The herpes simplex virus 1 UL36USP deubiquitinase suppresses DNA repair in host cells via deubiquitination of proliferating cell nuclear antigen

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Herpes simplex virus 1 (HSV-1) infection manipulates distinct host DNA-damage responses to facilitate virus proliferation, but the molecular mechanisms remain to be elucidated. One possible HSV-1 target might be DNA damage-tolerance mechanisms, such as the translesion synthesis (TLS) pathway. In TLS, proliferating cell nuclear antigen (PCNA) is monoubiquitinated in response to DNA damage-caused replication fork stalling. Ubiquitinated PCNA then facilitates the error-prone DNA polymerase η (polη)-mediated TLS, allowing the fork to bypass damaged sites. Because of the involvement of PCNA ubiquitination in DNA-damage repair, we hypothesized that the function of PCNA might be altered by HSV-1. Here we show that PCNA is a substrate of the HSV-1 deubiquitinase UL36USP, which has previously been shown to be involved mainly in virus uptake and maturation. In HSV-1-infected cells, viral infection-associated UL36USP consistently reduced PCNA ubiquitination. The deubiquitination of PCNA inhibited the formation of polη foci and also increased cell sensitivity to DNA-damage agents. Moreover, the catalytically inactive mutant UL36C40A failed to deubiquitinate PCNA. Of note, the levels of virus marker genes also increased strikingly in cells infected with wild-type HSV-1, but over, the catalytically inactive mutant UL36C40A failed to deubiquitinate PCNA. The deubiquitination activity supports HSV-1 virus replication during infection. These findings suggest a role of UL36USP in the DNA damage-response pathway.

During DNA replication, multiple environmental toxins and pathogen invasions cause DNA damages (1). High-fidelity DNA polymerase cannot accommodate these damaged bases, which leads to stalled and collapsed replication forks, resulting in DNA double-strand breaks (DSB) (2). DSB is a severe lethal damage that increases genome instability (3). To prevent DSB caused by collapsed replication forks, the translesion synthesis (TLS) pathway is activated to bypass DNA-damage sites, which is mediated by proliferating cell nuclear antigen (PCNA) monoubiquitination (2).

PCNA can form a homotrimer clamp that slides along the DNA chain to provide scaffold for replication complex assembly (4). Blockage of the replication fork results in prolonged single-stranded DNA that is rapidly coated by replication protein A (RPA). Then RPA triggers the recruitment of RAD6-RAD18 ubiquitination ligase to monoubiquitinate PCNA on lysine 164 (5). The monoubiquitinated PCNA then promotes the recruitment of polη that belongs to the error-prone Y-family polymerase used in TLS pathway. Polη contains three PCNA interaction protein (PIP) boxes and an ubiquitin-binding zinc finger domain. The PIP domains of polη are less efficient than canonical PIP domains in error-free DNA polymerase, which lead to a minor affinity between polη and PCNA during normal DNA replication. Polη has a flexible active site that can accommodate damaged DNA template, allowing replication complex to bypass the lesion sites, thus preventing replication fork collapse and leaving the damaged sites to be repaired by other error-free pathways (6, 7). These studies described a widely accepted model of the function of ubiquitinated PCNA in TLS. However, a debate on the role of PCNA ubiquitination in TLS arose from recent extensive quantitative studies by Hedglin et al. (8). Their results demonstrated that monoubiquitination of PCNA did not change the binding affinity between PCNA and polη, and the subsequent TLS across a DNA lesion was also independent of PCNA monoubiquitination. They proposed that PCNA monoubiquitination indirectly promotes DNA synthesis by increasing the residence time of polη within the damaged sites, likely through altering the chromatin structure around damaged sites, although further studies are needed to test this hypothesis (8).

