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14-3-3ζ Reduces DNA Damage by Interacting With and Stabilizing Proliferating Cell Nuclear Antigen

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ABSTRACT

Proliferating cell nuclear antigen (PCNA) is a processivity factor of DNA replication which plays critical roles in the regulation of DNA replication and repair. In this study, we show that PCNA interacts directly in vitro and in cells with 14-3-3ζ, an adaptor protein that regulates cell growth and response to DNA damage in eukaryotes. The interaction is mediated by at least two PCNA-binding sites on 14-3-3ζ, one of which is a novel non-canonical PIP (PCNA interacting protein) box. We find that DNA damages induced by UVC irradiation and MMS (methyl methanesulfonate) can enhance both the interaction of these two proteins and their co-localization with chromatin. Functional analyses suggest that 14-3-3ζ stabilizes PCNA possibly by regulating its ubiquitination, which impacts on DNA damage repair and cell viability. J. Cell. Biochem. 116: 158–169, 2015.

KEY WORDS: 14-3-3ζ; PCNA; PROTEIN-PROTEIN INTERACTION; STABILITY; DNA DAMAGE

Proliferating cell nuclear antigen (PCNA) is a so-called DNA sliding clamp protein which plays a critical role in the replication of the genome. During DNA replication, by forming a ring-shaped homotrimer that encircles, and slides along the double-stranded DNA, PCNA serves as a molecular platform that coordinates the complex interacting network around the DNA polymerase at the replication fork and enhances the processivity of the latter by tethering it to DNA [Maga and Hubscher, 2003; Qi and Martinez, 2003; Essers et al., 2005; Lo et al., 2012]. During DNA replication, the advance of the replication fork is often blocked by the lesions in DNA templates. In order to overcome this obstacle and avoid the replication fork collapse, the DNA replication should switch to the translesion synthesis (TLS) mode in which the highly faithful and rapid replicative polymerases are replaced by the polymerases with lower fidelity and processivity (the so-called TLS DNA polymerases), so that the damages in the DNA templates can be bypassed and subsequently repaired [Lehmann, 2005; Watson et al., 2006; Jansen et al., 2007]. This switch is controlled by the ubiquitination of PCNA. Upon DNA damage, the Lys164 of PCNA is mono-ubiquitinated by the Rad6-Rad18 complex, conferring a higher affinity for the TLS polymerases. As a consequence, the replicative DNA polymerases associated with PCNA are replaced by the translesion DNA polymerases, leading to the translesion bypass of the DNA damages [Prakash et al., 2005; Washington et al., 2009]. The mono-ubiquitinated PCNA could be further poly-ubiquitinated, which is associated to the error-free DNA repair by template switching [Zhang et al., 2011]. Interestingly, besides its role in the control of the switch between different modes of DNA replication and repair, recent studies suggested that the ubiquitination of PCNA might also be involved in its proteasomal degradation and thereby plays a role in the stability of this protein. Lo et al. reported that in human cells, polyubiquitination on Lys164 mediated by the E3 ligase Cullin 4A could trigger the proteasomal degradation of PCNA [Lo et al., 2012]. Thus, mono- and poly-ubiquitination mediated by

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various E3 ligases may be important for either the regulation of the function or the stability of PCNA.

The 14-3-3 proteins are a family of ubiquitously expressed 28–33 kDa acidic polypeptides. Seven 14-3-3 isoforms (α/β, γ, τ/θ, ε, η, σ, ζ/δ) have been found in mammals [Dougherty and Morrison, 2004; Aitken, 2006]. 14-3-3 proteins have no detectable catalytic domain or activity [Moreira et al., 2008], but have been reported to interact with over 300 partner proteins [Mackintosh, 2004]. By interacting with its partners, 14-3-3 proteins could affect various physical and functional aspects of these proteins, such as the binding ability to other proteins, the subcellular distribution, the stability, the catalytic activity, or it may also serve as an adaptor/scaffold protein [Tzivion and Avruch, 2002; Paul et al., 2012]. 14-3-3 proteins have been involved in a myriad of cellular processes, including cell cycle control, growth, differentiation, apoptosis, mitogenic or stress signaling, and response to DNA damage [Dougherty and Morrison, 2004; Jin et al., 2004; Meek et al., 2004; Tzivion et al., 2006; Moreira et al., 2008]. Although discovered since more than 40 years, 14-3-3 proteins continue to attract the attention of the scientists in the world by its multiple functions and complex mechanisms of action.

The ζ isoform of 14-3-3 is a potential prognostic and therapeutic target protein. It is overexpressed in various human cancers, including lung cancers [Shoji et al., 1994; Qi et al., 2005], oral squamous cell carcinomas [Arora et al., 2005], stomach cancers [Jang et al., 2004], breast cancer [Zang et al., 2004; Somiari et al., 2005] and papillomavirus induced carcinomas [Huber et al., 2004]. 14-3-3ζ overexpression occurred in 42% of breast cancers [Neal et al., 2009]. The elevated expression of 14-3-3ζ was correlated with the progression and grade of non-small cell lung cancers (NSCLC), and poor survival rates of the patients [Fan et al., 2007]. Knockdown of 14-3-3ζ by siRNA or antisense RNA sensitizes the cancer cells to cisplatin-, stress-, and ionizing radiation-induced cell death, which has been associated with the inhibition of cell proliferation, G2-M cell cycle arrest, and the increased apoptosis [Qi and Martinez, 2003; Fan et al., 2007; Neal et al., 2009].

In the present study, we demonstrate that 14-3-3ζ interacts with PCNA in various cells, and that DNA damages induced by UVC irradiation and MMS enhance the interaction and co-localization of these two proteins. Functional studies by PCNA overexpression and 14-3-3ζ knockdown suggests that 14-3-3ζ stabilizes PCNA and facilitates DNA repair.

MATERIALS AND METHODS

CELL CULTURE AND TRANSFECTION

HeLa and HEK 293T cells (Cell Resource Center, Institute of life science Chinese Academy of Sciences, Shanghai, China) were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand island, NY) supplemented with 10% fetal bovine serum (PAA Laboratories, Linz, Austria), 1% penicillin/streptomycin (Genom, China) at 37°C in a humidified atmosphere containing 5% CO_2. U266 cells (Cell Resource Center, Institute of life science Chinese Academy of Sciences, Shanghai, China) were maintained in 1640 medium (Gibco). Flag-PCNA (kindly provided by Prof. McGowan of The Scripps Research Institute in La Jolla) and HA-14-3-3ζ (kindly provided by Prof. Feng-Qian Li of State University of New York at Stony Brook) plasmids were transfected into cells using Lipofectamin 2000 according to the manufacturer (Invitrogen, Carlsbad, CA).

SHORT HAIRPIN RNAs (shRNAs)

Recombinant lentivirus carrying 14-3-3ζ-knockdown shRNA (ACGGTTCACATTCCATTAT) [Li et al., 2008] was produced with LV-3 (pGLVH1/GFP + Puro) vector (GenePharma, Shanghai, China). To prepare 14-3-3ζ-knockdown stable cell lines, infected 293T and U266 cells were selected in the presence of puromycin (3 μg/ml) for one month. Almost 100% of HEK293T and U266 cells carrying green fluorescent protein (GFP) were observed by fluorescence microscopy (Olympus, Japan).

CO-IMMUNOPRECIPITATION ASSAY

HeLa cells transfected with Flag-PCNA and HA-14-3-3ζ, or with Flag-PCNA and HA-vector were lysed in lysis buffer A (50 mM Tris at pH 7.5, 150 mM NaCl, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, 5 mM NaF, 2 mM Na_3VO_4, 1 mM PMSF, 1% protease inhibitor cocktail (Roche)) for 30 min on ice. The co-immunoprecipitation assay were performed using the protocols described previously [Gao et al., 2013] with the mouse monoclonal HA antibody (Sigma, St. Louis, MO). The immune complexes from the co-immunoprecipitation assay were then analyzed by Western blotting using specific antibodies against Flag (Sigma) and HA.

To detect the ubiquitination of Flag-PCNA, 14-3-3ζ-silenced 293T cells or control cells were co-transfected with Flag-PCNA and His-Ub plasmids and treated with MG132 (20 mM) for 12 h. Then, the cells were harvested and prepared for immunoprecipitation experiment using the mouse monoclonal Flag antibody (Sigma). The immune complexes were analyzed by Western blotting using specific antibodies against Flag and Ub (Sigma).

