

# Zinc-finger protein 545 is inactivated due to promoter methylation and functions as a tumor suppressor through the Wnt/ $\beta$ -catenin, PI3K/AKT and MAPK/ERK signaling pathways in colorectal cancer

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**Abstract.** The transcription factor, zinc-finger protein 545 (*ZNF545*), that belongs to the Kruppel-associated box zinc-finger protein (KRAB-ZFP) family, acts as a tumor suppressor and is inactivated by promoter methylation in cancers such as nasopharyngeal carcinoma, breast cancer, and gastric cancer, but its role in colorectal cancer (CRC) is unknown. The purpose of this study was to characterize the *ZNF545* expression, methylation status, biological function, and related molecular mechanisms in CRC. The results showed that *ZNF545* was expressed in adult normal colorectal tissues, but downregulated or silenced in CRC cell lines, and this mechanism was reversed by demethylation treatment with 5-aza-2'-deoxycytidine and trichostatin A. The results also showed that the expression of *ZNF545* in primary CRC tissues was significantly downregulated compared to adjacent tissues ( $p < 0.05$ ). Overexpression of *ZNF545* caused CRC cell cycle arrest and apoptosis, suppressed cell proliferation, and suppressed colony formation and migration *in vitro*, showing that *ZNF545* can function as a tumor suppressor. This function was also shown in nude mice. Furthermore, Wnt/ $\beta$ -catenin, phosphatidylinositol 3 kinase/protein kinase B (PI3K/AKT), and mitogen-activated protein kinases/extracellular signal-regulated kinase (MAPK/ERK) signaling pathways participated in the regulation of *ZNF545* in CRC cells. Together, the results suggested that *ZNF545* functions as a tumor suppressor in CRC and is frequently inactivated by promoter methylation.

## Introduction

Colorectal cancer (CRC) is a common malignant tumor involving approximately 14 million new cases per year, and is the cause of approximately 690,000 deaths worldwide each year (1). The incidence of CRC increases with age; 79% of CRC patients are >60 years of age when diagnosed, and 34% are >80 years of age when diagnosed (2). Furthermore, it is predicted that the incidence of CRC in elderly patients will increase in the future. The pathogenesis of CRC involves activation of an oncogene and inactivation of a tumor suppressor gene (3), and the tumor suppressor gene inactivation is closely related to epigenetic changes. These epigenetic changes include DNA methylation, histone acetylation, and chromatin remodeling (4). DNA methylation plays an important role in the development and progression of different cancers such as CRC. Increased evidence has suggested that promoter methylation of tumor suppressor genes can be used as a tumor marker in the early diagnosis of multiple cancers (5,6). When CRC is diagnosed, it is often already in an advanced stage, so it is important to identify markers for early diagnosis of CRC.

Zinc finger proteins containing the Kruppel associated box (KRAB-ZFPs) belong to the zinc finger protein family, which is the largest family of transcription factors in mammals (7,8). KRAB-ZFPs regulate cell proliferation, differentiation, apoptosis, and formation of tumor cells, and play an important role in biological evolution (9). KRAB-ZFPs are transcription factors that bind to the promoter of target genes to inhibit or activate their expression (10), and to either inhibit or promote carcinogenesis. *ZIC1*, *ZNF569* and *ZFP932* have been reported to be tumor suppressor genes (11-13), and *KLF5*, *SALL4* and *ZNFEB* have been reported to be oncogenes (14-16).

*ZNF545*, located on the 19q13 chromosome, is a member of the KRAB-ZFP family of tumor suppressor genes, and is downregulated and frequently methylated in multiple types of tumors (17-19). However, the effect of epigenetic inactivation of *ZNF545* in colorectal carcinogenesis and its possible use as a tumor marker remain unclear. We therefore characterized the methylation status and expression of *ZNF545* in

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CRC, together with its related functions and mechanism of action.

## Materials and methods

**Cell lines and tumor samples.** Five CRC cell lines (HT-29, SW480, HCT116, CaCo-2, and LoVo) were used. The CRC cell lines, HT-29 and HCT116, were provided by Professor Q. Tao at the Chinese University of Hong Kong, and the SW480, CaCo-2, and LoVo cell lines were purchased from the Chinese Academy of Sciences. The cell lines were cultured with RPMI-1640 medium (Gibco-BRL, Karlsruhe, Germany) containing 10% fetal bovine serum (FBS) (ExCell Bio, Shanghai, China), and cultured in a 5% CO<sub>2</sub> incubator at 37°C. The CRC tissues and paracarcinoma tissues, which were diagnosed by a pathologist, were obtained from patients during surgery at the First Affiliated Hospital of Chongqing Medical University (Chongqing, China). All patients signed an informed consent form, and the research protocol was approved by the Institutional Ethics Committee of the First Affiliated Hospital of Chongqing Medical University.

**RNA, DNA, and protein extraction.** Total RNA was extracted separately from 32 paired CRC tissues, surgical margin tissues, and five CRC cell lines using TRIzol<sup>®</sup> reagent (Life Technologies, Carlsbad, CA, USA). Genomic DNA was separately obtained from 24 CRC tissues, six normal colorectal tissues, and the five CRC cell lines using the QIAamp DNA Mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The concentration of DNA and RNA were measured using a NanoDrop 2000 Spectrophotometer (Thermo Scientific, Rockford, IL, USA) and stored at -80°C. The experimental and control group of the HT-29 and SW480 cells were lysed using a protein extraction reagent (Thermo Scientific) that contained the protease inhibitor, phenylmethane sulfonyl fluoride, and a phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA), and the lysate was then homogenized using a Ultrasonic Cell Grinder (Scientz, Ningbo). The supernatant was collected after centrifugation, and the concentration of protein in the supernatant was determined using the BCA protein kit (Thermo Scientific).

**Semiquantitative polymerase chain reaction (PCR) and real-time PCR.** *ZNF545* expression in CRC cells and tissues was determined using semiquantitative PCR and quantitative PCR. The RNA (1 µg) was reverse transcribed to 20 µg of cDNA using the Reverse Transcription system (Promega, Madison, WI, USA). For semiquantitative PCR, the *ZNF545* gene was amplified using GoTaq DNA polymerase (Promega) with 35 cycles, using GAPDH as an internal control. The primer sequences are listed in Table I. Quantitative PCR was performed with a SYBR mix green fluorescent reagent (Promega) using an ABI 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). β-actin was used as a control, and each sample was tested in triplicate.

**Methylation-specific PCR analyses of *ZNF545*.** As previously described (20), methylation-specific PCR (MSP) was used to detect the *ZNF545* promoter methylation status. bisulfite-treated DNA (50 ng) was mixed with AmpliTaq

Gold polymerase (Applied Biosystems), mgCl<sub>2</sub>, and deoxynucleotide triphosphates for the MSP amplification reaction. The methylation-specific primers are listed in Table I. The PCR amplification was performed for a total of 40 cycles with an annealing temperature of 60°C or 58°C for 30 sec. The final products were analyzed on a 2% agarose gel, and then recorded using a Molecular Imager (Bio-Rad, Hercules, CA, USA).

