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Spartan/C1orf124, a Reader of PCNA Ubiquitylation and a Regulator of UV-Induced DNA Damage Response

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SUMMARY

PCNA is a key component of DNA replication and repair machineries. DNA damage-induced PCNA ubiquitylation serves as a molecular mark to orchestrate postreplication repair. Here, we have identified and characterized Spartan, a protein that specifically recognizes ubiquitylated PCNA and plays an important role in cellular resistance to UV radiation. In vitro, Spartan engages ubiquitylated PCNA via both a PIP box and a UBZ domain. In cells, Spartan is recruited to sites of UV damage in a manner dependent upon the PIP box, the UBZ domain, and PCNA ubiquitylation. Furthermore, Spartan colocalizes and interacts with Rad18, the E3 ubiquitin ligase that modifies PCNA. Surprisingly, while Spartan is recruited by ubiquitylated PCNA, knockdown of Spartan compromised chromatin association of Rad18, monoubiquitylation of PCNA, and localization of Pol η to UV damage. Thus, as a "reader" of ubiquitylated PCNA, Spartan promotes an unexpected feed-forward loop to enhance PCNA ubiquitylation and translesion DNA synthesis.

INTRODUCTION

The proliferating cell nuclear antigen PCNA is a homotrimeric protein complex that functions as a sliding clamp on DNA during DNA replication and repair. In addition to its role as the processivity factor for DNA polymerases, PCNA plays a key structural role in DNA replication and repair machineries to recruit and organize a number of proteins in these complexes (Moldovan et al., 2007). In response to DNA damage that interferes with DNA replication, PCNA is mono- or polyubiquitylated in human cells, creating unique types of molecular marks that orchestrate postreplication DNA repair (PRR) and repair of DNA interstrand crosslinks (ICLs) (Lehmann, 2011). Understanding how the ubiquitin marks of PCNA are written and read by the proteins involved in PRR and ICL repair is a critical step toward elucidating the mechanisms of these processes.

Among the known PCNA-binding proteins, many interact with PCNA through their *P*CNA-*i*nteracting *p*eptides, or PIP boxes (Warbrick, 1998). The PIP boxes of FEN-1 and p21 are shown to bind directly to a hydrophobic pocket formed by the interdomain-connecting loop of PCNA (Gulbis et al., 1996; Hosfield et al., 1998). When PCNA is monoubiquitylated by the E3 ligase Rad18 at lysine 164, it promotes translesion DNA synthesis (TLS) or ICL

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SUPPLEMENTAL INFORMATION

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repair by interacting with Y-family DNA polymerases (Pols) η, ι, κ, and REV1 (Bi et al., 2006; Bienko et al., 2005; Garg and Burgers, 2005; Kannouche et al., 2004; Plosky et al., 2006; Watanabe et al., 2004). Monoubiquitylated PCNA may also interact with SNM1A, a protein involved in ICL repair, and contribute to monoubiquitylation of FANCD2 and FANCI (Geng et al., 2010; Wang et al., 2011; Yang et al., 2010). Polyubiquitylated PCNA is thought to promote an error-free PRR pathway, whose mechanism remains largely unknown (Lehmann, 2011). Although many proteins involved in the DNA damage response possess different types of *u*biquitin-*b*inding *d*omains (UBDs) (Dikic et al., 2009), only a subset of these proteins interacts with ubiquitylated PCNA specifically. Interestingly, several proteins that interact or potentially interact with ubiquitylated PCNA, including Pols η, ι, κ, and SNM1A, contain at least one PIP box and one UBD (Bienko et al., 2005; Haracska et al., 2001a, 2001b; Hishiki et al., 2009; Ogi et al., 2005; Vidal et al., 2004; Yang et al., 2010). The conservation of both PIP box and UBD in these proteins suggests that the two domains may function in concert to recognize ubiquitylated PCNA. To gain further insight into how ubiquitylated PCNA is read during the DNA damage response, we searched for uncharacterized proteins with both a PIP box and a UBD.

In this study, we found that a previously uncharacterized protein encoded by C1orf124 contains a PIP box, a *u*biquitin-*b*inding *z*inc finger (UBZ), and an SprT-like domain. We named this protein Spartan for its *SprT*-like domain at the *N* terminus. We show that Spartan localizes to sites of ultraviolet light (UV)-induced DNA damage in cells and is required for normal cellular resistance to UV radiation. Both the PIP box and the UBZ domain of Spartan are important for its binding to ubiquitylated PCNA, its localization to sites of UV damage, and its function in UV resistance. In addition to PCNA, Spartan also colocalizes and interacts with Rad18. Furthermore, Spartan is required for efficient association of Rad18 with chromatin, monoubiquitylation of PCNA, and localization of Pol η to sites of UV damage. Together, these findings suggest that Spartan is an important reader of ubiquitylated PCNA that enhances Rad18-mediated PCNA ubiquitylation and TLS, revealing a surprising feed-forward loop coupling a reader and a writer of PCNA ubiquitylation.

RESULTS

Spartan Localizes to Sites of UV Damage and Promotes Cell Survival

C1orf124 is predicted to encode a 489-amino acid protein containing a Rad18-like UBZ domain (amino acids 456–475) and an SprT-like domain of unknown function (amino acids 45–212) (Figure 1A). We have therefore named this putative gene *SPRTN* (*SprT*-like domain at the *N* terminus) and its protein product Spartan. Our further analysis of the predicted amino acid sequence of Spartan led to the identification of a putative PIP box at amino acids 325–332 (Figure 1A). The presence of both a PIP box and a UBZ domain in Spartan prompted us to investigate its potential involvement in the DNA damage response.

Since Spartan has a Rad18-like UBZ domain, we first asked whether Spartan and Rad18 are involved in similar DNA damage responses. Rad18 is important for cellular responses to both UV and ionizing radiation (IR) (Huang et al., 2009). To determine whether Spartan is also involved in the responses to UV and IR, we knocked down Spartan from U2OS cells using two independent siRNAs (Figures S1A and S1B). Similar to Rad18 knockdown cells, Spartan knockdown cells showed significantly elevated sensitivity to UV as compared to cells treated with control siRNA (Figure 1B). Spartan knockdown cells, however, were not hypersensitive to IR, suggesting a specific involvement of Spartan in the response to UV-induced DNA damage (Figure 1C).

Consistent with a role in UV-induced DNA damage response, SFB (S/FLAG/streptavidin-binding peptide)-tagged Spartan colocalized with PCNA at sites of UV damage (Figures 1D and S1C). Triton extraction completely removed Spartan from unirradiated cells, but the UV-induced Spartan foci were resistant to Triton extraction. These results are reminiscent of those previously described for Pol η (Kannouche et al., 2004), suggesting that Spartan, like Pol η, binds to chromatin in a UV-induced manner. To test whether Spartan is recruited to double-stranded DNA breaks (DSBs) like Rad18, we treated cells with IR and monitored Spartan localization. Spartan did not localize to IR-induced nuclear foci (IRIF) marked with γH2AX (Figure 1E), suggesting that, in contrast to Rad18, Spartan is specifically involved in the response to UV-induced DNA damage.

Spartan Binds Ubiquitin via Its UBZ Domain

To understand how Spartan contributes to UV damage response, we first focused our attention on its putative PIP box and UBZ domain. Alignment of Spartan sequences across different species revealed strong conservation of the UBZ domain in all Spartan homologs identified (Figure 2A). Its PIP box, on the other hand, was highly conserved among mammals, but exhibited somewhat weaker conservation in lower vertebrates (see Figure 3A). These findings suggest that Spartan may have acquired the UBZ domain and the PIP box sequentially during evolution.

We next asked if the conserved UBZ domain of Spartan is functional. We mutated two of the critical, zinc-coordinating cystines of the UBZ domain to alanines (Spartan$^{\Delta UBZ}$ in Figure 2A). In western blots, wild-type Spartan (Spartan^{WT}) migrated as a doublet, whereas Spartan$^{\Delta UBZ}$ was only detected in the fast-migrating band (Figure 2B). UBD-containing proteins are known to undergo self-ubiquitylation (Hoeller et al., 2006). Indeed, the UBZ-dependent slow-migrating band of Spartan^{WT} was detected by anti-ubiquitin antibody after Spartan^{WT} was isolated under denaturing conditions (Figure 2C). Consistent with this result, Spartan was found to be ubiquitylated by two recent proteomic studies (Emanuele et al., 2011; Kim et al., 2011).

