TRIM29 regulates the p63-mediated pathway in cervical cancer cells

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Abstract

Cell invasion and adhesion play an important role in cancer metastasis and are orchestrated by a complicated network of transcription factors including p63. Here, we show that a member of the tripartite motif protein family, TRIM29, is required for regulation of the p63-mediated pathway in cervical cancer cells. TRIM29 knockdown alters the adhesion and invasion activities of cervical cancer cells. TRIM29 knockdown and overexpression cause a significant decrease and increase of TAp63α expression, respectively. TRIM29 knockdown alters the expression pattern of integrins and increases ZEB1 expression. TRIM29 is required for suppression of an increase in the adhesion activity of cells by TAp63α. These findings suggest that TRIM29 regulates the p63-mediated pathway and the behavior of cervical cancer cells.
Highlights

- TRIM29 regulates cell adhesion and invasion.
- TRIM29 regulates TAp63α and integrin expression
- TRIM29 interacts with TA p63α in cervical cancer cells
1. Introduction

Invasion and migration are significant hallmarks of metastatic cancer cells. Epithelial to mesenchymal transition (EMT) allows cancer cells to acquire metastatic ability [1]. During the EMT process, interaction of integrins with the extracellular matrix (ECM) exerts a prominent role by regulating the function of matrix metalloproteinases (MMPs) [1]. The degradation of ECM components by MMPs contributes to cancer metastasis by changing the tumor microenvironment [1]. These biological processes are orchestrated by a network of transcription factors including SNAIL1/2, TWIST1/2 and ZEB1/2 [1]. In addition to these transcription factors, one of the members of the p53 family, p63, is involved in cancer metastasis by regulating the expression of integrins [2, 3].

Uterine cervical cancer is one of the common health problems for women worldwide [4]. Human papillomavirus (HPV) is a significant risk factor associated with invasive cervical cancer [5]. Among the various HPV types, HPV-18 is strongly related to adenocarcinoma of the uterine cervix, whereas HPV-16 is detected more frequently in squamous cell carcinoma than in adenocarcinoma [6, 7]. HPV infection enhances the invasion activity of cervical cancer cells by upregulation of MMPs [8]. In cervical cancer cells, epidermal growth factor promotes the transition of epithelial cells to mesenchymal cells, being dependent on SNAIL1 and α5β1 integrin [9]. Interaction of α5β1 integrin with fibronectin contributes to the migration with up-regulation of MMP-9 in cervical cancer cells [10]. Although EMT-related genes contribute to metastasis of cervical cancer, how cervical cancer cells gain invasive ability remains to elucidate.
TRIM29 has been identified as an ataxia telangiectasia group D-complementing gene in a functional complementation study with an AT cell line by transfection with a human cosmid library [11]. TRIM29 is a member of the tripartite motif (TRIM) protein family and is specifically expressed in squamous cells [12, 13]. There is increasing evidence that TRIM29 enhances or suppresses cell invasion depending on cell type. In breast cancer cells, TRIM29 suppresses invasiveness by down-regulating the expression of TWIST1 [14]. TRIM29 promotes cell invasion by regulating MMP-9 in lung cancer [15]. TRIM29 also regulates the invasion of pancreatic cancer cells by inducing EMT [16]. Furthermore, it has been reported that overexpression of TRIM29 is associated with malignancy of various cancers including bladder, colorectal, gastric lung and pancreatic cancers [12]. To date, it has been controversial whether TRIM29 functions as an oncogene or tumor suppressor. Although expression of TRIM29 is closely related to the characteristics and malignancy of cancer, the relationship between TRIM29 and cervical cancers remains incompletely understood.

In this study, we identified TRIM29 as a regulator of the p63-mediated pathway in cervical cancer cells. TRIM29 was required for the suppression of ZEB1 and integrin β1 (ITGB1). Depletion of TRIM29 suppressed an elevation of the adhesion activity of cells by TAp63α. We propose that TRIM29 regulates the p63-mediated pathway and the behavior of cervical cancer cells.
2. Materials and Methods

2.1. Plasmid

Full-length human TRIM29 (NM_012101.3) was amplified using total RNA from HeLa cells. TAp63α-FLAG was a gift from David Sidransky (Addgene plasmid # 27008).

2.2. Generation of stable cell lines

cDNAs encoding full-length TRIM29 and TAp63α were introduced by retroviral transduction into a HeLa S3 cell line stably expressing the mouse ectopic retrovirus receptor (mCAT-1) [17]. Retroviruses were produced with Plat-E packaging cells [18].