CONSTRUCTION, EXPRESSION AND PURIFICATION OF FUSION PROTEIN

pGEX-14-3-3ζ and pGEX were kindly provided by Prof. Piwnica-Worms of Harvard University. GST and GST-14-3-3ζ fusion proteins were expressed in *E. coli BL21* after induction with 0.2 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Promega) for 4 h at 37°C. Bacteria were resuspended in lysis buffer B (50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1mM EDTA, 1 mM PMSF) containing 100 μg/ml lysozyme. Bacterial extracts were sonicated for 20 min and centrifuged at 12,000 rpm for 30 min at 4°C to remove cell debris. GST and GST-14-3-3ζ fusion proteins were purified from bacterial lysates by affinity chromatography using Glutathione Sepharose 4B (GE Healthcare). The purified protein concentrations were determined using a BCA assay (Beyotime Biotechnology, China). Then, equal amount of GST and GST-14-3-3ζ fusion proteins was employed for the following GST pull-down assay.

Various truncated 14-3-3ζ cDNA were amplified by PCR from pGEX-14-3-3ζ using the appropriate primers flanked by appropriate restriction sites and ligated in-frame into the pGEX vector. GST-14-3-3ζ EF 3A mutant was generated using a pair of mutant primers: 5′-ATGGAATTCAATGCTTCAGCCGCAGAGAGCAAAGTCGCCGCTTT-GAAA-3′ and 5′-ATGGTCGACTTATGGTTGCATTTCCTTTTTGCTG-3′.

All truncated recombinant GST-14-3-3ζ peptides were then produced and purified as described above.

The full length PCNA was amplified by PCR from Flag-PCNA plasmid and inserted into pET-20b(+) vector (kindly provided by Prof. Xuesong Sun of Jinan University, China) with Bam HI and Xho I to produce His-PCNA recombinant expression vector. The yielded recombinant vectors were then verified by DNA sequencing. His-PCNA fusion protein was expressed in *Rosetta* cells after induction with 0.2 mM IPTG for 4 h at 37°C. After the lysis of cells as described above, His-PCNA fusion protein was purified by affinity chromatography using Ni-NTA Superflow (Qiangen). The concentration of purified protein was determined using a BCA assay.

GST PULL-DOWN ASSAY

To prepare the whole HeLa cell lysates for GST-14-3-3ζ pull-down assay, normal cells, UVC-treated (1 min, 25 J/m^2) or MMS-treated (methyl methanesulfonate, 1 μM, Sigma) cells were harvested and resuspended in ice-cold lysis buffer A for 30 min on ice. Then, the GST pull-down assays were performed as described previously [Gao et al., 2013]. Briefly, whole cell extracts (0.5 mg of proteins) were incubated overnight with glutathione sepharose beads coated with GST or with full-length or truncated GST-14-3-3ζ (50 μg of recombinant protein) at 4°C on a rocker. The specific antibodies against PCNA (Santa Cruz, CA, PC10: sc-56), β-actin (Protein Tech Group), GST (Santa Cruz) were used for detections. The levels of various recombinant GST proteins were analyzed by GST antibody or CBB (Coomassie Brilliant Blue) staining of SDS–PAGE gels.

For in vitro GST pull-down assay using two recombinant proteins, equal amount of GST or GST-14-3-3ζ (20 μg) was mixed with 20 μg His-PCNA protein in lysis buffer A for 4 h at 4°C on a rocker. After brief centrifugation, the pellets of GST or GST-14-3-3ζ beads were washed with lysis buffer, resuspended in SDS–PAGE sample buffer, and subjected to Western blot analysis. 5% of the input His-PCNA protein was loaded on the gel. The anti-His monoclonal antibody (ZSGB–BIO, China) was used to analyze and the CBB staining of SDS–PAGE gels was used to evaluate the amounts of recombinant proteins used in the assay.

HIS-TAG PULL-DOWN ASSAY

For semi-in cellulo His-tag pull-down assay, HEK 293T cells were harvested and lysed in ice-cold lysis buffer A for 30 min on ice. The cell suspension was centrifuged at 13,200 rpm for 30 min at 4°C, and precleared by incubation with 50% Ni-NTA Superflow (20 μl) for 20 min twice at 4°C. The precleared supernatants (200 μg of proteins) were then incubated with Ni-NTA Superflow beads coated or not with His-PCNA (25 μg of recombinant protein) respectively for 2 h at 4°C on a rocker. After brief centrifugation, the pellet of beads was washed four times with lysis buffer B. The fractions bound to Ni-NTA Superflow or His-PCNA were analyzed by Western blotting using specific antibodies against 14-3-3ζ. The amounts of recombinant His-PCNA were estimated by CBB staining of SDS–PAGE gels.

For in vitro His-tag pull-down assay using two recombinant proteins of GST-14-3-3ζ and His-PCNA, Ni-NTA Superflow or Ni-NTA Superflow coated with His-PCNA (50 μg) was mixed with 80 μg GST-14-3-3ζ protein in lysis buffer A for 2 h at 4°C on a rocker. After centrifugation and washing, the fractions bound to Ni-NTA Superflow or His-PCNA were subjected to Western blot analysis using specific antibody against GST (Santa Cruz). 10% of the input GST-14-3-3ζ protein was loaded on the gel. Similarly, the CBB staining of SDS–PAGE gels was used to evaluate the amounts of His-PCNA.

WESTERN BLOT ANALYSIS

Whole cell extracts were prepared with lysis buffer A for 30 min on ice. Same amounts of the extracts were separated by 10% SDS/PAGE, electrophoresed and transferred onto a PVDF membrane. Blots were probed with the corresponding antibodies at 4°C overnight with gentle agitation. The visualization of all immunoblots was detected using horseradish peroxidase-conjugated secondary antibodies (ProteinTech Group) and enhanced chemiluminescence (ECL kit, Beyotime Biotechnology, China). The quantization of the Western blot results were analyzed by ImageJ software.

CONFOCAL MICROSCOPY ASSAY

Twenty-four hours after transfection with Flag-PCNA expression construct, UVC-treated, MMS-treated cells, or untreated control cells, were washed twice with PBS and extracted with 0.4% NP-40 in PBS for 40 min, fixed with 4% paraformaldehyde for 60 min at 4°C, permeabilized with 0.2% Triton X-100 for 10 min, blocked with 10% goat serum in phosphate-buffered saline (PBS) and then immunostained with primary antibodies (Flag antibody (1:100) in PBS with 2% goat serum; 14-3-3ζ (1:50)) overnight at 4°C. After washing in precooled PBS, the Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibody (ZSGB–BIO, China) were then added to the culture. The cells were incubated for another 1 h at room temperature. DAPI staining was used to determine the morphology of cell nuclei.

For the detection of phospho-Ser139 H2AX (γ-H2AX), the stable 14-3-3ζ-silenced 293T cells or control cells were cultured on coverslips and transfected with the indicated plasmids. After 6 h of UVC (1 min, 25 J/m^2) treatment, the cells were washed twice in ice-cold PBS, fixed with 4% paraformaldehyde for 10 min at 4°C, permeabilized with Triton buffer (20 mM Hepes, pH 7.4, 300 mM sucrose, 50 mM NaCl, 3 mM MgCl2, and 0.5% Triton X-100) for 10 min on ice, blocked with 10% goat serum in PBS and then immunostained with rabbit monoclonal phospho-histone H2AX (Ser139) antibody (9718, Cell Signaling Technology, Inc.) in PBS with 2% goat serum overnight at 4°C. The secondary antibody conjugated with Alexa Fluor 594 (ZSGB–BIO, China) was then added to the culture. The imaging experiments were performed on laser scanning confocal microscopes (LSM700, Zeiss, Jena, Germany) as described previously [Gao et al., 2013]. The same settings were used for imaging experiments and ImageJ software (National Institutes of Health) was used for quantitation of images.

