson correlation was used to analyze the association between mRNA expression of TRIM28 and its related molecular in patient samples. Other comparisons were conducted by the Student's *t*-test. Data were presented as the mean ± SD (standard deviation). $P < 0.05$ was considered as statistically significant. All analyses were performed with SPSS version 22.

3. Results

3.1. Low expression of TRIM28 is associated with poor prognosis in CRC

To assess the clinical significance of TRIM28 in human colorectal cancer, we investigated the expression of TRIM28 in 15 paired colorectal tumor tissues and adjacent normal tissues. Western blot showed that TRIM28 was downregulated in tumor tissues compared to adjacent normal tissues in 66.7% of CRC patients (Fig. 1a, b), consistent with immunohistochemical staining of TRIM28 (Fig. 1c). Compared to immortalized colon mucosal cell line (NCM460), CRC cell lines had lower levels of TRIM28 mRNA and protein (Fig. 1d), which was in line with the data from GSE41258 (Fig. 1e). Moreover, CRC patients at the advanced stages expressed less TRIM28 than early staged patients (stage IV vs stage I in TCGA, stage III/IV vs stage I/II in GSE39582) (Fig. 1f). Kaplan–Meier survival analysis revealed that TRIM28 expression was positively associated with improved OS (Overall Survival) and RFS (Relapse-free Survival) of CRC patients from TCGA and GSE39582 datasets (Fig. 1g, h). Then, we divided CRC patients into low and high expression groups based on TRIM28 values in TCGA dataset. TRIM28 expression was associated with the extent of the tumor, lymphatic metastasis, distant metastasis, and tumor anatomical position, but not with age, gender, and tumor pathologic stage (Table S2 online). Multivariate cox regression analysis revealed low TRIM28 expression was an independent prognostic factor for poor survival in CRC patients in both TCGA (HR 2.433, 95% CI, 1.211–4.890; $P = 0.013$; Table S3 online) and GSE39582 (HR 1.464, 95% CI, 1.047–2.045; $P = 0.026$; Table S4 online). These results demonstrate that TRIM28 may act as an inhibitory factor in CRC progression.

3.2. TRIM28 suppresses the metastasis of CRC in vitro and in vivo

Next, we examined the functional roles of TRIM28 in CRC progression. TRIM28 overexpression suppressed cell migration ability in vitro (Fig. 2a–c). Conversely, knockdown of TRIM28 (Fig. 2d, f) significantly promoted cell migration and invasion in LoVo and HCT116 cells in vitro (Fig. 2e, g, h). Moreover, the stable knockdown of TRIM28 in SW48 cells (Fig. 2i) also significantly promoted cell migration and invasion in vitro, and enhanced the incidence and the number of pulmonary metastatic nodules in vivo (Fig. 2j, k). These findings indicate that TRIM28 suppresses the migration and invasion of CRC cells in vitro and in vivo.

3.3. TRIM28 interacts with CARM1 and suppresses CRC metastasis in a CARM1-dependent manner

We further investigated the molecular mechanism underlying the anti-metastasis effect of TRIM28 in CRC. CARM1 was previously suggested to be one of putative Trim28-interacting proteins via mass spectrometry analysis [20]. Their interactions were confirmed by reciprocal co-immunoprecipitation (Co-IP) experiments in HEK293T cells (Fig. 3a). Endogenous TRIM28 and endogenous CARM1 protein were also found to interact with each other in LoVo cells (Fig. 3b). To map the TRIM28 domain that bound to CARM1, we generated a series of Flag-tagged truncated TRIM28 by referring to its putative functional domains (Fig. 3c) and performed the Co-IP experiments with CARM1 with a Myc tag at C-terminus. Full length TRIM28 and its truncated forms with PHD/Bromo domain (C and △N) interacted with CARM1, while the truncations lacking PHD/Bromo domain (N and M) completely abolished the interactions (Fig. 3d). CARM1 contains a conserved EVH1 domain that is involved in protein-protein interactions. To determine whether EVH1 domain was responsible for the TRIM28-CARM1 interaction, we co-immunoprecipitated TRIM28 with either full length CARM1-Myc (FL) or the truncated form lacking EVH1 domain (CARM1-ΔN) (Fig. 3c). CARM1-ΔN was unable to interact with TRIM28 (Fig. 3e), indicating that EVH1 domain is required for the interaction.

CARM1 is reportedly a pro-oncogene or a tumor-inhibiting gene depending on cancer types [21,22]. However, the functional role of CARM1 in CRC metastasis was not yet determined. Kaplan-Meier survival analysis revealed that CRC patients with low levels of CARM1 had significantly poor outcomes in TCGA and GSE39582 datasets (Fig. S1a online). Knockdown of CARM1 promoted the migration ability of LoVo and HCT116 cells (Fig. S1b–d online), which was similarly observed for TRIM28 knockdown Fig. 2e, g,

