184–196 *Nucleic Acids Research, 2019, Vol. 47, No. 1* *Published online 24 October 2018*
doi: 10.1093/nar/gky975

Methylation of UHRF1 by SET7 is essential for DNA double-strand break repair

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Received July 16, 2018; Revised September 29, 2018; Editorial Decision October 02, 2018; Accepted October 06, 2018

ABSTRACT

Ubiquitin-like with PHD and RING finger domains 1 (UHRF1) is a key epigenetic regulator of DNA methylation maintenance and heterochromatin formation. The roles of UHRF1 in DNA damage repair also have been emphasized in recent years. However, the regulatory mechanism of UHRF1 remains elusive. In this study, we showed that UHRF1 is methylated by SET7 and demethylation is catalyzed by LSD1. In addition, methylation of UHRF1 is induced in response to DNA damage and its phosphorylation in S phase is a prerequisite for interaction with SET7. Furthermore, UHRF1 methylation catalyzes the conjugation of polyubiquitin chains to PCNA and promotes homologous recombination for DNA repair. SET7-mediated UHRF1 methylation is also shown to be essential for cell viability against DNA damage. Our data revealed the regulatory mechanism underlying the UHRF1 methylation status by SET7 and LSD1 in double-strand break repair pathway.

INTRODUCTION

Post-translational modifications (PTMs) of non-histone proteins are known to be essential for regulating cell signaling pathways. Since PTMs are closely related to protein stability, catalytic activity and protein–protein interaction, dysregulation of these modifications causes severe diseases such as cancer and inflammatory disorders. For this reason, the addition and removal of protein PTMs are essential for proteins to function properly and for cells to survive normally (1).

Some PTMs of non-histone proteins are well known to be necessary for promoting DNA damage repair. Since unrepaired DNA is sufficient to induce genome instability, chromosome rearrangement or cancer development, many proteins involved in DNA repair system are regulated by the modulation of PTMs for a rapid DNA damage response (DDR). For example, P300/CBP-associated factor (PCAF)-mediated acetylation of RPA1 has been reported to be essential for nucleotide excision repair and protein arginine N-methyltransferase 5 (PRMT5)-dependent methylation of RuvB Like AAA ATPase 1 (RUVBL1) for homologous recombination (HR) (2,3). Additionally, proliferating cell nuclear antigen (PCNA), which functions in DNA replication and cell cycle regulation, has been reported to be involved in DNA repair through post-translational regulation, such as ubiquitination for translesion synthesis (4–6).

Ubiquitin-like with PHD and RING finger domains 1 (UHRF1) is widely known as a key regulator of DNA methylation and histone modifications (7–9). By recruiting DNA methyltransferase to newly synthesized DNA, UHRF1 plays a critical role in the maintenance of DNA methylation, which is crucial for transmitting epigenetic information from cell to cell during cell division (10–13). UHRF1 is also important for cancer progression and overexpressed in various types of tumors, such as bladder, prostate or ovarian cancer (14–17). Additionally, previous studies have reported the essential roles of UHRF1 in DNA damage (18–21). In the studies on UHRF1 PTMs, phosphorylation and ubiquitination have been reported to be crucial for the function of protein in cellular senescence and regulation of its stability (22,23). A recent study revealed that phosphorylation of UHRF1, promoted in S phase, is required for interaction with BRCA1 (BRCA1, DNA repair associated) to activate DNA damage repair pathway, especially HR (24). However, the precise mechanism underlying UHRF1 PTMs in DNA repair or tumor progression needs to be elucidated.

Meanwhile, methylation of non-histone proteins has been highlighted as a prevalent PTM, with important regulatory roles in various cellular processes, such as DNA metabolism, transcriptional regulation and DNA repair (25–27). Among methyltransferases, SET7 has been reported as a prime methyltransferase for various non-histone proteins (28–30). In particular, SET7 has been reported to play critical roles in proper DDR by promoting the enzymatic activity of DDR proteins or regulating the binding affinity of DDR-associated transcription factors. For example, SET7-mediated methylation of PARP1 (poly [ADP-

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ribose] polymerase 1) shows improved enzymatic activity and catalytically activated PARP1 is required for activating the DDR proteins (31). E2F1 is also known to be methylated by SET7 and methylation of E2F1 is a crucial step in modulating the DDR pathway to regulate the transcription of various DNA repair proteins (32).

In this study, we found that UHRF1 is methylated by SET7 at K385 in response to DNA damage. We detected that LSD1 can catalyze the demethylation reaction. We also proved that phosphorylation of UHRF1 at S661 in S phase is prerequisite for interaction with SET7. Additionally, we revealed that methylation of UHRF1 promotes the interaction between PCNA and UHRF1. This interaction results in polyubiquitination of PCNA, which is required for inducing HR. Consequently, our findings suggest that UHRF1 is an essential DDR protein and provides the evidence that methylation of UHRF1 promotes the polyubiquitination of PCNA and involves in HR pathway.

MATERIALS AND METHODS

Immunoprecipitation and ubiquitination assays

For immunoprecipitation (IP) assay, HCT116, H1299 or DLD1 cells were lysed in lysis buffer (50 mM Tris–HCl [pH 7.5], 200 mM NaCl, 0.5% NP-40, 1× protease inhibitor cocktail) and incubated with indicated antibodies overnight at 4°C. Protein A/G agarose beads (GenDEPOT) were then added, and the mixture was rotated for 3 h at 4°C. Bound proteins were analyzed by immunoblotting with indicated antibodies. For ubiquitination assays, transiently transfected HCT116 or H1299 cells synchronized in S phase were lysed in modified RIPA buffer (10 mM Tris–HCl [pH 7.5], 150 mM NaCl, 0.025% sodium dodecyl sulfate [SDS], 1% sodium deoxycholate, 1% NP-40, 1× protease inhibitor cocktail, 5 mM ethylenediaminetetraacetic acid [EDTA]). The cell lysates were immunoprecipitated using anti-Flag. Protein A/G agarose beads were then added, and the mixture was rotated for 3 h at 4°C. Bound proteins were analyzed by immunoblotting using the indicated antibodies.

Chromatin immunoprecipitation (ChIP) assay for measuring the DSB recruitment

U2OS-DRGFP cells that have integrated I-SceI site in chromosome were used to detect DDR protein recruitment to I-SceI-induced double-strand break (DSB) sites as described previously (33). Briefly, 24 h after infection of shUHRF1 virus, cells were transfected with indicated constructs and I-SceI plasmids that induce DSBs. 48 h after transfection, cells were harvested and subsequently crosslinked with 1% formaldehyde, followed by the addition of 125 mM glycine for 5 min at room temperature. Harvested cells were resuspended in SDS lysis buffer [1% SDS, 10 mM EDTA, 50 mM Tris–HCl (pH 8.1)]. Cells were then sonicated, and the lysates were subjected to immunoprecipitation using the anti-UHRF1 antibodies. The immunoprecipitates were eluted and reverse crosslinked, after which the DNA fragments were purified for polymerase chain reaction (PCR) amplification. UHRF1-associated DNA was detected with real-time PCR analysis using the primer at the I-SceI site. The primer sequence was: forward, 5′-AACCATGTTCATGCCTTCTT-3′, reverse, 5′-CCTCGTGGGTCTT CTACTTT-3′. The thermal cycler conditions were as follows: 15 min of holding at 95°C followed by 45 cycles at 94°C for 15 s, 56°C for 30 s and 72°C for 30 s (Bio-Rad). Values represent mean ± SD of technical duplicates from a representative experiment. All experiments were performed three times with similar results.

LTQ-orbitrap mass spectrometry

Purified GST-UHRF1 was used as a substrate in *in vitro* methylation assay with GST-SET7 enzyme. After the reaction, the proteins were separated by SDS-PAGE and GST-UHRF1 was isolated. After an overnight trypsin digestion at 37°C, the eluted peptides were separated using a C18 column with a linear gradient (A: 100% H_2O, 0.1% formic acid and B: 100% ACN) at a flow rate of 300 nl/min. Typically, 2 μl of sample was injected. Mass spectrometry was performed with a dual-mass spectrometer (LTQ Orbitrap Velos; Thermo Scientific) coupled to a nano-LC system (EASY nLC; Thermo Scientific). This method consisted of a cycle combining one full MS scan (mass range: 150–2000 m/z). Proteins were identified by searching the MS/MS spectra using SEQUEST.