Besides multiple chemical or physical stimuli that induce DNA damage, many pathogens also cause cellular DNA damage and even manipulate DNA-damage repair proteins to facil-
ulate their own proliferation (9). HSV-1 is a large double-stranded DNA virus with an icosahedral capsid wrapped by an envelope. The tegument layer between the capsid and envelope contains proteins that are important for virus infection and capsid assembly. HSV-1 infection usually occurs at oral mucosa neurons. After primary infection, virus particles transport along the neuronal axons to the nucleus and establish a latent state. During the latent infection stage, virus DNA is packed into a repressed structure and most genes remain silent. Latent virus can be reactivated by multiple stimuli including UV exposure and transport from the neurons down to primary infection sites, resulting in herpetic stomatitis. The reactivation of HSV-1 can happen multiple times during one’s lifetime. Besides the oral mucosa, HSV-1 also infects genitals and corneal tissues, causing genital herpes or herpes keratitis, respectively (10, 11).

The infection of HSV-1 activates a cellular defense system including DNA-damage response. Multi-virus proteins coordinate these DNA-damage response (DDR) pathways elegantly to facilitate virus DNA replication. It has been reported that the ATM signaling pathway is activated during HSV-1 infection, whereas ATR and DNA-PKCs pathways are inhibited (1, 12). In addition to function in the DDR signaling pathway, PCNA and several other DDR proteins including RPA, DNA-PKCs, Rad50, Ku86, Ku70, PARP1, and Mre11 have also been observed to aggregate at the HSV-1 virus replication compartment (13–15). However, the putative roles of PCNA in viral replication remain poorly documented.

UL36 (VP1/2) is the largest tegument protein of HSV-1 containing more than 3000 amino acids, which helps HSV-1 viruses enter cells (16, 17). When most tegument proteins are released into the cytoplasm, UL36 remains attached to capsid and facilitate viral DNA entering the nucleus. This direct attachment enables UL36 to play a central role in the whole tegument structure assembly and virions maturation (18–20). In addition, the N-terminal of UL36 cleaved from the full-length protein has been reported to possess deubiquitination activity (21). This fragment contains around 500 amino acids and named as UL36 ubiquitin-specific protease (UL36USP). Cysteine at position 40 in the HSV-1 F strain has been recognized as the active site for UL36USP deubiquitination activity (22). This mutation of Cys at 40 to Ala (C40A) causes a significant loss of deubiquitination activity (22), next we explored whether the deubiquitination of PCNA by UL36USP was dependent on its deubiquitination activity. Cells were transfected with UL36USP or UL36C40A and the ubiquitination of PCNA was examined by a His pulldown assay after treatment with ultraviolet (UV) or H2O2. As expected, both UV and H2O2 promoted PCNA ubiquitination efficiently, and UL36USP inhibited PCNA ubiquitination completely, whereas the UL36C40A mutant exhibited no capability of deubiquitinating PCNA with or without DNA-damage stimuli (Fig. 1, C and D). The His pulldown experiment using UV as DNA-damage stimuli were repeated three times and quantified with Odyssey software. The intensity of ubiquitinated PCNA was normalized to that of the empty vector transfected but not stimulated group, and the deubiquitination effect of UL36USP on PCNA ubiquitination was significant with p value equaling 0.0076 (Fig. 1E).

To avoid the influence of ubiquitin overexpression in the His pulldown assay described above and confirm the deubiquitinating activity of UL36USP, we performed a chromatin fractionation assay. Cells transfected with UL36USP or C40A were subjected to UV or H2O2 treatments, and endogenous chromatin-associated proteins were isolated by using Triton X-100 containing buffer. Monoubiquitinated PCNA should be concentrated in the Triton X-100-insoluble fraction (TIF), as it is associated with the chromatin. Indeed, UL36USP reduced accumulation of ubiquitinated PCNA induced by UV (Fig. 1F) or H2O2 treatments (Fig. 1G), whereas the UL36C40A mutant had no such effect. Interestingly, we also found an unexpected
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A. His-Ubi, H₂O₂ 30 min

B. UL36

C. His-Ubi

D. His-Ubi

E. Normalized intensity of ubiquinated PCNA (%)

F. UV (8 h)

G. H₂O₂ (30 min)

H. 2 μg  5 μg

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band that migrated slower than UL36C40A itself but not wild-type UL36USP (Fig. 1, F and G), which was similar to the results of ubiquitinated VP1–2 NT1 C65A in a previous report (24). This observation suggests that UL36C40A might be ubiquitinated, which will be discussed later. These results demonstrate that UL36USP debiquitinates PCNA in vivo, whereas UL36C40A loses its debiquitinating activity on PCNA, suggesting that viral protein UL36USP is involved in the TLS pathway.

