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TRIM8 regulates the chemoresistance of colorectal cancer in a p53-dependent manner

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Abstract. Chemotherapy is an important treatment for colorectal cancer (CRC); however, colorectal adenocarcinoma cells often develop resistance to chemotherapeutic drugs, leading to relapse and poor patient prognosis. Tripartite motif containing 8 (TRIM8) is a new p53 modulator, and induces the reduction of cell proliferation; however, it is unclear whether TRIM8 expression has an effect on regulating drug sensitivity of CRC. The present study demonstrated that TRIM8 is downregulated in CRC tissues and cell lines. Two different colorectal adenocarcinoma cell lines were used, which exhibit different p53 protein levels: SW620 cells express wild-type p53 protein, while SW480 are p53-null cells. Overexpression of TRIM8 sensitized SW620 cells to 5-fluorouracil, a chemotherapeutic drug, while knockdown of TRIM8 promoted SW620 cell survival. However, this did not occur in SW480 cells. It was demonstrated that TRIM8 promoted CRC cell sensitivity to the above chemotherapeutic drug in a p53-dependent manner. Furthermore, it was observed that TRIM8 downregulation prevented p53 activation. Taken together, our findings suggested that TRIM8 may play a role in colorectal adenocarcinoma cancer cell drug resistance, and may be a therapeutic target against chemotherapy drug resistance in colorectal adenocarcinoma where p53 is wild type.

Introduction

Colorectal cancer (CRC) is one of the most common malignant tumors worldwide (1). Resistance of cancer cells to chemotherapy continues to be a major clinical obstacle to the successful treatment of cancer, including CRC (2,3). The most studied drug in CRC is the anti-metabolite 5-fluorouracil (5-FU), which was developed ~60 years ago (4). 5-FU monotherapy, or its combination with other conventional therapeutics, has become the standard chemotherapy regimen for CRC (5). However, an important issue in this context is therapy resistance (6). The beneficial effect of 5-FU exhibits short duration due to the development of resistance (7). Numerous patients relapse following an initial response to 5-FU-based chemotherapies, and these recurrent cancers exhibit acquired resistance to chemotherapy, resulting in progression and fatality (8). Therefore, the mechanisms by which tumor cells survive chemotherapy treatment are an active area of investigation (9).

Inactivation of the p53 pathway is a common feature of tumors, which accounts for ~50% of all human cancers (10). These tumors are associated with chemoresistance and, in general, predict a considerably worse patient prognosis in comparison with malignancies with functional p53 (11-13). The reactivation of p53 in cancer cells is a promising treatment strategy (14). Among cancers displaying rare p53 mutations and poor response to conventional anti-cancer treatments, CRC represents a remarkable example of the importance of p53 pathway alterations in therapy resistance (15).

Intrinsic and extrinsic cellular stresses, DNA damage or abnormal proliferative signals act upon the p53 pathway, activating different enzymes that modify the p53 protein (16). It has been established that mouse double minute 2 homolog (MDM2) can differentially catalyze either monoubiquitination or polyubiquitination of p53 in a dosage-dependent manner (17). Low dosage of MDM2 induces p53 monoubiquitination, which greatly promotes its mitochondrial translocation and apoptosis, whereas high levels of MDM2 promote the polyubiquitination and degradation of p53 (18).

Tripartite motif containing 8 (TRIM8) belongs to a subfamily of the really interesting new gene (RING) type E3 ubiquitin ligases, which is characterized by a tripartite motif, and a number of whose members function as important regulators of carcinogenesis (19). Each member appears to have a peculiar function relative to the p53 pathway, both antagonizing and enhancing p53 response to specific stimuli (20,21).

It has been reported that TRIM8 is expressed in various human tissues, including the brain, lung, breast, gut, placenta, kidney and muscle, as well as germinal center B cells (22,23). TRIM8 has been identified as a new key regulator of p53 in the cell cycle arrest vs. apoptosis decision (24). It has recently been reported that TRIM8 can regulate p53-mediated cellular responses to chemotherapeutic drugs in clear cell renal cell carcinoma (25). However, it is not clear whether TRIM8 truly regulates the p53 pathway and drug sensitivity of CRC.