ALKALINE COMET ASSAY

The alkaline comet assay was conducted according to the manufacturer (KeyGEN Biotech, China). Briefly, the cells after corresponding treatments were harvested and suspended in PBS with a concentration of 2×10^6 cells/ml. The mixture of ~20,000 cells in 10 μl volume with 75 μl of 0.7% low-melting point agarose was placed on the slide pre-dipped with 0.6% normal melting agarose and covered with coverslip. A third layer of gel of 75 μl of 0.7% low-melting point agarose was dipped on the top of cell layer. The

prepared slide was incubated with lysis buffer (2.5 mol/L NaCl, 100 mmol/L Na2EDTA, 10 mmol/L Tris, 1% sodium lauryl sarcosine, Ph 10, 1% Triton X-100 and 10% DMSO added prior to use) for 1.5 h at 4°C, and the sample was to unwind with fresh alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH 13) for 20 min at room temperature, then electrophoresed at 25 V (300 mA) for 20 min. After electrophoresis, the slides were incubated in the neutralizing solution (0.4 M Tris–HCl, pH 7.5) three times, 10 min each at 4°C. Slides were stained with 20 μl Propidium Iodide for 10 min at room temperature in the dark. And the slides were observed using fluorescence microscope (Olympus, Japan) through a 610IF emission filter excited by 545–580 excitation filter. The 20× objective lens and a 100-millisecond exposure time were used to record all the images.

GENE EXPRESSION BY QUANTITATIVE RT-PCR

After various treatments as indicated, total RNA of the corresponding HeLa cells or 293T cells was extracted with trizol (Invitrogen) and reversed to cDNA using iScript™ cDNA Synthesis Kit (Bio–Rad). The SsoFast EvaGreen Supermix (Bio–Rad) was used to perform qRT-PCR on qRT-PCR system (Mini Opticon, Bio–Rad). The following specific primers were used: PCNA, 5′-CCTGCTGGGATATTAGCT-3′ and 5′-AACTTTCTCCTGGTTTGG-3′; Flag-PCNA, 5′-ACAAGGATGACGAC-GATAAG-3′ and 5′-TCGAAGCCCTCAGACCGC-3′; ACTB, 5′-ACGTG-GACATCCGCAAAG-3′ and 5′-GACTCGTCATACTCCTGCTTG-3′. The procedure of qRT-PCR is the following: 94°C for 2 min, followed by 40 Cycles (94°C 15 s, 60°C 15 s, 72°C 20 s). Three independent repeat experiments were carried out and the data are expressed as means ± SEM of three independent experiments. β-actin was set as the internal control to correct for potential differences of mRNA in template in different groups.

STATISTICS ANALYSIS

Statistical analysis was performed using Student's paired *t*-test and the statistical significance was defined as $P < 0.05$.

RESULTS

14-3-3ζ INTERACTS WITH PCNA IN HUMAN CELLS AND IN VITRO

To demonstrate the interaction of 14-3-3ζ with PCNA protein in cells, we performed co-immunoprecipitation experiments with HeLa cells transfected either with Flag-PCNA and HA-14-3-3ζ, or with Flag-PCNA and HA-vector. As shown in Figure 1A, Flag-PCNA was specifically co-immunoprecipited with HA-14-3-3ζ by anti-HA antibody in the cells transfected with Flag-PCNA and HA-14-3-3ζ but not in those transfected with Flag-PCNA and HA-vector (Fig. 1A). The interaction between 14-3-3ζ and PCNA was also confirmed in vitro using firstly a semi-in cellulo GST pull-down assay (Fig. 1B) and a His-tag pull-down assay (Fig. 1C). As shown in Figure 1B, the endogenous PCNA in 293T cells, HeLa cells and U266 cells could be pulled down by GST-14-3-3ζ fusion protein but not by GST alone. In opposite direction, the endogenous 14-3-3ζ in 293T cells could be pulled down by His-PCNA fusion protein but not by Ni-NTA Superflow alone (Fig. 1C). These results demonstrated the interaction of 14-3-3ζ and PCNA in the cellular context of various cell lines.

We then carried out both GST pull-down assay and His-tag pull-down assay using the recombinant proteins GST-14-3-3ζ and His-PCNA in solution to detect the direct interaction between 14-3-3ζ and PCNA in a cell-free context in both directions (Figs. 1D and E). After co-incubation of the two recombinant proteins in lysis buffer A, His-PCNA could be detected in the bound fraction of GST-14-3-3ζ beads but not in GST, simultaneously, GST-14-3-3ζ appeared in the bound fraction of His-PCNA beads but not in Ni-NTA Superflow alone, suggesting that the direct interaction exists between these two proteins (Figs. 1D and E).

14–3–3ζ BINDS TO PCNA VIA TWO BINDING SITES, ONE OF WHICH IS A NON–CANONICAL PIP BOX

Many but not all PCNA interacting proteins bind to PCNA via the PCNA-interacting protein (PIP) box, with the canonical sequence: QXX(M/L/I)XX(F/Y)(F/Y) [27–29]. Sequence analysis showed that 14-3-3ζ does not contain a canonical PIP box. However, we have found a similar sequence QAESKVFY (amino acids 111–118 in 14-3-3ζ) with a Ser residue instead of a Met/Leu/Ile at the fourth position of the motif. In order to determine whether this sequence is a non-canonical PIP box, and to identify other potential binding domain of 14-3-3ζ protein involved in the 14-3-3ζ-PCNA interaction, we firstly designed several truncated 14-3-3ζ constructs, taking account of its structural organization (Fig. 2A). 14-3-3ζ protein is composed by nine antiparallel α-helices (αA–αI) organized into groups of two, two, two, and three helices [Xiao et al., 1995; Liu et al., 2002; Williams et al., 2011]. The first four helices are necessary for dimer formation [Yang et al., 2006], we have constructed and expressed three GST fused 14-3-3ζ truncated peptides, respectively corresponding to the αA to αD helices (including the amino acids 1–107, indicated hereafter as AD construct), αE to αF helices (EF construct, including the amino acids 108–162), and αG to αI helices (GI construct, including the amino acids 163–245) (Fig. 2A). These GST-14-3-3ζ truncations, together with full length GST-14-3-3ζ, were then incubated with 293T cell lysates in GST pull-down assays. Interestingly, EF and GI constructs were able to bind PCNA expressed in cell lysates as well as the full length GST-14-3-3ζ did, whereas a faint background of PCNA pulled down by AD construct was detected (Fig. 2B). Due to the low stability of this construct, which results in its significantly smaller quantity compared with other constructs, it is difficult to draw any clear conclusion about its binding ability to PCNA. Subsequently, the direct binding of EF, GI constructs with PCNA was reconfirmed by other GST pull-down assays against the purified His tagged PCNA recombinant protein instead of endogenous PCNA expressed in 293T cell lysate (data not shown).

The putative non-canonical PIP box is located in the EF construct. It comprises the two amino acids preceding the αE helix and its six first amino acids. In order to determine if the binding of EF to PCNA was really mediated by this non-canonical PIP box, we have mutated the three most critical amino acids of this motif, namely the Gln111, Phe117 and Tyr118 into alanine, and tested the impact of these mutations on the binding to PCNA. As shown in Figure 2C, the mutation of these three residues completely abrogated the binding

