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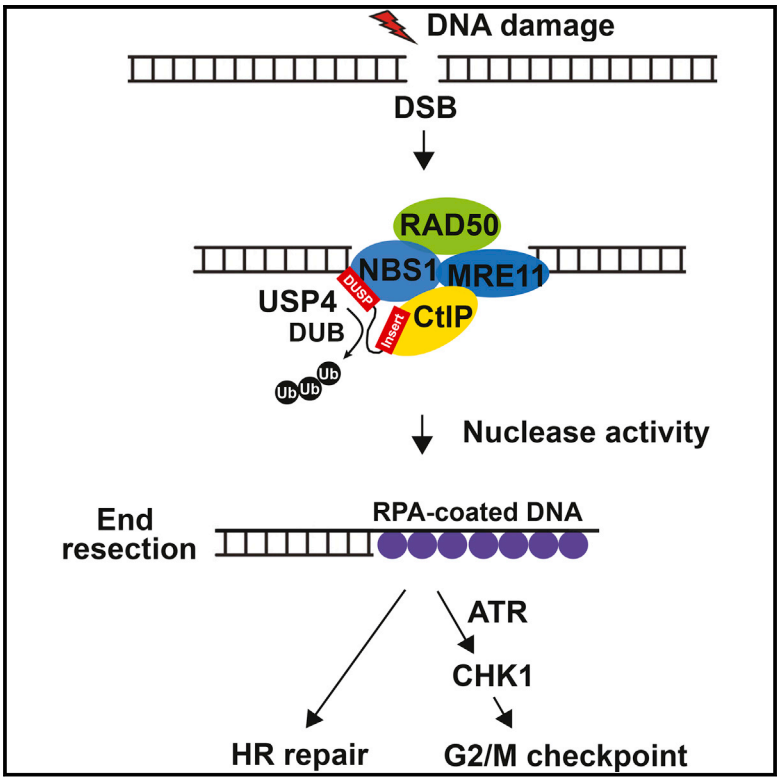
The Deubiquitylating Enzyme USP4 Cooperates with CtIP in DNA Double-Strand Break End Resection

Graphical Abstract



Highlights

- USP4 confers resistance to DNA damage-inducing agents
- USP4 interacts with CtIP and MRN via its conserved region and catalytic domain
- USP4 regulates CtIP recruitment, end resection, and homologous recombination
- USP4 autodeubiquitylation is essential for its HR functions

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In Brief

DNA end resection is a highly regulated and critical step in the response and repair of DNA double-strand breaks (DSBs). Liu et al. report that the deubiquitylating enzyme USP4 participates in DSB resection and HR.

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The Deubiquitylating Enzyme USP4 Cooperates with CtIP in DNA Double-Strand Break End Resection

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SUMMARY

DNA end resection is a highly regulated and critical step in DNA double-stranded break (DSB) repair. In higher eukaryotes, DSB resection is initiated by the collaborative action of CtIP and the MRE11-RAD50-NBS1 (MRN) complex. Here, we find that the deubiquitylating enzyme USP4 directly participates in DSB resection and homologous recombination (HR). USP4 confers resistance to DNA damage-inducing agents. Mechanistically, USP4 interacts with CtIP and MRN via a specific, conserved region and the catalytic domain of USP4, respectively, and regulates CtIP recruitment to sites of DNA damage. We also find that USP4 autodeubiquitylation is essential for its HR functions. Collectively, our findings identify USP4 as a key regulator of DNA DSB end resection.

INTRODUCTION

DNA is constantly challenged by physical and chemical threats that compromise its structure and function (Aguilera and Gómez-González, 2008; Davis and Chen, 2013). Such DNA lesions have to be corrected through DNA repair (Jimeno et al., 2015). Faithful replication and repair of DNA ensures that genomes remain stable enough during the lifetime of an organism and avoids compromising viability (Bakkenist and Kastan, 2004). In eukaryotic cells, NHEJ and homologous recombination (HR) are two key pathways that mediate double-stranded break (DSB) repair (Lieber, 2008; Panier and Boulton, 2014; Panier and Durocher, 2013; Pierce et al., 2001; San Filippo et al., 2008; Seluanov et al., 2010). NHEJ repairs DSBs by the re-ligation of broken DNA ends. NHEJ is considered error prone and mutagenic given that a homologous template is not used to guide repair (Jimeno et al., 2015). HR is considered an error-free mechanism for DSB repair that employs homologous sequence in the sister chromatid as a template to prime repair synthesis and restore chromosome integrity (Maher et al., 2011). Initiation of these processes is tightly regulated, and aberrant pathway activation results in genomic instability (Chapman et al., 2012a, 2012b; Doil et al., 2009; Mailand et al., 2007; Mattiroli et al., 2012).

The first control point for DNA repair pathway choice is the processing of the DNA break (Jimeno et al., 2015). DNA end resection inhibits NHEJ and allows HR (Huertas, 2010). It is believed that during HR, DNA ends are first resected in the 5′–3′ direction by nucleases (Buis et al., 2008; Cannavo and Cejka, 2014; Nimonkar et al., 2011; Sartori et al., 2007; Williams et al., 2008; Yamaguchi-Iwai et al., 1999; Yuan and Chen, 2009). The resulting single-stranded DNA (ssDNA) is rapidly bound by replication protein A (RPA) (He et al., 1995; Huertas, 2010; Zou and Elledge, 2003). Subsequently, RAD51 displaces RPA-ssDNA complexes with the help of its accessory factors to form a helical nucleoprotein filament that permits strand invasion and homology search (Davies et al., 2001; Forget and Kowalczykowski, 2010; Pellegrini et al., 2002; San Filippo et al., 2008). At the same time, the ssDNA-bound RPA can also recruit ATR, which phosphorylates CHK1 to trigger and activate cell-cycle checkpoints (Liu et al., 2000; Paulsen and Cimprich, 2007). Therefore, DNA end resection is considered as a key step that controls not only DNA repair but also DNA damage checkpoints (Yuan and Chen, 2010).

The MRN complex, which consists of MRE11, RAD50, and NBS1, and the nuclear protein CtIP have been suggested to operate together in the DNA end resection and DNA damage checkpoint activation. CtIP (also known as RBBP8) was originally identified as a protein that interacts with the transcriptional repressor CtBP, the retinoblastoma protein RB, and the tumor suppressor BRCA1 (Wu and Lee, 2006). CtIP can be recruited to DNA damage sites and control the DNA damage-induced G2/M checkpoint (Greenberg et al., 2006; Yu and Chen, 2004; Yu et al., 2006). More recently, the catalytic and noncatalytic roles of the CtIP endonuclease in DSB end resection have been unveiled (Makharashvili et al., 2014; Takeda et al., 2007; Wang et al., 2014). CtIP functions with the MRN complex to process DSB ends and generates ssDNA regions. Here, we further examine the regulation of CtIP on DNA end resection and the choice between different DSB repair mechanisms.

A

CtIP purification

Protein	Peptides	Coverage(%)
CtIP	67	77.80
BRCA1	38	41.3
LMO4	21	27.4
NBS1	18	18.4
BRIX	16	29.4
HP1	11	20.82
USP4	9	12.7
SIAH1	8	7.6
Mre11	5	6.4
XRCC1	6	4.8
PRS16	4	2.1

