Histone Ubiquitination Associates with BRCA1-Dependent DNA Damage Response[▽][†]

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Histone ubiquitination participates in multiple cellular processes, including the DNA damage response. However, the molecular mechanisms involved are not clear. Here, we have identified that RAP80/UIMC1 (ubiquitin interaction motif containing 1), a functional partner of BRCA1, recognizes ubiquitinated histones H2A and H2B. The interaction between RAP80 and ubiquitinated histones H2A and H2B is increased following DNA damage. Since RAP80 facilitates BRCA1's translocation to DNA damage sites, our results indicate that ubiquitinated histones H2A and H2B could be upstream partners of the BRCA1/RAP80 complex in the DNA damage response. Moreover, we have found that RNF8 (ring finger protein 8), an E3 ubiquitin ligase, regulates ubiquitination of both histones H2A and H2B. In RNF8-deficient mouse embryo fibroblasts, ubiquitination of both histones H2A and H2B is dramatically reduced, which abolishes the DNA damage-induced BRCA1 and RAP80 accumulation at damage lesions on the chromatin. Taken together, our results suggest that ubiquitinated histones H2A and H2B may recruit the BRCA1 complex to DNA damage lesions on the chromatin.

Cells encounter enormous DNA damage that is induced by both external and internal hazards. Among various types of DNA damage, DNA double-stand breaks are the most deleterious type of damage, which may substantially alter genetic information. The proper cellular response to DNA double-stand breaks, including activation of DNA damage checkpoint pathways and DNA repair systems, allows cells to repair damage lesions and to avoid genetic instability (16, 45, 46, 69). Following DNA double-stand breaks, a group of DNA damage response factors are accumulated at the DNA damage sites, which is essential to activate DNA damage checkpoints and repair damage lesions (53). One of these important DNA damage response proteins is BRCA1.

BRCA1 (breast cancer susceptibility gene 1) is an 1,873-amino-acid nuclear polypeptide that contains an N-terminal ring domain and a C-terminal BRCT domain. Accumulated evidence suggests that BRCA1 participates in the DNA damage response, including both DNA damage checkpoint activation and DNA damage repair (39, 50, 56). Following DNA double-strand breaks, BRCA1 is phosphorylated by upstream ATM and ATR kinases (8, 12, 13, 55) and controls downstream Chk1 kinase activity (65), which regulates the damage-induced intra-S-phase checkpoint and the G_2/M checkpoint (29, 63, 65). BRCA1 also associates with Rad51 (49) and mediates homologous recombination (37), which is an important

mechanism for DNA double-strand break repair in S and G_2 phases.

The prerequisite for BRCA1 to participate in these DNA damage responses is that BRCA1 recognizes DNA damage sites. Following DNA double-strand breaks, BRCA1 translocates to DNA damage sites and forms nuclear foci, which is also the most direct and obvious evidence of BRCA1 functioning in the DNA damage response (41, 48). However, the mechanism underlying this cellular phenomenon is not clear. The C-terminal BRCT domain of BRCA1, a phosphoprotein binding domain (34, 44, 66), is required for BRCA1's translocation and accumulation at the DNA damage sites (34). Recently, we and others identified two BRCT domain binding partners, CCDC98 (also known as Abraxas) and RAP80 (also known as UIMC1) (1, 25, 26, 32, 51, 59, 64). BRCA1, CCDC98, and RAP80 form a complex. Both CCDC98 and RAP80 are required for DNA damage-induced BRCA1 focus formation (1, 25, 26, 32, 51, 59, 64). Between these two BRCA1-associated proteins, the BRCA1 BRCT domain directly recognizes phosphorylated Ser406 of CCDC98 (26, 32, 59). While CCDC98 is a mediator between BRCA1 and RAP80, RAP80 indirectly binds to BRCA1 through its interaction with CCDC98 (26, 32, 59). In the absence of RAP80, neither BRCA1 nor CCDC98 could translocate to and accumulate at DNA damage sites, demonstrating that RAP80 is required for targeting this BRCA1 complex to DNA damage sites (26, 32).

RAP80 is a 719-amino-acid nuclear protein with an N-terminal UIM (ubiquitin-interacting motif) domain and a C-terminal zinc finger domain. Structural and functional studies indicate that the N-terminal UIM domain of RAP80 is important for the DNA damage-induced focus formation of RAP80 (1, 25, 51, 59, 64). This UIM domain contains tandem UIMs that potentially recognize ubiquitin or ubiquitinated proteins

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(1, 25, 51, 59, 64). Thus, we hypothesize that DNA damage-induced ubiquitination signals recruit the BRCA1 complex to DNA damage sites through the RAP80 UIM domain. Here, we have identified that the partners of the RAP80 UIM domain are ubiquitinated histones H2A and H2B. Histone H2A and H2B ubiquitination could be the molecular basis to load the BRCA1 complex to DNA damage lesions on the chromatin.

MATERIALS AND METHODS

Plasmids, antibodies, and other materials. S-Flag-biotin (SFB)-tagged full-length RAP80, a UIM domain deletion mutant of RAP80 (ΔUIM), a zinc finger domain deletion mutant of RAP80 (ΔZnF), and SFB-tagged full-length HSJ1A were described previously (25). The RAP80 UIM domain (amino acids [aa] 1 to 200), RAP80 ΔUIM (aa 73 to 126 deleted), RAP80 ΔUIM1 (aa 78 to 96 deleted), and RAP80 ΔUIM2 (aa 103 to 120 deleted) were cloned into the pGEX-4T-1 vector (Amersham) to generate glutathione S-transferase (GST) fusion proteins. H2A and H2B cDNAs were subcloned into a modified pCDNA3 vector to generate constructs encoding hemagglutinin (HA)-tagged H2A and H2B. The point mutants of H2A and H2B were generated by using the QuikChange site-directed mutagenesis kit (Stratagene). Rabbit anti-mouse RNF8, RAP80, and BRCA1 polyclonal antibodies were raised against GST-RNF8 (aa 1 to 324), GST-RAP80 (aa 1 to 354), and GST-BRCA1 (aa 1445 to 1812) fusion proteins, respectively. Rabbit anti-human RAP80, BRCA1, and phospho-H2AX antibodies were previously described (25). Antibodies to H2A, the ubiquitinated form of H2A (ub-H2A), H2B, and H4 antibodies were purchased from Upstate. Anti-HA and anti-β-actin antibodies were purchased from Covance and Sigma. Anti-RING1B and anti-RNF20 antibodies were purchased from MBL and Bethyl, respectively.

The small interfering RNA (siRNA) duplexes were purchased from Dharmacon Research (Lafayette, CO). The siRNA sequences targeting Ring1B and RNF20 were 5′-AAC UCA GUU UAU AUG AGU UAC-3′ and 5′-AAG AAG GCA GCU GUU GAA GAU-3′, respectively. siRNAs were transfected into the cells using Oligofectamine (Invitrogen) according to the manufacturer's instructions.