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Therefore, the present study aimed to investigate the effect of TRIM8 expression on the drug sensitivity of CRC, and to identify the mechanism behind these effects. Of note, our study attempted to identify the critical regulation of p53 by TRIM8 in CRC.

Materials and methods

Tissue samples and cell culture. In total, 11 pairs of CRC tissues and matched adjacent normal tissues located 5 cm away from the tumor were obtained from patients undergoing CRC surgery from June 2015 to October 2015 at Luoyang Central Hospital Affiliated Zhengzhou University (Luoyang, China), with written informed consent and agreement. The present study was approved by the Medical Ethics Committee of Luoyang Central Hospital Affiliated Zhengzhou University (Luoyang, China). Half of each specimen was snap frozen and stored at -80˚C, and the other half was fixed in formalin for histological assessment. Tumors were staged using the tumor-node-metastasis and the World Health Organization classification systems (26), as shown in Table I.

The human CRC cell lines (SW620 and SW480) and the non-tumoral colorectal cell line (CCD-841) were obtained from the American Type Culture Collection (Manassas, VA, USA). The cells were cultured in Dulbecco's modified Eagle's medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) plus 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.) supplemented with penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37˚C, 5% CO_2. 5-FU was purchased from Sigma-Aldrich (Merck Millipore, Darmstadt, Germany) and was reconstituted in PBS. Stock solutions were prepared at 5 mM.

Small interfering RNA (siRNA) preparation. TRIM8 siRNA was designed and synthesized according to the sequence information of theTRIM8 gene (GenBank accession #NM_030912.2) by Shanghai GenePharma Co., Ltd. (Shanghai, China). The siRNA sequence was 5'-AAAUCCAAAAGGAAAAGACCU-3' (sense) and 5'-GUCUUUUCCUUUUGGAUUUUG-3' (antisense).

Cell transfection. All transfections were carried out using Lipofectamine™ 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Luciferase assay was performed using a luciferase reporter gene assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocol. One day prior to transfection, $2x10^5$ cells were seeded in 6-well culture plates at 60-80% confluency. pcDNA3.1-TRIM8 (Invitrogen; Thermo Fisher Scientific, Inc.) and TRIM8-siRNA were transfected into SW620 and SW480 cells, while ectopic p53 (Invitrogen; Thermo Fisher Scientific, Inc.) was transfected into SW620 cells with TRIM8 downregulation or into the control vector group. The luciferase assay was performed after 30 h of transfection in order to determine the transfection efficiency. The experiment was performed in triplicate.

Proliferation assay by MTT. Cell growth was measured using an MTT Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). A total of $1x10^5$ cells/well were seeded onto 96-well plates for 1 day prior to the addition of dimethyl sulfoxide (DMSO). MTT reagent (10 μl) was added to each well and incubated at 37˚C for 0, 24, 48, 72 and 96 h. Then, 100 μl DMSO was added to each well, and the plates were agitated for 10 min in the dark. The absorbance values measured at 490 nm on day 0 were set as 100%, and the remaining measurements were then normalized relative to the value on day 0. Cell proliferation was monitored for ≤4 days. Experiments were conducted in triplicate.

Table I. Illustration of the clinicopathological factors of 11 colorectal cancer patients.

Variable	N
Age, years	
≥60	4
<60	7
Gender	
Male	8
Female	3
Stage	
I	1
II	5
III	3
IV	2
Distant metastasis	
Positive	2
Negative	9

RNA preparation from patients and cell lines, and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Tumor and paired adjacent non-tumor CRC samples from 11 patients were used for the present study. Immediately following surgery, half of the tissues were separately stored and frozen at -80˚C. Total RNA was extracted from 50-100 mg fresh frozen tissue using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.). All RNA samples were purified using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocol.