DNA repair assay (HR reporter assay)

Integrated DNA repair reporter systems were used to determine the HR efficiency. Briefly, 24 h after infection of shUHRF1 virus or transfection of siPCNA, U2OS cells integrated with an HR reporter were transfected with indicated constructs and I-SceI plasmids that induce DSBs. 48 h after transfection, cells were harvested and the percentage of GFP-positive cells were subjected to fluorescence-activated cell sorting analysis using a BD Accuri C6 cytometer (BD Biosciences). Data were analyzed using BD Accuri C6 software (BD Biosciences). HR efficiency is presented as the percentage of control cells. Repair frequencies are the mean of at least three independent experiments.

In vitro ubiquitination assay

The recombinant GST-UHRF1, GST-UHRF1 ΔRING, GST-PCNA and GST-PCNA K164R mutant were expressed in *Escherichia coli* and purified with Glutathion sepharose bead (GE Healthcare). *In vitro* ubiquitination assays were performed with 200 ng of ubiquitin-activating enzyme (UBE1) (Boston Biochem), 200 ng of purified UBC13/MMS2 (Boston Biochem), 2 μg of Myc-ubiquitin (Boston Biochem), 3 μg of GST-UHRF1 or GST-UHRF1 ΔRING and GST-PCNA or GST-PCNA K164R mutant in 50 μl of reaction buffer (50 mM Tris (pH 7.5), 2.5 mM $MgCl_2$, 2 mM ATP and 2 mM Dithiothreitol (DTT)). The reactions were carried out at 30 °C for 3 h and stopped by boiling in SDS sample buffer.

Immunofluorescence staining

Cells cultured on coverslips were treated with 2 mM of hydrogen peroxide (H_2O_2) for 30 min. After washing with

phosphate buffered saline (PBS), cells were fixed in 4% paraformaldehyde for 3 min and permeabilized in 0.2% Triton X-100 solution for 5 min at room temperature. Cells were blocked with 1% bovine serum albumin in PBS and incubated with primary antibody for 2 h. Subsequently, samples were washed and incubated with secondary antibody for 1 h. 4′,6-diamidino-2-phenylindole (DAPI) staining was performed to visualize nuclear DNA. The coverslips were mounted onto glass slides and visualized using a Nikon ECLIPSE Ti2 inverted microscope system.

Statistical analysis

Data are expressed as mean ± SEM of three or more independent experiments. Statistical significance ($P < 0.05$) was calculated using Microsoft Excel. Differences between groups were evaluated by one-way analysis of variance, followed by a Student's *t*-test or Bonferroni test, as appropriate.

RESULTS

SET7 methylates UHRF1 at K385 *in vitro* and *in vivo*

PTMs of UHRF1 including phosphorylation and ubiquitination have been reported to be important for its function in DNA damage repair and protein degradation (22,24). However, previous reports have not discussed the physiological effects of post-translational methylation of UHRF1. Here, we aimed to elucidate the methylation of UHRF1 and identify the physiological role of methylated UHRF1 inside the cell. Initially, we performed an *in vitro* methylation assay by incubating recombinant GST-UHRF1 and various methyltransferases. We found that UHRF1 is methylated by SET7, but not SET8 (PR-SET7), SUV39H1 or G9a (Figure 1A). To identify the precise residues that are methylated by SET7 in UHRF1, we performed an *in vitro* methylation assay with recombinant SET7 and partial fragments of UHRF1, which include UBI (UHRF1 #1), TTD and PHD (UHRF1 #2), SRA (UHRF1 #3) or RING (UHRF1 #4) domain. As shown in Figure 1B, we noticed that UHRF1 contains methylated residues in 370-685 amino acids region. Since the SET7-recognized sequence motif, K/R-A/T/K-K is well defined, we compared it with the UHRF1 sequence. We found three highly conserved SET7 target sequences in UHRF1, around K385, K408 and K670 (Supplementary Figure S1A). To identify the methylated residues in UHRF1, we measured the methylation level by scintillation counting using three peptides containing 11 amino acid residues; each of these peptides contained K385, K408 or K670. When incubated with SET7, peptides that consist of amino acids around K385 were strongly methylated compared to peptides containing amino acids around K408 or K670 (Supplementary Figure S1B). To further prove the accurate major methylation sites of UHRF1, we performed liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) on a high-resolution orbitrap instrument after *in vitro* methylation assay using recombinant SET7 and UHRF1 proteins. The result of mass spectrometry showed that the methylated lysine residues of UHRF1 were K385 and K670 (Figure 1C and Supplementary Figure S1C). To confirm the LC-MS/MS analysis, we performed *in vitro* methylation assays using SET7 and UHRF1 mutants in which K385, K670 or both K385 and K670 was replaced with arginine. The methylation levels of the UHRF1 K385R and K670R mutants were decreased compared to that of UHRF1 WT, indicating that both K385 and K670 are the major methylation sites in UHRF1 (Figure 1D).

Next, to identify the domains for interaction between UHRF1 and SET7 *in vitro*, we conducted a GST pull-down assay using partial constructs of the UHRF1. As shown in Supplementary Figure S1D, SET7 preferentially interacted with the UHRF1 partial construct that consists of 370–685 amino acids. Conversely, we confirmed that the N-terminal of SET7 (52–138 a.a) bound to UHRF1 (Supplementary Figure S1E). To prove the interaction between UHRF1 and SET7 in cells, we performed co-IP assays by overexpressing Flag-empty vector (EV) or Flag-UHRF1 and GFP-SET7, which showed that UHRF1 interacts with SET7 *in vivo* (Figure 1E). In addition to HCT116 cell line, interaction of UHRF1 and SET7 was also shown in H1299 cell line (Supplementary Figure S1F). To further validate whether K385 and K670 of UHRF1 can be methylated *in vivo*, we measured the methylation level of UHRF1 in SET7 overexpressed HCT116 cells using specific UHRF1 K385me1 or K670me1 antibodies. Interestingly, only K385 of UHRF1, but not K670 was shown to be methylated in SET7 overexpressing cells (Supplementary Figure S1G and H). Moreover, we observed that methylation of UHRF1 at K385 was decreased in SET7 knocked down cells, suggesting that SET7 is specifically responsible for methylation of UHRF1 at K385 *in vivo* (Figure 1F). Taken together, our data suggest that SET7 methylates K385 of UHRF1 both *in vitro* and *in vivo*.

H_2O_2-mediated DNA damage induces UHRF1 methylation by SET7

Recent studies showed that UHRF1 is an essential protein in DDR pathway, including HR and SET7 are closely related to DDR by catalyzing the methylation of DDR proteins (18,24,31). To validate whether methylation of UHRF1 plays an important role for DNA damage signal, we evaluated the methylation level of UHRF1 at K385 after exposing HCT116 cells to H_2O_2 that induces DNA DSBs. The methylation level of UHRF1 was dramatically increased by H_2O_2-induced DNA damage (Figure 2A, lane 3). Interestingly, when SET7 was knocked down, H_2O_2 did not induce UHRF1 methylation (Figure 2A). To investigate whether the interaction between UHRF1 and SET7 was affected by H_2O_2-induced DNA damage, we performed an IP assay in the presence or absence of H_2O_2. Notably, endogenous interaction between UHRF1 and SET7 was increased in response to DNA damage by H_2O_2 (Figure 2B). Among other DNA-damaging inducers tested, we found that interaction between UHRF1 and SET7 showed to be increased by UV and ionizing radiation damage (Supplementary Figure S2A and B). Both damages also induced the methylation of UHRF1 in a similar manner as H_2O_2-induced damage (Supplementary Figure S2C and D). In addition to HCT116 cell line, methylation levels of UHRF1 were also increased in both H1299 and DLD-1 cell lines in