To test directly whether the UBZ domain of Spartan binds ubiquitin, we incubated GST, a GST-tagged C-terminal fragment of Spartan^{WT} (GST-Spartan-C^{WT}), or its Spartan$^{\Delta UBZ}$ derivative (GST-Spartan-C$^{\Delta UBZ}$) with purified K63- or K48-linked ubiquitin chains, and pulled down the GST proteins. GST-Spartan-C^{WT}, but neither GST nor GST-Spartan-C$^{\Delta UBZ}$, bound to both types of ubiquitin chains efficiently (Figure 2D). Similar to Spartan's UBZ domain, the UBZ domains of Pols ι, η, and Rad18 all bind to both K63- and K48-linked ubiquitin chains in vitro (Huang et al., 2009; Plosky et al., 2006). We noted that GST-Spartan-C^{WT} preferentially captured short K48-linked ubiquitin chains (Figure 2D). The significance of this observation is currently unclear. In addition to purified ubiquitin chains, GST-Spartan-C^{WT} also captured endogenous K63-linked ubiquitin chains from cell extracts (Figure S2). Together, these results demonstrate that the UBZ domain of Spartan is capable of binding ubiquitin.

Spartan Interacts with Ubiquitylated PCNA via Its PIP Box and UBZ Domain

To test the function of Spartan's PIP box (Figure 3A), we first asked if Spartan binds to PCNA. In contrast to GST alone, GST-Spartan-C^{WT}, which contains both the PIP box and UBZ domain, captured endogenous PCNA from cell extracts efficiently (Figure 3B, lanes 2 and 3). Even from extracts of undamaged cells, GST-Spartan-C^{WT} captured some monoubiquitylated PCNA (Figure S2), consistent with the low levels of PCNA ubiquitylation in cycling cells (Terai et al., 2010). In extracts of UV-treated cells, GST-Spartan-C^{WT} bound to unmodified and ubiquitylated PCNA efficiently (Figure 3B). Both mono- and diubiquitylated PCNA were found to associate with GST-Spartan-C^{WT}. When

compared to their relative levels in input extracts, mono- and diubiquitylated PCNA were more enriched in GST-Spartan-C^{WT} pull-downs than unmodified PCNA, suggesting that Spartan preferentially binds ubiquitylated PCNA.

The interaction between the Spartan C-terminal fragment and PCNA was significantly reduced but not eliminated when the four conserved residues of Spartan's PIP box were mutated to alanines (GST-Spartan-$C^{\Delta PIP}$ in Figures 3A–3C), suggesting that Spartan interacts with PCNA via both PIP box-dependent and -independent mechanisms (see below). Importantly, while GST-Spartan-$C^{\Delta PIP}$ displayed drastically reduced binding to unmodified PCNA, its binding to ubiquitylated PCNA was only compromised modestly (Figures 3B, lanes 3 and 4, and 3C). These results suggest that the PIP box of Spartan is more important for the direct binding to PCNA than the binding to ubiquitin moieties. The low levels of unmodified PCNA captured by GST-Spartan-$C^{\Delta PIP}$ might come from PCNA trimers containing both ubiquitylated and unmodified subunits. To test if full-length Spartan interacts with PCNA in vivo, SFB-tagged full-length $Spartan^{WT}$ or $Spartan^{\Delta PIP}$ was expressed in cells and retrieved with streptavidin-coated magnetic beads. PCNA was captured efficiently by $Spartan^{WT}$, but not by $Spartan^{\Delta PIP}$ (Figure 3D). These results suggest that the PIP box of Spartan is important for efficient PCNA binding both in vivo and in vitro.

The binding of Spartan to ubiquitylated PCNA prompted us to investigate the role of its UBZ domain in this interaction. GST-Spartan-$C^{\Delta UBZ}$ bound unmodified PCNA slightly less efficiently than GST-Spartan-C^{WT}, but its ability to capture mono- and diubiquitylated PCNA was markedly reduced (Figures 3B, lanes 3 and 5, and 3C). Thus, while the main role of the PIP box is binding PCNA, the UBZ domain is important for recognizing ubiquitin conjugated to PCNA. A GST-tagged C-terminal fragment of Spartan with both the PIP box and UBZ domain mutated (GST-Spartan-$C^{\Delta PIP,UBZ}$) showed dramatically reduced binding to all species of PCNA (Figures 3B, lane 6, and 3C), suggesting that the two domains of Spartan function jointly to achieve efficient PCNA binding in vitro.

The Localization and Function of Spartan Are Regulated by Its PIP Box and UBZ

Given the ability of Spartan to bind PCNA and ubiquitin through its PIP box and UBZ domain, we next investigated the functional significance of these interactions in cells. First we tested whether disruption of the PIP box or UBZ domain affects the localization of Spartan to sites of UV damage. When U2OS cells expressing SFB-$Spartan^{WT}$ were irradiated with UV directly or through 5-micron filters, colocalization of $Spartan^{WT}$ with PCNA was evident (Figures 4A and S3A). In marked contrast to $Spartan^{WT}$, the $Spartan^{\Delta PIP}$ and $Spartan^{\Delta UBZ}$ mutants did not colocalize with PCNA in UV-irradiated cells (Figures 3A and S3A). Furthermore, unlike $Spartan^{WT}$, $Spartan^{\Delta PIP}$ and $Spartan^{\Delta UBZ}$ were completely removed from cells by Triton extraction even after UV damage. These results suggest that, although Spartan retains some ability to bind PCNA in vitro in the absence of either its PIP box or UBZ domain, both of these domains are indispensable for the localization of Spartan to sites of UV damage and its chromatin association in cells.

If Spartan is recruited by ubiquitylated PCNA, one would expect that the recruitment of Spartan should correlate with the efficiency of PCNA ubiquitylation. Indeed, when we used siRNA to knock down Rad18, the E3 ubiquitin ligase critical for PCNA monoubiquitylation, the percentage of cells with $Spartan^{WT}$ foci was reduced in untreated and UV-irradiated cell populations (Figures 4B and S3B). On the other hand, when Usp1, the deubiquitylase that removes ubiquitin from PCNA, was knocked down, an increased percentage of cells with UV-induced $Spartan^{WT}$ foci was observed (Figures 4B and S3B). As expected, while knockdowns of Rad18 and Usp1 decreased and increased PCNA monoubiquitylation, respectively (see Figure S4C), they did not affect the localization of PCNA (Figure S3B).

Together, these results suggest that Spartan is recruited to sites of UV-induced DNA damage by ubiquitylated PCNA.

To assess directly the functional significance of the PIP box and UBZ domain of Spartan, we next tested if the Spartan$^{\Delta PIP}$ and Spartan$^{\Delta UBZ}$ mutants are able to suppress the elevated UV sensitivity of Spartan knockdown cells. For these experiments, we generated U2OS-derived cell lines stably expressing SFB-tagged Spartan^{WT}, Spartan$^{\Delta PIP}$, or Spartan$^{\Delta UBZ}$. By treating these cell lines with siRNAs targeting either the *SPRTN* coding sequence (siSpartan-1) or the 3′UTR of *SPRTN* transcript (siSpartan-2), we sought to knock down both endogenous and exogenous Spartan or endogenous Spartan selectively. Consistent with Figure 1B, the UV sensitivity of parental U2OS cells was similarly increased by the two Spartan siRNAs. The UV sensitivity of Spartan^{WT} expressing cells was also increased by siSpartan-1, which targets both endogenous Spartan and exogenous Spartan^{WT} (Figure 4C). In contrast to siSpartan-1, siSpartan-2, which targets only endogenous Spartan, did not increase the UV sensitivity of Spartan^{WT}-expressing cells (Figure 4C). These results suggest that exogenous Spartan^{WT} suppressed the defect of UV response in the absence of endogenous Spartan. In contrast to Spartan^{WT}, neither Spartan$^{\Delta PIP}$ nor Spartan$^{\Delta UBZ}$ substantially suppressed the elevated UV sensitivity in the absence of endogenous Spartan (Figure 4C). Thus, both the PIP box and UBZ domain of Spartan are required for the repair and/or tolerance of UV-induced DNA damage.

