The Ubiquitin-Interacting Motif–Containing Protein RAP80 Interacts with BRCA1 and Functions in DNA Damage Repair Response

Jun Yan,[1] Yong-Sik Kim,[1] Xiao-Ping Yang,[1] Li-Ping Li,[2] Grace Liao,[1] Fen Xia,[2] and Anton M. Jetten[1]

[1]Cell Biology Section, Division of Intramural Research, National Institute of Environmental Health Sciences, NIH, Research Triangle Park, North Carolina and [2]Department of Radiation Oncology, Vanderbilt University Medical Center, Nashville, Tennessee

Abstract

In this study, we examine the potential role of receptor-associated protein 80 (RAP80), a nuclear protein containing two ubiquitin-interacting motifs (UIM), in DNA damage response and double-strand break (DSB) repair. We show that following ionizing radiation and treatment with DNA-damaging agents, RAP80 translocates to discrete nuclear foci that colocalize with those of γ-H2AX. The UIMs and the region of amino acids 204 to 304 are critical for the relocalization of RAP80 to ionizing radiation–induced foci (IRIF). These observations suggest that RAP80 becomes part of a DNA repair complex at the sites of IRIF. We also show that RAP80 forms a complex with the tumor repressor BRCA1 and that this interaction is mediated through the BRCA1 COOH-terminal repeats of BRCA1. The UIMs are not required for the interaction of RAP80 with BRCA1. Knockdown of RAP80 in HEK293 cells significantly reduced DSB-induced homology-directed recombination (HDR). Moreover, inhibition of RAP80 expression by small interfering RNA increased radiosensitivity, whereas increased radioresistance was observed in human breast cancer MCF-7 cells with overexpression of RAP80. Taken together, our data suggest that RAP80 plays an important role in DNA damage response signaling and HDR-mediated DSB repair. We further show that RAP80 can function as a substrate of the ataxia-telangiectasia mutated protein kinase *in vitro*, which phosphorylates RAP80 at Ser^{205} and Ser^{402}. We show that this phosphorylation is not required for the migration of RAP80 to IRIF. [Cancer Res 2007;67(14):6647–56]

Introduction

DNA double-strand breaks (DSB) are potential mutagenic/carcinogenic lesions that can lead to chromosomal aberrations and disruption of genomic integrity, and cause cancer (1–4). DSB triggers a series of cellular responses that are involved in the control of DNA damage repair and activation of cell cycle checkpoints. The regulation of these two processes is very closely coordinated (5–8). Early in DNA damage response signaling, nuclear proteins are recruited into multiprotein complexes at the site of the lesion. Some of these proteins are engaged in DNA repair, whereas others coordinate DNA repair and the activation of cell cycle checkpoints. The latter results in a block in cell cycle progression at the G_1-S, intra-S, or G_2-M checkpoints until the damage is repaired. If the damage is not properly repaired, this may lead to permanent cell cycle arrest, cell death, or oncogenesis (1, 4, 5).

Damage sensors, including Nijmegen Breakage Syndrome 1 (NBS1) and RAD50, play a role early in DNA damage responses and provide a scaffold for downstream proteins (5, 9). The nuclear protein kinase ataxia-telangiectasia mutated (ATM) also plays a critical role in the early responses to DNA damage and seems to be the primary player in response to ionizing radiation (10–12). Ionizing radiation and DNA damage–inducing agents induce dissociation of the inactive, dimeric ATM complex, autophosphorylation of Ser^{1981}, and ATM activation. Subsequently, active ATM localizes to ionizing radiation–induced foci (IRIF) and catalyzes the phosphorylation of many damage sensors as well as signal transducer and effector proteins. The ATM substrates include NBS1, H2AX, breast cancer susceptibility gene 1 (BRCA1), 53BP1, checkpoint kinase 2, and the tumor suppressor p53 (5, 10, 11, 13, 14). These effector proteins regulate cell cycle arrest, apoptosis, DNA repair, and transcription (4, 5).

