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USP7 Regulates the Stability and Function of HLTF Through Deubiquitination

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ABSTRACT

Human helicase-like transcription factor (HLTF) is a functional homologue of yeast Rad5 that regulates error-free replication through DNA lesions. HLTF promotes the Lys-63-linked polyubiquitination of proliferating cell nuclear antigen (PCNA) that is required for maintaining genomic stability. Here, we identified the deubiquitylating enzyme ubiquitin-specific protease 7 (USP7) as a novel regulator of HLTF stability. We found that USP7 interacted with and stabilized HLTF after genotoxic stress. Furthermore, USP7 mediated deubiquitination significantly prolonged the half-life of HLTF, which in turn increased PCNA polyubiquitination. More intriguingly, silencing of USP7 rendered A549 cells highly sensitive to DNA damage and over-expression of HLTF attenuated this sensitivity. Thus, our results delineate a previously unknown USP7–HLTF–PCNA molecular network controlling DNA damage response. J. Cell. Biochem. 112: 3856–3862, 2011.

KEY WORDS: USP7; HLTF; PCNA; UBIQUITINATION; DEUBIQUITINATION; DNA DAMAGE

The DNA damage response is a signal transduction pathway that senses DNA damage and replication stress and recruits serials of DNA repair proteins to the DNA lesions [Ciccia and Elledge, 2010]. Unless replication is rescued by the timely action of lesion bypass processes, stalled replication forks can collapse, leading to genomic instability [Ciccia and Elledge, 2010]. In eukaryotes the Rad6–Rad18 enzyme complex regulates lesion bypass processes that ensure the completion of replication [Bailly et al., 1997]. In response to DNA damage, Rad6–Rad18 monoubiquitinates proliferating cell nuclear antigen (PCNA) [Hoege et al., 2002], a protein that assembles into a trimeric ring which encircles DNA and recruits DNA polymerases [Stelter and Ulrich, 2003]. Monoubiquitination of PCNA and its subsequent polyubiquitination serves as a molecular switch between various DNA damage bypass processes [Stelter and Ulrich, 2003; Haracska et al., 2004].

Helicase-like transcription factor (HLTF), a SWI/SNF family protein and a candidate tumor suppressor gene, is a common target for methylation and epigenetic gene silencing in digestive tract cancers [Moinova et al., 2002; Kim et al., 2006]. HLTF has an ubiquitin E3 ligase activity that promotes the polyubiquitination of PCNA in collaboration with Rad6–Rad18 [Motegi et al., 2008; Unk et al., 2008]. Depletion of HLTF significantly reduced polyubiquitination of PCNA upon treatment of cells with DNA-damaging agents [Motegi et al., 2008]. Moreover, HLTF-deficient mouse embryonic fibroblasts showed elevated chromosome breaks and

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fusions after DNA-damaging agent treatment [Motegi et al., 2008]. A requirement of HLTF for error-free post-replication repair of damaged DNA is in keeping with its tumor suppression role [Moinova et al., 2002]. HLTF is tightly regulated by the ubiquitin-proteasome system and HLTF ubiquitination can be catalyzed by CHFR. However, the deubiquitination of HLTF is less studied and the identification of the deubiquitylating enzyme which regulates HLTF ubiquitination and stability may help us better understand the HLTF–PCNA molecular network controlling DNA damage response.

The removal of ubiquitin conjugates from target proteins by deubiquitylating enzymes (DUBs) has emerged as an important regulatory mechanism in a range of cellular processes including DNA damage response [Sowa et al., 2009]. An estimated 95 functional DUBs are encoded by the human genome, but only few of these have been assigned functions or substrates [Sowa et al., 2009]. Several DUBs have been found to function in the DNA damage response, including USP1 [Nijman et al., 2005], USP28 [Zhang et al., 2006], and USP7 [Meulmeester et al., 2005; Faustrup et al., 2009].

USP7, also known as the herpes simplex virus associated ubiquitin-specific protease (HAUSP), is an evolutionarily conserved protein which was originally isolated as a binding partner of the herpes simplex virus protein Vmw110/ICP0 [Everett et al., 1997]. In recent years, evidence of USP7 involvement in DNA damage response was mounting up [Faustrup et al., 2009]. However, the function of USP7 in DNA damage response appeared to be contradictory [Meulmeester et al., 2005]. For instance, USP7 stabilizes both p53 and MDM2 [Li et al., 2004]. Both MDM2 and p53 recognize the same site on the TRAF-like domain and may compete for interaction with USP7 [Sakuma and Yamaguchi, 2010]. Importantly, HLTF is one of the high-confidence candidate interacting proteins of USP7 [Sowa et al., 2009]. It will be interested to test the relationship between USP7 and HLTF.

