SIVA1 directs the E3 ubiquitin ligase RAD18 for PCNA monoubiquitination

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Introduction

The genomes of all living cells are under continuous attack from a variety of endogenous and exogenous DNA-damaging agents, which lead to many types of DNA lesions. These DNA lesions can block genome replication and transcription and, if not repaired, yield mutations or wilder scale genome aberrations that threaten the survival of the individual cells and the whole organism. To counteract the deleterious effects of damaged DNA, organisms have evolved a variety of surveillance and repair mechanisms (Jackson and Bartek, 2009; Ciccia and Elledge, 2010). Translesion DNA synthesis (TLS) is a universal DNA damage tolerance mechanism conserved from yeast to mammals. A key event in the regulation of TLS is the monoubiquitination of proliferating cell nuclear antigen (PCNA). Extensive evidence indicates that the RAD6–RAD18 ubiquitin-conjugating/ligase complex specifically monoubiquitinates PCNA and regulates TLS repair. However, the mechanism by which the RAD6–RAD18 complex is targeted to PCNA has remained elusive. In this study, we used an affinity purification approach to isolate the PCNA-containing complex and have identified SIVA1 as a critical regulator of PCNA monoubiquitination. We show that SIVA1 constitutively interacts with PCNA via a highly conserved PCNA-interacting peptide motif. Knockdown of SIVA1 compromised RAD18-dependent PCNA monoubiquitination and Pol η focus formation, leading to elevated ultraviolet sensitivity and mutation. Furthermore, we demonstrate that SIVA1 interacts with RAD18 and serves as a molecular bridge between RAD18 and PCNA, thus targeting the E3 ligase activity of RAD18 onto PCNA. Collectively, our results provide evidence that the RAD18 E3 ligase requires an accessory protein for binding to its substrate PCNA.

Translesion DNA synthesis (TLS) is a universal DNA damage tolerance mechanism conserved from yeast to mammals. A key event in the regulation of TLS is the monoubiquitination of proliferating cell nuclear antigen (PCNA). Extensive evidence indicates that the RAD6–RAD18 ubiquitin-conjugating/ligase complex specifically monoubiquitinates PCNA and regulates TLS repair. However, the mechanism by which the RAD6–RAD18 complex is targeted to PCNA has remained elusive. In this study, we used an affinity purification approach to isolate the PCNA-containing complex and have identified SIVA1 as a critical regulator of PCNA monoubiquitination. We show that SIVA1 constitutively interacts with PCNA via a highly conserved PCNA-interacting peptide motif. Knockdown of SIVA1 compromised RAD18-dependent PCNA monoubiquitination and Pol η focus formation, leading to elevated ultraviolet sensitivity and mutation. Furthermore, we demonstrate that SIVA1 interacts with RAD18 and serves as a molecular bridge between RAD18 and PCNA, thus targeting the E3 ligase activity of RAD18 onto PCNA. Collectively, our results provide evidence that the RAD18 E3 ligase requires an accessory protein for binding to its substrate PCNA.

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Abbreviations used in this paper: HU, hydroxyurea; MBP, maltose-binding protein; MMC, mitomycin C; PCNA, proliferating cell nuclear antigen; PIP, PCNA-interacting peptide; RFC, replication factor C; SUMO, small ubiquitin-like modifier; TLS, translesion DNA synthesis.

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Figure 1. Identification of SIVA1 as PCNA-binding partner. (A and D) HEK293T cells stably expressing either the vector control or SFB-tagged (S tag, Flag epitope tag, and streptavidin-binding peptide tag) PCNA/SIVA1 were used for tandem affinity purification of protein complexes. The final eluates were resolved on SDS-PAGE gels and Coomassie stained. (B and E) Tables are summaries of proteins identified by mass spectrometry analysis. Letters in bold on June 27, 2017 Downloaded from Published June 23, 2014
CRL4(Cdt2) E3 ubiquitin ligase in the absence of external DNA damage (Terai et al., 2010). In mammals, monoubiquitinated PCNA has been reported to have a much higher affinity than unmodified PCNA for Polh (Haracska et al., 2001a; Kannouche et al., 2004; Watanabe et al., 2004; Terai et al., 2010). This is in line with the identification of ubiquitin-binding domains in all Y family polymerases that might contribute to the increased interaction of PCNA and TLS polymerases after UV irradiation (Haracska et al., 2001b, 2002; Bienko et al., 2005; Plosky et al., 2006; Schmutz et al., 2010). Because untimely DNA synthesis by low-fidelity TLS polymerases could result in a higher mutagenesis rate, monoubiquitination of PCNA is kept in check by the USP1–UAF1 deubiquitinase complex (Huang et al., 2006; Cohn et al., 2007; Kim et al., 2009). Depletion of USP1 or UAF1 in human cells results in increased levels of monoubiquitinated PCNA both in the presence and absence of DNA damage (Huang et al., 2006; Cohn et al., 2007; Kim et al., 2009). There is growing evidence that the level of monoubiquitinated PCNA is closely linked with the DNA damage bypass to protect cells from a high level of mutagenesis. However, it still remains unclear how the level of monoubiquitinated PCNA is regulated.

SIVA1 is a small protein originally identified as an intracellular ligand for CD27 (Prasad et al., 1997; Xue et al., 2002). The structure of SIVA1 protein contains a death domain homology region in the central part and two zinc finger-like cysteine-rich domains in the C terminus (Prasad et al., 1997; Xue et al., 2002). SIVA1 plays a role both in extrinsic and intrinsic apoptotic pathways (Prasad et al., 1997; Xue et al., 2002; Resch et al., 2009). Recently, SIVA1 has also been shown to act as a suppressor of p53 (Du et al., 2009; Wang et al., 2013). Here, we identified SIVA1 as a critical regulator of PCNA monoubiquitination in response to UV-induced DNA damage. SIVA1 specifically interacts with RAD18 and PCNA in vivo and in vitro and displays properties of a substrate-specific adaptor. Depletion of SIVA1 compromised RAD18-dependent PCNA monoubiquitination and Polh focus formation, leading to increased UV sensitivity and mutation frequency. Our results provide new insights into the molecular mechanism that regulates PCNA monoubiquitination and TLS in response to UV damage.

Results
Identification of SIVA1 as a PCNA-binding protein
To identify previously undetected interacting partners of PCNA, we performed tandem affinity purification using lysates prepared from HEK293T cells ectopically expressing SFB (streptavidin-Flag–S protein)-tagged wild-type PCNA (Fig. 1 A). Mass spectrometry analysis identified many known PCNA-binding proteins, including PAF15, FEN1, and replication factor C (RFC) complex (Fig. 1 B; Emanuele et al., 2011; Povlsen et al., 2012). Interestingly, we also repeatedly identified SIVA1 as a putative PCNA-binding protein (Fig. 1 B). The presence of SIVA1 in PCNA–protein complexes was confirmed by Western blotting (Fig. 1 C).