2.3. Cell culture

HeLa, HeLa S3 and SiHa cell lines were obtained from American Type Culture Collection (Manassas, VA), which authenticates human cell lines in their collection, and were cultured in DMEM (Sigma-Aldrich Corp., St Louis, MO) supplemented with 10% fetal bovine serum (FBS) under an atmosphere of 5% CO₂ at 37°C. Unless otherwise indicated, cells were cultured in a 100-mm plastic dish (Corning 430167; Corning Co., NY, USA). For large-scale cultures, parental and FLAG-tagged TRIM29-expressing HeLa S3 cells were cultured in Joklik’s medium (Sigma) supplemented with 5% calf
serum (Invitrogen) using spinner culture.

2.4. Antibodies

Mouse anti-FLAG M2 was purchased from Sigma (Sigma). Mouse anti-TRIM29 (A-5: sc-166718), rabbit anti-p63 (H-137: sc-8343), mouse anti-p53 (DO-1: sc-126) rabbit anti-Pol II (N-20: sc-899) and mouse anti-actin (C-2: sc-8432) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA.). Rabbit anti-Integrin β4 (4707) was purchased from Cell Signaling Technology (Beverly, MA). Rabbit anti-Integrin β1 (ab52971) was purchased from Abcam (Cambridge, UK). Mouse anti-GAPDH (AM4300) was purchased from Ambion (Austin, TX). Rabbit anti-MED12 was purchased from Bethyl Laboratories (Bethyl Laboratories, Montbomery, TX).

2.5. siRNA transfections

HeLa S3, HeLa and SiHa cells in 10-cm dishes (∼2 × 10^6 cells/dish) were transfected with 25 nM siRNA ON-TARGETplus human TRIM29 siRNA SMART pool (Dharmacon, Pittsburgh, PA) or 25 nM siGENOME NON-TARGETING siRNA Pool #2 (Dharmacon) using Lipofectamine RNAi MAX (Invitrogen). After 48-h culture, cells were subjected to each analysis.

2.6. Quantitative real-time PCR

Total RNA was extracted using ISOGEN II (Nippon Gene, Tokyo, Japan). cDNA
was prepared by using oligonucleotide (dT) and a ReverTra-Plus kit (TOYOBO, Japan) for quantitative real-time PCR (qRT-PCR). The threshold cycle (Ct) values were determined by qRT-PCR using Express SYBRGreenER qPCR Supermix Universal (Invitrogen) on an ABI Prism 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA). ΔCt was obtained by subtracting the Ct value of the GAPDH gene from the Ct value of the respective gene. The relative expression level of mRNA was determined by $2^{-\Delta\Delta Ct}$. All PCR primers were as follows: TRIM29, 5'-AAAGGCTATCCCTCCCTCAT-3' (forward) and 5'-TATAATGGCCGGTAGTGAAGA-3' (reverse); p63, 5'-GCAGCTTTGCAAAACCATTAA-3' (forward) and 5'-GCTCGGAAGTCCTAGGTTATT-3' (reverse); TAp63, 5'-TGATCTCGCATGAGGACT-3' (forward) and 5'-CTGTGTTATACGGACTGGGAC-3' (reverse); ΔNp63, 5'-GAAAACAATGCCTCAGCTCAA-3' (forward) and 5'-TGCGCGTGTCTGTGTTTA-3' (reverse); p63α, 5'-ATTGATGCTGTGCGATTCACC-3' (forward) and 5'-TGTTGCTTTAGGGACTGGGAC-3' (reverse); p63β, 5'-AAGCCCTCCTCCTCAAC-3' (forward) and 5'-GACTTGCCAGATCTGCAATG-3' (reverse); p63γ, 5'-GCAGCACCAGCAGTTACACTCA-3' (forward) and 5'-TGTTTTGAGTTTCTCTCCGG-3' (reverse); SNAIL2, 5'-TTTCTGGGCTGGCAAAACATAGC-3' (forward) and 5'-ACACAAGGTATGTCGGGTGCAGA-3' (reverse); ZEB1, 5'-CAGGAAAGGAAGGGCAAGAA-3' (forward) and 5'-TCTGCATCTGACTCGATTTC-3' (reverse); p53,
5’-TTTCGACATAGTGTGGTGGTGTG-3’ (forward) and 5’-GCCCATGCAGGAACGTGTTA-3’ (reverse); ITGB1,

5’-CCTGAAAGTCCCAAGTGTGTCAT-3’ (forward) and 5’-ACCAACACGCCTTTCCATT-3’ (reverse); ITGB4,

5’-GAGCTGACATCGGCTCAA-3’ (forward) and 5’-AACACGTAGGAGTAGGAAGTGGTTG-3’ (reverse); ITGA3, 5’-AACTGTGGGAGGATGGAAG-3’ (forward) and 5’-CACTCCAGACCTAGGACCA-3’ (reverse); ITGA5,

5’-GGGACCTCAGATCCTGAAATG-3’ (forward) and 5’-CTGCAGACTTTGGCTCTCTT-3’ (reverse); ITGA6,

5’-AGACTCTTAAACTGTAGGAAGTGAAC-3’ (forward) and 5’-ATAAGAGACGCCTTGCTGTC-3’ (reverse); FN1,