Here we show that USP7 binds to and deubiquitinates HLTF in response to DNA damage, thereby stabilizing HLTF. We also demonstrate that USP7 promotes HLTF induced PCNA polyubiquitination. Our findings underscore the importance of deubiquitination in the regulation of HLTF and delineate a previously unknown USP7–HLTF–PCNA molecular network controlling DNA damage response.

MATERIAL AND METHODS

CELL CULTURE

HEK293T-, A549-, and A549-derived cell lines were cultured in Dulbecco modified Eagle's medium supplemented with 10% fetal calf serum, 100 IU/ml penicillin, and 100 mg/ml streptomycin.

REAGENTS

Etoposide (Vp16) was purchased from Merck, China. CHX, VP16, HU, and MMS were purchased from Sigma. The following antibodies were purchased: Anti-Flag (M2; Sigma), anti-Myc (Cell Signaling), anti-HA (Y11; Santa Cruz), anti-HLTF (Y20; Santa Cruz), anti-USP7 (ab4080; Abcam), anti-P53 (DO-1; Santa Cruz), anti-Chk1 (G-4; Santa Cruz), p-Chk1 (Ser 317; Cell Signaling), anti-PCNA (F-2; Santa Cruz), ubiquitin (A-5; Santa Cruz), USP1 (ab108104; Abcam), USP28 (4217; Cell Signaling), PARP-1 (F2; Santa Cruz), Cleaved Caspase-3 (Asp175; Cell Signaling).

CONSTRUCTS AND PLASMIDS

Full-length human cDNAs of USP1, USP7, USP28, PCNA, and Ubiquitin were purchased from Open Biosystems. HLTF and CHFR were amplified from A549 cells cDNA Laboratory by RT-PCR and subcloned into pCDNA3.1. USP7 was subcloned into pBabe-puro retroviral expression vector. PCNA was subcloned into pCMV-HA expression vector. Ubiquitin was subcloned into pCMV-His vector. All the plasmids studied here were confirmed by sequencing.

RETROVIRUS PRODUCTION AND INFECTION

Retrovirus production was performed as previously described [Lu et al., 2009]. Stably expressing cell lines were made by infecting A549 cells with VSV-G-pseudotyped retrovirus carrying the pbabe-control or pbabe-flag-USP7 vectors. The cells were selected with 2.5 mg/ml puromycin for about 7 days. In some experiments, cells were treated with 20 mM MG132 for 6 h before harvested to inhibit proteasome function.

TRANSIENT TRANSFECTIONS

HEK293T cells were transfected using the calcium phosphate method. A549 cells were transiently transfected using Lipofectamine 2000 (Invitrogen), according to the manufacturer's instructions.

RNA INTERFERENCE

To generate retroviral vectors expressing short hairpin RNAs directed against human USP7, the following oligos were annealed and ligated into the BamHI and EcoRI sites of RNAi-Ready pSIREN-puro retroviral vector (Clontech): ShRNA-1 5′-gatcc GCT CAG AAC CCT GTG ATC AAT TCA AGA GAT TGA TCA CAG GGT TCT GAG TTT TTTg-3′ and 5′-aattc AAA AAA CTC AGA ACC CTG TGA TCA ATC TCT TGA ATT GAT CAC AGG GTT CTG AGCg-3′, ShRNA-2 5′-gatcc GCC CAA ATT ATT CCG CGG CAA ATT CAA GAG ATT TGC CGC GGA ATA ATT TGG GTT TTTTg-3′ and 5′-aattc AAA AAA CCC AAA TTA TTC CGC GGC AAA TCT CTT GAA TTT GCC GCG GAA TAA TTT GGGCg-3'.

