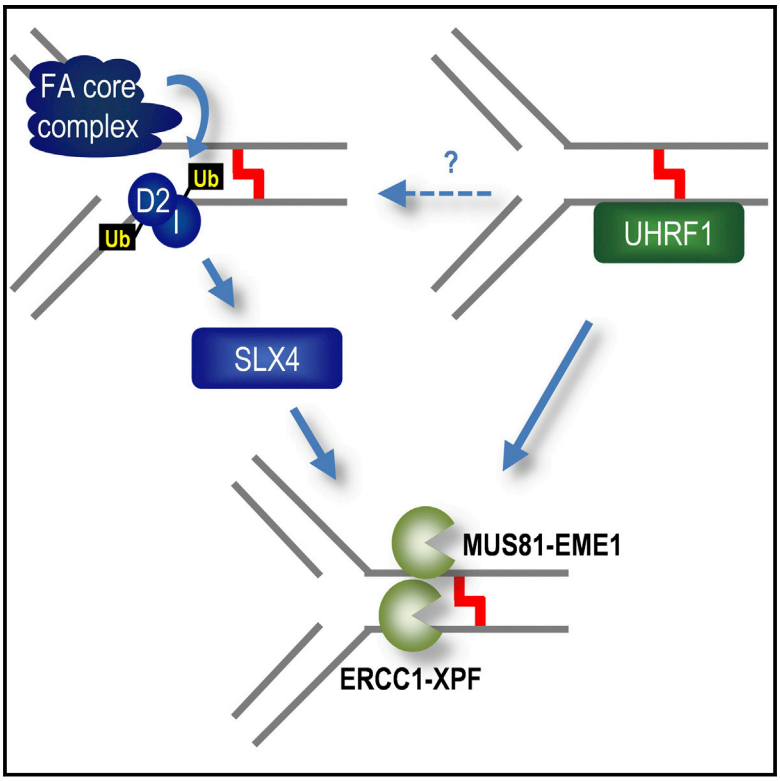
Report

Cell Reports

UHRF1 Contributes to DNA Damage Repair as a Lesion Recognition Factor and Nuclease Scaffold

Graphical Abstract



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In Brief

DNA interstrand crosslink (ICL) is a complex DNA lesion with severe cytotoxicity. Tian et al. identify ubiquitin-like with PHD and RING finger domain 1 (UHRF1) as a DNA crosslink-binding protein and find that UHRF1 deficiency leads to DNA-damage sensitivity and attenuated nucleolytic lesion processing. UHRF1 may serve as lesion-recognition factor and a nuclease scaffold for DNA-damage removal.

Highlights

- UHRF1 is identified as a DNA-damage recognition factor
- UHRF1 functions in DNA-damage response and lesion processing
- UHRF1 functions as a damage sensor and nuclease scaffold
- The function of UHRF1 in DNA repair is distinct from the FA pathway

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UHRF1 Contributes to DNA Damage Repair as a Lesion Recognition Factor and Nuclease Scaffold

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SUMMARY

We identified ubiquitin-like with PHD and RING finger domain 1 (UHRF1) as a binding factor for DNA interstrand crosslink (ICL) lesions through affinity purification of ICL-recognition activities. UHRF1 is recruited to DNA lesions in vivo and binds directly to ICL-containing DNA. UHRF1-deficient cells display increased sensitivity to a variety of DNA damages. We found that loss of UHRF1 led to retarded lesion processing and reduced recruitment of ICL repair nucleases to the site of DNA damage. UHRF1 interacts physically with both ERCC1 and MUS81, two nucleases involved in the repair of ICL lesions. Depletion of both UHRF1 and components of the Fanconi anemia (FA) pathway resulted in increased DNA damage sensitivity compared to defect of each mechanism alone. These results suggest that UHRF1 promotes recruitment of lesion-processing activities via its affinity to recognize DNA damage and functions as a nuclease recruitment scaffold in parallel to the FA pathway.

INTRODUCTION

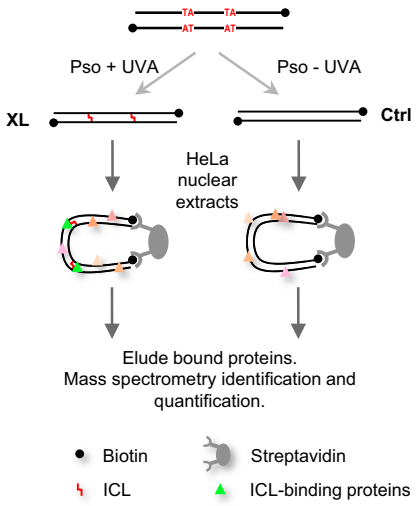
The DNA interstrand crosslink (ICL) is a complex DNA lesion arising from a variety of extrinsic and intrinsic bifunctional alkylating agents. ICL-inducing agents exhibit profound cytotoxicity and are among the most widely used chemotherapy drugs (McHugh et al., 2001). Deficiencies in repairing DNA ICLs have severe pathological consequences, as highlighted by the recessively inherited cancer-prone disease Fanconi anemia (FA) (D'Andrea, 2010; Kim and D'Andrea, 2012).

Repair of DNA ICLs is accomplished by two distinct pathways. The replication-dependent pathway operates primarily during S phase and is initiated by replication fork encountering with an ICL. Given that the formation of an ICL compromises both strands of the double helix, error-free repair most likely involves homologous recombination with the undamaged sister chromatid upon formation of DNA strand breaks (Knipscheer et al., 2009; Long et al., 2011). Consistently, defects in homologous recombination factors such as Brca2, Rad51C, and BACH1 render cells sensitive to crosslinking agents. On the other hand, ICLs in G1 and G0 phases or during early S phase of the cell cycle utilize a recombination-independent mechanism involving the combined actions of the nucleotide excision repair and lesion bypass synthesis (Sarkar et al., 2006; Williams et al., 2012; Zheng et al., 2003).

A key step in ICL repair is the loading of the appropriate nucleases to the damaged site to achieve the initial incision and unhooking of an ICL. Genetic and biochemical studies have identified several nucleases including ERCC1-XPF, MUS81-EME1, and SNM1A (Hodskinson et al., 2014; Wang et al., 2011) in the nucleolytic processing of ICLs. These structural-specific endonucleases act at different stages of ICL removal, generating intermediates of single- or double-strand breaks adjacent to the ICL lesion and allowing subsequent lesion bypass and homologous recombination to take place (Bhagwat et al., 2009; Hodskinson et al., 2014). However, the molecular mechanisms directing the recruitment of nucleases to the damaged sites are poorly understood. The main function of the FA pathway is presumed to be the loading of lesion-processing nucleases via FAND2/I monoubiquitination-mediated regulation of the SLX4/FANCP nuclease scaffold (Guervilly et al., 2015; Muñoz et al., 2009; Ouyang et al., 2015; Smogorzewska et al., 2010; Stoepker et al., 2011; Svendsen et al., 2009). However, it is unclear whether ICL-processing activities can be recruited through other mechanisms.