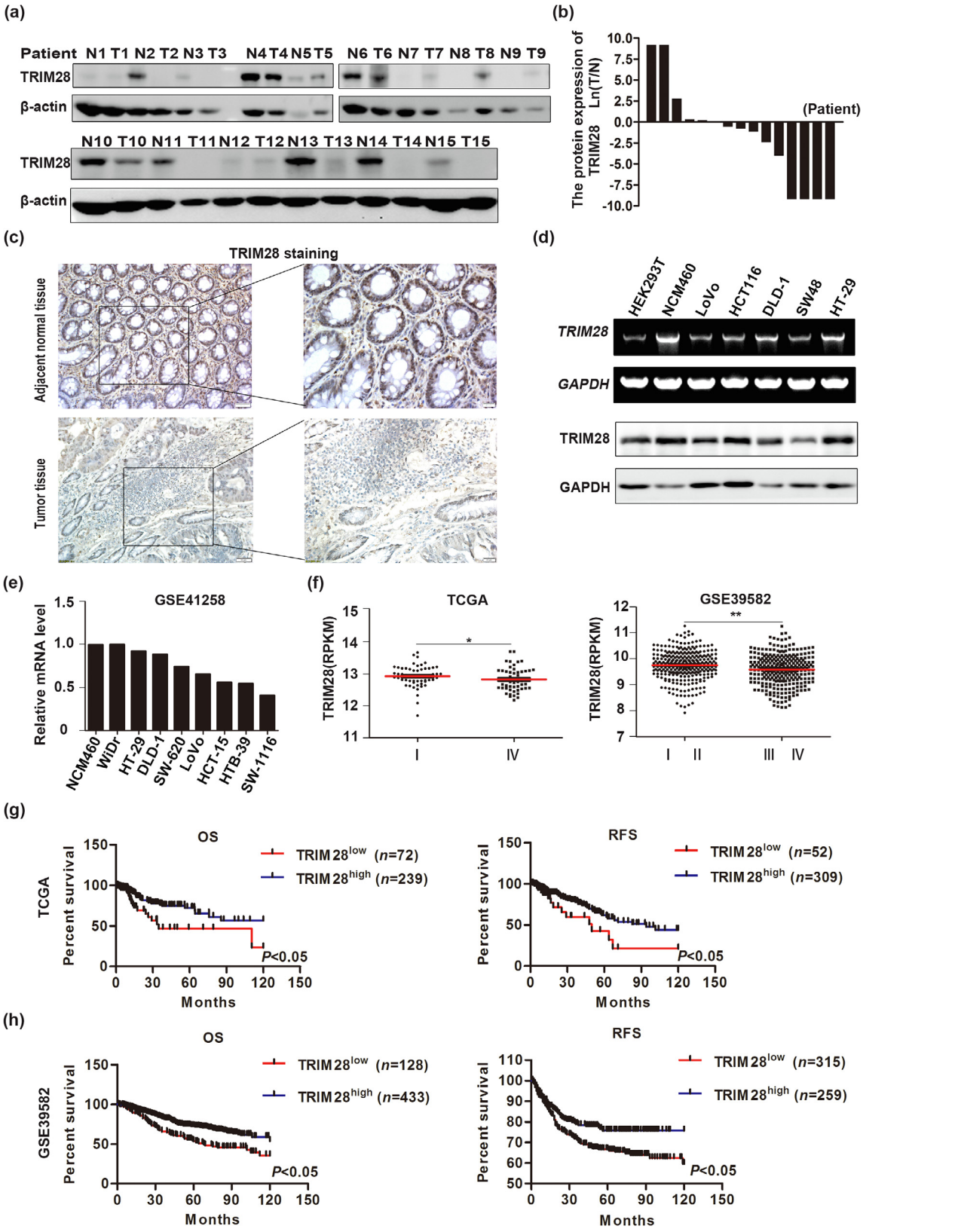

Fig. 1. Down regulation of TRIM28 is a poor prognostic factor in CRC. (a) Western blot analysis of TRIM28 expression in 15 paired CRC tissues. (b) Quantitative analysis of relative TRIM28 expression in 15 paired CRC tissues showed in (a). (c) Representative images of immunochemical staining of TRIM28 in CRC tissue and adjacent normal tissue. Left row: magnification ×200, scale bars, 50 μm; Right row: magnification ×400, scale bars, 20 μm. (d) RT-PCR analysis (upper) and western blot (lower) analysis of TRIM28 expression in the normal colon mucosal cell (NCM460) and CRC cell lines. (e) Analysis of TRIM28 mRNA level in NCM460 and CRC cell lines in GSE41258 dataset. (f) Analysis of TRIM28 mRNA level in different tumor stages of CRC patients from TCGA and GSE39582 datasets. (g, h) Kaplan-Meier survival analysis of overall survival (OS) and relapse-free survival (RFS) based on TRIM28 expression in CRC patients from TCGA and GSE39582 datasets.