SEMI-QUANTITATIVE RT-PCR ANALYSIS

Total RNAs were extracted from A549 cells by TRIzol reagent, and reverse transcriptions were performed by TaKaRa RNA PCR kit (Takara, Dalian, China) following manufacturer's instructions. HLTF cDNA and β-actin cDNA were amplified in the same tube by the following primers: GAA ATG GAA CCA GCT GAG GCT (forward) and TGT TCC CAG AAT GGT GGA AGT T (reverse) for HLTF; CAT CCT CAC CCT GAA GTA CCC (forward) and AGC CTG GAT AGC AAC GTA CAT G (reverse) for β-actin cDNA. PCR amplification was performed for 30 cycles with denaturing at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 45 s. All RT-PCR reactions were repeated at least three times.

CO-IMMUNOPRECIPITATION

Cells from two 10-cm tissue culture dishes at ~80% confluence were lysed in a total volume of 4 ml of lysis buffer (50 mM Tris-HCl pH

7.5, 150 mM NaCl, 0.5% Nonidet P40, Roche complete EDTA-free protease inhibitor cocktail) for 1 h with gentle rocking at 4°C. Lysates were cleared using centrifugation (13,000rpm, 10min); the supernatant was subjected to immunoprecipitation (IP) with 30 μl of immobilized anti-Flag or HA (Sigma) resin overnight at 4°C with gentle rotation. Resin containing immune complexes was washed with 1 ml ice cold lysis buffer four times followed by three 1 mL phosphate buffered saline washes. 2 × SDS loading buffer were added into the resin and boiled at 95 °C for 5 min, then the samples were loaded and separated by SDS–PAGE and detected by appropriate antibodies.

UBIQUITINATION AND DE-UBIQUITINATION ASSAYS

293T cells were cotransfected with 2 μg each of Myc-PCNA, HA-HLTF, and His-Ubiquitin plasmids with or without Flag-USP7. Cells were sonicated in lysis buffer containing 8 M urea and 10 mM imidazole. His-Ubiquitin-conjugated proteins were recovered with Ni-NTA resin (Qiagen), washed five times in urea lysis buffer containing 20 mM imidazole, and eluted with buffer containing 5% SDS, 0.72 M β-mercaptoethanol, and 200 mM imidazole. Proteins electrophoretically separated on denaturing gels were transferred to PVDF membranes (Millipore) and visualized with antibodies as indicated.

BACTERIAL PULL-DOWNS

E. coli BL21 (DE3) bacteria carrying pET-his-Con and pET-his-HLTF were grown at 30°C to OD600 = 0.3 and induced for 6 h with 0.1 mM IPTG. Cell pellets were resuspended in 1.5 ml of lysis buffer (50 mM Tris at pH 7.5, 500 mM NaCl, 10% glycerol, 0.5% Triton, 1 mM DTT, 10 mg/ml lysozyme) supplemented with protease inhibitors. Following sonication, cell lysates were centrifuged at 14,000 rpm for 30 min. Supernatants were then incubated for 2 h at 4°C 30 μl of Ni-NTA beads (Qiagen). Bead-bound proteins were then washed twice with lysis buffer and incubated for 1 h at 4°C with lysates from bacteria expressing USP1, USP7, or USP28. Protein complexes were then washed six times with lysis buffer, eluted in SDS loading buffer, and resolved on SDS–PAGE.

APOPTOSIS ASSAY

To assess the distribution of nuclear DNA content, cells were collected, rinsed and fixed overnight in 70% cold ethanol at −20 °C. Then, cells were treated with Tris-HCl buffer (pH 7.4) supplemented with 1% RNaseA and stained with 25 μg/ml propidium iodide (PI, Sigma). The samples were read on a Coulter Elite Flow Cytometer using Elite software program 4.0 for two-color detector (Beckman Coulter). The percentage of cells in the apoptotic sub-G1 phage was calculated using multicycle software (Phoenix Flow Systems).

STATISTICAL ANALYSIS

The Student's *t*-test was used to compare the difference between two different groups. A value of $P < 0.05$ was considered to be statistically significant.

RESULTS

USP7 INTERACTS WITH HLTF

To investigate whether HLTF is a direct target of USP7, we tested whether the two proteins co-purify in IP experiments. First, USP7 immunocomplex was purified from an A549 cell line that stably expressed Flag-USP7. Like p53, HLTF was also associated with USP7 (Fig. 1A). Then, USP7 and HLTF readily interacted under conditions where both proteins were over-expressed (Fig. 1B), and we could also detect association between the two endogenous proteins (Fig. 1C,D). In contrast, we did not observe binding of HLTF to USP1 or USP28 (data not shown), underscoring the specificity of the USP7–HLTF interaction. To investigate whether the interaction between USP7 and HLTF is direct, we utilized bacterial produced His-HLTF and USP family members to test their in vitro interaction. Among the DUBs we chose, USP7 was the only USP enzyme that physically interacted with HLTF (Fig. 1E). These observations suggest that USP7 interacts with HLTF both in vivo and in vitro.