Cell culture and treatment with ionizing radiation. 293T and HeLa cells were cultured in RPMI 1640 medium with 10% fetal bovine serum. RNF8-deficient mouse embryo fibroblasts (MEFs) were cultured in Dulbecco modified Eagle medium with 10% fetal bovine serum. For ionizing radiation (IR) treatment, cells were irradiated at the indicated doses by using a JL Shepherd ^{137}Cs radiation source. Cells were then maintained in the culture conditions for the time points specified in the figure legends. H2AX$^{-/-}$ MEFs were a gift from Andre Nussenzweig.

Cell lysis, immunoprecipitation, GST pull-down assay, and Western blotting. Cells were lysed with NETN buffer (0.5% NP-40, 50 mM Tris-HCl [pH 8.0], 2 mM EDTA, and 100 mM NaCl). For immunoprecipitation and GST pull-down assay, insoluble lysates were collected and sonicated, followed by micrococcal nuclease (Sigma) treatment at room temperature for 10 min. Chromatin-associated proteins were eluted for further analyses. For acid extraction of histones, insoluble pellets were resuspended in 0.2 M HCl; the acid was neutralized with 1 M Tris-HCl (pH 8.5) for further Western blot analysis. Immunoprecipitation, GST pull-down assay, and Western blotting were performed following standard protocols as described previously (25).

Immunofluorescence staining. Cells grown on coverslips were fixed in 3% paraformaldehyde for 20 min and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS) for 5 min at room temperature. Samples were blocked with 5% goat serum and then incubated with primary antibody for 60 min. Samples were washed three times and incubated with secondary antibody for 30 min. The coverslips were mounted onto glass slides and visualized with a fluorescence microscope. For ubiquitinated H2A staining, cells were treated 0.5% Triton X-100 for 5 min before fixation. To visualize IR-induced foci, cells were cultured on coverslips and treated with 10 Gy IR (1 Gy = 100 rads), followed by recovery for 4 h.

Construction of the lysine-less H2AX mutant. The lysine-less H2AX mutant (NOK) was constructed by overlap PCR to change all lysine resides to arginine residues using primer pairs KO1F (5′ ATG TCG GGC CGC GGC AGG ACT GGC GGC AGG GCC CGC GCC AGG GCC AGG TCG CGC TCG TCG CGC GCC GGC CTC CAG TTC CCA GTG GGC CGT GTA CAC CGG 3′) and KO1B (5′ AGC GGT GAG GTA CTC CAG CAC TGC CGC CAG GTA CAC TGG CGC GCC GGC GCC AAC GCG CTC GGC GTA GTG GCC CCT CCG CAG CAG CCG GTG TAC ACG G 3′), KO2F (5′ AGT ACC TCA CCG

CTG AGA TCC TGG AGC TGG CGG GCA ATG CGG CCC GCG ACA ACA GGA GGA CGC GAA TCA TCC CCC GCC ACC TGC AGC TGG 3′) and KO2B (5′ ATG TTG GGC AGG ACG CCT CCC TGG GCG ATC GTC ACG CCG CCC AGC AGC CTG TTG AGC TCC TCG TCG TTG CGG ATG GCC AGC TGC AGT GG 3′), and KO3F (5′ CGT CCT GCC CAA CAT CCA GGC CGT GCT GCT GCC GCC CAG GAG GAC CAG CGC CAC CGT GGG GCC GAG GGC GCC CTC G 3′) and KO3B (5′ TTA GTA CTC CTG GGA GGC CTG GGT GGC CCT CCT GCC GCC CGA GGG CGC CCT CGG 3′). Thereafter, wild-type H2AX, S139A mutant, or NOK mutant cDNA was PCR amplified using primers with flanking restriction sites and were subcloned into the pBabe-puro retroviral vector and packaged in BOSC23 cells for infecting H2AX-deficient cells.

Affinity purification of SFB-tagged RAP80 and mass spectrometry. SFB-tagged RAP80 was stably expressed in K562 cells. Two-liter cultures of cells were harvested and treated with or without 20 Gy IR. At 4 h after IR treatment, cells were lysed with 40 ml NETN buffer on ice for 10 min. Cell lysates were centrifuged at 12,000 × g at 4°C for 20 min. The soluble fraction was collected. The insoluble fraction was washed three times with PBS and then treated with 50 units of micrococcal nuclease on ice for 1 h and centrifuged at 12,000 × g at 4°C for 20 min. The supernatant was the chromatin fraction combined with the NETN soluble faction. The cell lysates were incubated with 500 μl streptavidin-conjugated beads (Amersham) at 4°C for 2 h. The beads were washed three times with NETN buffer, and then bead-bound proteins were eluted with 1 ml PBS containing 2 mM biotin (Sigma). The eluted supernatant was incubated with 50 μl S beads (Novagen) at 4°C for 2 hours. The beads were washed three times with NETN buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The gels were digested, and the peptides were analyzed by liquid chromatography-tandem mass spectrometry. (The common nonspecific associated proteins, including acetyl coenzyme A carboxylase 1, pyruvate carboxylase, methylcrotonoyl coenzyme A carboxylase subunit alpha, and heat shock protein 70, are not listed in the mass spectrometry results shown in Table 1, but these proteins were present in each purification.)

RESULTS

Histones H2A and H2B are ubiquitinated following DNA damage. To examine the molecular mechanism by which BRCA1 is recruited to DNA damage sites, we focused our studies on RAP80, since the RAP80 UIM domain plays a key role in BRCA1 complex focus formation following DNA damage. Previously, we have found that RAP80 associated with chromatin following DNA damage (25), suggesting that there are RAP80 binding partners on the chromatin. By using protein affinity purification, we analyzed RAP80-associated proteins. Besides three known RAP80 partners, CCDC98, BRCC45, and BRCC36, we identified redundant peptides of histones H2A and H2B from the same purification (Table 1), suggesting that RAP80 may interact with histones. In fact, histones H2A and H2B are the most abundant ubiquitinated proteins on the chromatin; 10 to 15% of histone H2A and 1 to 5% of H2B are ubiquitinated under normal conditions. Thus, we hypothesize that the RAP80 UIM domain recognizes ubiquitinated histones H2A and H2B at the DNA damage sites, which loads the BRCA1 complex to DNA damage lesions on the chromatin. To examine our hypothesis, we first determined whether histones H2A and H2B are ubiquitinated at DNA damage sites. HeLa cells were treated with 10 Gy of IR. By using immunofluorescence staining with an antibody against ubiquitinated H2A, we found that the ubiquitinated H2A formed DNA damage-induced foci, which colocalized with phospho-H2AX (γH2AX) foci, a marker of DNA damage sites (Fig. 1A). This suggests that H2A is highly ubiquitinated at the DNA damage sites, which also has been recently reported by other groups (5, 33, 40). H2AX, a variant of H2A that controls DNA damage-induced focus formation by various