5’-AGGAATATCTCGGTGCCATTT-3’ (forward) and 5’-CTTCGGGACTGGGTTCAC-3’ (reverse); VIM,

5’-CTTCGGGACTGGGTTCAC-3’ (forward) and 5’-CTCCAGACCTTTGGCTCTCTT-3’ (reverse); SOX2,

5’-ATCCAAGTTTTGCTGACCTTC-3’ (forward) and 5’-TACAGCATGATGCAGGACCA-3’ (reverse); SOX2,

5’-TACTGCAAGGCCGCTCAC-3’ (reverse); RB1,

5’-GGATGGAGTATTGGGAGGTTAT-3’ (forward) and 5’-ATCCAAGTTTTGCTGACCTTC-3’ (forward); RB1,

5’-AGTGAAACGATCTCCTACTAGG-3’ (reverse); FOXM1,

5’-AGGCTCCTCAAACCTTTAACC-3’ (reverse); E2F1,

5’-GGAGAAGTCAGCTATGAGAC-3’ (forward) and 5’-GTCCCTGAGTTCCCAACC-3’ (reverse); E2F2, 5’-GTCCCTGAGTTCCCAACC-3’ (forward) and 5’-GGAGAAGTCAGCTATGAGAC-3’ (forward) and 5’-GTCCCTGAGTTCCCAACC-3’ (reverse); E2F3,
5’-AGGCTGGAGCTAGGAGAAA-3’ (forward) and
5’-CGTAGTGCACTCTCTTCTT-3’ (reverse); E2F4,
5’-CCGGGAGATTGCTGACAAA-3’ (forward) and
5’-CCTTGTGCTGGTCTAGTTCTT-3’ (reverse); E2F5,
5’-ACCAATGTCTTAGAGGGAATTGA-3’ (forward) and
5’-TTTAGTATTACAGCCACCGACCT-3’ (reverse); E2F6,
5’-TCCAAGAACCATATTAGATGGATAGG-3’ (forward) and
5’-ATTAACTCATCCAAAGCATCTTCC-3’ (reverse); HOXB5,
5’-TCCGCAATATTTCCCTGGA-3’ (forward) and
5’-CGGTCAGGTAGCGGTTGAA-3’ (reverse); HOXB7,
5’-GACTTGGCGGCGGAGAGTA-3’ (forward) and
5’-CAGGGTCTGGTAGCGGTTG-3’ (reverse); HOXB9,
5’-CCACTGGCCTGGACTGGT-3’ (forward) and
5’-TATGACTGGGCCAGGTTCTTTG-3’ (reverse); BRCA1,
5’-GCATGGCCTGAATTCTCTACAAC-3’ (forward) and
5’-CGTACTTCTTGTAGGCTCCTT-3’ (reverse); GAPDH,
5’-GTCAACGGGATTGGTGCATTG-3’ (forward) and
5’-TG TAGATTGAGGTCAATGAAGGG-3’ (reverse).

2.7. Cell adhesion assay

Cell adhesion assay to fibronectin, collagen I, collagen IV, laminin I and vimentin was performed using a CytoSelect 48-well cell adhesion assay extracellular matrix (ECM) array (Cell Biolabs Inc., San Diego, CA) according to the manufacturer’s
instructions. Cells were incubated on the ECM array for 1 h under an atmosphere of 5% CO₂ at 37°C in a serum-free medium. Each well was gently washed to remove non-adherent cells. Adherent cells were stained by Cell Stain Solution. The stains were extracted by Extract Solution and quantified by measuring absorbance at 595 nm.

2.8. Cell invasion assay

Cell invasion assay was performed using a CytoSelect 24-well cell invasion assay kit (Cell Biolabs Inc.) according to the manufacturer’s instructions. Cells were incubated on the upper chamber on a polycarbonate membrane coated with basement membrane matrix in a serum-free medium. Cells were incubated for 1 h under an atmosphere of 5% CO₂ at 37°C. Non-invasive cells were gently removed from the interior of the chamber. Cells that had invaded to the bottom of the membrane were stained by Cell Stain Solution. The stains were extracted by Extract Solution and quantified by measuring absorbance at 595 nm.

2.9. Immunostaining

Cells were cultured on a cover glass in DMEM supplemented with 10% FBS under an atmosphere of 5% CO₂ at 37°C. The cells were fixed by 4% paraformaldehyde in PBS for 15 min at room temperature, washed with PBS three times, and then incubated with blocking buffer (5% normal goat serum and 0.1% Triton X-100 in PBS) for 1 h. After blocking, the cells were incubated with primary antibodies in blocking buffer overnight at 4°C and incubated with goat Alexa Fluor 488-labeled anti-mouse IgG or
goat Alexa Fluor 555-labeled anti-rabbit IgG antibody (Invitrogen Life Technologies, Carlsbad, CA) for 1 h at room temperature. DNA was counterstained with 0.4 µg/ml DAPI in PBS for 30 min at room temperature. Confocal images were taken using an upright microscope (FV1000, Olympus, Tokyo, Japan).