USP7 STABILIZES HLTF

Since USP7 is a ubiquitin-specific protease, it is possible that USP7 may stabilize HLTF. Over-expression of Flag-USP7 increased the protein levels of endogenous HLTF without any effect on its mRNA levels (Fig. 2A), suggesting that USP7 could up-regulate HLTF protein at post-translation levels. To confirm the role of USP7 in regulating HLTF stability, we depleted USP7 in A549 cells using USP7-specific short hairpin RNA (shRNA). Knockdown of USP7 decreased the protein levels of HLTF but not its mRNA levels (Fig. 2B and data not shown). Addition of proteasome inhibitor MG132 resulted in restoration of HLTF levels (Fig. 2B), suggesting USP7 protected HLTF from proteasome-mediated degradation. To clarify further whether HLTF is stabilized by USP7 dependent deubiquitination, we assessed the half-life of HLTF by adding cycloheximide to block protein synthesis in cells with manipulated USP7 expression levels. Over-expression of USP7 brought a robust increase in the stability of HLTF as compared with control cells (Fig. 2C,D). Silencing of USP7 using two different USP7-specific shRNAs both brought significant decrease in the stability of HLTF as compared with control shRNA transfection cells. (Fig. S1A and S1B). These results demonstrate that USP7 stabilizes HLTF in cells.

USP7 REGULATES THE HLTF UBIQUITINATION AND E3 LIGASE ACTIVITY

Recently, the E3 ligase responsible for the polyubiquitination of HLTF was identified as CHFR [Kim et al., 2010]. We next tested whether USP7 regulates the levels of HLTF ubiquitination in vivo. Cells were transfected with either CHFR or USP7 or both and treated with MG132 for the last 6 h, endogenous HLTF was purified by HLTF antibody, and the polyubiquitin chain of HLTF was confirmed by both HLTF and Ubiquitin antibody (Fig. 3A). HLTF was rapidly ubiquitinated in the presence of its E3 ligase CHFR (Fig. 3A lane 1–2). But over-expression of USP7 suppressed the appearance of polyubiquitinated HLTF species in cells (Fig. 3A lane 3). Moreover, in an in vitro binding assay, the interaction between HLTF and CHFR was almost not affected by the addition of USP7 suggesting USP7 did not affect the binding between HLTF and CHFR (Fig S2). So USP7