To ensure that SIVA1 indeed forms a complex with PCNA, we performed reverse tandem affinity purification using HEK293T cells stably expressing SFB-tagged SIVA1 and identified PCNA as a major SIVA1–associated protein (Fig. 1, D and E). The presence of PCNA in SIVA1 purification was also confirmed by Western blotting (Fig. 1 F). Collectively, these results indicate that SIVA1 is a bone fide PCNA-interacting protein.

SIVA1 interacts with PCNA in vivo and in vitro
To verify our tandem affinity purification results, we performed coimmunoprecipitation assays using SFB-tagged SIVA1 and Myc-tagged PCNA. We found that SIVA1, like p21, interacts strongly with PCNA (Fig. S1 A). In contrast, the unrelated control protein Morc3 (Takahashi et al., 2007) does not interact with PCNA (Fig. S1 A). We further performed coimmunoprecipitation experiments and confirmed the interaction between endogenous SIVA1 and PCNA (Fig. 1, G and H; and Fig. S1, B and C). The SIVA1–PCNA complex formation was DNA damage independent, and this preexisting complex could be detected in HeLa cells (Fig. S1 D) as well as in other cell lines including HEK293T cells (Fig. S1 E).

We next tested the possibility that there could be a direct protein–protein interaction between PCNA and SIVA1. Pull-down assays using recombinant maltose-binding protein (MBP)–tagged PCNA and GST-tagged SIVA1 purified from Escherichia coli demonstrated that SIVA1 binds directly to PCNA in vitro (Fig. 1 I). PCNA and several other proteins involved in DNA metabolism could form discrete foci in the nucleus. Given that SIVA1 forms a complex with PCNA, we speculate that SIVA1 may colocalize with PCNA at nucleus foci. As expected, SIVA1 could form discrete nuclear foci and colocalized with PCNA (Fig. 1 J). Collectively, these results established that SIVA1 binds PCNA and suggested an involvement for SIVA1 protein in PCNA-mediated cellular processes.

PCNA regulates SIVA1 protein stability
The stability of one component in a multiprotein complex often depends on the presence of other components so that when one

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indicate the bait proteins. (C and F) Confirmation of PCNA- or SIVA1-interacting proteins by Western blotting. PCNA/SIVA1 purification products were analyzed by Western blots using specific antibodies against Flag, SIVA1, or PCNA. (G and H) Association of endogenous SIVA1 with PCNA in HeLa cells was performed by coimmunoprecipitation using the anti-SIVA1 or anti-PCNA antibody. SIBNA-treated HeLa cells were lysed in the presence of Benzonase, cell lysates were then incubated with protein A agarose beads conjugated with the indicated antibodies, and Western blot analysis was performed according to standard procedures. siCon, control siRNA. (J) Direct in vitro binding between recombinant MBP-PCNA and GST-SIVA1 purified from E. coli. GST served as negative control for PCNA binding. (top) PCNA was detected by immunoblotting. (bottom) Purified proteins visualized by Coomassie staining. (J) SIVA1 colocalizes with PCNA at nucleus foci. HeLa cells were transfected with HA-Flag-tagged SIVA1 plasmids. 24 h after transfection, cells were treated with 50 J/m² UV or left untreated. After 1 h recovery, foci assembled by this fusion protein and by PCNA were detected by immunofluorescence using anti-Flag and anti-PCNA antibodies, respectively. HA-Flag–SIVA1 foci were detected in red, whereas PCNA foci were detected in green. A merged image shows colocalization. IP, immunoprecipitation. Bar, 10 µm.
In response to UV damage, PCNA can be monoubiquitinated at K164 by E3 ubiquitin ligase RAD18 (Hoege et al., 2002; Watanabe et al., 2004; Lehmann, 2011). We therefore tested whether the ubiquitination status of PCNA plays a role in stabilizing SIV A1. As shown in Fig. 2 E, depletion of RAD18, which abolishes PCNA monoubiquitination after UV treatment, has no effect on SIV A1 protein stability, indicating that RAD18-mediated PCNA monoubiquitination is not required for maintaining the steady-state levels of SIV A1.

protein is depleted, the others become unstable and show reduced expression levels. Indeed, reduction of PCNA expression by shRNAs in cells led to a dramatic decrease in SIV A1 protein level but not in mRNA level (Fig. 2, A and B). Moreover, this decrease was partially rescued by treatment with the proteasome inhibitor MG132 (10 µM) or DMSO for 6 h. Cell lysates were then subjected to immunoblotting using the indicated antibodies. (D) SIV A1 depletion has no effect on PCNA protein stability. HeLa cells were transfected twice with control siRNA or siRNAs specific for SIV A1. Cell lysates prepared 48 h after the second transfection were analyzed by immunoblotting with indicated antibodies. (E) RAD18 depletion has no effect on SIV A1 protein stability. HeLa cells transfected with control siRNA or siRNAs specific for RAD18 were treated with 50 J/m² UV for 1 h or left untreated. Cell lysates were then analyzed by immunoblotting with indicated antibodies.

ShCon, control shRNA; SiCon, control siRNA; ub, ubiquitin.
SIVA1 interacts with PCNA via a putative PCNA-interacting peptide (PIP) box

We next mapped the regions within SIVA1 responsible for its interaction with PCNA by using SFB-tagged wild-type SIVA1 and a series of SIVA1 deletion mutants (Fig. 3 A). Coimmunoprecipitation experiments revealed that a domain spanning amino acids 83–96 of SIVA1 contributes to its interaction with PCNA (Fig. 3 B). Consistently, whereas purified recombinant wild-type SIVA1 and the other mutants were able to pull-down endogenous PCNA from HEK293T cell extracts very efficiently, the SIVA1-Δ5 mutant failed to do so (Fig. 3 C). Interestingly, this region of SIVA1 (residues 83–96) has been highly conserved throughout evolution, suggesting that it may carry out an important function of SIVA1 (Fig. 3 D).

Many PCNA-associated proteins have been shown to interact with PCNA via their corresponding PIP box, which has the consensus motif QXX(M/L/I)XX(F/Y)(F/Y). During visual inspection of the SIVA1 amino acid sequence, we identified a potentially divergent PIP box that coincided with the region required for its interaction with PCNA (Fig. 3 D). To test whether SIVA1 might similarly interact with PCNA via its putative PIP box, we generated two point mutants with mutations within the consensus PIP box (Q85A and I88A; Fig. 3 D). As shown in Fig. 3 E, whereas wild-type SIVA1 was able to interact with PCNA, both the Q85A and I88A mutants failed to do so. Furthermore, pull-down experiments consolidated the aforementioned notion that SIVA1 interacts with PCNA via its PIP box (Fig. 3 F).