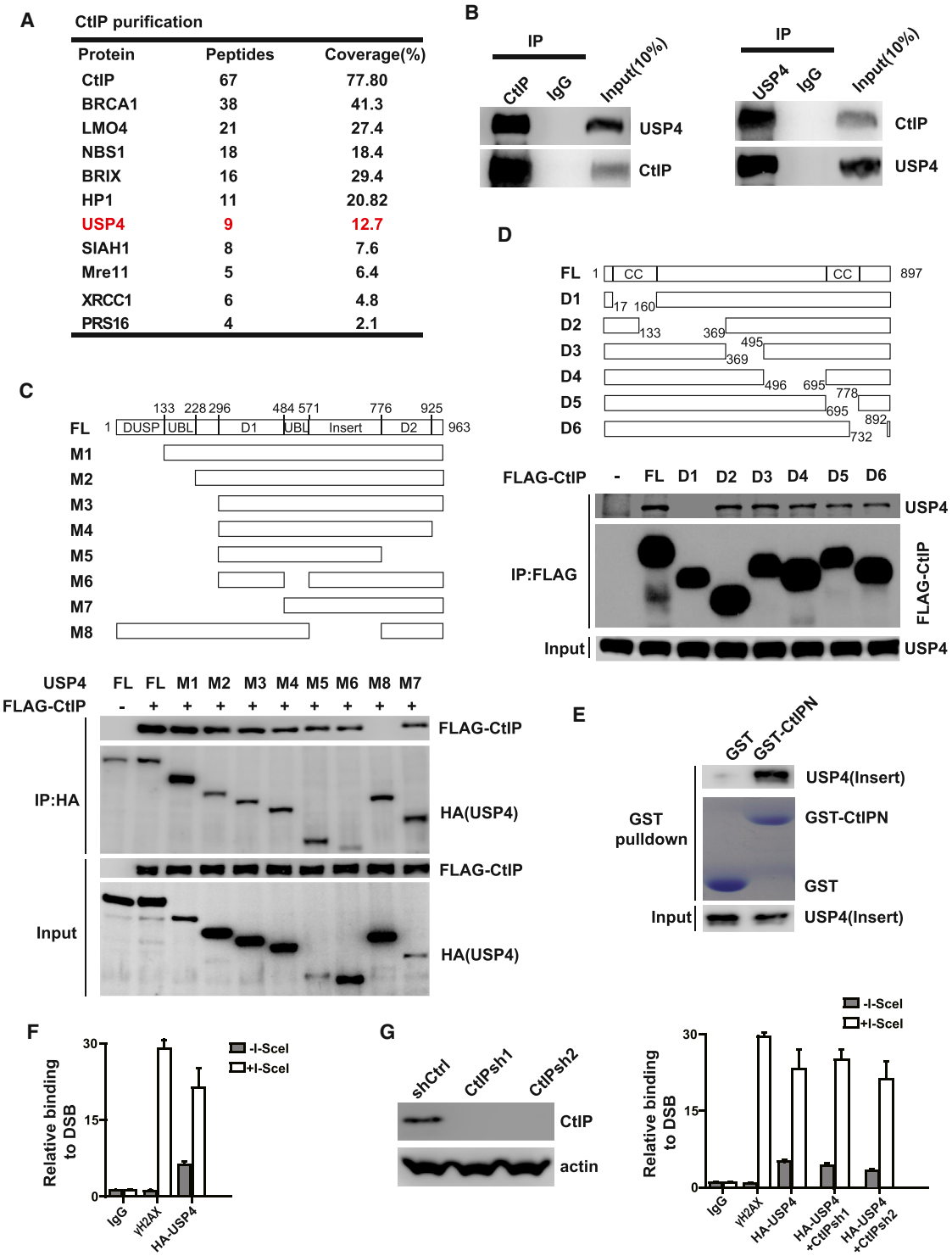

Figure 1. USP4 Interacts with CtIP

(A) Tandem affinity purification was performed with 293T cells stably expressing FLAG-SBP-tagged CtIP. The major hits from MS result were shown.

(B) Reciprocal co-immunoprecipitation (coIP) between USP4 and CtIP in U2OS cells after adding DNase was performed. (Left) IP with anti-CtIP antibody and blot with anti-CtIP or USP4 antibody, respectively, are shown. (Right) IP with anti-USP4 antibody and blot with anti-USP4 or CtIP antibody, respectively, are shown. IgG IP is a negative control.

(legend continued on next page)

Covalent post-translational modification of proteins by ubiquitin and ubiquitin-like factors has emerged as a general mechanism to modulate DNA damage response (DDR) pathways (Jackson and Durocher, 2013; Jacq et al., 2013). Ubiquitin-based DSB signaling by RNF8 and RNF168 has been well established in DDR (Doil et al., 2009; Huen et al., 2007; Kolas et al., 2007; Lukas et al., 2011; Mailand et al., 2007; Mattiroli et al., 2012). So far, however, we only have a relatively limited understanding of the DDR roles for deubiquitylation enzymes (DUBs), which mediate the processing of DNA end resection.

In this study, we show that the DUB USP4 promotes DSB resection and HR, thus contributing to cell survival upon exposure to DNA damage agents. Our findings establish USP4 as a regulator of the DDR pathway and explain how DUB-mediated autodeubiquitylation functions in repair choice and maintaining genomic integrity.

RESULTS

USP4 Interacts with CtIP

In order to better characterize the regulatory network that controls the end resection and the choice between DSB repair pathways, CtIP purification was performed using HEK293T cells stably expressing FLAG-SBP-tagged CtIP and subjected to mass spectrometry analysis. A number of known CtIP-associated proteins were co-purified with CtIP, including BRCA1, NBS1, and Mre11 (Figure 1A). Interestingly, we also identified USP4, a well-characterized deubiquitylating enzyme, as a CtIP-associated protein. Ubiquitin-specific protease USP4 is emerging as an important regulator of cellular pathways, including the TGF-β response, NF-κB signaling, and splicing, with possible roles in cancer (Fan et al., 2011; Song et al., 2010; Sowa et al., 2009; Xiao et al., 2012; Zhang et al., 2012; Zhou et al., 2012). However, its role in DDR is not clear. To confirm this interaction, we performed reciprocal coimmunoprecipitation (coIP) experiments with antibody against USP4 or CtIP. As shown in Figure 1B, endogenous USP4 and CtIP interact with each other in cells. We did the coIP experiment after DNase treatment; this result indicated that CtIP-USP4 interaction was not bridged by DNA.

To identify the regions of USP4 that are responsible for the USP4-CtIP interaction, we generated deletion mutants of USP4 (Figure 1C). Deletion of USP4 insert domain (residues 572–775) abolished the binding of USP4 with CtIP. Similarly, we generated deletion mutants of CtIP (Figure 1D). The USP4-binding region of CtIP was mapped to the N-terminal of CtIP (residues 17–160). A direct interaction between the insert domain of USP4 and the N-terminal of CtIP expressed in *E.coli* was confirmed by GST pull-down assay (Figure 1E).

USP4 Localizes to Sites of DNA Damage

CtIP is a key regulator of HR (Wang et al., 2013), so we hypothesized that USP4 is also involved in DDR. Many proteins involved in DDR can be recruited to DNA lesions. But we could not see USP4-formed foci upon IR by immunofluorescence staining, perhaps due to the USP4 antibody quality limitation (data not shown). To determine whether USP4 is recruited to DSBs, we use a cellular system (a U2OS clone carrying the DR-GFP HR reporter), in which expression of exogenous I-Sce1 endonuclease introduces a single DSB in the genome. As shown in Figure 1F, USP4 chromatin immunoprecipitation (ChIP) revealed that USP4 is recruited to the I-SceI-induced DNA damage site. We also investigated whether CtIP could affect USP4 recruitment to the DNA damage sites. As shown in Figure 1G, knockdown of CtIP by two different shRNAs did not affect USP4 recruitment to the DNA damage sites.

USP4 Binds NBS1 upon DNA Damage

The results that CtIP interacts with MRN complex and works together in DNA end resection prompt us to think whether USP4 could bind NBS1, which is indeed the case (Figure 2A). In cells, NBS1 and USP4 can form a complex, and the interaction between USP4 and NBS1 was upregulated by IR (Figure 2B). We then mapped the interaction regions of USP4 and NBS1. We found that the residues 530−630 of NBS1 and the intact catalytic domain of USP4 (DUSP domain) are responsible for their interaction (Figures 2C and 2D). Because MRN complex is a sensor of DNA damage (Uziel et al., 2003), we investigated whether NBS1 could affect USP4 recruitment to the DNA damage sites. As shown in Figure 2E, knockdown of NBS1 by two different shRNAs dramatically decreased USP4 recruitment to the DNA damage sites. These results indicated that MRN complex binds USP4 and recruits USP4 to the DNA damage sites upon DNA damage. To further confirm that NBS1-USP4 interaction is indeed involved in NBS1-mediated USP4 recruitment, we stably transfected DR-GFP cells with NBS1 shRNA and reconstituted these cells with shRNA-resistant NBS1-WT or the NBS1 (1–530) deletion mutant. As shown in Figure 2F, USP4 was recruited to DSBs in NBS1-WT cells, but the recruitment of USP4 was defective in NBS1(1–530) deletion mutant cells. On the other hand, USP4 DUSP domain deletion mutant could not be recruited to the DNA damage site (Figure 2G). The above results indicated that USP4-NBS1 interaction is indeed involved in USP4 recruitment.

(C) Schematic representation of USP4 constructs used in this study (top). Mapping the regions essential for the USP4-CtIP interaction in 293T cells (bottom) is shown. 293T cells were transiently transfected with the indicated constructs for 24 hr. Cell lysates were immunoprecipitated with anti-HA affinity gel, and western blot was performed with indicated antibodies.

(D) Schematic representation of CtIP constructs used in this study (top). Mapping the regions essential for the CtIP-USP4 interaction in 293T cells (bottom) is shown. 293T cells were transiently transfected with the indicated constructs for 24 hr. Cell lysates were immunoprecipitated with anti-FLAG M2 affinity gel, and western blot was performed with indicated antibodies.

(E) GST pull-down assay of the insert domain of USP4 with the N-terminal of CtIP expressed in *E. coli*.

(F) Recruitment of USP4 to I-SceI-induced DSBs measured by ChIP assay.