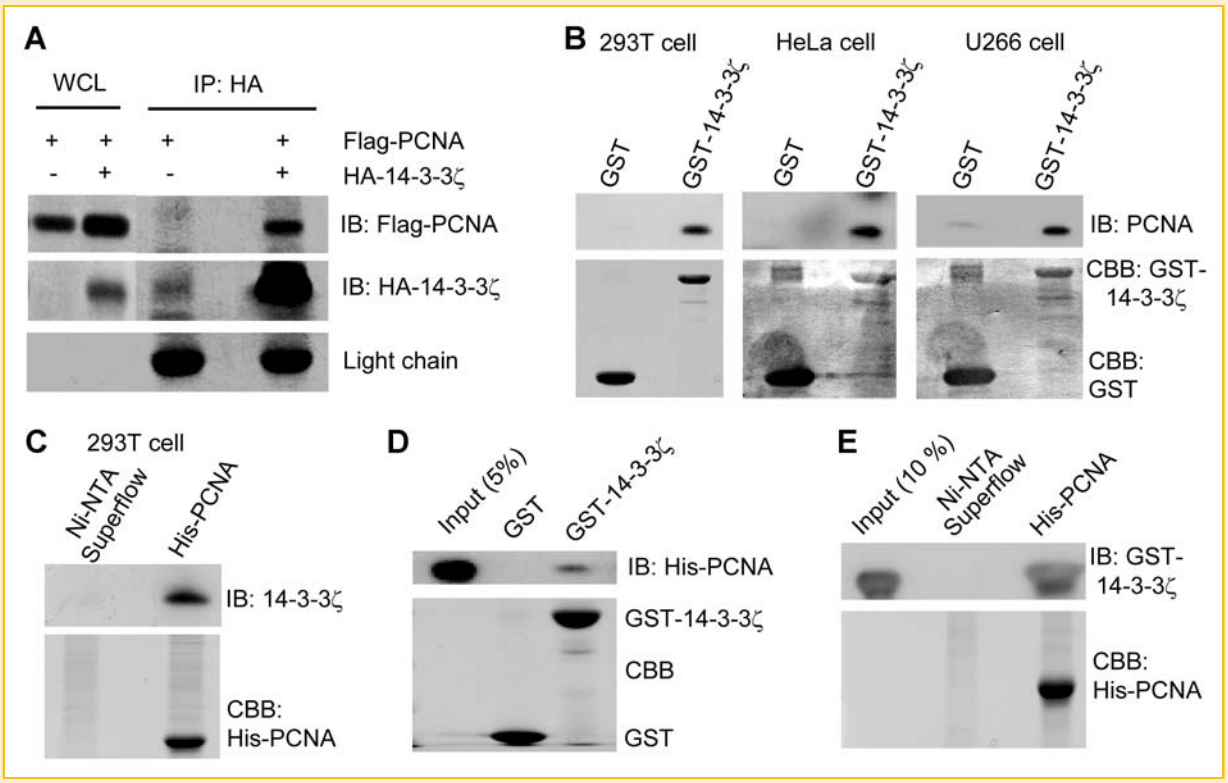

Fig. 1. 14-3-3ζ interacted with PCNA in cells and in vitro. A: Co-immunoprecipitation assay. HeLa cells were co-transfected either with Flag-PCNA and HA-14-3-3ζ, or with Flag-PCNA and empty HA-vector as indicated, then were lysed in lysis buffer A. The lysates were used for immunoprecipitation assay. The presence of both Flag-PCNA and HA-14-3-3ζ in the Sepharose-beads bound fractions were analyzed by immunoblot (IB) with antibodies against Flag and HA. The staining of IgG light chains indicate the equal amount of antibodies used for IP. WCL: whole cell lysates. B: GST pull-down assays for 14-3-3ζ-PCNA binding in cellular context. The lysates of 293T, HeLa or U266 cells were used for incubation with glutathione sepharose beads coated with GST or GST-14-3-3ζ as described. The bound proteins were analyzed by IB with PCNA antibody (the upper panels), whereas the amounts of GST or GST-14-3-3ζ recombinant proteins used in these assays are evaluated by CBB staining (293T cells) or GST antibody (HeLa and U266 cells). C: His-tag pull-down assay for 14-3-3ζ-PCNA binding in cellular context. The lysates of 293T cells were used for incubation with Ni-NTA Superflow beads coated or not with His-PCNA. The fraction bound to the beads were analyzed by IB with 14-3-3ζ antibody. D: 14-3-3ζ bound to PCNA directly in a cell-free GST pull-down assay. The proteins bound to beads were analyzed by IB with His monoclonal antibody. 1 μg of His-PCNA protein was loaded as input (5% of total His-PCNA used for pull-down assay). E: PCNA bound to 14-3-3ζ directly in a cell-free His-tag pull-down assay. The proteins bound to beads were analyzed by IB with GST antibody. 8 μg of GST-14-3-3ζ protein was loaded as input (10% of total GST-14-3-3ζ used for pull-down assay). CBB: Coomassie brilliant blue staining. All results are representative of at least three repeated experiments.

capacity of EF fragment to PCNA, demonstrating that this motif is indeed a non-canonical PIP box which mediates the binding of EF helices in 14-3-3ζ to PCNA. Sequence analyses showed that the G, H, and I helices of 14-3-3ζ do not contain the PIP-like motif, nor the APIM motif (AlkB homologue 2 PCNA-interacting motif, [KR]-[FYW]-[LIVA]-[LIVA]-[KR]), a novel and widespread PCNA-interacting motif recently discovered [Gilljam et al., 2009]. In addition, the tertiary structure of the region formed by these helices makes it difficult to further narrow the binding site in this region. Taken together, we conclude that at least two binding sites, one of which is a non-canonical PIP box, located respectively in αE–αF helices and αG–αI helices may mediate the interaction of 14-3-3ζ with PCNA.

DNA DAMAGES ENHANCED THE INTERACTION BETWEEN 14-3-3ζ AND PCNA

In light of the important role of PCNA in DNA repair, we investigated whether the interaction between 14-3-3ζ and PCNA could be associated with the process of DNA repair. We examined the 14-3-3ζ-PCNA interaction under DNA damage conditions. HeLa cells in culture were treated either by UVC irradiation or by MMS to induce DNA damages. At different time points after treatment, cells were lysed and subjected to GST pull-down experiments to examine the interaction between 14-3-3ζ and PCNA. In parallel, we performed confocal microscopy assays to assess the subcellular localization of these two proteins in HeLa cells under UVC- and MMS-treatments. Due to the difficulties we encountered using an anti-PCNA antibody for the immune-staining of the cells, we transfected HeLa cells with Flag-PCNA construct and used an anti-Flag antibody for the immuno-staining of Flag-PCNA in the cell.

Our results from GST pull-down assays showed that, in response to both UVC irradiation and MMS treatments, the amount of PCNA bound to GST-14-3-3ζ increased gradually with time, whereas the expression level of PCNA in these cells remained stable (Figs. 3A and B). These results indicate that DNA damages might enhance the interaction between 14-3-3ζ and PCNA in the cells, possibly via an augmentation of the binding affinity between these two proteins.

