Knockdown of TRIM9 attenuates irinotecan-induced intestinal mucositis in IEC-6 cells by regulating DUSP6 expression via the P38 pathway

WENJUN ZHAO¹ and QINGMING WANG²

¹Department of Anorectal Section, Shanghai Baoshan District Hospital of Integrated Traditional Chinese and Western Medicine, Shanghai 201999; ²Department of Anorectal Section, Shuguang Hospital, Shanghai University of Traditional Chinese Medicine, Shanghai 201203, P.R. China

Received March 19, 2021; Accepted September 1, 2021

DOI: 10.3892/mmr.2021.12507

Abstract. Intestinal mucositis is a common side effect of cancer chemotherapy and it limits the dose of chemotherapy given to a patient. Tripartite motif family (TRIM) proteins have been reported to be implicated in the regulation of cancer chemotherapy. The present study aimed to investigate the effect of TRIM9 on irinotecan-induced intestinal mucositis in the rat intestinal epithelial cell line IEC-6. The expression of several TRIMs, such as TRIM1, TRIM9, TRIM18, TRIM36, TRIM46 and TRIM67, was examined. After TRIM9 knockdown or overexpression by lentivirus infection, cell proliferation and apoptosis, epithelial barrier tight-junction proteins, inflammatory cytokines, transepithelial electrical resistance (TEER) and FITC dextran were measured. Treatment with irinotecan significantly inhibited cell proliferation and induced cell apoptosis, TRIM9 expression, intestinal mucosal barrier impairment, the levels of inflammatory cytokines and P38 phosphorylation in IEC-6 cells, while the expression levels of epithelial barrier tight-junction protein ZO-1 and claudin-4 were decreased. Knockdown of TRIM9 partly counteracted the effect of irinotecan treatment, and inhibition of P38 potently reversed the effect of TRIM9 overexpression in IEC-6 cells. Moreover, co-immunoprecipitation showed an interaction between TRIM9 and DUSP6 in IEC-6 cells, and overexpression of DUSP6 notably counteracted the effect of TRIM9 overexpression. The results demonstrated that TRIM9 knockdown may benefit patients with intestinal mucositis by inhibiting inflammatory cytokine expression and repairing intestinal barrier functions, which was probably due to inhibition of the activation of the P38 pathway via targeting DUSP6.

Introduction

Intestinal mucositis is a common side effect of cancer chemotherapy and it limits the dose of chemotherapy given to a patient (1). The incidence of intestinal mucositis is 40% in patients receiving standard-dose chemotherapy and nearly 100% in those who undergo high-dose chemotherapy (2). Mucositis is an important factor that determines morbidity and treatment compliance. Intestinal mucosal atrophy, imbalance in the intestinal flora, pseudomembranous colitis and severe diarrhea are the main manifestations of intestinal mucosal damage (3). It has been reported that chemotherapy can cause cytotoxic injury to crypt cells, which then causes mucositis (4,5).

Irinotecan, a topoisomerase 1 inhibitor (6), can be used specifically as a precursor drug to treat colorectal cancer, which is the third most common cause of cancer-related deaths in men and women in the United States (7). As a second-line therapy, irinotecan has been reported to improve the rate of overall survival in patients with advanced colorectal cancer (8). Irinotecan is an effective antitumor drug, but it is notorious for its tendency to cause mild and moderate diarrhea, which compared to its other major toxic effects, has a greater clinical impact (9). Irinotecan has been shown to align with mucosal injury, but it is unknown whether this is due to direct chemotherapy-mediated injury or secondary inflammatory injury. A previous study has shown the protective effect of curcumin against irinotecan-induced intestinal mucosal injury, which is due to inhibition of the activation of NF-κB, and suppression of oxidative stress and endoplasmic reticulum stress (10). Data from Wardill et al (11) showed that toll-like receptor 4 (TLR4)-dependent mechanisms control irinotecan-induced tight-junction disruption (12). However, how to effectively target these underlying mechanisms to inhibit its development remains unclear.

Tripartite motif protein 9 (TRIM9) is a member of the TRIM protein family, which are a highly conserved family of E3 ubiquitin ligases, and >70 members have been reported to...
be implicated in tumorigenesis and tumor progression (12,13). TRIM proteins are characterized by their structures that contain a tripartite motif, which constitutes the N-terminal region, and have a highly conserved sequence consisting of a RING domain, one or two B-Box domains and a coiled-coil domain (14,15). TRIM9 is an evolutionarily conserved class I TRIM protein and is a key regulator of netrin-dependent morphogenesis in cortical and hippocampal neurons (16-18). It has been demonstrated that TRIM9 is essential for resolving NF-κB-dependent neuroinflammation, thus promoting recovery and repair after brain injury (19). NF-κB is a key regulator of mucositis (20). In addition, disrupting TRIM9 function can abrogate the motility of macrophages in vivo (21). However, little is known about the effect of TRIM9 on irinotecan-induced inflammation and intestinal barrier impairment in rat IEC-6 cells.