(G) Knockdown of CtIP did not affect USP4 recruitment to I-SceI-induced DSBs measured by ChIP assay.

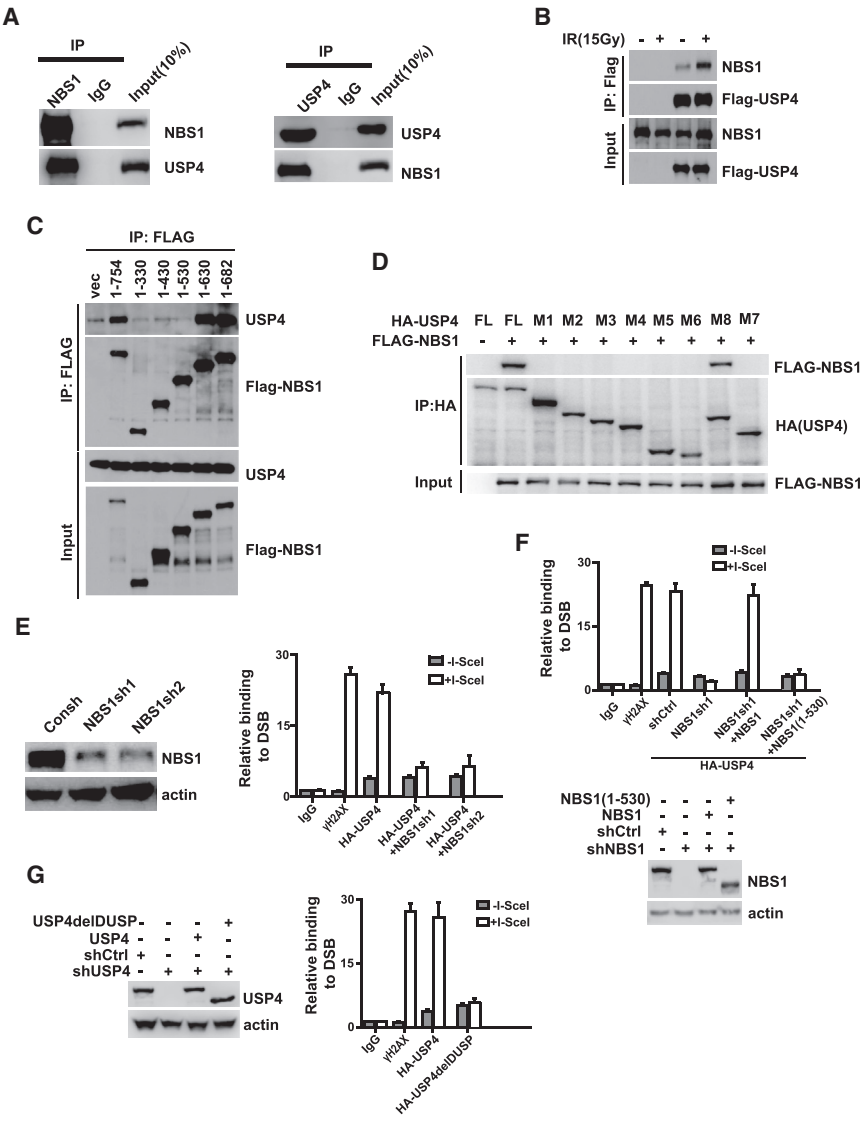

Figure 2. USP4 Binds NBS1 upon DNA Damage

(A) U2OS cells were immunoprecipitated after adding DNase and examined with the indicated antibodies. (Left) IP with anti-NBS1 antibody and blot with anti-NBS1 or USP4 antibody, respectively, are shown. (Right) IP with anti-USP4 antibody and blot with anti-USP4 or NBS1 antibody, respectively, are shown. IgG IP is a negative control.

(B) HEK293T cells were transfected with FLAG-USP4 following irradiation (15 Gy) and immunoprecipitated with anti-FLAG antibody and subjected to immunoblot with the indicated antibodies.

(C–D) Reciprocal coIP between the domains of USP4 and NBS1 in HEK293T cells was performed and subjected to immunoblot with the indicated antibodies.

(E) HeLa DR-GFP cells were transfected with the indicated shRNA, I-SceI, or HA-USP4 and chromatin were immunoprecipitated with the indicated antibody. qPCR was performed for the quantitative analysis of ChIP samples. All qPCR reactions were performed in triplicate, with the SEM values calculated from at least three independent experiments.

(F) HeLa DR-GFP cells were transfected with the indicated plasmids; USP4 and chromatin were immunoprecipitated with the indicated antibody. qPCR was performed for the quantitative analysis of ChIP samples. All qPCR reactions were performed in triplicate, with the SEM values calculated from at least three independent experiments.

(G) HeLa DR-GFP cells were transfected with the indicated shRNA, I-SceI, HA-USP4, or its deletion mutant, HA-USP4 and chromatin were immunoprecipitated with the indicated antibody. qPCR was performed for the quantitative analysis of ChIP samples. All qPCR reactions were performed in triplicate, with the SEM values calculated from at least three independent experiments.

Inactivation of USP4 Sensitizes Human Cells to DNA Damage

To explore a possible role for USP4 in the DDR, we first investigated whether USP4 depletion resulted in DNA damage sensitivity. HCT116 cells depleted with an shRNA targeting USP4 were compared with cells expressing the nontargeting shRNA (shCtrl) for their sensitivity to hydroxyurea (HU), mitomycin C (MMC), ionizing radiation (IR), camptothecin (CPT), and UV light. The viability of these cells after DNA damage treatment was measured using the MTS assay. USP4 depletion did not inhibit cell survival in the absence of DNA damage. However, exposure of the cells to HU, MMC, IR, CPT, and UV caused a reduction in the viability of cells depleted for USP4 (Figure 3A). To rule out off-target effects of the USP4 shRNA, we confirmed the DNA damage sensitivity of USP4 knocked down cells using an shRNA-resistant USP4, which rescued the damage sensitivity conferred by the shRNA (Figure 3A).

At DSB sites, γH2AX foci persist if DSBs are not repaired. To investigate whether USP4 has a role in DSB repair, we examined γH2AX foci formation in USP4-depleted cells that were either untreated or exposed to IR. As shown in Figure 3B, depletion of USP4 resulted in elevated levels of spontaneous γH2AX foci formation. Moreover, at 48 hr after IR, suppression of USP4 resulted in sustained γH2AX foci, in contrast to control. We conclude from these observations that USP4 contributes to the resistance of human cells to DNA damage.

DSBs can be repaired by either HR or NHEJ. Next, we examined how USP4 promotes DNA repair using well-established reporter assays for HR and NHEJ (Bennardo et al., 2008; Fattah et al., 2010; Moynahan et al., 2001). We found that USP4 depletion led to decreased HR frequency to a level similar to that achieved by depleting the key HR factor CtIP (Figure 3C). Conversely, we observed a very slight increase in NHEJ frequency in USP4-depleted cells (Figure 3D). In addition, similar to CtIP depletion, USP4-depletion rendered cells hypersensitive

to PARP inhibitor (AZD2281; Figure 3E). These results suggest that USP4 promotes DSB repair by HR.

USP4 Depletion Abolished DNA End Resection

HR is initiated by DNA end resection, which generates 3′ ssDNA tails that are coated by RPA (Chen et al., 2013). Subsequently, RAD51 displaces RPA-ssDNA complex to form a helical nucleoprotein filament (Stauffer and Chazin, 2004). Consistent with a role of USP4 in HR, we observed that USP4 depletion resulted in sharply decreased RPA recruitment and Rad51 loading to DSBs (Figures 4A and 4B). As USP4 directly interacts with CtIP, we also checked CtIP foci formation in USP4-depleted cells. As expected, depletion of USP4 dramatically inhibited CtIP foci formation (Figure 4C). The accumulation of upstream DNA repair factors (MDC1, RNF8, RNF168, 53BP1, BRCA1, Mre11, and NBS1) at DSBs remained unperturbed (Figures 4D–4F and S1A–S1D). These results suggest an important role for USP4 in regulating DNA end resection, by directly interacting with CtIP and regulating CtIP foci formation.

The generation of RPA-coated ssDNAs is also essential for CHK1 activation after DNA damage. Indeed, USP4 depletion abolished IR-induced CHK1 phosphorylation, but not CHK1 total level (Figure 4H). Importantly, knockout of USP4 has no significant effect on cell-cycle distribution in U2OS cells without DNA damage, indicating that USP4 functions in HR were not caused by cell-cycle change (Figure S2A).