SIVA1 is required for Polβ recruitment after UV damage

To investigate the cellular function of SIVA1, we knocked down its expression in human HeLa cells using two independent siRNAs specifically targeting SIVA1. Interestingly, SIVA1 knockdown cells showed significantly elevated sensitivity to UV as compared with cells treated with control siRNA (Fig. 4 A). SIVA1 knockdown cells, however, showed little or no hypersensitivity to mitomycin C (MMC) and hydroxyurea (HU), suggesting a specific involvement of SIVA1 in the response to UV damage (Fig. 4 B). To stabilize expression level of both the SIVA1 mutants defective in PCNA binding (Q85A and I88A), expression of wild-type SIVA1 was stably expressed in the cells transfected with control siRNA or SIVA1 siRNA#1 (Fig. 4 E). Notably, the expression level of both the SIVA1 mutants defective in PCNA binding (Q85A and I88A) is significantly lower than that of wild type (Fig. 4 E). This finding further supports our conclusion that SIVA1 is normally stabilized in vivo by PCNA (Fig. 2 A). Accordingly, the defect of Polβ foci formation in SIVA1-depleted cells was readily restored by the reexpression of wild-type SIVA1 but not the point mutants defective in PCNA binding (Fig. 4 F and Fig. S3 B).

Depletion of SIVA1 causes increased mutation frequency

Cells deficient in Polβ exhibit hypersensitivity to UV damage and display elevated mutation frequency. Given that SIVA1 is required for efficient recruitment of Polβ to sites of UV-induced DNA damage, we propose that SIVA1 may also be involved in mutagenesis suppression. To test this possibility, we measured the mutation frequency using the pZ189 shuttle vector system (Akasaka et al., 1992). As shown in Fig. 4 (G and H), the mutation frequency in SIVA1-depleted cells was dramatically elevated. Moreover, codepletion of SIVA1 and Polβ did not cause a further increase in mutation frequency, suggesting that SIVA1 and Polβ may operate in the same pathway (Fig. 4, G and H).

SIVA1 is required for efficient PCNA monoubiquitination

RAD18/RAD6-dependent monoubiquitination of PCNA at Lys164 (K164) is thought to promote direct lesion bypass by recruiting TLS polymerases to the stalled replication forks. The association of SIVA1 with PCNA and the requirement of SIVA1 for Polβ recruitment raised the intriguing possibility that SIVA1 may play a critical role in PCNA monoubiquitination. In agreement with this idea, SIVA1 depletion results in a dramatic reduction in the level of PCNA monoubiquitination (Fig. 5 A). Importantly, suppression of SIVA1 expression did not markedly alter the cell cycle distribution (Fig. S3 C), suggesting that these observed phenotypes in SIVA1 knockdown cells were not caused by any change in cell cycle.

It is well established that the level of PCNA monoubiquitination is regulated by both the RAD18–RAD6 ubiquitin ligase complex and the USP1–UAF1 deubiquitase complex. The requirement of SIVA1 for the efficient monoubiquitination of PCNA suggests that it either positively regulates ubiquitin ligation or negatively regulates USP1–UAF1 via direct or indirect mechanisms. If SIVA1 positively regulates ubiquitin ligation, depletion of SIVA1 will compromise PCNA monoubiquitination.
Figure 3. The PIP box of SIVA1 is responsible for PCNA binding. (A) Schematic representation of wild-type and deletion mutants of SIVA1 used in this study. (B) Residues 83–96 of SIVA1 are responsible for PCNA binding. HEK293T cells were transiently transfected with plasmids encoding tagged PCNA and SIVA1. Cell lysates were immunoprecipitated with S beads, and Western blot analysis was performed with anti-Flag and anti-Myc antibodies. (C) Purified GST, GST-tagged wild-type SIVA1, or its deletion mutants immobilized on Sepharose beads were incubated with HEK293T cell lysates. Endogenous bound PCNA was analyzed by anti-PCNA immunoblotting. Input GST or GST-SIVA1 proteins were shown at the bottom. (D) Sequence alignment of the PCNA-binding region in SIVA1 from different species. (E and F) The PIP box of SIVA1 is required for SIVA1 to bind to PCNA. HEK293T cells were transfected with plasmids encoding SFB-tagged wild-type SIVA1 or two PIP box mutants (Q85A and I88A) together with plasmids encoding Myc-tagged PCNA. (E) Coprecipitation was performed using S protein beads, and immunoblotting was performed using antibodies as indicated. (F) Purified GST or GST-tagged wild-type SIVA1 or two PIP box mutants (Q85A and I88A) immobilized on Sepharose beads were incubated with HEK293T cell lysates. Endogenous bound PCNA was analyzed by anti-PCNA immunoblotting. IP, immunoprecipitation; WT, wild type.
Figure 4. **SIVA1 regulates localization of Pol\(\gamma\).** (A) Clonogenic survival assays in SIVA1-depleted HeLa cells after UV, MMC, or HU treatment. RAD18 depletion is used as a positive control. Cells were permitted to grow for 14 d before staining. Experiments were performed in triplicates. Results shown are means of three independent experiments and were presented as means ± SD. (B–D) SIVA1 is required for Pol\(\gamma\) foci formation. A XP30RO cell line to express SFB-tagged Pol\(\gamma\) [XP30RO-Pol\(\gamma\)] under the control of a tetracycline-inducible promoter was generated. The resulting cell line was transfected twice with the indicated siRNAs and was induced by 1 µg/ml doxycycline addition for 24 h before 50 J/m² UV treatment. Cells were then fixed and processed for Pol\(\gamma\) immunofluorescence. (B) Representative Pol\(\gamma\) foci were shown. (C) Quantification results were the mean of three independent experiments and were presented as means ± SD. More than 100 cells were counted in each experiment. (D) Knockdown efficiency was confirmed by immunoblotting. (E and F) PIP box mutants of SIVA1 could not rescue Pol\(\gamma\) foci formation in SIVA1-depleted cells. XP30RO-Pol\(\gamma\)–derivative cell lines stably expressing HA-tagged siRNA#1-resistant wild-type SIVA1 or two PIP box mutants [Q85A and I88A] were generated. The resulting cell line was transfected twice with the indicated siRNAs and was induced by doxycycline addition for 24 h before 50 J/m² UV treatment. 4 h later, cells were fixed and processed for Pol\(\gamma\) immunofluorescence. (F) Representative Pol\(\gamma\) foci were shown. (E) The exogenous SIVA1 expression was confirmed by immunoblotting using the anti-HA antibody. (G and H) HEK293T cells transfected with the indicated siRNAs for 48 h were treated with UV-damaged supF plasmid. After a further 48 h, the supF plasmid was recovered, and supF mutation frequencies were measured from 10,000 colonies. (G) Results were the mean of three independent experiments and were presented as means ± SD. (H) Knockdown efficiency was confirmed by immunoblotting. SiCon, control siRNA; SiR, siRNA resistant; WT, wild type. Bars, 10 µm.
Figure 5. **SIVA1 is required for efficient PCNA monoubiquitination.**

(A) SIVA1 is required for UV-induced PCNA monoubiquitination. HeLa cells were transfected twice with control siRNA or siRNAs specific for SIVA1. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times. Chromatin fractions were isolated and immunoblotted with the indicated antibodies.