**UL36USP removes ubiquitin from PCNA in vitro**

The in vivo experiments indicate that UL36USP debiquitinates PCNA by its catalytic activity, to detect the direct debiquitination of PCNA by UL36USP, we performed an in vitro debiquitination experiment. Ubiquitinated PCNA was enriched by Ni²⁺ beads in Nonidet P-40 containing cell lysis buffer, and washed with debiquitinating buffer three times. UL36USP or -C40A recombinant proteins purified from Escherichia coli were incubated with ubiquitinated PCNA at 30 °C following an experimental procedure reported by Huang et al. (26). As shown in Fig. 1H, UL36USP-deubiquitinated PCNA significantly, whereas the UL36C40A mutant did not. The result of the in vitro experiment confirms that UL36USP debiquitinates PCNA directly by its catalytic activity but not through other proteins.

**DNA damage stimulates the interaction between UL36USP and PCNA**

The observations that UL36USP-deubiquitinated PCNA spurred us to determine whether UL36USP interacted with PCNA, we then performed a co-immunoprecipitation (co-IP) assay to verify this hypothesis. HEK293T cells transfected with FLAG-UL36USP and HA-PCNA were treated with or without UV irradiation, cell lysates were immunoprecipitated using anti-FLAG antibody. As shown in Fig. 2A, the interaction between UL36USP and PCNA was detected in the presence of DNA-damage stimuli, suggesting that DNA damage stimulates the interaction of UL36USP with PCNA. We further investigated the interaction between UL36USP and endogenous PCNA after UV treatment for different times by using anti-FLAG® M2 magnetic beads (Fig. 2B). The interaction increasing with time prolongs and reached the maximum at 9 h after UV treatment, consistent with the result in Fig. 2A. This result indicates that UL36USP could respond to DNA damage by increasing its affinity with PCNA. Next, we investigated the interaction of both exogenous (Fig. 2C) and endogenous (Fig. 2D) PCNA to UL36USP and UL36C40A after DNA-damage stimuli. Both wild-type UL36USP and UL36C40A mutant associated with PCNA (Fig. 2, C and D), suggesting that the deubiquitination activity of UL36USP has no effect on its binding to PCNA.

**UL36USP interacts with PCNA by PIP**

A PIP domain is found in UL36USP (Fig. 2E), which is conserved in herpes viruses (27). As the two conserved phenylalanines in the PIP domain play a crucial role in PIP-mediated PCNA interaction (27–31), we thus mutated these two residues to alanines (UL36PIP, Fig. 2E), and examined whether this mutant still interacted with PCNA. The result showed that mutation of the key residues in the PIP domain abolished its interaction with PCNA (Fig. 2E), suggesting that the PIP domain is critical in the UL36USP-PCNA interaction.

Moreover, to investigate whether mutation of the PIP domain will also affect the debiquitination of PCNA by UL36USP, HEK293T cells transfected with FLAG-UL36USP or FLAG-UL36PIP or FLAG-UL36C40A mutants together with His-ubiquitin were subjected to UV stimulation, and then the levels of ubiquitinated PCNA were detected by a His pulldown assay (Fig. 2F). As expected, UV induced a significant increase of PCNA ubiquitination in the empty vector expressing group, whereas no ubiquitinated PCNA was detected in cells transfected with wild-type UL36USP. Similar to UL36C40A, the UL36PIP mutant could not inhibit PCNA ubiquitination. This result suggests that the PIP domain contributes to the deubiquitination process by promoting the interaction between UL36USP and PCNA.

