RESEARCH ARTICLE

RNF8-ubiquitinated KMT5A is required for RNF168-induced H2A ubiquitination in response to DNA damage

Xiaopeng Lu1 | Min Xu1 | Qian Zhu1 | Jun Zhang1 | Ge Liu1 | Yantao Bao1 | Luo Gu2 | Yuan Tian1,3 | He Wen1 | Wei-Guo Zhu1,3,4

1Guangdong Key Laboratory of Genome Instability and Human Disease Prevention, Department of Biochemistry and Molecular Biology, Shenzhen University School of Medicine, Shenzhen, China
2Department of Physiology, Nanjing Medical University, Nanjing, China
3Shenzhen Bay Laboratory, Shenzhen University School of Medicine, Shenzhen, China
4International Cancer Center, Shenzhen University School of Medicine, Shenzhen, China

Abstract
Histone modifications play critical roles in DNA damage repair to safeguard genome integrity. However, how different histone modifiers coordinate to build appropriate chromatin context for DNA damage repair is largely unknown. Here, we report a novel interplay between the histone methyltransferase KMT5A and two E3 ligases RNF8 and RNF168 in establishing the histone modification status for DNA damage repair. KMT5A is a newly identified substrate of RNF8 in vitro and in vivo. In response to DNA double-strand breaks (DSBs), RNF8 promotes KMT5A recruitment onto damaged chromatin in a ubiquitination-dependent manner. RNF8-induced KMT5A ubiquitination increases the binding capacity of KMT5A to RNF168. Interestingly, KMT5A not only drives a local increase in H4K20 monomethylation at DSBs, but also promotes RNF168’s activity in catalyzing H2A ubiquitination. We proved that the interaction between the H2A acidic patch and KMT5A R188/R189 residues is critical for KMT5A-mediated regulation of H2A ubiquitination. Taken together, our results highlight a new role for KMT5A in linking H4K20 methylation and H2A ubiquitination and provide insight into the histone modification network during DNA damage repair.

KEYWORDS
DNA double-strand breaks, histone ubiquitination, KMT5A, RNF8, RNF168

Abbreviations: 53BP1, p53-binding protein 1; ATM, ataxia telangiectasia mutated; BRCA1, breast cancer 1; CtIP, CtBP-interacting protein; DNA-PKcs, DNA-dependent protein kinase catalytic subunit; DDR, DNA damage response; DSB, DNA double-strand break; FUCCI, fluorescence ubiquitin cell cycle indicator; HAT, histone acetyltransferase; HDAC, histone deacetylase; HR, homologous recombination; IR, irradiation; KMT5A, lysine methyltransferase 5A; KD, knockdown; KDM4A, lysine demethylase 4A; KO, knockout; L3MBTL1, L3MBTL histone methyl-lysine binding protein 1; MDC1, mediator of DNA damage checkpoint protein 1; NHEJ, nonhomologous DNA end joining; PCNA, proliferating cell nuclear antigen; RIF1, replication timing regulatory factor 1; RNF8, RING finger protein 8; RNF168, RING finger protein 168; SSA, single-strand annealing; SUV4-20H1/2, Su(Var)4-20 homolog ½; UHRF1, ubiquitin like with PHD and Ring finger domains 1; WCL, whole cell lysates; WT, wild-type.

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Mammalian genome fidelity is constantly threatened by DNA damage caused by environmental stressors and intracellular metabolites. Thus, cells have evolved surveillance and repair systems to eliminate these risks to ensure genomic and biological fitness. In response to DNA double-strand breaks (DSBs), histone modifiers are recruited to DNA lesions to change the chromatin context for the operation of DNA repair machinery. Histone modifiers include certain phosphokinases, E3 ligases, histone acetyltransferases (HATs), histone deacetylases (HDACs), and histone methyltransferases. Although all these histone modifiers are involved in the DNA damage response, their intrinsic connections in building a spatial and temporal chromatin context for appropriate DNA damage repair are largely unknown.

DNA double-strand breaks can be repaired through several different pathways, including nonhomologous DNA end joining (NHEJ), homologous recombination (HR), and single-strand annealing (SSA). Previous studies revealed that 53BP1-RIF1 and BRCA1-CtIP complexes antagonistically regulate the DNA damage repair pathway choice in a cell cycle-dependent manner. In mammalian cells, NHEJ represents the predominant DNA repair pathway that does not require a homologous DNA sequence and differs from error-free HR repair using sister chromatids for recombination. 53BP1 is a critical factor involved in NHEJ repair; it serves as a platform for the loading of other DNA repair factors necessary for the DNA damage response (DDR). 53BP1 recruitment onto DSBs is regulated by several histone modifications, such as H4K20me1, H4K16me1, H3K18ac, γ-H2AX, and H2AK15ub. To achieve efficient NHEJ repair, all these histone modification-related modifiers must cooperate organically to create an appropriate chromatin context for 53BP1 binding at DSBs.

RNF8 and RNF168 are two important E3 ligases that promote H2AK15ub for 53BP1 recruitment during DNA damage repair. In response to DSBs, ATM or DNA-PKcs phosphorylates H2AX at S139 (γH2AX), MDC1 binds to γH2AX through its BRCT domain and recruits RNF8 to DSBs to initiate the ubiquitin signaling pathway. RNF8 acts downstream of RNF8 and ubiquitinates H2AK15 for 53BP1 binding at DSBs. The importance of the ubiquitin signaling pathway in DSB repair is evidenced by the finding that RIDDLE (radiosensitivity, immunodeficiency, dysmorphic features, and learning difficulties) syndrome, a novel human immunodeficiency disorder associated with defective DSBR repair and cancer predisposition, occurs due to mutations in the gene encoding RNF168. In addition, RNF8 deficiency impairs class switch recombination, spermatogenesis, and genomic integrity and predisposes affected individuals to cancer. Interestingly, in RNF8 knockout (KO) mice, H4K20me1 was undetectable from the early pachytene stage to the mid-diplotene stage in meiotic prophase during spermatogenesis. Furthermore, on the male meiotic sex chromosomes during spermatogenesis, H4K20me1 accumulation is concomitant with ubiquitinated H2A from the early pachytene stage to the mid-diplotene stage. Recently, it was also suggested that the recruitment of the H4K20me1 methyltransferase KMT5A is regulated by the ubiquitin signaling pathway in DDR. However, the detailed mechanisms are still elusive. Because both KMT5A and RNF8/RNF168 are important regulators of 53BP1 in NHEJ repair, it is intriguing to explore whether and how KMT5A and RNF8/RNF168 interact to regulate chromatin ubiquitination and/or methylation in the DDR.