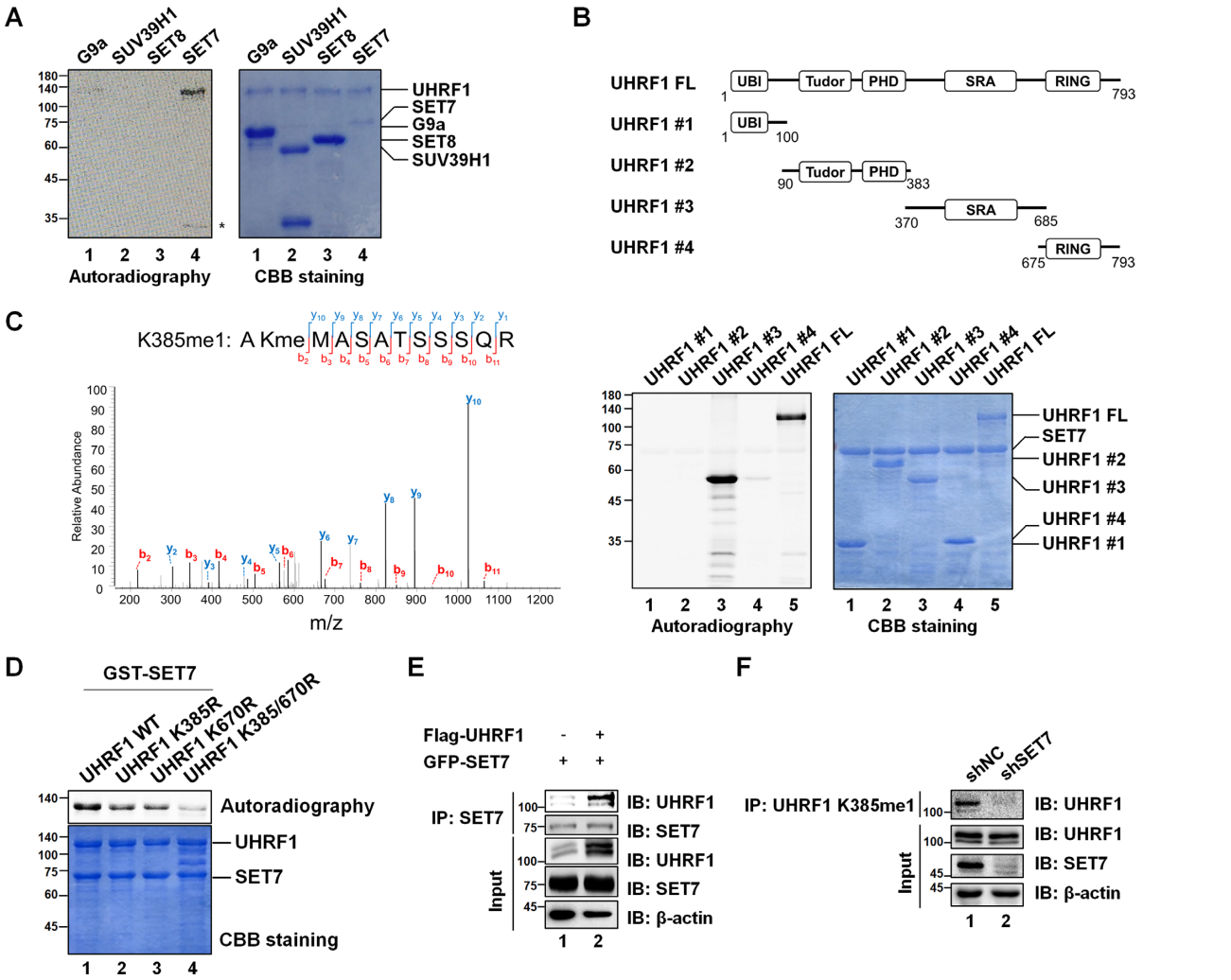

Figure 1. UHRF1 is methylated by SET7 *in vitro* and *in vivo*. (**A**) GST-UHRF1 was incubated with recombinant methyltransferases and [^{14}C]-SAM for 3 h at 30°C. The samples were separated by SDS-PAGE and stained by Coomassie brilliant blue (CBB) or exposed by autoradiography. * represents non-specific band. (left, autoradiography; right, CBB staining). (**B**) Partial recombinant constructs of UHRF1 were used as substrates for *in vitro* methylation assays with GST-SET7 (left, autoradiography; right, CBB staining). (**C**) GST-UHRF1 modified with methylation on K385. Mass spectrometry analysis (LC-MS/MS) was performed after *in vitro* methylation assay with full-length GST-UHRF1 and GST-SET7. (**D**) Each WT, K385R, K670R and K385/670R of UHRF1 protein was incubated with recombinant GST-SET7 and [^{14}C]-SAM for 3 h at 30°C. The samples were separated by SDS-PAGE and stained by CBB or exposed by autoradiography. (**E**) Flag-EV or Flag-UHRF1 and GFP-SET7 were overexpressed in HCT116 cells. The cell lysates were immunoprecipitated with SET7 antibody. Associated proteins were eluted, resolved by SDS-PAGE and immunoblotted using indicated antibodies. (**F**) SET7 was stably knocked down in HCT116 cells. Cell extracts of HCT116 shNC (negative control) and shSET7 were immunoprecipitated using an anti-UHRF1 K385me1 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE and immunoblotted using the indicated antibodies.

response to DNA damage by H_2O_2 (Supplementary Figure S2E and F).

Depending on stage in the cell cycle, different repair mechanisms including HR and non-homologous end joining (NHEJ) are activated to repair DSBs. UHRF1 has been reported to function in activating HR, which mainly occurs in S and G2 phases (34,35). To investigate the correlation between UHRF1 methylation and its role in HR, we first measured the UHRF1 methylation level in each phase of the cell cycle. As shown in Figure 2C, UHRF1 methylation was dramatically increased in the middle of S phase, implying that methylation of UHRF1 may play a role in HR (Figure 2C and Supplementary Figure S2G). More precisely, the methylation level of UHRF1 showed the gradual increase and decrease during S phase, peaking at 3 h after release (Supplementary Figure S2H and I). To further verify the UHRF1 methylation status in cells, we reconstituted wild-type SET7 or SET7 catalytic mutant (SET7 H297A) in SET7 stably knocked down cells and observed that methylated UHRF1 only in SET7 recovered cells, but not in SET7 mutant recovered cells (Figure 2D and E; Supplementary Figure S2J). Next, we asked whether the methylation affects the recruitment of UHRF1 to damaged lesion. We measured the recruitment of UHRF1 around DSBs by chromatin immunoprecipitation (ChIP) assay in U2OS-DRGFP cells. In this cell, a defective GFP cassette that contains an I-SceI enzyme recognition site is stably incorporated into genome and DSBs can be generated by ectopically expressed I-SceI constructs (Figure 2F, top) (36). The result showed that UHRF1 WT is recruited to damaged le-