USP4-CtIP Interaction Is Essential for HR

Because USP4 interacts with the N-terminal of CtIP through its insert domain, CtIP N-terminal is essential for its foci formation and depletion of USP4 decreased CtIP foci formation. We hypothesized that USP4-CtIP interaction is required for CtIP recruitment to the DNA damage sites. To test this, we stably transfected U2OS cells with USP4 shRNA and reconstituted these cells with shRNA-resistant USP4-WT or the insert domain deletion mutant. As shown in Figures 5A–5D, WT USP4, but not the insert domain deletion mutant, restored CtIP and downstream RAD51 and RPA foci in USP4-depleted cells after IR. We also found that WT USP4, but not the insert domain deletion mutant, restored the HR efficiency and CHK1 phosphorylation to their level in the wild-type cells (Figures 5E–5G). These results indicate that the USP4-CtIP interaction is required for its function in HR.

USP4 Regulates HR through Its Deubiquitylating Enzyme Activity

Because USP4 is a known deubiquitylating enzyme (Soboleva et al., 2005), we asked whether its deubiquitylating enzyme activity is required for its function in HR. We stably knocked down USP4 in cells using shRNA targeting the 3′ UTR region of USP4 and reconstituted cells with ectopically expressed WT USP4 or USP4 CA mutant (disabled USP4 deubiquitylating enzyme activity; Clerici et al., 2014; Soboleva et al., 2005). As shown in Figures 6A–6G, WT USP4, but not the CA mutant, restored HR efficiency, CtIP, RAD51, RPA foci, and CHK1 phosphorylation in USP4-depleted cells after IR. These results indicate that the deubiquitylating enzyme activity of USP4 is required for its function in HR.

USP4 Autodeubiquitination Promotes USP4-CtIP Interaction

Our results revealed that USP4 interacts with CtIP and USP4-CtIP interaction is important for HR. In addition, the deubiquitylating enzyme activity of USP4 is important for DNA end resection. However, the target(s) of USP4 in this process remain unclear. We reasoned that CtIP may be the substrate of USP4. So we knocked down USP4 in HCT116 cells, treated the cells with IR, and then checked the CtIP protein level and the ubiquitination modification state. Surprisingly, we did not find any CtIP protein level change in USP4 knockdown cells, with or without IR treatment (Figure S3A). The ubiquitination level of CtIP also did not change in control or USP4 knockdown cells with or without DNA damage (Figure S3B). To our surprise, we found that USP4 could autodeubiquitinate itself. As shown in Figure 7A, in USP4-depleted 293T cells, we put back wild-type USP4 and USP4CA mutant and then check the USP4 ubiquitination state; we found that the ubiquitination level of the wild-type USP4 is apparent lower than the USP4CA mutant. When we treated the cells with IR with different dose or harvested cells at different time points and then checked the USP4 protein level, we could not find any USP4 protein level change after damage treatment (Figure S3C). These results suggest that USP4 could autodeubiquitinate itself without affecting its levels.

Because USP4 autodeubiquitination did not affect its levels, the next question we asked was whether USP4 autodeubiquitination could affect USP4-CtIP interaction. As shown in Figure 7B, USP4 catalytic activity is essential for the USP4-CtIP interaction. USP4-CtIP interaction is a little increased after IR treatment, but in the USP4CA mutant cells, USP4-CtIP interaction is almost gone, even in IR-treated cells. USP4 also can form dimer itself in cells, and USP4 catalytically has no effect on its dimerization (Figure S3D). This result suggests that USP4 promotes USP4-CtIP interaction specifically through its deubiquitinase activity and likely through its autodeubiquitination.

Next, we mapped the ubiquitylated sites of USP4 based on public database and our own mass spectrum data. Perhaps USP4 has various ubiquitination sites (data not shown), but as shown in Figure 7C, K186, K632, K811, and K837 are the partial ubiquitination sites of USP4. More importantly, if we mutated all of these four sites in the catalytic dead mutant of USP4, this double mutant interacted with CtIP again (Figure 7D), and this double mutant also partially rescued HR efficiency and CtIP, Rad51, and RPA foci formation (Figures 7E–7I). These results clearly indicated that USP4 autodeubiquitination is indeed involved in DNA repair.

DISCUSSION

The tumor suppressor protein CtIP controls the decision to repair DSB damage by HR (You and Bailis, 2010; Yu et al., 2006). It does so by regulating the initiation of DSB end resection after integrating signals from the DNA damage checkpoint response and cell-cycle cues. However, how CtIP is recruited to DSBs has not been fully understood yet. Previous study showed that the tumor suppressor BRCA1 can interact with CtIP, regulate CtIP retention at DSBs, and accelerate CtIP-mediated DNA end resection (Cruz-García et al., 2014; Yu et al., 2006). But

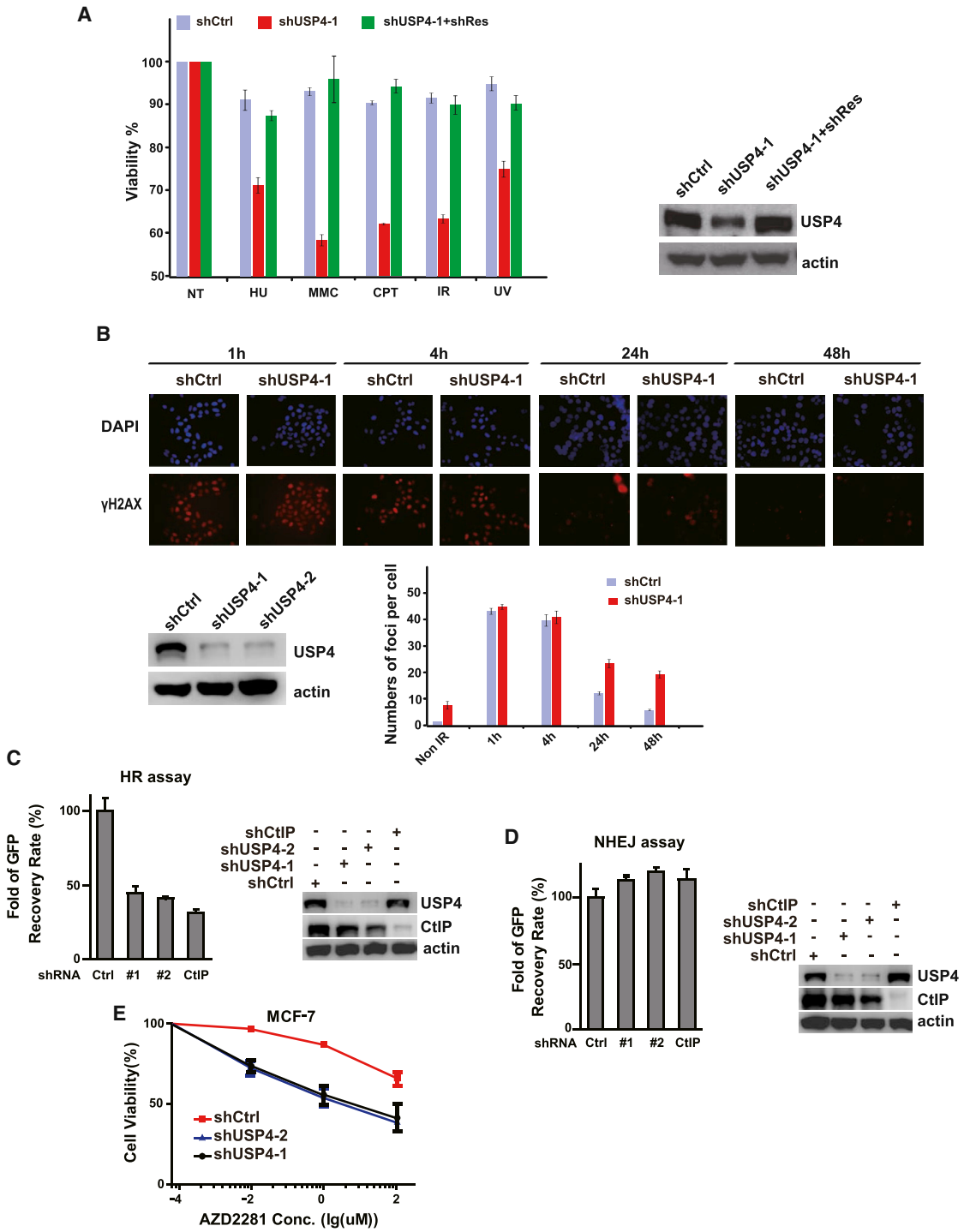

Figure 3. USP4 Is Involved in DNA Damage Response

(A) MTS assay of HCT116 cells with depleted USP4 compared with control after exposing to hydroxyurea (HU), mitomycin C (MMC), ionizing radiation (IR), camptothecin (CPT), and UV light. The DNA damage sensitivity of USP4 knocked down cells was rescued by expression of an shRNA-resistant USP4 (left). USP4 protein level in the indicated cells was confirmed by western blot (right). Results are the average of three independent experiments and presented as mean ± SD. (B) USP4 depletion inhibits DNA repair. Control or USP4-depleted U2OS cells were either mock treated or treated with IR (10 Gy) and allowed to recover for 1, 4, 24, or 48 hr before fixing and processed for γH2AX immunostaining. Representative γH2AX foci was shown in the upper panel. Knockdown efficiency was confirmed by western blot (lower left). Quantification results are the average of three independent experiments and presented as mean ± SD (lower right). More than 100 cells were counted in each experiment.