KMT5A, also known as SET8 or Pr-SET7, is the sole enzyme responsible for H4K20me1: it has important roles in gene regulation, DNA replication, and genome stability. In addition to H4K20me1, KMT5A was reported to methylate other nonhistone substrates, such as p53, Numb, and UHRF1. KMT5A protein levels change throughout the cell cycle, with extremely low expression at S phase. KMT5A is targeted for proteasomal degradation in S phase as well as upon UV-induced DNA damage in a PCNA-dependent manner. In response to DNA double-strand breaks, KMT5A is recruited to DSBs to induce H4K20me1, which is further catalyzed to H4K20me2 by SUV4-20H1/2 to ensure efficient 53BP1 binding to permit NHEJ repair. So far, it seems that KMT5A in DDR mainly serves to induce H4K20 methylation at DSBs. However, the major histone methylation for 53BP1 recruitment is H4K20me2, which is naturally highly abundant and exposed by RNF8/RNF168-mediated dissociation of proteins like KDM4A and L3MBTL1 in DDR. It is thus considered that KMT5A may have other functions in DNA damage repair apart from inducing H4K20me1.

In this study, we reveal that KMT5A has a new role in regulating histone ubiquitination in the DDR, which is independent of its methyltransferase activity. Mechanistically, KMT5A interacts with RNF168 and increases the activity of RNF168 in catalyzing H2A in a H2A acidic patch-dependent manner. In addition, KMT5A is identified as a new substrate of RNF8 and its loading onto damaged chromatin is dependent on both RNF8 and RNF168. Our findings suggest that KMT5A and RNF8/RNF168 cooperate to regulate histone modifications to permit DNA damage repair and provides a new insight into the study of DDR.

2 | MATERIALS AND METHODS

2.1 | Cell culture and transfection

HCT116 cells were grown in McCoy's 5a medium supplemented with 10% of fetal bovine serum and the appropriate amount of penicillin/streptomycin, and cultured in a
humidified atmosphere of 5% CO2 maintained at 37°C. HeLa cells were grown in Dulbecco’s Modified Eagles Medium under the same conditions. The cells were subcultured by trypsinization every 2 days, and seeded at the appropriate confluency. HeLa and HCT116 cells were obtained from the American Type Culture Collection. All knockout cell lines were generated following the published protocol. Transient transfections were performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), according to the manufacturer’s protocol.

2.2 | Antibodies

The antibodies used in this study were: anti-His-tag (D291-3, MBL, Aichi, Japan), anti-Myc-tag (M047-3, MBL), anti-GFP-tag (M048-3, MBL), anti-Flag-tag (F1804, Sigma-Aldrich, St. Louis, MO, USA), anti-GST-tag (C1303, APPLYGEN, Beijing, China), anti-H3 (ab1791, Abcam, Cambridge, UK), anti-H4 (ab10158, Abcam), anti-H4K20me1 (ab9051, Abcam), anti-phospho-Histone H2AX (Ser139) (05-636, EMD Millipore, Billerica, MA, USA), anti-FK2 (04-263, EMD Millipore), anti-ubiquitin (sc-8017, Santa Cruz Biotechnology), anti-Actin (sc-58673, Santa Cruz Biotechnology), anti-Pr-Set7 (sc-377034, Santa Cruz Biotechnology), anti-RNF8 (sc-271462, Santa Cruz Biotechnology), anti-RNF8 (H00165918-M01, Novus, Biologicals, CO, USA), and anti-KMT5A (C18B7, Cell Signaling Technology, Danvers, MA, USA).

2.3 | Plasmids

The cDNAs of human KMT5A, RNF8, and RNF168 were separately amplified and cloned into pET-28a, pGEX-6P-1, pCMV-Myc and p3xFLAG-CMV-10 vectors. The cDNAs of human RNF8 and Ub were separately inserted into a pCMV-HA vector. Human RNF8 and RNF168 cDNAs were subcloned into pEGFP-C1. All fragments and mutation constructs were generated with a Mut Express II Fast Mutagenesis Kit (Vazyme Biotech Co., Nanjing, China).

2.4 | DNA damage treatment

For X-ray irradiation, cells were cultured to 80% confluency, and then, subjected to the indicated dose of radiation, and then, re-cultured in fresh medium for the indicated time. X-ray irradiation was delivered using an RS2000pro Ras Source biological X-ray irradiator (Rad Source Technologies, Georgia, USA) with a radiation output of 160 KV, 25 mA at a dose rate of 4.125 Gy/min.

For etoposide treatment, cells were cultured to 80% confluency, and then, treated with 40 μM etoposide (E1383, Sigma-Aldrich) for the indicated time before being harvested for protein extraction.

2.5 | In vitro HMTase assay

For the in vitro HMTase assay, 2 μg of substrates were incubated with purified KMT5A in a methylation reaction buffer [50 mM Tris-HCl (pH 8.0), 5 mM MgCl2, 4 mM DTT, and 0.5 mM SAM] at 37°C for the indicated time before analysis by western blotting. Histone H4 was obtained from New England Biolabs, MA, USA. His-KMT5A, His-RNF8, and His-RNF168 were purified from E coli.

2.6 | In vitro ubiquitination assay

For KMT5A and KDM4A ubiquitination, assays were setup in a total volume of 25 μL in 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM MgCl2, 1 mM ZnCl2, 3 mM ATP, and 1 mM TCEP, 0.2 μM UBE1 (R&D Systems), 0.5 μM E2 (UBC13/MMS2 complex or UbcH5c; R&D Systems), 5 μM ubiquitin (Sigma), 1 μM recombinant E3 (His-RNF168/RNF8), and 0.25 μM substrates were incubated at 32°C for 3 hours or longer. For H2A ubiquitination, UbcH5c was used for E2, the indicated substrates were incubated in the reaction system as described above at 32°C for 2 hours, supplemented with 5 μM KMT5A or not as indicated.