(B and C) HeLa cells were transfected twice with the indicated siRNAs. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times. Chromatin fractions were isolated and immunoblotted with the indicated antibodies.

(D) Cells were transfected twice with the indicated siRNAs. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times. Chromatin fractions were isolated, and the proportion of cells with poly(ub)_PCNA was determined.

(E) HeLa cells were transfected twice with the indicated siRNAs. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times. Immunofluorescence analysis of Pol II and DAPI was performed.

(F) HeLa cells were transfected twice with the indicated siRNAs. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times. Immunofluorescence analysis of RAD18 and DAPI was performed.

(G) Cells were transfected twice with the indicated siRNAs. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times. Immunofluorescence analysis of RAD18 and DAPI was performed.
even in the absence of USP1. On the other hand, if SIVA1 negatively regulates USP1–UAF1, depletion of SIVA1 will not affect PCNA monoubiquitination in the absence of USP1. To test these possibilities, we first analyzed the monoubiquitination of PCNA in SIVA1 and USP1 single and double knockdown cells. Consistent with previous findings (Huang et al., 2006; Cohn et al., 2007; Kim et al., 2009), depletion of USP1 by using siRNA results in increased levels of monoubiquitinated PCNA both in the absence and presence of UV damage (Fig. 5, B and C). Interestingly, like that of RAD18, depletion of SIVA1 compromised PCNA monoubiquitination even when USP1 was simultaneously depleted (Fig. 5, B and C). Consistently, depletion of SIVA1 compromised Polα focus formation when USP1 was simultaneously depleted (Fig. 5, D and E; and Fig. S3 D). These results, collectively, with our observation that SIVA1 depletion did not affect the chromatin association of USP1 and UAF1 (Fig. 5 A), exclude the possibility that SIVA1 may act as an antagonist of PCNA deubiquitination by the USP1–UAF1 complex.

Next, we examined how SIVA1 facilitates the establishment of PCNA monoubiquitination. Because PCNA monoubiquitination occurs in a chromatin context, we asked whether SIVA1 regulates the chromatin association of RAD18 or RAD6, the ubiquitin-conjugating enzyme required for PCNA monoubiquitination. Surprisingly, however, depletion of SIVA1 had no effect on the levels of chromatin-bound RAD18 and RAD6 (Fig. 5 A). In addition, knockdown of SIVA1 did not affect the formation of RAD18 foci after UV treatment (Fig. 5, F and G). These results indicate that SIVA1 does not promote PCNA monoubiquitination by facilitating the binding and/or retention of RAD18 and RAD6 on chromatin.

**SIVA1 associates with and recruits RAD18 to PCNA**

Substrate-specific receptors dictate the specificity for the ubiquitination reaction by selectively recruiting substrates to the E3 ubiquitin ligase machinery. The observation that SIVA1 interacts with PCNA and is required for efficient PCNA monoubiquitination, despite normal RAD18 foci formation in SIVA1-depleted cells, suggested that SIVA1 may serve as a substrate receptor for RAD18 ubiquitin ligase. To test this possibility, we first examined whether SIVA1 physically interacts with RAD18. Coimmunoprecipitation experiments showed that SFB-tagged RAD18 interacted with Myc-tagged SIVA1 (Fig. 6 A). We further demonstrated that endogenous RAD18 interacts with SIVA1 and that the interaction between SIVA1 and RAD18 is enhanced after UV treatment (Fig. 6, A and B).

To identify the regions within SIVA1 responsible for its interaction with RAD18, we performed communoprecipitation experiments and found that a domain spanning amino acids 40–58 of SIVA1 is responsible for RAD18 binding (Fig. 6 C). Conversely, we generated a series of RAD18 deletion mutants to examine which domain of RAD18 might be required for its interaction with SIVA1. Interestingly, the RAD18 region that mediates its binding to SIVA1 is located at residues 331–375 and thus overlaps with the RAD18 binding region to RAD6 (Fig. S4; Bailly et al., 1997; Watanabe et al., 2004).

To test whether SIVA1 binding to RAD18 through a direct interaction, an in vitro GST pull-down assay was performed using GST-fused SIVA1 and His–small ubiquitin-like modifier (SUMO)–fused RAD18 recombinant proteins (Hibbert et al., 2011). Notably, His–SUMO–RAD18 could be pulled down by GST–SIVA1 but not by GST alone (Fig. 6 D), suggesting that SIVA1 directly interacts with RAD18. Moreover, consistent with the results of communoprecipitation experiments, the GST–SIVA1Δ2 fusion protein failed to pull down His–SUMO–RAD18 (Fig. 6 D).

The association of SIVA1 with RAD18 and PCNA through nonoverlapping regions and the similar effects of SIVA1 and RAD18 on PCNA monoubiquitination raised the intriguing possibility that SIVA1 might facilitate the RAD18–PCNA interaction in the cells, thus leading to PCNA monoubiquitination. In a mammalian overexpression system, RAD18 interacted with PCNA weakly, probably through endogenous SIVA1. This interaction was enhanced by ectopically expressed SIVA1 (Fig. 6 E). In untransfected cells, endogenous RAD18 interacted with PCNA in a UV-dependent manner, and this interaction was diminished by knockdown of SIVA1 (Fig. 6 F). Moreover, the SIVA1–PCNA interaction was not affected when RAD18 was ablated by siRNA (Fig. 6 G). These results suggest that SIVA1 is the central component of the RAD18–SIVA1–PCNA complex and physically connects RAD18 to PCNA in vivo. In agreement with this conclusion, the recombinant SIVA1 protein facilitated RAD18–PCNA complex assembly in vitro (Fig. 6 H).