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the previous studies from the mouse model also showed that loss of the CtIP-BRCA1 interaction does not detectably affect resection, maintenance of genomic stability, or viability (Reczek et al., 2013). In addition, MRN complex cooperates with CtIP in DNA DSB end resection (Buis et al., 2012). But the exact relationship between MRN complex and CtIP is still not clear. Recent study also showed that p75 interacts with CtIP and promotes DNA end resection and HR (Daugaard et al., 2012). Our data provided insights into the molecular basis by which MRN complex cooperates with CtIP in promoting DNA end resection. Here, we report that USP4 interacts with both MRN complex and CtIP, which is a positive regulator of DNA end resection, thus promoting HR.

Both the C terminus and the N terminus of CtIP protein are required in DSB end resection and DNA-damage-induced G2/M checkpoint control (Makharashvili et al., 2014; Wang et al., 2012, 2014; Yuan and Chen, 2009). Previous studies also showed that both termini of CtIP can interact with the MRN complex and that the N terminus of CtIP, especially residues 22–45, binds to MRN and plays a critical role in targeting CtIP to sites of DNA breaks (Yuan and Chen, 2009). On the other hand, the N terminus of CtIP could mediate its dimerization (Dubin et al., 2004). CtIP protein dimerization is critical for its recruitment to chromosomal DNA DSBs (Wang et al., 2012). These are consistent with our results; CtIP N-terminal could interact with USP4, and this interaction is essential for DNA end resection. So we put USP4 in the center of MRN-CtIP complex. USP4 interacts with CtIP and MRN via the C-terminal insert domain (residues 572–773) and intact catalytic domain of USP4, respectively. More importantly, USP4 ubiquitination can block USP4-CtIP interaction, whereas the USP4CA mutant could not mediate its autodeubiquitination, and sustained ubiquitination of USP4 would block its interaction with CtIP. Ubiquitination of USP4 could physically block its interaction with CtIP or induce conformational change of USP4. We also could not exclude other mechanisms; for example, there might be other factors that help the deubiquitinated USP4 interact with CtIP.

Ubiquitylation and sumoylation of proteins have a major role in the DDR and DSB repair, and they mainly facilitate HR to take place (Jackson and Durocher, 2013). So far, however, just several studies showed the limited understanding of the DDR roles for DUBs that mediate the processing and removal of ubiquitin. Although these DUBs have been previously suggested DDR connections (Clerici et al., 2014; Huang et al., 2006; Jacq et al., 2013; Nakada et al., 2010; Nicassio et al., 2007; Nijman et al., 2005; Nishi et al., 2014; Wiener et al., 2012), all of them were not directly involved in DNA end resection. The genetic screen in search of DUBs that are involved in DDR showed that USP4 perhaps is involved in DDR, but the detailed function and mechanism is not clear (Nishi et al., 2014). We found that USP4 can directly regulate DNA end resection. And its deubiquitylating enzyme activity is essential for its interaction with CtIP. This finding extends the list of DUBs directly promoting HR and highlights this mechanism for DNA end resection.

Previous studies have identified a correlation between USP4 and cancer progression and metastasis (Zhang et al., 2012). However, the exact mechanism underlying the USP4-dependent tumorigenesis remains elusive. One possible explanation is based on the important role of USP4 in the TGF-β response (Zhang et al., 2012). Here, we uncover USP4's function in DNA repair pathway. Elucidating the mechanisms for DSB repair pathway choice has important implications in understanding the pathogenesis of human diseases and cancer therapy. For example, similar to CtIP depletion, USP4-depletion rendered cells hypersensitive to PARP inhibitor (AZD2281; Figure 3E), which has been used in clinic for patients with breast, ovarian, and prostate cancer caused by some genetic flaws. It is thus timely to evaluate the potential for USP4 as DDR drug targets for therapeutic intervention. The synthetic lethal approach provides exciting opportunities for therapeutic targeting of cancers exhibiting high levels of DNA damage or which have underlying defects in DDR processes or chromatin components.

EXPERIMENTAL PROCEDURES

Plasmid Construction

Full-length and truncated USP4 were cloned into pIRES2-N-FLAG or pCMV-N-HA to generate various mammalian expression plasmids. USP4 C311A mutant without deubiquitinase activity was generated using the QuikChange site-directed mutagenesis kit (Stratagene) and verified by sequencing. USP4 K186R, K632R, K811R, K837R, and KAllR mutants were generated as described above.

Antibodies

Primary antibodies used in this study were as follows: rabbit anti-USP4 (2651; Cell Signaling Technology); rabbit anti-pS345Chk1 (2348; Cell Signal); rabbit anti-Chk1 (ab32531; Abcam); and mouse anti-FLAG (M2) (F1804; Sigma-Aldrich). BRCA1 antibodies were from Santa Cruz Biotechnology (sc-6954). Antibodies against the HA epitope, ubiquitin, γ-H2AX, RPA, CtIP, RAD51, and 53BP1 were previously described (Lou et al., 2003; Luo et al., 2012; Pei et al., 2011).

RNAi Target Sequences

For siRNA transfection, cells were transfected twice at 24-hr intervals with the indicated siRNA using Oligofectamine (Invitrogen) following the manufacturer's instructions. The sequence of CtIP shRNA was CGGCAGCAGAATCTTAAACTT. The sequences of USP4 shRNAs were no. 1 TTAAACAGGTGGUGAGAAA and no. 2 CGAAGAATGGAGAGGAACA. For lentiviral infection, shRNA lentiviral particles were packaged and transduced into the indicated cells according to the manufacturer's guidelines (Sigma-Aldrich).

ChIP Assay

To induce a single DSB in HeLa DR-GFP cells, transfection of the I-SceI expression plasmid was used. Twenty-four hours after transfection, cells were treated with 1% formaldehyde for 10 min at room temperature to cross-link proteins to DNA. Glycine (0.125 M) was added and incubated at room temperature for 5 min to stop the crosslinking. Cells were harvested, and the pellets were resuspended in cell lysis buffer (5 mM PIPES [KOH; pH 8.0], 85 mM KCl, and 0.5% NP-40) and incubated for 10 min on ice. Nuclei were

(C and D) USP4 was depleted with two independent shRNAs in HEK293 cells integrated with HR or NHEJ reporter. Cells reconstituted with the indicated constructs were subjected to HR assay (C) and NHEJ assay (D) as described in Experimental Procedures. Data were presented as the mean ± SD of three independent experiments.

(E) MCF-7 cells stably expressing the indicated shRNA were treated with increasing doses of PARP inhibitor (AZD2281). MTS assay was performed to determine the surviving fraction. Data are presented as mean ± SD of three independent experiments.