**5-Aza-2'-deoxycytidine (Aza) and trichostatin A (TSA) treatment.** Aza, a DNA methyltransferase (DNMT) inhibitor, makes DNMT inactivation through DNMT covalent bonding with thiol on cysteine residues, causing reactivation genes silenced by promoter methylation. TSA, a histone deacetylase inhibitor, plays an important role in controlling the tightness of DNA around histone. Combination treatment of Aza and TSA leads the synergistic activation of methylated genes.

CRC cell lines, SW480 and HT-29, were cultured and demethylated. The cells were treated with Aza (Sigma-Aldrich) in the dark at a final concentration of 10 mM/l for 3 days or TSA at a final concentration of 100 mM/l for 1 day, and further treated with or without 100 mM/l TSA (Cayman Chemical, Ann Arbor, MI, USA) for another 1 day.

**The establishment of stable cell lines.** A *ZNF545*-expressing plasmid was provided by Professor Q. Tao at the Chinese University of Hong Kong. After SW480 and HT-29 cells were plated into 6-well plates, the cells were transfected with pcDNA3.1-*ZNF545* or pcDNA3.1 (+) vectors using Lipofectamine<sup>®</sup> 2000 reagent (Invitrogen) and cultured with serum-free RPMI-1640 medium. After 4-6 h, the medium containing 10% FBS was changed to a selection medium containing 400 µg/ml G418 (Invitrogen/Gibco) and incubated for 48 h. Approximately 14 days later, the HT-29 and SW480 cells were stably expressing pcDNA3.1 and pcDNA3.1-*ZNF545*.

**The cell proliferation and colony formation assays.** SW480 and HT-29 cells stably expressing *ZNF545* or pcDNA 3.1 were seeded into 96-well plates at 2,000 cells/well, and the cell viability was measured using MTS (Promega) at 0, 24, 48 and 72 h using a Multiskan Spectrum<sup>™</sup> (Tecan, Switzerland). The stably expressing cells were counted and seeded into 6-cell plates at 500 cells/well. Visible cell clones appeared in ~2 weeks, then they were fixed with 4% paraformaldehyde for 30 min and then stained with crystal violet solution for 30 min. Finally, the cells were scanned using a CanoScan 8800F (Canon, Tokyo, Japan).

**Flow cytometry of the cell cycle and apoptosis.** The cell cycle and apoptosis were measured using flow cytometry as previously described (20). For the cell cycle measurements, SW480 and HT-29 cells stably expressing *ZNF545* or pcDNA 3.1 were digested and washed twice using precooled phosphate-buffered saline (PBS), then fixed with ice-cold 70% ethanol, stored at 4°C overnight, and then stained with propidium iodide (PI), followed by analysis using CellQuest software (BD Biosciences, San Jose, CA, USA). After the stably transfected cells were collected and washed twice with PBS, they were stained with Annexin V-FITC/PI in the dark, and the cell

Table I. The primers used in this study.

	Primer	Sequences (5'-3')	Product sizes (bp)	Annealing temperature (°C)	Cycles
RT-PCR	ZNF545-F	GAGCCTTGGAAGTTGTGAG	245	55	35
q-PCR	ZNF545-R	GGCATTTCACACTACTGAAG			
MSP	ZNF545-M1	TTTTTTTTTAGGTTTTGTGTCGCGTC	177	60	40
	ZNF545-M2	CTACTAAAAAACCGAACGCG			
	ZNF545-U1	TTTTTTTTTAGGTTTTGTTGTGTT	164	58	40
	ZNF545-U2	CCAAACACACTCACAAAATACA			

apoptosis was measured using CellQuest software, followed by data analyses.

*The analyses of wound healing.* Stably ZNF545- or vector-transfected SW480 or HT-29 cells were seeded into 6-well plates, and when the cells reached 95% confluency, a sterile P-20 pipette was used to scratch wounds across each well, followed by removal of cell debris using PBS. The cells were then cultured with medium without FBS to minimize cell proliferation. The images of the wound closure areas of SW480 cells were observed at 0, 48, and 60 h, and the images of the wound closure areas of HT-29 cells were observed at 0, 24, 48, and 84 h using a light microscope (CTR4000; Leica, Germany).

*Western blot analyses.* A total of 40 mg of protein lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then transferred to a polyvinylidene fluoride (PVDF) membrane (Bio-Rad). The primary antibodies were against cleaved caspase-3 (12742; Cell Signaling Technology, Danvers, MA, USA), cleaved poly(ADP-ribose) polymerase (PARP) (9541; Cell Signaling Technology), BAX (5023; Cell Signaling Technology), ZNF545 (sc-102235; Santa Cruz Biotechnology, Santa Cruz, CA, USA), PI3K (ab125571; Abcam, Cambridge, UK), phospho-PI3K (Abcam), Akt (4691; Cell Signaling Technology), phospho-Akt (ser473) (4060; Cell Signaling Technology), phospho-GSK3 $\beta$  (ser9) (9323; Cell Signaling Technology),  $\beta$ -catenin (2677; Cell Signaling), active  $\beta$ -catenin (4270; Cell Signaling), c-Myc (1472-1; Epitomic), cyclin D1 (2261; Epitomics),  $\beta$ -actin (LK-ab008-100; Liankebio, China), and Flag (F3165; Sigma-Aldrich).

*In vivo tumorigenicity.* Six groups of 4-week-old nude mice were used. Empty vector, stably transfected SW480 cells ( $3 \times 10^6$  cells suspended in 200  $\mu$ l of PBS) were injected subcutaneously into the left dorsal flank of nude mice, and ZNF545-transfected SW480 cells ( $3 \times 10^6$  cells suspended in 200  $\mu$ l of PBS) were injected subcutaneously into the right dorsal flank. After the tumor mass appeared, its length and width were measured every 3 days using a microcaliper. As previously described (23), the tumor volume (mm<sup>3</sup>) was calculated using the equation: volume = length x width<sup>2</sup>/2. The mice were euthanized and the tumors were removed when the tumor length reached 1.5 cm. All tumor tissues were weighed before

fixation in 4% paraformaldehyde and embedded in paraffin, followed by preparation of the sections for immunohistochemical analyses. All experimental procedures were approved by the Animal Ethics Committee of the Experimental Animal Center of the Chongqing Medical University, Chongqing, China.

*Immunohistochemical staining.* The samples of excised tumor tissues from nude mice were fixed in 4% paraformaldehyde before being embedded in paraffin, followed by sectioning into 4- $\mu$ m slices. After the sections were dewaxed for 2 h in a 60°C incubator, dewaxing and alcohol dehydration were performed in the following order: xylene I, xylene II, xylene III, xylene IV, absolute ethyl alcohol, 95% ethyl alcohol, 80% ethyl alcohol, and 70% ethyl alcohol. Antigen retrieval was performed by boiling the slides in citrate buffer solution for 20 min, followed by cooling at room temperature. The sections were then incubated in 3% hydrogen peroxide for 20 min to prevent endogenous peroxidase activity and washed three times with PBS, then incubated in sheep serum for 20 min. The sections were incubated at 4°C overnight with rabbit anti-PCNA monoclonal antibody. The next day, the sections were warmed to room temperature for 2 h, then washed three times with PBS and incubated with rabbit secondary antibody for 20 min. The slices were washed three times with PBS and incubated with horseradish peroxidase-labeled streptomycin anti-biotin antibody for 20 min. The sections were washed three times, followed by color development with DAB. The sections were washed with water, and the cell nuclei were dyed using hematoxylin, followed by observation using light microscopy.

