Ubiquitin-like protein FAT10 regulates DNA damage repair via modification of proliferating cell nuclear antigen

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Abstract. In response to DNA damage, proliferating cell nuclear antigen (PCNA) has an important role as a positive regulator and as a scaffold protein associated with DNA damage bypass and repair pathways by serving as a platform for the recruitment of associated components. As demonstrated in the present study, the ubiquitin-like modifier human leukocyte antigen F locus adjacent transcript 10 (FAT10), which binds to PCNA but has not previously been demonstrated to be associated with the DNA damage response (DDR), is induced by ultraviolet/ionizing radiation and VP-16 treatment in HeLa cells. Furthermore, DNA damage enhances FAT10 expression. Immunoprecipitation analysis suggested PCNA is modified by FAT10, and the degradation of FATylated PCNA located in the cytoplasm is regulated by the 26S proteasome, which is also responsible for the upregulation of nuclear foci formation. Furthermore, immunofluorescence experiment suggested FAT10 co-localizes with PCNA in nuclear foci, thus suggesting that FATylation of PCNA may affect DDR via the induction of PCNA degradation in the cytoplasm or nucleus. In addition, immunohistochemistry experiment suggested the expression levels of FAT10 and PCNA are enhanced in HCC tissues compared with healthy liver tissues; however, the expression of FAT10 is suppressed in regenerated liver tissues, which express high levels of PCNA, thus suggesting that the association between FAT10 and PCNA expression is only exhibited in tumor tissues. In conclusion, the results of the present study suggest that FAT10 may be involved in DDR and therefore the progression of tumorigenesis.

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Introduction

Human leukocyte antigen F locus adjacent transcript 10 (FAT10) is an 18-kDa ubiquitin-like modifier that functions as a proteasomal degradation signal (1,2). The FATylation mechanism involves a ubiquitin-activating enzyme, ubiquitin-like modifier activating enzyme 6 (3,4), and a ubiquitin-conjugating enzyme, unconventional soluble N-ethylmaleimide-sensitive factor activating protein receptor in the endoplasmic reticulum 1 (1), both of which resemble numerous other ubiquitin-like proteins that covalently modify their substrates. FAT10 has previously been suggested to function as a regulator of cell growth and survival, and alterations in FAT10 expression may induce abnormal cell growth, which is associated with neoplasm formation (5). Knockout of the FAT10 gene in mice has been revealed to prolong lifespan and reduce adiposity, thus suggesting that FAT10 has a role in the regulation of immune metabolism, which may affect progression of aging and chronic diseases (6,7). The majority of studies regarding FAT10 have investigated its overexpression in various cancer types, such as gastrointestinal cancer, hepatocellular carcinoma (HCC), pancreatic ductal adenocarcinoma and human glioma (5,8). Considering these studies, it can be suggested that FAT10 has an important role in cancer (9); however, the exact molecular mechanisms underlying the involvement of FAT10 in tumorigenesis have not yet been fully determined. Using proteomics, we recently identified 175 proteins as FATylated candidates in HeLa cells (10). As FAT10 highly expressed in HCC and cervical cancer, the present study aimed to investigate the association between FAT10 and liver or cervical cancer.

Proliferating cell nuclear antigen (PCNA), a substrate identified in our previous study (10), is not only associated with DNA replication (11), but also with other fundamental cellular processes, such as chromatin remodeling, DNA repair, sister chromatid cohesion and cell cycle control (12,13). Dysregulation of DNA damage repair and signaling at cell cycle checkpoints is referred to as the DNA damage response (DDR) (14). PCNA serves an important role by recruiting proteins involved in DNA replication and the DNA damage repair process (12,15). Following DNA damage, PCNA is modified in a post-transcriptional manner, such as being ubiquitinated or SUMOylated, in order to regulate DDR (16,17). A ubiquitin-like protein, interferon-stimulated protein 15 kDa, was recently demonstrated to modify PCNA

in a post-transcriptional manner in cells damaged by ultraviolet (UV) radiation (18). Numerous cancer-associated risk factors have been revealed to enhance the severity of DNA damage (19). Successful repair of the lesion and the reinitiation of replication, or alternatively, failure of the DNA repair machinery, may ultimately determine whether or not a patient will respond to anticancer therapy (19,20).

Ubiquitination has an important regulatory role in the DNA damage repair pathway (21-23). Ubiquitin-like proteins are involved in the regulation of numerous cellular processes, such as cell division, DNA repair, autophagy, signal transduction and embryonic development (17,18,24) The roles of ubiquitin and the ubiquitin-like modifiers (UBLs) in DDR has been widely studied over the last decade, however, the association of FAT10 with DDR has not yet been determined (8,16,17). The present study aimed to investigate whether FAT10 is involved in DDR via PCNA modification, and whether FAT10 is associated with tumorigenesis induced by DNA damage.

Materials and methods

Mouse and human tissues. All animal experiments were approved by the Animal Care and Use Committee of Beijing Institute of Transfusion Medicine (Beijing, China). In the present study, 9, male, 8-week-old C57 mice (20 g) with HCC (Bamai Biotechnology Co., Ltd, Chongqing, China) were used in specific-pathogen free level animal housing (20˚C, 50-60% relative humidity under a 12 h light/dark cycle; food and water was provided *ad libitum*), and the mice were then random divided into three groups (n=3): Normal healthy mice, HCC mice and partial hepatectomy mice. The partial hepatectomy was prepared as follows: Subsequent to anaesthetization using 70 mg/kg pentobarbital, a partial hepatectomy was performed only on mice within this group. Segments of the left, middle and right lobes of the liver were then removed, leaving only 30% of the liver remaining. The resulting wound was then stitched and following a further 48 h (the time determined for PCNA expression to reach the maximum level), the mice were then sacrificed and the remaining liver tissue was removed. Mice of the normal and HCC groups were sacrificed using 700 mg/kg sodium pentobarbital and liver tissue samples were collected and then embedded in paraffin, and sectioned to 4 μm. The experiment was performed in triplicate.

In addition, cancer tissue and healthy surrounding tissue were collected from 3 male patients (25 to 46-years-old) with T2N0M0 liver cancer and 3 female patients (48 to 72-years-old) with T1N0M0 cervical cancer from the 307th Hospital of Chinese People's Liberation Army. All samples were collected from July 2016 to August 2016, all experiments were approved by the Ethics Committee of the 307th Hospital of Chinese People's Liberation Army and all patients provided written informed consent.

