Deubiquitinase ubiquitin-specific protease 3 (USP3) inhibits HIV-1 replication via promoting APOBEC3G (A3G) expression in both enzyme activity-dependent and -independent manners

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Abstract

Background: Ubiquitination plays an essential role in many biological processes, including viral infection, and can be reversed by deubiquitinating enzymes (DUBs). Although some studies discovered that DUBs inhibit or enhance viral infection by various mechanisms, there is lack of information on the role of DUBs in virus regulation, which needs to be further investigated.

Methods: Immunoblotting, real-time polymerase chain reaction, *in vivo*/*in vitro* deubiquitination, protein immunoprecipitation, immunofluorescence, and co-localization biological techniques were employed to examine the effect of ubiquitin-specific protease 3 (USP3) on APOBEC3G (A3G) stability and human immunodeficiency virus (HIV) replication. To analyse the relationship between USP3 and HIV disease progression, we recruited 20 HIV-infected patients to detect the levels of USP3 and A3G in peripheral blood and analysed their correlation with $CD4^+$ T-cell counts. Correlation was estimated by Pearson correlation coefficients (for parametric data).

Results: The results demonstrated that USP3 specifically inhibits HIV-1 replication in an A3G-dependent manner. Further investigation found that USP3 stabilized 90% to 95% of A3G expression by deubiquitinating Vif-mediated polyubiquitination and blocking its degradation in an enzyme-dependent manner. It also enhances the A3G messenger RNA (mRNA) level by binding to A3G mRNA and stabilizing it in an enzyme-independent manner. Moreover, USP3 expression was positively correlated with A3G expression ($r = 0.5110$) and $CD4^+$ T-cell counts ($r = 0.5083$) in HIV-1-infected patients.

Conclusions: USP3 restricts HIV-1 viral infections by increasing the expression of the antiviral factor A3G. Therefore, USP3 may be an important target for drug development and serve as a novel therapeutic strategy against viral infections.

Keywords: APOBEC3G; Ubiquitin-specific protease 3; Deubiquitination; Human immunodeficiency virus-1 Vif; Human immunodeficiency virus; Deubiquitinase

Introduction

The ubiquitin–proteasome system (UPS) is widely exploited by human immunodeficiency virus (HIV) to antagonize the restriction of host defensive factors such as APOBEC3 (A3) family members, sterile alpha motif (SAM) domain and histidine-aspartate (HD) domain-containing protein 1 (SAMHD1), bone marrow stromal cell antigen 2 (BST-2), and helicase-like transcription factor (HLTF). HIV encodes viral accessory proteins Vif, Vpx, Vpu, and Vpr to form E3 ubiquitin ligase by recruiting host factors that induce polyubiquitination and degradation of the aforementioned host defensive factors.[1-4] Among them, A3G, which belongs to the cytidine deaminase family, was initially identified as a host restriction factor against HIV-1.[5] The mammal-specific A3 subfamily contains seven members: APOBEC3A (A3A), APOBEC3B (A3B), APOBEC3AC (A3C), APOBEC3DE (A3DE), APOBEC3F (A3F), APOBEC3G (A3G)

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APOBEC3H (A3H),[6,7] which possess evolutionarily conserved cytidine deaminase activity and edit single-stranded deoxyribonucleic acid (DNA) sequences by transforming deoxycytidine into deoxyuridine, resulting in virus restriction, cancer development, and immune responses.[8-14] As a well-studied host restriction factor, A3G was demonstrated to be a broad-spectrum inhibitor against DNA viruses, ribonucleic acid (RNA) viruses, and retrotransposons.[15-22] To overcome A3G-imposed restriction and facilitate viral replication, HIV-1 Vif induces A3G polyubiquitination and degradation by hijacking cellular proteins cullin 5 (Cul5), elongin B (ELOB), elongin C (ELOC), the transcription factor core-binding factor beta subunit (CBF-β), and a RING-box protein (Rbx) to form a Cul5-containing E3 ubiquitin ligase complex.[5,23-25] In addition, A3G has antiviral activity in a deaminase-independent manner, such as RNA binding activity.[15,18-20,26] The ability of A3G to lethally mutagenize HIV-1 in the absence of Vif indicates that this natural defense network can be reinstated if A3G is protected from degradation.[19]

Ubiquitination is a complicated and reversible process, in which reversal is implemented by an array of proteases termed deubiquitinating enzymes (DUBs) that reverse the process of ubiquitination by hydrolyzing the bonds between the ubiquitin moieties or between ubiquitin and the substrate.[27] Although there are numerous studies on ubiquitination, the functions of deubiquitination and DUBs in viral infection or virus-host interactions are much less explored. Moreover, DUB functions are complicated, and some host-encoded DUBs regulate viral infection by affecting important factors such as retinoic acid-inducible gene 1 (RIG-1) or arginine monomethylation precisely regulates the mitochondrial antiviral-signaling protein (MAVS) in the innate immune pathway or by stabilizing antiviral factors.[28-31] Some host-encoded DUBs are utilized by viruses to escape the innate immune system and complete their own replication.[32-34] Of course, viruses also encode DUBs to antagonize host restriction.[35-40]

HIV-1 is a well-studied pathogen,[41,42] and our studies and those of other groups have discovered several different modes of DUB regulation of HIV-1 replication by targeting either virus or host proteins.[43-48] For example, some DUBs have different effects on HIV-1 infectivity by targeting the HIV-1 Tat protein. Ubiquitin-specific protease 7 (USP7) promotes HIV-1 production by stabilizing Tat protein,[49] whereas our recent study showed that USP21 inhibits HIV-1 replication by downregulating Tat expression.[43] Several DUBs have been reported to inhibit HIV replication by stabilizing host antiviral factors.[44,47] USP49 inhibits Vif-mediated degradation of A3G.[35] USP8, USP37, USP7, and USP33 repress the ubiquitination and proteasomal degradation of Vif-, Vpr-, Vpu-, and Vpx-triggered host restriction factors.[43] DUBs have nearly 100 family members, while HIV-1 and its hosts have separately evolved various strategies to employ the host proteasomal machinery to interact with each other. Therefore, the function of DUBs in HIV-1 infection requires further investigation.

In the present study, we found that USP3 stabilizes A3G expression in two ways, regardless of the presence or absence of Vif, resulting in HIV-1 inhibition. Our study reveals a previously unrecognized interplay between host DUB USP3 and viral replication, and provides new targets for virus prevention and treatment.

Methods

Ethical approval

This study was approved by the Ethics Committee of the First Hospital of Jilin University (No. 21K143-001), and the procedures were carried out in accordance with approved guidelines. All research participants signed informed consent forms. HIV-1-infected patients were recruited at the Changchun Center for Disease Control and Prevention, Jilin, China, from December 2021 to January 2022.