2.10. Analysis of focal adhesion area

Cells were transfected with siControl or siTRIM29 and incubated on fibronectin-coated coverslips in serum-free medium. After 48 h, cells were fixed and stained with anti-actin and anti-ITGB1 antibodies. DNA was counterstained with DAPI (0.4 µg/ml). Cell adhesion area was calculated as the ratio of ITGB1-stained area to DAPI-stained area. ITGB1- and DAPI-stained areas were quantified using NIH ImageJ software (n = 20).

2.11. FLAG immunoprecipitation

Nuclear extracts of HeLa S3 cells expressing FLAG-tagged TRIM29 were prepared according to Dignam’s method [19]. Anti-FLAG affinity purifications were performed as described previously [20].
3. Results

3.1. TRIM29 regulates cell adhesion and invasion

To reveal the function of TRIM29 in cervical cancer cells, we knocked down TRIM29 in HeLa S3 (HPV-18-positive), HeLa (HPV-18-positive) and SiHa (HPV-16-positive) cells by siRNA transfection and then performed an in vitro cell adhesion assay using plates coated with fibronectin, fibrinogen, collagen I, collagen IV or laminin I. TRIM29 knockdown in HeLa S3 cells increased the adhesion activity of cells to fibronectin (4.4-fold), fibrinogen (2.6-fold), collagen IV (1.7-fold) and collagen I (1.5-fold) (Fig. 1A), and TRIM29 knockdown in HeLa cells increased in adhesion activity to fibronectin (2.2-fold), collagen IV (2.7-fold) and collagen I (3.8-fold) (Fig. 1B). TRIM29 depletion in SiHa cells did not have a significant effect on adhesion activity (Fig. 1C). It has been reported that TRIM29 regulates invasion of lung and breast cancer cells [14-16]. To examine whether TRIM29 regulates cell invasion, we performed an in vitro cell invasion assay using polycarbonate membranes coated with basement membrane matrix. TRIM29 knockdown reduced the invasion activity of HeLa S3 and SiHa cells (Fig. 1D). In addition, we confirmed a reciprocal relationship between cell morphology and TRIM29 knockdown in each cell line. Knockdown of TRIM29 caused a significant change in morphology of HeLa S3 cells but not morphology in HeLa and SiHa cells (Fig. 1E). These findings suggest that TRIM29 regulates adhesion and invasion depending on the type of cervical cancer cells.

3.2. Effect of TRIM29 knockdown on focal adhesion
ITGB1 plays a major role in binding to ECM components and contributes to the formation of focal adhesion [21]. To examine the effect of TRIM29 depletion on focal adhesion area of cells, we depleted HeLa S3, HeLa and SiHa cells of TRIM29 by siRNA transfection and then incubated the TRIM29-depleted cells on fibronectin-coated coverslips in serum-free medium for 24 h (Fig. 2A and B). We defined the ratio of ITGB1-stained area to DAPI-stained area as focal adhesion area. TRIM29 knockdown increased the focal adhesion area in HeLa S3 and HeLa cells but not in SiHa cells (Fig. 2C). These findings suggest that TRIM29 regulates focal adhesion depending on the type of cells.

3.3. TRIM29 transcriptionally regulates ZEB1 and TAp63α isoform

To reveal the relationship between TRIM29 and the behavior of cervical cancer cells, we examined the effects of TRIM29 knockdown on relative mRNA levels of p63 and EMT-related transcription factors. We also examined E2F and HOXB transcription factors, which are associated with cancer progression [22, 23]. We suppressed TRIM29 in HeLa S3, HeLa and SiHa cells by siRNA transfection and analyzed the expression of indicated genes by qRT-PCR. Among the indicated genes, TRIM29 knockdown decreased the mRNA level of p63, whereas the mRNA level of ZEB1 was elevated in TRIM29-depleted cells (Fig. 3A-C). We also found that p53 and SNAIL2 were significantly upregulated depending on the cell type (Fig. 3A-C).

The p63 gene has two distinct promoters that produce two subclasses of p63: transactivating p63 (TAp63) and N-terminal truncated p63 (ΔNp63) [24]. There are also
C-terminal splicing variants known as p63α, p63β and p63γ. To determine which isoforms of p63 are regulated by TRIM29, we performed qRT-PCR using previously reported primers that are specific for the isoforms of p63 (TAp63, ΔNp63, p63α, p63β and p63γ) [3]. While TAp63 isoforms were highly expressed, mRNA level of the ΔNp63 isoform was much lower than that of the TAp63 isoform (Fig. 3D-F). In addition, mRNA level of the p63α isoform was much higher than the levels of p63β and p63γ isoforms (Fig. 3D-F). TRIM29 knockdown in each cell line suppressed the expression of TAp63, p63α and p63γ isoforms (Fig. 3D-F). Taken together, these findings indicate that TRIM29 is a transcriptional regulator of TAp63α and ZEB1 in cervical cancer cells.