*Statistical analysis.* All statistical analyses were performed using SPSS statistical software for Windows, version 16. Student's t-test was used to compare the expression of ZNF545 in CRC tissues and adjacent normal tissues. The four table Chi-square test or the t-test were used to analyze categorical variables. A value of  $p < 0.05$  was considered statistically significant.

## Results

*ZNF545 is downregulated in CRC tissues and cell lines.* The messenger RNA (mRNA) expression of ZNF545 in CRC and its corresponding adjacent tissues was detected by quantitative PCR. The results showed that the expression of ZNF545 was

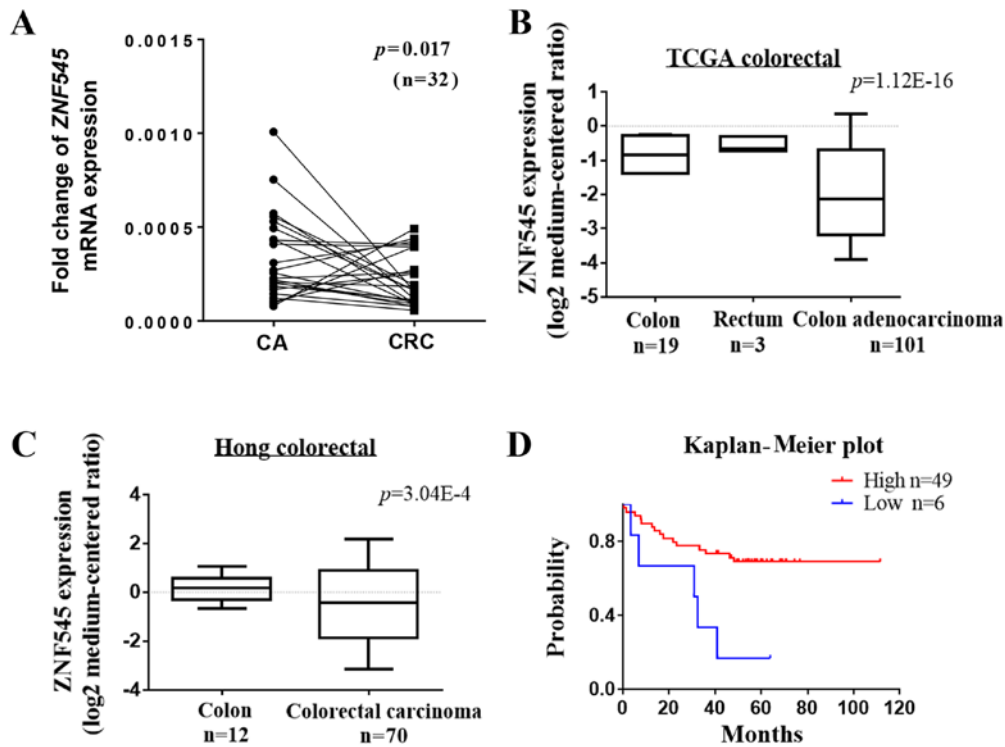


Figure 1. The expression and prognosis of *ZNF545* in CRC tissues. (A) Expression of *ZNF545* mRNA in human CRC and its corresponding adjacent tissues detected by qRT-PCR. (B and C) The expression of *ZNF545* (median of expression intensity) in CRC and normal colorectal tissues from Oncomine database. (D) The correlation between *ZNF545* expression and CRC patient prognosis. The Prognoscan database was used to compared the *ZNF545* expression and patient prognosis.

downregulated in tumor tissues compared with the adjacent tissue ( $p < 0.05$ , Fig. 1A). RT-PCR was then used to examine the expression of *ZNF545* in the HT-29, SW480, HCT116, CoCo-2, and LoVo colorectal cell lines. *ZNF545* expression was downregulated or silenced in all cell lines compared with the vector (Fig. 2A). In addition, analyses of an online microarray database ([www.oncomine.org](http://www.oncomine.org), Compendia Bioscience, Ann Arbor, MI, USA) showed that *ZNF545* was downregulated in CRC tissues but not in normal adult colorectal tissues ( $p < 0.001$ ; Fig. 1B and C). Prognostic analyses from the Prognoscan database (<http://www.prognoscan.org/>) showed that patients with higher expression of *ZNF545* mRNA had significantly increased relapse-free survival compared with those with lower *ZNF545* mRNA levels (Fig. 1D). Taken together, these results suggested that *ZNF545* was a cancer suppressor gene.

*The ZNF545 promoter was frequently methylated during CRC.* Aberrant promoter methylation is a common mechanism involved in tumor suppressor gene silencing in cancers (21,22). To identify whether the decreased expression of *ZNF545* was due to promoter methylation, we determined the methylation status of the *ZNF545* promoter in five CRC cell lines using MSP. The results showed that *ZNF545* was methylated in all cell lines that were associated with silenced or reduced *ZNF545* expression, while normal colorectal tissues expressed significantly higher levels of *ZNF545* mRNA (Fig. 2A). We then treated SW480 and HT-29 cells which have low-expression of *ZNF545* with Aza, TSA and combination, results showed that its expression was restored together with increasing unmeth-

ylated alleles of the *ZNF545* promoter (Fig. 2B). The results suggested that promoter methylation is directly responsible for *ZNF545* downregulation in colorectal cancer cells.

To investigate whether *ZNF545* promoter methylation existed in CRC tissues, MSP was used to examine 32 primary colorectal carcinomas and six normal colorectal tissues. The clinical information of colorectal carcinoma tissues are shown in Table II. We observed that *ZNF545* methylation was detected in 28 out of 32 (87.5%) CRC tissues, but in only two out of six (33.33%) normal colorectal tissues (Fig. 2C and D and Table III  $p = 0.0116$ ). Nine pairs of primary CRC tissues and para-carcinoma tissues which were randomly selected in the 32 CRC tissues, were also tested for *ZNF545* expression by qPCR. Most tumors have lower *ZNF545* mRNA expression compared with their paired adjacent tissues (Fig. 2E), while displaying a higher level of promoter methylation.