Cell lines, immunoblotting and immunoprecipitation. HeLa cells (Stem Cell and Tissue Engineering Lab, Beijing Institute of Transfusion Medicine) were cultured 5% CO_2 conditions at 37˚C, using Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum. FAT10 was induced within HeLa cells (10^4) upon stimulation with 25, 50, 40 and 100 ng/ml interferon γ (IFN-γ) and tumor necrosis factor α (TNF-α) for 24 h, which were cultured at 5% CO_2 at 37˚C. FAT10 is rapidly degraded by the 26S proteasome in mammalian cells; the purpose of adding the 26s proteasome inhibitor MG132 is to inhibit the degradation of FAT10 (10). MG132 (100 ng/ml) was added for 6 h prior to sample collection, which were cultured at 5% CO_2 at 37˚C. UV/ionizing radiation (IR) and VP-16 can induce DNA damage via double-strand breaks. Following a 12/24 h treatment with UV/IR and VP-16 (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) on 10^4 cells, respectively, at 37˚C. Hela cells untreated at the same cell densities, incubation temperatures and durations served as blank controls. FAT10 conjugates were subjected to immunoprecipitation. HeLa cells were washed three times with PBS and lysed in lysis buffer (Beyotime Institute of Biotechnology, Haimen, China). The lysates were then centrifuged (587 x g, 5 min at 4˚C). Following a 4-h incubation at 4˚C with PCNA antibodies (1:2,000; cat. no. 2586, Cell Signaling Technology, Inc., Danvers, MA, USA), Protein A/G agarose was added to the lysates and the samples were then incubated for a further 4 h at 4˚C. Following collection and washing of the bound proteins, the samples were boiled for 10 min and separated using 10% SDS-PAGE followed by western blot analysis.

Immunohistochemistry analysis. Paraffin-embedded tissues were dewaxed, submerged in antigen retrieval solution, and then heated in a microwave on high power (500-1,000 W) for 10 min until the solution came to a steady boil. The sample was allowed to cool at room temperature for 20-30 min. Slides were then immersed in 0.3% H_2O_2 for 15 min, and one or two drops of blocking agent (1 drop of horse serum (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) in 5 ml PBS) were then applied to the tissue sections. The slides were incubated for 60 min at 27˚C. The slides were incubated with an avidin solution for 15 min, followed by a biotin solution for 15 min (Vector Laboratories, Inc., Burlingame, CA, USA; Avidin/Biotin Blocking kit; #SP-2001) at 27˚C. Following this, the slides were incubated with the anti-PCNA (as aforementioned) and anti-FAT10 (1:100; sc-67203, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) antibody in blocking agent (Vecta-kit; 1 drop of horse serum in 5 ml PBS) overnight at 4˚C. Slides were then incubated with the secondary antibody in blocking agent [1:400; AB-horse anti-rabbit/mouse biotinylated immunoglobulin G (IgG), P-2001, Vector Laboratories, Inc.)] for 60 min at 27˚C, and the reaction with NovaRed was visualized using a light microscope (magnification, x40). Finally, the slides were incubated with hematoxylin QS (Vector #H-3404) for 15-20 sec at 27˚C and then sealed with a cover glass via resin bonding.

Immunofluorescence analysis. HeLa cells grown on cover slips were washed with PBS, fixed via incubation with 4% paraformaldehyde in PBS for 10 min at 27˚C, permeabilized with 0.25% Triton X-100 in PBS for 10 min, and blocked with 10% goat serum (Gibco; Thermo Fisher Scientific, Inc.) for 1 h at 27˚C. Following this, cells were incubated with primary antibodies against FAT10 and PCNA (1:50) overnight at 4˚C, and then incubated further with rhodamine-conjugated goat antibodies against mouse IgG (1:50; cat. no. B-2763, Invitrogen; Thermo Fisher Scientific, Inc.) and rabbit IgG (1:50; cat. no. B-2770, Invitrogen; Thermo Fisher Scientific, Inc.) for

1 h at 37˚C. The cover slips were then counterstained with 4',6-diamidino-2-phenylindole (Invitrogen; Thermo Fisher Scientific, Inc.) and images were captured using a confocal laser scanning microscope (magnification, x60 and x120).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was harvested from HeLa cells using the RNeasy micro kit (Qiagen GmbH, Hilden, Germany) according to the manufacturer's protocols. RT-qPCR was performed using the SYBR green universal mix PCR reaction buffer (Toyobo Life Science, Osaka, Japan). The following primers were used for amplification: FAT10 forward, 5'-GATGAGGAGCTGCCCTTGTT-3' and reverse, 5'-GCCTCTTTGCCTCATCACCT-3'; and β-actin forward, 5'-AGTCATTCCAAATATGAGATGCGTT-3' and reverse, 5'-TGTGGACTTGGGAGAGGACT-3'. Fragments were amplified according to the thermocycling conditions: Pre-denaturation, 95˚C for 3 min; denaturation, 95˚C for 10 sec; annealing, 60˚C for 35 sec (reading board) x40 cycles; elongation, 65˚C temperature gradient to 95˚C (reading board; over). The $2^{-\Delta\Delta Cq}$ method was employed for the quantitative analysis of amplified samples (25).

Western blot analysis. Cells were collected and harvested in radioimmunoprecipitation buffer (1X PBS, 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate and 1 mM sodium orthovanadate) supplemented with a protease inhibitor cocktail (Complete EDTA-free; Roche Diagnostics GmbH, Mannheim, Germany) on ice. In order to perform western blot analysis, equal amounts of cell lysates (30 μg/lane) determined via a Bicinchoninic Acid protein assay, were separated on a 10-12% SDS-PAGE gel, electrotransferred to polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA) and blocked using 5% skimmed milk for 40 min at 27˚C. Primary antibodies against FAT10 (1:500), PCNA (1:4,000) and β-actin (1:2,000; cat. no. 60008-1-Ig, ProteinTech Group, Inc., Chicago, IL, USA) were incubated with the membranes overnight at 4˚C. Following this, membranes were incubated with the corresponding horseradish peroxidase-conjugated anti-rabbit (Wuhan Sanying Biotechnology, Inc.), anti-mouse (Wuhan Sanying Biotechnology, Inc.) or anti-goat IgG antibodies (Wuhan Sanying Biotechnology, Inc.) for 40 min at 37˚C. Signals were then detected using an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc.). β-actin was used as a loading control. ImageJ Software v1.48 (National Institutes of Health, Bethesda, MD, USA) were used for densitometry analysis.