Dual-specificity phosphatases (DUSPs) are mitogen-activated protein kinase phosphatases (MKPs) characterized by their variable N-terminal mitogen-activated protein kinase (MAPK)-binding region, which contains the kinase interaction motif, and can govern the specificity of the substrate and stability of interactions (22). Notably, a previous study showed that DUSP1 is elevated in the differentiated villi cells of the adult intestine, but not in the proliferating crypt cells (23). DUSP6, also called MKP-3 or Pyst1, is mainly detected in differentiated epithelial cells of the mouse intestine (24), and it has been found to reverse activation of the ERK1/2 pathway by dephosphorylating tyrosine and threonine residues (25-27). In addition to ERK1/2, DUSPs also modulate the duration and magnitude of the phospho-activation of p38 and JNK1/2 (28-30). It has been reported that the manipulation of DUSP6 has potential in the treatment of acute inflammatory diseases (31). In addition, TRIM46, as a novel regulator of DUSP6 has potential in the treatment of acute inflammatory diseases, but not in the proliferating crypt cells (23). DUSP6, called MKP-3 or Pyst1, is mainly detected in differentiated epithelial cells of the mouse intestine (24), and it has been found to reverse activation of the ERK1/2 pathway by dephosphorylating tyrosine and threonine residues (25-27). In addition to ERK1/2, DUSPs also modulate the duration and magnitude of the phospho-activation of p38 and JNK1/2 (28-30). It has been reported that the manipulation of DUSP6 has potential in the treatment of acute inflammatory diseases. In addition, TRIM46, as a novel regulator of DUSP6 has potential in the treatment of acute inflammatory diseases, but not in the proliferating crypt cells (23). DUSP6, called MKP-3 or Pyst1, is mainly detected in differentiated epithelial cells of the mouse intestine (24), and it has been found to reverse activation of the ERK1/2 pathway by dephosphorylating tyrosine and threonine residues (25-27). In addition to ERK1/2, DUSPs also modulate the duration and magnitude of the phospho-activation of p38 and JNK1/2 (28-30). It has been reported that the manipulation of DUSP6 has potential in the treatment of acute inflammatory diseases.

In the present study, irinotecan was used to induce intestinal mucositis in rat IEC-6 cells in vitro. The aim was to investigate the effect of TRIM9 on irinotecan-induced intestinal mucositis in the rat intestinal epithelial IEC-6 cell line.

**Materials and methods**

**Cell culture.** The IEC-6 cell line was purchased from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Following extraction from the intestines of normal rats, the cell line was developed and characterized morphologically and immunologically, as Quaroni et al (32) previously described. IEC-6 cells retain the undifferentiated characteristics of epithelial stem cells. The test results of mucosa-negative were negative. IEC-6 cells were cultured in a 5% CO₂ incubator at 37°C with Dulbecco’s modified Eagle’s medium (DMEM; HyClone; Cytiva) that was supplemented with 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) and 1% antibiotics (penicillin streptomycin mixture; Beijing Solarbio Science & Technology Co., Ltd.). A single cell suspension was prepared and diluted to 1x10⁶ cells/ml. The cell suspension was mixed with 0.4% trypan blue solution (cat. no. C0040; Beijing Solarbio Science & Technology Co., Ltd.) at a ratio of 9:1. The cells were incubated for 3 min at room temperature, and the number of live and dead cells was quantified using a counting plate. The cells observed under a light microscope were adherent cells and the percentage of living cells stained by trypan blue was >95%.

**Plasmid construction.** The mRNA sequences for TRIM9 (NM_130420.1) were searched in the National Center for Biotechnology Information (NCBI; https://www.ncbi.nlm.nih.gov/nuccore/NM_130420.1) database. Then, the coding sequence of TRIM9 was synthesized using primers containing the restriction enzyme cutting sites for SfiI and EcoRI and integrated into pLVX-Puro (Clontech; Takara Bio USA) to increase TRIM9 expression (oeTRIM9): TRIM9-forward (F), 5'-CGGAAATTCATGGAAGATGGGAAGAGTTGAGT-3' (EcoRI) and -reverse (R), 5'-CGGGATCTCCTAGGCTAGTGG AAGCCTGCTGCT-3' (BamHI).

RNA interference (RNAi) sequences (shown in Table I) specific to the TRIM9 gene were cloned into the pLKO.1-puro plasmid (Addgene, Inc.) to knock down TRIM9 expression (shTRIM9).

The mRNA sequences of DUSP6 (NM_053883.2) were searched in the NCBI database. Then, the coding sequence of DUSP6 was synthesized using the primers containing the restriction enzyme cutting sites for Hind III and EcoRI and integrated into pCDNA3.1(+) (Addgene, Inc.) to increase DUSP6 expression (oeDUSP6): DUSP6-F, 5'-CCCCAAAGCTTTATGATGATCGCTAGCAGACCT-3' (HindIII) and -R, 5’-CGGAATTCTACGTAAGTGGCA GGGAGTC-3' (EcoRI).

Lentiviral constructs of pLKO.1-shTRIM9 (1 µg), pLVX-Puro-TRIM9 (1 µg), pCDNA3.1(+)-DUSP6 or pLVX-Puro, pLKO.1 and pCDNA3.1(+) empty vectors were co-transfected with packaging vector psPAX2 (0.1 µg; Addgene, Inc.) and envelope vector pMD2.G (0.9 µg; Addgene, Inc.) (lentiviral plasmid:packaging vectorenvelope vector, 10:1:9) into 3rd generation 293T (ATCC) cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Following transfection for 48 h, the lentiviral particles were collected via ultracentrifugation at 55,000 x g, at 4°C.
Table II. Primer sequences.

| Name | Sequences (5’→3’) |
|------|------------------|
| TRIM1 (NM_001191889.1) | F: GGCCAGCTAATTCATC  
R: CTTGGTTGTTCTAGTTC |
| TRIM9 (NM_130420.1) | F: GTGCCTGCTCCAGAACAAG  
R: GAGTCTGAAAGCTGGTTAGTC |
| TRIM18 (NM_022927.1) | F: GGTGGTGAACATACACAG  
R: GGTGGTGAAGTCCAAAG |
| TRIM36 (NM_001106147.1) | F: CATGCGTCCGAGTTGAG  
R: GCCCAAGAAGTGTATTCC |
| TRIM46 (NM_001107691.1) | F: GCCACCTTGCGTATGAC  
R: GACACGCGACGACATAAC |
| TRIM67 (NM_001135715.1) | F: TCCATCTGCGTTCATC  
R: ATAGGCGCTGTAGCTCTC |
| DUSP6 (NM_053883.2) | F: ACCCGCTGTTAATACTCC  
R: TACCAGTGTAAGAAATCC |
| β-actin (NM_031144.3) | F: CGGTCAGGTCTCATCTAC  
R: CAGGGCGATTAATCTCC |

Numbers in brackets represent the corresponding NCBI gene number. F, forward; R, reverse; TRIM, tripartite motif protein; DUSP, dual-specificity phosphatase.

for 2.5 h. Finally, the viral supernatant (MOI, 10) was used to transduce IEC-6 cells. After 24 h of transfection, the cells were cultured for 24 h with serum-free transfer solution as the complete medium.