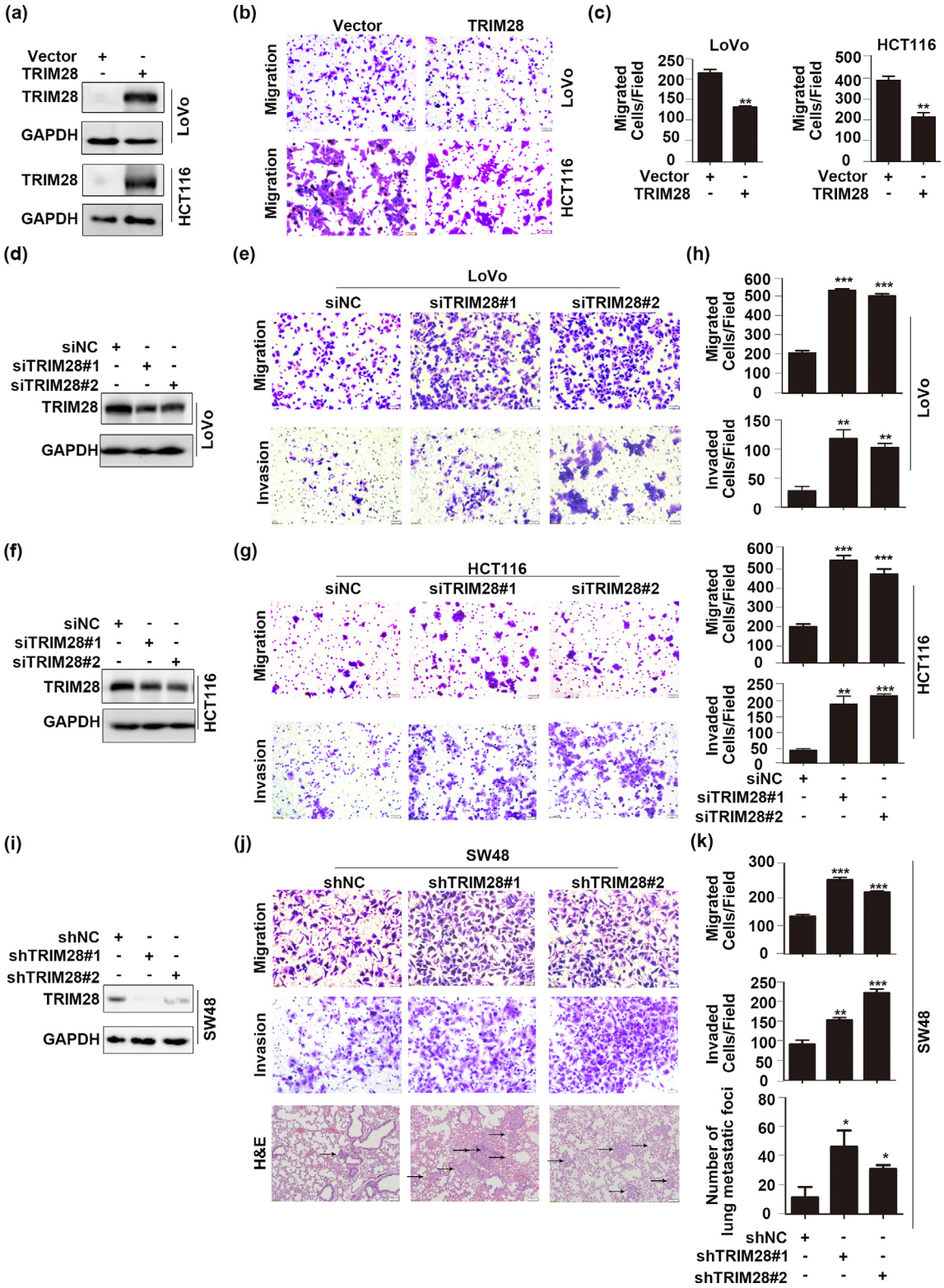

Fig. 2. TRIM28 inhibits the metastasis of CRC. (a) Western blot analysis of TRIM28 expression in LoVo and HCT116 cells transiently transfected with empty vector plasmid (Vector) or Flag-TRIM28 plasmid (TRIM28 OE) for 48 h. (b) The migration assay of LoVo and HCT116 cells transiently overexpressing Flag-TRIM28 or Vector. (c) The average number of migrated cells per field was calculated. $n = 3$ samples per group, four fields per sample; **, $P < 0.01$. (d, f) Western blot analysis of TRIM28 knockdown efficiency in LoVo (d) and HCT116 (f) cells transfected with negative control siRNA (siNC) or two independent siRNAs against TRIM28 (siTRIM28#1 and siTRIM28#2). (e, g) The migration assay (upper) and invasion assay (lower) of LoVo (e) and HCT116 (g) cells transiently transfected with siNC, siTRIM28#1, or siTRIM28#2. (h) The average number of cells per field was calculated. $n = 3$ samples per group, four fields per sample; **, $P < 0.01$; ***, $P < 0.001$. (i) Western blot analysis of TRIM28 knockdown efficiency in SW48 cells infected with lentiviruses containing negative control shRNA (shNC) or two independent shRNAs against TRIM28 (shTRIM28#1 and shTRIM28#2). (j) The migration assay (upper), invasion assay (middle) and animal experiment (bottom) of SW48 cells with TRIM28 stably knockdown. (k) The average number of cells per field (upper and middle in (j)) was calculated. $n = 3$ samples per group, four fields per sample; **, $P < 0.01$; ***, $P < 0.001$; the quantification of the lung metastatic colonization (bottom in (j)) in nude mice injected with SW48 cells (shNC, shTRIM28#1, or shTRIM28#2) at a dose of 2×10^6 cells/each mouse. Black arrows indicate metastatic foci. Scale bars, 100 μm. Data shown as mean ± SD, $n = 5$ per group; *, $P < 0.05$.