Spartan Promotes PCNA Ubiquitylation and Pol η Recruitment

We next further investigated how Spartan contributes to the repair and/or tolerance of UV damage. Two major DNA damage repair/tolerance pathways implicated in the UV response are TLS and nucleotide excision repair (NER). To monitor TLS, we analyzed the localization of Pol η, a TLS polymerase that bypasses UV-induced DNA lesions. Similar to Spartan, Pol η interacts with ubiquitylated PCNA. In cells irradiated with UV, eGFP-Pol η was recruited to sites of DNA damage and colocalized with PCNA in a Rad18-dependent manner (Figures 5A and 5B). The recruitment of eGFP-Pol η to UV damage was significantly reduced in Spartan knockdown cells (Figures 5A and 5B), suggesting that Spartan is an important regulator of TLS. In contrast to Pol η, XPA, a key factor in NER, was recruited to sites of UV damage in control and Spartan knockdown cells with similar efficiency (Figure 5C). Together, these results suggest a role for Spartan in TLS, but not NER.

The recruitment of Spartan to ubiquitylated PCNA and its role in Pol η localization suggest that Spartan may function downstream of PCNA ubiquitylation. Surprisingly, however, knockdown of Spartan by three independent siRNAs reduced UV-induced PCNA monoubiquitylation (Figure 6A). This reduction in PCNA ubiquitylation was observed over a range of UV doses (Figure 6B). Furthermore, this defect was completely suppressed by SFB-Spartan^{WT} (Figure 6C), suggesting that it was not due to siRNA off-target effects. Interestingly, following UV-induced PCNA ubiquitylation, the levels of Spartan^{WT} gradually declined (Figures 6B, 6C, S4A, and S4B), suggesting attenuation of Spartan function in the late stage of UV damage response. In contrast to Spartan^{WT}, the Spartan$^{\Delta PIP}$ and Spartan$^{\Delta UBZ}$ mutants were unable to suppress the reduction in PCNA ubiquitylation in Spartan knockdown cells (Figure 6D). Even in the presence of endogenous Spartan, Spartan$^{\Delta PIP}$ and Spartan$^{\Delta UBZ}$ exerted dominant negative effects on PCNA ubiquitylation (Figure S4B). Thus, while Spartan is recruited to ubiquitylated PCNA through its PIP box and UBZ domain, it is involved in a feed-forward loop required for normal levels of UV-induced PCNA ubiquitylation.

Spartan Is Functionally Linked to Rad18

Consistent with a role for Spartan in enhancing, but not initiating, PCNA ubiquitylation, the reduction in PCNA ubiquitylation in Spartan knockdown cells was less striking than that in Rad18 knockdown cells (Figure S4C, lanes 2 and 4). Also consistent with a role for Spartan in a feed-forward loop that enhances PCNA ubiquitylation, the defect of PCNA ubiquitylation in Spartan knockdown cells was more evident at late time points than early time points (Figure S4D). Even in cells lacking Usp1, knockdown of Spartan led to a reduction in PCNA ubiquitylation (Figure S4C, lanes 3 and 5), suggesting that Spartan is an enhancer of Rad18 function, rather than an inhibitor of Usp1. Together, these results suggest that Spartan and Rad18 may function in concert to form a feed-forward loop that enhances PCNA ubiquitylation.

To further investigate the functional link between Spartan and Rad18, we performed an epistasis analysis of the two proteins. Cells in which both Spartan and Rad18 were knocked down exhibited similar UV sensitivity to Spartan or Rad18 single knockdown cells (Figure 7A), suggesting that they function in the same epistasis group. It is important to note that Spartan knockdown did not impair PCNA ubiquitylation as severely as Rad18 knockdown, suggesting that even a partial defect in PCNA ubiquitylation compromises cell survival or that Spartan possesses additional functions required for UV resistance. Similarly to Pol η (Watanabe et al., 2004), SFB-Spartan^{WT} colocalized with GFP-Rad18 at sites of DNA damage in UV-irradiated cells (Figure 7B). Furthermore, like Pol η (Watanabe et al., 2004), SFB-Spartan^{WT} was coimmunoprecipitated by GFP-Rad18 from extracts of undamaged and UV-treated cells (Figure 7C). Immunoprecipitation of SFB-Spartan^{WT} captured not only GFP-Rad18, but also endogenous Rad18 (Figure S5). These results suggest that Spartan and Rad18 not only functionally, but also physically, interact with each other.

How does Spartan regulate Rad18? Since PCNA ubiquitylation occurs on chromatin (Kannouche et al., 2004), we asked whether Spartan influences the chromatin association of Rad18. Consistent with previous studies (Huang et al., 2009; Williams et al., 2011; Yang et al., 2008), in cycling cells, Rad18 was detected on chromatin even in the absence of DNA damage (Figure 7D). The levels of chromatin-bound Rad18, but not soluble Rad18, were reduced by Spartan knockdown in undamaged and UV-treated cells (Figure 7D). Coincident with the reduction in chromatin-bound Rad18 in UV-treated Spartan knockdown cells, the levels of ubiquitylated PCNA on chromatin, but not the overall levels of chromatin-bound PCNA, were reduced (Figure 7D). Thus, Spartan promotes PCNA ubiquitylation at least in part by facilitating the binding and/or retention of Rad18 on chromatin.

DISCUSSION

Protein ubiquitylation has emerged as an important type of post-translational modification that orchestrates the DNA damage response (DDR) (Bergink and Jentsch, 2009). A number of DDR proteins have been shown to function as “readers” of the ubiquitin marks on other proteins (Dikic et al., 2009). For example, FAN1 and SLX4 recognize monoubiquitylated FANCD2 (Kratz et al., 2010; Liu et al., 2010; MacKay et al., 2010; Smogorzewska et al., 2010; Yamamoto et al., 2011), and Rap80 binds to the polyubiquitin chains synthesized by RNF8 and RNF168 (Kim et al., 2007; Sobhian et al., 2007; Wang et al., 2007). How the ubiquitin readers can recognize their functional partners specifically is still an important question under investigation.

Part of the specificity of ubiquitin readers comes from their different UBDs (Dikic et al., 2009). For example, the *u*biquitin *i*nteracting *m*otifs (UIMs) of Rap80 bind to K63-linked ubiquitin chains more efficiently than K48-linked chains (Sato et al., 2009; Sobhian et al., 2007). In addition to their UBDs, a number of ubiquitin readers interact with their partners

through a second ubiquitin-independent contact, which presumably increases the affinity and specificity for the binding. For example, Pol η contains a PIP box and a UBZ domain, which contribute to the binding of Pol η to PCNA and ubiquitin, respectively (Bienko et al., 2005; Haracska et al., 2001a). In this study, we show that Spartan also recognizes ubiquitylated PCNA through its PIP box and UBZ domain, strengthening the model in which the concerted action of a PIP box and a UBD confers the specificity to readers of PCNA ubiquitylation.

Surprisingly, while Spartan is a reader of ubiquitylated PCNA, it facilitates chromatin association of Rad18 and is required for efficient PCNA monoubiquitylation. Unlike Spartan, Rad18 contains only a UBZ domain but not a PIP box. In contrast to Spartan's UBZ domain, the UBZ of Rad18 is dispensable for PCNA ubiquitylation (Huang et al., 2009). It has been suggested that Rad18 is recruited to stalled replication forks by RPA-coated single-stranded DNA (ssDNA) (Chang et al., 2006; Davies et al., 2008), but how Rad18 engages PCNA remains elusive. Our findings on Spartan reveal an unexpected feed-forward loop that couples a reader and a writer of PCNA ubiquitylation. The Rad18 recruited by RPA-ssDNA may initiate PCNA ubiquitylation, and the Spartan-mediated feed-forward loop may help Rad18 engage PCNA efficiently.