**Cell transfection.** In the logarithmic growth phase, IEC-6 cells were trypsinized and counted for a 1x10^6 cells/ml suspension, followed: 37˚C for 30 min, 85˚C for 5 min and 4˚C for 5 min.

DNA elimination. The thermocycling conditions used were as follows: 37˚C for 30 min, 85˚C for 5 min and 4˚C for 5 min.

**Western blotting.** Radioimmunoprecipitation assay lysis buffer (Beijing Solarbio Science & Technology Co., Ltd.) containing protease and phosphatase inhibitors were added to IEC-6 cells in a flask at 4˚C to fully lyse the cells. The extracted total proteins were quantified using a bicinchoninic acid (BCA) assay kit. Furthermore, proteins (25 µg/lane) were subjected to 10% SDS-PAGE, followed by transfer to a nitrocellulose membrane. At room temperature, the membrane was blocked for 1 h in 5% skimmed milk and then incubated with the following primary antibodies for 2 h at room temperature: Anti-DUSP1 (1:1000; cat. no. MA5-32480; Invitrogen; Thermo Fisher Scientific, Inc.), anti-DUSP5 (1:1000; cat. no. MA5-27383; Invitrogen; Thermo Fisher Scientific, Inc.), anti-DUSP10 (1:1000; cat. no. PA5-106794; Invitrogen; Thermo Fisher Scientific, Inc.), anti-Claudin1 (1:1000; cat. no. 32-9400; Invitrogen; Thermo Fisher Scientific, Inc.), anti-DUSP4 (1:1000; cat. no. ab216576; Abcam), anti-DUSP6 (1:500; cat. no. ab76310; Abcam), anti-zona occludens protein 1 (ZO-1; 1:1000; cat. no. ab96587; Abcam), anti-P38 (1:1000; cat. no. ab170099; Abcam), anti-phosphorylated (p)-p38 (1:1000; cat. no. ab47363; Abcam), anti-TRIM9 (1:500; cat. no. P0786-1-AP; Proteintech Group, Inc.) and anti-β-actin (1:1000; cat. no. 66009-1-Ig; ProteinTech Group, Inc.). Subsequently the membrane was washed with PBS + 0.05% Tween-20 (PBST) and probed with an HRP-conjugated goat anti-rabbit secondary antibody (1:1000; cat. no. A0208; Beyotime Institute of Biotechnology) for 1 h at 37˚C. Blots were developed using Immobilon Enhanced Chemiluminescent HRP Substrate (MilliporeSigma) for 5 min in the dark, and then the protein bands were visualized under an enhanced chemiluminescence imaging system (Tanon-5200; Tanon Science and Technology Co., Ltd.). ImageJ software, version 1.47 (National Institutes of Health), was used for semi-quantification of the relative grayscale, the relative grayscale = (the grayscale of each protein - the grayscale of the background) / (the grayscale of β-actin - the grayscale of the background).

**Reverse transcription-quantitative PCR (RT-qPCR).** The IEC-6 cells in a flask were treated with different concentrations of irinotecan (0, 30 and 100 µM) for 24 h at 37˚C and then the cells were harvested. Total RNA was isolated with TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and cDNA was obtained by RevertAid First Strand cDNA Synthesis kit (cat. no. K1622; Fermentas; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol after DNA elimination. The thermocycling conditions used were as follows: 37˚C for 30 min, 85˚C for 5 min and 4˚C for 5 min. The prepared cDNA was amplified with a SYBR Green PCR kit (cat. no. K0223; Thermo Fisher Scientific, Inc.) and the results were calculated using an ABI-7300 instrument with ABI Prism 7300 SDS Software v1.2.3 (Applied Biosystems; Thermo Fisher Scientific, Inc.). The following thermocycling conditions were used for qPCR: Initial denaturation for 10 min at 95˚C; followed by 40 cycles of denaturation, elongation and annealing for 15 sec at 95˚C and 45 sec at 60˚C. β-actin was used as the internal control. Using the 2^{-∆∆cq} method (33), the relative mRNA expression levels were determined using the ratio of the corresponding gene to the β-actin optical density. Following transfection, the relative expression levels of TRIM9 or DUSP6 were calculated. The primers used in RT-qPCR are shown in Table II.
Measurement of transepithelial electrical resistance (TEER). TEER is a common method used to monitor cell growth and evaluate cell-cell tight-junction integrity (34). Therefore, TEER was measured in this study using the following procedures. The resistance meter and electrode were calibrated. The electrode was rinsed with sterilized electrolyte solution after its functional detection. Cells from each group were inoculated into the upper chamber of a 24-well Transwell plate at a concentration of 1x10⁴ cells/well. A total of 100 and 600 µl DMEM medium (HyClone; Cytiva) were added to the upper and lower chambers, respectively, in a 5% CO₂ incubator at 37°C. The culture medium was changed 24 h later, and cells were overgrown at 48 h later. The TEER of cells in each group was measured using a resistor. Meanwhile, a blank well (without cells) was set up to determine the TEER (the diameter and area of the Transwell membranes were 8 µm and 0.6 cm²). The resistance per unit area was calculated using the following formula: TEER (resistance per unit area, Ω·cm²) = (R experiment - R blank) (resistance measurement, Ω) x effective membrane area (cm²) (35).