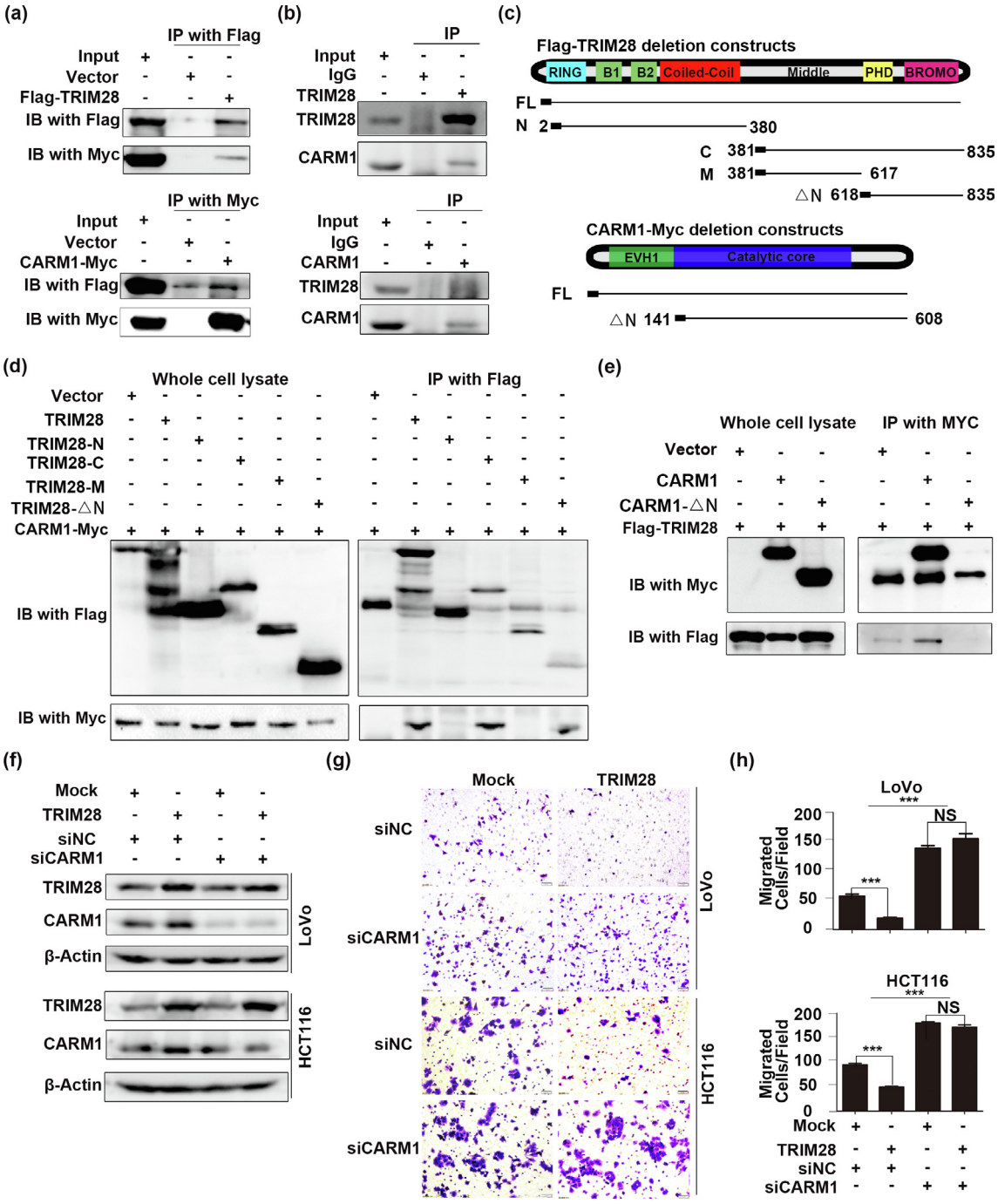

Fig. 3. TRIM28 interacts with CARM1 and suppresses CRC metastasis in a CARM1-dependent manner. (a) HEK293T cells were co-transfected with Flag-TRIM28 and CARM1-Myc. Total cell lysates were subjected to immunoprecipitation with either anti-Flag (upper) or anti-Myc antibody (lower). CARM1-Myc and FLAG-TRIM28 were detected by western blot. (b) Reciprocal Co-IP and western blot assays indicated the interaction between endogenous TRIM28 and CARM1 in LoVo cells. The endogenous TRIM28 or CARM1 was immunoprecipitated by the anti-TRIM28 antibody (upper) or anti-CARM1 antibody (lower). Rabbit immunoglobulin G (IgG) was taken as a negative control. (c) The schematic representation of full-length and deletion mutants of TRIM28 (upper) and CARM1 (lower) constructs. (d) HEK293T cells were co-transfected with full length or truncation mutants of FLAG-TRIM28 and CARM1-Myc. Cell lysates were immunoprecipitated with anti-FLAG antibody, and bound CARM1 was examined using anti-Myc antibody. (e) HEK293T cells were co-transfected with full length or truncation mutants of CARM1 and FLAG-TRIM28. Cell lysates were immunoprecipitated with anti-Myc antibody, and bound TRIM28 was examined using anti-FLAG antibody. (f–h) LoVo and HCT116 cells stably expressing empty vector (Mock) or TRIM28 were transfected with the control or CARM1 siRNA for 48 h. Cells were harvested and subjected to Western blot (f) and migration assay (g). The average number of cells per field was calculated. $n = 3$ samples per group, and four fields per sample; ***, $P < 0.001$ (h).

h). We sought to determine the functional role of TRIM28-CARM1 interaction in CRC metastasis. Depletion of CARM1 abolished the TRIM28-overexpression-induced suppression on CRC metastasis (Fig. 3f–h). Thus, TRIM28 inhibits CRC metastasis in a CARM1-dependent manner.

3.4. TRIM28 protects CARM1 from proteasome-mediated degradation through physical protein-protein interaction

Next, we examined whether the expression of CARM1 or TRIM28 was affected by their interactions. Knockdown of TRIM28

decreased CARM1 protein level without affecting its mRNA level (Fig. 4a, b). Moreover, depletion of CARM1 had no effect on TRIM28 protein levels (Fig. 4c). These results suggest that TRIM28 acts upstream of CARM1 to control CARM1 protein stability via post-translational regulation. The cycloheximide chase assay showed that knockdown of TRIM28 significantly reduced CARM1 protein's half-life in two CRC cell lines (LoVo and HCT116) (Fig. 4d, e). Of note, when TRIM28 knockdown cells were treated with a proteasome inhibitor (MG132), CARM1 protein levels were increased (Fig. 4f), suggesting that TRIM28 protects CARM1 protein from proteasome-mediated degradation. To validate this, we performed protein ubiquitination assays. The ubiquitination of CARM1 was enhanced by TRIM28 knockdown (Fig. 4g). Collectively, these results indicate that TRIM28 stabilizes CARM1 protein by inhibiting its ubiquitination.

As a E3 ubiquitin ligase, TRIM28 might ubiquitinate CARM1 without causing its degradation to suppress CRC metastasis, like HECTD3- and CUL7-mediated Caspase-8 ubiquitination [23,24]. To test this, we constructed TRIM28 (C65/68A) RING mutant that lacked the ubiquitin ligase activity [25,26], and then performed ubiquitination assays and functional assays. Similar to wild type TRIM28, the inactive TRIM28 (C65/68A) RING mutant decreased the ubiquitination of CARM1 (Fig. 4h). This indicates that the effect of TRIM28 on CARM1 ubiquitination is independent of the E3 ligase activity. Meanwhile, the TRIM28-△N mutant, containing the PHD/Bromo domain responsible for the interaction between TRIM28 and CARM1, also attenuated the CARM1 ubiquitination. However, the TRIM28-N mutant lacking PHD/Bromo domain did not affect CARM1 ubiquitination, suggesting that TRIM28-CARM1 interaction is required to prevent CARM1 ubiquitination. We further re-expressed empty vector, wild type TRIM28, the inactive TRIM28 (C65/68A) RING mutant, TRIM28-N mutant, and TRIM28-ΔN mutant in TRIM28 stable knockdown LoVo and HCT116 cells. Western blot analysis showed that wild type TRIM28, the inactive TRIM28 (C65/68A) RING mutant, and TRIM28-ΔN could partially restore CARM1 protein level that was decreased by TRIM28 knockdown. On the contrary, TRIM28-N failed to do so (Fig. 4i). Consistently, the migration assays showed that wild type TRIM28, the inactive TRIM28 (C65/68A) RING mutant, and TRIM28-ΔN, but not TRIM28-N mutant, attenuated metastasis enhanced by TRIM28 knockdown in LoVo and HCT116 cells (Figs. 4j and S2 online). Taken together, TRIM28 stabilizes CARM1 protein to suppress CRC metastasis independently of TRIM28 E3 ligase activity, but dependently on its physical interaction with CARM1.

3.5. TRIM28 dampens WNT/β-catenin signaling to suppress CRC metastasis

We next explored the potential mechanisms by which TRIM28-CARM1 axis suppressed the CRC progression. Western blot analysis showed no changes of MAPK/ERK and PI3K/AKT pathways in TRIM28 knockdown LoVo and HCT1116 cells (data not shown). It was previously reported that TRIM28 knockout led to aberrant accumulation of nuclear β-catenin in hepatocellular carcinoma [27]. Since WNT/β-catenin pathway played an important role in colorectal cancer progression, we wondered whether TRIM28 could regulate WNT/β-catenin pathway in CRC. GSE39582 and GSE21510 datasets showed a reverse correlation between TRIM28 and β-catenin in CRC (Fig. S3a online). Consistently, TRIM28 negatively regulated the protein level of β-catenin in both LoVo and HCT116 cells (Figs. 5a and S3b online). WNT-target genes (TCF4, C-Myc, Axin2) were obviously increased when TRIM28 was knockdown (Fig. 5b). The same results were also found in CARM1 knockdown CRC cells (Fig. S3c, d online). Moreover, CARM1 did not affect the methylation of β-catenin in CARM1 knockdown LoVo and HCT116 cells (Fig. S3e online), and β-catenin expression was also not changed in LoVo and HCT116 cells treated with a specific CARM1 methyltransferase inhibitor (Fig. S3f online). Since aberrant β-catenin nuclear accumulation is crucial to activation of WNT/β-catenin signaling [28], we examined the nuclear and cytoplasm localization of β-catenin. The nuclear of β-catenin was increased in LoVo and HCT116 cells with TRIM28 stable knockdown (Fig. 5c). To further assess the effect of TRIM28 on β-catenin activity, WNT signaling inhibitor XAV939 was used to suppress WNT pathway in TRIM28 knockdown cells. XAV939 diminished β-catenin's elevated transcriptional activity by TRIM28 knockdown (Fig. 5d). XAV939 also suppressed the migration promoted by TRIM28 knockdown in LoVo and HCT116 cells (Fig. 5e–g). we further knockdown CARM1 in TRIM28 stably overexpressed LoVo and HCT116 cells. As expected, CARM1 knockdown increased the protein level of β-catenin, and reversed the inhibited migration phenotype in TRIM28 stably overexpressed cells (Fig. 5h–j). Notably, such reversal was abolished by XAV939 (Fig. 5h–j). These results demonstrate that TRIM28 negatively regulates colorectal cancer metastasis through a TRIM28-CARM1-WNT/β-catenin axis.