Total RNA (1 μg) was used for RT with the iScript cDNA Synthesis kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) following the manufacturer's protocol. Amplification was performed for 40 cycles using iQ SYBR Green Supermix (Bio-Rad Laboratories, Inc.) on iCycler Thermal Cycler (Bio-Rad Laboratories, Inc.). A typical three-step running protocol was used, as follows: Denaturation program (10 min at 95°C); amplification and quantification programs, which were repeated 40 times (15 sec at 95°C for denaturation, 30 sec at 60°C for annealing with a single fluorescence measurement recorded and 30 sec at 72°C for elongation); melting curve program (at 60-99°C with a heating rate of 0.5°C/sec and a continuous measurement); and cooling program (down to 40°C). Oligonucleotide primers were synthesized to detect TRIM8 messenger RNA levels, with β-actin as an internal control. The relative quantitation value for each target gene compared to the calibrator for that target was expressed

as $2^{-\Delta\Delta Cq}$ (27), being Cq the mean quantification cycle upon normalizing to β-actin. The relative expression levels of the samples were presented by a semi-logarithmic plot.

Western blot analysis. Standard western blotting was performed to measure the expression levels of proteins. Briefly, cultured CRC cells were lysed in ice-cold radioimmunoprecipitation assay buffer (Cell Signaling Technology, Inc., Danvers, MA, USA). Protein samples were separated by 10% SDS-PAGE and then transferred to polyvinylidene fluoride membranes. The primary antibodies used were anti-p53 (1:100; sc-53861; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-p53-Ser15 phosphorylated (P) (1:100; sc-101762; Santa Cruz Biotechnology, Inc.), anti-p53-Ser20P (1:100; sc-55476; Santa Cruz Biotechnology, Inc.), anti-MDM2 (1:50; Ab-166; Sigma-Aldrich; Merck Millipore), anti-TRIM8 (1:100; sc-165791; Santa Cruz Biotechnology, Inc.) and anti-β-actin (1:100; clone EP1123Y; Calbiochem; Merck Millipore). Incubation with the above primary antibodies was conducted for 1 h at 37˚C. Upon washing the membranes three times with TBS containing Tween 20, the primary antibodies were detected with appropriate horseradish peroxidase-conjugated secondary antibodies for 1 h at 37˚C. Anti-mouse (A9044), anti-goat (A5420) and anti-rabbit (A0545) immunoglobulin G peroxidase antibodies (1:1,000; Sigma-Aldrich; Merck Millipore) were used as secondary antibodies. The bands were visualized using enhanced chemiluminescence (Beyotime Institute of Biotechnology).

Statistical analysis. Data were analyzed using SPSS 17.0 (SPSS Inc., Chicago, IL, USA). All data are expressed as the mean ± standard deviation from three independent experiments. The statistical significance of differences between two groups was analyzed by two-tailed Student *t* tests. Correlations were analyzed by Spearman's correlation analysis. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Downregulated TRIM8 expression in CRC tissues and cell lines. To understand the role of TRIM8 in CRC, the levels of TRIM8 expression were examined by RT-qPCR in 11 pairs of CRC tissues and their adjacent normal tissues. As shown in Fig. 1A, the expression of TRIM8 was significantly downregulated in CRC tissues compared with that in paired adjacent normal tissues ($P=0.0005$). The expression levels of TRIM8 in CRC cell lines (SW620 and SW480) and a non-tumoral colorectal cell line (CCD-841) were further assessed. Consistent with the results obtained in CRC tissues, TRIM8 levels were 20-30-fold lower in the CRC cell lines than those in the primary cell line (Fig. 1B), suggesting that TRIM8 is downregulated in CRC.