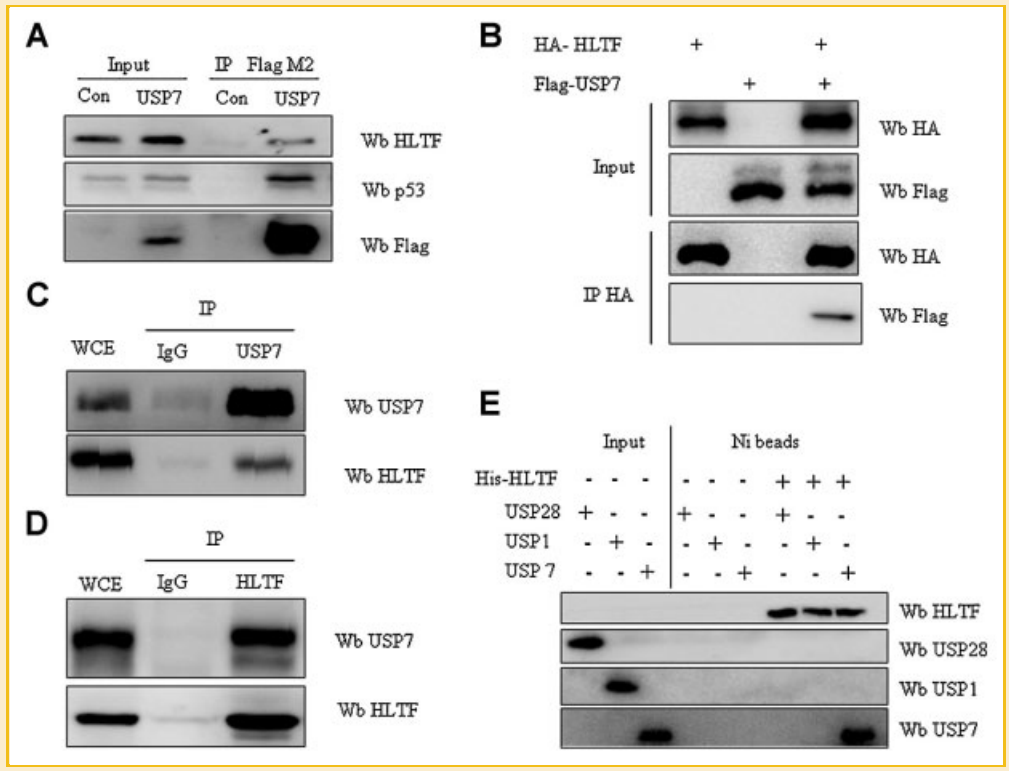

Fig. 1. USP7 interacts with HLTF. A: Lysates of A549 cells stably expressing Flag-USP7 were subjected to Flag immunoprecipitation (IP) followed by immunoblot (IB). p53 as a positive control. B: Lysates of 293T cells transfected with indicated plasmids for 24 h were subjected to HA IP followed by IB. C: Lysates of A549 cells were subjected to IP with USP7 antibody or preimmune rabbit serum (IgG) followed by IB. D: Lysates of A549 cells were subjected to IP with HLTF antibody or pre-immune rabbit serum (IgG) followed by IB. E: Western blot of protein complexes pulled down with nickel beads from BL21 bacteria expressing untagged USP1, USP7, or USP28, with or without His-tagged HLTF.

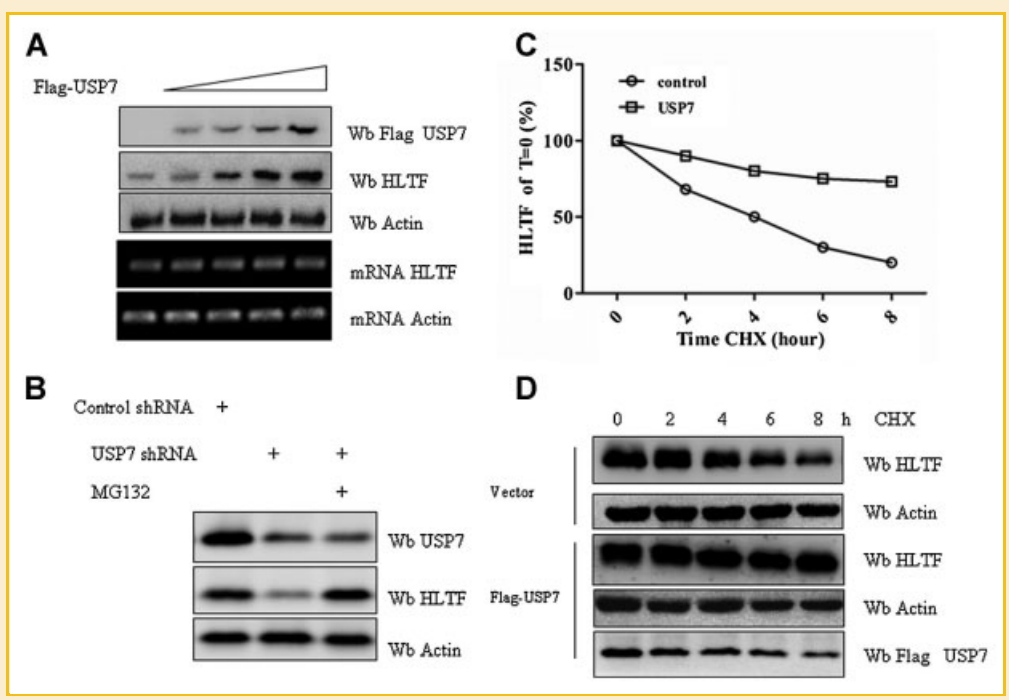

Fig. 2. USP7 stabilizes HLTF. A: A549 cells transfected with increased amounts of Flag-USP7 for 24 h; then, the indicated proteins (top panel, A), relative HLTF mRNA (bottom panel, A) were tested. B: A549 cells were transfected with indicated shRNAs for 72 h and analyzed by IB with the indicated antibodies. C: The graph shows quantification of HLTF half-life in A549 cells transfected with HLTF alone or in combination with USP7. The stability of HLTF protein was assayed by cycloheximide (CHX) chase. Lysates of the transfected cells were prepared at 2, 4, 6, 8 h and 0 h (control) after addition of CHX (20 μm), and subjected to immunoblotting with the indicated antibodies. D: A representative experiment of the CHX assay is shown.