TABLE 1. Mass spectrometry analysis of RAP80-interacting proteins

IR (Gy)	Protein	Peptide sequence[a]	Redundancy
0	CCDC98	K.INEM*YASLQEELK.S	3
		K.INEMYASLQEELK.S	3
		R.LEHSLYKPQK.G	3
		R.VPLVVANLGM*SEQLGYK.T	3
		R.VPLVVANLGMSEQLGYK.T	3
	BRCC45	K.LPVDFSNIPTYLLK.D	7
		R.DQPTLTFQSVYHFTNSGQLYSQAQK.N	4
		R.ISPM*LSPFISSVVR.N	6
		R.ISPMLSPFISSVVR.N	6
	BRCC36	K.DRVEISPEQLSAASTEAER.L	2
		K.IHNGSVFTK.N	1
		R.IHSLTHLDSVTK.I	3
		R.LAELTGRPMR.V	2
		R.VEISPEQLSAASTEAER.L	2
	H2A		
	H2B	K.AMGIMNSFVNDIFER.I	15
20	CCDC98	K.FFEEDGSLK.E	3
		K.INEM*YASLQEELK.S	3
		K.INEMYASLQEELK.S	3
		R.LEHSLYKPQK.G	3
		R.VPLVVANLGM*SEQLGYK.T	3
		R.VPLVVANLGMSEQLGYK.T	3
	BRCC45	K.DVNEDPGEDVALLSVSFEDTEATQVYPK.L	7
		K.LPVDFSNIPTYLLK.D	7
		K.NNWTGEFSAR.F	7
		K.VQYVIQGYHK.R	5
		R.DQPTLTFQSVYHFTNSGQLYSQAQK.N	4
		R.ISPM*LSPFISSVVR.N	6
		R.ISPMLSPFISSVVR.N	6
	BRCC36	K.DRVEISPEQLSAASTEAER.L	2
		K.FAYTGTEM*R.T	2
		K.IHNGSVFTK.N	1
		R.IEIPIHIVPHVTIGK.V	3
		R.IHSLTHLDSVTK.I	3
		R.VEISPEQLSAASTEAER.L	2
	H2A	R.AGLQFPVGR.I	33
	H2B	K.AMGIMNSFVNDIFER.I	31
		K.LLLPGELAK.H	37

[a] *, Met was oxygenated.

of other proteins, was also recently shown to be ubiquitinated following DNA damage (19, 22, 68). To exclude the possibility that the observed ubiquitinated H2A foci could be solely ubiquitinated H2AX foci, due to a potential risk that ubiquitinated H2A antibody might also recognize ubiquitinated H2AX, we utilized an H2AX mutant (NOK) with all lysine residues mutated to arginine, which could not be ubiquitinated before and after DNA damage (see Fig. S1 in the supplemental material). H2AX$^{-/-}$ cells were reconstituted with either wild-type H2AX or the NOK mutant. DNA damage-induced ubiquitinated H2A foci could still be observed in cells expressing the nonubiquitinatable NOK mutant, suggesting that ubiquitinated H2A contributes to at least part, if not all, of the foci detected by ubiquitinated H2A antibody (Fig. 1B). Moreover, these ubiquitinated H2A foci were controlled by γH2AX, since reintroducing the S139A mutant of H2AX into H2AX$^{-/-}$ cells that had H2AX phosphorylation abolished following DNA damage also disrupted damage-induced ubiquitinated H2A foci (Fig. 1B).