Our results also suggest that UV-induced PCNA ubiquitylation, like IR-induced H2AX phosphorylation (Lou et al., 2006), is a self-perpetuating process. It is possible that ubiquitylation of more than one subunit of the PCNA clamp, or ubiquitylation of multiple PCNA clamps around DNA lesions, is important for the full function of PCNA in UV-induced DNA damage response. Simultaneous ubiquitylation of multiple PCNA molecules in a trimeric complex may coordinate the functions of different readers or, alternatively, stabilize the interaction with readers containing multiple UBDs (Sale et al., 2012). The appearance of UV-induced PCNA nuclear foci suggests that a large number of PCNA molecules are present in close proximity at these sites. The colocalization of PCNA with certain DDR proteins, as well as the requirement of PCNA ubiquitylation for the localization of some of these proteins, suggest that robust PCNA ubiquitylation promotes accumulation of specific DDR proteins at sites of UV damage.

In addition to enhancing PCNA ubiquitylation, Spartan may have other roles in the response to UV damage. While Spartan is not as critical as Rad18 for PCNA ubiquitylation, knockdown of Spartan and Rad18 lead to similar UV sensitivity. It is possible that Spartan contributes to other DDR events important for UV resistance. The function of the SprT-like domain of Spartan is currently unknown. The role of Spartan in TLS suggests that it may be implicated in both PRR and ICL repair. The ability of Spartan to bind diubiquitylated PCNA and polyubiquitin chains in vitro raises the possibility that it may also be a reader of polyubiquitylated PCNA and regulate the error-free PRR pathway. Future experiments that investigate the potential enzymatic activity of Spartan and its effects on downstream DDR processes will help elucidate the full function of this reader of PCNA ubiquitylation.

EXPERIMENTAL PROCEDURES

Cell Culture and UV Irradiation

HEK293T, U2OS, and XP30RO-eGFP-Pol η cells were cultured in Dulbecco's modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS). U2OS and derived cell lines stably expressing SFB-Spartan or mutants were cultured in DMEM with 10% FBS and 1 μg/ml puromycin. UV treatment was performed with a 254 nm bulb (UVP) calibrated using a UVX Radiometer with a 254 nm probe (UVP).

Antibodies

In this study, we used the antibodies against PCNA (PC10, Millipore), FLAG (M2 and F7425, Sigma), K48- and K63-ubiquitin chains (Cell Signaling), Rad18 (Tsuji et al., 2008), GST (Abcam and Santa Cruz), FK2 (Eppendorf), GFP (Invitrogen), XPA (Santa Cruz), CPDs (Kamiya), γH2AX (Millipore), and Orc2 (BD Biosciences).

RNA Interference

siRNA transfections were performed using Lipofectamine RNAiMax (Invitrogen). siRNA sequences were as follows: Rad18 (5′CCAAGAAACAAGCGUA AUA); Spartan/C1orf124 (5′ACCGGACUUGCAGGCACUGUUUGUU, 5′AAUA CAGGUGGUACUUGAGACUUUG, 5′UAACCAAACACCUGACAGGAUUCCC); Usp1 (5′UCGGCAAUACUUGCUAUCUUA-UU); ATM (5′GCCUCCAGGCAGA AAAAGA).

Immunostaining and Microscopy

For immunofluorescence (IF) analyses, cells were extracted with 0.25% Triton prior to fixation with either 3% paraformaldehyde or ice-cold 100% methanol. Subsequently, cells were extracted again with 0.5% Triton, and blocked in PBS with 3% BSA and 0.05% Tween-20. To irradiate cells in subnuclear regions with UV, 5-micron Nucleopore Track-Etched membrane filters (What-man) were used. Microscopy was performed using NIS Elements software on a Nikon Eclipse 90i epifluorescence microscope with filter cubes for DAPI (UV-2E/C, Ex. 340–380 nm), FITC (FITC HQ, Ex. 460–500 nm), and Texas Red (Texas Red HQ, Ex. 532–587 nm), and a Q-Imaging 2000R camera. Final images were prepared with Adobe Photoshop CS5.1.

Immunoprecipitation

For coimmunoprecipitation of PCNA with SFB-tagged Spartan, HEK293T cells transfected with pSFB-Spartan, pSFB-Spartan$^{\Delta PIP}$, or empty vector were lysed in the NETN buffer (20 mM Tris-HCl [pH8.0], 100 mM NaCl, 1 mM EDTA, 0.5% NP40 and 1 × protease inhibitor cocktail [Sigma]). Pull-downs were performed using streptavidin-coated Dynabeads (Invitrogen) for 2 hr at 4°C. Beads were washed three times with lysis buffer and subsequently analyzed. For coIP of GFP-Rad18 with SFB-Spartan, HEK293T cells transfected with the plasmids were lysed in the GNZP lysis buffer (1 × PBS, 0.5% NP40, 10% glycerol, 10 μM $ZnCl_2$, 2 mM DTT, 1 mM PMSF, 1 mM NaF, 1 mM Na_3VO_4, and 1 × protease inhibitor cocktail [Sigma]). IPs were performed using anti-GFP antibody and Protein A-conjugated agarose beads (GE Healthcare) for 2 hr at 4°C. Beads were washed three times with lysis buffer prior to analysis. Pull-downs of Spartan under denaturing conditions were performed essentially as previously described (Choo and Zhang, 2009).

Cell Viability Assay

U2OS cells (or U2OS-derived mutant cell lines) were transfected twice with 20 μM siRNA using Lipofectamine RNAiMax (Invitrogen). Forty-eight hours after the first transfection, cells were trypsinized and replated in 24-well dishes at a density of 150–500 cells per well. On the following day, cells were irradiated with the indicated doses of UV or IR. Cell viability was measured using the fluorometric alamarBlue reagent (Invitrogen) 10 days after UV or IR irradiation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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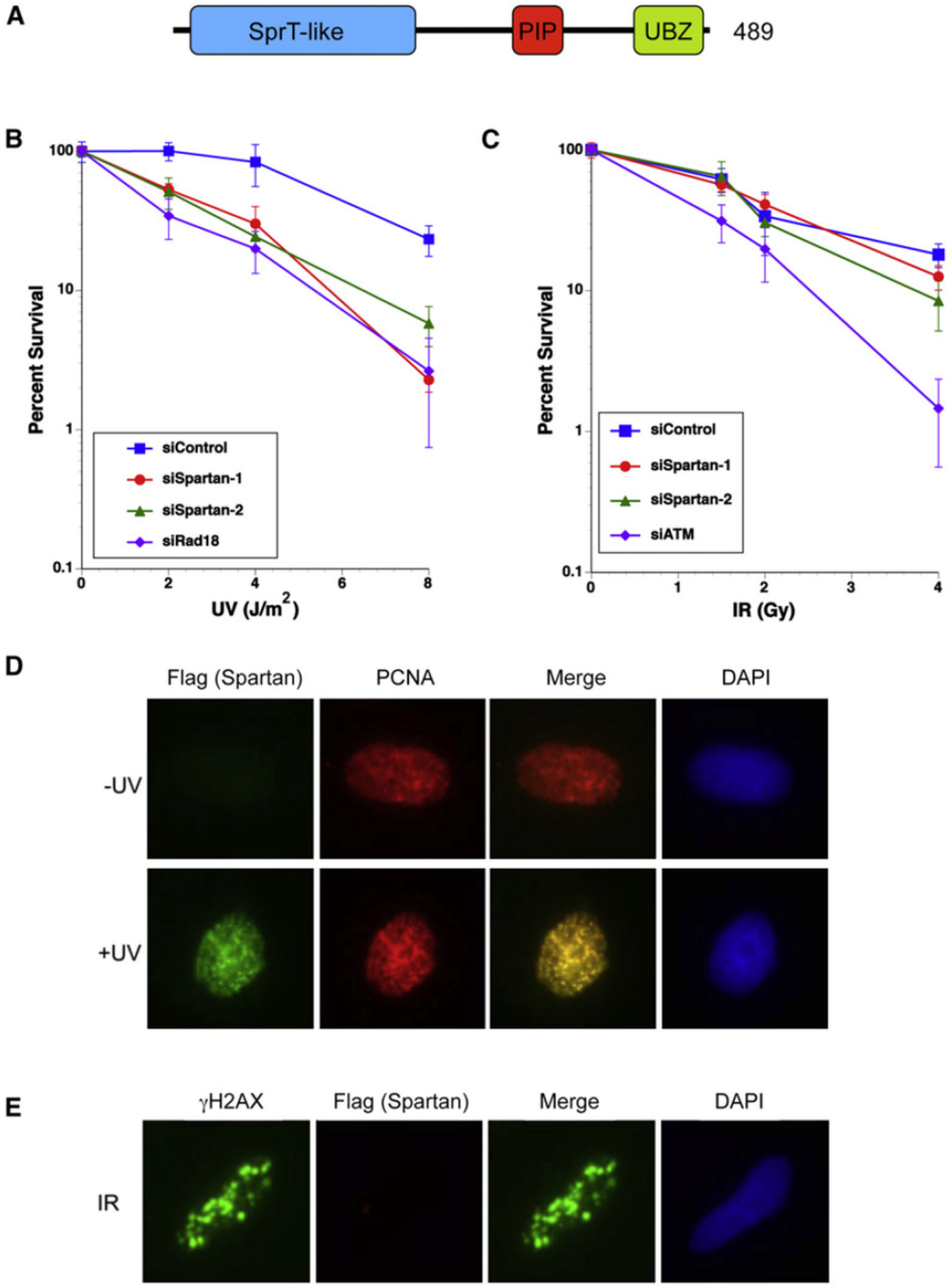