Ubiquitin-like with PHD and RING finger domain 1 (UHRF1) is a multi-domain protein important for the maintenance of cytosine methylation. It recognizes specific forms of histone modifications and DNA hemimethylation (Liu et al., 2013; Nishiyama et al., 2013) and facilitates the recruitment of Dnmt1 in order to catalyze the methylation reaction on hemimethylated CpG motifs. As expected, cells defective in UHRF1 have reduced amplitude and site accuracy of DNA methylation (Bostick et al., 2007). However, UHRF1 loss also causes cellular sensitivity to DNA-damaging agents (Muto et al., 2002). Mechanistic insights on

A



B

Substrate A		Substrate B	
Proteins	Ratio (XL/Ctrl)	Proteins	Ratio (XL/Ctrl)
UHRF1	43.3	UHRF1	26.0
UBTF	20.6	UBTF	15.1
HMGB3	18.3	HMGB3	11
HMGB2	8.0	HMGB2	6.0
KRT10	7.8	APOBEC3C	4.7
TFAM	5.6	CBF-beta	3.8
CUL4A	4.3	Helicase B	2.9

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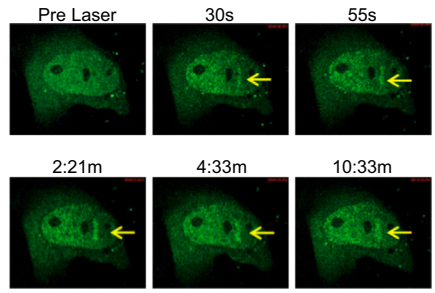

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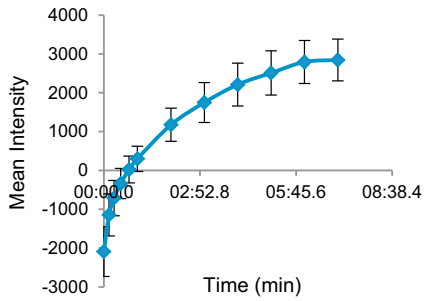

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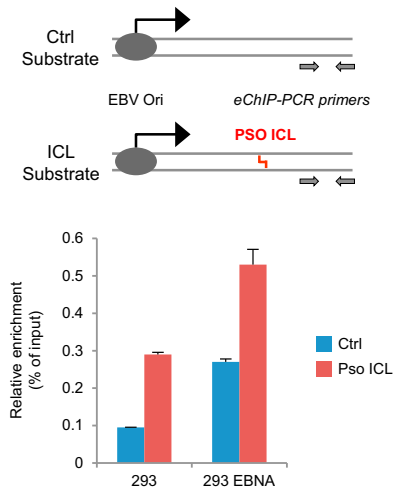

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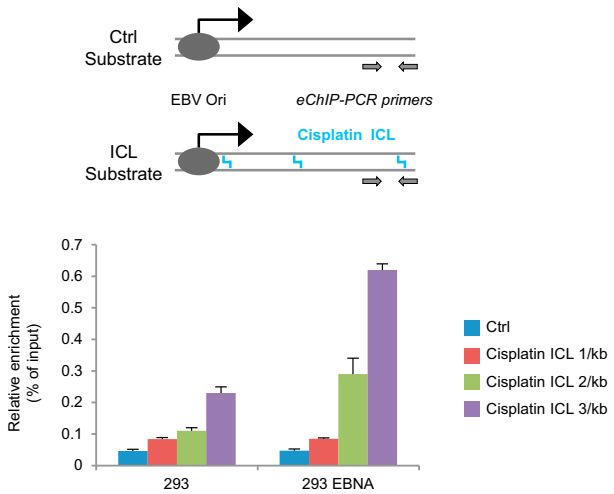

Figure 1. Identification of UHRF1 as an ICL-Interacting Protein

(A) A schematic of pull-down- and mass spectrometry-based purification of ICL-binding proteins. The 120-bp crosslinked substrate contains two psoralen ICLs at defined positions and is end-labeled with biotin for the pull-down assay.

(B) Candidate ICL-binding proteins identified by mass spectrometry analyses using two independent ICL-containing DNA substrates.

(C) Time-lapse images showing recruit of GFP-tagged UHRF1 to laser-localized psoralen ICLs in U2OS cell nuclei at indicated time points.

(D) Imaging quantification of nuclear strip intensity of GFP-tagged UHRF1 to laser-localized psoralen ICLs in U2OS cells.

(legend continued on next page)

this unexpected phenotype are unknown. In this report, we identified UHRF1 as an ICL-binding protein through an unbiased affinity purification of lesion-binding activities. Analyses of UHRF1-deficient cells revealed an unanticipated defect in lesion processing. We found that URHF1 interacts with lesion-processing nucleases ERCC1 and MUS81 and may serve as a recruitment factor for the repair of DNA lesions. These results suggest a UHRF1-dependent mechanism of directing structure-specific nucleases to the site of DNA damage.

RESULTS

Identification of UHRF1 as an ICL-Binding Protein

To isolate proteins that bind to DNA ICLs, we designed a 120-bp oligonucleotide duplex that has two TA nucleotide residues for the formation of site-specific, psoralen-based ICLs (Figures 1A and S1A). Upon psoralen and UVA treatment, crosslinked oligos were purified, end-labeled with biotin, and used as the crosslinked substrate for ICL-affinity purification. A control substrate was generated in parallel without the addition of psoralen. The crosslinked and control substrates were attached to streptavidin beads and incubated with HeLa nuclear extracts. Tightly bound proteins were eluded and subjected to mass spectrometry analyses. The amount of each protein bound to crosslinked substrate was normalized to that of the control substrate in order to yield a ratio (crosslinked:control) as a reflection of its affinity to the ICL. As shown in Figure 1B, we found that the UHRF1 protein is highly enriched by the crosslinked substrate.

To exclude the possibility that the identification of UHRF1 was biased toward sequence composition, we repeated the pull-down experiment with a second set of crosslinked and control substrates (substrate B, Figure S1A) in which only the two TA motifs and their relative positions are identical to the original substrate (substrate A). UHRF1 was again identified as the top candidate, suggesting that binding of UHRF1 to ICL-containing DNA does not rely on the sequence context of the ICL substrates.