3.4. TRIM29 regulates the expression of cell adhesion genes

p63 regulates the expression of cell adhesion genes including β1-integrin (ITGB1), β4-integrin (ITGB4), α3-integrin (ITGA3), α5-integrin (ITGA5), α6-integrin (ITGA6), fibronectin (FN1) and vimentin (VIM) [2]. To clarify the relationship between TRIM29 and p63, we analyzed the effect of TRIM29 depletion on the expression of these genes. Knockdown of TRIM29 in HeLa S3 and HeLa cells had similar effects on the expression of ITGB1, ITGB4, ITGA5 and FN1 (Fig. 4A and B). TRIM29 knockdown in SiHa cells caused an increase in the expression of ITGB4 and a decrease in the expression of FN1 (Fig. 4C). In addition, loss of TRIM29 in each cell line increased the mRNA level of VIM, which is a marker of mesenchymal cells (Fig. 4). These findings suggest that TRIM29 regulates the expression of cell adhesion molecules in cervical cancer cells. However, the transcriptional regulation of cell adhesion genes by TRIM29 is dependent on the type of cells.
3.5. *TRIM29 interacts with TAp63α in cervical cancer cells*

To confirm that TRIM29 regulates the p63-mediated pathway, we examined the effect of TRIM29 knockdown on the protein levels of p63, ZEB1, ITGB1 and ITGB4 by immunoblot analysis. HeLa S3, HeLa and SiHa cells were transfected with TRIM29 siRNA, and the whole cell lysates were subjected to SDS-PAGE followed by immunoblotting with the indicated antibodies. TRIM29 knockdown led to a slight decrease in the protein level of TAp63α in HeLa S3 cells but not in HeLa and SiHa cells (Fig. 5A). TRIM29 knockdown caused increased expression of ZEB1 in HeLa S3, HeLa and SiHa cells (Fig. 5A). While ITGB1 was increased in HeLa S3 and HeLa cells by TRIM29 knockdown, ITGB4 was decreased in HeLa cells and was slightly increased in SiHa cells by TRIM29 depletion (Fig. 5A).

Nuclear localization of p63 is required for its function as a transcription factor. To examine whether TRIM29 regulates the subcellular localization of p63, we incubated TRIM29-depleted cells on coverslips and then analyzed the subcellular localization of p63 by immunofluorescence staining. TRIM29 depletion did not alter the subcellular localization of p63 in each cell line (Fig. 5B).

Depletion of TRIM29 elevated the level of ZEB1 protein in each cell line (Fig. 5A). These findings raised the possibility that TRIM29 suppresses the expression of ZEB1 by interacting with TAp63α in cervical cancer cells. To determine whether TRIM29 physically interacts with TAp63α, we generated HeLa S3 cells stably expressing N-terminal FLAG-tagged TRIM29, prepared nuclear extracts from the cells, and purified FLAG-tagged TRIM29-binding proteins by anti-FLAG immunoprecipitation.
As expected, we found that TRIM29 interacted with TAp63α (Fig. 5C). A recent study showed that knockdown of MED12, which is a key subunit of the Mediator complex, upregulates the expression of EMT-related genes [25]. To further determine the role of TRIM29 in the transcription of EMT-related genes, we confirmed whether TRIM29 interacts with MED12 and RNA polymerase II (Pol II). Western blot analysis showed that TRIM29 binds to Pol II and MED12 (Fig. 5C). These findings suggest that TRIM29 regulates ZEB1 suppression and integrin expression through TAp63α, Pol II and MED12.

3.6. Forced expression of TRIM29 upregulates ITGB4

To address the reciprocal relationship between TRIM29 and TAp63α, we analyzed the effect of TRIM29 overexpression on the expression of p63 isoforms and integrins by qRT-PCR. We confirmed that the mRNA level of TRIM29 was greatly increased in FLAG-TRIM29-expressing HeLa S3 cells (Fig. 6A). TRIM29 overexpression caused an increase in the expression levels of TAp63, p63α, p63β and p63γ but not in that of ΔNp63 (Fig. 6B). Overexpression of TRIM29 also resulted in increases in ITGB4, ITGA3 and ITGA6 expression levels (Fig. 6C). Although cells expressing FLAG-TRIM29 showed an increase in ITGB1 expression, TRIM29 overexpression had less of an impact on ITGB1 expression than did TRIM29 knockdown (Fig. 4A and 6C). We also confirmed the effect of TRIM29 overexpression on protein levels of TAp63α, ITGB1 and ITGB4 by Western blotting. TRIM29 overexpression resulted in an increased level of ITGB4 but not in increased levels of TAp63α and ITGB1 (Fig. 6D).