FITC-dextran uptake. Meddings et al (36) showed that FITC was a large fluorescent substance and fluorescence values could reflect intestinal mucosal permeability. Each group of cells at the logarithmic growth phase were inoculated into the upper chamber of the 24-well Transwell plate (1x10⁵ cells/well). A total of 100 and 600 µl DMEM medium (HyClone; Cytiva) containing 10% fetal bovine serum (Thermo Fisher Scientific, Inc.) were added to the upper chamber and lower chambers, respectively, and then cultured in a 5% CO₂ incubator at 37°C. The culture medium was changed each day. Cells were cultivated to 80% confluence. Serum-starved cells in the upper chamber of the serum-free medium were replaced at 4 h before the experiment, and they were given corresponding treatment in line with the experimental groups. Subsequently, 1 mg/ml FITC-dextran (cat. no. sc-263323; Santa Cruz Biotechnology, Inc.) was added and then cells were cultured in an 5% CO₂ incubator at 37°C for 5 min. The basal culture medium was used as the base value, and the medium was supplemented for incubation for 24 h. The fluorescence intensity of FITC was detected by absorbing 200 µl from the substrate culture medium using a microplate analyzer (excitation wavelength, 490 nm; emission wavelength, 520 nm), and the permeability rate was compared to the standard curve.

Enzyme-linked immunosorbent assay (ELISA). ELISA kits (Beyotime Institute of Biotechnology) were used for the measurement of tumor necrosis factor-α (TNF-α; cat. no. PT516) and interleukin-1β (IL-1β; cat. no. PI303) levels in the supernatant of IEC-6 cells in a flask. Briefly, the ELISA plate was incubated with 50 µl capture mAb, and then washed with PBST (0.05% Tween-20) five times. Following treatment with 50 µl bovine serum albumin blocking solution (10 mg/ml; cat. no. ST025; Beyotime Institute of Biotechnology), the plate was incubated with serum (50 µl; Thermo Fisher Scientific, Inc.) and biotin-conjugated detector mAb (50 µl; cat. no. MSAB001; Sigma-Aldrich; Merck KGaA) for 30 min at 37°C. Following probing with avidin-HRP solution (10 mg/ml; Sigma-Aldrich; Merck KGaA), the plate was treated with 50 µl tetramethylbenzidine substrate solution (10 mg/ml; cat. no. T4444; Sigma-Aldrich; Merck KGaA), which triggered the color reaction of the Ag-Ab complex. After the final rinsing with PBST, using the automated ELISA reader (model 550; Bio-Rad Laboratories, Inc.), the absorbance at 450 nm was measured. The concentration was determined by comparing the optical density to a standard curve.

Co-immunoprecipitation (Co-IP) detection. The total protein of IEC-6 cells was groupd using radioimmunoprecipitation assay lysis buffer containing protease and phosphatase inhibitors and incubated with rabbit-IgG (cat. no. sc-2357; Santa Cruz Biotechnology, Inc.), IP-indicated antibodies and untreated proteins as an input control. The mixtures were then incubated with Protein A/G PLUS-Agarose (30 µl; cat. no. sc-2003; Santa Cruz Biotechnology, Inc.) to form an immune complex. After centrifugation at 1,000 x g at 4°C for 30 min, the supernatant was collected for western blot analysis. Anti-TRIM9 (1:200; cat. no. 10786-1-AP; ProteinTech Group, Inc.) and anti-DUSP6 (1:500; cat. no ab76310; Abcam) antibodies were applied for IP detection, while anti-TRIM9 (1:200; cat. no. PA5-40966; Invitrogen; Thermo Fisher Scientific, Inc.), anti-DUSP6 (1:500; cat. no. ab76310; Abcam) and anti-DUSP10 (1:1000; cat. no. PA5-106794; Invitrogen; Thermo Fisher Scientific, Inc.) were used for western blot analysis.

Cell apoptosis. IEC-6 cells were seeded in 6-well plates at 1x10⁴ cells per well and cultured for 24 h before use. IEC-6 cells were harvested after treatment with irinotecan (100 µM; cat. no. S1198; Selleck Chemicals) combined with TRIM9, TRIM9 overexpression combined with P38 inhibitor (SB203580; 20 µM; cat. no. S1076; Selleck Chemicals), TRIM9 overexpression combined with DUSP6 overexpression, or irinotecan (100 µM) combined with DUSP6 overexpression at

Figure 1. TRIM9 is highly expressed in irinotecan-induced intestinal mucositis in vitro. IEC-6 cells were used to induce intestinal mucositis in rat IEC-6 cells in vitro. After 24 h treatment, TRIM1, TRIM9, TRIM18, TRIM36, TRIM46 and TRIM67 mRNA expression levels were detected via reverse transcription-quantitative PCR in IEC-6 cells following treatment with 0 (control), 30 and 100 µM irinotecan. *P<0.05, **P<0.001 vs. 0 µM irinotecan. TRIM9, tripartite motif protein 9.
37°C for 48 h. Cells were prepared with the Annexin V-FITC Apoptosis Detection kit (cat. no. C1062; Beyotime Institute of Biotechnology) according to the manufacturer’s protocols, and incubated at room temperature in the dark for 20 min. The cell early apoptosis rate was measured using a Biosciences AccuriC6 flow cytometer (BD Biosciences) and analyzed using BD Accuri™ C6 Software (version 1.0.264.21; BD Biosciences).

Statistical analysis. Data are presented as the mean ± SD with three repeat independent experiments. Statistical comparisons were performed with a one-way ANOVA followed by Tukey’s post hoc test using GraphPad Prism 7.0 software (GraphPad Software, Inc.). P<0.05 was considered to indicate a statistically significant difference.