**UL36USP inhibits polγ foci formation after DNA damage stimuli**

Because UL36USP debiquitinated PCNA, we wondered whether it would compromise the accumulation of polγ at replication stalling sites. HeLa cells transfected with FLAG-UL36USP, -C40A, or empty vector plasmids were treated with or without UV, and then subjected to immunofluorescence staining. Endogenous polγ foci were detected by anti-polγ antibody, whereas UL36USP or -C40A were labeled using anti-FLAG monoclonal antibody. Images were taken by Zeiss 710...
confocal microscope. Cells with more than 10 pol\(\eta\) focus were considered positive, and the percentage of pol\(\eta\) foci-positive cells were counted, respectively, in three groups and then normalized to the empty vector group. This experiment was conducted three times and around 1000 cells for each group were counted and processed by using Bitplane Imaris software. Notice that due to low transfection efficiency in HeLa cells, only cells expressing UL36USP or -C40A (labeled green) were counted in each group (Fig. 3A). As expected, UL36USP decreased both the number of pol\(\eta\) foci and percentage of pol\(\eta\) foci-positive cells induced by DNA-damage stimuli. However, catalytically inactive UL36C40A expressing cells exhibited an equivalent percentage of pol\(\eta\) foci-positive cells to that of control (Fig. 3, A and B). The expression of UL36USP or UL36C40A
in cells that used immunofluorescence were detected at the same time. Quantification of ubiquitinated PCNA were normalized relative to Lamin B. Consistent with the reduced pol\(\gamma\)/H9257 foci, a decreased ubiquitinated PCNA level was observed in UL36USP-expressing cells in comparison to the control or UL36C40A group (Fig. 3C). The relatively moderate deubiquitinating effect of UL36USP might be due to a restricted transfection efficiency in HeLa cells compared with that in HEK293T cells. These results indicate that UL36USP inhibits the TLS pathway via deubiquitinating PCNA and inhibiting pol\(\gamma\) foci formation.

**Viral infection-associated UL36USP deubiquitinates PCNA**

Because UL36USP transient expression caused significant deubiquitination of PCNA (Fig. 1), we further explored the effect of viral infection-associated UL36USP on PCNA during HSV-1 virus infection. We infected HEK293T cells with wild-type or UL36C40A HSV-1 virus and then chromatin-associated PCNA was enriched in TIF after chromatin fractionation (Fig. 4A). The infection of the catalytically inactive UL36C40A mutant virus resulted in a significant increase of PCNA ubiquitination, suggesting activation of the TLS pathway. On the contrary, cells infected with wild-type HSV-1 virus exhibited an equivalent level of PCNA ubiquitination to that of the mock infection group. These data suggest that wild-type HSV-1 virus infection also induces a DNA-damage response, which is compromised by the deubiquitination activity of virus infection-associated UL36USP.

Because the level of ICP8 in cells infected by wild-type virus is higher than that in cells with HSV-1 UL36C40A mutant virus infection (Fig. 4A), we therefore assumed that HSV-1 facilitates its own replication through the deubiquitination activity of UL36USP. To assess this prediction, we performed quantitative RT-PCR to examine the replication rate of wild-type and UL36C40A mutant virus by determination of the levels of two virus DNA marker genes, thymidine kinase (\(tk\)) and \(vp16\). The levels of both \(tk\) (Fig. 4B) and \(vp16\) (Fig. 4C) increased strikingly in cells infected with wild-type HSV-1
but moderately in the UL36C40A mutant virus-infected group, indicating a higher replication rate of wild-type virus than C40A virus. These results demonstrate that the deubiquitinating activity of UL36USP facilitates HSV-1 virus DNA replication during infection.