Due to antibody limitation, we could not directly assess ubiquitinated H2B foci. Instead, we examined H2B ubiquitination following DNA damage by Western blotting. Both 293T and HeLa cells were treated with 0, 10, or 20 Gy of IR. Global H2B ubiquitination increased with the increase of IR dose (Fig. 1C), suggesting that DNA damage induces H2B ubiquitination. However, we did not observe any significant increase in H2A ubiquitination following DNA damage in the Western
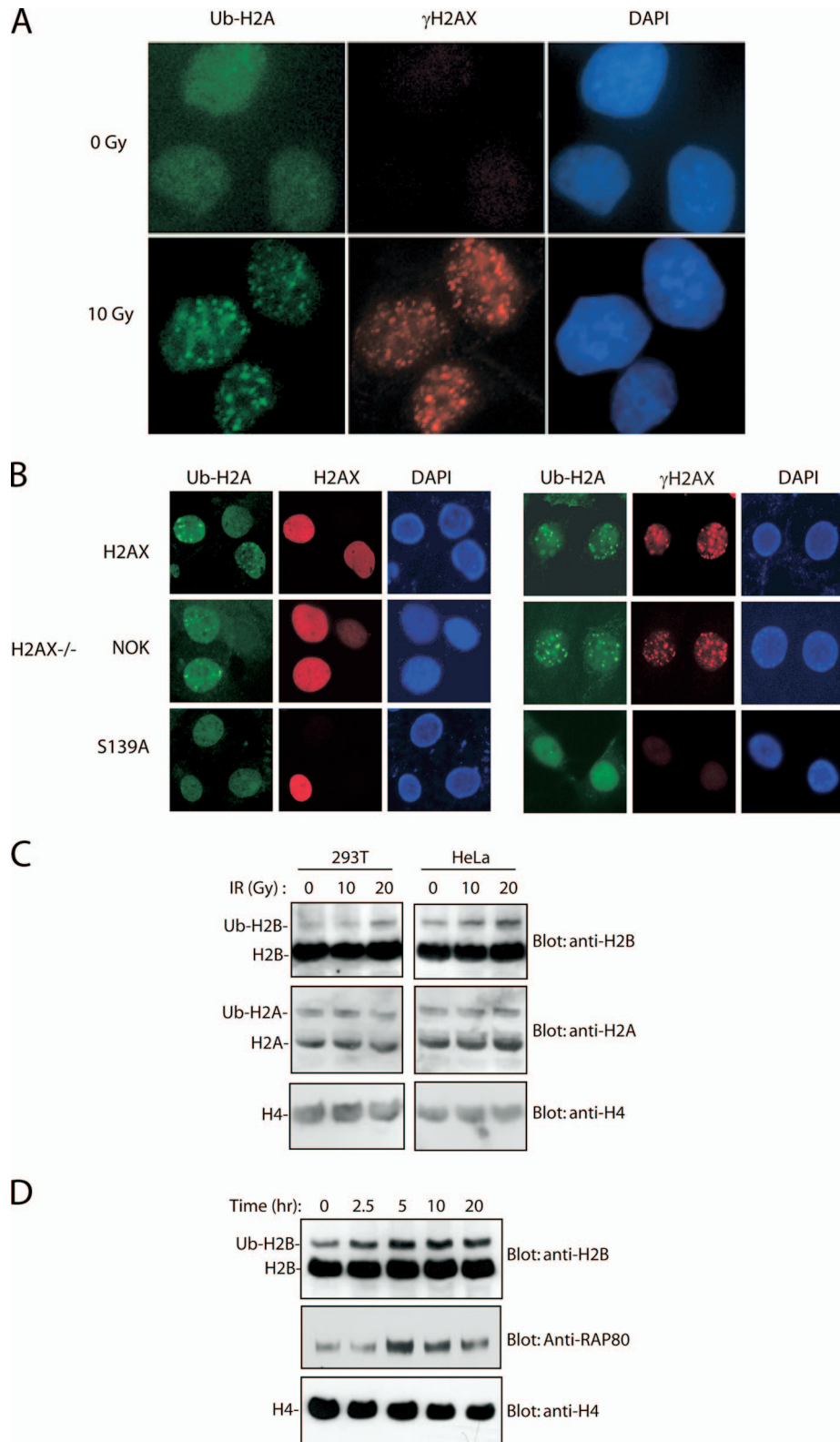
FIG. 1. Ubiquitinated H2A and H2B participate in the DNA damage response. (A) Ubiquitinated H2A forms DNA damage-induced foci and colocalizes with γH2AX. HeLa cells were exposed to 0 or 10 Gy of IR. Four hours after IR, cells were fixed and immunostained with anti-γH2AX polyclonal antibody and anti-ubiquitinated H2A (Ub-H2A) monoclonal antibody. DAPI, 4′,6′-diamidino-2-phenylindole. (B) γH2AX is required for ubiquitinated H2A focus formation after DNA damage. H2AX$^{-/-}$ cells were reconstituted with wild-type or S139A or NOK mutant H2AX and irradiated with 10 Gy of IR. Cells were fixed and immunostained with the indicated antibodies at 4 hours after IR treatment. (C) Histone H2B ubiquitination was induced following IR. 293T or HeLa cells were exposed to 0, 10, or 20 Gy of IR. Four hours after IR, chromatin fractions were analyzed by Western blotting with anti-H2B (upper panel) and anti-H2A (middle panel) antibodies. A blot with anti-histone H4 was used as protein loading control (lower panel). (D) Time course of H2B ubiquitination and RAP80 association with chromatin following IR. 293T cells were exposed to 20 Gy of IR. Chromatin fractions were analyzed by Western blotting with anti-H2B or anti-RAP80 antibodies at the indicated time points. A blot with anti-histone H4 was used as protein loading control.
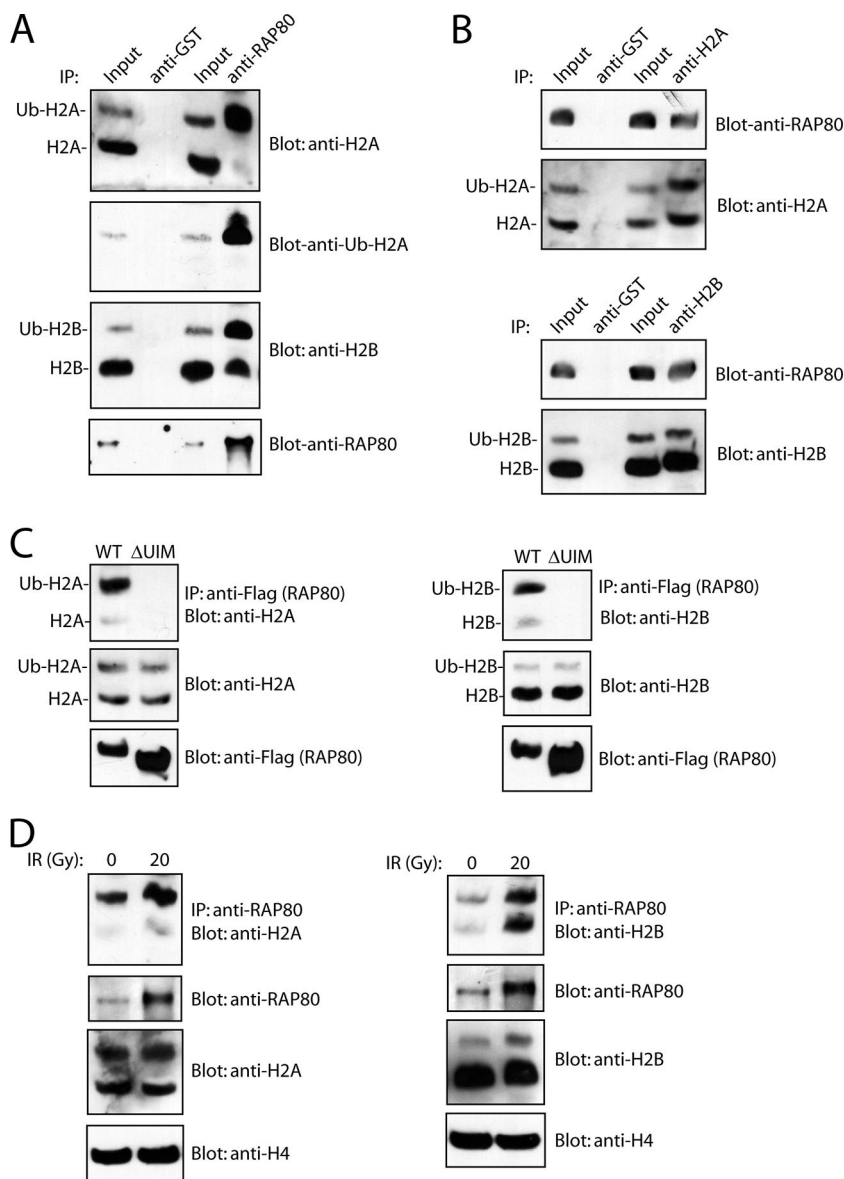
FIG. 2. RAP80 interacts with ubiquitinated H2A and H2B. (A) Endogenous RAP80 associates with ubiquitinated H2A or H2B in vivo. The chromatin fraction from 293T cell lysates was immunoprecipitated (IP) with anti-GST (control) or anti-RAP80 antibodies. The precipitated materials were subjected to Western blot analyses by using anti-H2A, anti-ubiquitinated H2A (Ub-H2A), anti-H2B, or anti-RAP80 antibodies. (B) Reverse coimmunoprecipitation was performed by using anti-GST or anti-H2A and -H2B antibody and Western blot analyses with the indicated antibodies. (C) The UIM domain of RAP80 is required for its association with ubiquitinated H2A and H2B. 293T cells were transfected with plasmids encoding SFP-tagged RAP80 or its UIM domain deletion mutant. Immunoprecipitation and Western blotting were performed with the indicated antibodies. (D) The interaction between RAP80 and ubiquitinated H2A and H2B is increased following IR. 293T cells were treated with or without 20 Gy of IR. Four hours after IR, chromatin fraction were extracted and used for coimmunoprecipitation and Western blotting with the indicated antibodies.

bolt analyses (Fig. 1C), which could probably be masked by a high endogenous H2A ubiquitination level without DNA damage. Nevertheless, the observed ubiquitinated H2A foci indicate that ubiquitinated H2A is highly concentrated at double-strand breaks after IR, making them distinct from background ubiquitinated H2A present before DNA damage. Since only ~1% of H2B was ubiquitinated before IR treatment, we could observe the increase in the total ubiquitinated H2B level with a relatively high dose of IR. We also examined the time course of H2B ubiquitination following DNA damage and found that