*Overexpression of ZNF545 inhibits cell proliferation and clonogenicity of CRC cells in vitro.* To determine whether *ZNF545* is a functional tumor suppressor gene in CRC we used the MTS assay and the colony formation assay to measure the growth-inhibiting effects of overexpressed *ZNF545* in SW480 and HT-29 cells. The transfection efficiencies of our *ZNF545* constructs were confirmed by RT-PCR and western blotting of tumor cell lines and stable cell lines that were selected, respectively (Fig. 3A and B). The MTS cell proliferation assay showed that the cell viability of the *ZNF545*-overexpressing cells significantly decreased at 48 and 72 h compared with cells transfected with the empty vector ( $p < 0.05$ ; Fig. 3C). The colony formation assay showed that the colony number

Table II. The clinical information of colorectal carcinoma tissues.

No.	Age	Size (cm)	Sex (1M2F)	Grade	Lymph metastasis	Distant metastasis	Localization	T-stage	N-status	Phase	Pathological type
CRC2	42	3,2	2	3	0	0	Middle,down	3	N0	IIA	Ad
CRC3	78	2	2	2	1	0	Middle	3	N1	IIIB	Ad
CRC4	65	2	1	2	0	0	Left	3	N1c	IIIB	Ad
CRC5	63	3	2	3	1	0	Left	4b	N1	IIIC	Ad
CRC6	82	3,2	1	2	0	0	Right, down	3	N0	IIA	Ad
CRC8	72	2	1	2-3	0	0	Middle	3	N0	IIA	Ad
CRC10	76	2	2	2	0	0	Right	3	N0	IIA	Ad
CRC14	67	2	1	2-3	0	0	Left	1	N0	I	Ad
CRC16	76	2	1	2	0	0	Left	3	N0	IIA	Ad
CRC17	79	2	2	2	4	0	Left	3	N2a	IIIB	Ad
CRC19	55	2	1	2	0	0	Left	3	N0	IIA	Ad
CRC23	76	2	2	2	2	0	Left	3	N1	IIIB	Ad
CRC24	44	2	2	X	0	0	Left	3	N0	IIA	Ad
CRC25	73	2	2	2	0	0	Left	3	N0	IIA	Ad
CRC26	62	3.2	1	2-3	7	0	Left	3	N2b	IIIC	Ad
CRC27	66	2	1	X	1	0	Left	4b	N1	IIIC	MN
CRC29	65	2	2	2	5	0	Right	3	N2a	IIIB	Ad
CRC30	78	3	1	2	1	0	Left	3	N1	IIIB	Ad
CRC31	66	2	1	2	0	1	Left	3	N1c	IVA	Ad
CRC33	78	2	2	2	0	0	Right	3	N1	IIIB	Ad
CRC34	70	2	1	2-3	0	0	Right	2	N0	I	Ad
CRC35	59	2	2	2	0	0	Left	3	N0	IIA	Ad
CRC36	45	2	1	2	0	0	Left	3	N0	IIA	Ad
CRC37	67	2	2	2	0	0	Right	3	N0	IIA	Ad
CRC38	76	2	2	2	1	0	Right	3	N1	IIIB	Ad
CRC39	64	2	1	2	0	0	Left	3	N0	IIA	Ad
CRC40	43	3	1	2	5	0	Right	3	N2a	IIIB	Ad
CRC41	72	3	1	2	0	0	Right	3	N0	IIA	Ad
CRC42	68	2	1	2	0	0	Left	2	N0	I	Ad
CRC43	57	3	1	2	0	0	Right	3	N0	IIA	Ad
CRC44	80	2,2,2	1	2	2	0	Left, down	3	N1	IIIB	Ad
CRC45	54	2	2	2	0	0	Right	3	N0	IIA	Ad

Ad, adenocarcinoma; MN, mixed neurosecretion.

Table III. Methylation status of the *ZNF545* promoter in colorectal cancer.

Samples	<i>ZNF545</i> promoter		Frequency of methylation
	Methylation	Unmethylation	
CRC (n=32)	28	4	87.5%
CN (n=6)	2	4	33.3%

CRC, colorectal cancer; CN, colorectal normal tissues.

of cells transfected with *ZNF545* was reduced by ~45% when compared with that transfected with the empty vector in SW480 and HT-29 cells ( $p < 0.001$ ; Fig. 3D), indicating that expression of *ZNF545* decreased the cloning efficiency. Together, these results showed that *ZNF545* inhibited cell proliferation in CRC.

*Overexpression of ZNF545 induces cell cycle arrest and apoptosis of CRC cells.* Further studies characterized the effects of *ZNF545* on apoptosis and the cell cycle of CRC cells. Fig. 4A shows that the stably-transfected SW480 and HT-29 cells showed a significantly increased number of cells