The receptor-associated protein 80 (RAP80) or ubiquitin-interacting motif containing 1 (UIMC1; as approved by the HUGO Gene Nomenclature Committee) is a nuclear protein containing two ubiquitin-interacting motifs (UIM) at its NH_2 terminus (15, 16). A recent study showed the importance of these UIMs in the binding of K63-linked (poly)ubiquitin and the regulation of the stability and transcriptional activity of the estrogen receptor α by RAP80 (16). UIMs have been found in a variety of proteins with roles in endocytosis, (de)ubiquitination, replication, transcription, and DNA repair (17, 18). The potential role of (de)ubiquitination in DNA repair (19, 20) and the observed association of RAP80 with several proteins involved in the activation of cell cycle checkpoints[3] suggested a possible role for RAP80 in DNA damage repair responses. In this study, we provide evidence that supports this hypothesis. We show that RAP80 translocates to IRIF after ionizing radiation and that the UIMs are essential for this relocalization of RAP80. We provide evidence showing that RAP80 and BRCA1 are associated with the same protein complex and that this interaction is dependent on the BRCA1 COOH-terminal (BRCT) repeats of BRCA1. We further show that RAP80 is a target of ATM phosphorylation. Knockdown of RAP80 expression by small interfering RNA (siRNA) reduced DSB-induced homology-directed recombination (HDR) and the cell sensitivity to ionizing radiation–induced cytotoxicity. These observations suggest that RAP80 may play a critical role in the regulation of BRCA1 function(s) and DNA damage response signaling.

Note: Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org/).

Requests for reprints: Anton M. Jetten, Division of Intramural Research, Cell Biology Section, NIEHS, NIH, MD2-01, Building 101, Research Triangle Park, NC 27713. Phone: 919-541-2768; Fax: 919-541-4133; E-mail: jetten@niehs.nih.gov.

doi:10.1158/0008-5472.CAN-07-0924

[3] J. Yan and A.M. Jetten, unpublished observations.