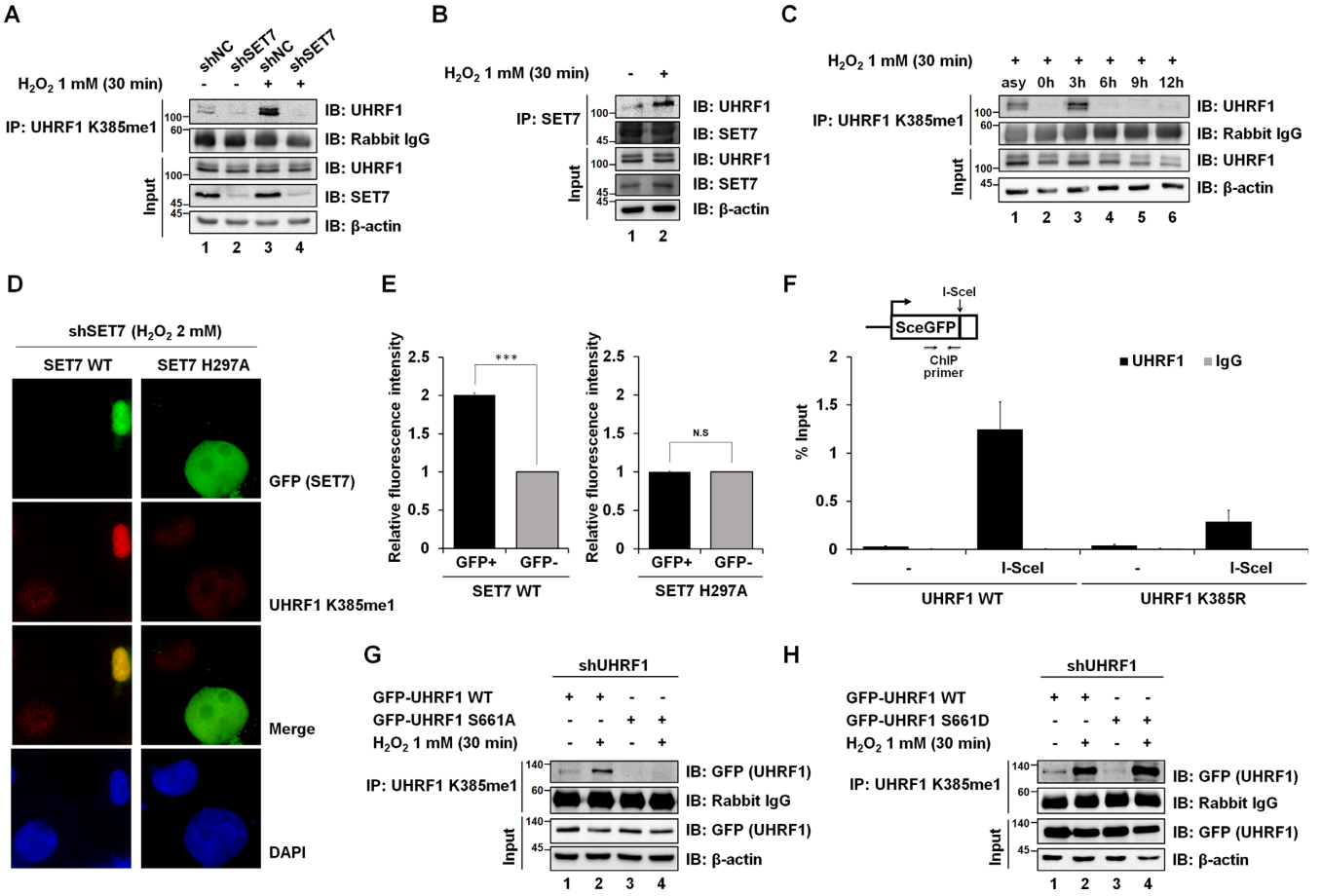

Figure 2. H_2O_2-mediated DNA damage induces UHRF1 methylation. (**A**) HCT116 shNC and shSET7 cells treated with 1 mM of H_2O_2 treatment for 30 min were immunoprecipitated using an anti-UHRF1 K385me1 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE and immunoblotted using the indicated antibodies. (**B**) HCT116 cells were treated with 1 mM of H_2O_2 for 30 min. Cell extracts of control and damaged cells were immunoprecipitated using anti-SET7 antibodies and associated proteins were pulled down with A/G agarose beads. Beads were washed extensively and bound proteins were resolved by SDS-PAGE and immunoblotted using the indicated antibodies. (**C**) HCT116 cells were arrested at G1/S boundary by double thymidine block/release, and the cells were then treated with 1 mM of H_2O_2 for 30 min. The cell lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody. (**D**) H1299 cells stably expressing SET7 shRNA that targets 3′-UTR were recovered with the indicated constructs and synchronized in S phase. UHRF1 K385me1 signals were examined following H_2O_2 treatment (2 mM for 30 min). (**E**) The relative fluorescence intensity of UHRF1 methylation (K385me1) was quantified with ImageJ, and the data were normalized to the SET7 untransfected cells. Results were shown as mean ± SD, $n >$ 70. Values represent mean ± SD of technical duplicates from a representative experiment. All experiments were performed three times with similar results. ***$P < 0.001$, N.S: no significant difference. (**F**) U2OS-DRGFP cells were transiently knocked down with shUHRF1 RNA for 24 h and transfected with UHRF1 WT or UHRF1 K385R and I-SceI. Forty-eight hours later, cells were harvested and crosslinked for ChIP assay. ChIP assay was performed using anti-UHRF1 antibody, followed by q-PCR. Values represent mean ± SD of technical duplicates from a representative experiment. All experiments were performed three times with similar results. (**G** and **H**) UHRF1 knocked down HCT116 cells overexpressed with GFP-UHRF1 WT, GFP-UHRF1 S661A or GFP-UHRF1 S661D were treated with 1 mM of H_2O_2 for 30 min. The lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody and immunoblotted with indicated antibodies.

sions after transfection of I-SceI, but methylation-deficient UHRF1 is not (Figure 2F, bottom).

Given the report that UHRF1 is phosphorylated by cyclinA2/CDK2 in S phase (34), we hypothesized that UHRF1 phosphorylation is required for the interaction between SET7 and UHRF1 in response to DNA damage. To verify this hypothesis, we generated UHRF1 phosphorylation-deficient (UHRF1 S661A) and phosphorylation-mimic (UHRF1 S661D) mutants and overexpressed each DNA construct in UHRF1 knocked down stable cells (Supplementary Figure S3A). Compared to the methylation level in the absence of H_2O_2, UHRF1 WT showed a strong methylation signal after H_2O_2 treatment, but phosphorylation-deficient mutant, UHRF1 S661A did not show any change in methylation level (Figure 2G). Surprisingly, UHRF1 S661D, which mimics phosphorylated UHRF1, showed strong methylation signal, similar to that of UHRF1 WT in response to H_2O_2-induced DNA damage (Figure 2H). Consistent with the pattern of methylation levels, UHRF1 WT and UHRF1 S661D overexpressing UHRF1 knocked down HCT116 cells showed strong interaction with SET7 in response to H_2O_2 damage, whereas phosphorylation-deficient mutant (UHRF1 S661A) did not (Supplementary Figure S3B and C). Not only H_2O_2-induced DNA damage, phosphorylation-dependent methylation of UHRF1 was also observed after UV exposure (Supplementary Figure S3D). In addition, phosphorylation-deficient UHRF1

could not be methylated in H1299 cell line, whereas wild-type UHRF1 showed strong methylation signal (Supplementary Figure S3E). Taken together, these results suggest that UHRF1 K385 methylation by SET7 is crucial for recruitment at DSB lesion in response to DNA damage and that phosphorylation of UHRF1 at S661 is required for SET7-mediated methylation of UHRF1.

Methylation of UHRF1 promotes polyubiquitination of PCNA

PCNA, DNMT1 and UHRF1 affect the regulatory mechanism of each other by comprising PCNA/DNMT1/UHRF1 complex that is essential for maintaining DNA methylation in mammalian cells (10,37–38).

In addition to recent studies about the roles of UHRF1 in DDR, we showed that SET7-mediated UHRF1 methylation is induced in response to DNA damage and methylated UHRF1 is recruited to DSB sites (Figures 1 and 2). PCNA also has been widely studied for DDR and various PTMs of PCNA have been elucidated to be involved in DNA damage repair pathway, such as ubiquitination and sumoylation (39,40). To investigate whether UHRF1 and PCNA collaborate in DDR, we observed the interaction between PCNA and UHRF1 with and without DNA damage. Compared to normal condition, UHRF1 and PCNA showed higher binding affinity after H_2O_2 treatment (Figure 3A). Surprisingly, whereas the interaction between UHRF1 and PCNA was dependent on DNA damage mediated by H_2O_2 in normal cells, two proteins did not interact in SET7 knockdown cells (Supplementary Figure S4A). Furthermore, interaction between UHRF1 and PCNA was decreased in methylation-deficient UHRF1 K385R recovered cells, compared to that of UHRF1 WT, suggesting that interaction of these two proteins was dependent on the UHRF1 methylation (Supplementary Figure S4B). Since polyubiquitination of PCNA involving DDR mechanism has not been clearly elucidated, we hypothesized that E3 ligase UHRF1 might be one of the E3 ligases of PCNA and ubiquitination of PCNA by UHRF1 might be induced for DDR. To verify this hypothesis, we induced DSBs in control cells and UHRF1 knocked down cells. Remarkably, polyubiquitination of PCNA was dramatically elevated in the control cells by H_2O_2 treatment, while UHRF1 knocked down cells showed a low signal of polyubiquitin chains on PCNA (Figure 3B). To further confirm whether UHRF1 directly catalyzes the polyubiquitination of PCNA, we performed *in vitro* ubiquitination assay by incubating UHRF1 and PCNA with ATP, E1 and E2. Ubiquitination assay showed that UHRF1 could catalyze the polyubiquitination of PCNA with E1, E2 and ATP (Figure 3C, lane 6), whereas UHRF1 could not promote the polyubiquitination of PCNA without E1, E2, Ub or ATP (Figure 3C, lanes 1–5). In addition, compared to wild-type UHRF1, catalytic-inactive mutant, UHRF1 ΔRING could not activate the polyubiquitination of PCNA, indicating that E3 ligase activity of UHRF1 is essential for polyubiquitination of PCNA (Supplementary Figure S4C). Since significance of ubiquitination of PCNA on K164 has been reported in DDR (41–43), we investigated the effect of UHRF1 on PCNA polyubiquitination at K164. Unlike wild-type PCNA, UHRF1 could not promote the polyubiquitination of ubiquitination-deficient mutant of PCNA (PCNA K164R) (Figure 3C, lanes 6 and 7). Moreover, whereas wild-type PCNA showed strong polyubiquitination, signal on PCNA K164R was diminished (Figure 3D, lanes 1 and 2). However, in UHRF1 knockdown stable cells, polyubiquitin chains on wild-type PCNA and PCNA K164R did not detected (Figure 3D, lanes 3 and 4), suggesting that UHRF1 is an E3 ligase of PCNA on DDR. Next, we investigated whether the methylation of UHRF1 by SET7 regulates polyubiquitination of PCNA. The polyubiquitination of PCNA increased by H_2O_2 treatment, whereas polyubiquitin chains at PCNA was decreased in SET7 knocked down cells (Figure 3E). To further confirm whether UHRF1 methylation is necessary for PCNA polyubiquitination, we performed IP assays using UHRF1 WT and methylation-deficient UHRF1 K385R recovered UHRF1 stably knocked down cells. Compared to UHRF1 WT, polyubiquitination of PCNA was decreased in methylation-deficient UHRF1 K385R recovered cells in response to DNA damage (Figure 3F). Consistent with H_2O_2-induced DNA damage, interaction of UHRF1–PCNA and UHRF1-mediated polyubiquitination of PCNA were promoted in response to UV exposure (Supplementary Figure S4D and E). Furthermore, UHRF1-dependent PCNA polyubiquitination was verified in H1299 cell line (Supplementary Figure S4F and G). Together, these data indicate that methylation of UHRF1 promotes polyubiquitination of PCNA in response to DNA damage.