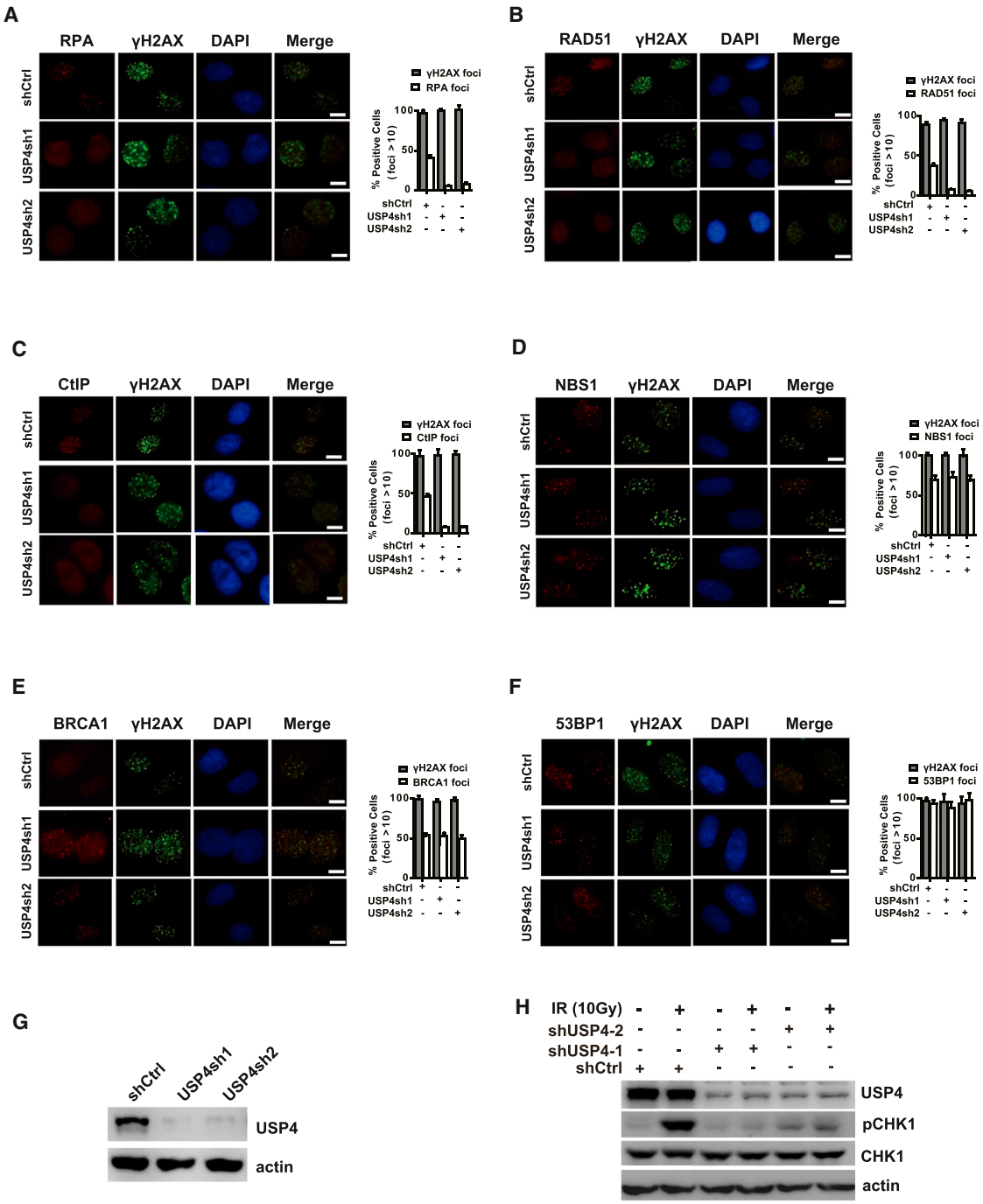

Figure 4. USP4 Regulates DNA End Resection

(A–C) USP4 is required for RPA, Rad51, and CtIP foci formation. USP4-depleted U2OS cells were treated with IR (10 Gy) and allowed to recover for 4 hr before fixing and processed for CtIP, Rad51, and RPA immunofluorescence. Quantification results were presented as the mean ± SD of three independent experiments. More than 100 cells were counted in each experiment.

(D–F) USP4 is not required for γH2AX, NBS1, BRCA1, and 53BP1 foci formation. USP4-depleted U2OS cells were treated with IR (10 Gy) and allowed to recover for 1 hr before fixing and processed for γH2AX, NBS1, BRCA1, and 53BP1 immunofluorescence. Representative NBS1 foci (D), BRCA1 foci (E), and 53BP1 foci (F) were shown. Quantification results were presented as the mean ± SD of three independent experiments. More than 100 cells were counted in each experiment. The scale bar represents 10 μm.

(G) Knockdown efficiency for above U2OS cells was confirmed by western blot.

(H) USP4 depletion abolished IR-induced CHK1 phosphorylation. USP4-depleted U2OS cells were treated with or without IR (10 Gy) and allowed to recover for 1 hr before cell lysis and analysis by western blot.

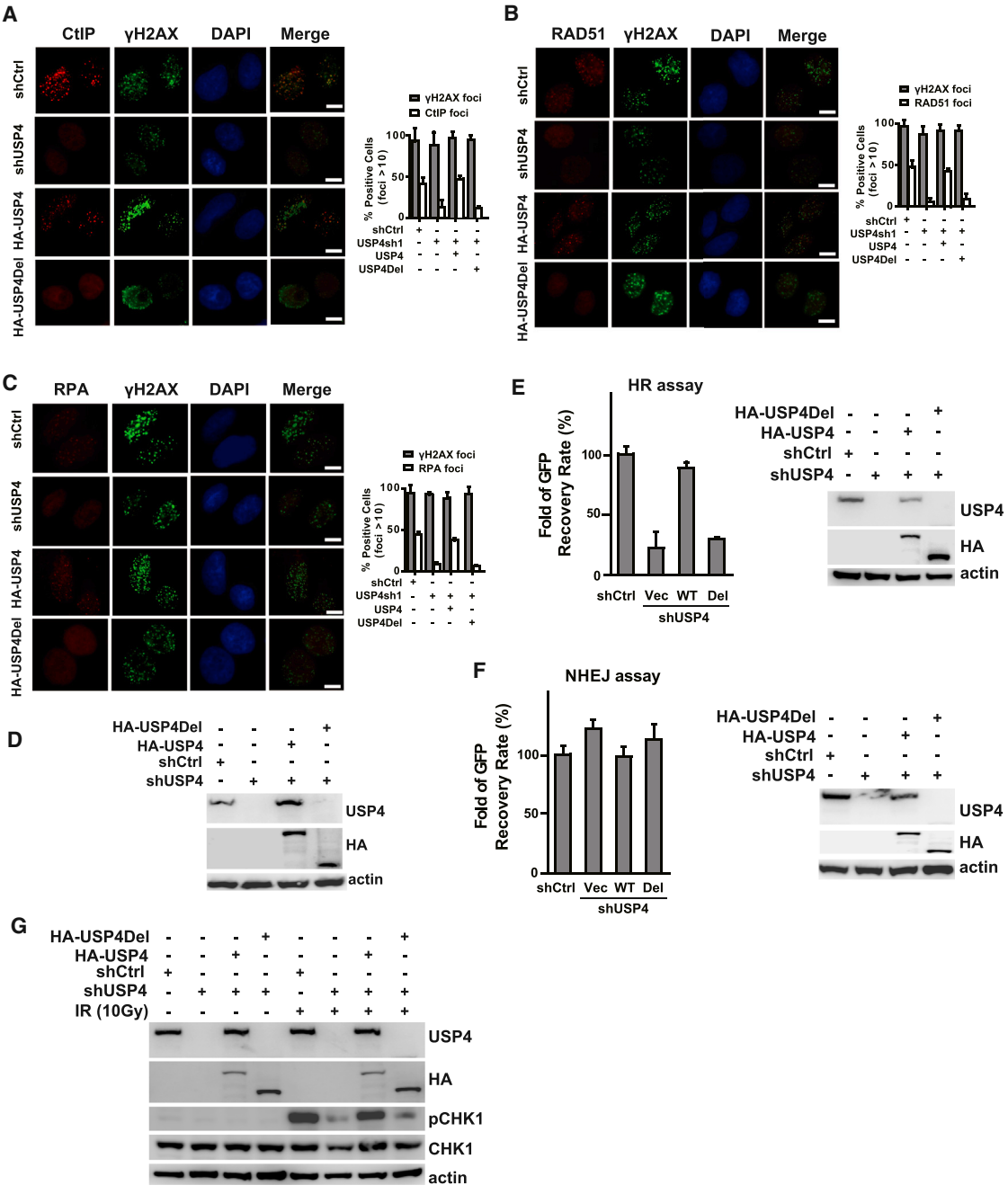

Figure 5. USP4-CtIP Interaction Is Essential for USP4 Functions in HR

(A–C) WT USP4, but not the insert domain deletion mutant, restored CtIP (A), Rad51 (B), and RPA (C) foci formation. USP4-depleted U2OS cells, reconstituted with shRNA-resistant USP4-WT or the insert domain deletion mutant, were treated with IR (10 Gy) and allowed to recover for 4 hr before fixing and processed for CtIP, Rad51, and RPA immunofluorescence. Quantification results were presented as the mean ± SD of three independent experiments. More than 100 cells were counted in each experiment. The scale bar represents 10 μm.

(D) USP4 protein level for above U2OS cells was confirmed by western blot.

(E and F) USP4-depleted HEK293 cells, integrated with HR or NHEJ reporter, reconstituted with the indicated constructs were subjected to HR assay (E) and NHEJ assay (F) as described in Experimental Procedures. Data were presented as the mean ± SD of three independent experiments.

(G) WT USP4, but not the insert domain deletion mutant, restored CHK1 phosphorylation level. The indicated cells were treated with IR (10 Gy) and allowed to recover for 4 hr, and then the cells were harvested for western blot analysis.