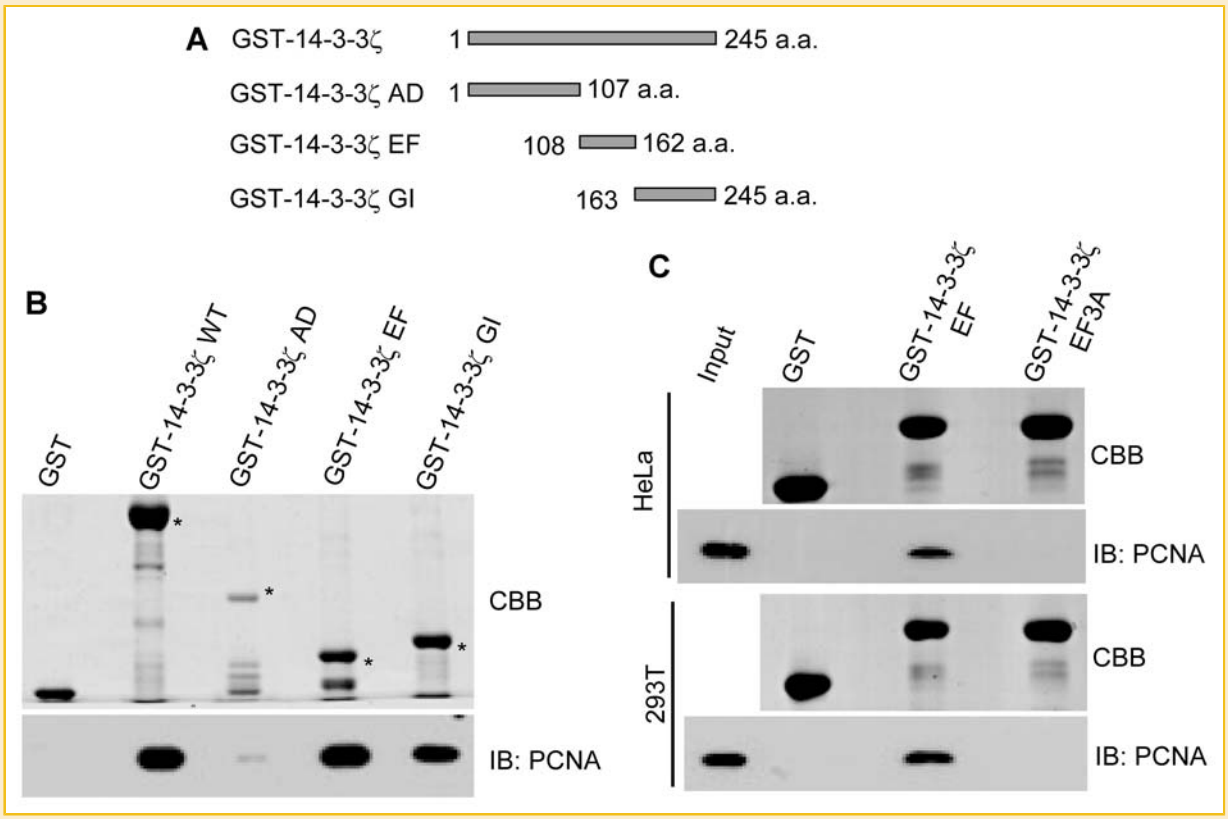

Fig. 2. Interaction of truncated GST-14-3-3ζ protein with PCNA in GST pull-down assay. A: Design of the GST-14-3-3ζ truncated constructs. AD: αA to αD helices; EF: αE to αF helices; GI: αG to αI helices. B: Binding of the truncated GST-14-3-3ζ peptides to PCNA in GST pull-down assays. The presence of the truncated GST-14-3-3ζ peptides immobilized on the Sepharose-GSH beads were revealed by CBB (upper panel, proteins bands are indicated by asterisks), while the bound PCNA was analyzed by IB with PCNA antibody (lower panel). Same molar amount of these peptides (except for AD due to its instability) was used in the experiments. C: Binding of the GST-14-3-3ζ EF wt or GST-14-3-3ζ EF3A mutant to PCNA in GST pull-down assay with lysates of 293T cells and HeLa cells. CBB: Coomassie brilliant blue staining. All results are representative of at least three repeated experiments.

The results from our confocal microscopy experiments showed that Flag-PCNA was located exclusively in cell nucleus with a punctate pattern, as described previously by other researchers (Fig. 3C, the upper panel). Interestingly, in these cells, few 14-3-3ζ molecules were co-located with Flag-PCNA in cell nucleus, since more separated red (14-3-3ζ) or green (Flag-PCNA) color staining was observed than the yellow color staining which would have resulted from the co-localization of the two proteins (the rightest upper panel in Fig. 3C). Six hours after the stimulation by either UVC or MMS treatments, we observed that more 14-3-3ζ molecules were co-localized with Flag-PCNA at the same loci in nucleus compared to the untreated cells (Fig. 3C, arrows).

It is of interest to note that, in the experiments with UVC irradiation and MMS treatment (Fig. 3C), cells have been pretreated with 0.4% NP-40 for 40 min before fixation and immuno-staining. This treatment was destined to mainly remove the soluble proteins in cell nucleus. After the treatment, the remaining NP40-insoluble fraction should correspond to the nuclear foci with chromatin structures [Andersen et al., 2011]. Thus, the immuno-labeled 14-3-3ζ and Flag-PCNA that we observed in these cells should be the molecules associated with chromatin. Taken together, our results suggest that DNA damages induced by both UVC irradiation and MMS treatment could strengthen the interaction between 14-3-3ζ and PCNA in chromatin fraction.

14-3-3ζ STABILIZED PCNA IN THE CELLS

During our investigation, we have observed that, when co-expressed with HA-14-3-3ζ, Flag-PCNA displayed a higher protein level in the co-transfected cells than when it was transfected alone (Figs. 4A and B). A 2.8-folds increase of Flag-PCNA protein level in HA-14-3-3ζ transfected cells versus the HA-vector transfected cells could be detected in the example given in Figure 4A. In order to exclude the possibility that the augmentation of PCNA protein level induced by 14-3-3ζ overexpression might result from an up-regulation of PCNA transcription, we have performed real-time quantitative PCR to assess the mRNA quantity of FLAG-PCNA in cells overexpressing HA-14-3-3ζ or not. Our results showed that the overexpression of HA-14-3-3ζ did not affect significantly the mRNA levels of Flag-PCNA (Fig. 4C). Together with our finding that 14-3-3ζ interacts directly with PCNA, these results suggest that 14-3-3ζ might stabilize PCNA protein by interacting with it rather than regulate its expression at transcriptional or translational level in the cells. To further confirm the stabilizing effect of 14-3-3ζ on PCNA, we have knocked down 14-3-3ζ expression in both HEK293T and U266 cells

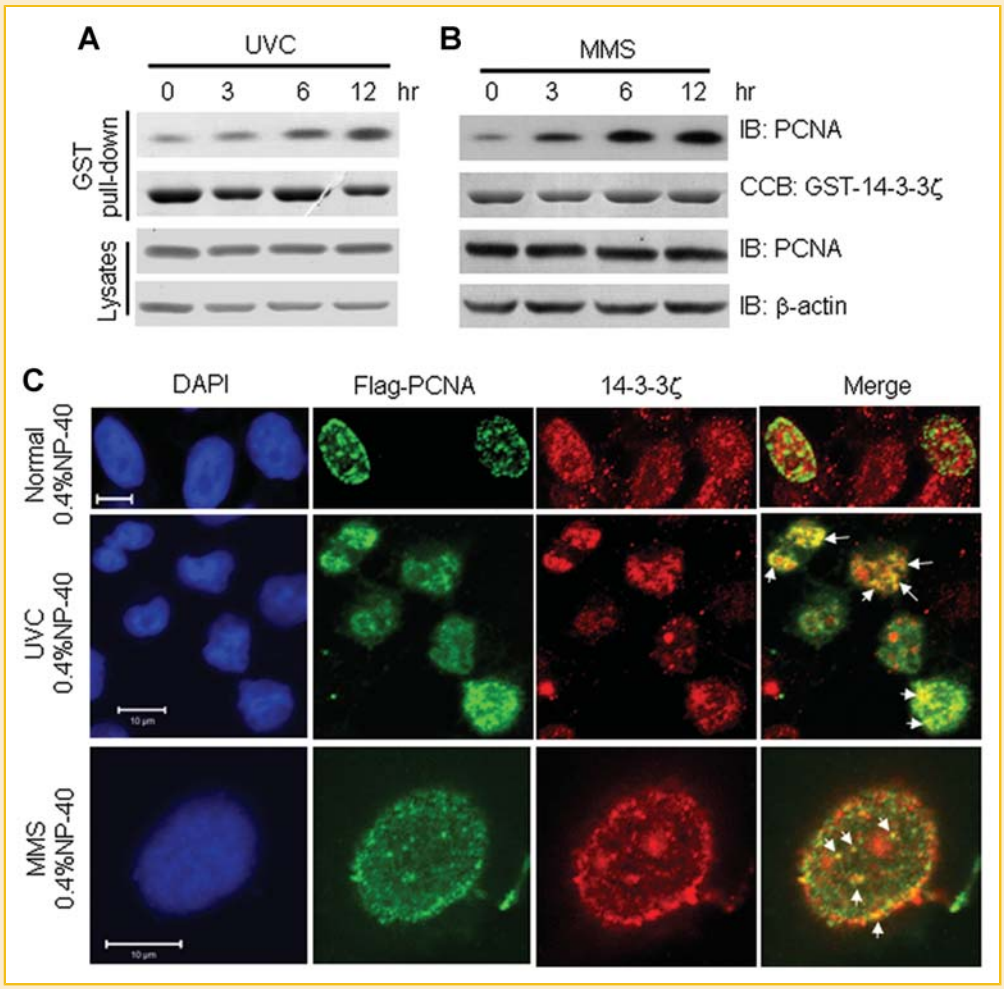

Fig. 3. UVC irradiation and MMS treatment enhanced 14-3-3ζ and PCNA interaction and co-localization. A: GST pull-down analysis of the interaction between 14-3-3ζ and PCNA after UVC irradiation. Lysates of HeLa cells treated with UVC for 0, 3, 6, and 12 h were incubated with glutathione sepharose beads coated with GST or GST-14-3-3ζ as described. The bound proteins were analyzed by IB with antibody against PCNA. The expression levels of PCNA and β-actin in cell lysates were analyzed by IB with appropriate antibodies. CBB: Coomassie brilliant blue staining. B: GST pull-down analysis of the interaction between 14-3-3ζ and PCNA after MMS treatment. HeLa cells treated with 1 μM MMS after 3, 6, and 12 h were analyzed using the same method as described in (A). C: Subcellular localization of 14-3-3ζ and PCNA in HeLa cells after UVC irradiation and MMS treatment. HeLa cells transfected with Flag-PCNA were treated with UVC (1 min, 25 J/m^2) or MMS (1 μM). After 6 h, cells were treated with 0.4% NP-40 for 40 min on ice and subsequently fixed and stained with both Flag and 14-3-3ζ antibodies. The Alexa Fluor 488 and Alexa Fluor 594 conjugated secondary antibody were used. Arrows: the co-localization of the two proteins. The nucleus was stained with DAPI. Bar: 10 μm.

by using a lentiviral-mediated shRNA. As expected, the interference of 14-3-3ζ expression resulted in a decrease of PCNA protein level in both HEK293T and U266 cells (Fig. 4D).