Results

TRIM9 is highly expressed in irinotecan-induced intestinal mucositis in vitro. Irinotecan was used to induce intestinal mucositis in rat IEC-6 cells in vitro. Due to the more current reports concerning the roles of TRIM1, TRIM9, TRIM18, TRIM36, TRIM46 and TRIM67 in irinotecan-induced intestinal mucositis (37-39), the mRNA expression levels of these TRIM members were detected via RT-qPCR in IEC-6 cells following treatment with 0 (control), 30 and 100 µM irinotecan. The results presented in Fig. 1 show that the mRNA expression of TRIM9 was significantly increased in irinotecan-induced IEC-6 cells in a dose-dependent manner compared with the 0 µM group, whereas the expression levels of TRIM1, TRIM18, TRIM36, TRIM46 and TRIM67 at the mRNA level showed no statistically significant differences, thus TRIM9 may play an important role in irinotecan-induced intestinal mucositis.

Interference and overexpression efficiency of TRIM9. TRIM9 interference and overexpression lentivirus vectors were infected into IEC-6 cells, and then RT-qPCR and western blotting were performed to detect transfection efficiency. As shown in Fig. 2A, in the TRIM9 group, the expression of TRIM9 mRNA was significantly higher compared with the vector group. Whereas the expression of TRIM9 mRNA in the shTRIM9-1, -2 and -3 groups was significantly inhibited compared with the shNC group, which was the most significant in the shTRIM9-1 and shTRIM9-2 groups (Fig. 2A). The western blotting results were consistent with the RT-qPCR results (Fig. 2B). Therefore, shTRIM9-1 and shTRIM9-2 were selected for the follow-up experiments.

Knockdown of TRIM9 partly inhibits the irinotecan-induced inhibition of cell proliferation, intestinal barrier function impairment and inflammatory factors expression levels. Transfected IEC-6 cells were treated with 100 µM irinotecan. The results demonstrated that treatment with irinotecan significantly inhibited cell proliferation (Fig. 3A) and increased intestinal mucosal permeability, as indicated by the decreased TEER and increased fluorescence intensity of FITC compared with the vehicle group (Fig. 3B and C). Furthermore, irinotecan significantly promoted cell apoptosis compared with the vehicle group (Fig. 3D). In addition, compared with the vehicle group, the levels of inflammatory cytokines, such as IL-1β and TNF-α were significantly increased (Fig. 3E), the protein expression levels of TRIM9 and p-P38/P38 (Fig. 3F) were also significantly increased, while the expression levels of ZO-1 and Claudin-4 (Fig. 3F) were significantly decreased. On the contrary, knockdown of TRIM9 significantly reversed the effects of irinotecan compared with the vehicle and shNC group.

TRIM9 regulates cell proliferation and intestinal barrier function in IEC-6 cells likely via the P38 pathway. A P38 inhibitor, SB203580 (20 µM), was applied to treat IEC-6 cells following the overexpression of TRIM9. The results revealed that cell proliferation, TEER and the expression levels of ZO-1 and Claudin-4 in the oeTRIM9 + Vehicle group were significantly lower than that in the Vector group (P<0.01, P<0.001; Fig. 4A, B and F), while cell apoptosis, fluorescence intensity of FITC, and IL-1β, TNF-α and p-P38/P38 expression in the oeTRIM9 + Vehicle group were all significantly increased (P<0.01, P<0.001; Fig. 4C-F). On the contrary, inhibition of P38 using SB203580 significantly reversed the effects of TRIM9 overexpression (Fig. 4A-F).
TRIM9 directly interacts with DUSP6 in IEC-6 cells. Due to the more current reports concerning the roles of DUSP members in irinotecan-induced intestinal mucositis (22,40–42), DUSP1, DUSP4, DUSP5, DUSP6 and DUSP10 were investigated in the current study. It was found that the protein expression levels of DUSP1, DUSP4, DUSP5, DUSP6 and DUSP10 were decreased in IEC-6 cells transfected with oeTRIM9. The results presented in Fig. 5A indicated that DUSP6 and DUSP10 protein expression levels were reduced in IEC-6 cells transfected with oeTRIM9 compared with the Vector group. Co-IP showed that TRIM9 interacted with DUSP6 (Fig. 5B). DUSP6 protein expression levels in IEC-6 cells transfected with shTRIM9-1 and shTRIM9-2 were significantly increased compared with the shNC group and in IEC-6 cells transfected with oeTRIM9 they were significantly decreased compared with the Vector group (Fig. 5D), while DUSP6 mRNA expression showed no changes (Fig. 5C). Additionally, DUSP6 protein expression was decreased in IEC-6 cells transfected with oeTRIM9, and treatment of proteasome inhibitor MG132 significantly inhibited the effect of TRIM9 expression. DUSP6 protein showed no significant difference between the Vector and oeTRIM9 groups following treatment with the proteasome inhibitor MG132 (Fig. 5E). Overexpression of TRIM9 notably enhanced the ubiquitination of DUSP6 in the IEC-6 cells (Fig. 5F).

TRIM9 regulates cell proliferation and intestinal barrier function in IEC-6 cells likely via the modulation of DUSP6 expression. IEC-6 cells were pre-transfected with oeTRIM9 and oeDUSP6 overexpression. The expression of DUSP6 was upregulated in IEC-6 cells following lentivirus infection (P<0.01; Fig. 6A and B). Overexpression of TRIM9 significantly inhibited cell proliferation, TEER, and the expression levels of ZO-1.
and Claudin-4 (P<0.05, P<0.01; Fig. 6C, E and H), whereas cell apoptosis, fluorescence intensity of FITC, and IL-1β, TNF-α and p-P38/P38 expression were all significantly increased (P<0.01, P<0.001; Fig 6D and F-H). On the contrary, overexpression of DUSP6 significantly reversed the effects of TRIM9 overexpression (Fig. 6C-H). In addition, irinotecan-induced inhibition of cell proliferation, and upregulation of intestinal mucosal permeability and cell apoptosis were significantly reversed by transfection with oeDUSP6 (Fig. 6I-L).