TRIM8 promotes CRC cell sensitivity to 5-FU in SW620 cells, but not in SW480 cells. In order to test the effect of TRIM8 on CRC resistance to a chemotherapeutic drug, CRC cell lines were transfected with full-length TRIM8 or with TRIM8 siRNA. Overexpression or inhibition of TRIM8 was confirmed by western blotting and RT-qPCR (Fig. 2A and B). Upon transfection, the cells were treated with the chemotherapeutic drug 5-FU. To determine the effect of TRIM8 expression on cancer cell drug sensitivity, cell survival was analyzed once the cells had been treated with 5-FU. When TRIM8 was overexpressed in SW620 cells, which express wild-type p53 protein, a strong increase in cell death was observed (Fig. 2C), demonstrating that the overexpression of TRIM8 sensitized SW620 cells to the aforementioned chemotherapeutic agent. Notably, cell death did not occur in SW480 cells, which are p53 null (Fig. 2C), indicating that TRIM8 regulated cell death in a p53-dependent manner. On the contrary, downregulation of TRIM8 resulted in increased cell proliferation in SW620 cells, which are p53 wild type, but not in SW480 cells, which are p53 null, when treated with 5-FU (Fig. 2D). These results indicated that the overexpression of TRIM8 sensitized SW620 cells to the chemotherapeutic drug 5-FU, and that knockdown of TRIM8 promoted SW620 cell survival, which was dependent on p53 expression.

Overexpression of p53 impairs the tumor-suppressor effect of TRIM8. It was next tested whether the effect of growth suppression mediated by TRIM8 was dependent on its p53 tumor-suppressor activity. It was observed that TRIM8 knockdown induced the chemoresistance of SW620 cells. Ectopic p53 was transfected into SW620 cells once TRIM8 was downregulated (Fig. 3A). As expected, the reintroduction of p53 could inhibit cell survival and drug sensitivity, whereas the expression of a control plasmid could not (Fig. 3B). Thus, these data suggested that p53 restoration partially counteracted the tumor-suppressor effect of TRIM8, and that TRIM8 promoted CRC cell sensitivity to 5-FU in a p53-dependent manner.

TRIM8 downregulation prevents p53 activation following 5-FU treatment. To further investigate the mechanism of TRIM8 expression on the p53-dependent cellular response to chemotherapeutic drugs, SW620 cells were transfected with TRIM8 siRNA or with an unspecific control vector (Fig. 2A). Once TRIM8 was downregulated, the cells were treated with 5-FU. TRIM8 downregulation resulted in p53 protein degradation as a result of MDM2 protein stabilization (Fig. 4). As it is well known that 5-FU induces p53 activation (28,29), the present study observed that 5-FU treatment induced p53 activation in SW620 cells, while TRIM8 downregulation decreased the endogenous protein levels of p53 (Fig. 4). These results strongly suggested that TRIM8 regulated CRC cells chemotherapeutic drug resistance via p53-mediated cellular responses.

Discussion

CRC is the most common subtype of gastrointestinal tumor, accounting for ~80% of all surgical cases, and is characterized by marked high resistance to radiation and chemotherapy despite harboring wild-type p53 (30). The p53 protein is a promising target in fighting cancer due to its marked ability to mediate tumor suppression, including irreversible growth suppression, induction of apoptosis, inhibition of angiogenesis, and blockade of cellular invasion and metastasis (31). The stabilization and activation of wild-type p53 are thus crucial to prevent cells from becoming cancerous. Therefore, one of the most important challenges in cancer research is the discovery of additional p53-inactivating pathways that may account