Small interfering (siRNA) transfection. The following primers were used for siRNA-mediated FAT10 silencing: Sense, 5'-GAGACUAAGACGGGUAUAATT-3' and antisense, 5'-UUAUACCCGUCUUAGUCUCTT-3'. RNA duplexes were synthesized by Guangzhou RiboBio Co., Ltd. (Guangzhou, China). VP-16-treated HeLa cells were transfected with 2.5 nmol siRNAs using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 12 h until subsequent experimentation.

Statistical analysis. All experiments were performed in triplicate, and statistical analyses were performed using SPSS 15.0 statistical software (SPSS, Inc., Chicago, IL, USA). Data are expressed as the mean ± standard deviation and analyzed using one-way analysis of variance followed by the Newman Keul's method. $P<0.05$ was considered to indicate a statistically significant difference.

Results

FAT10 is specifically expressed in tumor tissues. The FAT10 protein has previously been revealed to be expressed in a number of breast and gastrointestinal tumor tissues (8). The expression levels of FAT10 and PCNA in cervical cancer tissue samples and surrounding tumor tissue samples obtained from human patients were investigated via IHC analysis using anti-FAT10 and anti-PCNA antibodies. In addition, the expression levels of FAT10 and PCNA in healthy liver tissues and HCC tissues from human patients and mice were investigated via IHC analysis using anti-FAT10 and anti-PCNA antibodies. FAT10 and PCNA were revealed to be expressed at significantly increased levels in tumor tissues compared with surrounding healthy tissues (Fig. 1). Furthermore, the expression of FAT10 expression in regenerated mouse liver tissues, which exhibit similarities to tumor tissues as they are rich in stem cells (26), was investigated, and it was revealed that PCNA was expressed at high levels, whereas FAT10 expression levels barely detectable in the regenerated tissues compared with the tumor tissues (Fig. 1B). Thus, FAT10 was revealed to be specifically expressed in tumor tissues.

DNA damage induces FAT10 expression. Long-term accumulation of DNA damage has previously been suggested to promote tumorigenesis and tumor development (19). Furthermore, FAT10 expression is induced by IFN-γ and TNF-α (27). The present study investigated whether UV radiation or IR enhanced FAT10 expression in HeLa cells. HeLa cells were treated with UV radiation and IR for 12 h, and the results demonstrated that the two treatments significantly enhanced FAT10 expression in HeLa cells compared with that in the non-treated HeLa cells (Fig. 2A and B, respectively). In addition, the expression of FAT10 was revealed to increase in a time-dependent manner with regards to UV radiation treatment, as determined by western blotting (Fig. 2C and D). Furthermore, HeLa cells were treated with VP-16 in order to further investigate the association between DDR and FAT10 expression. The results demonstrated that the expression levels of FAT10 mRNA and protein were increased following treatment with increasing doses of VP-16 (Fig. 2E and F). Considering these results, it can be suggested that FAT10 expression is enhanced by DDR.

DNA damage induces FATylation of PCNA. The involvement of FAT10 in DDR following treatment with VP-16 was investigated. As revealed by our previous liquid chromatography-tandem mass spectrometry (LC-MS/MS) studies, PCNA is conjugated to FAT10, and PCNA regulates DDR via SUMOylation or ISGylation (16,18), and thus, in the present study, the function of FAT10-PCNA conjugation in HeLa cells treated with UV/IR and VP-16 was investigated. Co-immunoprecipitation analyses regarding FAT10 and PCNA were performed, and the results revealed that there were covalent interactions between