Discussion
The intestinal barrier has efficient and selective functions in the intestine. The present study verified the inhibitory effect of the TRIM9/DUSP6/P38 pathway on the irinotecan-induced increase in cell apoptosis, and inhibition of cell proliferation, intestinal barrier function impairment and the expression of inflammatory cytokines in IEC-6 cells, thus promoting the repair of the intestinal mucosal barrier.

A previous study revealed that TRIM9 is involved in paraneoplastic cerebellar degeneration (43). The absence of TRIM9 has been observed to lead to decreased dendritic density in adult-born neurons, excessive dendrite arborization and mis-localization, thus causing impairment of memory and learning (43). Do et al (43) demonstrated the importance of TRIM9 in regulating the function of neurons and uncovered the weak expression of TRIM9 in the brains of patients with Parkinson's disease and dementia (43). Furthermore, TRIM9...
has been speculated to be associated with carcinogenesis and could serve as a marker for detecting tumor DNA (44). The present research revealed that knockdown of TriM9 significantly inhibited irinotecan-induced cell apoptosis increases, cell proliferation inhibition, intestinal mucosal barrier repair, and the levels of inflammatory cytokines in IEC-6 cells. It has been reported that ZO-1 and Claudin-4 are the important components responsible for paracellular permeability (45). Moreover, alveolar epithelial barrier function has been found to be altered following the downregulation of ZO-1 and Claudin-4 expression (45). Claudin-4 in the small intestinal villus tips of MTX-treated rats may play a role in drug-induced intestinal barrier dysfunction (46). Consistently, the present study found that the intestinal mucosal barrier was repaired after the expression of epithelial barrier tight-junction proteins ZO-1 and Claudin-4 were increased. A previous study also showed that inflammatory cytokines (such as IL-1β and TNF-α) were significantly expressed after irinotecan administration (47). The increase in TNF-α contributed to the subsequent inhibition of ZO-1/Claudin-4 (46). These findings were in agreement with the current results that TriM9 knockdown partly decreased the irinotecan-induced expression of IL-1β and TNF-α and increased the irinotecan-induced inhibition of ZO-1 and Claudin-4 expression. Therefore, these results indicated that TriM9 knockdown may promote intestinal mucosal barrier repair by modulating ZO-1 and Claudin-4 expression levels.

The present study also investigated the signaling pathways of TriM9 that regulate intestinal mucosal barrier repair. Studies have found that the MAPK superfamily, including P38, is related to cardiovascular disease because P38 MAPK activation can stimulate cell growth, differentiation and cell death (48-50). Studies have reported that in intestinal mucositis, activation of the P38 pathway can inhibit cell proliferation (4,51). P38-MAPK has been shown to be activated by platelet factor-4, leading to intestinal damage and intestinal apoptosis (52). The present study found that irinotecan-induced expression of p-P38 was significantly decreased by TRIM9 knockdown, and inhibition of P38 significantly reversed the TriM9-induced increase in cell apoptosis, inhibition of cell proliferation and intestinal mucosal barrier damage. These results indicated that TriM9 regulated cell proliferation and intestinal barrier function in IEC-6 cells, likely through activation of the P38 pathway.

Furthermore, the downstream target genes of TriM9 regulating intestinal mucosal barrier repair were also analyzed. The results revealed that overexpression of TriM9 obviously enhanced the ubiquitination of DUSP6, which led to the rapid degradation of DUSP6, probably by proteasomes in the IEC-6 cells. DUSP6, a negative feedback mechanism of the MAPK
Figure 6. TRIM9 regulates cell proliferation and intestinal barrier function in IEC-6 cells likely through modulating DUSP6 expression. The overexpression efficiency of DUSP6 in IEC-6 cells was detected via (A) reverse transcription-quantitative PCR and (B) western blotting. "P<0.01, ***P<0.001 vs. Vector. IEC-6 cells were transfected with oeTRIM9 combined with oedUSP6. (C) Cell proliferation at 0, 12, 24 and 48 h was detected using a CCK-8 assay. (D) Cell apoptosis was detected with a flow cytometer. (E) TEER was measured in each group. (F) The fluorescence intensity of FITC was tested in each group. (G) The levels of IL-1β and TNF-α expression were detected with an ELISA. (H) The protein expression levels of ZO-1, DUSP6, Claudin-4 and p-P38/P38 were detected via western blotting. *P<0.05, **P<0.01, ***P<0.001 vs. Vector; #P<0.05 vs. irinotecan + Vector. TRIM9, tripartite motif protein 9; DUSP, dual-specificity phosphatase; oe, overexpression; CCK-8, Cell Counting Kit-8; TEER, transepithelial electrical resistance; TNF-α, tumor necrosis factor-α; IL-1β, interleukin-1β; ZO-1, zona occludens protein 1; p-, phosphorylated.
superfamily, which includes MAPK/ERK, SAPK/JNK and P38, are expressed differently in different types of cancers (53). For instance, in myeloma, melanoma and glioma, DUSP6 has been found to be significantly increased (54). On the other hand, in pancreatic invasive cancer, primary lung cancer and ovarian cancer, the expression of DUSP6 has been reported to be decreased (55-57). In addition, DUSP6 can regulate the inflammatory response of the colon and protect the intestinal epithelium from carcinogenic stress via activation of the ERK1/2 pathway (22). Consistent with these previous findings, the present results showed that the overexpression of DUSP6 significantly reversed the TRIM9-induced increase in cell apoptosis, inhibition of cell proliferation and intestinal mucosal barrier damage, concurrent with decreased P38 phosphorylation. Together, these results indicate that TRIM9 regulates irinotecan-induced intestinal mucositis probably via modulation of the ubiquitination of DUSP6.