To test whether UHRF1 binds to ICLs in vivo, laser-localized psoralen ICLs were introduced into the nuclei of U2OS cells (Muniandy et al., 2009) expressing GFP-UHRF1. Beginning at 30 s after ICL induction, GFP-UHRF1 accumulated at the ICL track and appeared to peak around 8 min (Figures 1C and 1D). This result indicates that UHRF1 is specifically enriched at the sites of ICL lesions and that its recruitment is an early event in ICL response in comparison to the recruitment of FANCA (Yan et al., 2012).

Next, we performed the episomal chromatin immunoprecipiation (eChIP) assay (Shen et al., 2009) with psoralen- or cisplatin-derived ICL lesions. The eChIP substrate contains a defined psoralen ICL positioned 488 bases downstream of the Epstein-Barr virus (EBV) replication origin (Figures S1B and 1C). Therefore, protein recruitment to the site of the ICL can be analyzed in the absence or presence of DNA replication to determine whether a blocked replication fork is required. We found that the presence of a single-defined psoralen ICL yielded significant UHRF1 enrichment onto the crosslinked substrate (Figure 1E). The enrichment was not significantly increased when substrate replication was enabled in the 293EBNA cells, suggesting that stalled replication fork is not essential for UHRF1 binding to the ICL. The increased UHRF1 recruitment in both control and ICL substrates mostly likely reflects a replication-coupled enrichment of UHRF1 (Nishiyama et al., 2013). Consistently, the eChIP substrate containing randomly introduced cisplatin ICLs showed a significant enrichment of UHRF1 in a dose-dependent manner in both replicated and unreplicated sites of ICLs (Figure 1F). These results suggested that UHRF1 is recruited to sites of DNA ICLs in vivo.

UHRF1 Binds Directly to ICLs through the SRA Domain

Although UHRF1 is shown to be associated with ICLs in nuclear extracts and in vivo, such association could be mediated by other factors. Therefore, we tested whether UHRF1 directly bound DNA ICLs. An MBP-UHRF1 recombinant protein was purified via amylose beads and incubated with DNA containing psoralen- or cisplatin-induced ICLs. As shown in Figure 2A, MBP-UHRF1 retained specifically crosslinked, but not control, DNA, whereas maltose-binding protein (MBP) alone showed no detectable binding to either DNA probes.

We further validated the affinity of UHRF1 to ICL substrates in a competition assay. Immobilized MBP-UHFR1 fusion protein was pre-incubated with ^{32}P-labeled oligonucleotide containing psoralen or cisplatin ICLs followed by the addition of increasing amounts of unlabeled ICL-containing or control oligo (Figures 2B and 2C). The results showed that the ICL-containing oligonucleotide was much more efficient in competing for UHRF1 binding than the control. These results demonstrated that UHRF1 exhibits direct affinity for DNA ICLs.

UHRF1 contains five conserved motifs that include UBL, tandem tudor (TUDOR), plant homeodomain (PHD), SET and RING-associated (SRA), and RING domains (Figure 2D). To determine whether the ICL-binding activity could be localized to a specific region or domain(s), we generated five truncation mutants. Each mutant has one of the five conserved domains removed. MBP-fusion proteins of each mutant were purified and tested in the ICL pull-down assay with psoralen or cisplatin ICL DNA. As shown in Figure 2E, only deletion of the SRA domain completely abolished UHRF1 binding to ICL-containing DNA, indicating that the SRA domain is most critical for UHRF1's ability to recognize DNA crosslinking lesions.

Loss of UHRF1 Function Leads to DNA Damage Sensitivity

The association of UHRF1 with ICL lesions suggested that it may play a role in cellular response to ICLs. To test this premise, we

(E) Top: Schematic representation of the DNA substrate used in the eChIP assay. The presence of a single-defined psoralen-ICL is indicated. The arrow indicates the direction of replication fork movement. Small arrows indicate the region of qPCR amplification. Bottom: eChIP assay measuring the recruitment of the UHRF1 protein in 293 and 293EBNA cells. Error bars are SD from four independent experiments.

(F) Top: Schematic representation of the DNA substrates used in the eChIP assay. The presence of random cisplatin-ICLs is illustrated. Small arrows indicate the region of qPCR amplification. Bottom: eChIP assay measuring the recruitment of the UHRF1 protein in 293 and 293EBNA cells. Numbers of cisplatin ICL per kb represent average distributions. Error bars are SD from three independent experiments.