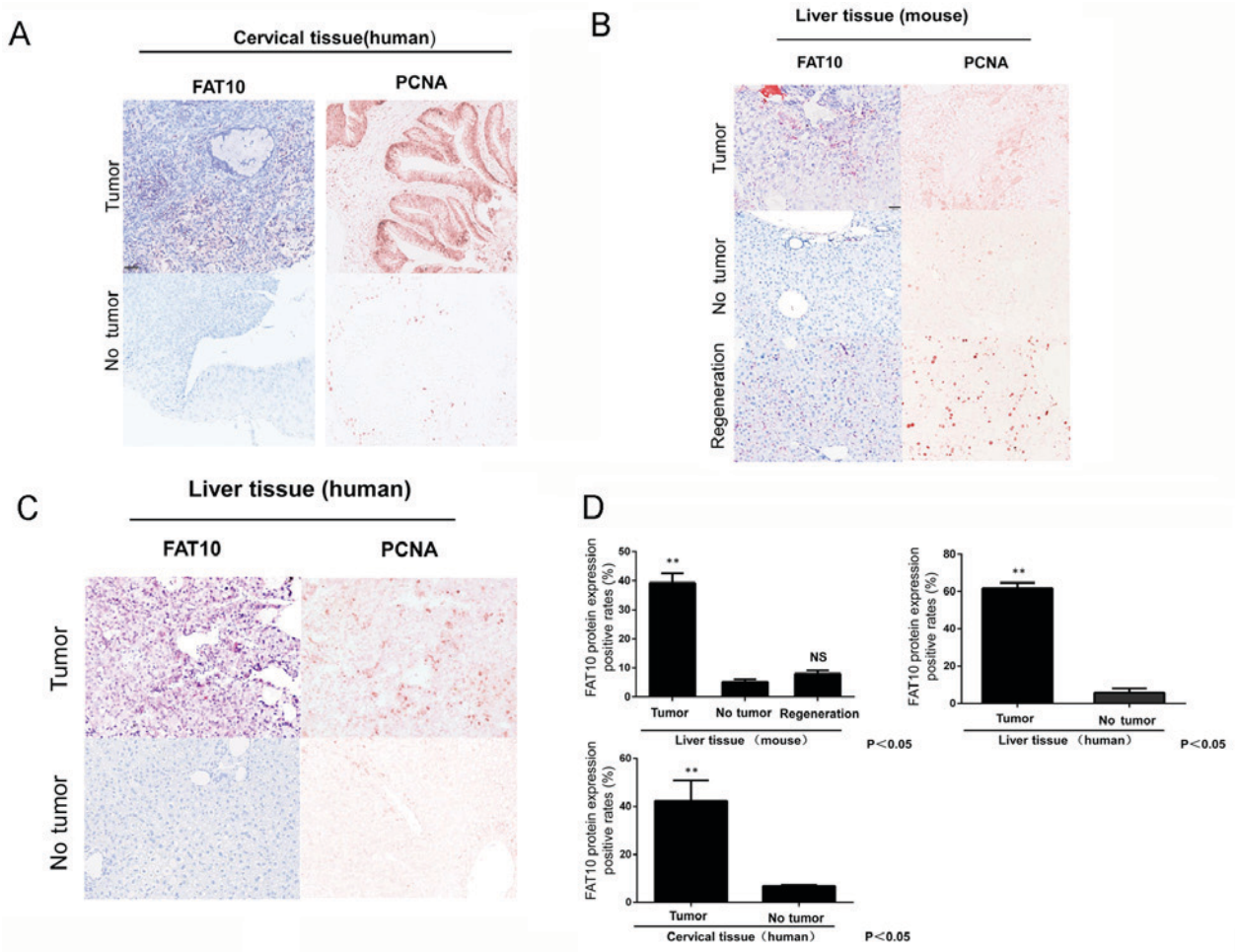

Figure 1. (A) Expression levels of FAT10 and PCNA in cervical cancer tissues and surrounding tumor tissues isolated from human patients were investigated via IHC using anti-FAT10 and anti-PCNA antibodies. Hematoxylin was used to dye the nucleus. (B and C) Expression levels of FAT10 and PCNA in mouse (C57) and human liver cancer tissues and surrounding tumor tissues were investigated via IHC using anti-FAT10 and anti-PCNA antibodies. Hematoxylin was used to stain nuclei. (D) The statistical analysis associated with IHC analyses were performed. Data are presented as the mean ± standard deviation (**P<0.01 vs. non-tumor). IHC, immunohistochemistry; FAT10, human leukocyte antigen F locus adjacent transcript 10; PCNA, proliferating cell nuclear antigen.

these proteins in HeLa cells treated with 10 and 20 J/m^2 UV irradiation (Fig. 3A and B). Furthermore, the extent of DNA damage was revealed to increase as the energy of the UV radiation increased. In addition, the extent of DNA damage was demonstrated to increase in HeLa cells following treatment with IR and VP-16 (Fig. 3C and D). Notably, PCNA expression increased following DNA damage. These results suggest that FAT10 is involved in DDR via FATylation of PCNA. Lysates of cells were analyzed using the same antibodies, as the positive control group in Co-Immunoprecipitation experiment (Fig. 3E and F).

PCNA is degraded by FAT10 via the 26S proteasome. The eventual fate of FATylated PCNA was investigated in order to determine the molecular mechanisms underlying the FATylation of PCNA. The results revealed that treatment with increasing cytokine concentrations (25, 50 and 100 ng/ml) suppressed the expression of PCNA in a dose-dependent manner (Fig. 4A). By contrast, the expression of PCNA was unchanged in cells treated with MG132 (100 ng/ml), the 26S proteasome inhibitor (Fig. 4A, lanes 6-8). In addition, it was revealed that treatment with MG132 inhibited the degradation of PCNA and FAT10 in VP-16-treated cells (Fig. 4B). As demonstrated by the results of the immunofluorescence staining analyses, PCNA expression levels were suppressed following induction of FAT10 expression by treatment with TNF-α/IFN-γ (40 ng/ml) or VP-16 (200 μM; Fig. 4D); however, in the presence of MG132 (100 ng/ml), the degradation of PCNA was markedly suppressed (Fig. 4D). Notably, in these analyses, FAT10 degradation was revealed to be sensitive to MG132 treatment (4,28). Thus, it can be suggested that FAT10 induces proteolysis-dependent PCNA degradation following PCNA modification by FAT10. In addition, a FAT10 siRNA was transfected into VP-16-treated cells in order to knock down endogenous FAT10, and the results revealed that PCNA degradation was subsequently inhibited (Fig. 4E and F), also observed with MG132 treatment. Immunoblotting experiments also proved the same results (Fig. 4G). Considering these results, it can be suggested that FAT10 regulates PCNA degradation via the 26S proteasome following DNA damage.

PCNA colocalizes with FAT10 in the cytosol. Considering that PCNA is predominantly localized in the nucleus, the subcellular location of the interaction between FAT10 and PCNA was investigated. Subcellular localizations of FAT10 and PCNA were determined via immunofluorescence staining,