at 5 h after IR treatment, H2B ubiquitination increased by a maximum of twofold (Fig. 1D; see Fig. S2 in the supplemental material). Taken together, these results demonstrate that histone H2A and H2B are ubiquitinated following DNA damage. DNA double-strand breaks also induce most RAP80 association with chromatin (25). The increase of chromatin-bound RAP80 correlates with IR-induced H2B ubiquitination, indicating that RAP80 may recognize ubiquitinated histones following DNA damage (Fig. 1D; see Fig. S2 in the supplemental material).
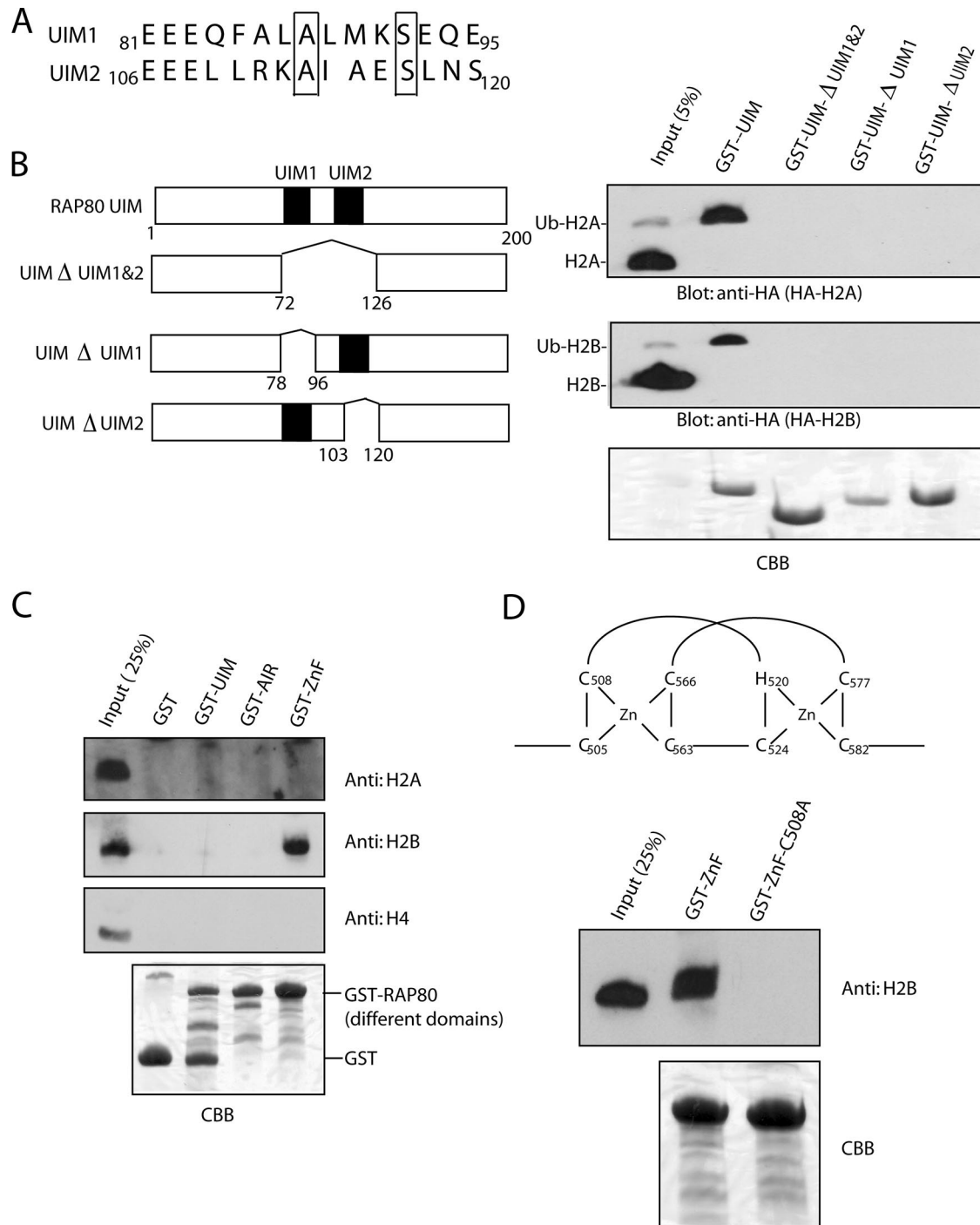
FIG. 3. Both UIMs of RAP80 are required for interaction with monoubiquitinated histones H2A and H2B. (A) Primary sequence alignment of the UIM1 and UIM2 of RAP80. (B) Both UIMs are required for interaction with ubiquitinated H2A and H2B. HA-tagged H2A and H2B were expressed in 293T cells. The chromatin fraction of the lysates was incubated with purified GST-RAP80 UIM domain (aa 1 to 200), RAP80 ΔUIM 1&2 (aa 73 to 126 deleted), RAP80 ΔUIM1 (aa 78 to 96 deleted), or RAP80 ΔUIM2 (aa 103 to 120 deleted). The UIM-associated proteins were analyzed by immunoblotting with the indicated antibodies. Coomassie brilliant blue (CBB) staining was done to ensure the equal loading of recombinant GST fusion protein. (C) The zinc finger (ZnF) domain interacts with nonubiquitinated H2B. Free histone was incubated with purified GST, GST-RAP80 UIM domain (aa 1 to 200), RAP80 AIR domain (aa 200 to 400), or RAP80 ZnF domain (aa 400 to 600). The associated proteins were analyzed by immunoblotting with the indicated antibodies. (D) Zinc binding is critical for ZnF domain associates with H2B. Histone was incubated with purified GST-ZnF or GST-C508A. The associated proteins were analyzed by immunoblotting with anti-H2B antibody. (E) The ZnF domain is important for RAP80 focus formation. HeLa cells transiently expressed Flag-tagged wild-type, ΔZnF mutant, or ΔUIM mutant RAP80. Cells were treated with IR (10 Gy), fixed, and immunostained with anti-Flag (positive transfectants) and anti-γH2AX polyclonal antibody at the indicated time points. One thousand positive transfectants from each transfection were examined by fluorescence microscopy. The percentage of focus-positive cells is shown.
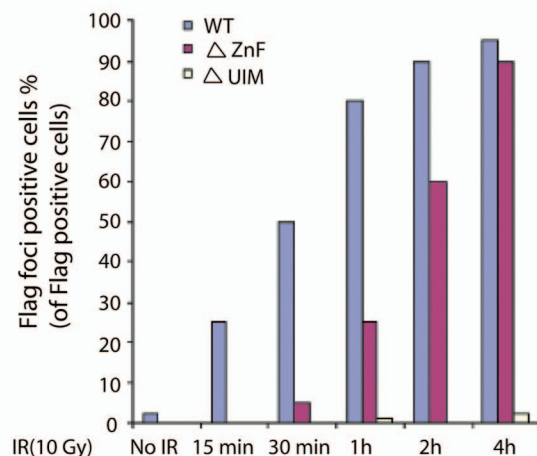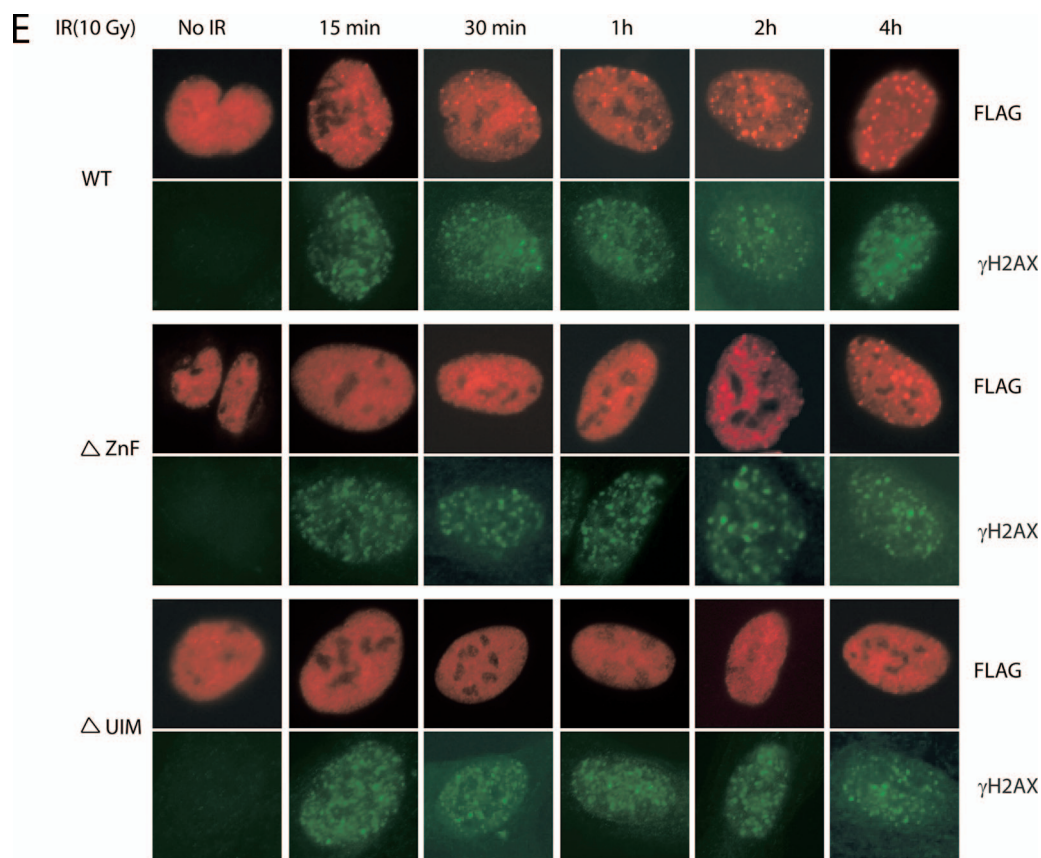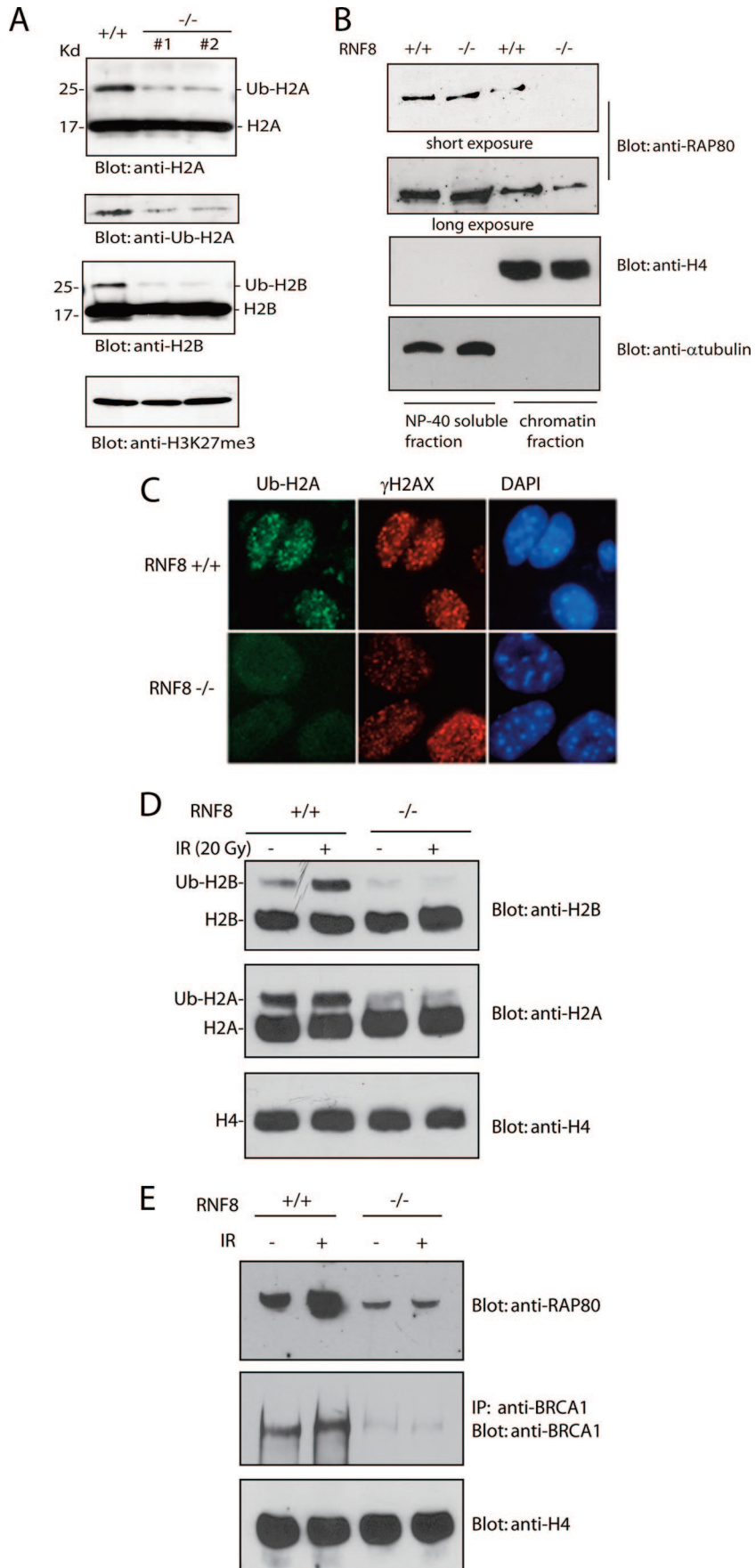
FIG. 3—Continued.