A limitation of the current study is that all data were derived from in vitro experiments and in vivo experiments were not performed. Another limitation is that IEC-6 cells, which are derived from rats (unlike T84, caaco2 and HT29 cells), were used to construct the cell model. Additionally, this study also lacks in-depth research into other members of the TRIM and DUSP families, if possible, we would like to conduct experiments on this aspect in the future. The current results indicated that TRIM9 significantly inhibited the irinotecan-induced increase in cell apoptosis and inhibition of cell proliferation, which suggested that this approach may also impair the antitumor efficacy of irinotecan. Therefore, it is necessary to conduct further research in the future to have a more comprehensive understanding of the mechanisms and effect on TRIM9 and irinotecan.

In conclusion, the results of this study demonstrated that the knockdown of TRIM9 significantly suppressed the irinotecan-induced increase in cell apoptosis, inhibition of cell proliferation and intestinal mucosal barrier impairment with elevated levels of inflammatory cytokines in IEC-6 cells, which probably occurred through the inhibition of P38 activation via targeting DUSP6. Although further research is needed to verify these findings, this study lays a theoretical foundation for TRIM9 as a potential therapeutic target for intestinal mucositis.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors’ contributions

QW conceived and designed the present study. QW and WZ performed the experiments. QW wrote the manuscript. All authors read and approved the final manuscript. QW and WZ confirm the authenticity of all the raw data.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Sougiannis AT, VanderVeen BN, Davis JM, Fan D and Murphy EA: Understanding chemotherapy-induced intestinal mucositis and strategies to improve gut resilience. Am J Physiol Gastrointest Liver Physiol 320: G712-G719, 2021.
2. Wu QZ, Han XD, Wang Y, Yuan KL, Jin ZM, Di JZ, Yan J, Pan Y, Zhang P, Huang XY, et al: Interleukin-1 receptor antagonist reduced apoptosis and attenuated intestinal mucositis in a 5-fluorouracil chemotherapy model in mice. Cancer Chemother Pharmacol 68: 87-96, 2011.
3. Murata Y, Hirose T, Yamaoka T, Shirai T, Okuda K, Sugiyama T, Kusumoto S, Nakashima M, Ohmori T and Adachi M: Phase II trial of the combination of carboplatin and irinotecan in elderly patients with small-cell lung cancer. Eur J Cancer 47: 1336-1342, 2011.
4. Gao J, Gao J, Qian L, Wang X, Wu M, Zhang Y, Ye H, Zhu S, Yu Y and Han W: Activation of p38-MAPK by CXCL4/CXCR3 axis contributes to p53-dependent intestinal apoptosis initiated by 5-fluorouracil. Cancer Biol Ther 15: 982-991, 2014.
5. Thomsen M and Vitetta L: Adjunctive treatments for the prevention of chemotherapy- and radiotherapy-induced mucositis. Integr Cancer Ther 17: 1027-1047, 2018.
6. Lu H, Qin J, Han N, Xie F, Gong L and Li C: Banxia Xiexin decoction is effective to prevent and control irinotecan-induced delayed diarrhea in recurrent small cell lung cancer. Integr Cancer Ther 17: 1109-1114, 2018.
7. Paulik A, Nekvindová J and Filip S: irinotecan toxicity during treatment of metastatic colorectal cancer: Focus on pharmacogenomics and personalized medicine. Tumori 106: 87-94, 2020.
8. Ottaiano A,Scala S, Normanno N, Napolitano M, Capozzi M, Rachiglio AM, Roma C, Trotta AM, D’Alterio C, Portella L, et al: Cetuximab, irinotecan and fluorouracile in IRSt-line treatment of immunologically-selected advanced colorectal cancer patients: The CIFRA study protocol. BMC Cancer 19: 899, 2019.
9. Spyropoulos BG: Interleukin-18 as a target for modulation of irinotecan-induced intestinal toxicity: a step towards a better therapeutic index?: Commentary on Lima-Junior et al., Br J Pharmacol 171: 2335-2350, Br J Pharmacol 172: 4779-4781, 2015.
10. Ouyang M, Luo Z, Zhang W, Zhu D, Lu Y, Wu J and Yao X: Protective effect of curcumin against irinotecan-induced intestinal mucosal injury via attenuation of NF-kB activation, oxidative stress and endoplasmic reticulum stress. Int J Oncol 54: 1376-1386, 2019.
11. Wardill HR, Gibson RJ, Van Sebille YZ, Secombe KR, Coller JK, White IA, Manavis J, Hutchinson MR, Staiopoulos V, Logan RM, et al: Irinotecan-induced gastrointestinal dysfunction and pain are mediated by common Tlr4-dependent mechanisms. Mol Cancer Ther 15: 1376-1386, 2016.
12. Hatakeyama S: TRIM family proteins: Roles in autophagy, immunity, and carcinogenesis. Trends Biochem Sci 42: 297-311, 2017.
13. Jaworska AM, Wlodarczyk NA, Mackiewicz A and Czerwinska P: The role of TRIM family proteins in the regulation of cancer stem cell self-renewal. Stem Cells 38: 165-173, 2020.
14. Espósito D, Koloppoulos MG and Rüttiger K: Structural determinants of TRIM protein function. Biochem Soc Trans 45: 183-191, 2017.
15. Fiorentini F, Esposito D and Rittinger K: Does it take two to tango? RING domain self-association and activity in TRIM E3 ubiquitin ligases. Biochem Soc Trans 48: 2615-2624, 2020.

16. Tanji K, Kamitani T, Mori F, Kakita A, Takahashi H and Wakabayashi K: TRIM5, a novel brain-specific E3 ubiquitin ligase, is repressed in the brain of Parkinson's disease and dementia with Lewy bodies. Neurol Biol Dis 38: 210-218, 2010.

17. Winkle CC, Olsen RH, Kim H, Moy SS, Song J and Gugton SL: Trim9 deletion alters the morphogenesis of developing and adult-born hippocampal neurons and impairs spatial learning and memory. J Neurosci 36: 4940-4958, 2016.