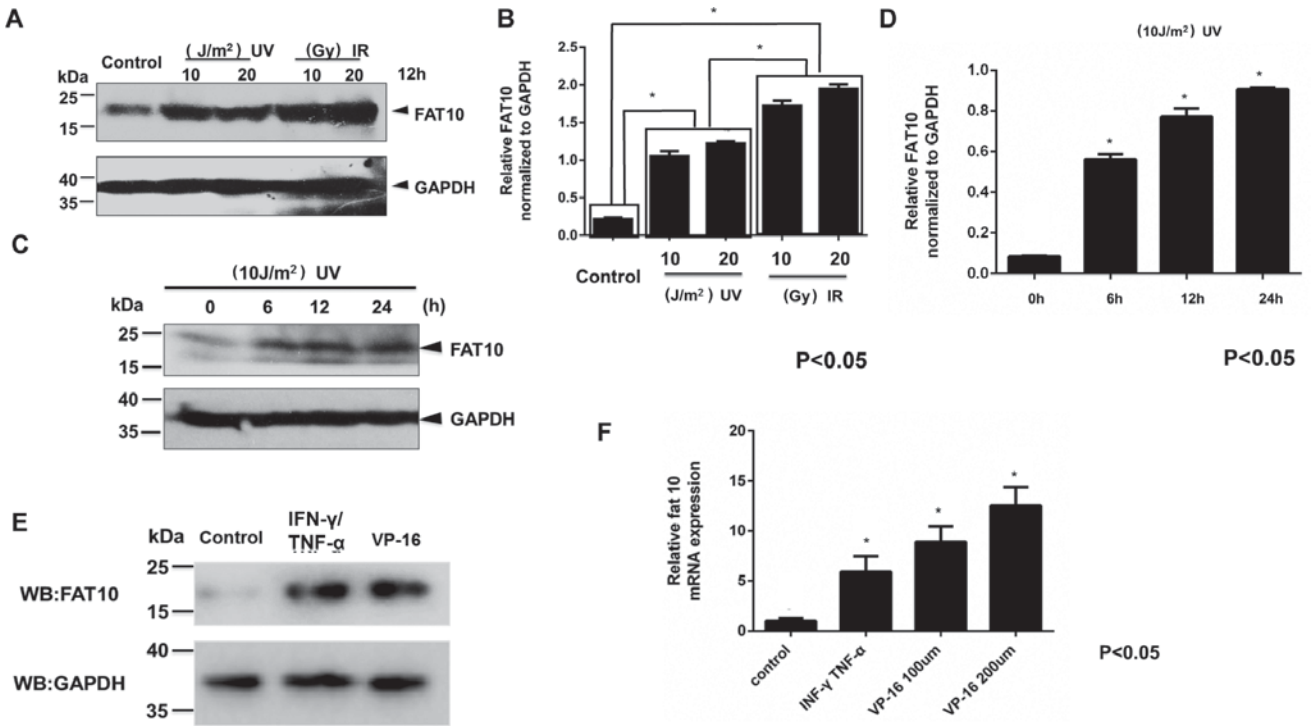

Figure 2. (A) HeLa cells were exposed to UV (10 and 20 J/m^2) and IR (10 and 20 Gy) and incubated for 12 h. (B) Statistical analysis of WB analyses presented in (A). (C) HeLa cells that had been exposed to UV (10 J/m^2) were incubated for differing time periods. Cells expressing the FAT10 protein were subjected to western blotting analysis using anti-FAT10 antibodies. (D) Statistical analysis of WB analyses presented in (C). (E) HeLa cells treated with VP-16 (200 μM) and INF-γ/TNF-α (50 ng/ml) were incubated for 24 h. Cell lysates were then subjected to western blot analyses using anti-FAT10 antibodies. (F) Total mRNA was prepared from the lysates of HeLa cells treated with VP-16 (100 and 200 μM) and subjected to reverse transcription-quantitative polymerase chain reaction analysis. Data are presented as the mean ± standard deviation (*P<0.05 vs. control or as indicated). UV, ultraviolet; IR, ionizing radiation; FAT10, human leukocyte antigen F locus adjacent transcript 10; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α; WB, western blotting.

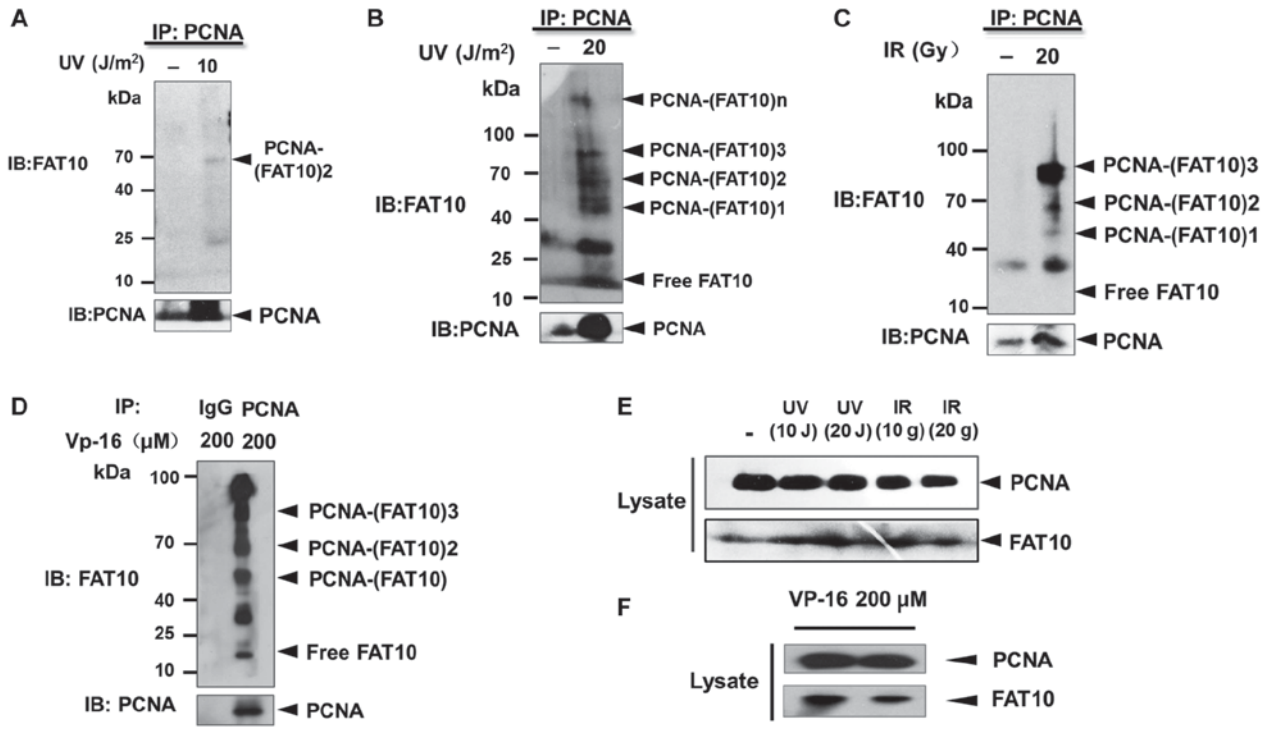

Figure 3. HeLa cells were exposed to (A) UV (10 J/m^2), (B) UV (20 J/m^2) or (C) IR (20 Gy), and cell lysates were then subjected to IP using an anti-FAT10 antibody, followed by western blot analysis using anti-FAT10 and anti-PCNA antibodies. Lysates were also analyzed using the same antibodies. (D) HeLa cells were treated with VP-16 (200 μM) for 24 h. In addition, lysates were subjected to immunoprecipitation using an anti-PCNA antibody followed by western blot analysis using anti-FAT10 and anti-PCNA antibodies. (E and F) Lysates of cells were analyzed using the same antibodies. UV, ultraviolet; IR, ionizing radiation; FAT10, human leukocyte antigen F locus adjacent transcript 10; PCNA, proliferating cell nuclear antigen; IP, immunoprecipitation; IgG, immunoglobulin G; IB, immunoblotting.