2.7 | Chromatin acid extraction

Cells were lysed in 1 mL hypotonic lysis buffer [10 mM Tris-HCl (pH 8.0), 1 mM KCl, 1.5 mM MgCl2, 1 mM DTT, and protease inhibitors], and the intact nuclei were pelleted by centrifugation at 12 000 rpm (13 523 g) at 4°C for 10 minutes. The supernatant was discarded and the nuclei were resuspended in 400 μL of 0.2 M sulfuric acid and incubated for at least 30 minutes at 4°C. The samples were again collected by centrifugation at 12 000 rpm (13 523 g) at 4°C for 10 minutes, and the supernatant containing the histones was collected. Trichloroacetic acid was added to a final concentration of 33% and the samples were incubated on ice for 30 minutes. The pellet was collected by centrifugation at 12 000 rpm (13 523 g) at 4°C for 10 minutes, washed with acetone, air-dried, and then, dissolved in ddH2O.
2.8 | Protein extraction and western blotting

For whole cell lysate extraction, equal numbers of harvested cells were washed with PBS by centrifugation at 10 000 rpm (9391 g) at 4°C for 30 sec and the cell pellet was resuspended in 30 μL (per 10^6 cells) 2X protease inhibitor buffer containing one cocktail protease inhibitor pellet (Roche Holding AG, Basel, Switzerland) in 3.5 mL PBS. An equal volume of 2X SDS loading buffer (950 μL Laemmle buffer + 50 μL 2-mercaptoethanol) was added to the resuspended cells. The samples were boiled for 10 minutes with a pulse vortex every 5 minutes, and then, pelleted by centrifugation at 12 000 rpm (13 523 g) at 4°C for 15 minutes.

For chromatin protein extraction, the cells were resuspended in buffer I [50 mM Hepes (pH 7.5), 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, and protease inhibitors] on ice for 3 minutes. After centrifugation at 13 000 rpm (15 871 g) at 4°C for 3 minutes, the supernatant (soluble fraction) was discarded. The pellet was washed with buffer I and resuspended in Buffer II (Buffer I containing 2% RNase A, protease inhibitors) at 25°C for 30 minutes. After centrifugation at 13 000 rpm (15 871 g) at 4°C for 3 minutes, the supernatant (containing RNA-binding proteins) was discarded, and the pellet was collected as the chromatin fraction (Chr).

Western blotting was used to evaluate protein expression following the protocol supplied by Abcam, with minor modifications. Equal amounts of proteins were size fractionated on a 6%-15% of SDS-PAGE gel.

2.9 | Protein purification and pull-down assays

GST-fusion proteins were expressed in E. coli BL21 cells and purified using glutathione-sepharose 4B beads (GE Healthcare, Kings Park, NY, USA), according to the manufacturer’s protocol. His-tagged proteins were expressed in E. coli BL21 (DE3) cells and purified using a Ni (ii)-Sepharose Affinity kit (GE Healthcare). Isolated proteins were further purified using a molecular sieve.

For GST pull-down, GST and GST-tagged proteins were incubated with the indicated targets in binding buffer [20 mM Tris-Cl (pH 8.0), 200 mM NaCl, 1 mM EDTA (pH 8.0), 0.5% Nonidet P-40, and protease inhibitors] at 4°C for 3 hours. After three washes in washing buffer, the beads were boiled and subjected to western blotting.

2.10 | RNA interference (RNAi)

The RNAi oligonucleotide sequences were as follows:

nonspecific small interfering RNA (siRNA) sense strand: 5’-UUUCUCCGAAACGUGUCAGCU-3’;
KMT5A siRNA sense strands: #1 5’-ACCCGUGGCUGAAGCAUAUUATT-3’; #2 5’-GCAACUAAGAGACAAAUCUUTT-3’;
RFN8 siRNA sense strands: #1 5’-GGGAAUAUUGGACAACATT-3’; #2 5’-CAGAGACCUUACAGAUGUT-3’;
RFN168 siRNA sense strands: #1 5’-GGCGAAGAGCAGAACATT-3’; #2 5’-GACACUUUCUCCACAGAUATT-3’;

All RNAi oligonucleotides were purchased from Suzhou GenePharma Company. RNAi oligonucleotides were transfected using a Lipofectamine 2000 transfection kit (Invitrogen), according to the manufacturer’s instructions. After transfection, the cells continued to grow in fresh medium for 48 hours before DNA damage treatment.

2.11 | Immunofluorescent analysis

Cells were cultured on slides to ~ 80% confluence. After DNA damage treatment, the cells were washed twice with PBS and fixed with 4% of paraformaldehyde at room temperature for 10 minutes. After washing twice with PBS, the cells were permeabilized with 100% of methanol at −20°C for 15 minutes. The slides were then washed once and incubated with blocking buffer (0.8% bovine serum albumin in PBS) at room temperature for 1 hour, and incubated overnight with the indicated primary antibody (1:100-1:500 dilution) at 4°C. After three washes with blocking buffer, the slides were exposed to secondary antibodies conjugated to Alexa Fluor 488 or 594 or 647 dye. After incubation for 1 hour with secondary antibody and three washes with blocking buffer, the samples were embedded in DAPI. Immunofluorescent images were captured under an Andor confocal microscope.

2.12 | Co-immunoprecipitation (Co-IP) assay

For whole cell lysate Co-IP assay, cells were collected and washed twice with PBS, and then, lysed in NP-40 lysis buffer [20 mM Tris-HCl (pH 8.0), 137 mM NaCl, 10% glycerol, 1% Nonidet P-40, and protease inhibitors] for 30 minutes on ice. The samples were then sonicated 15 times on ice, each for 1 sec at 30% intensity. After sonication, the samples were treated with benzonase (EMD Millipore) at a final concentration of 50 U/mL supplemented with 2 mM MgCl2 to digest the DNA at 4°C for 3 hours. After centrifugation at 13 000 rpm (15 871 g) at 4°C for 30 minutes, the supernatant was collected for immunoprecipitation. For the chromatin fraction Co-IP, samples were collected as described above. For anti-Flag immunoprecipitation,
50 μL precleared anti-Flag M2 beads (Sigma) was added and incubated at 4°C for 3 hours. After washing with lysis buffer and centrifugation at 1000 rpm (94 g) at 4°C for 1 minutes three times, the immunoprecipitated proteins were eluted with Flag peptides and analyzed by western blotting.