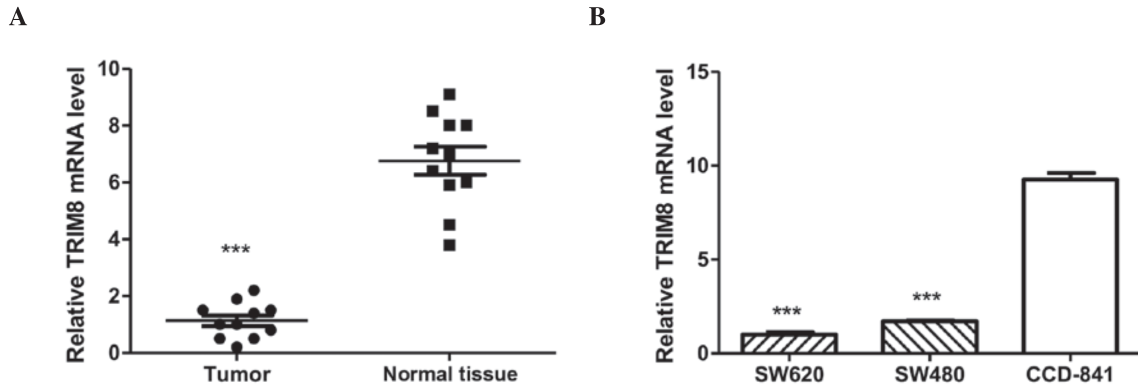

Figure 1. TRIM8 expression in CRC tissues and cell lines. (A) TRIM8 mRNA expression in 11 CRC samples and their paired non-tumor tissues. The expression levels of TRIM8 were determined by reverse transcription-quantitative polymerase chain reaction. The mean expression ± standard error is represented by a black line. The relative TRIM8 expression levels are shown as fold-change in normal tissues compared with those in tumor tissues, which are set to 1. (B) TRIM8 mRNA expression in different cell lines. The relative TRIM8 expression levels are shown as fold-change in the non-tumoral cell line CCD-841 and in the CRC cell line SW480 compared with those in SW620 cells, which are set to 1. ***P<0.001. TRIM, tripartite motif containing 8; mRNA, messenger RNA; CRC, colorectal cancer.