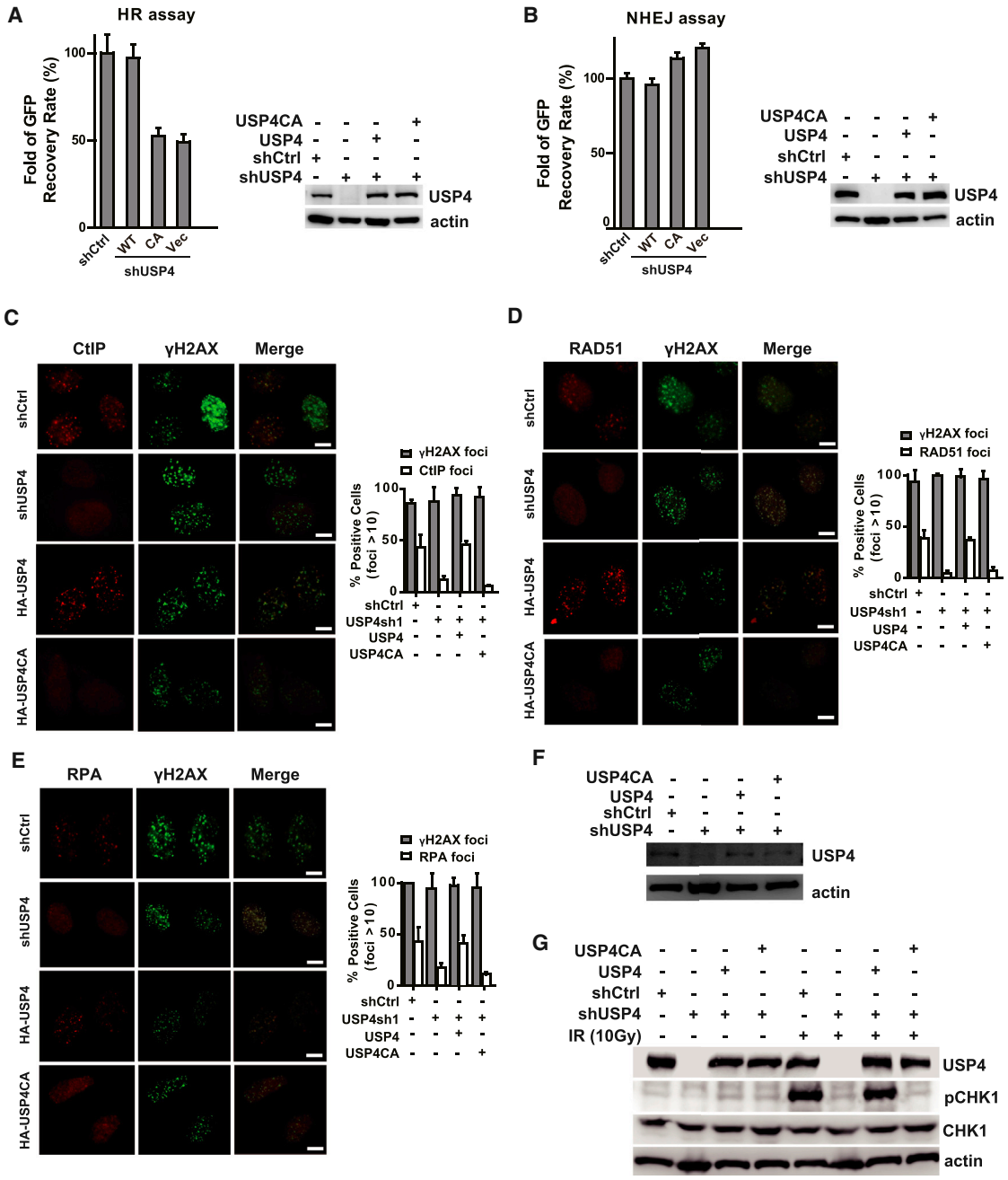

Figure 6. USP4 Regulates HR through Its Deubiquitylating Enzyme Activity

(A and B) HEK293 cells integrated with HR or NHEJ reporter were transfected with the indicated constructs and subjected to the HR assay (A) or NHEJ assay (B) as described in the Experimental Procedures. Data are presented as mean ± SD of three independent experiments.

(C–E) CtIP, RPA, and Rad51 foci formation were examined in the indicated cells following irradiation (10 Gy). Cells were fixed and immunostained with the indicated antibodies. Quantification results were presented as the mean ± SD of three independent experiments. For each condition, randomly selected cells (n = 400) were counted. The scale bar represents 10 μm.

(F) Cells from (C)–(E) were immunoblotted with the indicated antibodies.

(G) U2OS cells were transfected with the indicated constructs following irradiation (10 Gy) and subjected to immunoblot with the indicated antibodies.

pelleted by centrifugation. Nuclei were then resuspended in nuclear lysis buffer (50 mM Tris [pH 8.1], 10 mM EDTA, and 1% SDS containing the same protease inhibitors as in cell lysis buffer) and sonicated to shear chromatin to an average size of 0.6 kb. Once centrifuged until clear, the lysates were precleared overnight with salmon sperm DNA/protein-A agarose slurry. Twenty percent of each supernatant was used as input control and processed with the crosslinking reversal step. The rest of the supernatant was incubated with 5 μg of the indicated antibody overnight at 4°C with rotation. Complexes were washed

four times, once in high salt buffer (50 mM Tris-HCl [pH 8.0], 500 mM NaCl, 0.1% SDS, 0.5% deoxycholate, 1% NP-40, and 1 mM EDTA), once in LiCl buffer (50 mM Tris-HCl [pH 8.0], 250 mM LiCl, 1% NP-40, 0.5% deoxycholate, and 1 mM EDTA), and twice in TE buffer (10 mM Tris-HCl [pH 8.0] and 1 mM EDTA [pH 8.0]). Beads were resuspended in TE containing 50 μg/ml of RNase and incubated for 30 min. Beads washed with water and elution buffer (1% SDS and 0.1 M $NaHCO_3$) was added for 15 min. Crosslinks were reversed by adding 10 mg/ml RNase and 5 M NaCl to a final concentration of 0.3 M to the elutants and incubated in a 65°C water bath for 4–5 hr. Two volumes of 100% ethanol were added to the precipitate overnight at −20°C. DNA was pelleted and resuspended in 100 ml of water, 2 ml of 0.5 M EDTA, and 4 ml 1 M Tris (pH 6.5), and 1 μl of 20 mg/ml Proteinase K was added and incubated for 1–2 hr at 45°C. DNA was then purified and used in PCR reactions. The PCR primers for ChIP, about 220 bp away from the I-SceI cut site, were as follows:

forward: 5′-TACAGCTCCTGGGCAACGTG-3′;
reverse: 5′-TCCTGCTCCTGGGCTTCTCG-3′.

Quantitative Analysis of ChIP Samples

qPCR was performed on a 7500RT-PCR System (Applied Biosystems) using the SYBR Green detection system with the following program: 95°C for 5 min, one cycle; 95°C for 45 s and 62°C for 45 s, 40 cycles. As an internal control for the normalization of the specific fragments amplified, a locus outside the region of the DSB was amplified, in this case FKBP5, using the input control sample as template. The internal control (FKBP5) primers were as follows:

forward: 5′-CAGTCAAGCAATGGAAGAAG-3′;
reverse: 5′-CCCGTGCCACCCCTCAGTGA-3′.

All qPCR reactions were performed in triplicate, with the SEM values calculated from at least three independent experiments.

Sensitivity to DNA-Damaging Reagents

HCT116 cells were transfected as indicated, plated onto 96-well plates, and treated with MMC, CPT, HU, IR, and UV as indicated. Two days later, the viability of the cells was determined using the CellTiter-Blue reagent (Promega), and the average of three experiments was plotted. Data were presented as mean ± SD of three independent experiments.

Immunoprecipitation and GST Pull-Down Assay

Cells were lysed with NETN buffer (20 mM Tris-HCl [pH 8.0], 100 mM NaCl, 1 mM EDTA, and 0.5% NP-40) containing protease inhibitors on ice for 30 min. Following sonication, cell lysates were clarified by centrifugation and incubated with protein G or protein A agarose beads coupled with antibody against the indicated proteins for 8 hr at 4°C. Beads were then washed with NETN buffer three times and analyzed by western blot. For tagged protein IP, cell lysates were incubated with Anti-FLAG M2 Affinity beads (Sigma-Aldrich) for 4 hr at 4°C. Precipitates were then washed and immunoblotted with the indicated antibodies. For the CtIP N-terminal GST pull-down assay, GST-CtIP N-terminal fragment fusion protein was expressed in *E. coli*. Purified fusion protein was immobilized on glutathione Sepharose 4B beads and incubated with cell lysates at 4°C. The samples were separated by SDS-PAGE and analyzed by western blot.

DNA Repair Assay

Integrated DNA repair reporter systems were used to determine the HR and NHEJ efficiency as previously reported (Bennardo et al., 2008). Briefly, HEK293 cells integrated with HR or NHEJ reporters were infected with the indicated viruses. Forty-eight hours after infection, 4-hydroxytamoxifen (4OHT) was added at 3 mM for 24 hr. Three days after 4OHT was added, the percentage of GFP-positive cells was analyzed by FACS as previously described (Bennardo et al., 2008). HR efficiency is presented as the percentage of control cells. Repair frequencies are the mean of at least three independent experiments, and error bars represent the SD from the mean value. Statistical analysis was performed by the Student's t test for two groups and by ANOVA for multiple groups. $p < 0.05$ was considered significant.