2.13 | Chromatin immunoprecipitation (ChIP)

For ChIP assay, DR-GFP U2OS cells were cross-linked with formaldehyde, and then, lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 5 mM EDTA, and 1% sodium dodecyl sulfate] on ice for 30 minutes. After sonication three times on ice, each for 10 seconds at 30% intensity, the supernatant was collected by centrifugation at 12 000 rpm (13 523 g) at 4°C for 10 minutes, and precleared in dilution buffer [20 mM Tris-HCl (pH 8.0), 2 mM EDTA, 150 mM NaCl, and 1% Triton X-100] containing protein G or A sepharose beads (GE Healthcare, Kings Park, NY, USA) and salmon sperm DNA (Sigma-Aldrich, St. Louis, MO, USA) by rotation at 4°C for 2 hours. A total of 5% of the precleared samples was used as the input, and then, each remaining sample was divided into two parts and incubated with IgG or the indicated antibody at 4°C overnight. Protein G or A sepharose was then added to the sample and incubated at 4°C for 3 hours. The beads were washed sequentially in TSE I, TSE II, and Buffer III once and TE twice (detailed below). The samples were then eluted from the beads in elution buffer (1% SDS, 0.1 M NaHCO3) at 37°C for 30 minutes, and heated at 65°C overnight to reverse the cross-links. The DNA was purified using a NucleoSpin Gel and PCR Clean-up kit (Macherey Nagel, Düren, Germany) and real-time PCR was performed on an ABI7500 Real-Time PCR System with the following primers (~2 kb away from the I-SceI cutting site): 5'-GCCCATATATGGAGTTCCGC-3' (sense) and 5'-GCCCATATATGGAGTTCCGC-3' (antisense). For each sample, three PCR replicates were taken and the average Ct was used to calculate the IP/input (2−ΔCt). Three independent assays for each experiment were performed and the means ± SD of IP/input% (100/2ΔCt) from parallel experiments are presented to show the enrichment. TSE I: 0.1% SDS, 1% Triton X-100, 2 mM EDTA (pH 8.0), 20 mM Tris-HCl (pH 8.0), 150 mM NaCl; TSE II: 0.1% SDS, 1% Triton X-100, 2 mM EDTA (pH 8.0), 20 mM Tris-HCl (pH 8.0), 500 mM NaCl; Buffer III: 250 mM LiCl, 1% NP-40, 1% deoxycholate, 1 mM EDTA pH (8.0), 10 mM Tris-HCl (pH 8.0); TE: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).

2.14 | Colony formation assay

Cells were treated with or without 3 Gy X-ray irradiation, and then, plated into 6-well plates in equal number. After 2 weeks, the cells were fixed with 4% of paraformaldehyde and stained with Crystal Violet Staining Solution to identify colonies. Three independent experiments were performed.

2.15 | Statistical analyses

The data are expressed as the means ± SD. Significant differences between means were analyzed by two-tailed, unpaired Student’s t-test, and differences were considered statistically significant at *P < .05, **P < .01, ***P < .001 or ****P < .0001. Microsoft Excel 2016 was used to analyze all data.

3 | RESULTS

3.1 | KMT5A is required for H2A mono-ubiquitination in the DDR

To investigate whether and how KMT5A regulates the ubiquitin signaling pathway in the DDR, we constructed a CRISPR-Cas9 KMT5A knockout (KO) HeLa cell line, which we then subjected to X-ray irradiation before immunostaining with an anti-FK2 (ubiquitination) antibody. We found that FK2 foci formation was significantly impaired in KMT5A KO cells (Figure 1A and B). To rule out the possibility that the impaired FK2 foci formation comes from altered cell cycle profiles as a result of KMT5A deficiency, we introduced a Fluorescence Ubiquitin Cell Cycle Indicator (FUCCI) system into HeLa cells to monitor KMT5A-regulated FK2 foci formation in different cell cycle phases. Here, we saw impaired FK2 foci formation in both G1 and S/G2 phases in KMT5A KO cells, suggesting that KMT5A is indeed involved in the ubiquitin signaling pathway in the DDR (Figures S1A,B).

We subsequently investigated the influence of KMT5A on histone ubiquitination. Here, KMT5A overexpression induced an increase in the ubiquitination of acid-extracted chromatin, in which most of the proteins were histones (Figure S1C). Of note, the mono-ubiquitination of acid-extracted chromatin was markedly increased (Figure 1C). Interestingly, overexpression of Myc-KMT5A R295G, a catalytically dead mutant, also efficiently increased the ubiquitination signals, suggesting that this ubiquitination-related function of KMT5A is methyltransferase activity independent (Figure S1D).

Among all the histone subunits, H2A alone was detected to have an obviously increased mono-ubiquitination after KMT5A overexpression (Figure 1D). We then focused on the effect of KMT5A on the mono-ubiquitination of H2A. Overexpression of either KMT5A WT or KMT5A R295G efficiently increased H2A mono-ubiquitination in HCT116...
cells (Figure 1E). In response to X-ray irradiation, we observed an impaired induction of H2A ubiquitination in KMT5A KO cells, indicating a role of KMT5A in regulating H2A ubiquitination in the DDR (Figure 1F). Because H2A is frequently mono-ubiquitinated at lysine 119, we wondered whether KMT5A can regulate H2AK119ub. As shown in Figure S1E, we did not see changes in H2AK119ub levels after KMT5A overexpression. Apart from H2AK119ub,
H2AK15ub induced by RNF168 also has important roles in DNA damage repair.\textsuperscript{21,29} We found that KMT5A was involved in regulating H2AK15ub, as KMT5A overexpression had an impaired effect on the ubiquitination of the H2AK15R mutant (Figure 1G). All these data suggest that KMT5A is a newly identified regulator of H2AK15ub in the DDR.

3.2 KMT5A interacts with RNF8 and RNF168 in vitro and in vivo

Because RNF8 and RNF168 are two important E3 ligases promoting H2AK15ub in DDR, and KMT5A can regulate H2AK15ub, we were interested in investigating the relationship between KMT5A and RNF8/RNF168. First, by performing a mass spectrometry analysis, we observed that both RNF8 and RNF168 interacted with KMT5A after X-ray irradiation (Figures S2A,B, Table S1). In addition, we successfully detected the interactions between HA-RNF8 and Flag-KMT5A (Figure 2A), and between Myc-RNF168 and Flag-KMT5A (Figure 2B) under normal conditions in HeLa cells. Endogenous co-immunoprecipitation (Co-IP) assays confirmed their physiological interactions in HCT116 cells (Figure 2C-E).

To check whether KMT5A directly interacts with RNF8/ RNF168 or not, we performed GST pull-down assays using GST-tagged KMT5A and His-tagged RNF8 or RNF168. Here, KMT5A directly interacted with RNF8 as well as RNF168 in vitro (Figure 2F). In addition, both RNF8 and RNF168 interacted with the KMT5A N-terminus in vitro (Figure 2G-H). We subsequently incubated different His-RNF8 fragments with GST-KMT5A and found that after deleting either the FHA or the RING domain of RNF8, RNF8 still maintained the ability to interact with KMT5A (Figure 2I). We then generated various His-RNF8 deletion constructs (ΔRING domain, ΔUMI domain, ΔMIU1 domain, and ΔMIU2 domain) and incubated them with GST-KMT5A. None of these deletions impaired the interaction between RNF168 and KMT5A (Figure 2J). All these data suggest that KMT5A interacts with both RNF8 and RNF168 in vitro and in vivo.