To confirm the effect of TRIM29 overexpression on cell invasion and adhesion, we
performed in vitro cell invasion and adhesion assays. TRIM29 overexpression caused a 1.9-fold increase in the invasion activity of HeLa S3 cells (Fig. 6E). Although TRIM29 overexpression decreased the adhesion activity of cells to collagen IV, there was no significant difference in the adhesion activity of cells to other ECM components (Fig. 6F). HeLa S3 cells expressing FLAG-TRIM29 also exhibited a marked change with tight cobblestone-like structures in cell morphology (Fig. 6G). These findings suggest that TRIM29 overexpression enhances cell invasion and cell-cell interactions in HeLa S3 cells.

3.7. TRIM29 is required for regulation of the p63-mediated pathway

To clarify the functional relationship between TRIM29 and TAp63α, we generated HeLa S3 cells stably expressing FLAG-tagged TAp63α and knocked down TRIM29 in the cells by siRNA transfection. We confirmed that FLAG-tagged TAp63α was expressed at a higher level than that of endogenous TAp63α (Fig. 7A and B). Although TRIM29 depletion increased the levels of ZEB1 and ITGB1, overexpression of TAp63α did not suppress the elevation of ZEB1 and ITGB1 levels (Fig. 7A and B). These findings suggest that TRIM29 regulates the expression of ZEB1 and ITGB1 independently of TAp63α.

We showed that TRIM29 depletion increases the adhesion activity of HeLa S3 cells to ECM components (Fig. 1A). To examine the effect of TAp63α overexpression on cell adhesion to the ECM, we performed in vitro cell adhesion assays. In the presence of endogenous TRIM29, overexpression of TAp63α had no effect on the adhesion activity of cells to ECM components (Fig. 7C). Conversely, overexpression of TAp63α
increased the adhesion activity of TRIM29-depleted cells to fibronectin, collagen I and collagen IV (Fig. 7C). These findings suggest that TRIM29 negatively regulates an increase in the adhesion activity of HeLa S3 cells by TAp63α.
4. Discussion

In this study, we showed that TRIM29 is an important regulator of the p63-mediated pathway in cervical cancer cells (Fig. 7D). p63 has a critical role in cell adhesion through the regulation of integrin expression [2], and it inhibits the expression of ZEB1 in pancreatic and bladder cancer cells [30, 31]. TRIM29 knockdown increased ZEB1 and ITGB1 levels in cervical cancer cells. However, TAp63α overexpression did not suppress the elevation of ZEB1 and ITGB1 levels in TRIM29-depleted cells, indicating that TRIM29 suppresses the expression of ZEB1 and ITGB1 independently of TAp63α. We also identified MED12, which is a key component of the Mediator complex [28, 29], as a binding partner of TRIM29. Given that loss of MED12 upregulates EMT-related genes in lung cancer cells [25], our observations raise the possibility that TRIM29 suppresses the expression of ZEB1 and ITGB1 through MED12. Furthermore, knockdown and overexpression experiments showed that TRIM29 regulates the mRNA level of TAp63α, suggesting that mRNA levels of TAp63α are regulated through the interaction with TRIM29 and MED12. This is important for understanding the detailed mechanism by which TRIM29 regulates TAp63α via MED12.

In cervical cancer cells, depletion and overexpression of TRIM29 affected the expression pattern of ITGB1 and ITGB4 and adhesion activity to the ECM. ITGB1 is essential for interaction of basal epithelial cells with the ECM [32]. ITGB4 expression disrupts adhesive activity and enhances invasive activity of epithelial cells [33]. However, loss of TRIM29 exerted different effects on integrin expression and cell adhesion in HPV-18-positive cells (HeLa and HeLa S3) and HPV-16-positive cells.
(SiHa). In cervical cancer cells, HPV genomes are integrated into the host genome, followed by chromosomal rearrangement and instability [34]. The integration of HPV genomes into the host genome disrupts several genes [35, 36]. Previous studies showed that HPV-18 and HPV-16 genomes are integrated into chromosomes 8 and 13, respectively [35, 36]. Thus, the difference in HPV integration sites may cause an alteration in the expression of cell adhesion genes. Further detailed studies on the mechanism by which TRIM29 regulates the expression of EMT-related genes in HPV-18 and HPV-16-positive cells might be useful for understanding the phenotypes of cervical cancers.

In this study, we found that TRIM29 enhances invasion of cervical cancer cells. While TRIM29 inhibits invasion of breast cancer cells [14], it enhances invasion of pancreatic and lung cancer cells [15, 16]. Degradation of the ECM by MMPs has an important role in cell invasion and metastasis [37]. It has been reported that HPV infection increases the expression of various MMPs in cervical cancer cells [8]. Thus, the invasive activity of cervical cancer cells may be regulated by the interplay between the function of TRIM29 and the effect of HPV infection. Further studies will be required for determination of the mechanism by which TRIM29 regulates the invasive activity of cervical cancer cells.