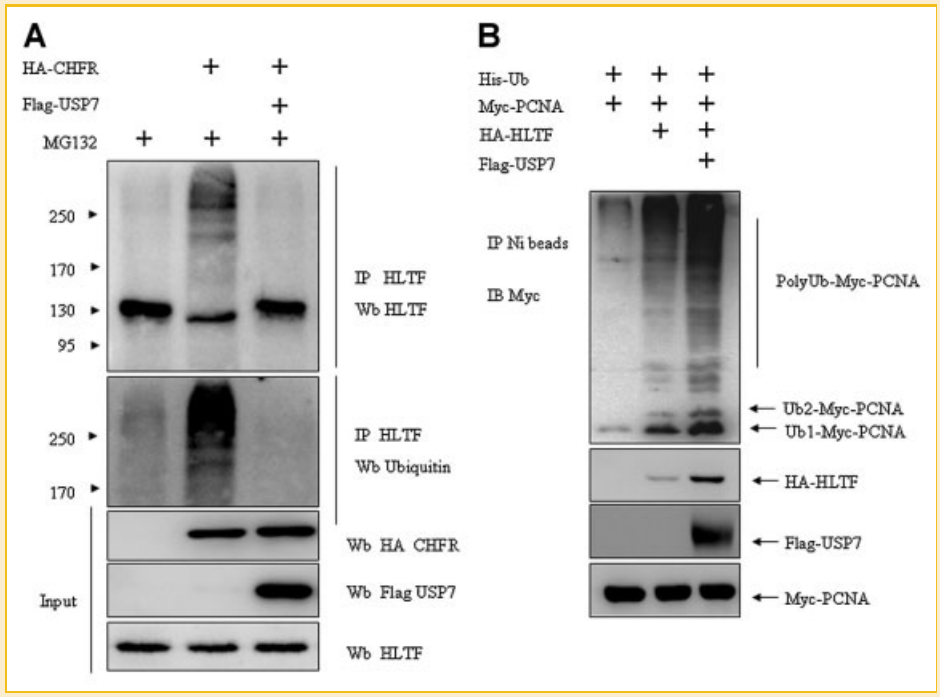

Fig. 3. USP7 regulates the HLTF ubiquitination and activity. A: Lysates of A549 cells transfected with indicated plasmids, treated with 20 μm MG-132 for 6 h before harvesting, were subjected to Flag IP followed by IB. The input immunoblotting data were performed with the indicated antibodies. B: Lysates of 293T cells transfected with indicated plasmids for 24 h were subjected to Ni-beads purification followed by IB. Among the bound proteins, ubiquitinated forms of PCNA were visualized by IB using anti-Myc antibody. The differences in the levels of monoubiquitinated PCNA in different lanes were presented as indicated. Ub1 and Ub2 indicate mono- and di-biquitinated species of PCNA, respectively. The input immunoblotting data were performed with the indicated antibodies.

prevents HLTF degradation may not through competition with CHFR.

HLTF functions as a ubiquitin ligase for PCNA polyubiquitination. The role of USP7 in maintaining steady-state levels of HLTF suggested that modulation of USP7 activity might affect PCNA regulation. To test this, we assessed the ability of USP7 over-expressed cells to activate PCNA. Agreed with the previous reports, HLTF over-expression induced polyubiquitination of PCNA. The polyubiquitination of PCNA was further increased when USP7 was co-expressed (Fig. 3B). On the contrary, knock down of USP7 decreased HLTF induced endogenous PCNA polyubiquitination (Fig S3). Hence, these data suggest that USP7 is required for HLTF activation in response to DNA damage.