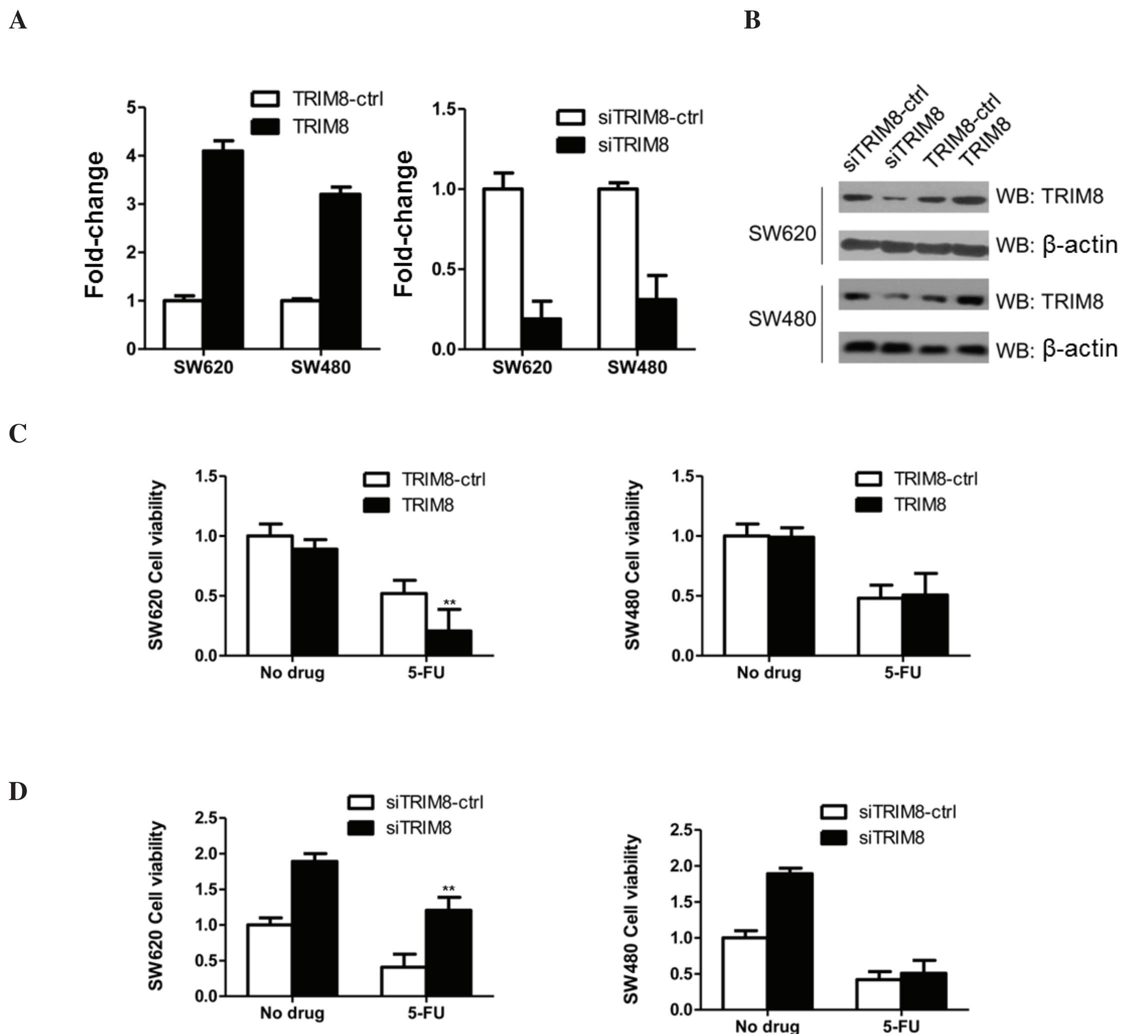

Figure 2. TRIM8 promotes colorectal cancer cell sensitivity to 5-FU in SW620 cells, but not in SW480 cells. (A) Reverse transcription-quantitative polymerase chain reaction was performed to detect the TRIM8 levels in SW620 and SW480 cells treated with pcDNA3.1-TRIM8 (left) and TRIM8 siRNA (right). TRIM8-ctrl and siTRIM8-ctrl represent the corresponding empty vector controls. (B) WB for TRIM8 was conducted to analyze the protein levels of the cells described in (A). (C) Cell viability was assessed using an MTT assay. SW620 (left) and SW480 (right) cells with upregulated TRIM8 expression were plated in 96-well plates. After 24 h of incubation, cells were exposed to 10 μM (SW620 cells) or 5 μM (SW480 cells) 5-FU for 48 h. Cell viability was assessed using an MTT assay. Data are represented as the survival rate relative to that of the untreated control. (D) Cell viability of cells with downregulated TRIM8 expression. SW620 (left) and SW480 (right) cells with downregulated TRIM8 expression were plated in 96-well plates. Drug treatment and cell viability were determined as in (C). **P<0.01. TRIM, tripartite motif containing 8; ctrl, control; 5-FU, 5-fluorouracil; si, small interfering; WB, western blotting.