Stably expressed UL36USP decreases cellular viability after DNA damage

When suffering from DNA damage, cells with an activated TLS pathway would survive better than those with a deficient TLS pathway (6). To detect whether UL36USP affected cellular sensitivity to DNA damage, we constructed HeLa cells stably expressing FLAG-UL36USP or FLAG-UL36C40A using the pLVX-IRES-zsGREEN lentivirus system as described under “Experimental procedures.” Monoclonal cells stably expressing UL36USP or -C40A were selected and stimulated with or without UV, and then the level of PCNA ubiquitination and cell viability were assessed. The UL36USP expressing cells showed a significant decrease of PCNA ubiquitination after exposure to UV irradiation, whereas UL36C40A expressing cells exhibited a much higher level of PCNA ubiquitination, which is similar to the control cells (Fig. 5A). Next, we examined the cell viability in these monoclonal cell lines treated with UV irradiation in different doses. 12 days later, the cell survival rates were calculated and normalized to that of cells without damage, respectively. DNA damage induced a decrease of cell numbers in all cell lines with the increase of UV intensity. However, UL36USP stably expressing cells exhibited a significantly lower survival rate compared with that of wild-type or UL36C40A-expressing cells (Fig. 5B). These results indicate that wild-type UL36USP leads to decreased cell viability by interrupting the cellular DNA damage repair ability.
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Because the ubiquitin-proteasome system is a classic pathway for protein degradation, the unexpected band that migrated slower than UL36C40A itself observed in Figs. 1, F and G, and 2, C and D, prompted us to examine whether UL36USP or -C40A is ubiquitinated and degraded as an invading virus protein. The result of the His pulldown revealed multiple ubiquitinated bands of UL36C40A, whereas the level of ubiquitinated wild-type UL36USP was much lower. Also, the ubiquitination of UL36USP and -C40A was not affected by DNA damage stimulus (Fig. 6A). It is possible that ubiquitinated UL36USP could be deubiquitinated by itself, whereas the catalytic null mutant UL36C40A lost its deubiquitinating activity and maintained ubiquitin conjugates. Next, to further detect the degradation of UL36USP and -C40A, HEK293T cells stably expressing UL36USP or -C40A were treated with cycloheximide (CHX) for different times, and then the amount of UL36C40A was assessed. As shown in Fig. 6B, the level of UL36C40A decreased with a prolonged treatment time, whereas UL36USP abundance had no change (Fig. 6B). These results suggest that UL36C40A is degraded, whereas UL36USP stays stable after CHX treatment. Moreover, to explore whether the degradation of UL36C40A is executed by a ubiquitin-dependent proteasome system, UL36USP or -C40A stably expressing cells were treated with the proteasome-specific inhibitor MG132 and protein abundance was examined by Western blotting (Fig. 6C). UL36USP had no significant increase after 1 h of MG132 treatment, whereas ubiquitinated UL36C40A increased obviously (Fig. 6C). These results demonstrate that UL36USP deubiquitinates itself, whereas ubiquitinated UL36C40A is more sensitive to CHX and MG132 treatment, suggesting that the deubiquitination activity of UL36USP is required for its own stability.

Discussion

As the largest tegument protein in HSV-1, the exploration of UL36 function has mainly focused on its role in viral maturation. Mounting evidence demonstrates that UL36 participates in virus uptake and the tegument assembly process (18, 32–36). Moreover, UL36USP, a deubiquitinating enzyme embedded in the N-terminal of UL36, could target both K48 and K63 polyubiquitin chains of TRAF3 and shows self-deubiquitinating activity (21, 22, 24). However, whether UL36USP manipulates other cellular pathways such as DDR remains unclear. In this study, we demonstrate PCNA as a new substrate of HSV-1 UL36USP. The DNA damage-induced PCNA ubiquitination
is suppressed by wild-type UL36USP but not UL36C40A mutant, suggesting that PCNA is a cellular substrate of HSV-1 UL36USP.