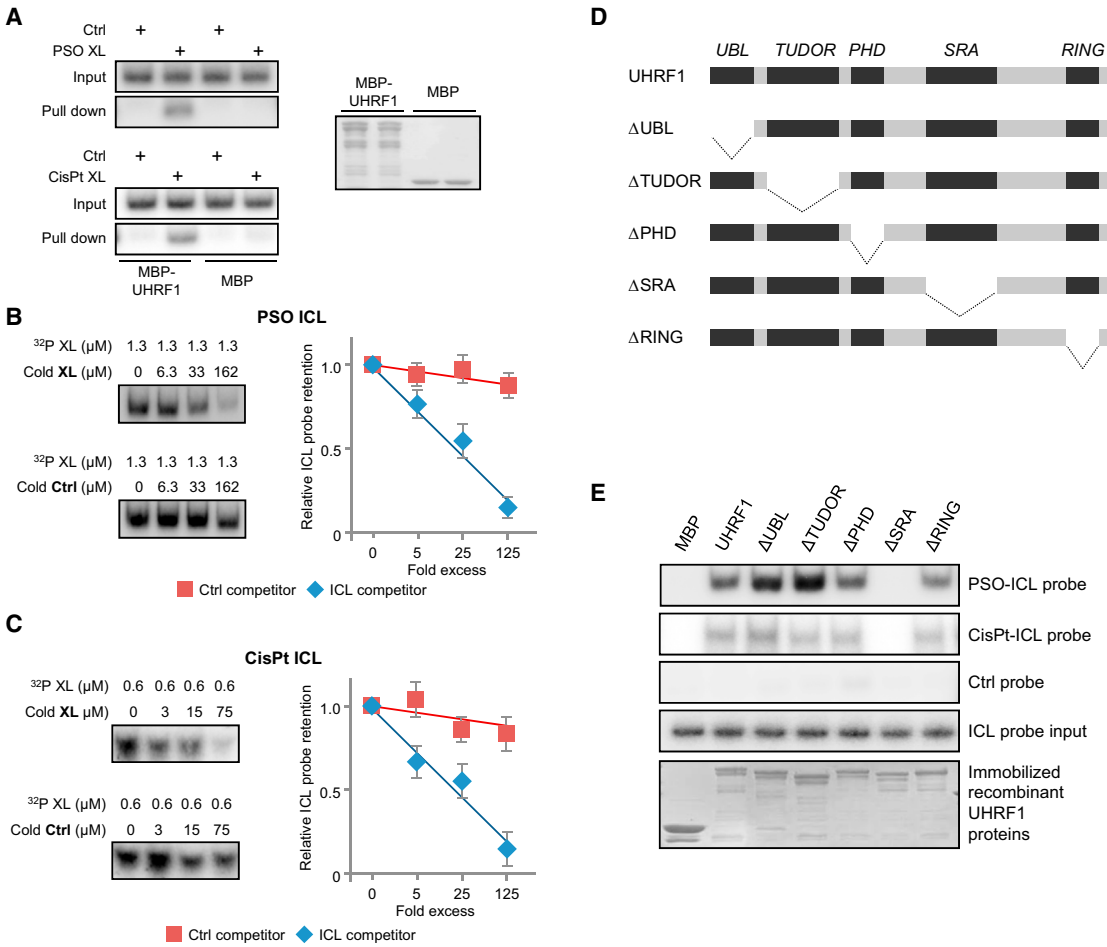

Figure 2. UHRF1 Binds to ICLs Directly to ICLs through the SRA Domain

(A) Binding of MBP-tagged UHRF1 to crosslinked (XL) and control (Ctrl) probes with ^{32}P end-labeling (left). MBP or MBP-UHRF1 were immobilized onto amylose beads and incubated with PSO ICL- or cisplatin ICL-containing probes. Bound DNA was eluded with maltose, and resolved on 2% agarose gel. Aliquots of amylose beads bound proteins were eluded and resolved by PAGE as the loading control (right).

(B) Binding affinity of UHRF1 toward psoralen DNA ICL. Left: MBP-tagged UHRF1 was preincubated with ^{32}P-labeled PSO-ICL probe prior to the addition of cold PSO-ICL competitor probe (top) or cold control competitor probe (bottom) with the indicated concentrations. Bound DNA was recovered by amylose beads and resolved on 2% agarose gel to reveal the retention of ^{32}P-labeled PSO-ICL probe. Right: Quantification of relative ICL retention as a function of excess amount of control or ICL competitors. Error bars are SD from three independent experiments.

(C) Binding affinity of UNRF1 toward cisplatin DNA ICL performed as in (B).

(D) Illustration of UHRF1 domain structure and UHRF1 domain deletion mutants. UBL, ubiquitin-like domain; TUDOR, tandem tudor domain; PHD, plant homeodomain; SRA, SET and RING-associated domain.

(E) Binding of UHRF1 domain deletion mutants to PSO-ICL and cisplatin-ICL probes. Bottom: Input recombinant proteins for the ICL binding assay as performed in (A).

established a UHRF1 conditional allele in the HCT116 background via somatic cellular targeting (Figure S2A). Although homozygous deletion of UHRF1 leads to severe proliferation defect (Figures S2B–S2D), we obtained two independent hypomorphic mutants (UHRF1$^{-/Neo}$-1 and UHRF1$^{-/Neo}$-2) in which one UHRF1 allele was inactivated and the other was rendered hypomorphic by the insertion of a Neo^{R} cassette upstream of exon 4. As a result, both hypomorphic mutants express UHRF1 protein at significantly reduced levels in comparison to wild-type (WT) *UHRF1*$^{+/+}$ cells but maintain normal growth characteristics.

Next, we analyzed UHRF1$^{-/Neo}$-1 and UHRF1$^{-/Neo}$-2 mutants for their sensitivity to DNA damage exposure. As shown in Figures 3A–3C, both hypomorphic mutants displayed increased sensitivity to mitomycin C, UV, or Pso-UVA treatments. Complementation of both mutants with WT UHFR1 restored cell survival to that of the WT HCT116 cells. Knockdown of UHRF1 in 293T and HeLa cells also significantly increased their sensitivity to mitomycin C and UV (Figures S2E and S2F). When compared to FANCL- or SLX4-null mutants, UHRF1 hypomorphic cells are considerably less sensitive (Figures 5B and 5C). However, in comparison to isogenic FANCM$^{-/-}$ and FAAP24$^{-/-}$ mutants, the UHRF1 hypomorphic mutants displayed similar sensitivities to mitomycin C (Figure S2G). These result suggested that UHRF1 is functionally involved in cellular resistance against DNA damage.