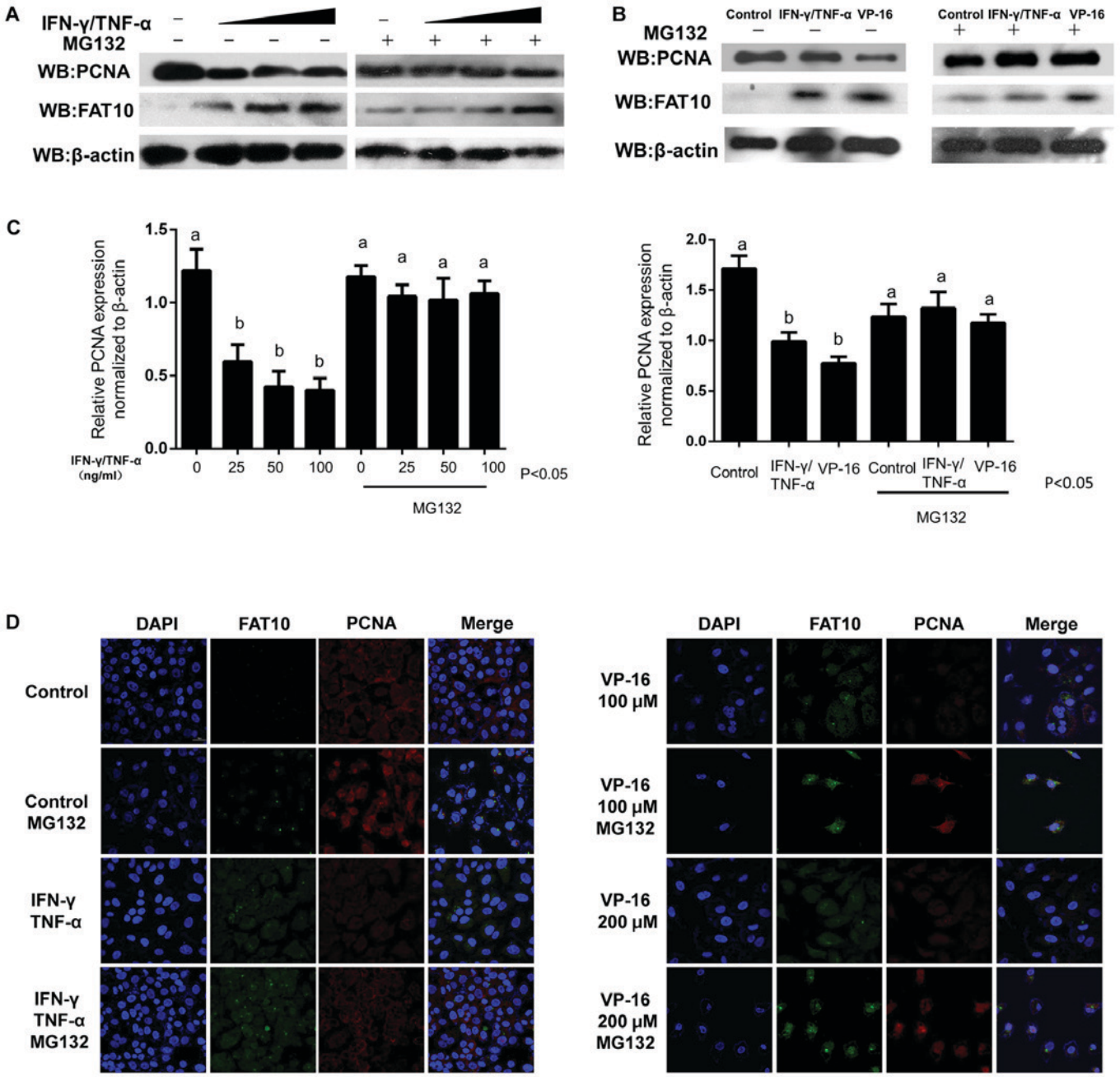

Figure 4. (A) HeLa cells (lanes 2-4) were treated with TNF-α and IFN-γ (25, 50 and 100 ng/ml) for 24 h in order to induce FAT10 expression. Cell lysates were subjected to WB analysis using anti-FAT10 and anti-PCNA antibodies. HeLa cells (lanes 6-8) were treated with TNF-α and IFN-γ as aforementioned, and MG132 (100 ng/ml) was then added for 6 h prior to sample collection. (B) HeLa cells were treated with equal volumes of TNF-α and IFN-γ (40 ng/ml) or with VP-16 (200 μM) for 24 h. Cell lysates were subjected to western blot analysis using anti-FAT10 antibodies and anti-PCNA antibodies. MG132 (100 ng/ml) was added for 6 h prior to sample collection. (C) Statistical analysis of WB analyses presented in (A and B). (D) Cells were fixed 24 h post-stimulation with equal volumes of TNF-α and IFN-γ (40 ng/ml), immunostained with anti-FAT10 and anti-PCNA, and then observed using a confocal microscope. Cells were fixed 24 h after treatment with VP-16 (100 and 200 μM), treated with MG132 (100 ng/ml) for 6 h, and then immunostained using anti-FAT10 and anti-PCNA antibodies and then observed using a confocal microscope (x60). Data are presented as the mean ± standard deviation. Groups with different small letter superscripts represent a significant difference (P<0.05), whereas groups with the same letter superscripts do not represent a significant difference (P>0.05). FAT10, human leukocyte antigen F locus adjacent transcript 10; PCNA, proliferating cell nuclear antigen; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α; WB, western blotting.

using anti-FAT10 and anti-PCNA antibodies and confocal microscopy. As revealed by Fig. 5A, a marked quantity of FAT10 colocalized with PCNA in the nucleus of VP-16-treated cells; however, in the presence of MG132, FAT10 and PCNA colocalized in both the nucleus and cytosol. Notably, PCNA was demonstrated to be predominantly located in the nucleus compared with the cytosol; however, the levels of PCNA in the cytosol were enhanced in cells following treatment with MG132. In addition, the cytoplasm and nuclei from cells were isolated and the expression levels of PCNA and FAT10 were determined by western blotting. The results of western blot analyses revealed that the majority of PCNA accumulated in the nucleus compared with the cytosol in the presence and absence of MG132. In addition, PCNA degradation was