DNA DAMAGE REGENTS ENHANCE THE INTERACTION BETWEEN USP7 AND HLTF

Given the fact that PCNA polyubiquitination is induced by DNA damage, the effect of USP7 on HLTF stability during DNA damage response was analyzed. HLTF was slightly up-regulated in A549 cells treated with various DNA damage reagents, including vp16, HU, and MMS (Data not shown, Fig. 4A). To elucidate the underline mechanism, we performed co-immunoprecipitation experiments in A549 cells co-expressed both USP7 and HLTF. Remarkably, the interaction between the two proteins was enhanced after treatment with DNA damage reagents (Fig. 4A). To investigate whether endogenous USP7 is also more efficiently bound to HLTF during DNA damage, we treated A549 cell with or without UV and lysates from both cells were analysed by IP followed by immunoblotting. Similar to overexpression observation, endogenous USP7 and HLTF interacted with each other more efficiently during UV treatment (Fig. 4B). These results suggest that USP7 regulates the stability and activity of HLTF in a DNA damage response dependent manner.

KNOCKDOWN OF USP7 ENHANCES DNA DAMAGE INDUCED APOPTOSIS IN A549 CELLS

In response to genotoxic stress, HLTF preventing mutagenesis and carcinogenesis partially by directly promoting error-free replication of damaged DNA [Motegi et al., 2008; Unk et al., 2008] and knockdown of HLTF in HCT116 cells enhances sensitivity to DNA damage reagents [Motegi et al., 2008]. To investigate whether silencing of USP7 in cells affects DNA damage sensitivity, we found knockdown of USP7 in A549 cells caused increased apoptosis induced by DNA damage reagent VP16 (Fig. 4C). Moreover, the sensitivity was partially restored by HLTF over-expression (Fig. 4C). Indeed, in response to VP16 treatment, increased caspase-3 and PARP-1 cleavage and decreased HLTF protein level were observed in both USP7-depleted cells when compared with that of control cells (Fig. 4D). While re-expression of HLTF in USP7-depleted cells inhibited abnormal activation of caspase-3 and prevented excess cleavage of PARP-1(Fig. 4D). These data indicate that USP7 regulates the biological function of HLTF.