Immunofluorescence Staining

Immunofluorescence staining was conducted as described previously (Luo et al., 2012; Pei et al., 2011). Briefly, cells cultured on coverslips were treated with 2 Gy IR followed by recovery for the indicated times. After washing with PBS, cells were fixed in 3% paraformaldehyde for 15 min and permeabilized in 0.5% Triton X-100 solution for 5 min at room temperature. Cells were blocked with 5% goat serum and incubated with primary antibody for 60 min. Subsequently, samples were washed and incubated with secondary antibody for 60 min. DAPI staining was performed to visualize nuclear DNA. The coverslips were mounted onto glass slides with anti-fade solution and visualized using a Nikon ECLIPSE E800 fluorescence microscope.

In Vivo Deubiquitination Assay

Transfected HEK293T cells were treated with MG132 (20 μg/ml) for 6 hr followed by irradiation (10 Gy). After 1 hr, cell lysates were prepared with 120 ml of 62.5 mM Tris-HCl (pH 6.8), 2% SDS, 10% glycerol, 20 mM NEM, and 1 mM iodoacetamide; boiled for 15 min; diluted ten times with NETN buffer containing protease inhibitors, 20 mM NEM, and 1 mM iodoacetamide; and clarified by centrifuge (16,000 *g*; 10 min; 4°C). The lysates were immunoprecipitated with the indicated antibody at 4°C with agitation. The precipitates were eluted in SDS sample buffer and analyzed by western blot with the indicated antibodies.

Tandem Affinity Purification

HEK293T cells stably expressing SFB-CtIP were used for tandem affinity purification. Cells stably expressing SFB-CtIP were lysed with NETN buffer on ice for 20 min. After removal of cell debris by centrifugation, crude lysates were incubated with Streptavidin Sepharose beads for 4 hr at 4°C. The bead-bound proteins were washed three times with NETN buffer and eluted twice with 2 mg/ml biotin (Sigma-Aldrich) for 1 hr at 4°C. The eluates were combined and then incubated with S-protein agarose (Novagen) for 4 hr at 4°C. The S-protein agarose beads were washed three times with NETN buffer. The proteins bound to S-protein agarose beads were separated by SDS-PAGE and visualized by Coomassie Blue staining.

Mass Spectrometry

After staining proteins in SDS-PAGE gels with Coomassie blue, gel lanes were sliced into different bands and in-gel digested overnight at 37°C with trypsin. After digestion, peptides were extracted twice in 200 μl of acetonitrile with resuspension in 20 μl of 2% formic acid prior to second extraction, dried in a Savant SpeedVac, and dissolved in a 5% methanol/0.1% formic acid solution. Tryptic peptides were separated on a C18 column and were analyzed by LTQ-Orbitrap Velos (Thermo). Proteins were identified by using the NCBI search engine against the human or mouse NCBI RefSeq protein databases.

Statistical Analysis

The statistical data are from three independent experiments. Statistical analysis was performed by the Student's t test for two groups and by ANOVA for multiple groups. $p < 0.05$ was considered significant.

SUPPLEMENTAL INFORMATION

Supplemental Information includes three figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.08.056.

AUTHOR CONTRIBUTIONS

Conceptualization, H.P.; Methodology, H.L., H.Z., and X.W.; Investigation, H.L., H.Z., and X.W.; Writing – Original Draft, H.P.; Writing – Review & Editing, H.L., H.Z. and H.P.; Funding Acquisition, H.P.; Supervision, P.Z. and H.P. All authors discussed the results and comments on the manuscript.

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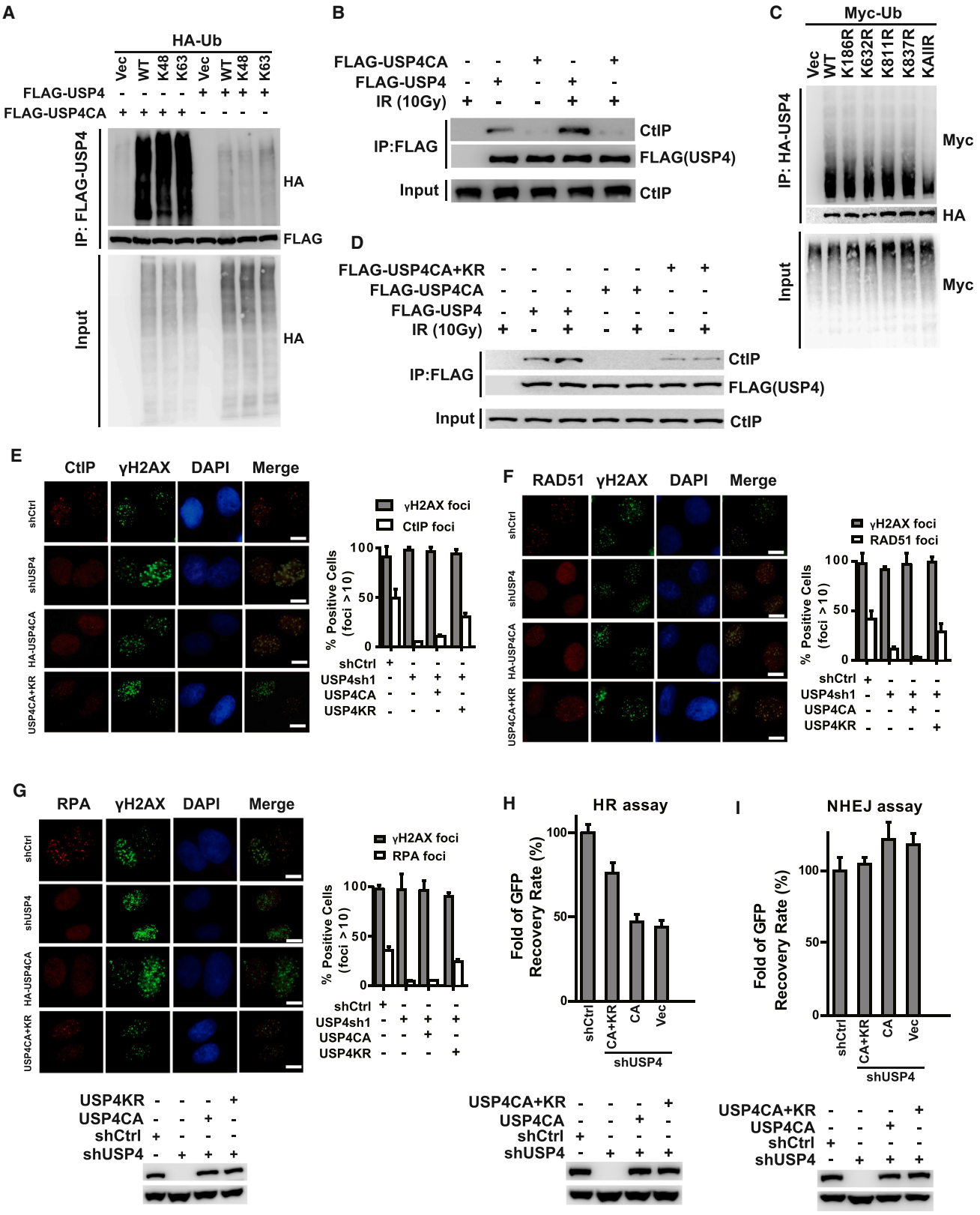

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Figure 7. USP4 Autodeubiquitination Promotes USP4-CtIP Interaction

(A) HEK293T cells were transfected with the indicated constructs and then immunoprecipitated with anti-FLAG antibody and subjected to immunoblot with the indicated antibodies.

(B) USP4-depleted HEK293T cells were transfected with FLAG-USP4 or FLAG-USP4CA mutant and then treated with irradiation (10 Gy) and immunoprecipitated with FLAG antibody and subjected to immunoblot with the indicated antibodies.

(C) USP4-depleted HEK293T cells were transfected with wild-type USP4 or different KR mutant and then immunoprecipitated with FLAG antibody and subjected to immunoblot with the indicated antibodies.

(D) USP4-depleted HEK293T cells were transfected with indicated plasmids and then treated with irradiation (10 Gy) and immunoprecipitated with FLAG antibody and subjected to immunoblot with the indicated antibodies.

(E–G) CtIP, RPA, and Rad51 foci formation were examined in the indicated cells following irradiation (10 Gy). Cells were fixed and immunostained with the indicated antibodies. Quantification data were presented as mean ± SD of three independent experiments. For each condition, randomly selected cells (n = 400) were counted. The scale bar represents 10 μm.

(H and I) HEK293 cells integrated with HR or NHEJ reporter were reconstituted with the indicated constructs and then were subjected to HR assay (H) and NHEJ assay (I) as described in Experimental Procedures. Data were presented as the mean ± SD of three independent experiments.

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