Figure 1. Spartan Is Important for the Cellular Response to UV-Induced DNA Damage

(A) A depiction of the conserved domains of Spartan.

(B and C) Spartan knockdown cells are sensitive to UV, but not IR. U2OS cells transfected with control, Spartan, Rad18, or ATM siRNA were treated with the indicated doses of UV (B) or IR (C). Cell viability was determined as described in the Experimental Procedures section. ATM knockdown cells are hypersensitive to IR and serve as a positive control in (C). Error bars: SD, $n \geq 2$.

(D) Spartan colocalizes with PCNA at sites of UV-induced DNA damage. U2OS cells stably expressing SFB-Spartan were treated with 50J/m^2 UV or left untreated. Cells were subsequently extracted with Triton at 1 hr, fixed, and stained with the indicated antibodies.

(E) Spartan does not localize to sites of DSBs. Cells stably expressing SFB-Spartan were treated with 2 Gy IR and analyzed with the indicated antibodies.

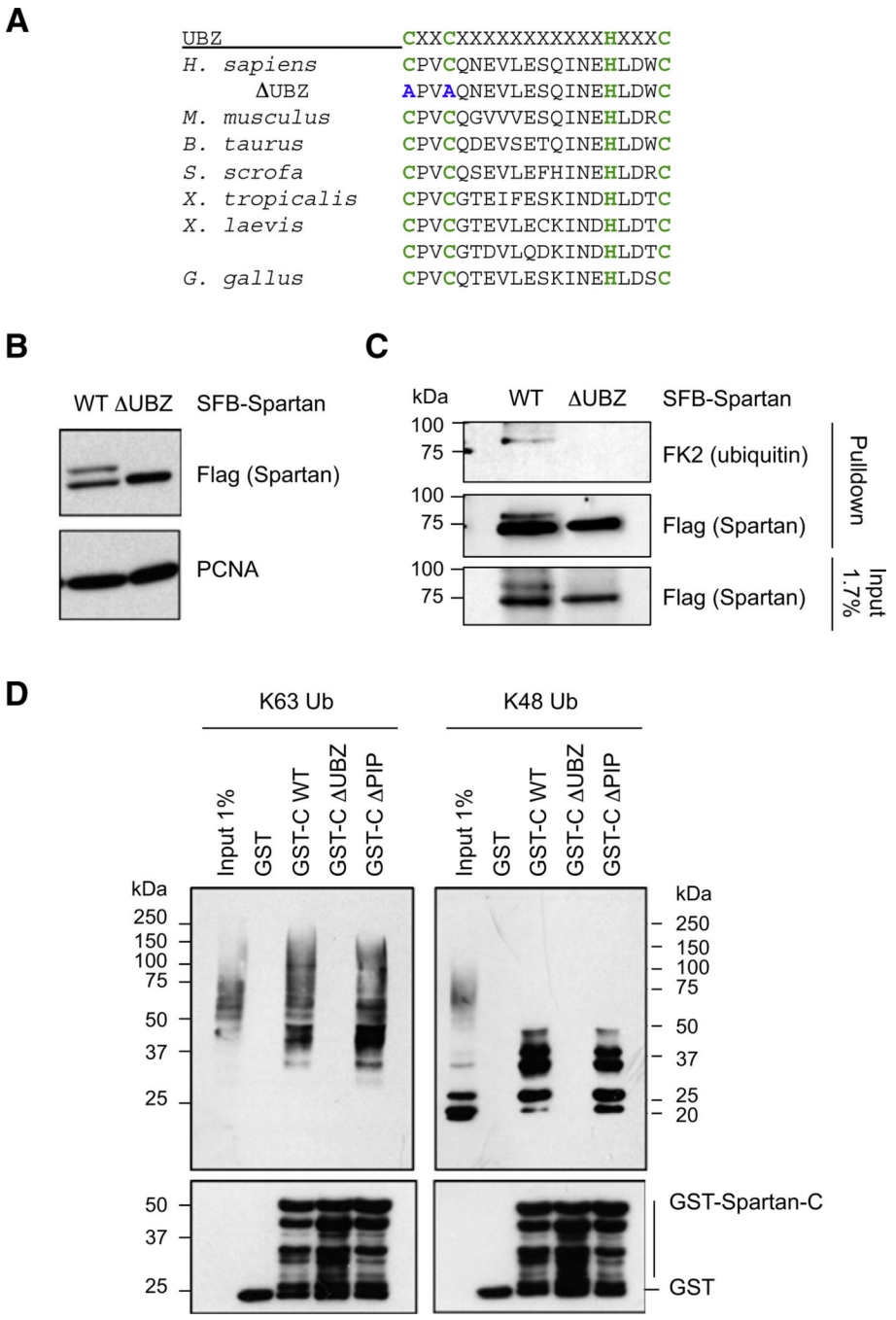

Figure 2. Spartan Contains a Functional UBZ Domain

(A) Sequence alignment of the UBZ domain of Spartan across different species. Green denotes important residues for ubiquitin binding; blue denotes residues mutated in the ΔUBZ mutant used in this study. Note that *X. laevis* uniquely contains two UBZ domains.

(B) SFB-Spartan^{WT} and SFB-Spartan$^{\Delta UBZ}$ were analyzed by western blot.

(C) Spartan is ubiquitylated in a UBZ-dependent manner. SFB-Spartan^{WT} was isolated under denaturing conditions and analyzed with the indicated antibodies.

(D) Spartan's UBZ domain binds ubiquitin chains directly. Purified GST, GST-Spartan-C^{WT}, GST-Spartan-C$^{\Delta PIP}$, and GST-Spartan-C$^{\Delta UBZ}$ were incubated with purified K63- or K48-linked ubiquitin chains and subsequently retrieved with glutathione beads. The

ubiquitin chains in input and pull-downs were analyzed using K63- or K48-Ub-specific antibodies.