Plasmid construction

USP3, USP3-zinc finger (ZNF), and USP3-USP catalytic domain (UCH) were constructed by polymerase chain reaction (PCR) amplification from USP3-HA/Flag (#22582; Addgene) and then were inserted between the *Sal*I and *Bam*HI sites of a C-terminal HA tag VR1012 vector. The viral assembly of the HIV-1NL4-3 laboratory strain (HIV-1 WT, HIV-ΔVif) was obtained from the acquired immune deficiency syndrome (AIDS) Research and Reference Reagents Program, Division of AIDS, National Institute of Allergy and Infectious Diseases, National Institutes of Health (NIH). The following expression vectors were used as described previously: A3G-V5 and HIV-1 NL4-3-Vif-HA (Vif-HA), A3C-HA, A3F-HA, A3H hapII-HA, and SAMHD1-Myc, HLTF-Flag.[43,44]

Cell culture, transfection, and antibodies

Jurkat (TIB-152, American Type Culture Collection [ATCC] and H9 (CRM-CCL-2, ATCC) cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium (1640, HyClone, Logan, UT, USA) with 10% fetal bovine serum (FBS, 04-001-1, Biological Industries) and 100 μg/mL penicillin/streptomycin. HEK293T (CRL-11268, ATCC), HepG2 (HB-8065, ATCC), and TZM-bl (PTA-5659, ATCC) cells were obtained from American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in Dulbecco's modified Eagle medium (DMEM, HyClone, Logan, UT, USA) containing 10% fetal bovine serum (FBS, PAN Seratech, Aidenbach, Germany) and 100 μg/mL penicillin/streptomycin (Biological Industries, Israel), and DNA transfections were carried out by Lipofectamine 2000 (Thermo, Waltham, MA, USA) according to the manufacturer's instruction. H9 cells were transfected using the Amaxa Cell Line Nucleofector Kit V (Lonza, Switzerland) with the program G-014 according to the manufacturer's instructions. The antibodies used in this study are listed as follows: Anti-HA (901513) monoclonal antibody (mAb) was purchased from BioLegend (San Diego, CA, USA). Anti-Myc (AHO0052) mAb and anti-V5 (R960-25) mAb were purchased from Invitrogen (Shanghai, China). Anti-A3G (D221663) rabbit polyclonal antibody was purchased from Sangon Biotech (Shanghai, China). Anti-USP3 (12490-1-AP) rabbit polyclonal antibody was

purchased from Proteintech (Rosemont, IL, USA). Anti-CAp24 mAb (1513) was purchased from NIH AIDS Reagents Program. Anti-tubulin (RM2002) mouse monoclonal antibody was obtained from Beijing Ray Antibody Biotech (Beijing, China). Secondary antibodies were horseradish peroxidase (HRP)-conjugated anti-rabbit (NA934 V, GE, Boston, MA, USA) and anti-mouse (sc-2005; Santa Cruz Biotechnology, CA, USA). All antibodies were used following the manufacturers' protocols.

Immunoblot analysis and co-immunoprecipitation (Co-IP)

These experiments were performed, as previously described.[43,44] Cell lysates were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to nitrocellulose membranes (10401396; GE, Boston, MA, USA) and reacted with appropriate antibodies, as described in the text. The cell lysates were used for Co-IP assays. Precleared cell lysates were mixed with anti-V5 antibody, anti-Myc antibody, anti-HA antibody, or anti-Flag antibody-conjugated protein G agarose beads and incubated at 4°C overnight. The next day, the beads were washed six times with washing buffer (20 mmol/L Tris, 100 mmol/L NaCl, 0.1 mmol/L ethylenediaminetetraacetic acid [EDTA], 0.05% Tween-20, pH 7.5) and centrifuged at 800 × *g* for 1 min each time. The eluted materials were then analyzed by SDS-PAGE and immunoblotting, as previously described.

RNA extraction and quantitative real-time reverse transcription-polymerase chain reaction (RT-qPCR)

Total RNA was isolated from cells with TRIzol reagent (15596-026; Invitrogen, Carlsbad, CA, USA) and then was reserve-transcribed with the EasyScript First-Strand complementary DNA (cDNA) Synthesis SuperMix (AE301; TransGen Biotech, Beijing, China). The cDNA was amplified using the Power SYBR® Green PCR Master Mix(2x) (4367659; ABI, Carlsbad, CA, USA). The primers used in this study are listed in Supplementary Table 1, http://links.lww.com/CM9/B372. Data were normalized to housekeeping *GAPDH* gene, and the relative abundance of the transcripts was calculated by the Ct models.

HIV-1 production and infection

HIV-1 virus was produced by transfecting pNL4-3 (wild-type [WT] or ΔVif) plasmids into HEK293T cells with Lipofectamine 2000 (Thermo, Waltham, MA, USA) according to the manufacturer's instructions. After 48 h, supernatants were collected. TZM-bl cells containing an integrated HIV-1 long terminal repeat (LTR) promoter, which was derived from HeLa cells, were used to assess the infectious HIV-1 production. Virus production was assessed using TZM-b1 indicator cells. The infected cells were harvested, and luciferase activity was measured with a GloMax 20/20 Luminometer (Promega, Madison, WI, USA).

Lentiviral production, transduction, and infection

Lentiviruses were produced by transfection of HEK293T cells with pLKO.1-sh control or pLKO.1-shUSP3 (sh control 5′-CAACAAGATGAAGAGCACCAA-3′; sh human USP3 5′-CCACTGTGGAAGGTATGTGAA-3′) together with pRSV-Rev (Rev cDNA expressing plasmid in which the joined second and third exons of HIV-1 rev are under the transcriptional control of RSV U3 promoter), pMDLg/RRE (Rev-responsive element), and pCMV-VSVG (the vesicular stomatitis virus glycoprotein). The assembled virus-like particles (VLPs) in the culture supernatants were used to infect fresh HEK293T, H9, or Jurkat cells. At 48 h post-infection, HEK293T cells were selected with 5 μg/mL puromycin (P8833; sigma, St. Louis, MO, USA), USP3 expression of the messenger RNA (mRNA) level was monitored by RT-qPCR, and the protein level was assessed by immunoblotting.

Luciferase assay

HIV-1 production was assessed using TZM-b1 indicator cells. LTR-luciferase was activated when TZM-b1 cells were infected by HIV-1. Cells were collected and lysed 48 h post-infection. LTR-luciferase activity was measured using the Dual-Luciferase Reporter Assay System (E1910; Promega, Madison, WI, USA) according to the manufacturer's protocol with the GloMax 20/20 Luminometer.