3.3 RNF8 ubiquitinates KMT5A in vitro and in vivo

Although KMT5A was originally identified as a histone methyltransferase, KMT5A also has nonhistone substrates.\textsuperscript{42-44} RNF8 and RNF168 also ubiquitinate several other proteins (apart from histones) under various conditions.\textsuperscript{50,51,56-59} We thus investigated whether these proteins can be modified by each other by performing in vitro methylation and in vitro ubiquitination assays. We detected no obvious methylation signals of RNF8 and RNF168 after KMT5A catalyzation (Figures S3A,B), whereas histone H4 (positive control) was successfully methylated by KMT5A (Figure S3C). Interestingly, in the in vitro ubiquitination assay, we found that RNF8 actively catalyzes KMT5A ubiquitination in vitro; we also detected weak ubiquitinated KMT5A signals after RNF168 catalyzation (Figure 3A, S3D).

To assess the effects of RNF8 and RNF168 on KMT5A ubiquitination in vivo, we transfected HCT116 cells with Flag-KMT5A, together with pcDNA or HA-RNF8 or Myc-RNF168 separately. We detected KMT5A ubiquitination levels with an anti-FK2 antibody. We found that only overexpression of RNF8, not RNF168, increased the ubiquitination of KMT5A (Figure 3B). In addition, overexpression of the RNF8 E3 ligase dead mutant (C406S) could no longer increase the ubiquitination of KMT5A (Figure 3C). Consistently, KMT5A ubiquitination levels were much lower in RNF8 KO HCT116 cells, as compared with the wild-type cells (Figure 3D). By contrast, KMT5A ubiquitination levels did not markedly change in the RNF168 KO HCT116 cells (Figure 3E).

Next, we investigated whether the RNF8-induced ubiquitination chain of KMT5A was K48-linked or K63-linked. To do so, we transfected HCT116 cells with HA-Ub WT, K48R, or K63R vectors with Flag-KMT5A, and with or without GFP-RNF8. Here, the cells transfected with HA-Ub K63R showed no obvious increase in HA-tagged KMT5A ubiquitination by GFP-RNF8 overexpression, suggesting that RNF8 mainly induces K63-linked ubiquitination of KMT5A in vivo (Figure 3F). Taken together, KMT5A is a newly identified substrate of RNF8 both in vitro and in vivo.

3.4 RNF8 promotes KMT5A recruitment onto the damaged chromatin

In view of the interactions between KMT5A and RNF8/RNF168, and the ubiquitination of KMT5A by RNF8 in vitro and in vivo, we were interested in investigating whether the recruitment of KMT5A onto the damaged chromatin in DDR is regulated by RNF8, RNF168, or neither. To address this question, we irradiated HCT116 WT, RNF8 KO, or RNF168 KO cells, and then, extracted the chromatin fractions for immunoblotting. We detected impaired KMT5A recruitment onto the damaged chromatin in both the RNF8 KO and the RNF168 KO cells (Figure 4A). We obtained consistent results when we knocked down RNF8 or RNF168 in HeLa cells (Figure S4A). Subsequently, we studied the local H4K20me1 changes in DR-U2OS reporter cells, where transfection of endonuclease I-SceI can specifically induce DSBs at the restricted sites.\textsuperscript{60,61} Consistent with defective KMT5A recruitment onto damaged chromatin upon RNF8 or RNF168 knockdown, the increase in H4K20me1 at I-SceI-induced DSBs was also impaired...
when RNF8 or RNF168 was knocked down individually or together (double knockdown) in the DR-U2OS reporter cells (Figure S4B).

To check the interaction between RNF8 and KMT5A in the DDR, we irradiated HCT116 cells transfected with Flag-KMT5A, and then, performed an anti-Flag Co-IP assay.

**FIGURE 2** KMT5A interacts with RNF8 and RNF168 in vitro and in vivo. A. HeLa cells were transfected with HA-RNF8, with or without Flag-KMT5A, for 48 hours before cell collection for anti-Flag immunoprecipitation. The immunoprecipitated proteins were eluted and detected by western blotting. B. A similar experiment as described in A was performed with the indicated vectors. C. Endogenous Co-IP was performed with an anti-RNF8 antibody to detect the interaction between RNF8 and KMT5A. D. An endogenous Co-IP assay was performed with an anti-RNF168 antibody to detect the interaction between RNF168 and KMT5A. E. An endogenous Co-IP assay was performed with an anti-KMT5A antibody to detect the interaction between KMT5A and RNF8/RNF168. F-H, GST pull-down assays were performed to check the direct interaction between different GST-KMT5A constructs and His-RNF8/RNF168 (F), His-RNF8 (G), and His-RNF168 (H). The GST-tagged proteins were stained with Coomassie brilliant blue (CBB) and indicated with “#.” I, GST pull-down assays were performed to check the interactions between different GST-RNF8 constructs and His-KMT5A: RNF8 ΔFHA, Δaa17-111; RNF8 ΔRING, Δaa402-444. The GST-tagged proteins were stained with CBB and indicated with “#.” J, GST pull-down assays were performed to check the interactions between different GST-RNF168 constructs and His-KMT5A: RNF168 ΔRING, Δaa1-55; RNF168 ΔUmi, Δaa136-166; RNF168 ΔMIU1, Δaa168-191; RNF168 ΔMIU2, Δaa439-462. The GST-tagged proteins were stained with CBB and indicated with “#.”
KMT5A showed an increased interaction with RNF8 in response to X-ray irradiation (Figure 4B). In addition, KMT5A ubiquitination also increased after X-ray irradiation in HeLa cells (Figure 4C). We observed a defective increase of KMT5A ubiquitination in RNF8 KO cells, suggesting that RNF8 is responsible for the increased KMT5A ubiquitination in response to DNA damage (Figure 4D). In addition, overexpression of wild-type but not mutant RNF8 rescued the KMT5A recruitment deficiency in RNF8 KO cells (Figure 4E).

Apart from RNF8, KMT5A also showed an increased interaction with RNF168 after X-ray irradiation (Figure 4F). Interestingly, RNF168 markedly interacts with ubiquitinated KMT5A in the DDR (Figure 4F). By performing a sequential in vitro ubiquitination-GST pull-down assay, we found that RNF168 preferred to bind the RNF8-ubiquitinated KMT5A in vitro (Figure S4C). In addition, RNF8 overexpression not only increased ubiquitination of KMT5A, but also increased the interaction between KMT5A and RNF168 in HCT116 cells (Figure 4G). Taken together, KMT5A recruitment to DNA breaks depends on RNF8-induced ubiquitination, which promotes the interaction between KMT5A and RNF168.