RAP80 interacts with monoubiquitinated histones H2A and H2B. Next we examined whether RAP80 recognizes ubiquitinated H2A and H2B. Even without DNA damage, a small portion of RAP80 already associates with chromatin (25). Thus, we first examined whether RAP80 interacts with ubiquitinated H2A and H2B on the chromatin under normal conditions. Coimmunoprecipitation results indicated that RAP80 associated only with the ubiquitinated form of H2A and not with unmodified H2A (Fig. 2A; see Fig. S3 in the supplemental material). Although a small amount of unmodified H2B associated with RAP80, RAP80-associated ubiquitinated H2B was dramatically enriched in this coimmunoprecipitation assay (Fig. 2A), suggesting that RAP80 associates with both ubiquitinated histones H2A and H2B on the chromatin. The reverse coimmunoprecipitation also confirmed these results (Fig. 2B). Since the UIM domain of RAP80 recognizes ubiquitinated proteins (1, 25, 51, 59, 64), we deleted the UIM domain (aa 73 to 126) and examined whether a UIM domain deletion mutation of RAP80 (ΔUIM) could abolish its interaction with ubiquitinated histones H2A and H2B. As shown in Fig. 2C, only wild-type RAP80, and not the ΔUIM mutant, interacted with ubiquitinated histones H2A and H2B, suggest-