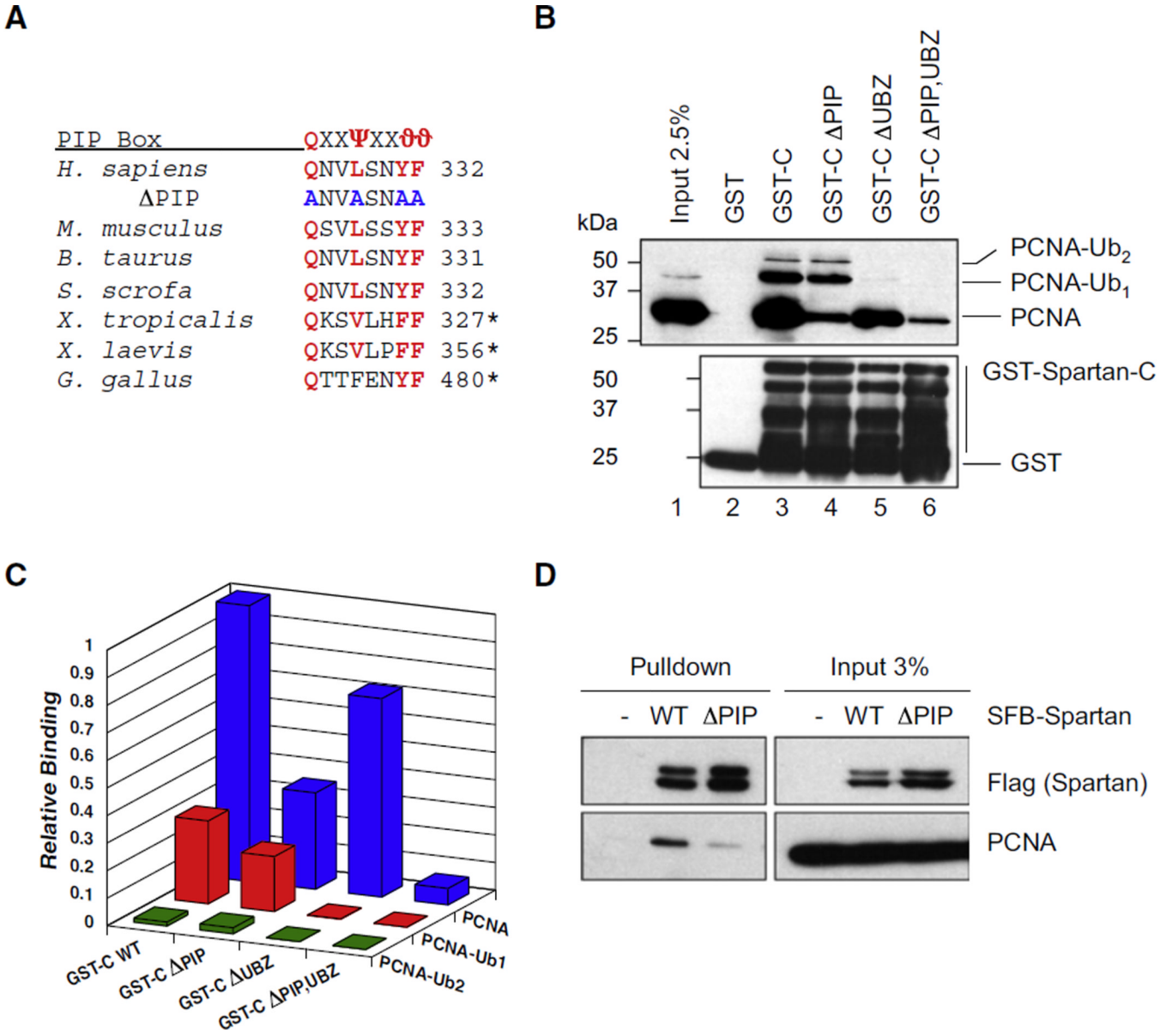

Figure 3. Spartan Binds Ubiquitylated PCNA via Its PIP Box and UBZ

(A) Sequence alignment of the PIP box of Spartan across different species. *: putative PIP boxes in locations different from the PIP box of human Spartan. Red denotes important residues for PCNA binding; blue denotes residues mutated in the ΔPIP mutant used in this study.

(B) Spartan's PIP box and UBZ domain are important for efficient PCNA binding in vitro. 293T cells were treated with 50J/m^2 UV and lysed after 5 hr. GST, GST-Spartan-C^{WT}, GST-Spartan-$C^{\Delta PIP}$, GST-Spartan-C^{UBZ}, and GST-Spartan-$C^{\Delta PIP,UBZ}$ were incubated with cell extracts and subsequently retrieved with glutathione beads. The PCNA in input and pull-downs was analyzed by western blot.

(C) Quantification of the relative abundance of unmodified and ubiquitylated PCNA in Figure 3B.

(D) Spartan's PIP box is important for PCNA binding in cells. 293T cells were transiently transfected with empty vector, SFB-Spartan, or SFB-Spartan$^{\Delta PIP}$. Cell extracts were subjected to streptavidin-coated Dynabead pull-down, followed by western blot.

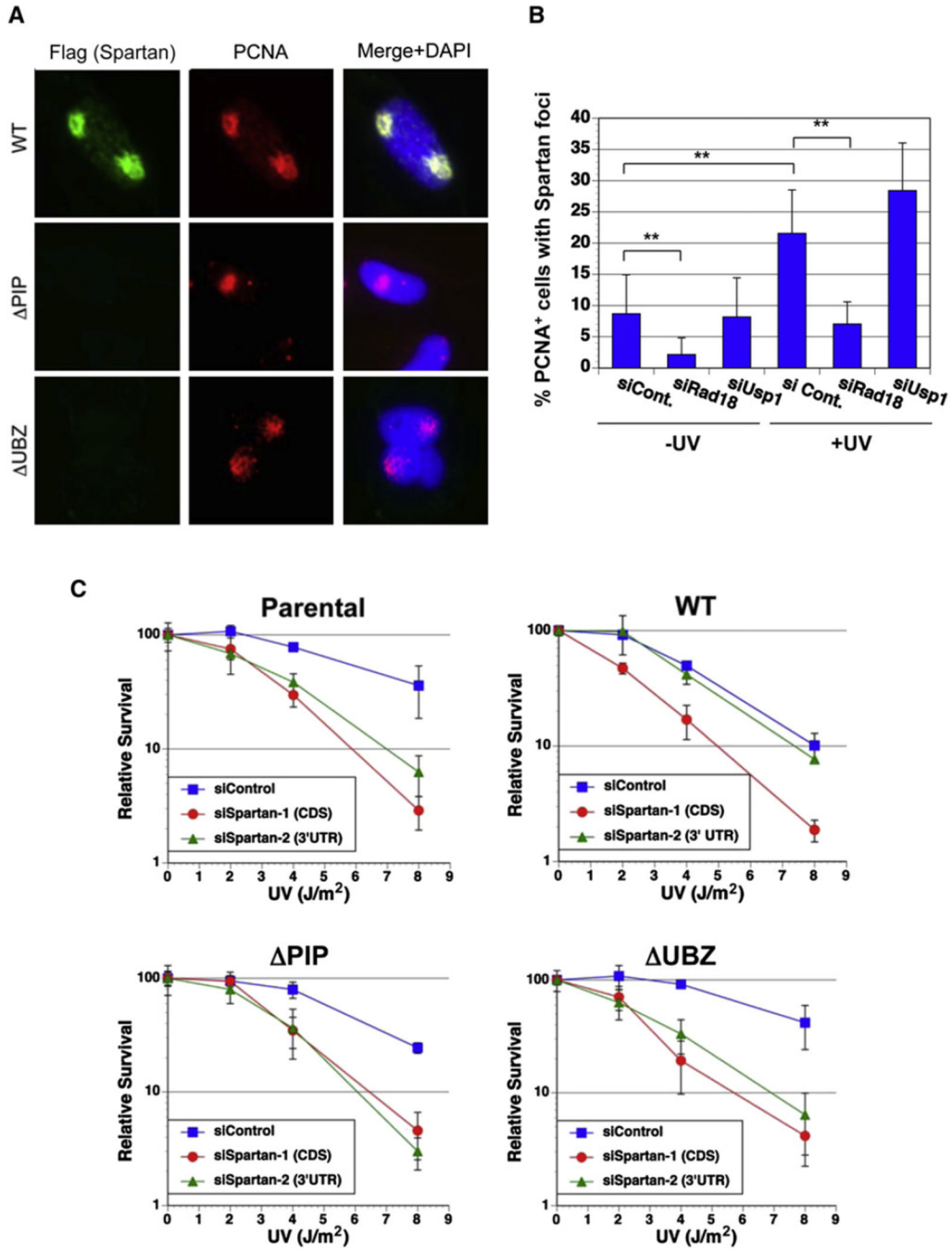

Figure 4. The PIP Box and UBZ Domain of Spartan Are Important for Its Function In Vivo

(A) Colocalization of Spartan with PCNA requires both its PIP box and UBZ domain. U2OS cells stably expressing SFB-Spartan^{WT}, Spartan$^{\Delta PIP}$, or Spartan$^{\Delta UBZ}$ were irradiated with UV through 5-micron filters and subsequently analyzed by immunofluorescence after 1 hr as in Figure 1D.