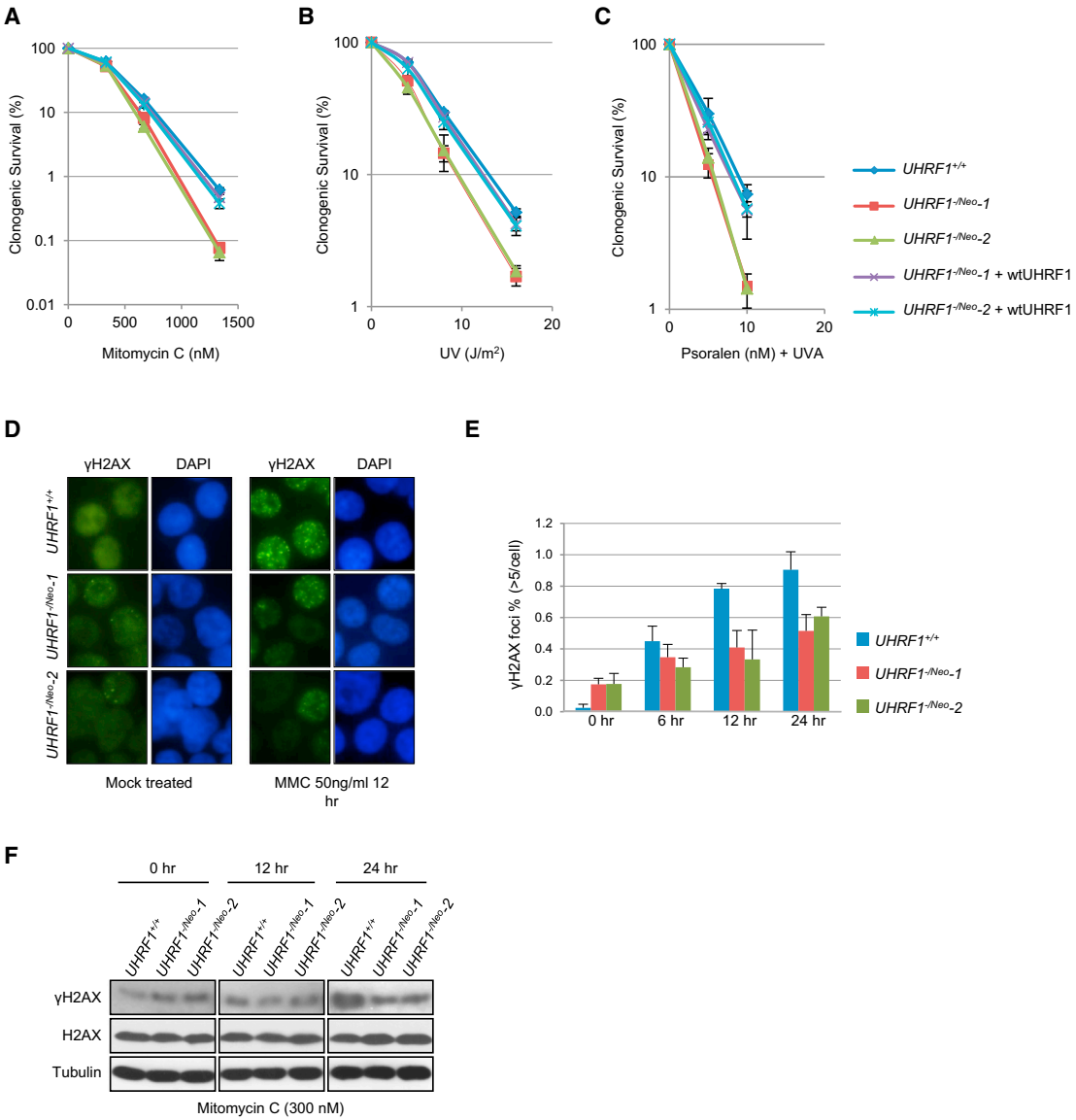

Figure 3. UHRF1 Deficiency Sensitizes Cells to DNA Damage and Attenuates Lesion Processing

Clonogenic survival of *UHRF1*$^{-/Neo}$*-1* and *UHRF1*$^{-/Neo}$*-2* and their complemented derivatives exposed to mitomycin C (A), UV (B), and psoralen plus UVA (C) treatment. Error bars are SD across three or more technical replicates.

(D) Formation of mitomycin C (MMC)-induced γH2AX nuclear foci in WT (*UHRF1*$^{+/+}$) and hypomorphic mutant (*UHRF1*$^{-/Neo}$*-1* and *UHRF1*$^{-/Neo}$*-2*) cells 12 hr after treatment.

(E) Percentage of γH2AX foci-positive nuclei from MMC-treated *UHRF1*$^{+/+}$ and *UHRF1*$^{-/Neo}$*-1* and *UHRF1*$^{-/Neo}$*-2* cells at indicated time points after MMC exposure.

(F) Immunoblot detecting γH2AX in *UHRF1*$^{+/+}$ and *UHRF1*$^{-/Neo}$ cells treated with mitomycin C and collected at indicated time points.

As an important factor in maintaining DNA cytosine methylation (Bostick et al., 2007), the UHRF1 RING-domain-dependent E3 ligase activity has been found essential for the recruitment of DNMT1 and subsequent replication of cytosine methylation patterns (Nishiyama et al., 2013). When two UHRF1 E3 ligase mutants, C724A and H741A (Jenkins et al., 2005), were used to complement the UHRF1$^{-/Neo}$ cells, the DNA damage resistance was largely restored (Figure S2H). This result indicated that disruption of methylation maintenance was unlikely the primary cause for the damage sensitivity phenotype and that UHRF1 may have functions directly linked to the DNA damage response.

The DNA damage sensitive phenotype suggests that UHRF1 mutant cells may be deficient in the removal of ICL lesions efficiently, resulting in a higher level of residue damage and decreased cell survival. Thus, we analyzed γH2AX foci formation in UHRF1$^{-/Neo}$ and UHRF1$^{+/+}$ cells exposed to mitomycin C. Under unperturbed growth conditions, UHRF1$^{-/Neo}$ cells exhibit a higher percentage of γH2AX foci-positive nuclei (Figure 3D). This result suggests that cells lacking UHRF1 function indeed

accumulate DNA damage. However, upon mitomycin C treatment, both the UHRF1$^{-/Neo}$-1 and UHRF1$^{-/Neo}$-2 mutants showed a markedly reduced γH2AX foci formation in comparison to WT UHRF1$^{+/+}$ cells, especially at later time points (12 and 24 hr; Figure 3E). Similarly, immunoblotting of γH2AX (Figure 3F) also confirmed the attenuated onset of γH2AX induction upon DNA damage and a higher level of basal level γH2AX in the absence of exogenous DNA damage. To further validate these results, we analyzed 53BP1 foci formation in UHRF1$^{-/Neo}$ mutants exposed to mitomycin C. Consistent with the γH2AX results, mitomycin C-mediated 53BP1 foci formation also decreases in *UHRF*$^{-/Neo}$ mutant cells (Figures S3A and S3B). These results suggest that UHRF1 plays a role in the repair of both endogenous and exogenous lesions.

UHRF1 Function Is Involved in the Recruitment of ICL Damage-Processing Activities

Because the formation of DNA strand breaks is a primary signal that triggers the accumulation of γH2AX and 53BP1, attenuated foci formation in UHRF1-deficient cells may reflect a potential lack of ICL processing that creates DNA strand breaks. Therefore, we tested the premise that impaired recruitment of lesion-processing nuclease activities contributes to the diminished induction of γH2AX and 53BP1 foci and to the DNA damage sensitivity phenotype from UHRF1-deficiency. First, we asked whether UHRF1 interacts directly with ERCC1-XPF or MUS81-EME1, two structure-specific endonucleases that are required in processing ICL lesions. As shown by coimmunoprecipitation of both tagged and endogenous proteins (Figures 4A–4C and S3B), UHRF1 interacts with both ERCC1/XPF and MUS81/EME1. Moreover, recombinant MBP-UHRF1 was able to pull down MUS81 and ERCC1 from HeLa nuclear extract. An N-terminal UHRF1 truncation lacking the SRA and RING domains disrupted the association with both ERCC1 and MUS81, whereas a C-terminal truncation retaining these domains was able to bind MUS81 and ERCC1 (Figure 4D). Together, these results suggest a direct interaction between UHRF1 and ICL-processing nucleases.