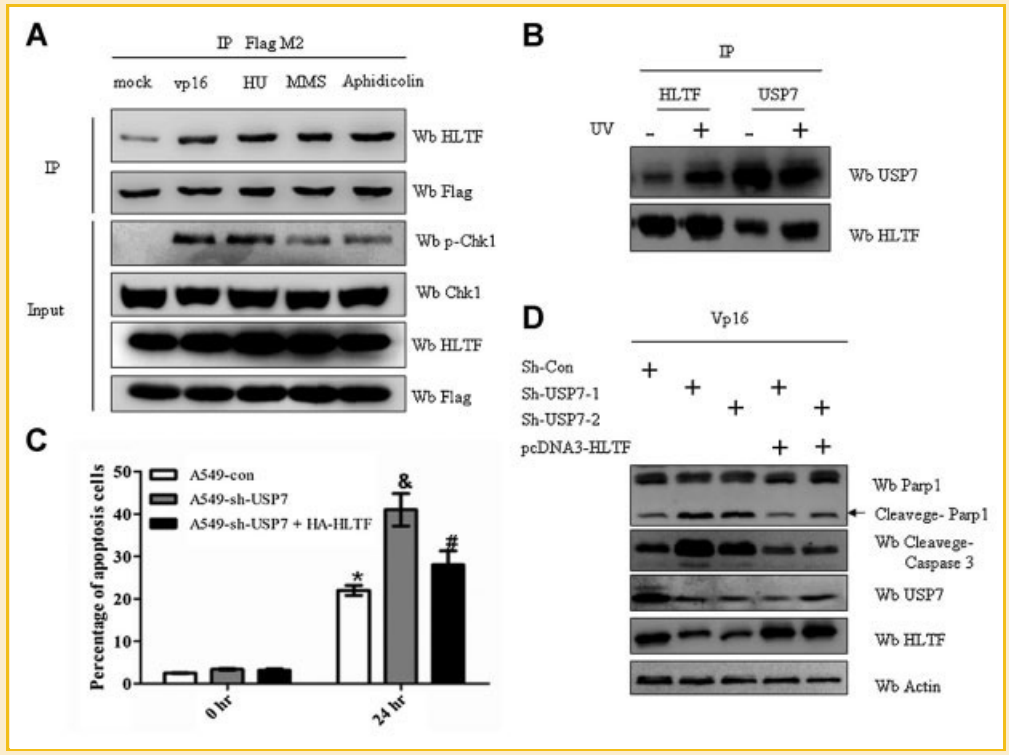

Fig. 4. USP7 regulates HLTF function after genotoxic stress. A: A549 cells stable expressing Flag-USP7 was untreated or treated with vp16, Hu, or MMS for 12 h. Cell lysates were analysed by immunoprecipitation or immunoblotting as indicated. The phosphorylation status of Chk1 serves as a marker of DNA damage. B: A549 cells were untreated or irradiated by 150 J UV; Cell lysates were analysed by immunoprecipitation or immunoblotting as indicated. C: A549 control cells and A549 USP7-shRNA cells transfected with indicated plasmids were either untreated (0 hr) or treated with 20 μm VP16 for 24 h. Apoptotic cells were analyzed by a sub-G1 DNA content assay. Error bars represent the standard deviation. $^{*}P < 0.01$ versus control cells 0 h group, $^{\&}P < 0.01$ versus control cells 24 h group, $^{\#}P < 0.01$ versus A549 USP7-shRNA cells 24 h group. D: A549 cells stably expressed that the indicated shRNA vectors were transfected with or without pcDNA3-HLTF and treated with vp16 for 24 h; cell lysates were analysed by immunoblotting with indicated antibodies.

DISCUSSION

In the present study, our results uncover a dynamic and complex mode of controlling HLTF stability upon DNA damage stimuli involving both ubiquitination and deubiquitination activities. Our data clearly demonstrated that HLTF stability and activity could be regulated by USP7 through a direct protein–protein interaction. USP7 forms a stable complex with HLTF and positively regulates its stability and activity. In DNA-damaged cells, PCNA is mono-ubiquitinated at its K164 residue by Rad6–Rad18 and then polyubiquitinated by Mms2–Ubc13 via a Lys-63-linked ubiquitin chain [Bailly et al., 1997; Hoege et al., 2002]. HLTF has a ubiquitin ligase activity that promotes the polyubiquitination of PCNA in collaboration with Rad6–Rad18 and Mms2–Ubc13. PCNA poly-ubiquitination may provide a signal for the removal of all of the DNA replication machinery and mark the DNA damage until the necessary DNA repair proteins were recruited to fix the DNA damage [Prakash et al., 2005]. Over-expression of USP7 amplified the polyubiquitination of PCNA, while silencing of USP7 caused decreased polyubiquitination of PCNA. The positive effect of USP7 on PCNA polyubiquitination may be the explanation for the highly DNA damage sensitivity of USP7-depleted cells. In this regard, USP7 may act as a tumor suppressor, and loss of USP7 would result in the increase of genomic instability and mutagenesis and promote apoptosis. Collectively, our results delineate a previously unknown USP7–HLTF–PCNA molecular network controlling DNA damage response.

It was reported that USP7 also binds to and controls CHFR which regulates HLTF [Oh et al., 2007]; it will be interesting to test whether USP7 competes with CHFR for binding to HLTF. However, in our present data, we failed to observe the existence of a mutually elusive competition between USP7 and CHFR with HLTF. It is possible that USP7 regulates numerous substrates and these substrates were transiently interacted with USP7 during diverse stimuli. It is also possible that the interaction between HLTF and CHFR may be weakened during DNA damage response. Further work will be required to understand this potential mechanism.

Cellular responses to DNA damage are mediated by a number of protein kinases, including ATM (ataxia telangiectasia mutated), ATR (ATM and Rad3-related), and DNA-PK [Ciccia and Elledge, 2010]. As previous research has shown, depletion of ATM or ATR does not affect the polyubiquitination of PCNA in response to DNA damage [Motegi et al., 2008], suggesting that the enhanced interaction between USP7 and HLTF would not be mediated by ATM or ATR. Future work will be focused on the molecular mechanism of the upstream signaling that enhancing the interaction between USP7 and HLTF in response to DNA damage as well as the relationship between USP7 and HLTF in clinical tumor samples.

The tumor suppressor protein p53 was reported to be an important target of USP7 [Li et al., 2002]. Knockdown of USP7 may also affect the protein level of p53 in A549 cells that harbor wide-type p53 gene. USP7-depleted A549 cells are highly apoptotic and this phenomenon is partially rescued by exogenous HLTF transfection. As p53 also participates in a wide range of DNA damage and repair response, and is also regulated by USP7, additional work will be needed to clarify whether HLTF is separated or combined with p53 to prevent USP7-depleted A549 cells from DNA damage induced apoptosis.

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