A

+/+ -/-
Kd   #1 #2
25-   Ub-H2A
17-   H2A
Blot: anti-H2A

Blot: anti-Ub-H2A

25-   Ub-H2B
17-   H2B
Blot: anti-H2B

Blot: anti-H3K27me3

B

RNF8   +/+ -/- +/+ -/-

short exposure   Blot: anti-RAP80

long exposure

Blot: anti-H4

Blot: anti-αtubulin

NP-40 soluble   chromatin
fraction   fraction

C

Ub-H2A   γH2AX   DAPI

RNF8 +/+

RNF8 -/-

D

RNF8   +/+   -/-
IR (20 Gy) -   +   -   +

Ub-H2B-   Blot: anti-H2B
H2B-

Ub-H2A-   Blot: anti-H2A
H2A-

H4-   Blot: anti-H4

E

RNF8   +/+   -/-
IR   -   +   -   +

Blot: anti-RAP80

IP: anti-BRCA1
Blot: anti-BRCA1

Blot: anti-H4

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ing that it is the RAP80 UIM domain that recognizes ubiquitinated H2A and H2B. Ubiquitinated histones are the most abundant ubiquitinated proteins in the cell. To examine the specificity of the interaction between the RAP80 UIM domain and ubiquitinated histones, we also checked HJS1A, a UIM domain-containing protein in the nucleus. However, only RAP80, and not HJS1A, could associate with ubiquitinated histones, indicating that the UIM domain of RAP80 specifically recognized ubiquitinated histones (see Fig. S4 in the supplemental material).

Following IR treatment, most RAP80 associates with chromatin (25). Since the RAP80 UIM domain recognizes ubiquitinated histones H2A and H2B, we examined whether the interaction between RAP80 and ubiquitinated H2A and H2B was increased after DNA damage. As expected, DNA damage induced RAP80's association with ubiquitinated H2A and H2B in the chromatin fraction (Fig. 2D). This is consistent with the purification results for RAP80-interacting proteins (Table 1), suggesting that DNA damage-induced histone ubiquitination recruits RAP80 to the chromatin.

In this RAP80 UIM domain, there are two tandem UIMs. These two UIMs have conserved residues to interact with ubiquitin, but they differ in the flanking regions (Fig. 3A), indicating that these two UIMs may have different affinities for different ubiquitinated histones (18). We performed a GST pull-down assay to examine this possibility. The GST-UIM domain, but not its mutant with both UIMs deleted (GST-ΔUIM 1&2), could pull down ubiquitinated histones H2A and H2B, further confirming that RAP80 UIMs interact with ubiquitinated histones. However, deletion of either UIM abolished the interaction between the UIM domain and ubiquitinated H2A and H2B (Fig. 3B), suggesting that both UIMs have to cooperate together to interact with either ubiquitinated H2A or ubiquitinated H2B.

In the coimmunoprecipitation assay, we noticed that RAP80 associated with a small amount of nonubiquitinated H2B (Fig. 2A), suggesting that other regions of RAP80 could also interact with H2B regardless of whether it is ubiquitinated or not. Such interaction may determine the binding specificity of the RAP80 UIM domain with ubiquitinated H2A and H2B over other ubiquitinated proteins. Besides the N-terminal UIM domain, RAP80 also contains two other domains: the Abraxas/CCDC98-interacting region (AIR) in the middle and the C-terminal zinc finger domain (58). We generated GST fusion proteins of all three domains and performed a pull-down assay by using nonubiquitinated free histones. We found that only the zinc finger domain, and not the others, specifically interacted with H2B, but not H2A or H4 (Fig. 3C). Moreover,

abolishing the Zn binding ability of this zinc finger domain by mutating a conserved cysteine to alanine (C508A) totally disrupted this interaction (Fig. 3D). We postulate that the interaction between the RAP80 zinc finger domain and H2B further increases the affinity between RAP80 and ubiquitinated H2B. To further analyze the function of the zinc finger domain of RAP80 in the DNA damage response, we examined IR-induced focus formation of mutant RAP80 in the absence of the zinc finger domain (ΔZnF). Compared with wild-type RAP80, which formed IR-induced foci within 15 minutes, the ΔZnF mutant had significantly delayed IR focus formation and started to relocate to DNA damage sites after 1 hour of DNA damage (Fig. 3E). Consistent with our and other previous reports (1, 25, 51, 64), mutant RAP80 without UIM failed to relocate to DNA damage sites (Fig. 3E). These results indicate that the RAP80 zinc finger domain facilities IR-induced RAP80 focus formation, possibly by additional interaction with H2B other than with the UIM domain.

Histone ubiquitination controls RAP80/BRCA1 complex association with chromatin following DNA damage. To further examine the role of histone ubiquitination in targeting the RAP80/BRCA1 complex to DNA damage sites, we planned to abolish H2A and H2B ubiquitination in vivo. Recent studies have identified that an E3 ligase, RNF8, controls H2A ubiquitination, especially DNA damage-induced H2A ubiquitination (19, 33). We have generated RNF8-deficient MEFs (35). To our surprise, not only 80% of H2A ubiquitination but also 90% of H2B ubiquitination was lost in RNF8$^{-/-}$ MEFs (Fig. 4A), suggesting that RNF8 not only controls H2A ubiquitination but also regulates H2B ubiquitination. The loss of ubiquitinated H2A and H2B correlated with the reduction of chromatin-associated RAP80 in RNF8$^{-/-}$ cells (Fig. 4B), further confirming the interaction between RAP80 and ubiquitinated histones H2A and H2B. Consistently, DNA damage-induced H2A and H2B ubiquitination was also abrogated in the absence of RNF8 (Fig. 4C and D), and this in turn abolished the damage-induced association of RAP80 and BRCA1 with chromatin in RNF8-deficient cells (Fig. 4E). These data together suggest that ubiquitinated H2A and H2B could be docking sites for recruiting the RAP80/BRCA1 complex to the chromatin following DNA damage.

DISCUSSION

In this study, we have identified that ubiquitinated histones H2A and H2B are upstream functional partners of the BRCA1/RAP80 complex in the DNA damage response. The RAP80 UIM domain associated with ubiquitinated H2A and

FIG. 4. Histone H2A and H2B ubiquitination controls RAP80/BRCA1 complex association with chromatin following DNA damage. (A) H2A and H2B monoubiquitination is dramatically reduced in RNF8-deficient cells. Two different lines of RNF8$^{-/-}$ MEFs were generated. Ubiquitinated histones H2A and H2B were analyzed with the indicated antibodies. A blot for trimethylated histone H3K27 was used as protein loading control. (B) Chromatin-associated RAP80 is reduced in RNF8$^{-/-}$ cells. The NP-40-soluble fraction and chromatin fraction were prepared from wild-type or RNF8$^{-/-}$ MEFs and subjected to Western blot analysis with the indicated antibodies. (C and D) IR does not induce histone H2A and H2B ubiquitination in RNF8$^{-/-}$ cells. RNF8$^{+/+}$ and RNF8$^{-/-}$ MEFs were treated with or without 20 Gy IR. Four hours later, the cells were fixed and immunostained with anti-ubiquitinated H2A (Ub-H2A) and anti-γH2AX antibodies (C). DAPI, 4′,6′-diamidino-2-phenylindole. Alternatively, the chromatin fraction was subjected to Western blotting with the indicated antibodies (D). (E) IR does not induce RAP80/BRCA1 complex association with chromatin in the absence of RNF8. RNF8$^{+/+}$ and RNF8$^{-/-}$ MEFs were treated with or without IR (20 Gy). Cells were harvested 4 h later. The chromatin fraction was subjected to Western blotting with the indicated antibodies. Anti-H4 was used as a loading control.