In vitro deubiquitination assay

Ubiquitinated A3G was isolated from HEK293T cells transfected with expression vectors of A3G-V5, ubiquitin (Ub)-Flag, and Vif-HA and then purified from the cell extracts with anti-V5 antibody-conjugated protein G agarose beads. USP3 or its mutant was purified from HEK293T cells overexpressing USP3-HA or its mutant, using anti-HA-Agarose antibody in immunoprecipitation (IP) buffer (50 mmol/L Tris–HCl pH 7.4, 150 mmol/L NaCl, 50 mmol/L EDTA, 1% [v/v] Triton X-100, 10 μmol/L NaF, 10% glycerol, and fresh protease inhibitor cocktail). For the *in vitro* deubiquitination assay, ubiquitinated A3G protein was incubated with USP3 or its mutant in deubiquitination buffer (20 mmol/L Tris–HCl pH 8.0, 200 mmol/L NaCl, 1 mmol/L EDTA, 10 μmol/L dithiolthreitol (DTT), and 5% glycerol) for 12 h at 37°C. Ubiquitinated A3G was analyzed by immunoblotting.

Statistical analysis

Statistics analysis was performed with GraphPad Prism 6 (GraphPad Software Inc., San Diego, California, USA). All data represent the results of three independent experiments and are presented as mean ± standard deviation. Correlation was estimated by Pearson correlation coefficients (for parametric data). Statistical significance was calculated using Student's *t*-test and a $P < 0.05$ was considered statistically significant.

Results

USP3 suppresses HIV-1 production by increasing A3G expression

After initial screening, we observed that USP3 was able to inhibit HIV-1 replication. To determine whether USP3 inhibition of HIV-1 replication is associated with the

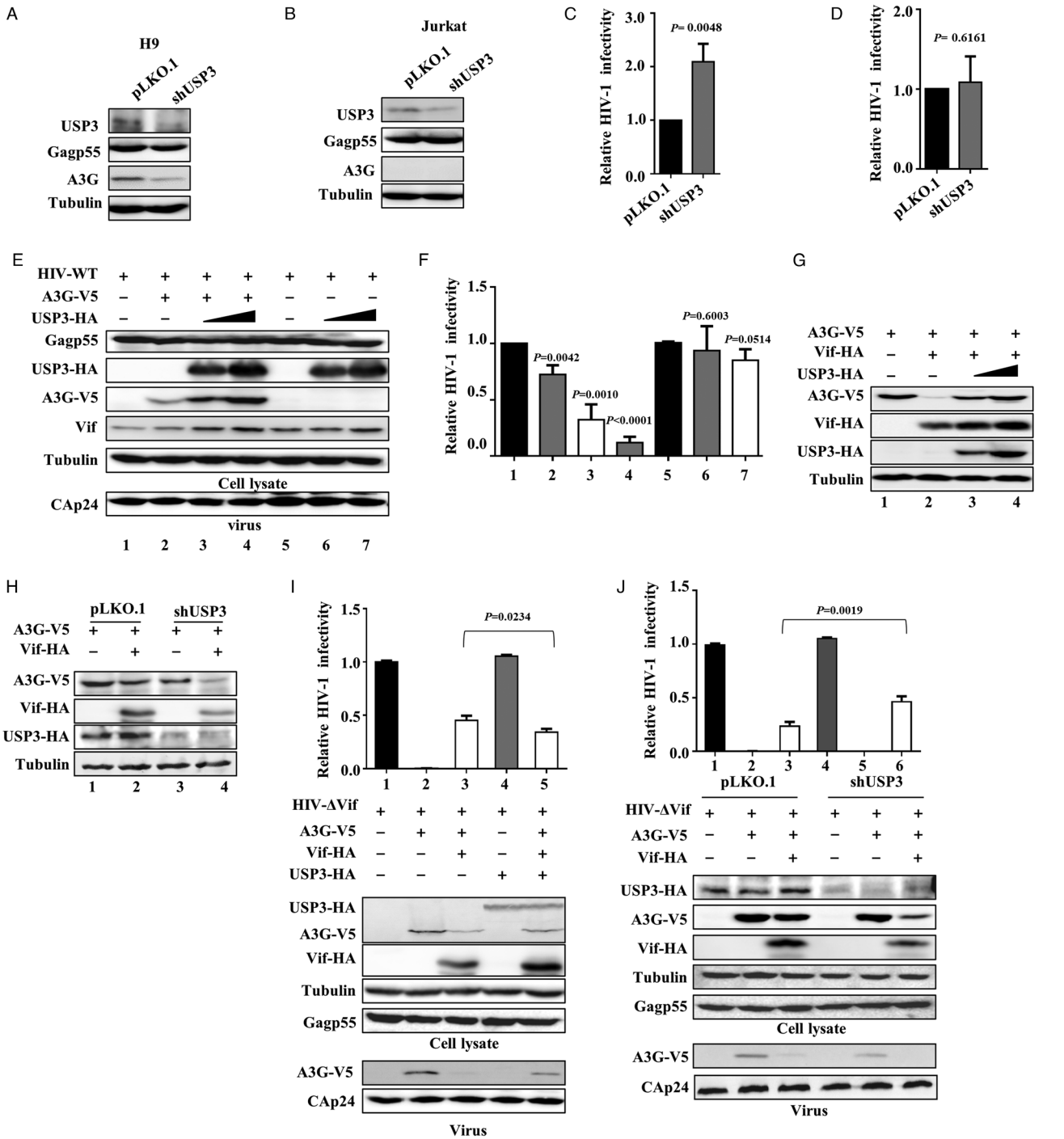

Figure 1: USP3 inhibits HIV-1 infection by increasing A3G packaging into the virus. (A, C) Effect of USP3 silencing on HIV production in H9 cells. (A) USP3 was knocked down in H9 cells. Endogenous USP3 were analyzed by immunoblotting. (C) USP3 silencing and control pLKO.1 cells were infected with HIV-1 NL4-3 viruses for 30 h. The cells were then washed three times with phosphate buffered saline (PBS) and placed in fresh RPMI-1640 medium with 10% FBS. Cell supernatants were then harvested after 48 h of infection. Virus production was assessed using TZM-b1 indicator cells. (B, D) Effect of USP3 silencing on HIV production in Jurkat cells. The experimental process was consistent with that in H9 cells. (E, F) HIV-1 production was significantly reduced when USP3 was co-expressed with A3G. (E) WT HIV-1 and A3G-V5 or control vector were co-transfected into HEK293T cells with increasing amounts of USP3-HA. After 48 h, cells were harvested and analyzed by IB, and tubulin was used as a loading control. (F) HIV-1 production was assessed by TZM-bl indicator cells, and HIV-1 WT production alone was set to 100%. (G) USP3 overexpression inhibits Vif-mediated A3G degradation. (H) USP3 knockdown promotes Vif-mediated A3G degradation. (I) Overexpression of USP3 inhibits HIV-1 production by increasing A3G packaging into virions. HEK 293T was transfected, as indicated. After 48 h, cells were harvested and analyzed by IB. Virion particle-containing supernatants were harvested and filtered through a 0.45-mm filter and then concentrated by ultracentrifugation. Virion pellets were immunoblotted with anti-CAp24 and anti-V5 antibody targeting A3G-V5. HIV-1 ΔVif production was assessed by TZM-b1 indicator cells. HIV-1 ΔVif production alone was set to 100%. (J) USP3 knockdown promotes HIV-1 production. The experimental process was similar to USP3 overexpression. Column results were from $n = 3$ independent experiments (C, D, F, I, J), and IB results are representative of $n = 3$ experiments (A, B, E, I, J). The statistical significance analyses were performed using Student's *t*-test. A3G: APOBEC3G; CAp24: HIV-1 viral capsid protein p24; FBS: Fetal bovine serum; Gagp55: HIV-1 Gag protein precursor; HIV: Human immunodefificiency virus; IB: Immunoblot; USP3: Ubiquitin-specifific protease 3; Vif: HIV-1 viral infectivity factor; WT: Wild type.