DNA-damage response is used as a defense to viral infection. On the contrary, to inhibit the cellular antiviral defense, viruses will manipulate cellular pathways to change the hostile cellular environment to support its own replication. Previous studies indicate that HSV-1 regulates DDR pathways when the viral genome is transported to the cell nucleus. HSV-1 infection is found to increase single strand annealing and the Fanconi anemia (FA) pathway while decreasing homologous recombination (HR), non-homologous end joining (classic NHEJ), and microhomology-mediated end joining (MMEJ) pathways, although the detailed mechanisms remained to be elucidated (37, 38). In addition to these pathways, our data here indicates that the TLS pathway is also suppressed by HSV-1 through the deubiquitination activity of UL36USP, adding a new member into the cellular DDR network manipulated by HSV-1 infection. DDR pathways usually function coordinately with each other. For example, interstrand DNA cross-link repair requires cross-talk among TLS, FA network, and HR repair pathways. It is possible that TLS suppression by HSV-1 infection may coordinate with other DDR pathways. Therefore, additional studies are required to explore how UL36USP-mediated TLS correlates with other pathways including HR and FA in HSV-1-infected cells.

To overcome the cellular defense system and facilitate virus replication, as we found in this study, one possible mechanism is to inactivate the TLS pathway and hijack DNA-damage repair proteins to aid its own proliferation. In accordance with this scenario, during HSV-1 virus infection, certain cellular proteins including PCNA that interacts with ICP8 have been observed at the virus DNA replication center (13, 14). Besides, PCNA has been found to be important for HSV-1 virus DNA replication and histone deposition on the virus genome (25). In this study, we found that cells infected with the UL36C40A mutant virus showed a lower viral DNA replication rate than that with wild-type HSV-1. Consistently, we also found that cells infected by HSV-1 wild-type or UL36C40A mutant virus exhibited a significant difference in the accumulations of ubiquitinated PCNA on chromatin (Fig. 4). Our mechanistical studies revealed that in response to DNA damage, UL36USP interacts with and deubiquitinates PCNA, resulting in a reduced number of polη foci and decreased cell viability (Fig. 7). Although the role of PCNA ubiquitination in TLS is controversial, our findings here indicate that HSV-1 infection-associated UL36USP suppresses the cellular TLS pathway by deubiquitinating PCNA, and facilitates viral DNA replication as well, providing insight for understanding how viruses fight against the cellular defense system to benefit its own replication (Fig. 7). However, additional studies such as efficient detection of mutation frequency (39–42) will further help us to elucidate the effect of UL36USP on the function of polη in TLS. Moreover, as mentioned in our working model, whether deubiquitination of PCNA by UL36USP is directly associated with viral DNA replication or if UL36USP...
targets other host proteins, these questions remain to be elucidated in the future.

The UL36USP catalytic motif is conserved among the herpesvirus family including Kaposi’s sarcoma-associated herpesvirus (KSHV), Epstein-Barr virus (EBV), human cytomegalovirus, and pseudorabies virus (23). Similar to what we found here, BPLF1, the UL36USP homolog in EBV, has also been reported to deubiquitinate PCNA during EBV infection (27). The E3 ubiquitin ligase of PCNA, Rad18, is also a substrate of BPLF1, which is stabilized by BPLF1 deubiquitinating activity during EBV infection (43). It is interesting that both PCNA and its E3 ligase are substrates of the same deubiquitinase. Whether HSV-1 UL36USP could also deubiquitinate Rad18 is worthy to be tested. Different from UL36USP that only possesses deubiquitinating activity, BPLF1 also acts as a deeddylase in addition to its deubiquitinating activity, which inhibits culling-ring ligase activity and leads to a deregulation of the cell S phase that benefits virus replication (44). Besides, the deubiquitinating activity of UL48, a homogenous protein of UL36 in human cytomegalovirus, also plays a crucial role in virus replication (45). Together, these studies indicate that through their ubiquitin-specific protease, herpes viruses modulate DDR pathways to facilitate virus proliferation.

A previous report (24) indicated that loss of UL36 deubiquitinating activity resulted in a drastic reduction in the full-length protein level, which could be stabilized by proteasome inhibition, and studies of the stability of different HSV-1 UL36 truncations suggest that functional USP is required for stabilizing the fragments longer than the core USP domain. Consistently, here we also showed that UL36USP, which possesses deubiquitinating activity, could deubiquitinate and stabilize itself, whereas the strongly ubiquitinated UL36C40A is highly sensitive to CHX and MG132 treatment. These works together suggest that the deubiquitinating activity of UL36USP contributes, at least partially, to its own stability. This work identified a new cellular substrate for UL36USP, demonstrated that the deubiquitinase of HSV-1 contributes to viral replication, and therefore enriched the knowledge of HSV-1 manipulation of cellular DNA damage pathways.