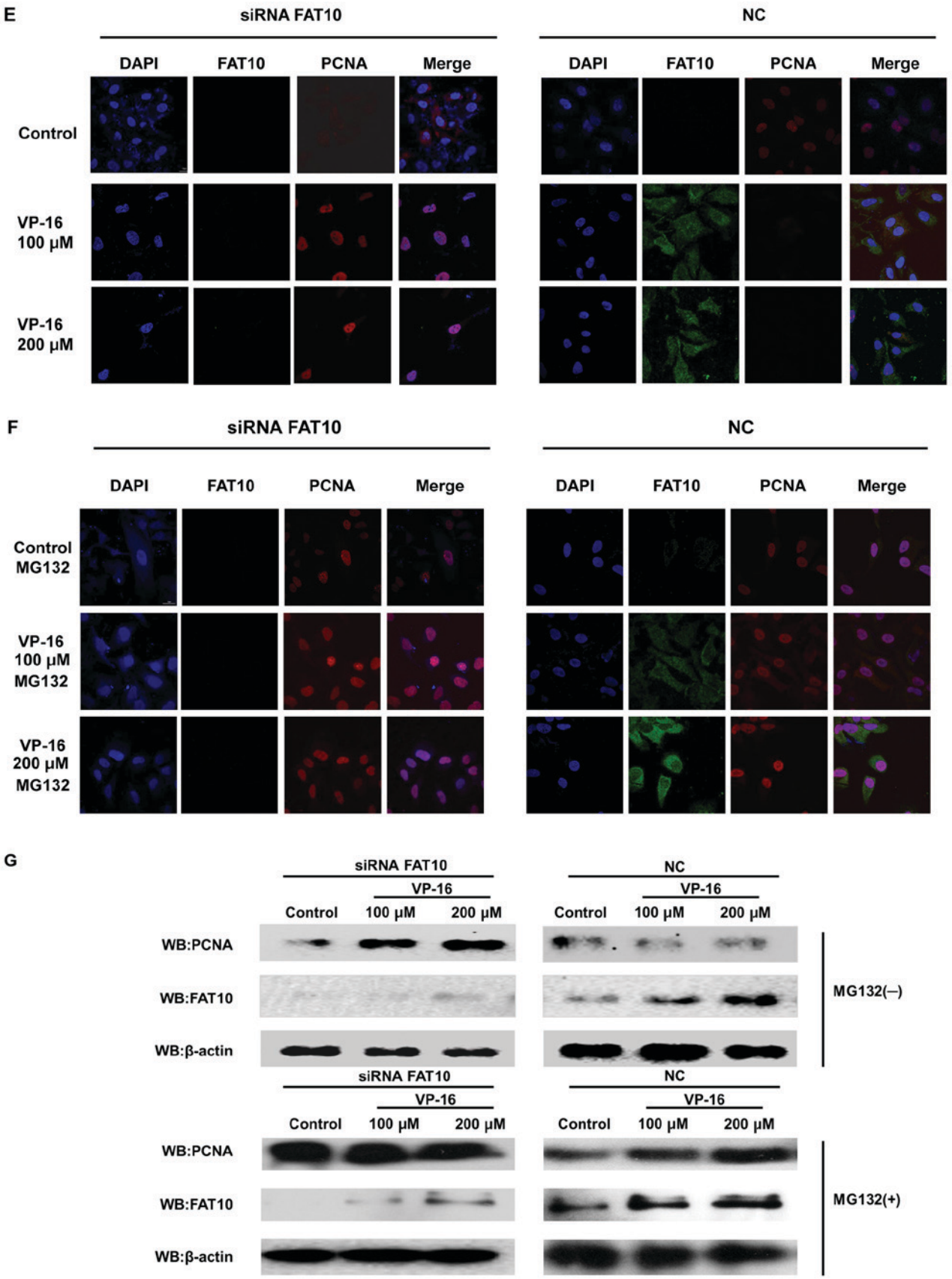

Figure 4 continued. (E and F) HeLa cells were treated with VP-16 (100 and 200 μM) and incubated for 24 h. Following this, a FAT10 siRNA and a universal siRNA were transfected into the treated cells. MG132 (100 ng/ml) was then added 6 h prior to sample collection. (G) HeLa cells were treated with VP-16 (100 and 200 μM) and incubated for 24 h. Following this, FAT10 siRNA and universal siRNA were transfected into the treated cells. MG132 (100 ng/ml) was then added 6 h prior to sample collection. Cell lysates were subjected to WB analysis using anti-FAT10 and anti-PCNA antibodies. Data are presented as the mean ± standard deviation. Groups with different small letter superscripts represent a significant difference ($P<0.05$), whereas groups with the same letter superscripts do not represent a significant difference ($P>0.05$). FAT10, human leukocyte antigen F locus adjacent transcript 10; PCNA, proliferating cell nuclear antigen; siRNA, small interfering RNA; NC, negative control; WB, western blotting.