3.6. TRIM28 has a high clinical correlation with CARM1 in CRC

We next explored the clinical relevance between TRIM28 and CARM1 in CRC. We studied CARM1 expression in 15 paired CRC tumor and adjacent normal tissues and found that the expression of TRIM28 was positively correlated with CARM1 (Fig. 6a–c). Moreover, the patients with high levels of TRIM28 and CARM1 ($TRIM28^{high}CARM1^{high}$) had the best RFS (Relapse-free Survival), whereas the $TRIM28^{low}CARM1^{low}$ patients had the poorest outcomes (Fig. 6d). Taken together, TRIM28 and CARM1 in CRC are highly and clinically related in predicting survival of CRC patients.

4. Discussion and conclusion

TRIM28 is significantly upregulated and acts as an oncogene in various types of tumors, including breast, gastric, cervical, ovarian and brain cancers and contributes to tumor progression [29–33]. However, opposite conclusions were also reported in lung cancer and TRIM28 liver-specific knockout mice. High levels of TRIM28 predicted improved overall survival of patients with early stage lung tumors and TRIM28 inhibits lung cancer cells proliferation through E2F interactions [13]. Liver-specific TRIM28 knockout in mice led to hepatic tumors with sex-specific transcriptional dysregulation in a wide range of metabolic genes [27,34]. To date, the cellular functions of TRIM28 and the underlying mechanisms remain unexplored in CRC. In this study, we showed that the TRIM28 was downregulated in CRC and tightly associated with improved survival of CRC patients. TRIM28 was able to inhibit CRC metastasis in vitro and in vivo. Therefore, unlike its oncogenic roles in other types of cancer, TRIM28 acted as a tumor metastasis suppressor in CRC.

Ubiquitination is a common post-translational protein modification that targets substrate proteins for proteasomal degradation [35]. As an E3 ubiquitin ligase, TRIM28 promoted the ubiquitination and degradation of many cancer related molecules, such as BCL2A1 and P53 [25,26]. TRIM28 could also interact with TRIM24 and protected it from E3 ubiquitin ligase SPOP-mediated ubiquitination degradation [10]. CARM1, also known as PRMT4, is a type I protein arginine methyltransferase (PRMT) that asymmetrically dimethylates arginine residues of protein substrates [36]. CARM1 methylates histones and other proteins to exert oncogenic or anti-cancer functions in tumor [37,38]. However, the precise regulatory mechanisms on CARM1 are still not well understood. Most of studies focused on the regulation of CARM1 by miRNAs, including