**Experimental procedures**

**Cell lines, culture, and transfection**

HEK293T, HeLa, and VERO cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco). Cells were grown at 37 °C in a humidified CO₂ (5%) incubator, and transfected with polyethylenimine.

**Plasmids and reagents**

The 1485 bp encoding the deubiquitinase at the N terminus of UL36 protein was amplified by PCR and inserted into pcDNA3.0–3FLAG and pET28a vectors. UL36C40A and UL36CIP mutants were constructed by the point mutation method. The following antibodies were used in this study: anti-PCNA (BE0029, EASYBIO; 1:1000), anti-pol1 (BS6695, Bio-world; 1:100), mouse monoclonal anti-FLAG antibody (F3165, Sigma; 1:2000), anti-HA antibody (H9658, Sigma; 1:2000), mouse monoclonal anti-His antibody (D291–3, MBL, Nagoya, Japan; 1:2000), and anti-HSV-1 ICP8 antibody (A0914, Santa Cruz Biotechnology; 1:100).

**HSV-1 virus infection and propagation**

Wild-type HSV-1 F strain and UL36C40A mutant strain were propagated in VERO cells and cultured in DMEM containing 10% FBS. When cell density reached 90%, DMEM was removed and HSV-1 virus were added to the cells at a m.o.i. of 5–10, and then incubated at 37 °C. One h later, fresh DMEM containing 10% FBS was added and continuously cultured until a clear cytopathic effect was shown. Cells were collected in DMEM and placed at −80 °C for 1 day, and then thawed on ice and the freeze-thaw cycle was repeated for three times. The virus was stored at −80 °C after titration.

**Co-immunoprecipitation**

In Fig. 2A, HEK293T cells transfected with HA-PCNA and FLAG–UL36USP plasmids were lysed in Nonidet P-40 cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 1 mM EDTA-2Na) and sonicated on ice using a Bioruptor Plus device. Immunoprecipitation and Western blotting were performed as described previously (46). In Fig. 2B, HEK293T cells transfected with HA-PCNA and FLAG–UL36USP or UL36C40A plasmids were lysed and sonicated as previously described. Cell lysates were centrifuged at 12,000 rpm for 15 min. FLAG-M2 beads were incubated with the supernatants for 3 h and then washed with cell lysis buffer 3 times. After being centrifuged at 3,000 rpm for 5 min, the supernatants were discarded, and the beads were rinsed with FLAG peptide for 3 h and centrifuged at 3,000 rpm for 5 min. The supernatants were collected and boiled with 6× SDS loading buffer. Then samples were subjected to SDS-PAGE and Western blot analyses.

**His-ubiquitin pulldown assay**

HEK293T cells were transfected with His-ubiquitin and FLAG–UL36/C40A plasmids, and then lysed with His pulldown lysis buffer containing 6 M guanidinium chloride, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0, 5 M imidazole, and 10 mM β-mercaptoethanol. Cell lysates were incubated with nickel-nitrilotriacetic acid beads (Qiagen) for 4 h, and then washed with His pulldown wash buffer I (6 M guanidinium chloride, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0), buffer II (8 M urea, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 8.0), buffer III (8 M urea, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 6.3, 0.2% Triton X-100), and buffer IV (8 M urea, 0.1 M Na₃HPO₄/NaH₂PO₄, 0.01 M Tris-HCl, pH 6.3, 0.1% Triton X-100), respectively. The samples were centrifuged and the supernatant was denatured and loaded on a SDS-PAGE gel. Western blotting was performed using the indicated antibody. For detailed procedures see our previous article (47).