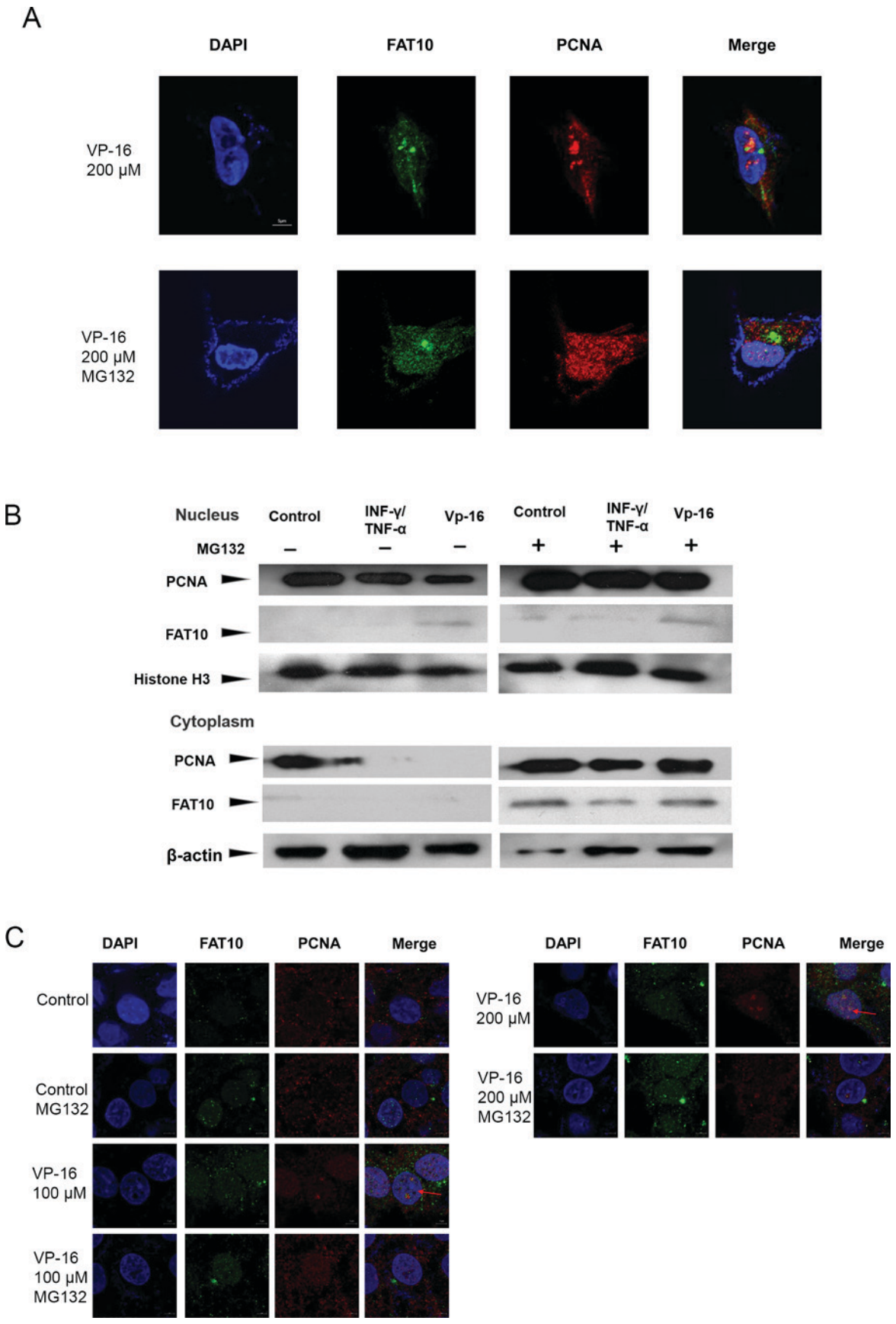

Figure 5. (A) Cells were fixed 24 h post-treatment with VP-16 (200 μM), immunostained with anti-FAT10 and anti-PCNA antibodies, and observed using a confocal microscope (x120). MG132 (100 ng/ml) was added 6 h prior to sample collection. (B) Cells were fixed 24 h post-treatment with VP-16 (200 μM) or with equal volumes of TNF-α and IFN-γ (200 ng/ml) for 24 h. Following this, the nuclei and cytoplasm were separated from the cell lysate. These nuclear and cytoplasmic lysates were then subjected to western blot analysis with anti-FAT10, anti-β-Actin, anti-HistoneH3 and anti-PCNA antibodies. MG132 (100 ng/ml) was then added to the cells 6 h prior to sample collection. (C) Cells were fixed 24 h post-treatment with VP-16 (100 and 200 μM) and immunostained with anti-FAT10 and anti-PCNA antibodies and then observed using a confocal microscope (x120). FAT10, human leukocyte antigen F locus adjacent transcript 10; PCNA, proliferating cell nuclear antigen; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α; DAPI, 4',6-diamidino-2-phenylindole.

revealed to be sensitive to MG132 (Fig. 5B). By contrast, FAT10 was detected in both the nuclear and cytoplasmic fractions and was sensitive to MG132. Considering these results, it can be suggested that FAT10 primarily regulates PCNA degradation via the 26S proteasome in the cytoplasm of VP-16 treated cells.

PCNA FATylation upregulates nuclear foci formation. Nuclear foci of treated cells were investigated using laser confocal microscopy in order to determine whether PCNA degradation affects DNA damage-induced formation of nuclear foci at sites of DNA damage. FAT10 and PCNA were revealed to be colocalized at the damage site, and the number of nuclear foci was increased in VP-16-treated cells. Furthermore, PCNA expression in the cytoplasm was simultaneously suppressed. In addition, the number of nuclear foci in cells was demonstrated to decrease following treatment with MG132, and PCNA expression was demonstrated to return to normal levels. FAT10 and PCNA were colocalized at the damage site (Fig. 5C). Therefore, the results suggest that PCNA FATylation increases the formation of nuclear foci. The role of PCNA FATylation in DDR may not be limited to PCNA degradation in the cytoplasm, but may also have a nuclear role.

Discussion

FAT10 is overexpressed in numerous types of malignancies, including HCC, colorectal tumors, gastric tumors and gynecological tumors (8,9,29). Previous studies have suggested that FAT10 has a pathological role in cancer via involvement in mechanisms other than its proteasome targeting function (29). However, the role of FAT10 in tumorigenesis remains undetermined. Failure to repair DNA damage has been well established to induce tumorigenesis. To the best of our knowledge, the present study is the first to demonstrate that FATylation is significantly associated with DDR and thus has an important role in the process of tumorigenesis.

The process of DDR is strongly regulated by reversible protein post-translational modifications (PTMs), including phosphorylation, poly (ADP-ribosylation, ubiquitylation, SUMOylation, methylation and acetylation (16). These PTMs regulate protein stability, localization and activity, which represent a number of physiological processes that rely on functional DNA repair pathways (16). In recent studies, SUMOylation, NEDDylation and ISGylation, representing various modifications of PCNA, were demonstrated to have important roles in DDR (16,17). PCNA has an important role as a positive regulator and a scaffold protein responsible for the recruitment of replication machinery. In addition, PCNA has an important role in DNA damage bypass and repair by serving as a platform for the recruitment of essential components associated with DDR (11,18). As revealed in our previous LC-MS/MS study, PCNA is a substrate of FAT10 (10). In the present study, HeLa cells were treated with UV/IR and VP-16, and it was demonstrated that FAT10 expression was enhanced following DNA damage. In addition, FAT10 and PCNA were revealed to be covalently linked in HeLa cells following UV radiation treatment. As the severity of DNA damage increased, the quantity of PCNA modified by FAT10 increased. In addition to ubiquitin, FAT10 is the only member of the ubiquitin-like family known to enhance proteasomal degradation (30). In the present study, FAT10 was demonstrated to regulate PCNA degradation via the 26S proteasome in response to DNA damage. Furthermore, it was revealed that following DNA damage, FAT10 regulated PCNA degradation via the 26S proteasome in the cytoplasm, thus suggesting that FAT10 interacts with PCNA following the post-translational modification of PCNA. In addition, the formation of nuclear foci was markedly upregulated following PCNA FATylation. Therefore, the results of the present study suggest that FAT10 is involved in DDR and affects foci formation via modification of PCNA.

Considering that the failure of the DNA repair process may result in tumor formation (19), it can therefore be suggested that PCNA is not associated with DNA repair, as degradation by FATylation induces tumorigenesis. In addition, at the organismal level, the expression of FAT10 and PCNA was enhanced in HCC tissues compared with healthy liver tissues (Fig. 1B and C). However, FAT10 was demonstrated as being expressed at low levels in regenerated tissues, which PCNA is also highly expressed in. Thus, FAT10 was revealed to be specifically expressed in tumor tissues and to affect DDR via regulation of PCNA degradation. FAT10 was also demonstrated to colocalize with PCNA in nuclear foci, therefore suggesting that PCNA FATylation may affect the DDR via induction of PCNA degradation in the cytoplasm or nucleus.

In the present study, modification of PCNA by FAT10 following DNA damage was investigated, and the results suggest that the modification of PCNA by FAT10 regulates important signaling pathways involved in DDR. Future studies should investigate whether other proteins are also modified by FAT10, and whether such modifications are associated with DNA damage repair.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LL designed experiments. ZC, LL and WZ performed experiments and analyzed data. ZC and LL prepared the figures. SJ, FG and YW contributed intellectual expertise and provided relevant innovations for experiments. ZY and XZ helped establish the mouse liver regeneration model.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of the 307th Hospital of Chinese People's Liberation Army and all patients provided written informed consent.

Consent to publication

Written informed consent was obtained from all patients.

Competing interests

The authors declare that they have no competing interests.

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