**SIVA1 promotes RAD18-mediated PCNA monoubiquitination in vivo and in vitro**

As shown in Fig. 5 A and Fig. 6 (E–H), SIVA1 mediates the interaction between RAD18 and PCNA and is required for efficient PCNA monoubiquitination. Therefore, it would be interesting to test whether SIVA1 can promote RAD18-mediated PCNA monoubiquitination. We first investigated RAD18-mediated monoubiquitination of PCNA in cells overexpressing SIVA1. As we expected, RAD18-mediated monoubiquitination of PCNA was significantly enhanced in SIVA1-overexpressed cells compared with that in control cells both in the presence and absence of UV treatment (Fig. 7 A).
Figure 6. **SIVA1 associates with and recruits RAD18 to PCNA.** (A) SIVA1 interacts with RAD18. HEK293T cells were transiently transfected with plasmids encoding SFB-tagged Morc3 or RAD18 together with plasmids encoding Myc-tagged SIVA1. 24 h after transfection, cells were left untreated or treated with 50 J/m² UV for 1 h. Coprecipitation was performed using S protein beads, and immunoblotting was performed using antibodies as indicated.

(B) Association of endogenous SIVA1 with RAD18 in HeLa cells was analyzed by immunoprecipitation using the anti-SIVA1 antibody. Cells transfected with the indicated siRNAs were treated with 50 J/m² UV for 1 h or left untreated and then lysed with NETN buffer containing Benzonase. Cell lysates were...
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wild-type SIVA1, but not the deletion mutants defective in RAD18 binding, was able to restore PCNA monoubiquitination and Pol\(\eta\) foci formation in SIVA1-depleted cells. These results indicate that the RAD18-binding activity is required for SIVA1 to function in vivo.

To further define the in vivo functional link between SIVA1 and RAD18, we performed an epistasis analysis of the two proteins. As shown in Fig. 8 (D and E), the UV hypersensitivity of cells depleted of both RAD18 and SIVA1 was not greater than when each protein was depleted individually, indicating that SIVA1 and RAD18 function in the same pathway.

Discussion

In this study, we have provided several lines of evidence to show that SIVA1 is a critical regulator of PCNA monoubiquitination in response to UV damage. First, SIVA1 constitutively interacts

incubated with protein A agarose beads conjugated with indicated antibodies, and Western blot analysis was performed as indicated. (C) Residues 40–58 of SIVA1 are responsible for RAD18 binding. HEK293T cells were transiently transfected with the indicated plasmids. 24 h after transfection, cells were treated with 50 J/m\(^2\) UV for 1 h. Coproportionation was performed using S protein beads, and immunoblotting was performed using antibodies as indicated. (D) Direct in vitro binding between recombinant GST-SIVA1 and His-SUMO–RAD18 purified from E. coli. GST served as negative control for RAD18 binding. (top) RAD18 was detected by immunoblotting. (bottom) Purified proteins visualized by Coomassie staining. (E) Overexpression of SIVA1 enhances the interaction between RAD18 and PCNA. HEK293T cells were transiently transfected with the indicated plasmids. 24 h after transfection, cells were left untreated or treated with 50 J/m\(^2\) UV for 1 h. Coproportionation was performed using S protein beads, and immunoblotting was performed using antibodies as indicated. (F) The RAD18–PCNA interaction is diminished in SIVA1-depleted cells. HEK293T cells were transiently transfected with siRNA specific for SIVA1. 48 h after transfection, cells were left untreated or treated with 50 J/m\(^2\) UV for 1 h. Cells were then Triton X-100 extracted and formaldehyde fixed, and cell lysates were immunoprecipitated with protein A agarose beads conjugated with anti-PCNA antibody. Western blot analysis was performed as indicated. (G) RAD18 depletion does not affect SIVA1–PCNA interaction. HeLa cells transfected with the indicated siRNAs were treated with 50 J/m\(^2\) UV for 1 h or left untreated and then lysed with NETN buffer containing Benzonase. Cell lysates were then incubated with protein A agarose beads conjugated with anti-SIVA1 antibody, and Western blot analysis was performed according to standard procedures. (H) SIVA1 facilitates RAD18–PCNA complex assembly in vitro. (top) RAD18 and SIVA1 were detected by immunoblotting. (bottom) Purified proteins were visualized by Coomassie staining. Con, control; IP, immunoprecipitation; ub, ubiquitin; WT, wild type.

Figure 7. SIVA1 promotes PCNA monoubiquitination in vivo and in vitro. (A) SIVA1 promotes RAD18-mediated PCNA monoubiquitination in vivo. HeLa cells were transiently transfected with the indicated plasmids. 48 h after transfection, cells were left untreated or treated with 50 J/m\(^2\) UV for 1 h. Chromatin fractions were isolated and immunoblotted with the indicated antibodies. (B) SIVA1 promotes RAD18-mediated PCNA monoubiquitination in vitro. 0.4 \(\mu\)M PCNA was incubated in the absence or presence of various combinations of 50 \(\mu\)M ubiquitin, 50 nM Uba1, 0.2 \(\mu\)M RAD6, 0.2 \(\mu\)M RAD18, and SIVA1 (0.5 \(\mu\)M or 1 \(\mu\)M) as indicated. The assay was performed in reaction buffer containing 25 mM Tris, pH 8.0, 50 mM NaCl, and 0.1 mM DTT. The reactions were activated with 2 mM ATP and incubated at 30°C for 1 h. Proteins were resolved by SDS-PAGE and immunoblotted with the indicated antibodies. ub, ubiquitin.
Figure 8. **SIVA1 is functionally linked to RAD18.** (A) The mutant defective in RAD18 binding failed to rescue PCNA monoubiquitination in cells with SIVA1 depletion. A HeLa cell line stably expressing HA-tagged siRNA#1-resistant wild-type SIVA1 (SiR-WT) or its deletion mutant defective in RAD18 binding (SiR-Δ2) was generated. The resulting cell lines were transfected twice with control siRNA or SIVA1 siRNA#1. 48 h after the second transfection, cells were left untreated or treated with 50 J/m² UV for 1 h. Chromatin fractions were isolated and immunoblotted with the indicated antibodies. (B and C) The mutant
with PCNA through a highly conserved putative PIP box. Second, cells depleted of SIVA1 showed marked increases in cellular sensitivity to UV treatment. Third, SIVA1 depletion largely prevented the formation of Polh foci after UV treatment. Fourth, SIVA1 promotes RAD18-mediated PCNA monoubiquitination in vivo and in vitro. Finally, depletion of SIVA1 resulted in a marked decrease in PCNA monoubiquitination, which was accompanied by reduced RAD18–PCNA complex formation. Our data are consistent with a model in which SIVA1 acts as a molecular bridge between RAD18 and PCNA, thus targeting the E3 ligase activity of RAD18 onto PCNA (Fig. 8 F).