We found that TAp63α is maintained at a constant protein level independently of the mRNA level of TAp63α. There are several reports that ubiquitination of TAp63α by E3 ubiquitin ligase plays an important role in its stability. Ectopically expressed TAp63α is ubiquitinated and degraded in an Itch-dependent manner [26]. In contrast, it has been reported that βTrCP1 ubiquitinates and stabilizes TAp63α [27]. These findings suggest that TAp63α is maintained at a constant level in a ubiquitination-dependent
manner specific for cervical cancer cells.

Our results demonstrate that TRIM29 regulates the p63-mediated pathway and the behavior of cervical cancer cells. Further studies on the mechanism by which TRIM29 regulates the characteristics of cervical cancer cells may contribute to the establishment of new therapies for cervical cancers.
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Figure legends

**Fig. 1.** TRIM29 regulates cell adhesion and invasion. (A,B,C) Effect of TRIM29 knockdown on cell adhesion. HeLa S3 (A), HeLa (B) and SiHa (C) cells transfected with siCTRL or siTRIM29 were incubated on wells coated with fibronectin, fibrinogen, collagen I, collagen IV and laminin I for 1 h. Cells that had adhered to basement membrane proteins were stained and extracted to measure absorbance at 595 nm. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student’s *t* test (*, *P* < 0.05). Error bars show s.d. (D) Effect of TRIM29 knockdown on invasion to the basement membrane matrix. HeLa S3, HeLa and SiHa cells transfected with siCTRL or siTRIM29 were incubated on membranes coated with basement membrane matrix for 1 h. Invasive cells were stained and extracted to measure absorbance at 595 nm. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student’s *t* test (*, *P* < 0.05). Error bars show s.d. (E) Effect of TRIM29 depletion on cell-cell interactions. HeLa S3, HeLa and SiHa cells were transfected with siCTRL and siTRIM29 and then incubated for 48 h.

**Fig. 2.** TRIM29 regulates focal adhesion. (A) Effect of TRIM29 knockdown on focal adhesion. HeLa S3, HeLa and SiHa cells were transfected with siCTRL or siTRIM29 and incubated on fibronectin-coated coverslips in a serum-free medium. After 48 h, cells were fixed and stained with anti-actin and anti-ITGB1 antibodies. DNA was counterstained with DAPI. Scale bar, 40 μm. (B) Immunoblot analysis to confirm knockdown of TRIM29. HeLa S3, HeLa and SiHa cells were transfected with siCTRL.
or siTRIM29. After 48 h, whole cell lysates of TRIM29-depleted cells were subjected to SDS-PAGE and analyzed by immunoblotting. Actin was used as a loading control. (C) Quantification of focal cell adhesion area. Focal cell adhesion area was calculated as the ratio of ITGB1-stained area to DAPI-stained. ITGB1- and DAPI-stained areas were quantified using NIH ImageJ software (n = 20). P values were determined by Student’s t test (*, P < 0.05). Error bars show s.d.

**Fig. 3.** TRIM29 regulates the expression of TAp63α and EMT-related genes. HeLa S3 (A and D), HeLa (B and E) and SiHa (C and F) cells were transfected with siCTRL and siTRIM29 and then incubated for 48 h. The relative mRNA levels of TRIM29, p63 and the indicated genes were analyzed by quantitative RT-PCR. Standard deviations of each result were calculated from three independent experiments. P values were determined by Student’s t test (*, P < 0.05). Error bars show s.d.

**Fig. 4.** Effect of TRIM29 knockdown on expression of integrins, fibronectin and vimentin. Quantitative RT-PCR analysis was performed to measure mRNA levels of integrins, FN1 and VIM in TRIM29-knockdown HeLa S3 (A), HeLa (B) and SiHa (C) cells. Using the same procedure as that described in the legend of Fig. 3, mRNA levels of ITGB1, ITGB4, ITGA5, ITGA6, FN1 and VIM were analyzed by quantitative RT-PCR. Standard deviations of each result were calculated from three independent experiments. P values were determined by Student’s t test (*, P < 0.05). Error bars show s.d.