EFFECT OF 14-3-3ζ ON THE UBIQUITINATION OF PCNA

We then tested whether 14-3-3ζ stabilized PCNA by affecting its ubiquitination. Firstly, we have observed a similar increase of PCNA in the cells overexpressing 14-3-3ζ and those treated by 20 mM MG132, a proteasome inhibitor (Fig. 5A). We assessed the effect of 14-3-3ζ-silencing on the ubiquitination of PCNA in the MG132-treated cells using the co-expressed Flag-PCNA and His-ubiquitin. Stable 14-3-3ζ-silenced cells and control cells were co-transfected by Flag-PCNA and His-ubiquitin expression vectors, and Flag-PCNA immunoprecipitation assays were performed using anti-Flag antibody. The levels of Flag-PCNA and ubiquitinated Flag-PCNA in the immune-complexes were examined by Western blotting using respectively the anti-Flag and anti-Ub antibodies (Fig. 5B). Our result showed that higher ubiquitinated-PCNA levels were detected in 14-3-3ζ-silenced cells compared with control cells, especially for the polyubiquitinated forms (Fig. 5B).

OVEREXPRESSED FLAG-PCNA REDUCED DNA DAMAGE IN 14-3-3ζ-SILENCED CELLS

Since PCNA plays a key role in the process of DNA damage detection and repair [Ellison and Stillman, 2003; Maga and Hubscher, 2003; Essers et al., 2005; Lo et al., 2012], and 14-3-3ζ-PCNA interaction seemed to be strengthened under DNA damage conditions, we asked if the stabilization of PCNA by 14-3-3ζ could play a role in the DNA damage repair. To this end, we compared the degree of DNA damage in 293T cells with and without 14-3-3ζ silencing, and tested if the

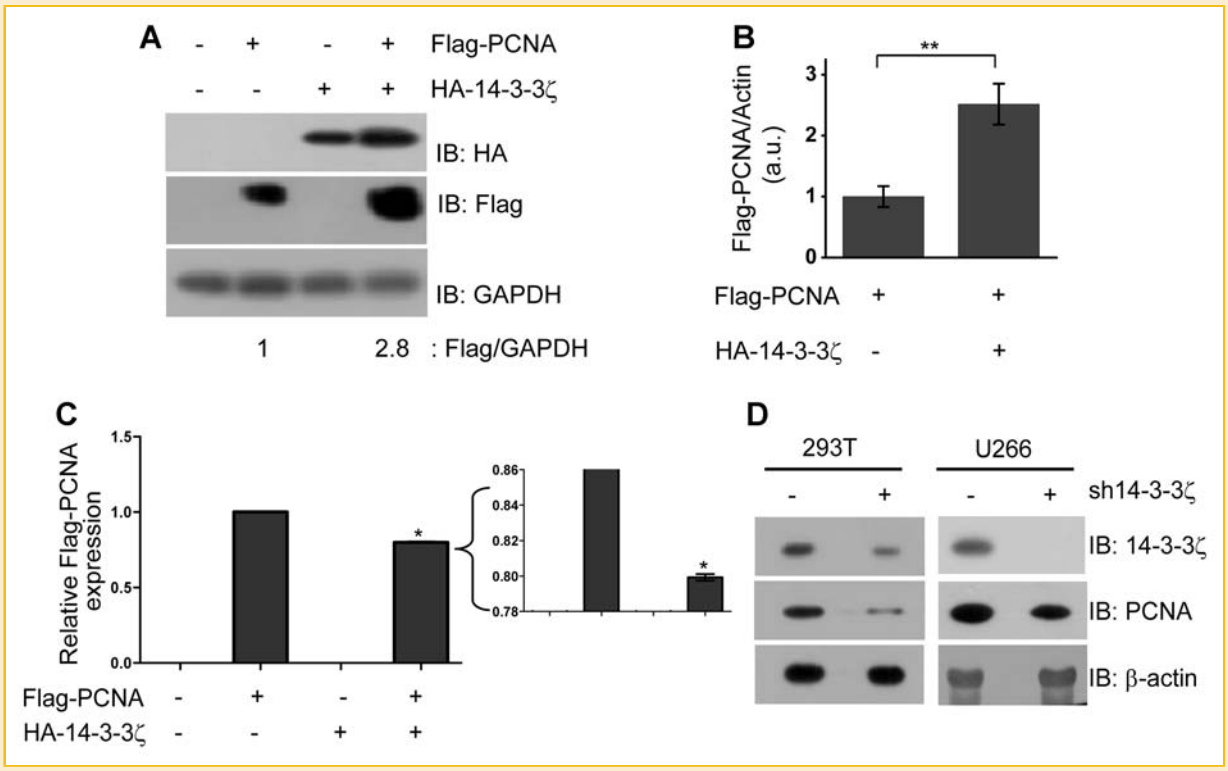

Fig. 4. 14-3-3ζ stabilized PCNA in cells. A: HA-14-3-3ζ overexpression up-regulates Flag-PCNA protein level. 293T cells co-transfected with the indicated plasmids were immunoblotted with Flag, HA and GAPDH antibodies. B: The quantified results of immunoblots of three repeated experiments. $^{**}P < 0.05$. C: Relative mRNA level of Flag-PCNA. 293T cells co-transfected with the indicated plasmids were used to perform qRT-PCR experiments. The mRNA level of β-actin was set as an internal control. The data are expressed as means + SEM of three independent experiments. The right panel was the zoom-in image destined to better visualize the SEM (indicated by the asterisk). D: 14-3-3ζ silencing down-regulated the protein level of PCNA. The lysates of 14-3-3ζ-silenced 293T and U266 cells were immunoblotted with 14-3-3ζ, PCNA and β-actin antibodies. The results are representative one of three independent experiments.

overexpression of PCNA could rescue the effect of 14-3-3ζ-silencing on DNA damage. Firstly, we used immunofluorescence with an antibody against phospho-Ser139 in H2AX (γ-H2AX), a marker of DNA breaks [Rogakou et al., 1999; Sedelnikova et al., 2003], to evaluate the degree of DNA damage in the cells after diverse treatments. As indicated by the results in Figures 6A and B, under UVC irradiation, 14-3-3ζ-silenced 293T cells displayed markedly enhanced γ-H2AX staining compared with the control cells, suggesting a less efficiency of DNA repair. However, Flag-PCNA overexpression reversed significantly the effect of 14-3-3ζ silencing (Figs. 6A and B). Similar results were also obtained by performing the comet assays to further assess the degree of DNA breaks upon UVC treatment. 14-3-3ζ silencing in 293T cells significantly increased the percentage of cells containing comet tail, and Flag-PCNA overexpression abrogated this effect (Figs. 6C and D). It is worthy to note that much fewer chromosomes were labeled for 14-3-3ζ-silenced cells under UVC irradiation, compared with the control shNC cells and those over-expressing Flag-PCNA, even though same number of cells was used for the four groups before the treatments. This indicated a higher mortality of cells upon UVC irradiation when 14-3-3ζ was knocked down, and also confirmed that ectopic expression of Flag-PCNA rescued the functional loss of 14-3-3ζ (Fig. 6E). Finally, in both immunofluorescence and comet assays, the overexpression of Flag-PCNA had not obvious effect on DNA repair and cell viability when 14-3-3ζ expression was not altered (Figs. 6B, D and E). This may be explained by the fact that, in this experimental condition, the level of endogenously expressed PCNA was sufficient to cover the requirement of the cell so that the ectopic expression of PCNA was in excess. However, when 14-3-3ζ was knocked down, the level of endogenous PCNA decreased, and the overexpressed exogenous PCNA permit to offset the loss of the endogenous PCNA. Taken together, our studies by using two different methods to evaluate the functions of 14-3-3ζ and PCNA in DNA repair suggest that the functions of these two proteins might be linked. This provides functional evidences for our hypothesis that 14-3-3ζ might be able to regulate the DNA repair by stabilizing PCNA.