antiviral factor A3G, we generated USP3 silencing stable cell lines in HIV-1 natural target T cells H9 and Jurkat [Figures 1A and 1B], in which A3G is expressed or not, respectively.[50,51] The negative control pLKO.1 and shUSP3 cells were infected with HIV-1 NL4-3 viruses, as indicated. The infectivity of HIV-1 was detected using TZM-b1 indicator cells. The data showed that USP3 significantly inhibited HIV-1 infectivity in H9 cells [Figure 1C], but had no effect on Jurkat cells [Figure 1D]. Therefore, we speculated that A3G might be a major determinant of USP3-mediated HIV-1 inhibition. To further examine the effect of USP3 on HIV-1 production in the absence or presence of A3G, we overexpressed A3G in 293T cells that do not express endogenous A3G. By detecting CAp24 in the viral supernatant [Figure 1E lanes 1–4], we found that USP3 significantly inhibited HIV-1 infectivity in the presence of A3G, whereas it had no effect in the absence of A3G [Figure 1E lanes 5–7]. Interestingly, we observed that USP3 increased A3G expression in a dose-dependent manner [Figure 1E lanes 1–4]. As expected, virus infectivity in the cell supernatant assessed by TZM-b1 indicator cells showed similar results [Figure 1F], suggesting that USP3 inhibition of HIV-1 progeny virus infectivity is closely associated with A3G [Figures 1E, 1F].

Because HIV-1 Vif antagonizes A3G antiviral function by forming viral-specific cullin 5-RING ligase (CRL5) E3 ubiquitin ligase to promote the polyubiquitination and degradation of A3G,[5,52,53] we next determined whether the loss or gain of USP3 affected A3G expression in the presence of Vif. We found that increasing the level of USP3 expression effectively inhibited HIV-1 Vif-induced A3G degradation [Figure 1G], while silencing endogenous USP3 enhanced it [Figure 1H]. To further validate the function of USP3 in HIV-1 infection, we examined the effect on HIV-1 infectivity of loss or gain of USP3 in the presence or absence of Vif. When USP3 was overexpressed, intracellular A3G expression and virion incorporation of A3G were increased, even in the presence of HIV-1 Vif [Figure 1I lanes 3 and 5]. Accordingly, USP3 reduced the released HIV-1 virion infectivity [Figure 1I, upper panel]. When USP3 was silenced, Vif-induced A3G degradation was promoted, and less A3G was packaged into virion particles, resulting in increased virus infectivity [Figure 1J lanes 3 and 6]. Taken together, these results indicate that USP3 exerts potent anti-HIV-1 activity by antagonizing Vif-mediated A3G degradation, resulting in enhanced antiviral activity of A3G.

USP3 reduces Vif-triggered A3G polyubiquitination

To explore whether increased A3G expression induced by USP3 is due to reverse Vif-CRL5 E3-mediated A3G polyubiquitination, we employed a Co-IP assay to determine A3G polyubiquitination in the presence of USP3. *In vivo* deubiquitination assay results showed that HIV-1 Vif-induced polyubiquitination of A3G [Figure 2A, lane 2] was significantly reduced in the presence of USP3 [Figure 2A, lane 4]. By contrast, knockdown of USP3 increased Vif-induced A3G polyubiquitination compared to the control group [Figure 2B, lanes 2 and 4]. In addition, USP3 did not disrupt Vif-CRL5 E3 ubiquitin ligase complex formation, and Vif still interacted with host proteins Cul5, ELOB/C, and CBF-β to form the E3 ubiquitin ligase complex that is required for A3G polyubiquitination [Figure 2C]. Interestingly, we observed that USP3 also interacted with Vif. These data suggest that USP3 inhibits Vif-mediated A3G degradation by specifically removing A3G polyubiquitylation, not by disrupting the interaction between Vif and the host factors Cul5, ELOB/C, and CBF-β.

USP3 contains two conserved domains, a zinc finger Ub-binding domain (ZNF-UBP) and a catalytic domain of the ubiquitin-specific protease (USP) class.[54,55] To identify whether the deubiquitinase activity of USP3 is required for Vif-mediated A3G degradation, we generated two mutants containing the ZNF-UBP domain (ZNF) or USP catalytic domain (UCH) of USP3 [Figure 2D] and assessed their ability to inhibit Vif-mediated A3G degradation. Compared with full-length USP3, the UCH enzyme activity domain of USP3 strongly inhibited Vif-mediated A3G degradation [Figure 2E lane 5]. Interestingly, the USP3 ZNF domain without deubiquitination activity also effectively inhibited Vif function [Figure 2E lane 4]. To further investigate whether both the ZNF and UCH domains of USP3 are required for the inhibitory function of USP3, we validated the effect of USP3 mutants on A3G inhibition of HIV infection. HEK293T cells were transfected with HIV-1, A3G-V5, USP3 wild type (WT), or mutants, as indicated. The results showed that USP3 WT, ZNF domain, and UCH domain all stabilized the expression of A3G [Figure 2F] and inhibited HIV-1 infection [Figure 2G]. To examine why the ZNF domain of USP3 lacking deubiquitination activity also stabilizes A3G and inhibits HIV-1 infection, we performed deubiquitination experiments *in vivo* and *in vitro*. The results showed that both USP3 WT and the UCH domain effectively removed A3G polyubiquitination in *in vivo* and *in vitro* experiments, but the ZNF domain of USP3 had no effect [Figures 2H and 2I]. Altogether, these results indicate that both the ZNF and UCH domains of USP3 can stabilize A3G and inhibit HIV-1 infection, and the deubiquitination activity of USP3 is not the only reason for Vif-mediated A3G degradation and HIV-1 inhibition.