SET7-mediated UHRF1 methylation promotes HR for DSB repair

Our data demonstrated that methylation of UHRF1 is induced by SET7 in response to DNA damage and promotes PCNA polyubiquitination (Figures 2 and 3). Given that PCNA has been reported to be involved in DDR and that UHRF1 was known to affect HR process, we hypothesized that methylation of UHRF1 is required for activating PCNA to function in HR. We examined whether UHRF1 methylation has an effect to HR efficiency, by an integrated reporter assay (Figure 4A). As expected, SET7 depletion showed the deficiency in HR and reconstituted SET7 could recover HR efficiency, whereas SET7 catalytic mutant could not (Figure 4B). Next, we tested the effect of UHRF1 methylation on HR and showed UHRF1 depletion led to significantly compromised HR. Furthermore, UHRF1 knocked down cells recovered with wild-type UHRF1 restored the level of efficiency in HR. However, we observed further HR deficiency in UHRF1 K385R overexpressed cells (Figure 4C). Since methylation of UHRF1 induced HR process by catalyzing the ubiquitination of PCNA, we investigated whether PCNA ubiquitination-deficient mutant also could promote HR. HR reporter assay showed that UHRF1 promoted HR in wild-type PCNA overexpressing cells, but not in PCNA K164R expressing cells (Figure 4D; Supplementary Figure S5A and B). Finally, we observed the effect of UHRF1 on Rad51 foci formation to DNA damage sites using H_2O_2-treated shUHRF1

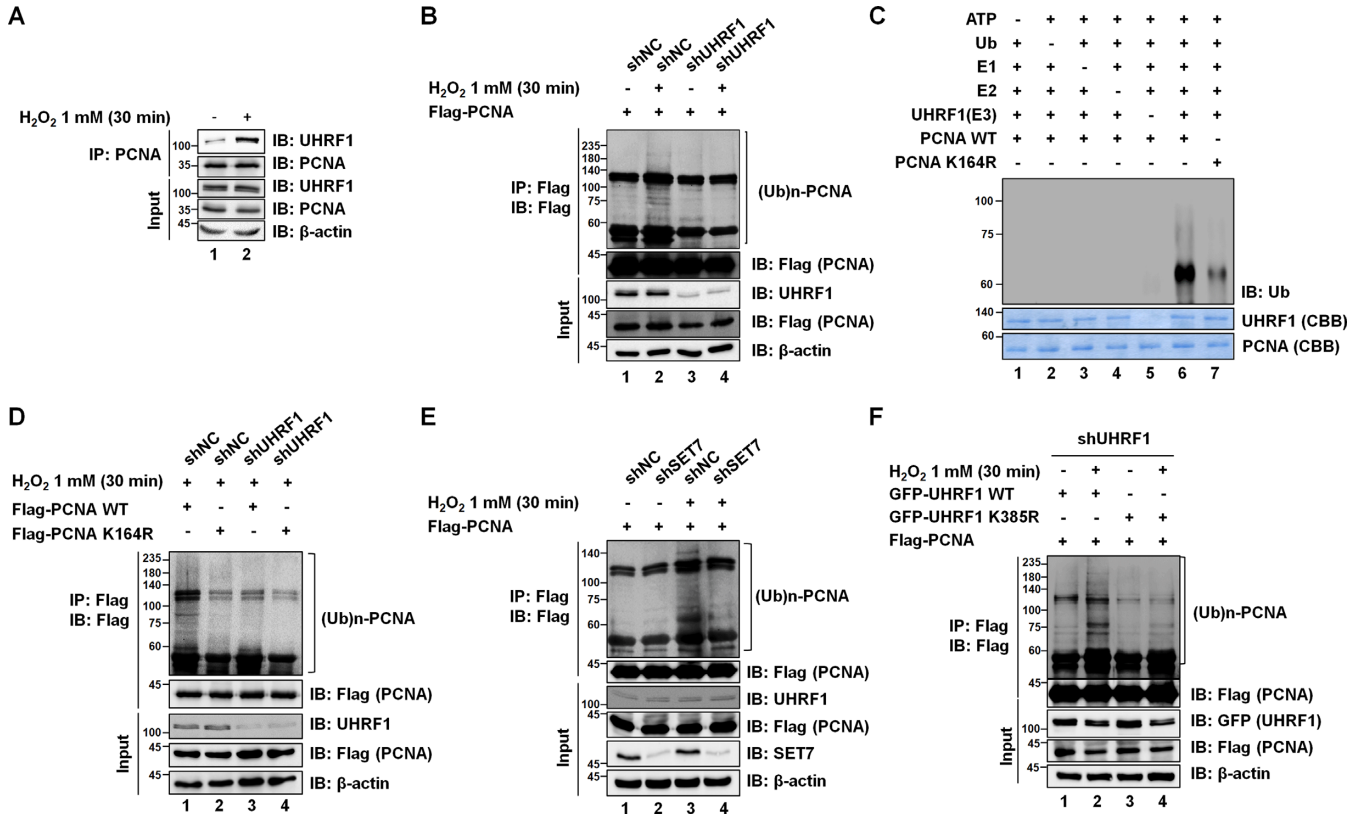

Figure 3. UHRF1 methylation by SET7 is important for polyubiquitination of PCNA. (**A**) HCT116 cells were treated with 1 mM of H_2O_2 for 30 min. Cell extracts of control and damaged cells were immunoprecipitated using anti-PCNA antibodies and associated proteins were pulled down with A/G agarose beads. Beads were washed extensively and bound proteins were resolved by SDS-PAGE, and immunoblotted using the indicated antibodies. (**B**, **D** and **E**) HCT116 shNC and shUHRF1 or shSET7 cells transfected with Flag-PCNA or PCNA K164R and synchronized in S phase were treated with 1 mM of H_2O_2 for 30 min. Each cell extracts were immunoprecipitated using an anti-Flag antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE and immunoblotted using the indicated antibodies. (**C**) *In vitro* ubiquitination assay. Recombinant UHRF1, PCNA WT, PCNA K164R, ubiquitin, UBC13/Mms2 and UBE1 were incubated at 30°C for 3 h. (**F**) Cell extracts of UHRF1 knocked down HCT116 cells transfected with GFP-UHRF1 WT or GFP-UHRF1 K385R and Flag-PCNA were treated with 1 mM of H_2O_2 for 30 min. The lysates were immunoprecipitated using an anti-Flag antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE and immunoblotted using the indicated antibodies.

stable cells. Contrary to the effect of UHRF1 WT, Rad51 foci were blocked in methylation-deficient mutant recovered cells (Figure 4E).

LSD1 mediates demethylation of UHRF1

For the maintenance of genomic homeostasis, the balance between addition and removal of epigenetic signals is tightly regulated in cells (1). In this regard, demethylation of protein is also as important as methylation for the functional regulation of proteins. To fully understand the methylation-mediated regulatory mechanism underlying the role of UHRF1 in DNA damage, we tried to identify the demethylase of SET7-mediated UHRF1 methylation. Since SET7 has been known as a histone H3K4 methyltransferase (44), we speculated that H3K4 demethylase LSD1 might have a role in the demethylation of UHRF1. To verify whether LSD1 can catalyze demethylation of UHRF1, we first performed IP assay, which showed that endogenous UHRF1 interacts with LSD1 in HCT116 cells (Figure 5A). Intriguingly, we found that the methylation level of UHRF1 at K385 was increased in cells treated with LSD1 inhibitor, GSK-LSD1 (Figure 5B). To further demonstrate whether LSD1 is responsible for demethylation of UHRF1, we overexpressed empty vector or Flag-tagged LSD1 in LSD1 knocked down HCT116 cells and conducted IP assays with anti-UHRF1 K385me1 antibody. Similar to the treatment of GSK-LSD1, LSD1 knocked down cells showed the increase of UHRF1 methylation and reconstitution of LSD1 alleviated the increase of UHRF1 methylation at K385 (Figure 5C). Furthermore, immunocytochemistry analysis showed that overexpression of LSD1 resulted in a loss of UHRF1 methylation, in contrast with the UHRF1 K385 methylation staining signals observed in adjacent non-transfected cells (Figure 5D). We observed demethylation of UHRF1 catalyzed by LSD1 both in HCT116 and H1299 cell lines (Supplementary Figure S6A and B). To verify if LSD1 affects UHRF1 recruitment at DSBs, we performed ChIP assay in U2OS-DRGFP cells. ChIP assay revealed that LSD1 attenuated the recruitment of UHRF1 WT, whereas LSD1 could not affect the recruitment of UHRF1 K385R (Figure 5E). Since SET7-mediated methylation of UHRF1 promotes the polyubiquitination of PCNA, we examined whether LSD1 blocks polyubiquitination of PCNA. Despite of the H_2O_2 treatment, polyubiquitination of PCNA was dramatically de-