Both ERCC1-XPF and MUS81-EME1 are known to be associated with a common scaffold protein, SLX4/FANCP (Fekairi et al., 2009; Muñoz et al., 2009; Svendsen et al., 2009). We asked whether the observed interactions are mediated by SLX4. To this end, we generated a HeLa SLX4-null mutant and examined the interactions between UHRF1 and the two nucleases by coimmunoprecipitation (Figure 4E). We found that deletion of SLX4 does not abolish the interactions, suggesting that binding of UHRF1 to the lesion-processing nucleases is independent of SLX4. Consistently, reciprocal immunoprecipitation between Myc-SLX4 and SFB-UHRF1 showed no detectable interaction between the two proteins (Figure 4F).

Given UHRF1's lesion-binding affinity and its direct interactions with lesion-processing nucleases, a likely function for UHRF1 in DNA damage response may be to recruit lesion-processing nucleases to the site of damage. We tested this notion by eChIP in the *UHRF1*$^{-/Neo}$ hypomorphic mutant cells. As shown in Figure 4G, enrichment of Mus81 onto cisplatin-adducted DNA substrate is significantly reduced in both UHRF1 mutants in comparison to the parental *UHRF*$^{+/+}$ cells. Similarly, enrichment of ERCC1 is also reduced in the *UHRF1*$^{-/Neo}$ hypomorphic mutant cells (Figure 4H), suggesting a role for UHRF1 in the processing of ICL lesions by recruiting structure-specific endonucleases.

UHRF1 Function in DNA Damage Response Is Not Redundant with the FA Pathway

Although UHRF1 interacts with ERCC1 and MUS81 independently of SLX4, it is unclear whether UHRF1 is functionally distinct from the FA pathway. To address this question, we tested whether activation of the FA pathway is compromised in the UHRF1-deficient cells (Figure 5A). We found that both UHRF1 hypomorphic mutants exhibited mitomycin C-induced FANCD2 monoubiquitination similar to WT controls, suggesting that activation of the FA pathway is not significantly affected by UHRF1 depletion.

To functionally determine whether UHRF1's role in DNA damage response is distinct from the FA mechanism, we knocked down UHRF1 in SLX4$^{-/-}$ HeLa cells and analyzed clonogenic survival against cisplatin (Figures 5B and S4A). In comparison to cells lacking UHRF1 or SLX4 alone, the combined loss of UHRF1 and SLX4 acquired additional hypersensitivity to DNA damage, suggesting that the damage repair function of UHRF1 is parallel to that of SLX4.

To substantiate this result further, *FANCL*$^{-/-}$ cells constructed in HeLa background were depleted of UHRF1 via small hairpin RNA (shRNA) knockdown. Clonogenic survival indicated that UHRF1 knockdown in HeLa cells yielded increased sensitivity to mitomycin C, although it was less profound than in FANCL knockout cells. However, UHRF1 knockdown in *FANCL*$^{-/-}$ HeLa cells produced enhanced sensitivity to mitomycin C (Figures 5C and S4B). Consistently, UHRF1 knockdown in *FANCL*$^{-/-}$ HCT116 cells also rendered additional cell killing by mitomycin C (Figure 5D). These results suggest that UHRF1 provides ICL repair functions independent of the FA pathway components most likely through its lesion-binding capability and recruitment of lesion-processing nucleases.

DISCUSSION

Each type of DNA damage, such as bulky adducts, mismatches, and DNA double-strand breaks, is recognized by a lesion-specific damage-binding protein to initiate the repair process. In the case of DNA ICLs, it has been unclear whether an ICL-specific lesion recognition factor exists. We approached this question by biochemical purification of ICL binding affinities from nuclear extracts and identified UHRF1. Previous studies have shown that loss of UHRF1 renders cells sensitive to DNA-damage exposure (Jenkins et al., 2005; Muto et al., 2002). In this study, we constructed genetic and knockdown mutants of UHRF1 and analyzed its function in DNA damage response. Our experimental evidence suggests that UHRF1 possesses activities recognizing ICL lesions while also interacting with lesion processing nucleases, suggesting that it is a candidate factor in promoting DNA damage processing.

DNA Damage Affinity of UHRF1

The lesion-binding ability of UHRF1 was examined by three independent approaches: cell biology with laser-localized ICLs