USP3 also increases A3G expression in the absence of Vif by stabilizing A3G mRNA level

Our observations that the USP3 ZNF domain without deubiquitination activity could increase A3G expression suggest that USP3 might also increase A3G expression in a different manner. To verify this hypothesis, We co-transfected HEK293T cells with A3G, USP3 and Vif expression plasmids in the absence of Vif. The results showed that USP3 directly increased the expression of A3G in the absence of Vif [Figure 3A]. Moreover, overexpression of USP3 in H9 cells obviously increased the expression of endogenous A3G [Figure 3B] and prolonged its half-life [Figure 3C]. USP3 mutant ZNF and UCH domains increased A3G expression, similar to USP WT [Figure 3D], confirming that USP3 increases A3G expression in a different manner. In HepG2 cells which express endogenous A3G, we also observed that USP3 mutants increased A3G expression at the protein level [Figure 3E] and significantly increased A3G expression at the mRNA level [Figure 3F].

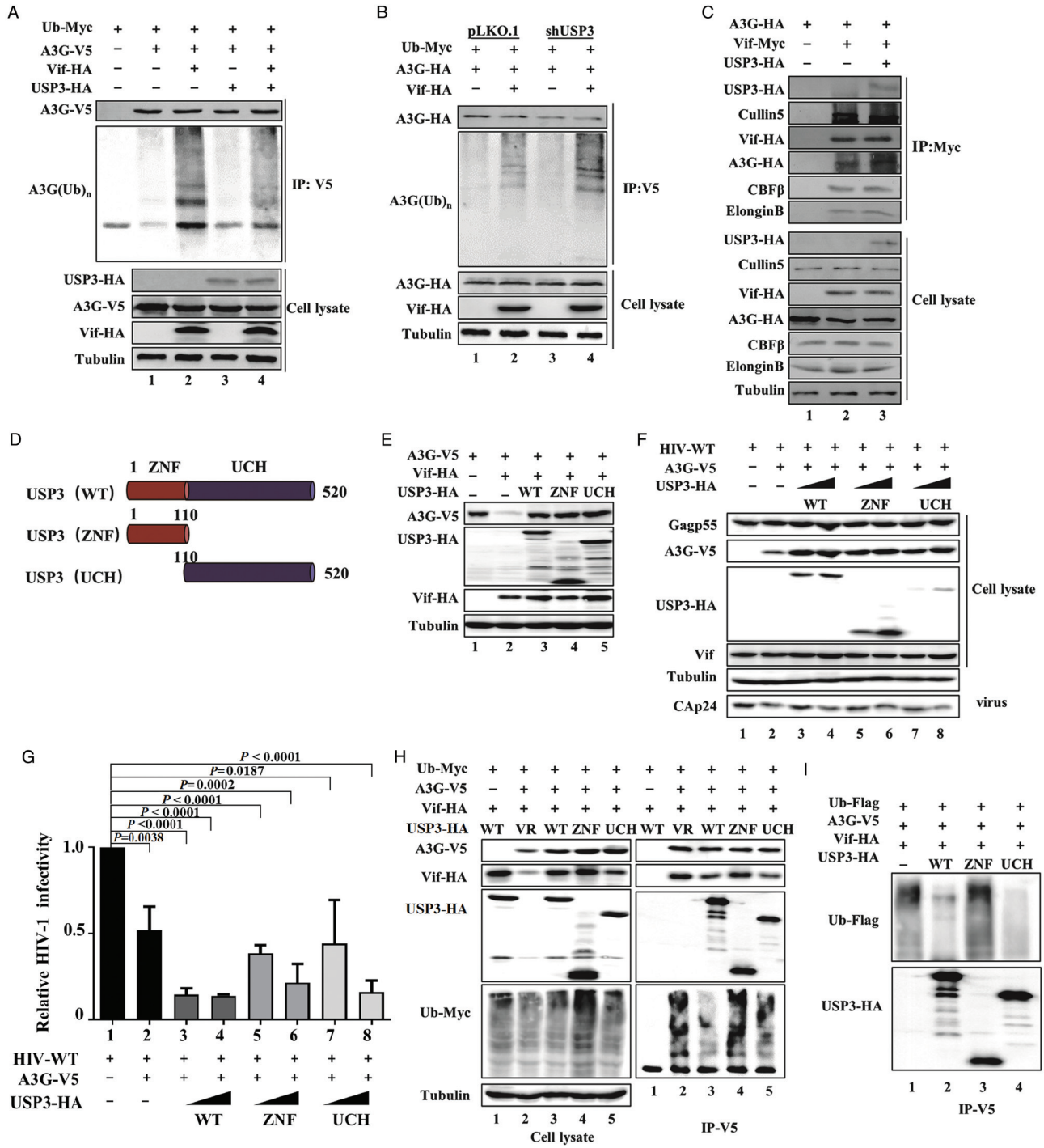

Figure 2: USP3 reduces Vif-mediated A3G polyubiquitination in a deubiquitination enzyme activity-dependent manner. (A) Overexpression of USP3 inhibits Vif-induced A3G ubiquitination. HEK293T cells were transfected as indicated. Cells were treated with 10 μmol/L MG132 for 12 h before harvesting. Cell lysates were prepared and immunoprecipitated using anti-V5 antibody conjugated to agarose beads 48 h post-transfection. Cell lysates and precipitated samples were analyzed by IB with the corresponding antibodies. (B) Knocking down USP3 increases Vif-induced A3G ubiquitination. (C) USP3 does not affect Vif-CRL5 E3 ubiquitin ligase formation. HEK293T cells were transfected, as indicated. Cell lysates were immunoprecipitated with anti-Myc antibodies conjugated to agarose beads. Cell lysates and precipitated samples were analyzed by IB. (D) Schematic diagram representing USP3 WT and mutants used in the study. (E) USP3 mutants inhibit Vif-mediated degradation of A3G like USP3 WT. (F, G) USP3 WT and the two mutants inhibit HIV-1 infection. (F) HEK 293T was transfected, as indicated. After 48 h, cells were harvested, and protein expression was analyzed by IB. (G) Virus production was assessed using TZM-b1 indicator cells. HIV-1 production alone was set to 100%. (H) USP3 WT and mutants deubiquitinate A3G *in vivo*. HEK293T cells were transfected, as indicated, and treated as (A). (I) USP3 WT and mutants deubiquitinate A3G *in vitro*. Ubiquitinated A3G was purified from HEK293T cells transfected with Ub-Myc, Vif-HA, and A3G-V5 using anti-V5 affinity purification. HA-tagged USP3 WT, ZNF, or UCH was purified from HEK293T cells using anti-HA affinity purification. Ubiquitinated A3G-V5 was incubated with HA-tagged USP3 or mutants for 1 h, followed by IB using antibodies against Ub-Myc and USP3-HA. The statistical significance analyses were performed using Student's *t*-test. A3G: APOBEC3G; CAp24: HIV-1 viral capsid protein p24; CBF-β: Core-binding factor beta subunit; Gagp55: HIV-1 Gag protein precursor; HIV: Human immunodeficiency virus; IB: Immunoblot; IP: Immunoprecipitation; MG132: Proteasome; Ub: Ubiquitin; UCH: USP3- USP catalytic domain; USP3: Ubiquitin-specific protease 3; Vif: HIV-1 viral infectivity factor; WT: Wild type; ZNF: USP3- zinc finger.