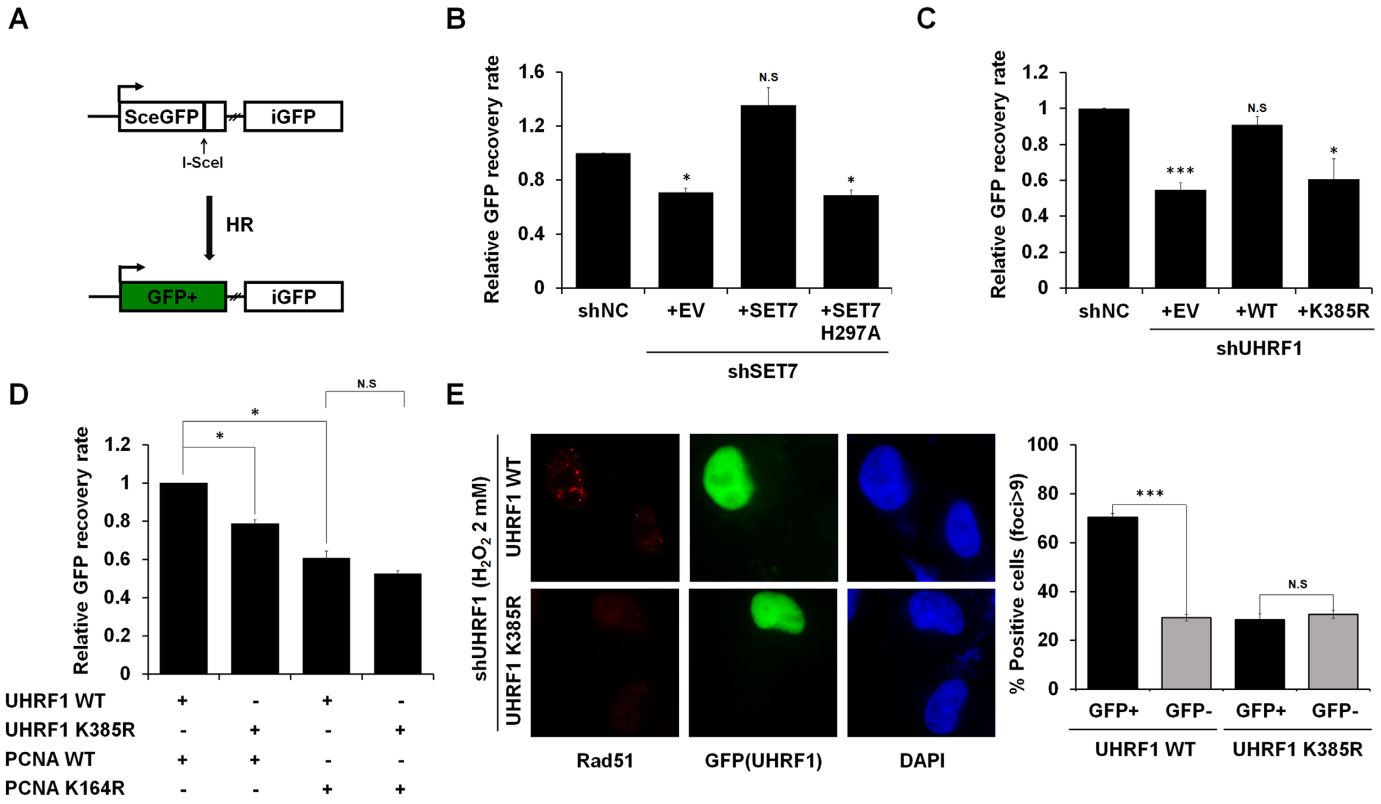

Figure 4. SET7-dependent methylation of UHRF1 is required for HR DSB repair pathway. (**A**) Schematic diagram of HR reporter. (**B**) U2OS cells integrated with HR reporter were transiently knocked down with the indicated shNC or shSET7 RNA and recovered with indicated SET7 constructs for measuring HR efficiency. Results were shown as mean ± SEM, $n = 3$; $^{*}P < 0.05$, N.S: no significant difference. (**C** and **D**) Flag-PCNA WT, Flag-PCNA K164R, Flag-UHRF1 WT or Flag-UHRF1 K385R was subjected to the HR assay. Results were shown as mean ± SEM, $n = 3$; $^{*}P < 0.05$, $^{***}P < 0.001$, N.S: no significant difference. (**E**) H1299 cells stably expressing UHRF1 shRNA that targets 3′-UTR were recovered with the indicated constructs and synchronized in S phase. Rad51 foci were examined following 2 mM H_2O_2 treatment for 30 min, followed by incubation in fresh media for 1 h. Results were shown as mean ± SD, $n > 70$. Values represent mean ± SD of technical duplicates from a representative experiment. All experiments were performed three times with similar results. $^{***}P < 0.001$, N.S: no significant difference.

creased in LSD1 overexpressing cells, in contrast with control cells whose PCNA was strongly polyubiquitinated, implying that loss of UHRF1 methylation by LSD1 inhibits the polyubiquitination of PCNA (Figure 5F). To investigate the effect of demethylation of UHRF1 by LSD1 on HR, we confirmed HR efficiency using HR reporter assay. As expected, whereas knockdown of LSD1 showed the increase of HR efficiency, reconstitution of LSD1 diminished HR efficiency compared to that of un-reconstituted cells (Figure 5G). In detailed HR reporter assay, LSD1 reduced HR efficiency by affecting wild-type UHRF1, whereas LSD1 could not affect the HR efficiency in UHRF1-K385R recovered cells (Figure 5H). Together, these data showed that methylation of UHRF1 is tightly regulated by two methylation counterparts, SET7 and LSD1, in DNA damage repair process. We also showed that LSD1 could affect HR efficiency by eliminating the methyl-group on UHRF1.

Methylation of UHRF1 in response to DNA damage is required for cell survival

Our results revealed that UHRF1 methylation is strictly regulated by the counteractive effects of SET7 and LSD1, and methylated UHRF1 catalyzes the polyubiquitination of PCNA in response to DNA damage. To discover the importance of UHRF1 methylation and demethylation in DDR, we first observed the cell viability in UHRF1 WT and methylation-deficient mutant overexpressed cells with treatment of 1 mM H_2O_2 for 30 min. Compared to control cells, UHRF1 knockdown cells exhibited a significant decrease in cell viability. However, UHRF1 knocked down cells recovered with wild-type UHRF1 had relatively higher viability than that of UHRF1 knockdown cells, whereas methylation-deficient mutant (UHRF1 K385R) recovered cells showed higher sensitivity to DNA damage (Figure 6A). To further validate the effect of UHRF1 methylation on damage tolerance, we performed colony formation assay, which can assess cell viability. As shown in Figure 6B and Supplementary Figure S7A, cells overexpressing wild-type UHRF1 showed increased cell viability whereas methylation-deficient mutants showed decreased cell viability, indicating the consistent effect of UHRF1 methylation on cell survival. Additionally, we evaluated the rate of apoptosis to measure the effect of UHRF1 methylation on DNA damage tolerance. Consistent with the result described in Figure 6A and B, we showed that depletion of UHRF1 promotes apoptotic cell death and recovery of UHRF1 suppresses the apoptosis. However, the blockage of methylation with reconstituted UHRF1 K385R or LSD1 overex-