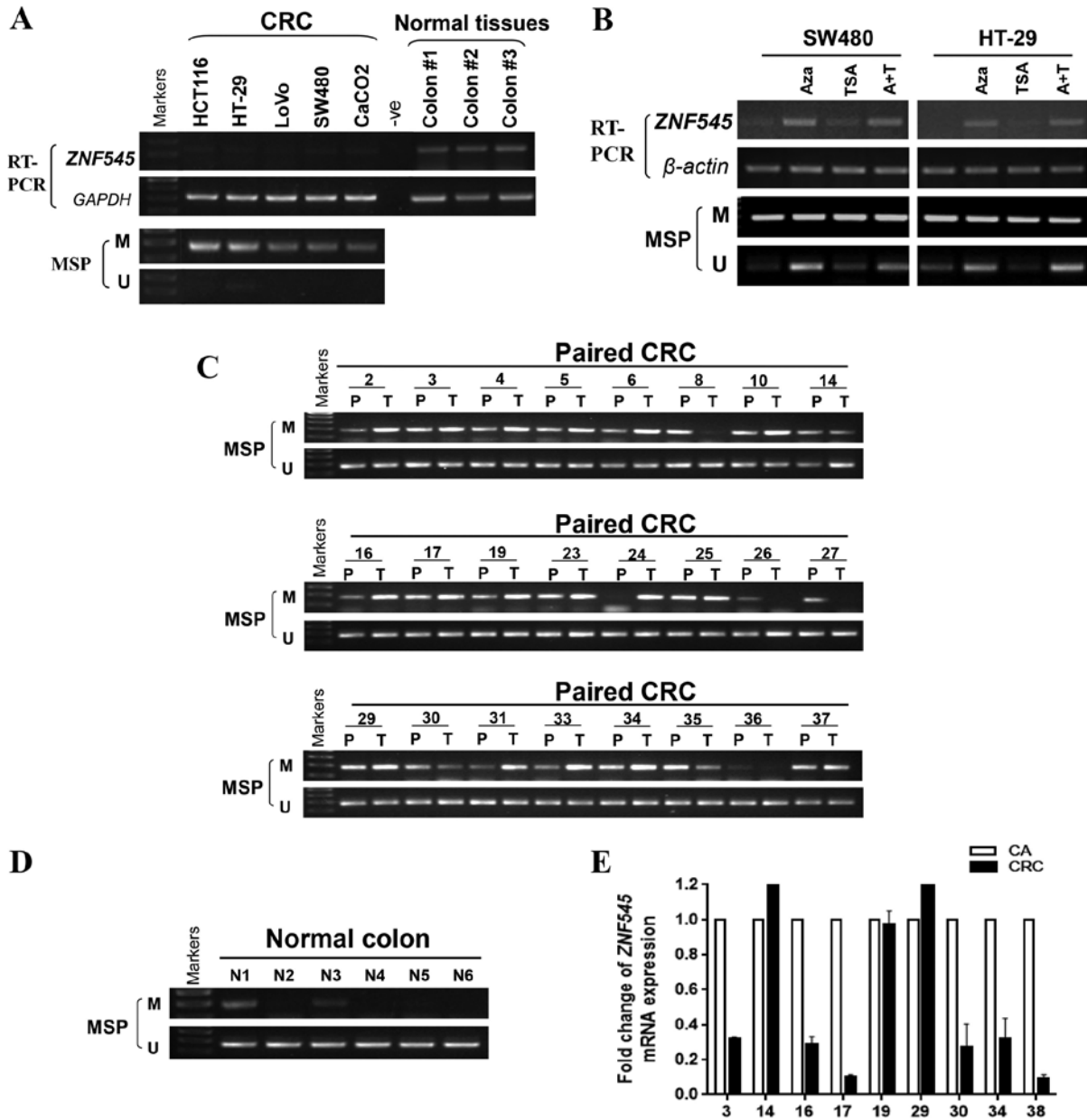


Figure 2. The expression and methylation status of *ZNF545* in CRC cell lines and colorectal tissues. (A) Expression of *ZNF545* mRNA in CRC cell lines and normal colorectal tissues, and the methylation status of *ZNF545* in CRC cell lines. (B) Pharmacological demethylation of *ZNF545* by Aza (A) or TSA (T) restored its expression. *ZNF545* expression before and after drug treatment was determined by RT-PCR, and demethylation was confirmed by MSP. (C) The methylation status of the *ZNF545* promoter in CRC tissues, CRC adjacent tissues, and (D) normal colon tissues. M, methylated; U, unmethylated. (E) *ZNF545* expression levels in 9 paired CRC tissues and adjacent tissues were detected by qPCR. CA, colorectal tumor adjacent tissues; CRC, colorectal cancer tissues.

accumulating in the G0/G1 phase when compared with control cells ( $p < 0.01$ ), suggesting that the inhibition of cell proliferation by *ZNF545* was likely mediated by a cell cycle arrest at the G0/G1 interface. In addition, flow cytometry showed that the percentage of apoptotic Annexin V-PI-positive cells increased with the overexpression of *ZNF545* (Fig. 4B and C).

We also determined the apoptosis of CRC cell lines by western blot analyses of Bax, the active form of caspase-3, and PARP. Fig. 4D shows the increased expression of Bax, cleaved-caspase-3, and cleaved-PARP in both cell lines when compared with cells transfected with the control vector. Together, these results suggested that *ZNF545* inhibited cell proliferation by apoptosis and by mediating the cell cycle arrest at the G0/G1 interface.

*ZNF545* inhibits CRC cell migration. A wound-healing assay was performed to test the effect of *ZNF545* on CRC cell migration. Cells expressing *ZNF545* spread along the wound edges significantly more slowly than the control cells (Fig. 5), suggesting that *ZNF545* suppressed the migration of SW480 and HT-29 cells.

*ZNF545* inhibits the growth of tumor xenografts in nude mice. We also determined if *ZNF545* could suppress the growth of CRC cells *in vivo*. SW480 cells stably transfected with *ZNF545* or an empty vector formed tumors in nude mice (Fig. 6A). The subcutaneous tumor growth curve of SW480 cells stably-transfected with *ZNF545* or with empty vector showed a significant difference ( $p < 0.05$ ; Fig. 6B), and the tumor volumes from the

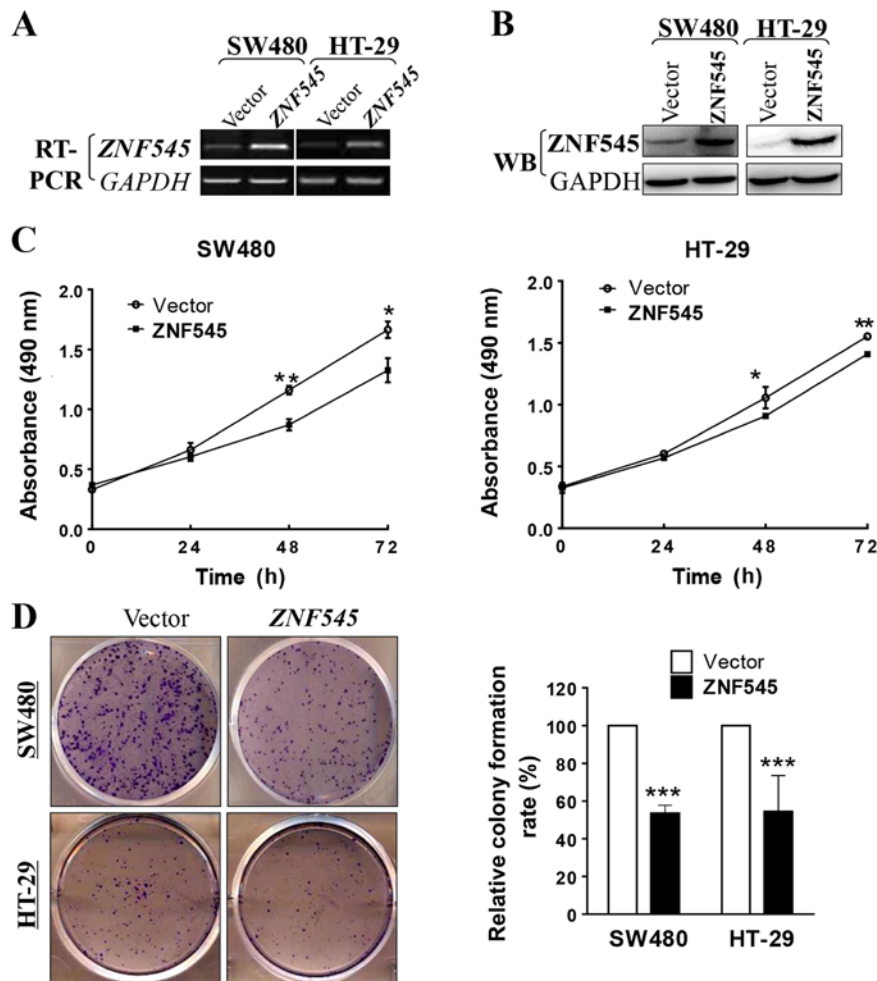


Figure 3. Overexpression of *ZNF545* inhibits the cell proliferation and colony formation of CRC cells. (A and B) Expression of *ZNF545* by RT-PCR and western blot analysis in vector- and *ZNF545*-transfected SW480 and HT-29 cells. (C) MTS cell proliferation assay for vector- and *ZNF545*-transfected SW480 and HT-29 cells. Asterisks indicate a significant level of proliferation compared to controls (\* $p < 0.05$ ; \*\* $p < 0.01$ ). (D) Representative colony formation assay in vector- and *ZNF545*-transfected SW480 and HT-29 cells. The numbers of the colonies in vector-transfected controls were set to 100%. The values are expressed as mean  $\pm$  SD from three experiments (\*\* $p < 0.001$ ).