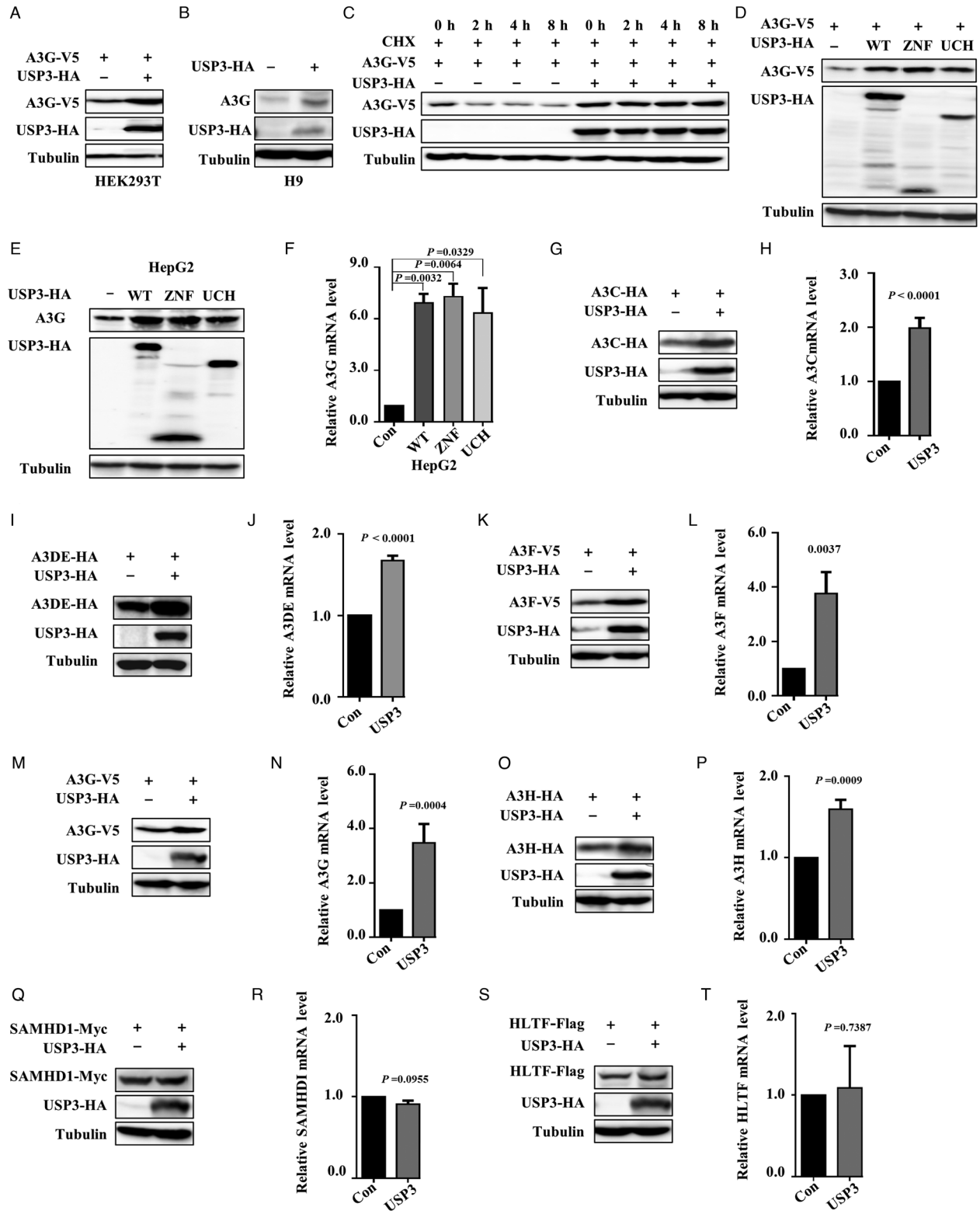

Figure 3: USP3 directly stabilizes A3G expression without Vif. (A) USP3 increases A3G expression without Vif in HEK293T cells. HEK293T cells were transfected with A3G-V5 and USP3-HA and then were analyzed by IB. (B) USP3-HA or control vector was electro-transfected into H9 cells and then analyzed by IB. (C) Overexpression of USP3 prolongs the half-life of A3G. HEK293T cells were transfected and treated with 50 μg/mL of CHX and harvested at the indicated time. (D) USP3 and the two mutants stabilize A3G expression in A3G-transfected HEK293T cells. (E, F) USP3 and the two mutants increase A3G expression at both protein level (E) and RNA level (F) in HepG2 cells. (G–P) USP3 promotes A3C(G-H)/A3DE (I,J)/A3F (K,L)/A3G (M,N)/A3H (O,P) expression in the protein level and mRNA level. (Q,R) USP3 had no effect on the SAMHD1 protein level and mRNA level. (S,T) USP3 had no effect on the HLTF protein level and mRNA level. The statistical significance analyses were performed using Student's *t*-test. A3C: APOBEC3AC; A3DE: APOBEC3DE; A3G: APOBEC3G; A3F: APOBEC3F; A3H: APOBEC3H; CHX: Cycloheximide; Con: Control; HLTF: Helicase-like transcription factor; IB: Immunoblot; mRNA; Messenger ribonucleic acid; SAMHD1: Sterile alpha motif (SAM) domain and histidine-aspartate (HD) domaincontaining protein 1; UCH: USP3-USP catalytic domain; USP3: Ubiquitin-specifific protease 3; Vif: HIV-1 viral infectivity factor; WT: Wild type; ZNF: USP3- zinc finger.