**Fig. 5.** TRIM29 suppresses ZEB1 expression through interaction with TAp63α. (A)
Effect of TRIM29 knockdown on protein levels of TRIM29, TAp63α, ZEB1, ITGB1 and ITGB4 in HeLa S3, HeLa and SiHa cells. HeLa S3, HeLa and SiHa cells were transfected with siCTRL and siTRIM29 and then incubated for 48 h. Cell lysates were subjected to SDS-PAGE. Protein levels of TRIM29, p63, ZEB1, ITGB1 and ITGB4 were analyzed by immunoblotting. Actin was used as a loading control. (B) Effect of TRIM29 knockdown on subcellular localization of p63. HeLa S3, HeLa and SiHa cells were transfected with siCTRL or siTRIM29 and incubated on coverslips. After 48 h, cells were fixed and stained with anti-actin and anti-p63 antibodies. DNA was counterstained with DAPI. Scale bar, 40 µm. (C) Western blot analysis of proteins associated with FLAG-tagged TRIM29. M2-agarose was incubated with nuclear extracts of HeLa S3 cells stably expressing FLAG-tagged TRIM29. The bound proteins were washed, eluted by 3×FLAG peptides, and then analyzed by Western blotting. Cells that were not transfected were used as control cells.

**Fig. 6.** Effect of overexpression of TRIM29 on p63 and integrin expression. (A-C) Quantitative PCR analysis of the expression levels of TRIM29 (A), p63 isoforms (B) and integrins (C) in HeLa S3 cells overexpressing TRIM29. *P* values were determined by Student’s *t* test (*, *P* < 0.05). Error bars show s.d. (D) Effect of TRIM29 overexpression on protein levels of p63, ITGB1 and ITGB4 in HeLa S3. Cell lysates from HeLa S3 cells expressing FLAG-tagged TRIM29 were subjected to SDS-PAGE. Protein levels of p63, ITGB1 and ITGB4 were analyzed by immunoblotting. (E) Effect of TRIM29 overexpression on cell invasion to the basement membrane matrix. HeLa S3 cells stably expressing FLAG-tagged TRIM29 were incubated on membranes coated with basement membrane matrix for 1 h. Invasive cells were stained and extracted to
measure absorbance at 595 nm. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student’s *t* test (*, *P* < 0.05). Error bars show s.d. (F) Effect of TRIM29 overexpression on cell adhesion to ECM proteins. HeLa S3 cells stably expressing FLAG-tagged TRIM29 were incubated in wells coated with fibronectin, fibrinogen, collagen I, collagen IV and laminin I for 1 h. Cells that had adhered to basement membrane proteins were stained and extracted to measure absorbance at 595 nm. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student’s *t* test (*, *P* < 0.05). Error bars show s.d. (G) Effect of TRIM29 overexpression on cell-cell interactions. Images of HeLa S3 cells expressing FLAG-tagged TRIM29 are shown.

**Fig. 7.** Functional relationship between TRIM29 and TAp63α. (A) Quantitative PCR analysis of the expression levels of TRIM29, TAp63, ZEB1 and ITGB1 in HeLa S3 cells overexpressing TAp63α. HeLa S3 cells stably expressing FLAG-tagged TAp63 were transfected with siCTRL and siTRIM29 and then incubated for 48 h. The relative mRNA levels of the indicated genes were analyzed by quantitative RT-PCR. Standard deviations of each result were calculated from three independent experiments. *P* values were determined by Student’s *t* test (*, *P* < 0.05). Error bars show s.d. (B) Effect of TRIM29 knockdown on cells overexpressing TAp63α. HeLa S3 cells expressing FLAG-tagged TAp63 were depleted of TRIM29 by siRNA transfection. Cell lysates from the cells were subjected to SDS-PAGE followed by Western blotting with the indicated antibodies. GAPDH was used as a loading control. (C) Effect of TAp63α overexpression on cell adhesion to ECM proteins. Cells were incubated in wells coated with fibronectin, fibrinogen, collagen I, collagen IV and laminin I for 1 h. The cells that
had adhered to basement membrane proteins were stained and extracted to measure absorbance at 595 nm. Standard deviations of each result were calculated from three independent experiments. $P$ values were determined by Student’s $t$ test (*, $P < 0.05$). Error bars show s.d. (D) Schematic representation of a putative role of TRIM29 in the p63-mediated pathway. TRIM29 binds to TAp63α in cervical cancer cells and regulates the expression of ZEB1 and integrins.
Figure 1
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ITGB1

Actin

DAPI

Merge

B

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C

![Graph](image19.png)

Figure 2
Figure 3

A

HeLa S3

Relative mRNA level

- siCTRL
- siTRIM29

B

HeLa

Relative mRNA level

- siCTRL
- siTRIM29

C

SiHa

Relative mRNA level

- siCTRL
- siTRIM29

D

HeLa S3

Relative mRNA level

- siCTRL
- siTRIM29

E

HeLa

Relative mRNA level

- siCTRL
- siTRIM29

F

SiHa

Relative mRNA level

- siCTRL
- siTRIM29

Figure 3
Figure 4

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Relative mRNA level
Figure 5

A

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TRIM29  TAp63α  ZEB1  ITGB1  ITGB4  GAPDH  Actin

B

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p63  Actin  DAPI  Merge

C

IP: FLAG  Input

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Figure 6
Figure 7