*ZNF545*-treated animals were significantly smaller than that of the control group ( $p < 0.05$ ; Fig. 6C). Moreover, the mean weight of the tumors formed by *ZNF545*-transfected cells was significantly lower than that of tumors formed from the empty vector control cells ( $p < 0.05$ ; Fig. 6D), suggesting that *ZNF545* inhibited the growth of CRC cells *in vivo*. After using PCNA to stain the tissue sections from nude mice, immunohistochemical analyses showed that cell proliferation in the tumors from empty vector-treated cells was greater than that of tumors from the *ZNF545*-treated animals (Fig. 6E).

The tumor suppressive property of *ZNF545* was mediated by the Wnt/ $\beta$ -catenin, PI3K/AKT, and MAPK/ERK signaling pathways. Multiple zinc finger family members have been reported to interact the  $\beta$ -catenin activity, and we investigated whether *ZNF545* functions as a tumor suppressor through Wnt/ $\beta$ -catenin signaling pathway. The results showed that the expression of active-catenin was downregulated. Moreover, we characterized the effects of *ZNF545* on the PI3K/Akt and ERK signaling pathways. The results showed that phospho-AKT and phospho-ERK1/2 were downregulated, indicating that the two pathways also participated in the

carcinogenic process. GSK3 $\beta$  is a downstream target gene of the PI3K/AKT pathway and *ZNF545* resulted in a profound reduction of GSK3 $\beta$  (Fig. 7A).

## Discussion

Increasing evidence has shown that *ZNF545* plays an important role in the development of various cancers. *ZNF545* is abnormally expressed, and acts as a suppressor gene in some tumors. For example, *ZNF545* was poorly expressed in breast cancer (19) and gastric cancer (23), and suppressed breast cancer and gastric cancer proliferation. Furthermore, the Oncomine database showed that *ZNF545* was significantly downregulated in multiple types of CRC tissues when compared to normal adult colorectal tissues. In the present study, we found that the expression of *ZNF545* was frequently downregulated in CRC cells, as well as in primary colorectal tumor tissues when compared with surgical margin tissues, suggesting that *ZNF545* may be a tumor suppressor in CRC.

It has been reported that the decreased expression of *ZNF545* is related to its methylation, and it is generally accepted that promoter methylation is a major mechanism involved in



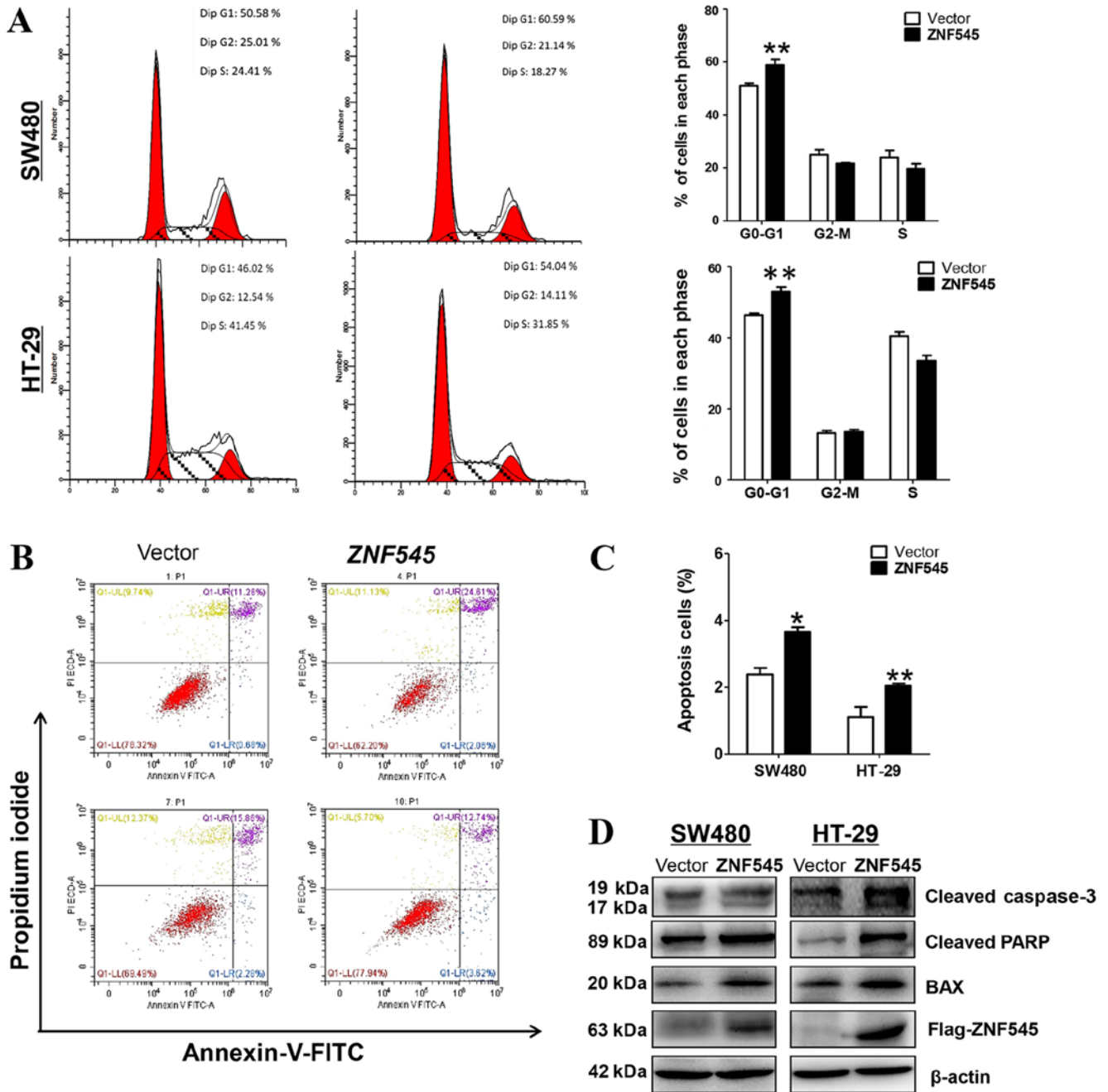


Figure 4. Re-expression of *ZNF545* induces cell cycle arrest and apoptosis of SW480 and HT-29 cells. (A) The effect of cell cycle distribution of vector- and *ZNF545*-transfected SW480 and HT-29 cells was detected by flow cytometry analysis. Left, representative flow cytometry plots. Right, histograms of cell cycle alterations. (B) Induction of apoptosis detected by flow cytometric analysis with Annexin V-FITC and PI-staining. (C) Quantity analysis of apoptotic cells. Statistical significance was assessed by Student's t-test. The graph displays the mean  $\pm$  SD of three independent experiments. \* $p < 0.05$ ; \*\* $p < 0.01$ . (D) Western blot analysis of apoptosis-related proteins in vector-, *ZNF545*-transfected SW480 and HT-29 cells.  $\beta$ -actin was used as a control.

gene changes such as gene silencing or gene downregulation that have been found in human malignant tumors (21). Using MSP and a demethylation treatment, we found that the reduced expression of *ZNF545* was associated with methylation of the promoter, suggesting that DNA methylation is a regulatory mechanism of *ZNF545* inactivation in CRC.