H2B, which recruit the BRCA1/RAP80 complex to DNA damage lesions on the chromatin. In addition, we have also found that RNF8 controls histone H2A and H2B ubiquitination before and after DNA damage, which regulates the BRCA1 complex's translocation to DNA damage sites.

Histone ubiquitination has been known for more than 30 years (15). In fact, histone H2A was the first protein known to be ubiquitinated (15). Monoubiquitinated histones H2A and H2B have been shown to participate in gene transcriptional regulation (3, 23, 28, 31, 67) and the DNA damage response (4, 5, 14, 24, 40). However, the molecular mechanisms underlying these histone ubiquitination-dependent functions are not clear. Here, we have shown that histone ubiquitination, like histone phosphorylation and methylation, may function as a platform to recruit the DNA damage response factor BRCA1 to damage lesions on the chromatin. These histone ubiquitination events do not catalyze protesome-dependent protein degradation. Instead, they may resemble other protein monoubiquitinations during endocytosis and protein sorting (38), which function as protein-protein interaction motifs recognized by other ubiquitin binding domains (10). In this study, we may have identified the first ubiquitinated histone binding protein, RAP80. Since ubiquitinated histones are much more abundant than RAP80 in the nucleus, we postulate that many other ubiquitin binding proteins may also recognize monoubiquitinated histones H2A and H2B and participate in gene transcription regulation and the DNA damage response.

We have characterized the interaction between the RAP80 UIM domain and monoubiquitinated histones H2A and H2B. Interestingly, the RAP80 UIM domain contains two tandem UIMs. Previous structure analyses suggested that one UIM could bind to one or two ubiquitins (17, 18, 42, 54). Thus, theoretically, the RAP80 UIM domain could recognize at least two ubiquitins. To our surprise, deleting either UIM abolished the interaction between the UIM domain of RAP80 and ubiquitinated H2A or H2B, suggesting that these two tandem UIMs are likely to cooperate together and recognize one ubiquitinated protein. It is consistent with previous results that abolishing either UIM will disrupt RAP80 focus formation following DNA damage (1, 25, 51, 59, 64). Further structural analyses are needed to examine the molecular details of the interaction between RAP80 and ubiquitinated histones.

Recent publications showed that H2A and H2AX could be polyubiquitinated following DNA damage (19, 22, 33, 68). To avoid antibody cross-reaction, we confirmed that the HA-tagged H2AX could be diubiquitinated (see Fig. S1 in the supplemental material). Moreover, we have mutated all the lysine residues in H2AX to arginine (NOK mutant). The NOK mutant is phosphorylated at Ser139, but ubiquitination is abolished. This mutation still does not affect ubiquitinated H2A focus formation (Fig. 1B), suggesting that H2AX ubiquitination could only be part of H2A ubiquitination that regulates the activation of the DNA damage response. Although we could not detect major diubiquitination or polyubiquitination of HA-tagged H2A and H2B (see Fig. S5 in the supplemental material), we could not rule out that a small amount of histones H2A and H2B was polyubiquitinated following DNA damage. Indeed, RAP80 also recognizes K6- or K63-based polyubiquitin at the DNA damage sites (36, 43, 51). Thus,

RAP80 might also recognize polyubiquitinated histones or other targets at the DNA damage sites.

Recent studies from our group and others have shown that RNF8 controls histone H2A and H2AX ubiquitination following DNA damage (19, 33). We have generated RNF8-deficient MEFs. To our surprise, this single E3 ligase controls not only histone H2A ubiquitination but also histone H2B ubiquitination. To ensure the function of RNF8 in histone ubiquitination, we have generated two different RNF8 knockout mouse cell lines and two different deficient MEF lines. Both lines of RNF8$^{-/-}$ MEFs dramatically lose ubiquitinated histones H2A and H2B. Previously, the Ring1/Ring2/Bmi1 complex has been shown to ubiquitinate histone H2A (2, 7, 9, 60), while the RNF20/RNF40 complex ubiquitinates histone H2B (21, 27, 61, 70). However, in Ring2 and RNF20 depletion cells, although ubiquitinated H2A and H2B were dramatically reduced, IR treatment still could induce H2A and H2B ubiquitination (see Fig. S6 in the supplemental material). This IR-induced histone ubiquitination is controlled by RNF8 and H2AX (Fig. 4C and D; see Fig. S1 and S7 in the supplemental material). Thus, it is likely that RNF8 is important not only for basal level histone ubiquitination but also for IR-induced histone ubiquitination. Moreover, both ubiquitinated H2A and RAP80 formed IR-induced foci in Ring2 and RNF20 depletion cells (see Fig. S6 in the supplemental material), suggesting that RNF8-dependent IR-induced histone ubiquitination is critical to recruit the RAP80/BRCA1 complex to DNA damage sites. Since the Ring2 complex and the RNF20 complex are known E3 ligases for histone ubiquitination, regulation of histone ubiquitination could be more complicated than in previously proposed models.

Besides RNF8, Ubc13, an E2 conjugase, coordinates together with RNF8 to regulate RAP80/BRCA1 focus formation following DNA damage (20, 58, 68). Ubc13 is known for catalyzing K63 polyubiquitin chains on its targets (68). However, two known E2 conjugase partners of Ubc13, MMS2 and UEV1A, are dispensable for ubiquitination foci at the DNA damage sites (20). Thus, it is likely that only a portion of Ubc13 associates with RNF8 and generate ubiquitination signals at the DNA damage sites.

Following DNA damage, a group of DNA damage checkpoint proteins and DNA damage repair proteins, including BRCA1, translocate and accumulate at DNA damage sites. However, the molecular mechanisms by which these DNA damage response factors are recruited to damaged lesions are not clear. Recent evidence indicates that DNA damage induces substantial chromatin remodeling, including histone phosphorylation, acetylation, and ubiquitination (11, 30, 57). In this study, we found that histone ubiquitination functions as the platform to recruit DNA damage response factor BRCA1. Besides this finding, it has been shown that MDC1 and MCPH1 recognize phosphorylated Ser139 of histone H2AX (52, 62) and that Crb2 and 53BP1 recognizes histone H4 Lys20 methylation (6, 47). Thus, various DNA damage-induced histone modifications could function as docking sites to host DNA damage response factors at damage lesions on the chromatin.

Taken together, our results demonstrate the molecular mechanism by which ubiquitinated histones participate in the DNA damage response.

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