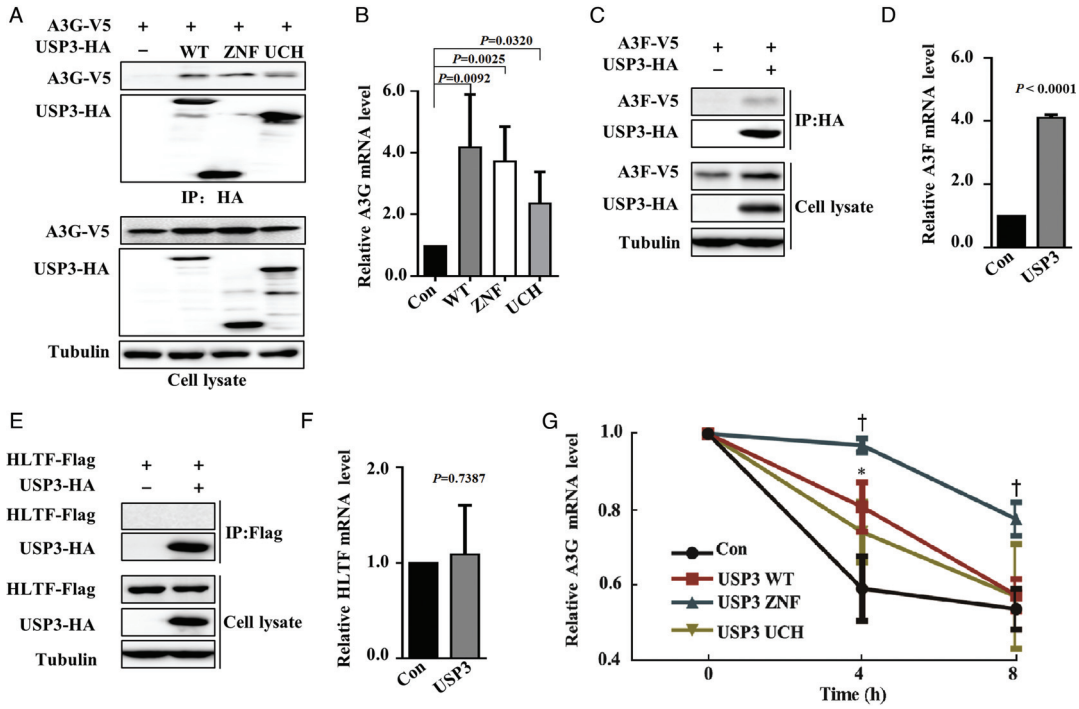

Figure 4: USP3 interacts and stabilizes the A3G mRNA level. (A, B) USP3 interacts with A3G mRNA. HEK293T cells were transfected with A3G-V5, USP3-HA, or mutants, as indicated. Cells were harvested and lysed in lysis buffer supplemented with 20 U Protector RNase inhibitor (Roche) 48 h post-transfection. Cell lysates were immunoprecipitated using anti-HA antibody conjugated to agarose beads. Partial precipitated samples were analyzed by IB (A), and another part of precipitated samples was extracted RNA for RT-qPCR analysis (B). (C, D) USP3 interacts with A3F mRNA. (E, F) USP3 does not interact with HLTF mRNA. (G) Overexpression of USP3 WT or mutants prolongs the half-life of A3G mRNA. HepG2 cells were transfected with empty vector, USP3 WT, or the two mutants; treated with 5 μg/mL of RNA polymerase inhibitor actinomycin D (59144, MCE, Shanghai, China); and harvested at the indicated time. The A3G mRNA level was analyzed by RT-qPCR. The statistical significance analyses were performed using Student's *t*-test. A3F: APOBEC3F; A3G: APOBEC3G; HLTF: Helicase-like transcription factor; IP: Immunoprecipitation; mRNA: Messenger ribonucleic acid; RT-qPCR: Reverse transcription quantitative real-time polymerase chain reaction; UCH: USP3-USP catalytic domain; USP3: Ubiquitin-specifiic protease 3; WT: Wild type; ZNF: USP3-zinc finger. $^{*}P < 0.05$, $^{\dagger}P < 0.01$.

In addition to A3G, the A3 family contains six other members, namely, A3A, A3B, A3C, A3DE, A3F, and A3H, of which A3C, A3DE, A3F, and A3H have various degrees of anti-HIV-1 activity.[12,56] HIV-Vif counteracts their antiviral activity via the same mechanism.[57] Interestingly, we found that USP3 specifically promoted A3 expression at both the protein and mRNA levels in the absence of Vif [Figures 3G–P]. Because other host antiviral factors such as SAMHD1 and HLTF can be degraded by HIV accessory proteins through the UPS,[3,58-61] we then examined the effect of USP3 on the expression of SAMHD1 and HLTF and found that it had no effect at either the protein or mRNA level [Figures 3Q–T]. In summary, USP3 specifically promotes the expression of the A3 family of proteins.

Our data showed that USP3 increased A3G expression not only at the protein level but also significantly at the mRNA level [Figures 3E, F], suggesting that USP3 might affect the transcription of A3G. We examined the interaction between USP3 and the mRNA of different host factors. The results showed that USP3 could directly bind to A3G mRNA [Figures 4A, B] and to A3F mRNA [Figures 4C, D], but not to HLTF mRNA [Figures 4E, F]. These data suggest that USP3 may affect the expression of A3G by regulating or splicing A3G mRNA. The USP3 ZNF mutant showed stronger binding ability with A3G mRNA than with the USP3 UCH mutant and prolonged the A3G mRNA half-life [Figure 4G], indirectly explaining why ZNF mutants can promote A3G expression [Figures 3D–F].

USP3 expression correlates with A3G expression and HIV-1 disease progression

Studies have shown that the expression of A3G in HIV-1 patients is correlated with some important clinical indicators, such as viral load and $CD4^+$ T-cell counts in peripheral blood.[62-65] Here, we found that USP3 can increase A3G expression. To verify whether there is any correlation between USP3 and A3G expression or $CD4^+$ T-cell counts in HIV patients. We performed a correlation analysis and observed positive correlations between USP3 mRNA and A3G mRNA levels [Figure 5A], A3G mRNA level and $CD4^+$ T-cell counts [Figure 5B], and USP3 mRNA levels and $CD4^+$ T-cell counts [Figure 5C]. These results suggest that the expression level of USP3 in $CD4^+$ T cells of HIV-1-infected individuals is involved in HIV-1 disease progression.

Discussion

As the largest subfamily of DUBs, the USP family members have attracted considerable attention. Studies have shown that USP3 plays a crucial role in numerous biological processes by deubiquitinating some host factors.[54,66-70] For example, USP3 regulates cancer progression and metastasis by deubiquitinating the Kruppel-like factor 5 (KLF5), stabilizing p53 or deubiquitination-dependent COL9A3/COL6A5.[53,71,72] USP3 has also been identified as a novel regulator of histone H2A and H2B ubiquitination, highlighting its role in preventing replication stress