3.5 | KMT5A promotes RNF168-catalyzed H2A ubiquitination in a H2A acidic patch-dependent manner

Because KMT5A was found involved in the ubiquitin signaling pathway in DDR, we investigated whether the functions
of RNF8/RNF168 were impaired in KMT5A-deficient cells. In contrast to the effect of RNF8/ RNF168 deficiency on KMT5A recruitment, we did not see any obvious changes in RNF8 and RNF168 recruitment or foci formation after X-ray irradiation when KMT5A was knocked down in HeLa cells (Figures S5A-C). Interestingly, we found that KMT5A was important for ensuring RNF168 activity toward histones in cancer cells. Specifically, RNF168 overexpression-induced ubiquitination of acid-extracted chromatin was obviously impaired in KMT5A KO HeLa cells (Figure 5A). We obtained similar results when we knocked down KMT5A in different cell lines (Figures S6A,B). In addition, when we co-overexpressed KMT5A and RNF168, we saw a further increase in acid-extracted chromatin ubiquitination (Figure 5B).

FIGURE 4  RNF8 promotes KMT5A recruitment onto damaged chromatin. A, Wild-type and RNF8 KO and RNF168 KO HCT116 cells were subjected to X-ray irradiation at 5 Gy. The cells were collected at the indicated time point and the proteins within the chromatin fraction were extracted for western blotting with the indicated antibodies. B, HCT116 cells were transfected with Flag-KMT5A for 48 hours before X-ray irradiation at 5 Gy. The cells were collected after 5 minutes and Flag-KMT5A proteins were immunoprecipitated from whole cell lysates. The immunoprecipitated proteins were eluted to detect changes in the RNF8-KMT5A interaction by western blotting. C, HeLa cells were subject to 5 Gy X-ray irradiation and collected after 5 minutes. KMT5A proteins were immunoprecipitated from the chromatin fraction and the KMT5A ubiquitination levels were detected by western blotting. D, Wild-type and RNF8 KO HCT116 cells were transfected with Flag-KMT5A for 48 hours, and then, treated or not treated with 5 Gy X-ray irradiation. Cells were collected after 5 minutes and the Flag-KMT5A proteins were immunoprecipitated from the chromatin fraction and eluted for analysis western blotting to detect the ubiquitination levels. E, Wild-type and RNF8 KO HCT116 cells were transfected with Flag-RNF168 WT, or Flag-RNF168 Mut vector as indicated for 48 hours. The cells were treated with or without 5 Gy X-ray irradiation and collected 10 minutes later to extract the chromatin proteins. The protein levels were detected by western blotting. F, HCT116 cells were transfected with Flag-RNF168 WT, or Flag-RNF168 Mut vector as indicated for 48 hours. The cells were treated with or without 5 Gy X-ray irradiation and collected 10 minutes later to extract the chromatin proteins. The protein levels were detected by western blotting. G, HCT116 cells were transfected with the indicated vectors for 48 hours. Whole cell lysates were extracted for anti-Flag immunoprecipitation to check changes in the interaction between KMT5A and RNF168.
RNF168-induced H2B ubiquitination and RNF8 activity toward H2A and H2B were not obviously affected by KMT5A (Figure 5C). Contrary to the influence of KMT5A on RNF168-catalyzed H2A ubiquitination, we did not find such regulation when KDM4A was used as the substrate (Figure S6C). In addition, KMT5A overexpression showed no marked effect on KDM4A ubiquitination levels in HCT116 cells (Figure S6D), suggesting that KMT5A might selectively regulate the activity of RNF168 toward H2A in cells.

A previous structure-based study suggested that KMT5A R188 and R189 residues interact with the acidic patch of
KMT5A promotes RNF168-catalyzed H2A ubiquitination in vitro and in vivo. A, Wild-type and KMT5A KO HeLa cells were transfected with HA-Ub, together with pcDNA or Myc-RNF168 for 48 hours. The whole cell lysates (WCL) and acid-extracted chromatin were analyzed by western blotting with the indicated antibodies. B, HeLa cells were transfected with HA-Ub, Myc-RNF168, and Flag-KMT5A as indicated for 48 hours. The whole cell lysates (WCL) and acid-extracted chromatin were analyzed by western blotting with the indicated antibodies. C, In vitro ubiquitination assays were performed to check the activity of RNF8 or RNF168 on mononucleosomes with or without KMT5A. D, HCT116 cells were transfected with Flag-H2A and subjected to 5 Gy X-ray irradiation. The cells were collected 15 minutes later and Flag-H2A proteins were immunoprecipitated from the chromatin fraction. The levels of the KMT5A interaction were detected by western blotting. E, HCT116 cells were transfected with pcDNA, Flag-KMT5A WT, or Flag-KMT5A 2RA vectors for 48 hours. The Flag-KMT5A proteins were immunoprecipitated from the chromatin fraction to detect their interaction with H2A. F, HCT116 cells were transfected with the indicated vectors to see the influence of KMT5A WT or 2RA overexpression on H2A ubiquitination. G, KMT5A KO HeLa cells were transfected with Flag-H2A, GFP-RNF168, Myc-KMT5A WT, and 2RA vectors as indicated for 48 hours. The Flag-H2A proteins were immunoprecipitated from the chromatin fraction, and the RNF168-induced changes in H2A ubiquitination were detected by western blotting. H, In vitro ubiquitination assays were performed to check the influence of the interaction between KMT5A R188/R189 and the H2A acidic patch, and the effect of RNF8-catalyzed KMT5A ubiquitination on RNF168-induced H2A ubiquitination.

H2A.62 By performing a Co-IP assay, we detected a notable increase in the interaction between H2A and KMT5A after X-ray irradiation (Figure 5D). This H2A acidic patch is considered important for RNF168-induced H2A ubiquitination.63,64 We therefore constructed a KMT5A R188A/R189A (2RA) mutant to investigate the effects of the KMT5A-H2A interaction on KMT5A-regulated histone ubiquitination. As expected, KMT5A 2RA showed an impaired interaction with H2A in HCT116 (Figure 5E) and HeLa cells (Figure S6E). In addition, the interaction between KMT5A and RNF8/RNF168 was not disrupted by the 2RA mutation as detected in vitro (Figures S6F,G) and in HCT116 cells in vivo (Figures S6H-6I). We subsequently transfected HCT116 cells with HA-Ub, together with pcDNA or Flag-KMT5A WT or Flag-KMT5A 2RA separately to monitor changes in the ubiquitination of acid-extracted chromatin. Differing from the result of overexpressing wild-type KMT5A, overexpression of KMT5A 2RA failed to increase the ubiquitination (Figure S6J). In addition, KMT5A 2RA overexpression did not increase H2A ubiquitination levels in HCT116 cells (Figure 5F).