DISCUSSION

In order to counteract the constant aggression from a variety of agents that may damage their DNA, eukaryotic cells have developed a complex system coordinating numerous factors involved in DNA synthesis, signal transmission, and cell cycle check points to detect and repair the DNA lesions at any time point of its life cycle, and particularly during the replication of the genome [Ljungman, 2005; Houtgraaf et al., 2006; Hübscher and Maga, 2011]. By virtue of its roles as the processivity factor of DNA replication and the

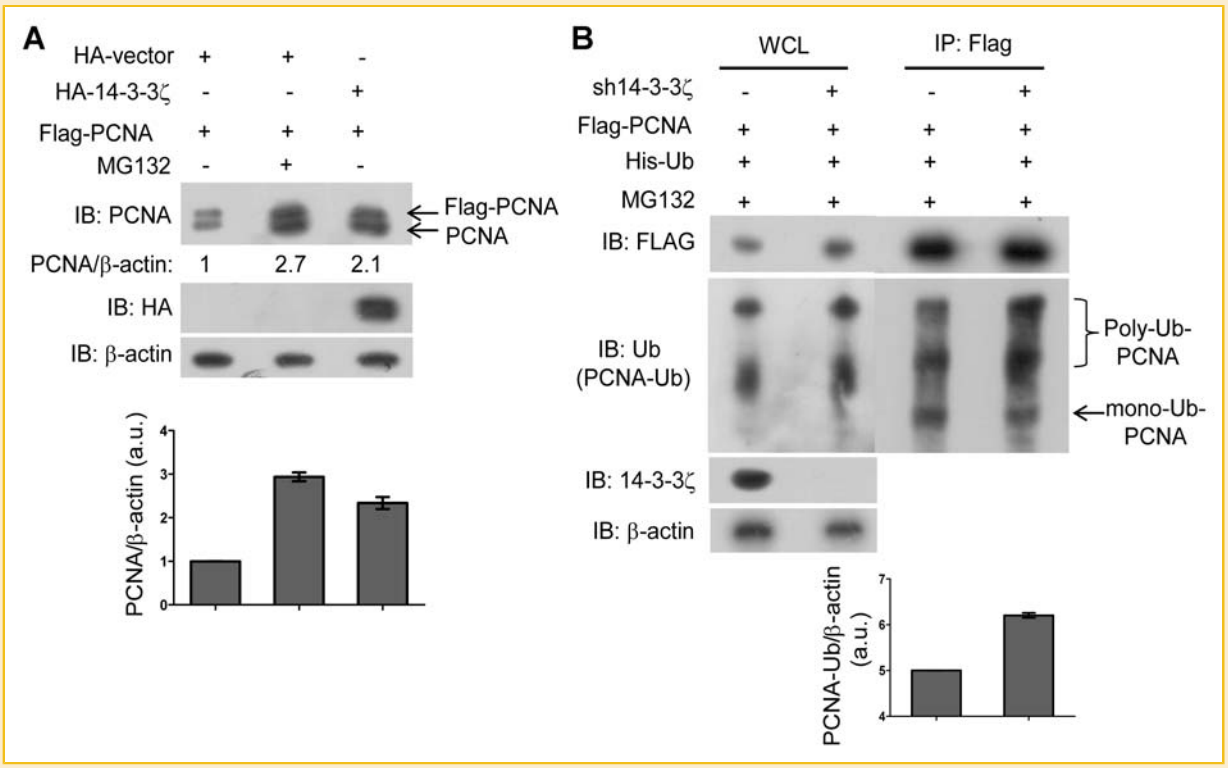

Fig. 5. 14-3-3ζ silencing facilitated the ubiquitination of PCNA. A: Flag-PCNA levels under MG132 treatment or HA-14-3-3ζ overexpression. 293T cells were transfected with the indicated plasmids and treated with or without MG132 (20mM, 12 h). Then immunoblotting was performed to assess the protein level of PCNA, HA-14-3-3ζ, and the internal control β-actin with appropriate antibodies. Quantification of total PCNA/β-actin were shown in the bottom panel of (A). The data are expressed as means ± SEM of three independent experiments. B: Detection of the ubiquitination of Flag-PCNA by immunoprecipitation assay. 14-3-3ζ-silenced 293T cells or control cells were co-transfected with Flag-PCNA and His-Ub plasmids and treated with MG132 (20 mM) for 12 h. The cells were lysed to perform immunoprecipitation assay using Flag antibody. The immune complexes were analyzed by Flag and Ub antibodies. The whole cell lysates (WCL) were also analyzed by immunoblot. All results are representative of three repeated experiments. Quantification of total PCNA-Ub/β-actin were shown in the bottom panel of (B). The data are shown as means ± SEM of three repeated experiments.

coordinator of the complex interactions around DNA polymerases, PCNA is one of the most important proteins involved in the regulation of DNA replicative and reparative syntheses. The investigation of protein–protein interactions between PCNA and other proteins is determinant for the understanding of the molecular mechanisms of DNA replication and repair.

In the present study, we demonstrate that PCNA binds directly to 14-3-3ζ, an adaptor protein associated to the regulation of many cellular processes, including the response to DNA damage, cell apoptosis, and survival [Tzivion and Avruch, 2002; Paul et al., 2012]. More interestingly, we found that DNA damage induced by UVC or MMS treatment stimulated the binding of these two proteins and their co-localization with chromatin. This strongly suggests that the interaction between these two proteins may be involved in DNA repair. The strengthening of the interaction between 14-3-3ζ and PCNA induced by DNA damage could possibly be mediated by a structural alteration/modification in one or both of these two proteins. However, even though DNA damages can induce the ubiquitination of PCNA, this modification may not be able to impact on the interaction between 14-3-3ζ and PCNA, since we have observed a comparable increase of binding to 14-3-3ζ for both non-ubiquitinated PCNA and mono-ubiquitinated PCNA upon the UVC irradiation or MMS treatment (data not shown). On the other hand, the participation of others interacting partners in the complex of interaction might also facilitate the binding between 14-3-3ζ and PCNA. It would be of interest, in the future, to identify the causing factor of this change.

More than one PCNA-binding site has been identified in 14-3-3ζ. One of them is probably a non-canonical PIP box located in the 14-3-3ζ EF fragment, the other one is located in the 14-3-3ζ GI fragment (Fig. 2). Indeed, several proteins have been reported to interact with PCNA through multiple contacts. Besides the classical PIP box, contributing to the primary contact, other secondary contacts outside the PIP motif were also identified in these proteins. They are found to interact with different binding sites in different subunits of PCNA trimer. Human PCNA Flap endonuclease 1 (FEN1) and the DNA polymerase B in *P. furiosus* are two of these proteins. It has been shown that three FEN1 molecules bind to PCNA homotrimer at different sites of different PCNA subunits whereas one single molecule of DNA polymerase B interacts with two different binding sites located at different subunits of PCNA trimer [Dieckman et al., 2012]. Similar situation could be imagined for 14-3-3ζ-PCNA interaction. 14-3-3ζ exists mainly as homodimer while PCNA is in trimer. Asymmetric conformation of 14-3-3ζ-PCNA interacting