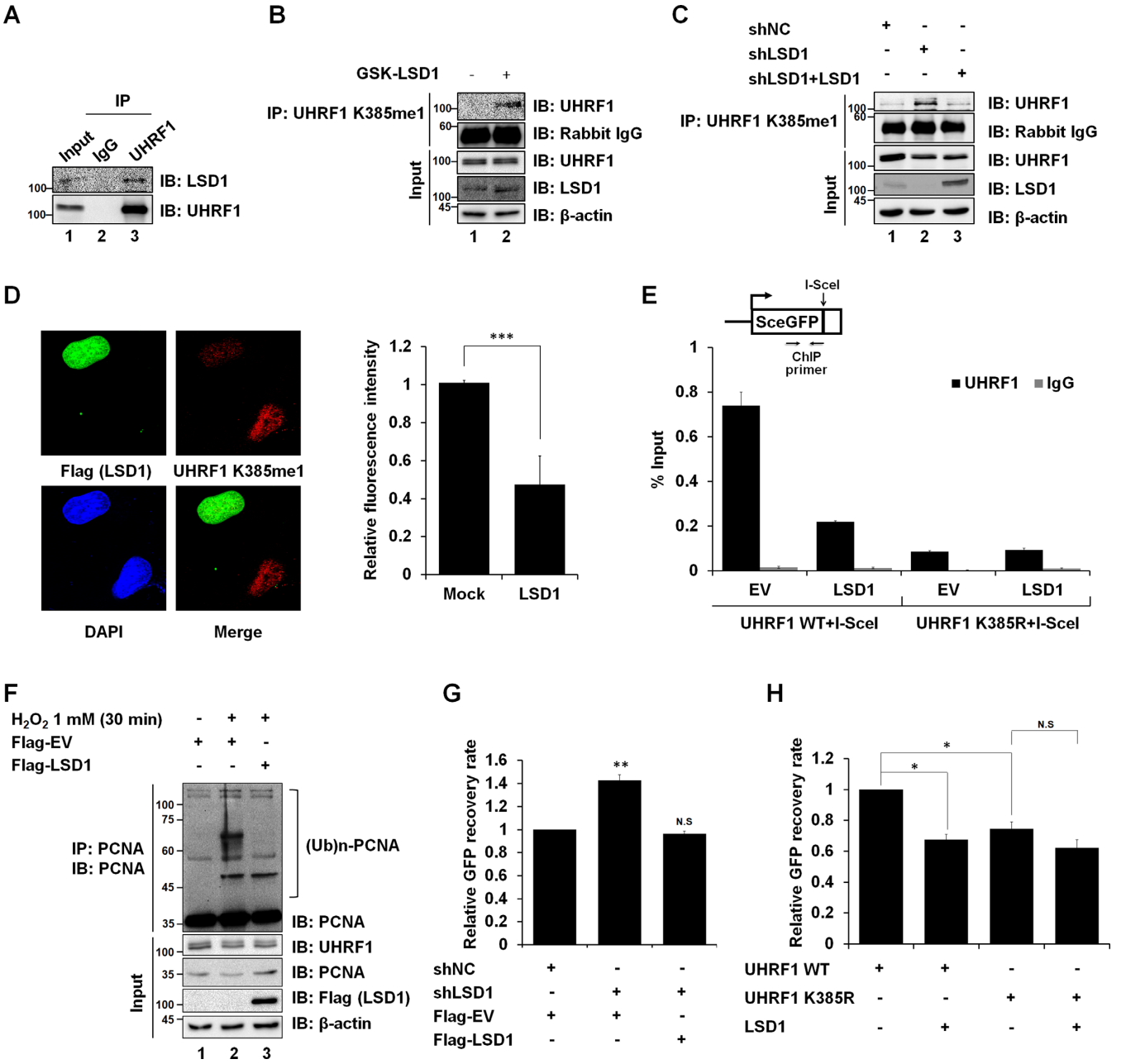

Figure 5. Histone demethylase LSD1 demethylases UHRF1. (**A**) Cell extracts from HCT116 cells were immunoprecipitated with anti-UHRF1 antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE and immunoblotted. (**B**) HCT116 cells treated with 500 nM GSK-LSD1 for 24 h, and control cells were immunoprecipitated with anti-UHRF1 K385me1 antibody and analyzed with immunoblotting. (**C**) HCT116 cells transfected with Flag-EV or Flag-LSD1 in control or LSD1 stably knocked down cells. The lysates were immunoprecipitated using an anti-UHRF1 K385me1 antibody. (**D**) H1299 cells were transfected with flag-LSD1 and synchronized in S phase. Intensity of methylated UHRF1 was examined following H_2O_2 treatment (2 mM for 30 min). The relative fluorescence intensity of UHRF1 methylation (K385me1) was quantified with ImageJ, and the data were normalized to LSD1 untransfected cells. Values represent mean ± SD of technical duplicates from a representative experiment. All experiments were performed three times with similar results. $^{***}P < 0.001$. (**E**) U2OS-DRGFP cells were transiently knocked down by shUHRF1 RNA, 24 h later transfected with UHRF1 WT or UHRF1 K385R, Flag-LSD1 and I-SceI, and 48 h later, cells were harvested and crosslinked for ChIP assay. ChIP assay was performed using anti-UHRF1 antibody, followed by q-PCR. Values represent mean ± SD of technical duplicates from a representative experiment. All experiments were performed three times with similar results. (**F**) Cell extracts of UHRF1 knocked down HCT116 cells transfected with Flag-EV of Flag-LSD1 and synchronized in S phase were treated with 1 mM of H_2O_2 for 30 min. The lysates were immunoprecipitated using an anti-PCNA antibody. Immunoprecipitates were eluted, resolved by SDS-PAGE and immunoblotted using the indicated antibodies. (**G**) U2OS cells integrated with HR reporter were transiently knocked down with the indicated shNC or shLSD1 RNA and recovered with indicated LSD1 constructs for measuring HR efficiency. Results were shown as mean ± SEM, $n = 3$; $^{**}P < 0.01$, N.S: no significant difference. (**H**) Flag-LSD1, Flag-UHRF1 WT or Flag-UHRF1 K385R was subjected to the HR assay. Results were shown as mean ± SEM, $n > 3$; $^{*}P < 0.01$, N.S: no significant difference.