18. Menon S, Boyer NP, Winkle CC, McClain LM, Hanlin CC, Pandey D, Rothenfusser S, Taylor AM and Gugton SL: The E3 ubiquitin ligase TRIM9 is a filopodia off switch required for mitotically dependent axon guidance. Dev Cell 35: 698-712, 2015.

19. Zeng J, Wang C, Yoon YS, Van H, Chou Y, Xie X, Deverman BE, Grudanaru V, et al: TRIM9-mediated resolution of neuroinflammation confers neuroprotection upon ischemic stroke in mice. Cell Rep 27: 549-560.e6, 2019.

20. Wu J, Gan Y, Li M, Chen L, Liang J, Zhuo L, Hou X, Wu X, Wu Q, et al: Patchouli alcohol attenuates 5-fluorouracil-induced intestinal mucositis via TLR2/MyD88/ NF-κB pathway and regulation of microbiota. Biomed Pharmacother 124: 109883, 2020.

21. Tokarz DA, Heffelfinger AK, Jima DD, Gerlach J, Shah RN, Law JM et al: Disruption of Trim9 function abrogates macrophage maturation motility in vivo. J Leukoc Biol 102: 1371-1380, 2017.

22. Beaudry K, Langlois M-J, Montagne A, Cagnol S, Carrier JC and Riard N: Dual-specificity phosphatase 6 deficiency regulates gut microbiome and transcriptome response against diet-induced obesity in mice. Nat Microbiol 2: 1620, 2016.

23. Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W and Arkinstall S: MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J Biol Chem 271: 4319-4326, 1996.

24. Mula M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A and Arkinstall S: The dual specificity phosphatases M3/6 and MKP-3 are highly selective protein-tyrosine phosphatases. FEBS Lett 315: 545-550, 1993.

25. Ruan JW, Statt S, Huang CT, Tsai YT, Kuo CC, Chan HL, Law JM, et al: Disruption of Trim9 function abrogates macrophage maturation motility in vivo. J Leukoc Biol 102: 1371-1380, 2017.

26. Beaudry K, Langlois M-J, Montagne A, Cagnol S, Carrier JC and Riard N: Dual-specificity phosphatase 6 deficiency regulates gut microbiome and transcriptome response against diet-induced obesity in mice. Nat Microbiol 2: 1620, 2016.

27. Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W and Arkinstall S: MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J Biol Chem 271: 4319-4326, 1996.

28. Ruan JW, Statt S, Huang CT, Tsai YT, Kuo CC, Chan HL, Law JM, et al: Disruption of Trim9 function abrogates macrophage maturation motility in vivo. J Leukoc Biol 102: 1371-1380, 2017.

29. Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W and Arkinstall S: MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J Biol Chem 271: 4319-4326, 1996.

30. Mula M, Theodosiou A, Rodrigues N, Boschert U, Camps M, Gillieron C, Davies K, Ashworth A and Arkinstall S: The dual specificity phosphatases M3/6 and MKP-3 are highly selective protein-tyrosine phosphatases. FEBS Lett 315: 545-550, 1993.

31. Ruan JW, Statt S, Huang CT, Tsai YT, Kuo CC, Chan HL, Law JM, et al: Disruption of Trim9 function abrogates macrophage maturation motility in vivo. J Leukoc Biol 102: 1371-1380, 2017.

32. Beaudry K, Langlois M-J, Montagne A, Cagnol S, Carrier JC and Riard N: Dual-specificity phosphatase 6 deficiency regulates gut microbiome and transcriptome response against diet-induced obesity in mice. Nat Microbiol 2: 1620, 2016.

33. Muda M, Boschert U, Dickinson R, Martinou JC, Martinou I, Camps M, Schlegel W and Arkinstall S: MKP-3, a novel cytosolic protein-tyrosine phosphatase that exemplifies a new class of mitogen-activated protein kinase phosphatase. J Biol Chem 271: 4319-4326, 1996.

34. Ruan JW, Statt S, Huang CT, Tsai YT, Kuo CC, Chan HL, Law JM, et al: Disruption of Trim9 function abrogates macrophage maturation motility in vivo. J Leukoc Biol 102: 1371-1380, 2017.

35. Beaudry K, Langlois M-J, Montagne A, Cagnol S, Carrier JC and Riard N: Dual-specificity phosphatase 6 deficiency regulates gut microbiome and transcriptome response against diet-induced obesity in mice. Nat Microbiol 2: 1620, 2016.
53. Piya S, Kim JY, Bae J, Seol DW, Moon AR and Kim TH: DUSP6 is a novel transcriptional target of p53 and regulates p53-mediated apoptosis by modulating expression levels of Bcl-2 family proteins. FEBS Lett 586: 4233-4240, 2012.
54. Bermúdez O, Pagès G and Gimond C: The dual-specificity MAP kinase phosphatases: Critical roles in development and cancer. Am J Physiol Cell Physiol 299: C189-C202, 2010.
55. Furukawa T, Fujisaki R, Yoshida Y, Kanai N, Sunamura M, Abe T, Takeda K, Matsuno S and Horii A: Distinct progression pathways involving the dysfunction of DUSP6/MKP-3 in pancreatic intraepithelial neoplasia and intraductal papillary-mucinous neoplasms of the pancreas. Mod Pathol 18: 1034-1042, 2005.
56. Chan DW, Liu VW, Tsao GS, Yao KM, Furukawa T, Chan KK and Ngan HY: Loss of MKP3 mediated by oxidative stress enhances tumorigenicity and chemoresistance of ovarian cancer cells. Carcinogenesis 29: 1742-1750, 2008.
57. Okudela K, Yazawa T, Woo T, Sakaeda M, Ishii J, Mitsui H, Shimoyamada H, Sato H, Tajiri M, Ogawa N, et al: Down-regulation of DUSP6 expression in lung cancer: Its mechanism and potential role in carcinogenesis. Am J Pathol 175: 867-881, 2009.