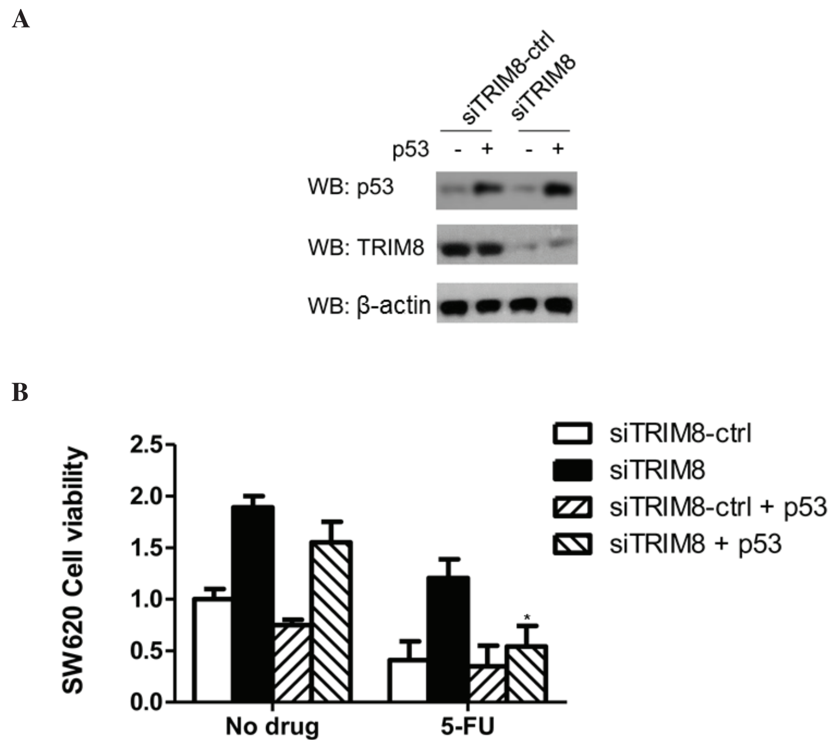

Figure 3. Overexpression of p53 impairs the tumor-suppressor effect of TRIM8. (A) Ectopically expressed p53 and control vector were transfected into SW620 cells, with or without TRIM8 suppression. The protein levels were detected by WB. (B) Cell viability was assessed using an MTT assay. Drug treatment and cell viability were determined as in Fig. 2. *P<0.05. TRIM, tripartite motif containing 8; ctrl, control; 5-FU, 5-fluorouracil; si, small interfering; WB, western blotting.

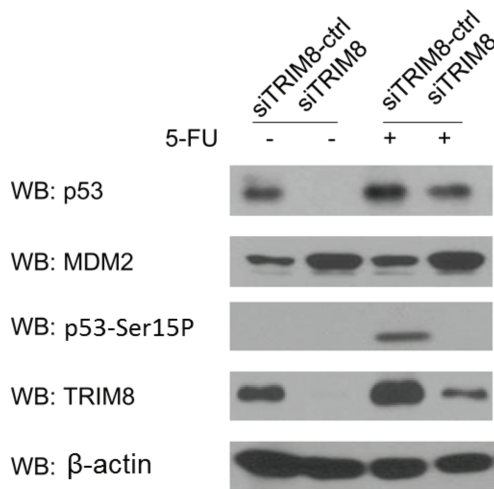

Figure 4. TRIM8 silencing prevents p53 activation following chemotherapeutic drug treatment. The levels of the indicated proteins were determined by WB in SW620 cells. At 24 h post-transfection with TRIM8 siRNA or control vector, cells were treated with or without 5-FU (10 μM). TRIM, tripartite motif containing 8; ctrl, control; 5-FU, 5-fluorouracil; si, small interfering; WB, western blotting; P, phosphorylated; MDM2, mouse double minute 2 homolog.

for the escape of cells from p53 control in malignancy with wild-type p53.

Our study suggests a model in which the loss of TRIM8 would contribute to p53 inactivity following chemotherapeutic treatment in CRC cells. The present study analyzed how TRIM8 expression levels may influence the cellular response to chemotherapeutic drugs, and it was noticed that TRIM8 was downregulated in CRC tissues and cell lines. Via ectopic overexpression and RNA interference, it was observed that the overexpression of TRIM8 sensitized SW620 cells to chemotherapeutic agents, and that knockdown of TRIM8 promoted SW620 cell survival. However, these findings did not occur in p53-null CRC cells, suggesting that this regulation may be dependent on p53 expression. In addition, it was observed that TRIM8 was responsible for both p53 stabilization/activation and MDM2 degradation. It was identified that TRIM8 promoted drug sensitivity of CRC in a p53-dependent manner.

TRIM8 is a member of the TRIM family of proteins, and it has been reported that a large number of members of this family act as E3-ubiquitin ligases (32-34). Other TRIMs are involved in p53 regulation (35). For instance, the RING domain of TRIM24 functions as an E3-ubiquitin ligase that targets p53 for degradation, and its depletion induces p53-dependent apoptosis (35,36). In the present study, it was demonstrated that TRIM8 is a new regulator of p53 in response to chemotherapeutic drugs. The loss of TRIM8 may be one of the mechanisms that enable acquired resistance to 5-FU, suggesting that histochemical analysis of TRIM8 may be used as a biomarker to determine drug sensitivity in CRC patients undergoing 5-FU treatment.

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