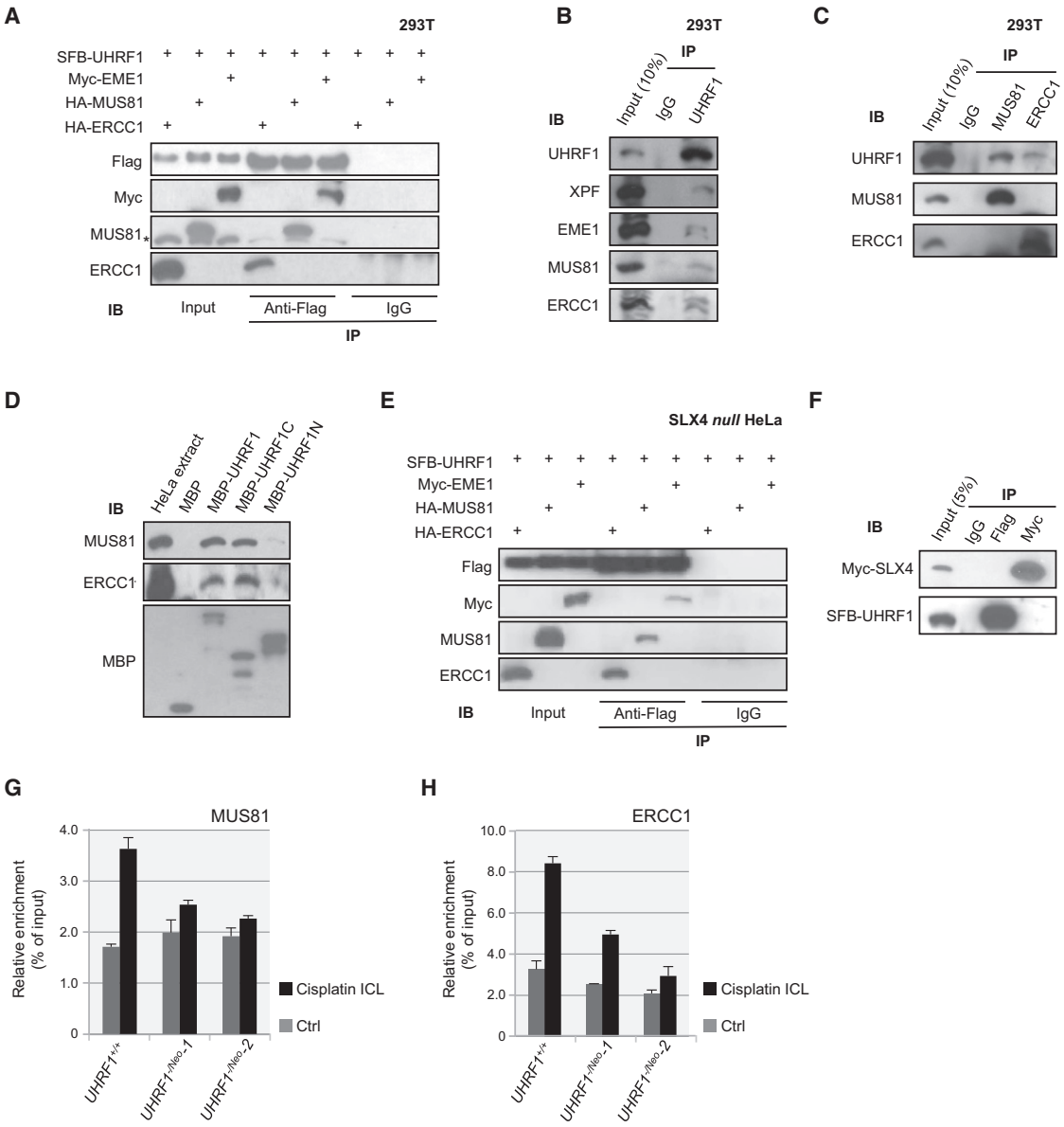

Figure 4. UHRF1-Dependent Recruitment of ICL-Processing Nucleases

(A) Co-immunoprecipitation between UHRF1 and ICL-processing nucleases. 293T cells co-transfected with SFB-tagged UHRF1 and indicated nucleases were co-immunoprecipitated with anti-Flag antibody and immunoblotted (IB) with indicated antibodies. The asterisk marks a nonspecific band in 293 cells.

(B) Immunoblot detecting indicated proteins from control (IgG) and UHRF1 co-immunoprecipitation of MMC-treated 293T cells.

(C) Immunoblot detecting UHRF1 from control (IgG), MUS81, and ERCC1 antibody co-immunoprecipitation of MMC-treated 293T cells.

(D) Pull down of MUS81 and ERCC1 from HeLa extract. MBP-UHRF1 and two UHRF1 truncations, UHRF1C (ΔUBL-TUDOR-PHD) and MBP-UHRF1N (ΔSRA-RING), were immobilized onto amylose beads and incubated with HeLa nuclear extract. Bound proteins were eluted with maltose for immunoblot detection of ERCC1 and MUS81.

(E) Co-immunoprecipitation between UHRF1 and ICL-processing nucleases. SLX4-null HeLa cells co-transfected with SFB-tagged UHRF1 and indicated nucleases were in co-immunoprecipitation with anti-FLAG antibody as immunoblotted with the indicated antibodies.

(F) Reciprocal immunoprecipitation between UHRF1 and SLX4. 293T cells co-transfected with SFB-tagged UHRF1 and Myc-tagged SLX4 were subjected to reciprocal IP with anti-Myc and anti-Flag antibodies.

(G) eChIP assay measuring the recruitment of Mus81 to cisplatin ICLs in *UHRF1*$^{+/+}$, *UHRF1*$^{-/Neo}$*-1*, and *UHRF1*$^{-/Neo}$*-2* cells. Error bars are SD derived from three independent experiments.

(H) eChIP assay measuring the recruitment of ERCC1 to cisplatin ICLs in *UHRF1*$^{+/+}$, *UHRF1*$^{-/Neo}$*-1*, and *UHRF1*$^{-/Neo}$*-2* cells. Error bars are SD derived from three independent experiments.