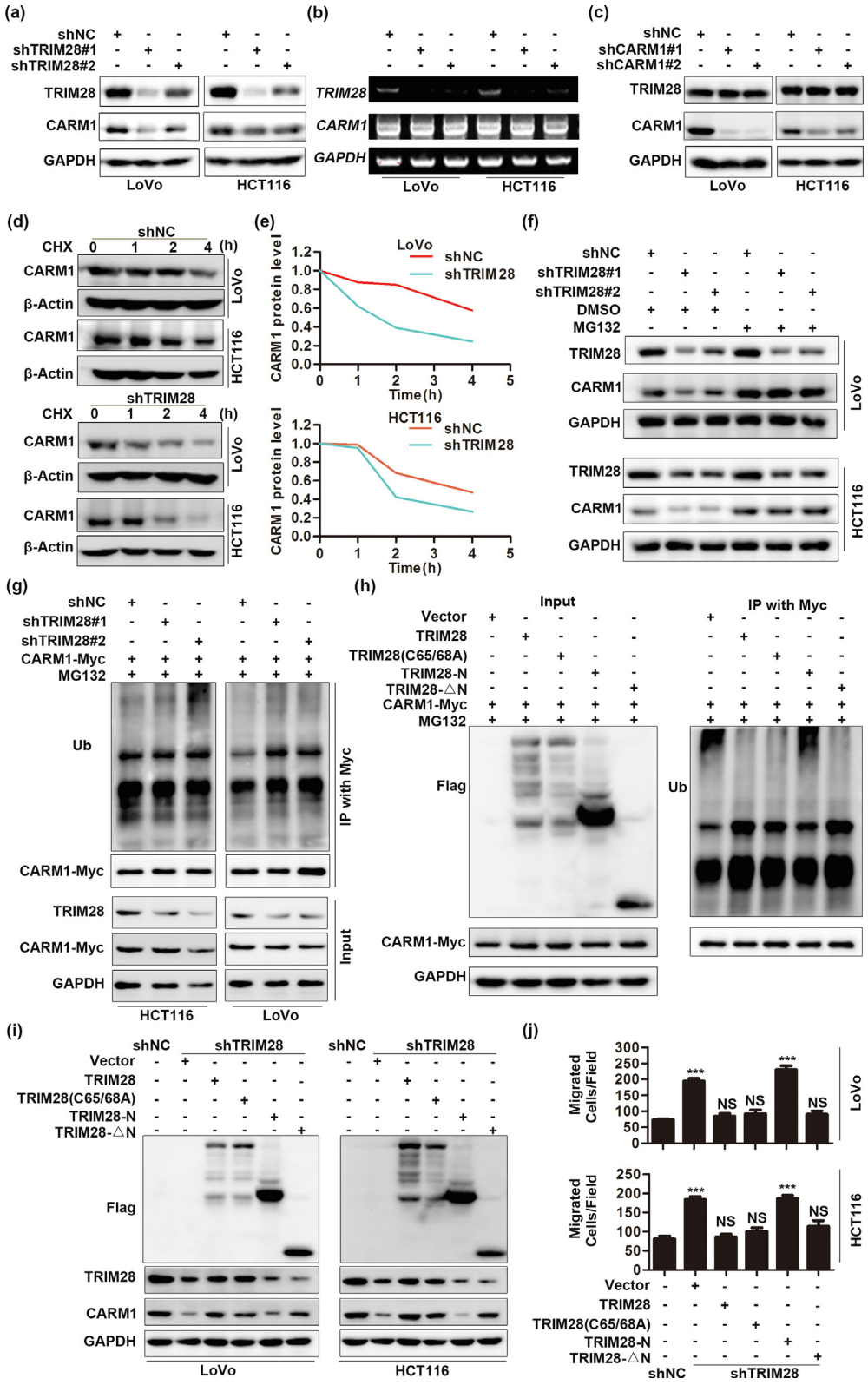

Fig. 4. TRIM28 protects CARM1 from proteasome-mediated degradation through protein-protein interaction. (a, b) Western blot (a) and RT-PCR (b) analysis of CARM1 expression in TRIM28 stably knockdown LoVo and HCT116 cells. (c) Western blot analysis of TRIM28 expression in CARM1 stably knockdown LoVo and HCT116 cells. (d) Cycloheximide chase analysis of CARM1 protein half-life in TRIM28 knockdown LoVo and HCT116 cells. (e) The band intensity of CARM1 for each time point in (d) was quantified by ImageJ and plotted. (f) LoVo (upper) and HCT116 (lower) cells (shNC or shTRIM28) were treated with DMSO or 10 μmol/L MG132 for 12 h, and protein lysates were subjected to western blot analysis. (g) LoVo (right) and HCT116 (left) cells (shNC or shTRIM28) were transfected with CARM1-Myc plasmid for 48 h and treated with MG132 (10 μmol/L) for another 12 h. Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblots with anti-ubiquitin and anti-Myc antibody. (h) CARM1-Myc was co-transfected with empty vector, Flag-TRIM28 (WT), an inactive RING mutant C65/68A of TRIM28, TRIM28-N or TRIM28-△N into HEK293T cells for 48 h and treated with MG132 (10 μmol/L) for another 12 h. Cell lysates were immunoprecipitated with anti-Myc antibody and immunoblotted with anti-ubiquitin and anti-Myc antibody. (i, j) TRIM28 stable knockdown LoVo and HCT116 cells were transfected with empty vector, Flag-TRIM28 or its mutants as indicated for 48 h. Transfected cells were subjected to western blot analysis (i) and migration assay (j).

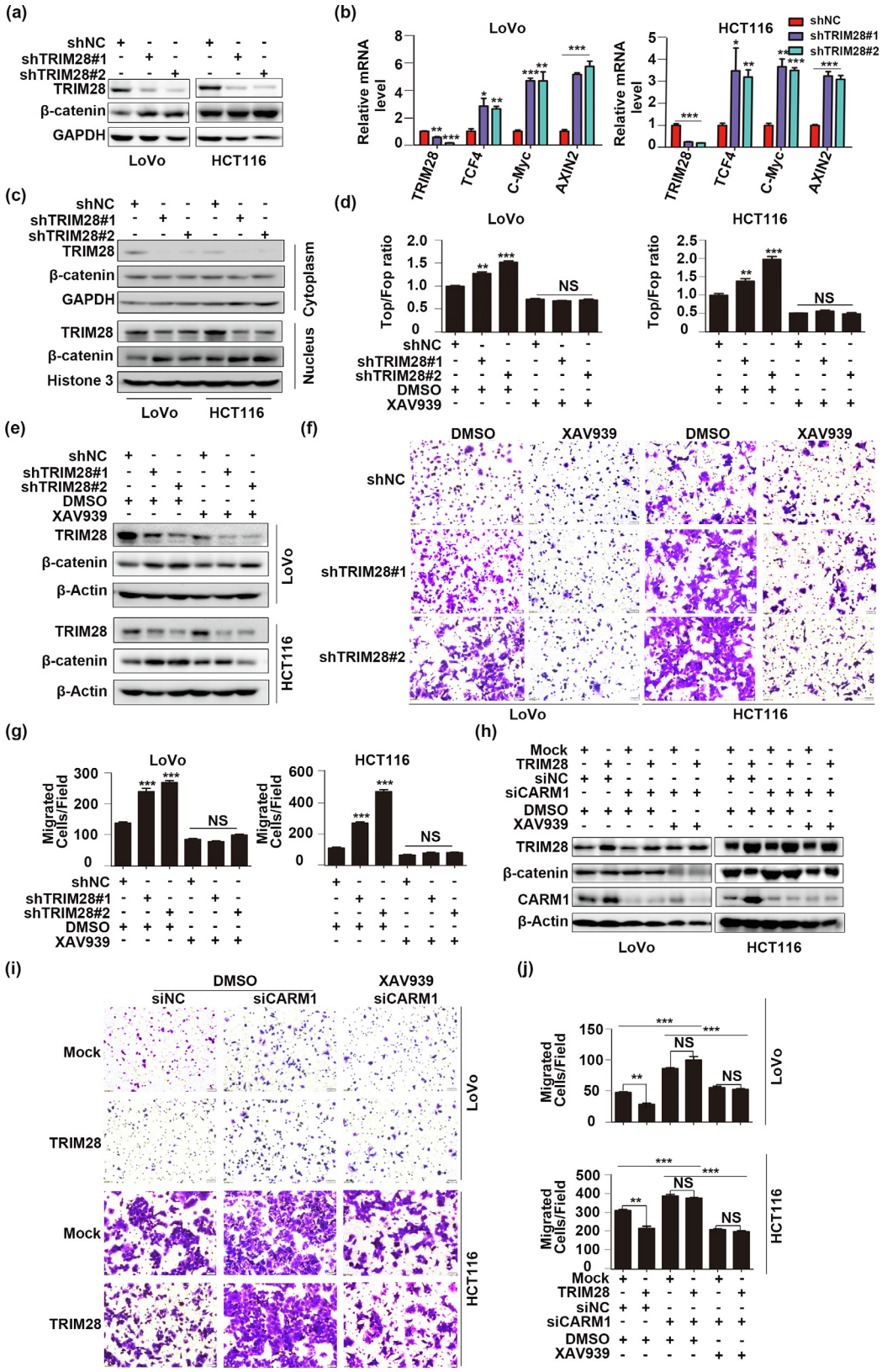

Fig. 5. TRIM28 suppresses the metastasis of colorectal cancer via inactivating WNT/β-catenin pathway. (a) Western blot analysis of β-catenin expression in TRIM28 stably knockdown LoVo and HCT116 cells. (b) qRT-PCR analysis of WNT/β-catenin pathway downstream target genes in TRIM28 stably knockdown LoVo and HCT116 cells. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. (c) Western blot analysis of β-catenin expression in cytoplasmic (upper) and nuclear (lower) extraction from TRIM28 stable knockdown LoVo and HCT116 cells. (d–g) TRIM28 stably knockdown LoVo and HCT116 cells were treated with DMSO or 5 μmol/L XAV939 for 12 h. Cells were harvested for TOP/FOP flash luciferase reporter assays (d), Western blot (e) and migration assay (f). The average number of migrated cells per field was calculated. $n = 3$ samples per group, and four fields per sample; ***, $P < 0.001$ (g). (h–j) LoVo and HCT116 cells stably expressing TRIM28 were transfected with the control or CARM1 siRNA for 48 h, then cells were treated with DMSO or 5 μmol/L XAV939 for another 12 h and harvested for Western blot (h) and migration assay (i). The average number of migrated cells per field was calculated. $n = 3$ samples per group, and four fields per sample; **, $P < 0.01$; ***, $P < 0.001$ (j).