A key event in the regulation of TLS is the monoubiquitination of PCNA, a homotrimeric protein that acts as an auxiliary factor for DNA polymerases. Upon UV damage, PCNA is monoubiquitinated by the RAD18–RAD6 complex. This modification thus provides a platform for recruiting repair factors as well as specialized translesion DNA polymerases, which operate to bypass bulky DNA adducts during DNA replication. Besides the RAD18–RAD6 complex, many other proteins, including RPA (Davies et al., 2008), Claspin (Yang et al., 2008), CHK1 (Yang et al., 2008), C1orf124/Spartan/DVC1 (Centore et al., 2012; Davis et al., 2012; Ghosal et al., 2012; Juhasz et al., 2012; Machida et al., 2012; Mosbech et al., 2012), NBS1 (Yanagihara et al., 2011), and Polh (Durando et al., 2013), are also required for efficient PCNA monoubiquitination. Most of these proteins are involved directly or indirectly in recruiting RAD18 to stalled replication forks. In contrast, SIVA1 serves as an adaptor that bridges RAD18 to PCNA and is not required for RAD18 nuclear foci formation and chromatin recruitment. Based on these findings, we propose that, in response to UV damage, single-stranded DNA regions are exposed and are coated with RPA at stalled replication forks. Through its interaction with RPA, NBS1, and/or other auxiliary factors, RAD18 is recruited to these stalled replication forks. SIVA1 then serves as a molecular bridge between RAD18 and PCNA, thus targeting the E3 ligase activity of RAD18 onto PCNA (Fig. 8 F). However, in the absence of SIVA1, although RAD18 is still efficiently recruited to UV damage sites, it fails to interact efficiently with PCNA, leading to defects in PCNA monoubiquitination and subsequent Polh recruitment and TLS activation (Fig. 8 F).

Equally important as PCNA monoubiquitylation is PCNA deubiquitylation, which is performed by USP1 in a heterodimeric complex with its cofactor UAF1 (Huang et al., 2006; Cohn et al., 2007; Kim et al., 2009). Depletion of USP1 or UAF1 results in increased levels of monoubiquitinated PCNA both in the presence and absence of DNA damage. PCNA deubiquitylation also requires the PCNA-binding protein hELG1 (Lee et al., 2010; Parnas et al., 2010; Yang et al., 2011). Several studies have demonstrated that hELG1 specifically directs the USP1–UAF1 complex to PCNA at the damage site and in doing so initiates the switch from the TLS polymerases to the replicative polymerases (Lee et al., 2010; Parnas et al., 2010; Yang et al., 2011). It is apparent that the two scaffold proteins SIVA1 and hELG1 have opposing functions in the regulation of PCNA monoubiquitination and are important for determining the ultimate ubiquitination status of PCNA. The mechanisms that regulate this balance require further investigation.

Although most known factors involved in TLS are conserved from yeast to humans, the counterpart of SIVA1 protein is only found in higher eukaryotes. The reason for this difference between yeast and human cells is not clear but is likely to reflect a need for additional levels of control in mammalian cells. Previous studies have shown that limited PCNA monoubiquitination could be achieved in the absence of DNA, and significant PCNA monoubiquitination occurred only when PCNA is loaded onto DNA by RFC (Watanabe et al., 2004; Garg and Burgers, 2005; Haracska et al., 2006; Unk et al., 2006, 2008; Hibbert et al., 2011). In an in vitro ubiquitination assay using purified proteins, we showed that SIVA1 could promote RAD18-dependent PCNA monoubiquitination in the absence of DNA. Given that SIVA1 facilitates RAD18–PCNA complex assembly in vivo and in vitro, we speculate that SIVA1 may also enhance RAD18-dependent monoubiquitination of RFC-loaded PCNA. Further studies will be conducted to consolidate this working model.

It has been previously reported that SIVA1 regulates cell proliferation and participates in p53-dependent apoptosis (Du et al., 2009; Resch et al., 2009; Iorio-Morin et al., 2012; Wang et al., 2013). In contrast, we found that SIVA1 depletion does not significantly affect cell proliferation and cell cycle distribution (Fig. S3, A and C; and Fig. S5). The basis for these discrepancies is not clear, and additional experiments will be required to clarify the issue.

Using the SupF suppressor tRNA gene as the mutagenesis reporter gene, we found that SIVA1 depletion resulted in a hypermutator phenotype. Although it is not currently clear how SIVA1 suppresses mutagenesis, given that both SIVA1 and Polh (Durando et al., 2013) forms a complex with RAD18, we speculate that SIVA1 may bind directly or indirectly to Polh and may favor the recruitment of Polh to monoubiquitinated PCNA, thereby facilitating the error-free bypass of UV lesions. However, we cannot exclude the possibility that SIVA1 may have other roles in suppressing mutagenesis.

In summary, we have identified a function for SIVA1 as an adaptor for the E3 ligase RAD18, showing that collaboration of SIVA1 and RAD18 controls the level of PCNA monoubiquitination. These findings will provide new insights into the...
molecular mechanisms of the replicative bypass of base damage to DNA by TLS, which is critically important for the maintenance of genomic integrity and tumor suppression.

**Materials and methods**

**Antibodies**

Rabbit polyclonal anti-SIVA1, anti-USP1, and anti-UAF1 antibodies were generated by immunizing rabbits with MBP-SIVA1 (residues 1–175), MBP-USP1 (residues 351–785), or MBP-UAF1 (residues 400–677) fusion proteins expressed and purified from E. coli, respectively. Anti-sigma was affinity purified using an immobilization and purification kit (AminoLink Plus, Thermo Fisher Scientific). Anti-RAD18 and anti-RAD6A antibodies were purchased from Bethyl Laboratories, Inc. Anti-PCNA and anti-Myc (PE10) antibodies were purchased from Santa Cruz Biotechnology, Inc. and Covance, respectively. Anti-GAPDH, anti-H3, and anti-HA antibodies were purchased from Sigma-Aldrich.

**Cell culture and transfection**

HEK293T and Hela cells were maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin and streptomycin. Cell transfection was performed using Lipofectamine 2000 (Invitrogen) following the manufacturer’s protocol.

**Constructs**

SIVA1, PCNA, and RAD18 cDNAs were obtained from Thermo Fisher Scientific. The PCR-amplified DNA fragment containing wild-type SIVA1, RAD18, or PCNA was subcloned into the pDONR201 vector using Gateway Technology (Invitrogen). Site-directed mutagenesis was performed according to standard procedures to obtain the SIVA1 mutants. For transient expression of PCNA, RAD18, SIVA1, or its mutants, the corresponding fragment in the entry vector was transferred into a Gateway-compatible destination vector (Invitrogen), which harbors either an N-terminal triple-epitope tag (S protein tag, Flag epitope tag, and Streptavidin-binding peptide tag) or an N-terminal Myc tag or HA tag. All clones were sequenced to verify desired mutations.