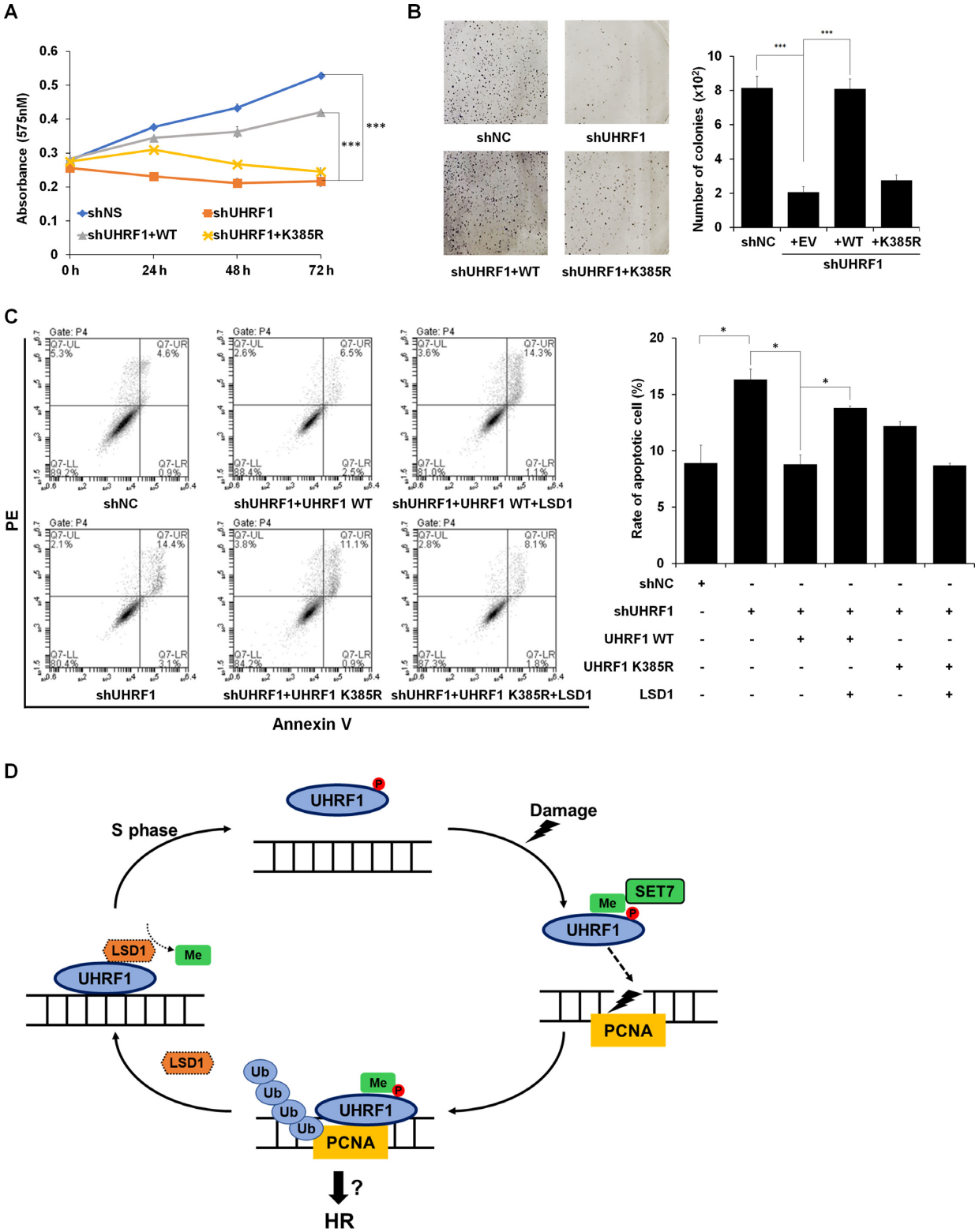

Figure 6. Methylation of UHRF1 is essential for cell viability. (**A**) Cell viability was determined using the MTT assay. HCT116 UHRF1 knocked down cells were transfected with UHRF1 WT and UHRF1 K385R. Cells were treated with 1 mM H_2O_2 for 30 min and incubated in fresh media for 0–72 h. Results were shown as mean ± SEM, $n = 3$; ***$P < 0.001$. (**B**) Representative colony formation assay using HCT116 UHRF1 knocked down cells transfected with UHRF1 WT and UHRF1 K385R. Cells were treated with 1 mM H_2O_2 for 30 min and incubated in fresh media for 7 days. Results were shown as mean ± SEM, $n = 3$; ***$P < 0.001$. (**C**) HCT116 cells were treated with 1 mM H_2O_2 for 30 min. Flow cytometry analysis of annexin-V and propidium iodide (PI) staining of apoptotic cells transfected with UHRF1 WT, UHRF1 K385R or LSD1 in UHRF1 knocked down cells or control cells, showing % of apoptotic cells (Annexin-V positive + Annexin-V and PI double positive cells). Results were shown as mean ± SEM, $n = 3$; *$P < 0.05$. (**D**) Model illustrating UHRF1 methylation induces polyubiquitination of PCNA and promotes HR progression.

pression could not suppress the apoptotic cell death (Figure 6C; Supplementary Figure S7B and C). Collectively, our data showed that SET7-dependent methylation of UHRF1 is essential for cell viability, suggesting UHRF1 methylation involves in DNA repair pathway for cell survival.

Overall, our results indicate that methylation of UHRF1 regulated by SET7 and LSD1 is essential for recruitment of UHRF1 to DNA damaged lesion, and phosphorylation of UHRF1 at S661 during S phase is prerequisite for SET7-mediated UHRF1 methylation. Moreover, our data suggest that methylation of UHRF1 promotes polyubiquitination of PCNA and could facilitate HR repair (Figure 6D).

DISCUSSION

Our data provided novel perspective on epigenetic regulation of UHRF1 function. Post-translational methylation of UHRF1 catalyzes the polyubiquitination of PCNA and promotes DSB repair process, HR. Previously, ubiquitination of PCNA has been identified to be essential for DNA repair and each modification of PCNA functions in different types of DNA damage (4). The Rad6/Rad18 complex catalyzes monoubiquitination of PCNA at K164; these results in replacement of replicative polymerase with an alternative DNA polymerase, promoting the error-prone repair pathway, translesion synthesis (45). In contrast, polyubiquitination of PCNA at K164 has been known to promote 'error-free repair' to DNA damage (5,46), but this pathway is currently poorly defined (6). The E3 ligases, HLTF and SHPRH, known to catalyze polyubiquitination of PCNA, are not sufficient for polyubiquitination of PCNA, since Hltf-/- and Shprh-/- double-knockout mouse embryonic fibroblasts seem to display some residual PCNA polyubiquitylation (47). This study proposed the possibility that additional E3 ligases may also contribute to polyubiquitylating PCNA. We focused on the properties of UHRF1 as an E3 ligase that forms a complex with PCNA and identified that methylated UHRF1 promotes the polyubiquitination of PCNA. Moreover, we showed that methylation of UHRF1 could affect the progression of HR, suggesting the possible role of UHRF1 methylation in HR.

Since regulation on the level of transcription takes a relatively long time, PTMs of protein could allow to respond rapidly to environmental changes such as DDR. Phosphorylation of DDR proteins such as BRCA1, p53 and RPA by ATR or ATM is the representative PTM-dependent regulatory mechanism of DDR. Among PTMs of UHRF1, phosphorylation of UHRF1 by CDK2/cyclin A was reported in zebrafish and a recent study identified that phosphorylated UHRF1 showed an increased interaction with BRCA1 (24,34). In the current study, our data showed that phosphorylation of UHRF1 is a prerequisite for methylation by SET7. How can SET7 methylate only phosphorylated UHRF1? We suggest two possible hypotheses: (i) A structural change of UHRF1 that is optimal for SET7-mediated methylation could occur by the phosphorylation. For example, this alteration in protein structure could give SET7 higher accessibility to UHRF1 for the addition of methyl-groups. (ii) It is possible that BRCA1 acts as a mediator and brings UHRF1 to SET7. In the previous study, it was shown that SET7 methylates PARP1, and that SET7-mediated PARP1 methylation enhances its recruitment to damaged lesions, implying that SET7 can be recruited to damage sites (31). Moreover, another study implicated that UHRF1 recruitment to damage sites is dramatically decreased in BRCA1 knockdown cells, suggesting that BRCA1 recruits phosphorylated UHRF1 to DNA damage sites (24). Recruitment of UHRF1 to DNA damage sites by BRCA1 might affect the interaction with SET7 and UHRF1, which is thought to be located in DNA-damaged lesions and finally promotes the methylation of UHRF1.

In this study, we found that UHRF1 is methylated by SET7 in response to DNA damage, and that LSD1 as a counterpart of SET7 reduces UHRF1 methylation. We showed that methylated UHRF1 catalyzes attachment of the polyubiquitin chain to PCNA. In a similar context to our data, a recent study suggested a relationship between LSD1 and DSB repair (48). They showed that HR is promoted in LSD1 knocked down cells, and that LSD1 promotes NHEJ. We also identified that demethylation of UHRF1 by LSD1 results in removal of polyubiquitin-chains on PCNA and blockage of HR progression, despite treatment with a damage inducer. Though we could suggest that it might be possible through the blockage of UHRF1 methylation by LSD1, the relationship between UHRF1, SET7 and LSD1, and how they regulate methylation status of UHRF1 in DNA repair pathway remains unclear. Additionally, their regulatory roles in cell cycle-dependent contexts such as interaction should be identified in further studies.

For many years, UHRF1 has been mainly studied as an epigenetic regulator for maintaining DNA methylation during cell division. Recently, functional roles of UHRF1 in DNA damage repair pathway have been revealed (18–21). Occupancy of UHRF1 on DNA strands in S phase to maintain DNA methylation could be advantageous for access to damaged lesion for DNA damage repair. It is possible that UHRF1 methylation might have a role in ensuring DNA methylation maintenance after damaged DNA has been repaired.

In this study, we discovered that UHRF1 is methylated by SET7 and that phosphorylation of UHRF1 in S phase is required for it to be methylated in response to DNA damage. We proved that modulation of UHRF1 methylation is regulated by SET7 and LSD1. Also, we provided evidence that UHRF1 is a new E3 ligase of PCNA, which catalyzes the conjugation of polyubiquitin chains on PCNA. Furthermore, we concluded that SET7-mediated UHRF1 methylation induces polyubiquitination of PCNA and suggested a potential correlation between methylation of UHRF1 and HR progression. In this study, we suggest a novel mechanism of DNA damage repair system through the exquisite modulation of UHRF1 methylation status by SET7 and LSD1.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

ACKNOWLEDGEMENTS

We appreciate Dr Keun P. Kim (Chung-Ang University) for helpful comments and insightful discussions. We also thank

Dr Sung Hee Back (Seoul National University) for generously providing the pCMV-Flag-LSD1 and Dr Jung Min Kim (Chonnam National University) for the integrated DNA repair reporter system. We thank National Center for Inter-University Research Facilities (NCIRF) for assistance with LTQ-orbitrap analysis.

FUNDING

National Research Foundation of Korea (NRF) grant, Ministry of Science, ICT & Future Planning [NRF-2016R1A4A1008035, 2013R1A2A2A01068788]; NRF grant, Korean Government [NRF-2015H1A2A1033488]. Funding for open access charge: National Research Foundation of Korea [NRF-2016R1A4A1008035].
Conflict of interest statement. None declared.

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