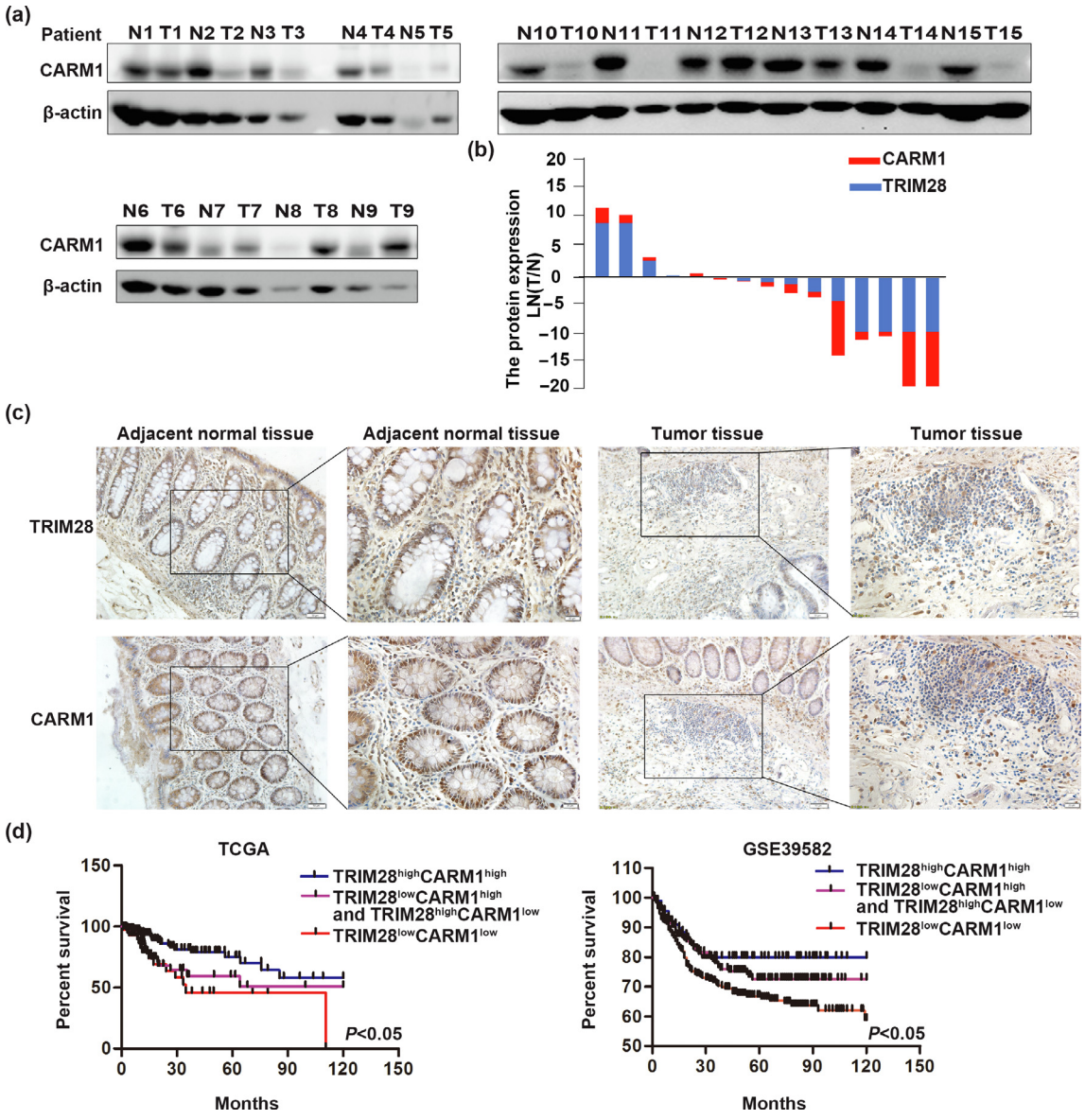

Fig. 6. TRIM28 and CARM1 show a high clinical correlation in CRC. (a) Western blot analysis of CARM1 expression in 15 paired CRC tissues. (b) Quantitative analysis of relative TRIM28 and CARM1 protein levels in 15 paired CRC tissues. (c) Representative images of immunochemical staining of TRIM28 and CARM1 in CRC tissue and adjacent normal tissue. Left row: magnification ×200, scale bars, 50 μm; Right row: magnification ×400, scale bars, 20 μm. (d) Kaplan-Meier survival analysis of RFS based on TRIM28 and CARM1 expression in the CRC patients from TCGA and GSE39582 datasets. Patients were classified as $TRIM28^{high}CARM1^{high}$, $TRIM28^{low}CARM1^{high}$, $TRIM28^{high}CARM1^{low}$, and the $TRIM28^{low}CARM1^{low}$ group.

miR-15a, miR-181c and miR-195 [39–41]. A study showed that CARM1 was a ubiquitination substrate of SKP2-containing E3 ligase complex [42]. In our study, we observed that TRIM28 interacted with CARM1 and protected it from ubiquitin-mediated degradation. Non-degradative ubiquitination of substrates reportedly plays crucial roles in cellular processes. For instance, both HECTD3 and CUL7 mediated Caspase-8 non-degradative ubiquitination at K215 was involved in cell apoptosis [23,24]. Our data revealed that TRIM28 protects CARM1 protein from ubiquitination degradation independently of the TRIM28 E3 ligase activity. We further found that the PHD/Bromo domain of TRIM28 and the EVH1 domain of CARM1 were essential for the interaction. EVH1 domain is a member of pH domains subfamily that binds proline-rich sequences. The N-terminal EVH1 domain of CARM1 has been reported to be necessary for methylation substrate recognition [43]. This interaction may prevent the binding between CARM1 and other E3 ubiquitin ligases that mediate the ubiquitylation and degradation of CARM1.