**The establishment of stable cell lines and affinity purification of SFB-tagged protein complexes**

HEK293T cells were transfected with plasmids encoding SFB-tagged SIVA1 or PCNA. Cell lines stably expressing tagged proteins were selected by culturing in medium containing 2 μg/ml puromycin and confirmed by immunoblotting and immunostaining. For affinity purification, HEK293T cells stably expressing tagged proteins were lysed with NETN buffer (20 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, 10 mM 2-mercaptoethanol, and 1 μg/ml of each of leupeptin, aprotinin, and pepstatin). The extract was centrifuged at 18,000 rpm for 40 min. The supernatant was collected and incubated with amylase resins for 2 h at 4°C. The bound protein was eluted with lysis buffer containing 10 mM maltose. Full-length RAD18 was cloned into the pET28-NH-His-SUMO vector (EMD Millipore) for the expression of His-SUMO-tagged RAD18 in E. coli. The SUMO tag on RAD18 was able to improve its solubility to allow expression and purification in the absence of RAD6 (Hilbert et al., 2011). Cells were harvested and resuspended in lysis buffer (20 mM Hepes, 300 mM NaCl, 1% Triton X-100, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin). After sonication, the extract was centrifuged at 18,000 rpm for 40 min. The supernatant was collected and incubated with cobalt agarose for 2 h at 4°C. After washing the beads with washing buffer (20 mM Hepes, 500 mM NaCl, 1% Triton X-100, 5 mM imidazole, and 1 μg/ml each of leupeptin, aprotinin, and pepstatin), the bound protein was eluted with lysis buffer containing 200 mM imidazole.

**Coimmunoprecipitation and Western blotting**

For whole-cell extracts, the cells were solubilized in NETN lysis buffer supplemented with 50 U/ml Benzonase, protease inhibitors, and phosphatase inhibitors. After removal of cell debris by centrifugation, the soluble fractions were collected. For Flag immunoprecipitations, a 0.8-ml aliquot of lysate was incubated with 1 μg of the Flag monoclonal antibody and 25 μl of a 1:1 slurry of protein A-Sepharose for 2 h at 4°C. For endogenous immunoprecipitations, 1 μg of the whole-cell extract was incubated with 25 μl of a 1:1 slurry of protein A-Sepharose coupled with 2 μl of the indicated antibodies for 2 h at 4°C. The Sepharose beads were washed three times with NETN buffer, boiled in 2× SDS loading buffer, and resolved on SDS-PAGE. Membranes were blocked in 5% milk in TBST (TBS with Tween) buffer and then probed with antibodies as indicated. To detect endogenous interaction between PCNA and RAD18, coimmunoprecipitation was conducted as described previously with modification (Kannouche et al., 2004). Hela cells treated with the indicated siRNA were left untreated or treated with 50 J/m² UV and recovered for 1 h. Cells were then rinsed twice in cold PBS, incubated for 5 min on ice with gentle shaking in buffer A (100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, 10 mM Pipes, pH 6.8, 1 mM EDTA, 0.2% Triton X-100, and protease inhibitors). After Triton X-100 extraction, the adherent cellular material was fixed by shaking with 1% formaldehyde in PBS for 10 min at 4°C. Glycerol was added to a final concentration of 50% for 5 min to quench the cross-linking. Cellular material were then washed twice with cold PBS, harvested, centrifuged, and dissolved in 200 μl buffer B (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, and 0.1% SDS). After 10-min incubation on ice, the samples were sonicated to shear the DNA to 200–1,500 bp and diluted five times with dilution buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 5 mM EDTA, and 0.2% Triton X-100). Redissolved proteins were incubated with monoclonal mouse anti-PCNA antibody overnight at 4°C and then with 30 μl of protein A-Sepharose. 2 h later, the beads were washed three times with dilution buffer and boiled in 2× SDS loading buffer, and the bound proteins were analyzed by electrophoresis and immunoblotting.

**RNAi**

All siRNAs were synthesized by Thermo Fisher Scientific. The siRNAs were 21 bp, and sequences are as follows: SIVA1 siRNA#1, 5'-CCCGUGUGGCU-CUGUGGAdTdT-3'; SIVA1 siRNA#2, 5'-GGGGCCUGCCUGGCAGCU-GUdTdT-3'; RAD18 siRNA, 5'-ACUCAGUGCCAAUCUUGUGUUU-3'; USP1 siRNA, 5'-GAAACACACACACAAUAAUU-3'; and control siRNA, 5'-UUCAUUAAUAUAUCUUCAGAGGUGUUU-3'. The siRNA-resistant wild-type and mutant SIVA1 constructs were generated by changing six nucleotides in the SIVA1 siRNA#1-targeting region (C453T, G456A, T459C, C462T, C465A, and G468A substitutions). The siRNA transfection was performed with 100 nM siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer’s instructions. Transfection was repeated twice with an interval of 24 h to achieve the maximal RNAi effect. Lentiviral nonsilencing control shRNA and shRNA target sets were purchased from Thermo Fisher Scientific. The PCNA targeting sequences were #1, 5'-AGACAGAATGATGTGAAAA-3', and #2, 5'-TCAGATGATGTCGAGATT-3'. The siRNAs were packaged into lentiviruses by cotransfecting with packaging plasmids pMD2G and pSPAX2 (provided by S. Zhou, Baylor College of Medicine, Houston, TX) into HEK293T cells. After a 48-hr transfection, the supernatant was collected for infection of HeLa cells. Infection was repeated twice with an interval of 24 h to achieve maximal infection efficiency.

**Immunofluorescence staining**

Indirect immunofluorescence was performed as previously described (Huang et al., 2009; Liu et al., 2010; Wan et al., 2013). HeLa or XP30RO (gift from C. Guo, Beijing Institute of Genomics, Chinese Academy of Sciences, June 23, 2014, JCB • VOLUME 205 • NUMBER 6 • 2014
Beijing, China) cells cultured on coverslips were treated with 50 J/m\(^2\) UV and recovered for the indicated times. Cells were then washed with PBS, preextracted with buffer containing 0.5% Triton X-100 for 5 min, and fixed with 3% paraformaldehyde for 10 min at room temperature. For PCNA and SIVA1 coimmunoﬂuorescence staining, HeLa cells were denatured by 2.5 M HCl for 1 h at room temperature after 3% paraformaldehyde ﬁxation. Cells were then incubated in primary antibody for 30 min at room temperature. After 3 min in washes with PBS, secondary antibody was added at room temperature for 30 min. Cells were then stained with DAPI to visualize nuclear DNA. Images were captured with a Zeiss LSM 700 laser scanning confocal microscope equipped with a Plan Fluor 60× oil objective lens (NA 1.38–1.45, Zeiss) and an Orca ER camera (Hamamatsu). Nuclei were then recovered and resuspended in NETN buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) and incubated on ice for 15 min. Nuclei were then recovered and resuspended in 0.2 M HCl. The soluble fraction was neutralized with 1 M Tris-HCl, incubated on ice for 15 min. Nuclei were then recovered and resuspended in NETN buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, and 0.5% Nonidet P-40) and incubated on ice for 15 min. Nuclei were then recovered and resuspended in 0.2 M HCl. The soluble fraction was neutralized with 1 M Tris-HCl, pH 8.0, for further analysis.