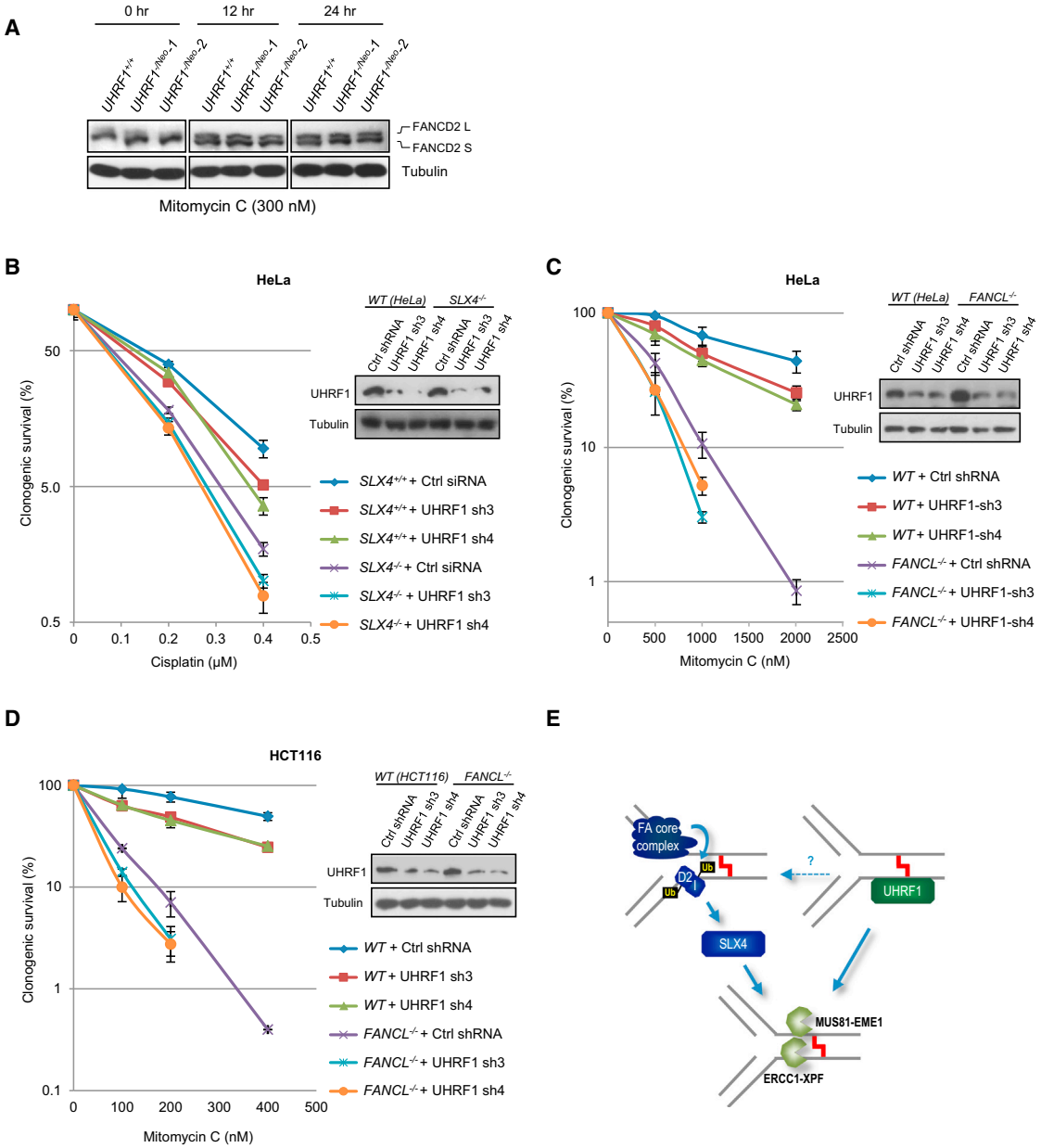

Figure 5. UHRF1-Dependent Recruitment of ICL-Processing Nucleases

(A) Immunoblots detecting FANCD2 monoubiquitination in cells with indicated genotypes treated or mock-treated with MMC (300 ng/ml). L and S represent monoubiquitinated and native forms of FANCD2, respectively. Note that the tubulin loading control is shared with Figure 3F because they are from the same blot.

(B) Clonogenic survival of *SLX*/HeLa cells infected with lentivirus expressing control (Ctrl) or UHRF1 shRNA (sh3 and sh4).

(C) Clonogenic survival of *FANCL*/HeLa cells with UHRF1 knockdown (UHRF1 sh3 and sh4).

(D) Clonogenic survival of HCT116 (WT) and *FANCL*/HCT116 cells with UHRF1 knockdown (UHRF1 sh3 and sh4).

(E) A model depicting UHRF1 acts as a damage recognition protein and nuclease scaffold in parallel to the FA-SLX4-mediated ICL DNA damage response.

in vivo, ChIP-based ICL damage enrichment at the molecular level, and biochemical binding assays in vitro. Results from these experiments consistently indicated that UHRF1 exhibited strong and direct affinity toward DNA ICL damage. The structural basis for UHRF1's ICL binding affinity is not clear. Using a panel of UHRF1 domain deletion mutants, we found that the SRA domain is required for the binding to ICL lesions (Figure 2E). The SRA domain has been reported to exhibit a moderate affinity toward hemimethylated CpG duplex DNA and was considered as a hemimethylation recognition mechanism for the recruitment of DNMT1 (Avvakumov et al., 2008). However, recent studies indicate that the Tudor and E3 ligase domains play more critical roles in directing DNMT1 to hemimethylated DNA (Arita et al., 2012; Liu et al., 2013; Nishiyama et al., 2013). It is conceivable that

the UHRF1 SRA domain exhibits structural flexibility and can recognize a broad range of damage-induced helix distortions, thus enabling it to bind to a variety of damages.

UHRF1 as a Nuclease Scaffold

Lack of DNA damage-induced γH2AX and 53BP1 foci accumulation in the UHRF1 mutant cells hinted at an impaired lesion processing that could arise from a deficit of nuclease activity at the site of the lesion. Accordingly, we examined UHRF1 protein-protein interactions and found both ERCC1/XPF and MUS81/EME1 are associated with UHRF1, suggesting that structure-specific endonucleases interact with UHRF1 in their heterodimeric forms. A protein-protein interaction between UHRF1 and EME1 was previously detected by a yeast two-hybrid screen (Mistry et al., 2008). This observation also suggests a direct interaction between UHRF1 and lesion-processing enzymes.

The functionality of the interactions is supported by the reduced nuclease recruitment to the sites of DNA lesions. The dual activities of UHRF1 in damage-binding and nuclease association functions may allow it to recruit lesion-processing activities to promote DNA damage removal. Such a property is akin to the NER protein XPA, which has lesion-binding affinity and acts as a scaffold for nuclease recruitment (Li et al., 1994; Orelli et al., 2010). The dual activities of UHRF1 is also functionally analogous to that of budding SAW1 protein which binds flap structures and promotes the recruitment of Rad1/Rad10 through direct interactions (Li et al., 2008).

Interestingly, the nuclease recruitment function of UHRF1 seems non-epistatic to that of SLX4/FANCP. This is supported by the experiments examining DNA damage sensitivity in cells with combined UHRF1 and SLX4 depletions. SLX4 is considered a main downstream effector of FA pathway activation, which is presumed to guide lesion-processing and Holliday junction resolution lesion-processing nucleases (Hodskinson et al., 2014; Klein Douwel et al., 2014; Wan et al., 2013). The enhanced phenotype from a combined loss of SLX4 and UHRF1 suggests that UHRF1 provides a parallel mechanism for the enrichment of lesion processing nucleases (Figure 5E). Such a notion is further supported by the epistatic analyses indicating that UHRF1 function is non-redundant to FANCL. However, given the early lesion recognition function of UHRF1, it remains possible that a portion of UHRF1 function is projected through promoting FA pathway activation (Liang et al., 2015). Together, our findings revealed a DNA damage response function of UHRF1 and an FA-pathway-independent mechanism of nuclease recruitment to the site of lesions.

EXPERIMENTAL PROCEDURES

Antibodies and Plasmids

Commercial antibodies used in this study were purchased as follows, anti-human UHRF1 (Abgent, NP-037414), anti-γH2AX (Upstate, 07-164), anti-human mus81 (Thermo Scientific, MA1-5837), anti-human ERCC1 (NeoMarker, MS-671-P0), and anti-MCM5 (Bethyl Laboratories, A300-195A). WT UHRF1 cDNA, ΔPHD, ΔSRA, and ΔRING constructs in pPyCAGIP-FLAG vector were a kind gift from Dr. Jiemin Wong. pENTER-UHRF1C724A and pENTER-UHRF1H741A were a kind gift from Dr. Yonchu Jenkins (Rigel Pharmaceuticals).

eChIP Assay

The eChIP assay protocol and substrate preparation were described earlier (Shen et al., 2009). Cisplatin-adducted plasmid substrates were prepared by incubating the pOriP plasmid with different dose of cisplatin for 3 hr at 37°C in the dark.

Clonogenic Survival Assay

Prior to treatment, 1–3 × 10^5 cells were seeded in a 100 mm culture plate 24 hr. Cells were exposed to various doses of DNA-damage agents for 1 hr and then seeded with appropriate cell number in triplicates for each dosage. After 14 days, colonies were fixed with 6% glutaraldehyde (v/v) and stained with 0.5% crystal violet (w/v).

SUPPLEMENTAL INFORMATION

Supplemental Information contains Supplemental Experimental Procedures and four figures and can be found with this article online at http://dx.doi.org/10.1016/j.celrep.2015.03.038.

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