We also overexpressed Myc-RNF168 with Flag-KMT5A WT or Flag-KMT5A 2RA separately to monitor ubiquitination levels of acid-extracted chromatin in KMT5A KO HeLa cells. Consistently, only overexpression of wild-type KMT5A enhanced the efficiency of RNF168 in inducing acid-extracted chromatin ubiquitination in KMT5A KO cells (Figure S6K). In addition, overexpression of wild-type KMT5A, but not the 2RA mutant, improved RNF168-induced H2A ubiquitination in KMT5A KO cells (Figure 5G). By performing in vitro ubiquitination assays, we found that the role of KMT5A in promoting RNF168-catalyzed H2A ubiquitination was impaired by the 2RA mutation (Figure 5H). In addition, the RNF8-ubiquitinated KMT5A was more efficient than unmodified KMT5A in promoting RNF168-induced H2A ubiquitination (Figure 5H). Taken together, these data suggest that KMT5A can promote RNF168-mediated H2A ubiquitination, which is dependent on the interaction between KMT5A R188/R189 and the H2A acidic patch and can be further improved by RNF8-induced KMT5A ubiquitination.

3.6 | KMT5A promotes cell survival with RNF8 and RNF168 in response to DNA damage

Because KMT5A is an important factor for DNA damage repair and cell survival, we considered that the role of KMT5A on cell survival may be regulated by RNF8 and RNF168. First, we saw that KMT5A overexpression improved the cell survival rate after X-ray irradiation (Figure 6A,B, S7A). However, this effect was impaired when RNF8 and RNF168 were knocked down individually or together (double knockdown) in HCT116 cells. We also checked whether the effects of RNF8 and RNF168 on cell survival were influenced by KMT5A. In HCT116 WT cells, RNF8 and/or RNF168 overexpression lead to a higher survival rate after X-ray irradiation (Figure 6C,D, S7B); however, this effect was largely impaired in KMT5A KO cells. This result suggested that the RNF8/RNF168-promoted cell survival was also dependent on KMT5A in response to DNA damage.

To further clarify the effect of the interaction between KMT5A R188/R189 and the H2A acidic patch in DNA damage repair and cell survival, we first performed immunofluorescent assays to detect FK2 foci formation with KMT5A KO HeLa cells transfected with pcDNA, KMT5A WT, KMT5A R295G, or KMT5A 2RA vectors separately. As shown in Figures S7C,D, unlike KMT5A WT and the KMT5A R295G mutant, the KMT5A 2RA mutant failed to rescue the deficiency of FK2 foci formation in KMT5A KO cells. To determine the role of the interaction between KMT5A R188/R189 and H2A acidic patch
**FIGURE 6** KMT5A promotes cell survival with RNF8 and RNF168 in response to DNA damage. A-B, HCT116 cells were transfected with RNF8 siRNA#1 or RNF168 siRNA#1 separately or together. After 12 hours, the cells were transfected with pcDNA or Flag-KMT5A as indicated and cultured for a further 36 hours. After 3 Gy X-ray irradiation, the cells were counted and seeded for colony formation assays. For IR non-treated cells, 500 cells were seeded in each plate. For IR-treated cells, 10,000 cells were seeded. Consistent results were obtained from three independent assays. The representative images are shown in A, and the statistical analysis is shown in B. The data represent the means ± SD. Student’s t-test: ***, P < .001; n.s., not significant. C-D, RNF8 and RNF168 were overexpressed in wild-type and KMT5A KO HeLa cells individually or simultaneously as indicated, for 48 hours. The cells were exposed to 3 Gy X-ray irradiation and seeded for colony formation as described in A. Representative images are shown in C, and the statistical analysis from three independent experiments is shown in D. The data represent the means ± SD. Student’s t-test: *, P < .05; **, P < .01; ***, P < .001; n.s., not significant. E-F, Wild-type and KMT5A KO HeLa cells were transfected with pcDNA, KMT5A WT, or KMT5A 2RA vectors for 48 hours before X-ray irradiation. Colony formation assays were performed as described in A. Representative images are shown in E, and the statistical analysis from three independent experiments is shown in F. The data represent the means ± SD. Student’s t-test: **, P < .01; ***, P < .001; n.s., not significant. G, A model illustrating how KMT5A and RNF8/RNF168 cooperate during the DNA damage response.
in cell survival after DNA damage, we performed colony formation assays with wild-type and KMT5A KO HeLa cells, separately transfected with pcDNA, KMT5A WT, or KMT5A 2RA vectors. In wild-type cells, KMT5A WT rather than KMT5A 2RA mutant overexpression promoted cell survival after X-ray irradiation (Figure 6E,F, S7E). In addition, in KMT5A KO cells, only the overexpression of wild-type KMT5A rescued the KMT5A deficiency-induced phenotype (Figure 6E,F, S7E). Taken together, KMT5A promotes cell survival with RNF8 and RNF168 in response to DNA damage.

4  |  DISCUSSION

In this study, we describe a new role for KMT5A in regulating the ubiquitin signaling pathway. We have revealed important interplay between KMT5A and RNF8/RNF168 in DNA damage repair, suggesting a coordinated response of histone modifiers to establish the chromatin context surrounding DSBs. We would like to highlight two points in this regulation. First, KMT5A is a newly identified substrate of RNF8 that is required for KMT5A recruitment onto damaged chromatin to promote DNA damage repair. Second, RNF168 activity toward H2A is promoted by the recruited KMT5A at DSBs, indicating a new role for KMT5A in regulating the ubiquitin signaling pathway for DNA damage repair. These findings show that certain histone modifiers function cooperatively in the DDR and provide new insights into how cells respond to DNA damage (Figure 6G).