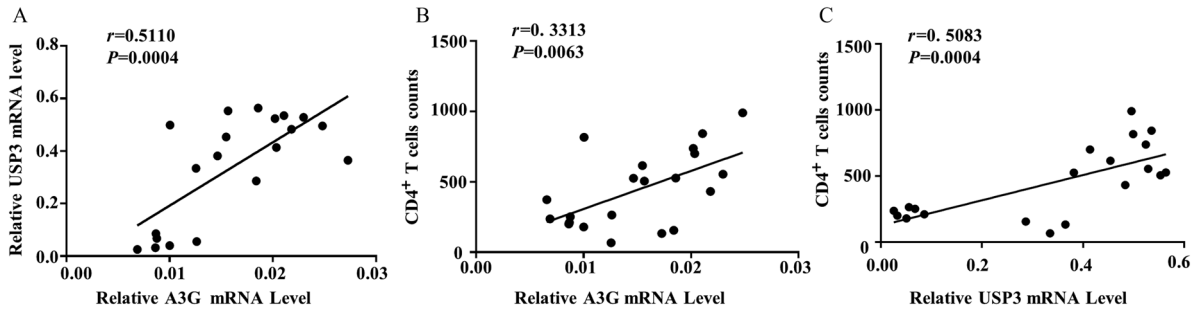

Figure 5: USP3 expression correlates with A3G expression and HIV-1 disease progression. (A) USP3 mRNA level was positively correlated with the A3G mRNA level in the $CD4^+$ T cells isolated from the newly diagnosed HIV-1-infected individuals ($n = 20$). (B) Correlation between the count of $CD4^+$ T cells and A3G mRNA level in the $CD4^+$ T cells isolated from the newly diagnosed HIV-1-infected individuals ($n = 20$). (C) Correlation between the count of $CD4^+$ T cells and USP3 mRNA level in the $CD4^+$ T cells isolated from the newly diagnosed HIV-1-infected individuals ($n = 20$). Pearson correlation coefficient and *P*-value are listed. A3G: APOBEC3G; CD4: Cluster of differentiation 4; HIV: human immunodeficiency virus; mRNA: Messenger RNA; USP3: ubiquitin-specific protease 3.

and suggesting its involvement in the response to DNA double-strand breaks.[55,70] USP3 negatively regulates the activation of type 1 interferon (IFN-I) signaling by specifically targeting Retinoic acid-inducible gene I lysine 63(RIG-I K63)-linked polyubiquitin chains and removing them, resulting in IFN-I inhibition.[54] However, the role of USP3 in viral infections has not been extensively studied.

In our study, we found that knockdown of USP3 in different HIV-1 target cells, A3G-expressing H9 and no-A3G-expressing Jurkat cells, had different effects on HIV-1 production. HIV-1 production in USP3 silencing H9 cells was significantly increased compared to that in the control group, but no significant effect was observed in USP3 silencing Jurkat cells. Because endogenous A3G expression is the major difference between H9 and Jurkat cells, we speculated that USP3 might affect HIV-1 production by stabilizing the antiviral factor A3G. Further experiments found that USP3 indeed inhibited HIV-1 infection by stabilizing A3G in a dose-dependent manner, although it had no effect on HIV production in the absence of A3G. HIV-1 Vif recruits host factors to form a viral-specific CRL5 E3 ubiquitin ligase, which induces poly-ubiquitination and degradation of A3G. Our data showed that as a DUB, USP3 specifically removes A3G poly-ubiquitylation, thereby antagonizing Vif-mediated A3G degradation, resulting in increased A3G expression and enhanced antiviral activity of A3G against Vif-containing HIV-1 [Figure 6]. At the same time, we demonstrated that USP3 inhibition of HIV-1 had no effect on the interaction of Vif with Cul5, ELOB/C, or CBF-β.

According to the functional domain of USP3, we constructed two truncated mutants with or without enzyme activity. Intriguingly, the USP3 ZNF domain without DUB activity also stabilized A3G expression when compared to the WT USP3 or UCH enzyme activity domain of USP3. As expected, the USP3 ZNF domain did not decrease A3G polyubiquitination but still increased the expression of A3G, suggesting that USP3 may affect A3G expression through other mechanisms. We next examined the effect of USP3 on mRNA levels of A3G and found that USP3 upregulated mRNA levels of endogenous A3G in HepG2 cells, even in ZNF and UCH (with weaker ability to increase A3G mRNA) truncated mutants,

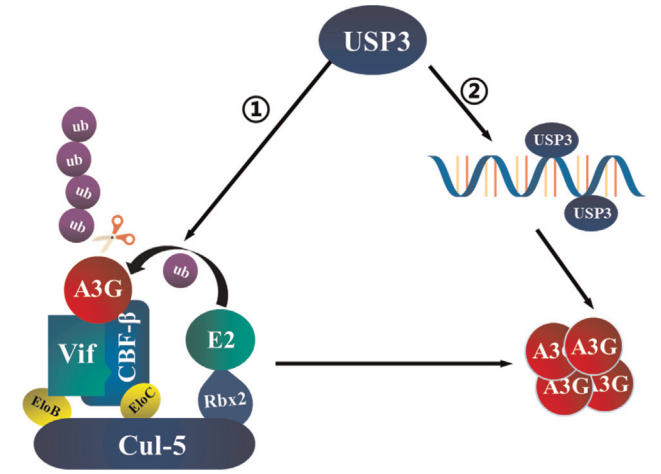

Figure 6: Schematic diagram of USP3 inhibiting HIV replication. Proposed inhibitory mechanism of USP3 on HIV-1 replication occurs through the increase of A3G expression via two mechanisms. First, USP3 deubiquitinates polyubiquitinated A3G resulting in A3G stability, and second, USP3 binds and stabilizes A3G mRNA. A3G: APOBEC3G; CBF-β: Core-binding factor beta subunit; CUL5: Cellular proteins cullin 5; ELOB: Elongin B; ELOC: Elongin C; HIV: Human immunodeficiency virus; mRNA: Messenger RNA; UB: Ubiquitination; USP3: Ubiquitin-specific protease 3.

explaining the reason why ZNF increased A3G expression. Further investigation showed that USP3 and USP3 mutants interacted with A3G mRNA. It is well known that mRNA synthesis, its post-transcriptional modification, and its translation into proteins are complicated processes *in vivo*.[66,73,74] DUBs have been reported to be involved in gene transcription regulation.[43] For example, USP12 aggravates angiotensin II (Ang II)-induced cardiac hypertrophy by enhancing methyltransferase-like 3 (METTL3) expression, which catalyzes N6-methyladenosine modification of mRNA and acts as a harmful factor in pathological cardiac hypertrophy.[20] Surprisingly, we found that USP3 directly increased A3G expression, both exogenous or endogenous, suggesting that USP3 does this through unknown pathways but is not a promoter. Further experiments found USP3 ZNF mutant showed stronger binding ability with A3G mRNA than the USP3 UCH mutant and prolonged the A3G mRNA half-life. The molecular mechanisms by which USP3 specifically regulates the expression of A3 family proteins are interesting and need to be further investigated.

To verify whether USP3 can be an effective target for HIV-1 therapy, we next studied the possible correlation between A3G expression and USP3 expression and disease progression. According to case statistics, we found a positive correlation between A3G mRNA levels and USP3 mRNA levels *in vivo*. $CD4^+$ T-cell count is known to be closely related to HIV viral load and disease progression,[75-78] and our preliminary analysis showed that both A3G and USP3 were positively correlated with $CD4^+$ T-cell count. Overall, our data suggest that USP3 protein expression levels in HIV-1-infected individuals can be used to indicate HIV viral load and may serve as a new biomarker for evaluating the prognosis of HIV-1-infected patients.

In summary, we found that USP3 restricts HIV-1 viral infections by increasing the expression of antiviral factor A3G. Therefore, USP3 may be an important target for drug development and novel therapeutic strategies against viral infections.

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Conflicts of interest

None.

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