It has been reported that the methylation of *ZNF545* can be used as a prognostic marker for early stage hepatocellular carcinoma (HCC) after thermal ablation (24), and low expression of *ZNF545* was determined positively correlated with the poor prognosis in CRC patients through analyzing

the PrognScan database. However, there was an insufficient number of clinical samples of CRC in our study, so we could not determine the possible relationship between *ZNF545* methylation and the pathological features and survival prognoses of CRCs. Further analyses of more clinical samples is therefore necessary to determine if *ZNF545* is indeed a prognostic marker for CRC.

The biological functions of *ZNF545* in CRC were also studied by *in vitro* and *in vivo* overexpression of *ZNF545*. Previous studies reported that expression of *ZNF545* inhibited clone formation and induced apoptosis in HCT116 CRC



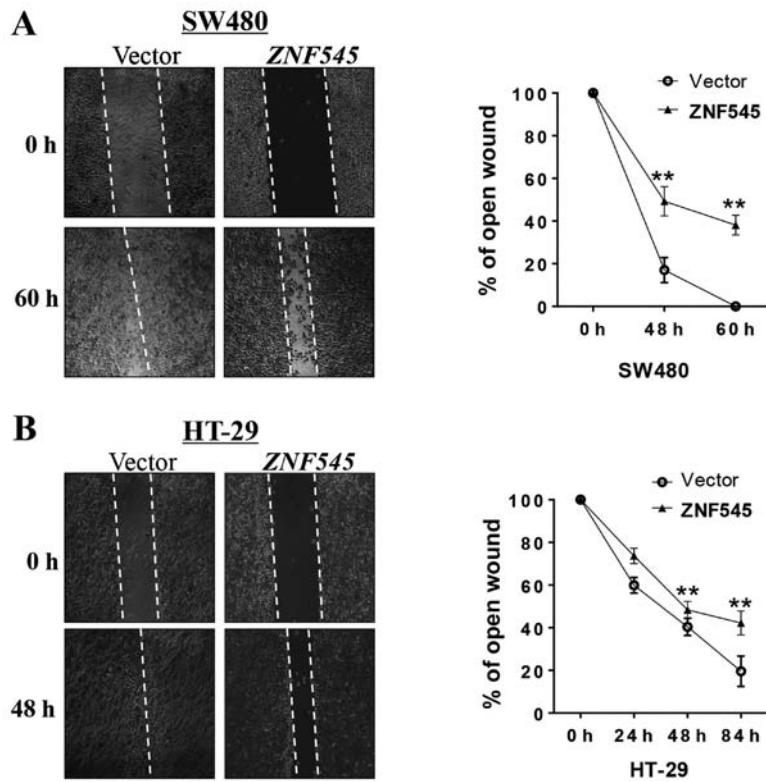


Figure 5. *ZNF545* suppresses the cell motility in SW480 and HT-29 cells. (A and B) Left, representative images of wound healing assay. Right, the percentage of wound healing compared with that of controls at each time-point as indicated, asterisks indicate a significant level of cell motility compared to controls, Mean  $\pm$  SD of three independent experiments (\* $p$ <0.01).

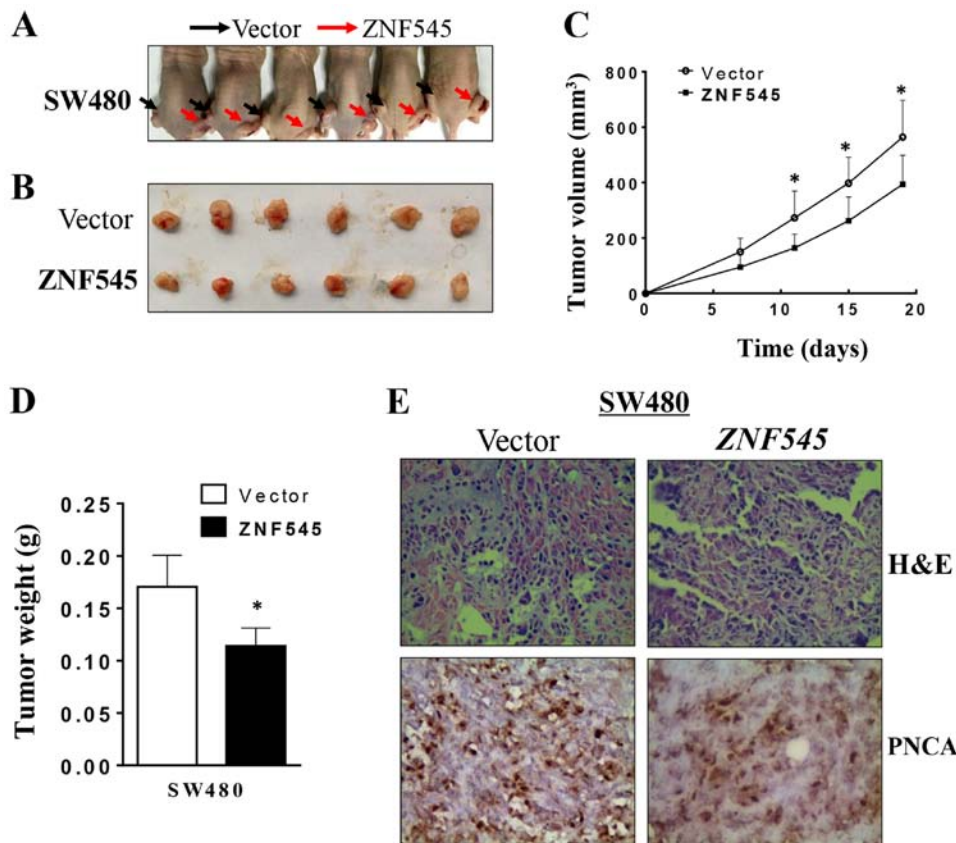


Figure 6. *ZNF545* inhibits tumorigenicity of CRC *in vivo*. (A and B) Representative images of tumor growth in nude mice. Left dorsal flank of nude mice is the vector group, right dorsal flank of nude mice is overexpression *ZNF545* group. (C) Growth curve for the *ZNF545*-expressing tumor compared to the control in nude mice. (D) Tumor weight of *ZNF545* expressing cells in nude mice compared with control tumors, \* $p$ <0.05. (E) Representative images of H&E staining and IHC analyses of the expression of PCNA in tumors from nude mice.