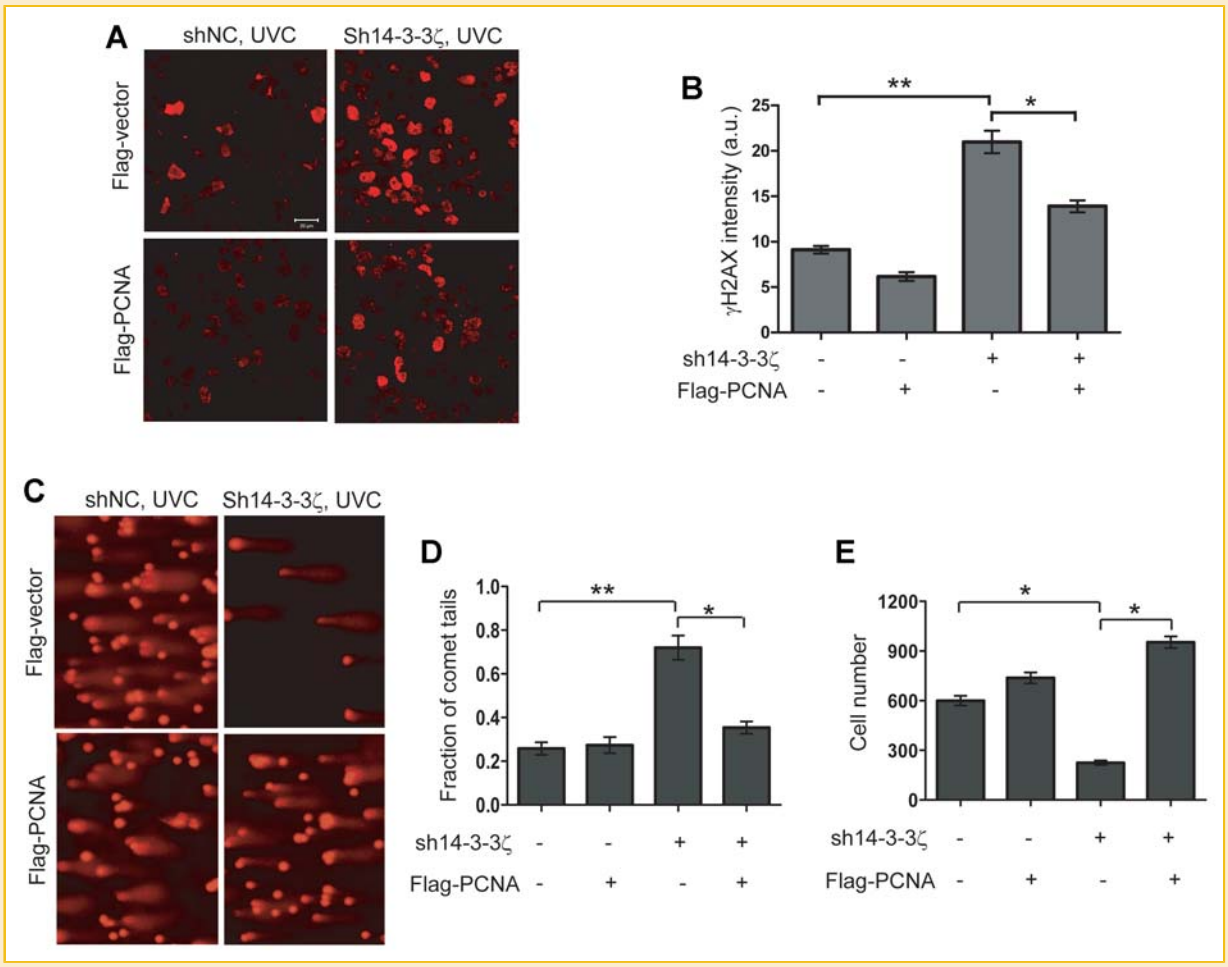

Fig. 6. Overexpressed Flag–PCNA decreased the DNA damage in 14–3–3ζ–silenced cells. 14–3–3ζ–silenced 293T cells or control cells transfected with the indicated plasmid were irradiated by UVC (1 min, 25 J/m^2). After 6 h, cells were subjected to analyses as following. A: Analysis of DNA damage by γ–H2AX immunofuorescence. The cells were fixed, immunostained with anγ–H2AX antibody and secondary antibody conjugated with Alexa Fluor 594, and observed by laser scanning confocal microscopes. Bar: 20 μm. B: The quantitation of γ–H2AX intensity of three independent experiments was performed using ImageJ software. Error bar: SEM. *$P < 0.05$. **$P < 0.01$. C: Analysis of DNA damage by comet assay as described. DNA was stained with Propidium Iodide (PI). D: Fraction of cells with comet tail. 238–988 cells/group were counted, and the means were shown ($n = 3$). Error bar: SEM. *$P < 0.05$. **$P < 0.01$. E: The number of cells present on images of comet assay. The total number of chromosomes (cells) labeled with PI staining were counted ($n = 3$). Error bar: SEM. *$P < 0.05$.

complex which involves different sites of contact on both proteins could therefore be possible.

Ottmann et al. [2007] have suggested that there might exist three mechanisms by which 14-3-3 proteins bind to their partners. The first one depends mainly on the interaction of the canonical 14-3-3 binding motifs (such as RSXpSXP and RX(Y/F) XpSXP) of its binding partners with the amphipathic groove of 14-3-3 (composed of the hydrophobic surface from helices G to I and the polar face from helices C to E) in a phosphorylation-dependent manner [Yaffe et al., 1997; Williams et al., 2011]. The C-terminal loop of 14-3-3ζ was shown to inhibit 14-3-3/ligand interactions [Truong et al., 2002]. The second mechanism has been demonstrated by the binding of the artificial peptide R18 to the amphipathic groove of 14-3-3 in a phosphorylation-independent manner [Ottmann et al., 2007]. Where the aspartate and glutamate of R18 substitute for the phosphorylated residues of canonical 14-3-3 binding motifs [Petosa et al., 1998]. The third mechanism involves the association of hydrophobic residues of its binding partners with the amphipathic groove of 14-3-3 independent of phosphorylation [Ottmann et al., 2007]. For example, the interaction of ExoS with 14-3-3ζ through the contact of four hydrophobic leucine residues of ExoS with the hydrophobic "roof" of the amphipathic groove of 14-3-3ζ, composed by the residues Pro165, Ile166, Leu216, Ile217, Leu220, and Leu172 located in the helices G to I [Ottmann et al., 2007]. It is possible that 14-3-3ζ GI fragment interacts with PCNA using the mechanism similar to the third one with its hydrophobic "roof" or other unidentified mechanism.

Our studies by 14-3-3ζ overexpression or silencing suggest that 14-3-3ζ may stabilize PCNA. In our study, we have observed a decrease of mRNA level of PCNA upon UVC irradiation (non-published data) while the PCNA protein level remained stable (Figs. 3A and B). This might result from the stabilization of PCNA protein by 14-3-3ζ through physical interaction with it. One of the

most plausible mechanisms underlying this effect might be the obstruction of the ubiquitination of PCNA, which has been shown to be important for the proteasomal degradation of PCNA [Davies et al., 2010; Lo et al., 2012]. By using the co-expression of Flag-PCNA and His-Ub, we have been able to show that 14-3-3ζ silencing could indeed facilitate the polyubiquitination of PCNA (Fig. 5B). It is possible that 14-3-3ζ binding alters the conformation of PCNA or/and obstructs the contact with its E3 ligases for polyubiquitination. For example, the E3 ligase CUL4A has been shown to facilitate the polyubiquitination and degradation of PCNA, whereas the phosphorylation of PCNA at Tyr-211 by the EGF receptor (EGFR) impeded these processes [Lo et al., 2012]. We have tested whether E3 ligase CUL4A was involved in the polyubiquitination and degradation of PCNA induced by 14-3-3ζ silencing; however, our unpublished data showed that knockdown of CUL4A had no obvious effects on this decrease of PCNA protein expression, suggesting that 14-3-3ζ stabilizes PCNA through interfering PCNA polyubiquitination mediated by other E3 ligases. There are reports showed that 14-3-3ζ binding could stabilize a number of interacting partners, such as β-catenin in the cytoplasm through impeding its binding to proteasome system [Tian et al., 2004]. Furthermore, many 14-3-3 target proteins have been stabilized by 14-3-3 after Akt phosphorylation [Tian et al., 2004]. However, the contribution of phosphoserine of PCNA to 14-3-3ζ binding and PCNA stability might need further investigation.

However, the mechanisms and functions of the ubiquitination of PCNA are complex and sometimes confusing. Mono- and polyubiquitinations mediated by Rad6-Rad18 and Ubc13/Mms2/Rad5 complexes on the K164 residue have been generally associated to the switches between different modes of DNA replication and repair [Prakash et al., 2005; Washington et al., 2009], whereas controversies exist regarding the role of the ubiquitination at the same K164 residue mediated by the cullin-RING family member $CRL4^{Cdt2}$. Indeed, It has been reported to be involved either in translesion DNA synthesis [Terai et al., 2010], or in the proteasomal degradation of PCNA [Lo et al., 2012]. Further investigation aiming to confirm the regulatory role of 14-3-3ζ in the ubiquitination of PCNA and to elucidate its precise mechanisms should take account of all these facts.

In conclusion, the delicate balance between DNA replication and repair is finely regulated by various proteins. The underlying mechanisms are complex and need to be elucidated. The demonstration of the interaction between two scaffold proteins, 14-3-3ζ and PCNA, which play important roles in this process may provide critical clues for future basic and cancer therapeutic studies.

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