(B) Effects of Rad18 and Usp1 knockdown on Spartan focus formation. Cells expressing SFB-Spartan^{WT} were transfected with control, Rad18, or Usp1 siRNA. Three days after transfection, cells were irradiated with UV and analyzed as in (A). Quantification of the percentage of PCNA-positive cells with Spartan foci is plotted. **: a p value of < 0.005; error bars: SD, >750 cells per condition.

(C) Spartan's PIP box and UBZ domain are important for normal UV resistance. Parental U2OS cells or derivatives stably expressing SFB-Spartan^{WT}, Spartan$^{\Delta PIP}$, or Spartan$^{\Delta UBZ}$ were transfected with control, Spartan-1 (targeting the coding sequence; CDS), or Spartan-2 (targeting the 3′UTR) siRNA. Cell survival was analyzed after UV irradiation. Error bars: SD, $n \geq 2$.

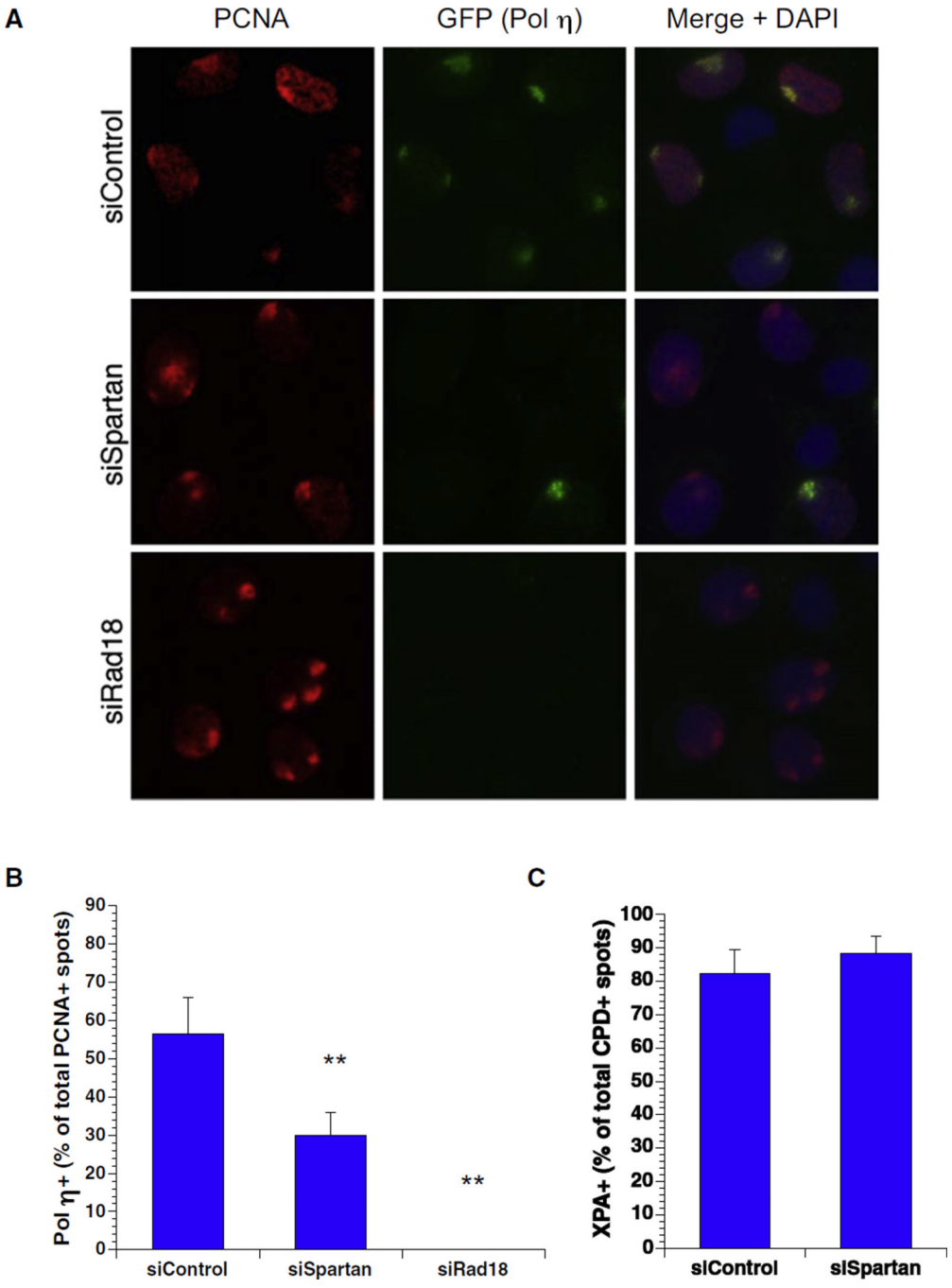

Figure 5. Spartan Regulates Localization of Pol η but Not XPA

(A) Spartan is necessary for the efficient recruitment of DNA polymerase η to sites of local UV-induced DNA damage. XP30RO cells stably expressing eGFP-Pol η were transfected with control, Spartan, or Rad18 siRNA, and irradiated with UV through 5-micron filters. After 5 hr, cells were extracted with Triton, fixed, and stained with antibodies against GFP and PCNA.

(B) Quantification of the percentage of cells with PCNA-positive spots that are also positive for eGFP-Pol η. **:$p < 0.05$. Error bars: SD, n = 3 independent experiments each, in which >50 PCNA-positive spots were counted for each condition.

(C) Spartan knockdown has no effect on the localization of XPA to sites of UV-induced DNA damage. U2OS cells were transfected with control or Spartan siRNA and UV-irradiated through filters. After 1 hr, cells were stained with antibodies for CPDs and XPA. The percentage of cells with CPD-positive spots that were also positive for XPA are plotted. Error bars: SD, n > 350 CPD spots counted for each condition.