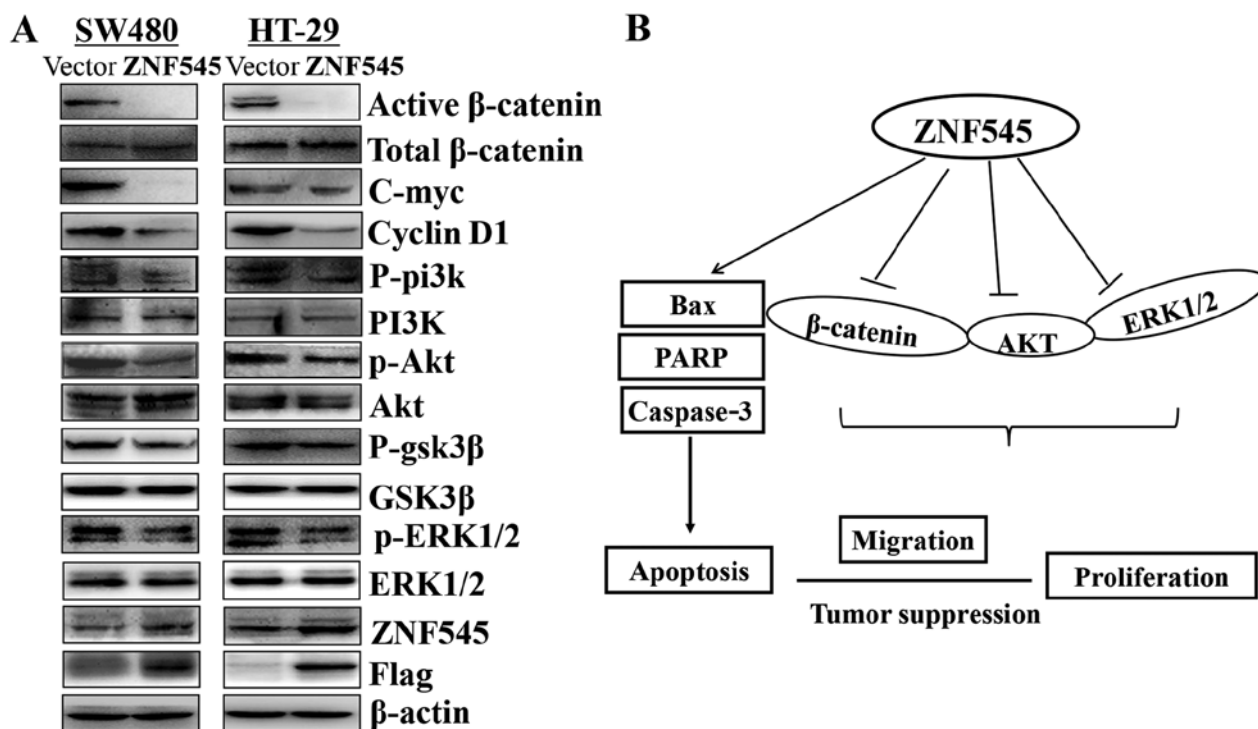


Figure 7. The mechanism of *ZNF545* acts on CRC cell lines. (A) Overexpression of *ZNF545* in SW480 and HT-29 cells disrupted Wnt/ $\beta$ -catenin, PI3K/AKT, and MAPK/ERK1/2 signaling pathways. Western blot analysis of  $\beta$ -catenin, AKT, ERK1/2, and the related downstream targets in SW480 and HT-29 cells.  $\beta$ -actin was used as an internal control. (B) The schematic diagram of antitumorigenesis function mechanisms of *ZNF545* deriving from the western blot analysis data.

cells (18). The results of the present study showed that *ZNF545* affected apoptosis and G0/G1 cell cycle arrest to inhibit cell proliferation and clone formation *in vitro*. Furthermore, using the wound healing assay, we showed that overexpression of *ZNF545* markedly inhibited cell migration. The inhibition of tumor growth by overexpression of *ZNF545* was also tested *in vivo*, by monitoring the formation of tumor xenografts in nude mice. Flow cytometry and western blot analyses were used to show the induction of apoptosis by expression of *ZNF545* in CRC cells. Flow cytometry showed that expression of *ZNF545* resulted in early apoptosis in CRC cells, and western blot analyses showed increased expression of Bax, cleaved caspase-3, and cleaved PARP. Taken together, the results suggested that *ZNF545* functions as a tumor suppressor in CRC.

The members of KRAB-ZFPs family have N-terminal KRAB domain and C-terminal C2H2 domain, and the C2H2 domain can function as structural motif to bind to the promoter of DNA or RNA. Previous studies have reported that many ZFPs influence cell biological function through Wnt/ $\beta$ -catenin pathway, such as ZNF488, ZNF191, KLF4 and ZIC2. ZNF488 acts as an oncogene via the Wnt/ $\beta$ -catenin pathway to induce EMT in nasopharyngeal carcinoma (25). ZNF191 can directly bind to the  $\beta$ -catenin promoter and invoke the expression of  $\beta$ -catenin to promote cell proliferation in hepatoma cell lines (26). Recent report showed that the KLF4 binds to the  $\beta$ -catenin through its C-terminus which contains three zinc-finger domains, inhibits Wnt/ $\beta$ -catenin signaling pathway and suppresses cell proliferation in colorectal cancer (27). The zinc finger domain of ZIC2 is required for the interaction with TCF4, and ZIC2 can modulate Wnt pathway in 293T cells (28). Whether *ZNF545*

performs a similar mechanism that acts on Wnt/ $\beta$ -catenin pathway in colorectal cancer remains unclear.

Wnt signaling plays an important role in the formation and maintenance of human intestinal epithelium (29). The deregulated Wnt signaling is an early event in colon carcinogenesis (30). Our data showed that re-expression of *ZNF545* inhibited the activated  $\beta$ -catenin and its downstream target genes in colorectal cancer cell lines.

It has been reported that the PI3K/AKT and MAPK/ERK pathways can promote the biological function of proliferation, and migration in multiple cancer cells (31-33). AKT is a key component in the PI3K pathway, where it suppresses cell survival and growth (34). Our results showed that when compared to the empty vector group, the expression level of phosphorylated AKT and phosphorylated GSK-3 $\beta$  were decreased in the *ZNF545* group. The total amount of ERK1/2 protein was unchanged in the *ZNF545*- and vector-transfected cells, but phosphorylated ERK1/2 levels decreased in the *ZNF545*-transfected cells. Thus, *ZNF545* may inhibit the PI3K/Akt/GSK3 $\beta$  and MAPK/ERK pathways to reduce the cell proliferation and migration ability in colorectal cancer cells. Identification of the target gene of *ZNF545* (RNA sequence or Chip sequence) will therefore assist in identifying its role in the progression of CRC.

In conclusion, these results suggested that *ZNF545* functions as a tumor suppressor in CRC, and that its inactivation results from promoter methylation. *ZNF545* may be an epigenetic biomarker for colorectal tumors. Overall, *ZNF545* can regulate cell proliferation, apoptosis, and metastasis by affecting the Wnt/ $\beta$ -catenin, PI3K/AKT, and MAPK/ERK signaling pathways.

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