**Lentivirus packaging and infection**

The pMD2G and pSPAX2 plasmids (pMD2G and pSPAX2) were provided by S. Zhou (Baylor College of Medicine, Houston, TX). Poly(ADP-ribose) polymerase (poly(ADP-ribose) polymerase, Poly(ADP-ribose) polymerase), and the pCL-Eco and VSV-G packaging plasmids (pMD2G and pSPAX2) into HEK293T cells. XP30RO cells were subsequently transfected with pMD2G and pSPAX2 into HEK293T cells. XP30RO cell line, and Drs. Zongping Xia and Sheng Ye for technical help.

**Online supplemental material**

Fig. S1 shows that SIVA1 interacts with PCNA and demonstrates the speciﬁcity of the anti-SIVA1 antibody. Fig. S2 shows that SIVA1 depletion affects Polh to form foci. The primers were used as follows: GAPDH forward, 5′-CGACCACTTTGT-CAAAGCTCA-3′; reverse, 5′-TGACGTTGCTGGAAGCAGCTA-3′; SIVA1 forward, 5′-CAATCTGAGGAAACTTGTG-3′; reverse, 5′-CATGAGGAACAACTGATG-3′; and Polh forward, 5′-CTGAGCTTCAAGCTCAGTTC-3′; reverse, 5′-TGCCCATATCAGGAATTTT-3′.

**References**


Bi, X., D.M. Slater, H. Ohmori, and C. Vaziri. 2005. DNA polymerase kappa is speciﬁcally required for recovery from the benzo[a]pyrene-dihydrodiol
Figure S1. **SIVA1 interacts with PCNA.** (A) HEK293T cells were transiently transfected with plasmids encoding Myc-tagged PCNA together with plasmids encoding SFB-tagged SIVA1, p21, or Morc3. Cell lysates were immunoprecipitated with S beads, and Western blot analysis was performed with anti-Flag and anti-Myc antibodies. (B) Quantitative RT-PCR showing SIVA1 mRNA levels are down-regulated by siRNAs. Bars represent the mean of three experiments, and error bars are SDs. (C) Representative immunoblotting of cells transfected with the indicated siRNAs. The asterisk indicates a nonspecific band. (D and E) SIVA1–PCNA interaction was UV damage independent. HeLa (D) or HEK293T (E) cells were treated with 50 J/m² UV for 1 h or left untreated and then lysed with NETN buffer containing Benzonase. Cell lysates were then incubated with protein A agarose beads conjugated with anti-SIVA1 antibody, and Western blot analysis was performed according to standard procedures. IP, immunoprecipitation; SiCon, control siRNA; ub, ubiquitin.
**Figure S2.** SIVA1 is required for Polβ foci formation. (A and B) A XP30RO cell line to express SFB-tagged Polβ (XP30RO-Polβ) under the control of a tetracycline-inducible promoter was generated. The resulting cell line was mock induced or induced by 1 µg/ml doxycycline (Dox) addition for 24 h before 50 J/m² UV treatment. 4 h later, cells were fixed and processed for Polβ immunofluorescence. (A) Representative Polβ foci were shown. (B) The exogenous Polβ expression was confirmed by immunoblotting using the anti-Flag antibody. (C–E) A HeLa-derivative cell line stably expressing HA-Flag–tagged Polβ was generated. The resulting cell line transfected twice with indicated siRNAs was treated with 50 J/m² UV for the indicated times. Cells were then fixed and processed for Polβ immunofluorescence. (C) Representative Polβ foci were shown. (D) Quantification results were the mean of three independent experiments and were presented as means ± SD. More than 100 cells were counted in each experiment. (E) Knockdown efficiency was confirmed by immunoblotting. SiCon, control siRNA. Bars, 10 µm.
**Figure S3. SIVA1 depletion does not markedly affect cell proliferation.** (A) SIVA1 depletion results in prolonged CHK1 phosphorylation. HeLa cells transfected with the indicated siRNAs were untreated or treated with 50 J/m² UV and recovered for the indicated times. Cell lysates were then analyzed by immunoblotting with indicated antibodies. (B) PIP box mutants of SIVA1 could not rescue Polũ foci formation in SIVA1-depleted cells. XP30RO-Polũ–derivative cell lines stably expressing siRNA#1-resistant wild-type SIVA1 or two PIP box mutants (Q85A and I88A) were generated. The resulting cell line was transfected twice with the indicated siRNAs and was induced by 1 µg/ml doxycycline addition for 24 h before 50 J/m² UV treatment. 4 h later, cells were fixed and processed for Polũ immunofluorescence. Quantification results were the mean of three independent experiments and were presented as means ± SD. More than 100 cells were counted in each experiment. (C) SIVA1 depletion does not markedly affect cell proliferation. HeLa cells were transfected twice with control siRNA or siRNAs specific for SIVA1. 48 h after the second transfection, BrdU incorporation assays were performed as described in the Materials and methods section. E refers to S phase, F refers to G1 phase, G refers to G2/M phase, and the corresponding numbers represent the percentage of each phase. (D) A HeLa cell line to stably express HA-Flag–tagged Polũ was generated. The resulting cell line was transfected twice with the indicated siRNAs. 48 h after transfection, cells were untreated or treated with 50 J/m² UV for the indicated times, and knockdown efficiency was confirmed by immunoblotting. SiR, siRNA resistant; SiCon, control siRNA; WT, wild type.
Figure S4. **Residues 331–375 of RAD18 are responsible for SIVA1 binding**. (A) Schematic representation of wild-type and deletion mutants of RAD18 used in this study. (B) Residues 331–375 of RAD18 are responsible for SIVA1 binding. HEK293T cells were transiently transfected with plasmids encoding tagged RAD18 and SIVA1. 24 h after transfection, cells were treated with 50 J/m² UV for 1 h. Cell lysates were then immunoprecipitated with S beads, and Western blot analysis was performed with anti-Flag and anti-Myc antibodies. IP, immunoprecipitation; WT, wild type.
Figure S5. SIVA1-depleted cells complemented with wild-type or Δ2 mutant of SIVA1 have comparable cell cycle profiles. A HeLa cell line stably expressing siRNA#1-resistant wild-type SIVA1 (SiR-WT) or its deletion mutant defective in RAD18 binding (SiR-Δ2) was generated. The resulting cell lines were transfected twice with control siRNA or SIVA1 siRNA#1. 48 h after the second transfection, BrdU incorporation assays were performed as described in the Materials and methods section. E refers to S phase, F refers to G1 phase, G refers to G2/M phase, and the corresponding numbers represent the percentage of each phase.