**Chromatin fractionation assay**

HEK293T cells were transfected with UL36USP or UL36C40A plasmids, at 42 h after transfection, cells were lysed with chromatin isolation buffer A containing 10 mM HEPES, pH 7.8, 10 mM KCl, 1.5 mM MgCl₂, 0.34 mM sucrose, 10% glycerol, 1 mM DTT, 0.1% Triton X-100, and 0.5% protease inhibitor, and incubated on ice for 30 min, and then centrifuged at 5,000 rpm for 5
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min. The insoluble fraction was recovered and resuspended in buffer B (3 mM EDTA, 0.2 mM EGTA, 1 mM DTT, 0.5% protease inhibitor) and incubated on ice for 30 min. After centrifugation at 5,000 rpm for 5 min, the insoluble fraction was recovered and resuspended in buffer C (50 mM Tris, 150 mM NaCl, 2% Nonidet P-40, 1 mM EDTA-2Na), and incubated on ice for 10 min. Samples were denatured in 2× SDS loading buffer and separated by SDS-PAGE, and subjected to Western blot analysis.

In vitro deubiquitination assay

HEK293T cells were transfected with HA-PCNA and His-ubiquitin plasmids and cultured for 48 h. Cells were treated with 40 J/m² for 8 h to promote the level of ubiquitinated PCNA, and lysed in cell lysis buffer using a ultrasonic cell disruption system. After centrifugation at 13,000 rpm for 10 min, the supernatant was collected and divided into six equal groups. The ubiquitinated PCNA was concentrated by incubating with 30 μl of nickel-nitritotriacetic acid beads for 4 h. The beads were washed with deubiquitinating buffer (60 mM HEPES, 5 mM MgCl₂, 4% glycerol, pH 7.6) three times and incubated with UL36USP or -C40A protein at 30 °C overnight (26). The reaction mixture was boiled in 2× SDS loading buffer and subjected to SDS-PAGE and Western blotting.

Immunofluorescence staining

HeLa cells cultured on coverslips were transfected with FLAG-UL36USP or -C40A plasmid and cultured for 48 h. Cells were treated with or without 40 J/m² for 8 h and washed with cold PBS, and then fixed with methyl alcohol at −20 °C for 10 min. After washing with PBS, cells were blocked with 1% BSA in PBS, and then incubated with primary antibody at 37 °C for 1 h. Cells were washed with PBS and incubated with secondary antibody at 37 °C for 1 h. DAPI were used to stain the nucleus. Images were captured by Zeiss LSM710 and analyzed with Bitplane Imaris software.

Quantitative RT-PCR

VERO cells were infected with HSV-1 wild-type or C40A mutant virus, and incubated with lysis buffer containing proteinase K at a final concentration of 100 μg/ml at 37 °C overnight. DNA was extracted using phenol/chloroform and precipitated with ethanol, and resuspended with TE buffer. qRT-PCR was performed using Bio-Rad system. The primers used were: vp16-F, GCCGCCCGTACCTCGTGAC; vp16-R, CAGCGCGCCTCGCTTTCG; tk-F, GTATGATGACACA-TGAA; tk-R, GAGGTTCACCGCACAAGA; GAPDH-F, TCTCTGCCCTCTGCGT; GAPDH-R, ATGGTTCACCCATGACGA.

Construction of cells lines stably expressing UL36USP or UL36C40A

HEK293T cells were transfected with pLVX-IRES-ZsGreen1 carrying FLAG-UL36USP or -C40A genes together with pLP2/R-1, pLP/VSVG, and pLP1/pRRE. The medium was changed at 6 and 24 h, respectively, after transfection. Another 10 ml of DMEM containing 5% FBS was added at 48 h, and the medium containing virus was collect 72 h after transfection. The medium containing virus was used to infect HeLa cells, at 48 h after infection, cells stably expressing UL36USP or -C40A were isolated with fluorescence-activated cell sorting (FACS).

Cell viability

HeLa cells stably expressing UL36USP or -C40A were seeded into six-well plates. 24 h later, cells were subjected to gradient UV stimulation and cultured for 12 days, and then stained with crystal violet. Colony numbers were counted.

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