Previous studies reported that CARM1 acted as an oncogene in several cancer types, such as breast cancer and osteosarcoma [17,44]. Conversely, some studies identified CARM1 as a tumor inhibitor in liver and pancreatic cancers [18,19]. These observations suggest that the function of CARM1 is dependent on type of cancers. CARM1 has been reported to promote cell growth and impair the radiosensitivity of CRC cells [41]. We found that CARM1 had a similar anti-metastasis phenotype with TRIM28 in CRC. TRIM28 mediated suppression of CRC metastasis was largely dependent on CARM1. More importantly, TRIM28-mediated regulation of CARM1 could be observed in clinical samples as there was a positive correlation between the expression of TRIM28 and CARM1 in CRC tissues. TRIM28 and CARM1 in CRC were clinically related in predicting survival of CRC patients. Our findings may suggest a novel combination strategy of targeting the TRIM28–CARM1 axis for CRC treatment.

Deregulation of WNT/β-catenin pathway is a common feature of solid cancers, especially in CRC [45–50]. Our results reveal that

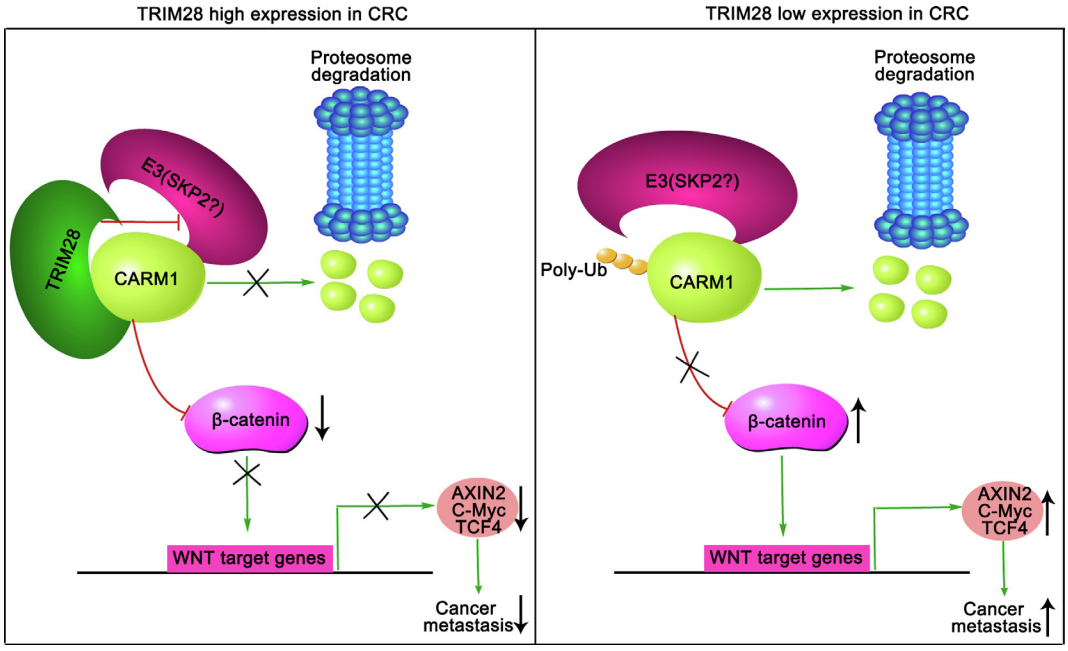

Fig. 7. Schematic diagram of the molecular mechanism of TRIM28 suppressing CRC metastasis. High expression of TRIM28 protects CARM1 from degradation by proteasome to inactivate WNT/β-catenin pathway and further to suppress CRC metastasis (left). CARM1 is degraded by ubiquitination to enhance WNT/β-catenin signaling and to promote CRC metastasis while low expression of TRIM28 (right).

TRIM28 negatively regulated WNT/β-catenin pathway in a CARM1-dependent manner to suppress CRC metastasis, which however was inconsistent with a previous study reporting that knockdown of TRIM28 inhibited the migration and invasion of ovarian carcinoma cells through downregulation of WNT/β-catenin pathway [51]. The contradictory results may due to the tumor heterogeneity of CRC and ovarian carcinoma. As a type I protein arginine methyltransferase, CARM1 did not affect the methylation of β-catenin and regulated β-catenin protein with no requirement for CARM1′s methyltransferase activity. The exact mechanisms by which TRIM28 modulates WNT/β-catenin pathway through CARM1 in CRC need further exploration.

In conclusion, we display for the first time that CARM1 protein stability is promoted by TRIM28. The orchestrated interaction between TRIM28 and CARM1 can inactivate WNT/β-catenin pathway that may contribute to suppress CRC metastasis (Fig. 7). Our findings underscore the roles of TRIM28 in CRC metastasis, contribute significantly to the understanding of CARM1 regulation, and highlight the high clinical correlation between TRIM28 and CARM1 in CRC. The newly defined TRIM28-CARM1-WNT/β-catenin regulatory axis may open new avenues for CRC therapy.

Conflict of interest

The authors declare that they have no conflict of interest.

Acknowledgments

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Author contributions

J.-Y.C. and Z.-W.; performed experiments and analyzed data, J.-Y.C., J.-H., Z.-L.Y., Y.-L.F., Y.-Q.L.; data curation, J.-Y.C.; writing—original draft, J.-Y.C., J.H. and Z.-W.; supervision, G.-B.W., L.-W. and Z.-W. All authors read and approved the final manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scib.2019.05.024.

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Jinyuan Cui is currently pursuing his Ph.D. degree of medicine under the supervision of Prof. Zheng Wang at Department of Gastrointestinal Surgery and Research Center for Tissue Engineering and Regenerative Medicine, Union Hospital, Tongji Medical College, Huazhong University of Science and Technology. His research mainly focuses on the biological role and related molecular mechanism of TRIM28 in colorectal cancer metastasis.

Guobin Wang, M.D./Ph.D., is a fellow of American College of Surgeons and a professor of Department of General Surgery, Union Hospital, Huazhong University of Science and Technology. His research mainly covers oncogenesis, cancer immunology, and inflammation-cancer transition.

Lin Wang, Ph.D., is currently a professor in Research Center for Tissue Engineering and Regenerative Medicine at Union Hospital, Huazhong University of Science and Technology. She is interested in solving medical problems using multi-interdisciplinary approaches. In recent years, she concentrates on research and development of naturally derived biomaterials and their applications in tissue regeneration and cancer therapy and her findings during the years have laid foundation for future clinic translation.

Zheng Wang, M.D./Ph.D., is a full professor and associate director of Department of Gastrointestinal Surgery and Research Center for Tissue Engineering and Regenerative Medicine, Union Hospital, Huazhong University of Science and Technology. His research focuses on elucidating molecular mechanisms underlying pathogenesis and metastasis of colorectal cancer and utilize cancer bioengineering approaches to develop novel therapeutic strategies towards colorectal precision medicine.