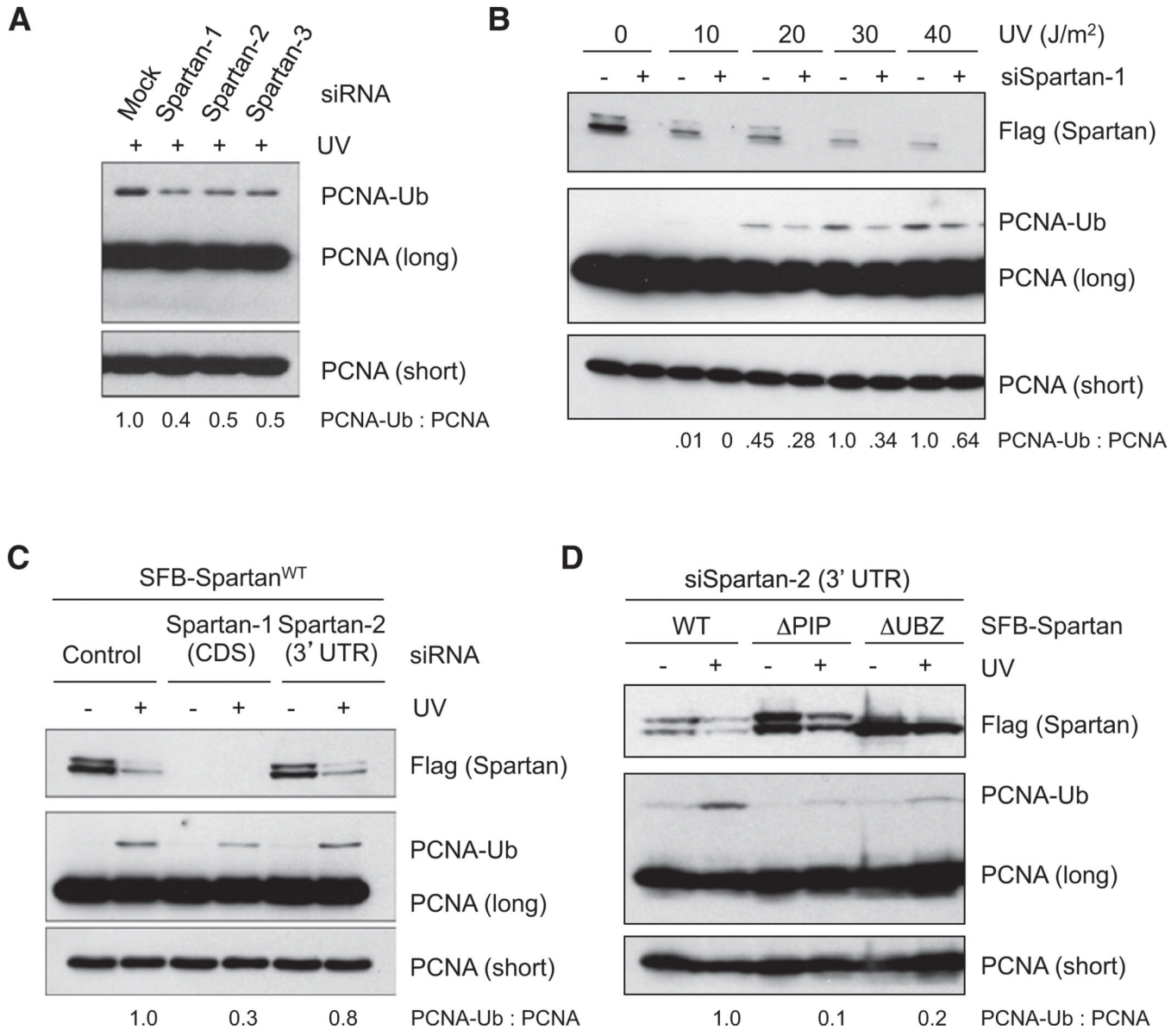

Figure 6. Spartan Promotes UV-Induced PCNA Ubiquitylation

(A) Spartan is required for efficient UV-induced PCNA ubiquitylation. U2OS cells were transfected with control or three independent Spartan siRNAs. Three days after transfection, cells were treated with 50J/m^2 UV and analyzed after 5 hr. Levels of unmodified PCNA (PCNA) and monoubiquitylated PCNA (PCNA-Ub) were analyzed by western blot and quantified using ImageJ.

(B) Defective UV-induced PCNA ubiquitylation in Spartan knockdown cells is observed over a range of UV doses. Cells stably expressing SFB-Spartan^{WT} were transfected with control or Spartan siRNA, irradiated with the indicated UV doses, and analyzed as in (A).

(C) The defect of PCNA ubiquitylation in Spartan knockdown cells is suppressed by SFB-Spartan^{WT}. Cells stably expressing SFB-Spartan^{WT} were transfected with the indicated siRNAs and analyzed as in (A).

(D) Spartan's PIP box and UBZ domain are required for efficient PCNA ubiquitylation. Cells stably expressing SFB-Spartan^{WT}, Spartan$^{\Delta PIP}$, or Spartan$^{\Delta UBZ}$ were transfected with Spartan-2 siRNA and were analyzed as in (A).

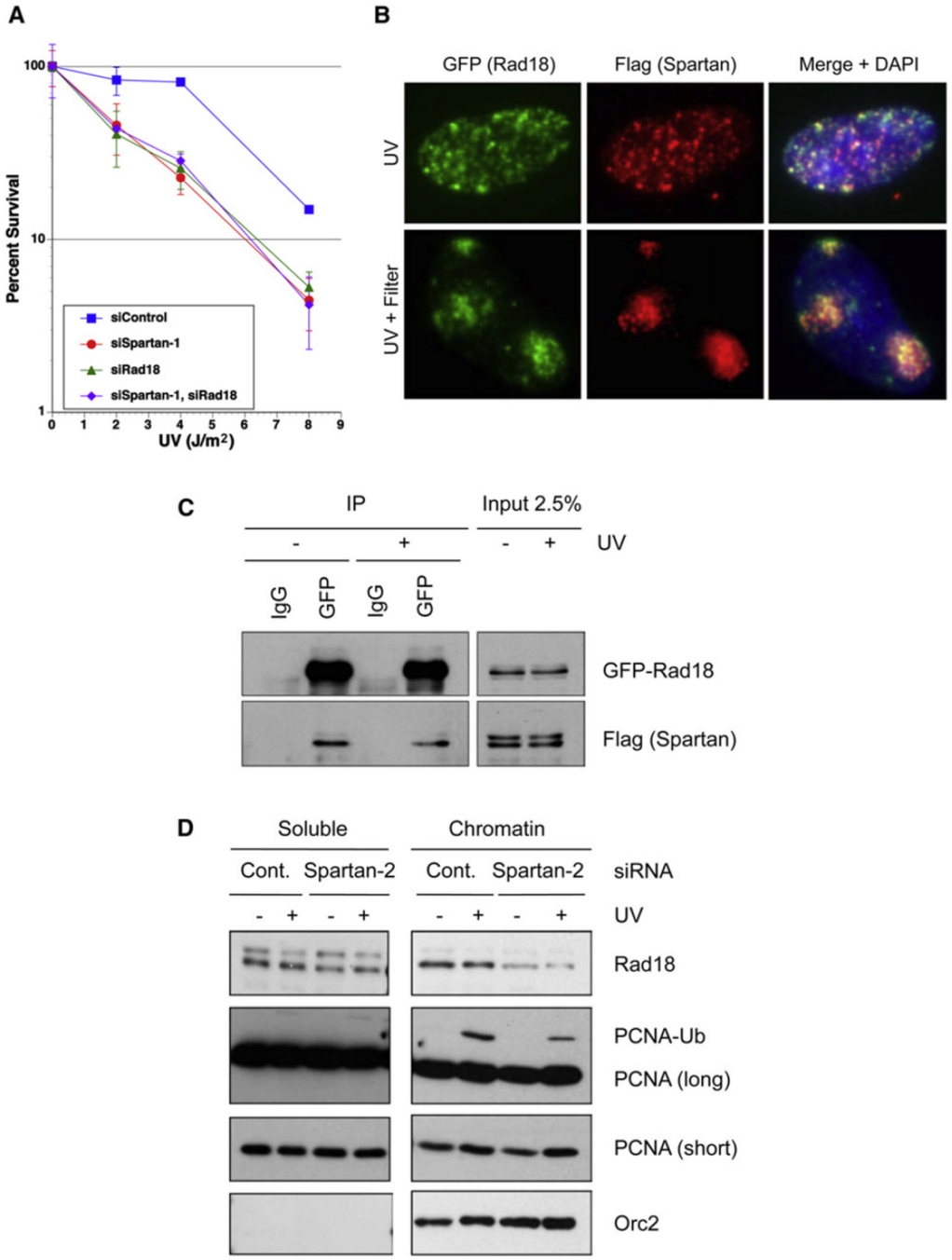

Figure 7. Spartan Is Functionally and Physically Linked to Rad18

(A) Spartan and Rad18 function in the same pathway to confer cellular UV resistance. U2OS cells were transfected with the indicated siRNAs, individually or in combination, and cell survival was measured after UV irradiation as described in the Experimental Procedures section. Error bars: SD, $n \geq 2$.

(B) Rad18 and Spartan colocalize at sites of UV damage. U2OS cells stably expressing SFB-Spartan^{WT} were nucleofected with GFP-Rad18 and irradiated with UV directly or through filters. After 1 hr, cells were extracted with Triton, fixed, and stained with GFP and FLAG antibodies.

(C) Spartan and Rad18 physically interact with each other. 293T cells were transfected with plasmids expressing SFB-Spartan and GFP-Rad18, treated with UV or mock treated, and analyzed by IP after 1 hr.
(D) Spartan is required for efficient chromatin association of Rad18. U2OS cells transfected with control or Spartan-2 siRNA were treated with UV or left untreated and subsequently subjected to chromatin fractionation after 5 hr. The indicated proteins in the chromatin and soluble fractions were analyzed by western blot. Orc2 serves as a loading control for the chromatin fractions.