A previous study suggested that KMT5A methyltransferase activity is important for 53BP1 recruitment in the DDR. Because KMT5A-catalyzed H4K20me1 at DSBs is the substrate of other histone methyltransferases like SUV4-20H1/H2 to induce H4K20me2, KMT5A deficiency can undoubtedly decrease H4K20me2 that further leads to impaired 53BP1 binding to DSBs. However, the real effects of KMT5A recruitment to DSBs might have been underestimated. In this study, we found that KMT5A is a histone ubiquitination regulator that functions with RNF168 to promote H2A ubiquitination in the DDR. This finding suggests that KMT5A plays at least two roles in DNA damage repair. First, as a histone methyltransferase, the recruited KMT5A can naturally induce the monomethylation of the unmodified H4K20 at specific DSB sites, which can be further methylated to H4K20me2 by the relevant methyltransferase for 53BP1 binding. Second, KMT5A is a ubiquitination regulator that can promote RNF168-induced H2AK15ub in response to DNA damage. Of note, although we found that RNF168 is a KMT5A-regulated E3 ligase, it is possible that KMT5A might regulate other E3 ligases and other ubiquitinated proteins apart from RNF168 and H2A. We noticed that KMT5A deficiency did not completely abolish the FK2 foci formation in the DDR. KMT5A may thus be dispensable for some other E3 ligases’ functions in the DDR. In addition, although KMT5A promotes RNF168’s activity toward H2A in the DDR, it is unlikely that KMT5A is essential for all RNF168-induced FK2 foci after irradiation. According to the role of KMT5A in the DDR, we consider that KMT5A is important for genome integrity and cell fitness in a normal physiological state, whereas the high expression of KMT5A in certain cancers may cause chemoresistance and promote malignancy. Some studies already showed that KMT5A promotes EMT and metastasis in breast and prostate cancer cells.

KMT5A is not the sole histone methyltransferase involved in H2A ubiquitination in the DDR. Histone methyltransferase EZH2 (KMT6A), a subunit of the PRC2 complex, is related to H2AK119ub in both gene expression regulation and DNA damage repair. KMT6A-induced H3K27 methylation may recruit the PRC1 complex to certain gene promoters, then, the PRC1 subunits RNF2 and BMI1 can induce H2AK119ub to repress gene expression. In response to DNA damage, members of the PRC2 and PRC1 complexes are recruited to DNA lesions and subsequently induce H2AK119ub, which is important for local transcription repression and HR repair. In this study, we did not detect obvious H2K119ub changes after KMT5A overexpression; we thus consider that KMT5A and KMT6A have different H2A ubiquitination targets in the DDR, and the histone methyltransferases-regulated ubiquitin signaling pathway is far more complicated than we originally thought. Consequently, we consider that this pathway warrants further investigation in the future.

RNF8 was first found as an important E3 ligase that ubiquitinates histones at DSBs to promote the assembly of DNA repair proteins. Subsequently, several other DNA repair factors have been reported to be the substrates of RNF8 in the DDR. For example, RNF8-induced KDM4A and L3MBTL1 poly-ubiquitination results in the dissociation of these proteins from H4K20 methylation and exposure of H4K20 methylation for 53BP1 binding. In addition, RNF8 removes Ku80 from DSBs at the appropriate stage of the DNA damage repair process by direct ubiquitination. In this study, we identified RNF8 as an E3 ligase ubiquitinating KMT5A both in vitro and in response to DNA damage repair in vivo, suggesting a broader ubiquitination behavior of RNF8 in DNA damage repair. In addition, we consider that there might be other E3 ligases or chromatin modifiers in addition to RNF8 that can regulate KMT5A function on RNF168-mediated H2A ubiquitination in the DDR. Based on our results, RNF8 is also responsible for KMT5A ubiquitination under normal conditions in addition to conditions of DNA damage. A previous study reported that H4K20me1 accumulation from the early pachytene stage to the mid-diplotene stage in meiotic prophase during spermatogenesis was dependent on RNF8.
It is considered that RNF8-regulated KMT5A ubiquitination can thus participate in other cellular processes, such as gene regulation and embryo development, which is an intriguing area for future research.

While KMT5A recruitment is regulated by RNF168, the contrary may not be true: we saw that RNF168 recruitment onto damaged chromatin was not markedly affected in KMT5A-deficient cells. Previous studies suggested that RNF168 recruitment mainly depended on RNF8-ubiquitinated X factors, such as H1 and L3MBTL2. Although KMT5A is also a substrate of RNF8 in the DDR, KMT5A ubiquitination is considered dispensable for RNF168 binding at DSBs. In contrast, because RNF168 has a higher affinity to bind RNF8-ubiquitinated KMT5A, this interaction is likely to be beneficial for the binding of KMT5A to chromatin at DSBs. In a recent report, Dulev et al also observed an interaction between KMT5A and RNF168 under normal conditions and after DNA damage treatment. However, they did not detect the increased interaction between KMT5A and RNF168 in the DDR, which might be due to differences in the co-overexpression procedure or the different detection time point monitored after DNA damage. In our study, we checked the interaction between Flag-RNF168 and the endogenous KMT5A during the early stages of the DDR, which we consider to be a more sensitive approach to detect their interaction changes.

A previous study showed that the nucleosome acidic patch plays a critical role in the RNF168-dependent ubiquitination of histone H2A. However, the acidic patch is not involved in the interaction between the RNF168 ubiquitination machinery and the histone target in vitro. Interestingly, results from a structural-based study showed that the acidic patch of H2A has an important role in ensuring KMT5A access to nucleosome. Because KMT5A has both increased interactions with H2A and RNF168 after DNA damage, it is possible that KMT5A can help RNF168 quickly target H2A at DSBs for ubiquitination in the DDR. Alternatively, a conformational change in the local chromatin context with KMT5A might orientate RNF168 to H2A to catalyze ubiquitination. In addition, KMT5A may enhance the ubiquitin transfer by forcing a different orientation of ubiquitin with respect to the E3/E2 machinery or by altering the interaction forces between RNF168 and H2A. The importance of the interaction between KMT5A R188/R189 and the H2A acidic patch is highlighted by the finding that the KMT5A 2RA mutant loses the ability to promote RNF168-mediated H2A ubiquitination and cannot improve cell survival after DNA damage. We thus consider that the KMT5A R188/R189-H2A acidic patch interaction may be a valuable drug target in the combined chemotherapy-radiotherapy to treat cancer.

In summary, our findings provide important insights into the interplay between histone modifiers in DNA damage repair. We have delineated a role for KMT5A in controlling histone ubiquitination in the DDR, which opens new avenues for developing new cancer treatments in the future.

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CONFLICT OF INTEREST
The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS
X. Lu and W-G. Zhu designed research; X. Lu performed experiments with help from M. Xu, Q. Zhu, and J. Zhang; G. Liu and Y. Bao analyzed the data; L. Gu, Y. Tian, and H. Wen provided professional suggestions. X. Lu wrote the paper. All authors approved the manuscript before submission.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section.

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