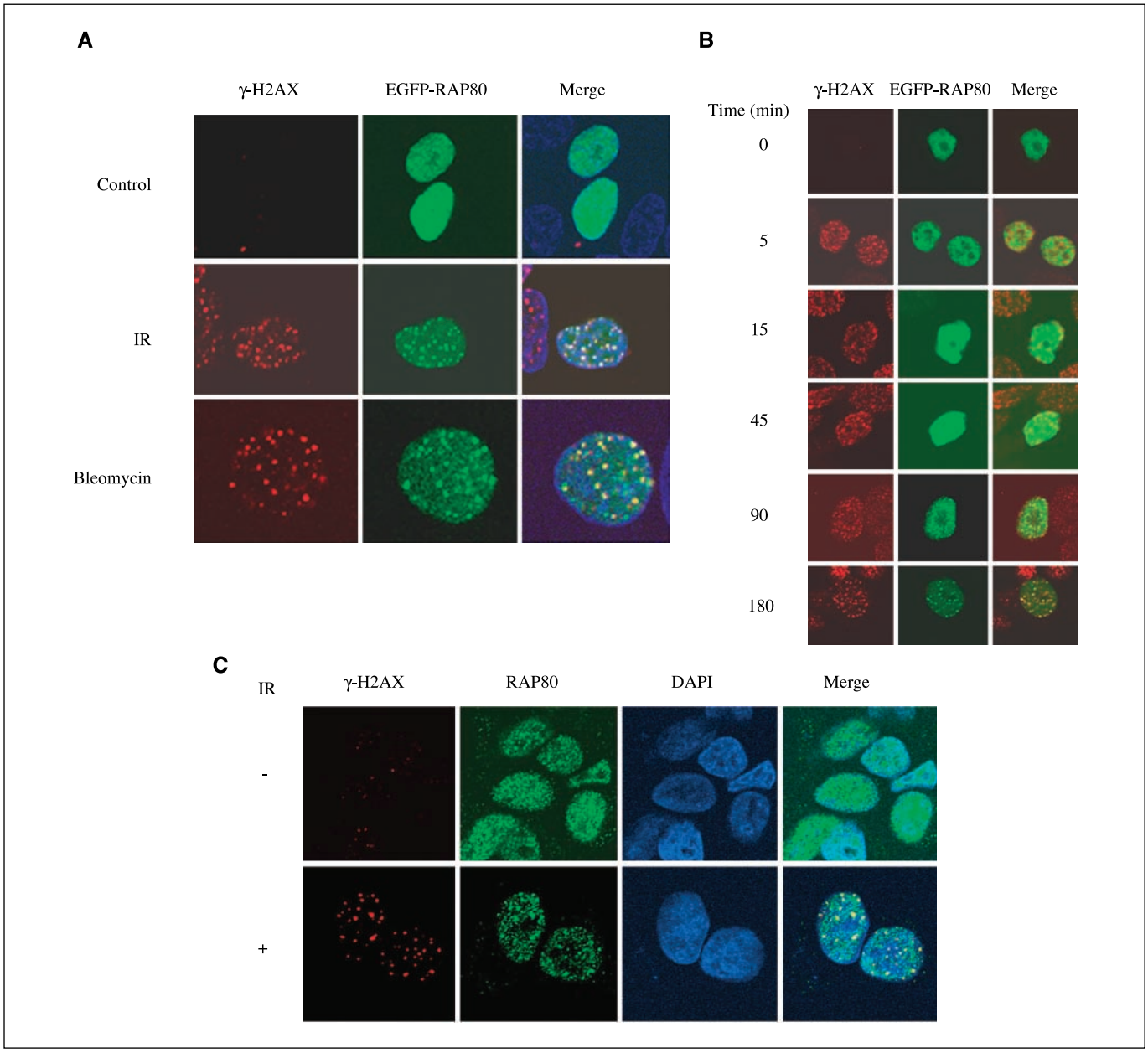

Figure 1. Irradiation and treatment with DNA damage–inducing chemicals induce translocation of RAP80 to IRIF. *A,* MCF-7 cells were transfected with pEGFP-RAP80 and, 48 h later, treated with ionizing radiation (*IR*; 10 Gy) or bleomycin (0.06 units/mL). After 3-h incubation, cells were fixed and subsequently stained with anti–γ-H2AX and Alexa 595–conjugated goat anti-mouse immunoglobulin G (*IgG*) antibody. Nuclei were identified by DAPI staining. Localization of EGFP-RAP80 and γ-H2AX was examined by confocal microscopy. *B,* time-dependent translocation of RAP80 to IRIF. MCF-7 cells were transfected with pEGFP-RAP80 and, 48 h later, were γ-irradiated (10 Gy). At the times indicated, cells were fixed and stained with anti–γ-H2AX antibody and DAPI. *C,* translocation of endogenous RAP80 to IRIF. MCF-7 cells were treated with 10-Gy γ-irradiation and, 3 h later, the localization of endogenous RAP80 and γ-H2AX was examined by confocal microscopy with anti-RAP80 and anti–γ-H2AX antibodies, respectively.

Materials and Methods

Plasmids. pLXIN-3×FLAG-RAP80, pLXIN-3×FLAG-RAP80ΔUIM, pLXIN-3×FLAG-RAP80 mutants (amino acids 1–582, 1–504, 1–404, 1–204, and 1–122), and pEGFP-RAP80 were previously described (15, 16). Details on other mutant RAP80, ATM, and BRCA1 plasmids used in this study are provided in Supplementary data.

Generation of MCF-7-RAP80. MCF-7-RAP80, which stably expresses 3×FLAG-RAP80, was obtained by transfecting MCF-7 cells with pLXIN-3×FLAG-RAP80, followed by selection with 750 μg/mL geneticin. Resistant clones were pooled and expression of 3×FLAG-RAP80 was verified by Western blot analysis and confocal microscopy.

Retroviral infection. PT67 cells were transfected with pLXIN-3×FLAG-RAP80, geneticin-resistant cells were isolated, and virus-containing conditioned medium was collected. Normal human fibroblasts (GM05757) and ataxia-telangiectasia cells (GM05823; Coriell) were then infected with the retrovirus and, 48 h later, examined by confocal microscopy.

SiRNA knockdown. MCF-7 cells were transfected with control or RAP80 siRNA SMARTpool (Dharmacon) following the manufacturer's suggestions. MCF-7 cells were seeded at 10^5/mL in antibiotic-free RPMI 1640 containing 10% fetal bovine serum. On the second day, siRNA was mixed with Dharmafect 1 (Dharmacon) in antibiotic- and serum-free medium for 20 min and then added to the cells at a final concentration of 100 nmol/L. Twenty-four hours later, the transfection was repeated. After an additional

48-h incubation, cells were harvested for Western blot analysis or colony-forming assay.

Confocal microscopy. MCF-7 cells were transiently transfected with wild-type or mutant pEGFP-RAP80 plasmid DNA and, 48 h later, irradiated at the dose indicated. At different time intervals after irradiation, cells were fixed for 20 min in 4% paraformaldehyde and subsequently treated for 7 min with 0.2% Triton X-100. Cells were washed in PBS and then incubated for 15 min in Superblock Blocking Buffer (Pierce). Cells were subsequently incubated for 2 h with anti–γ-H2AX antibody (Upstate Biotech.), and finally for 40 min with antimouse Alexa 595 (Molecular Probes). Endogenous RAP80 and BRCA1 were detected with an anti-RAP80 antibody (Bethyl) and an anti-BRCA1 antibody (Calbiochem), respectively. FLAG-RAP80 in MCF-7-RAP80 cells was detected with a rabbit anti-3×FLAG antibody (gift from Dr. Yue Xiong, University of North Carolina, Chapel Hill, NC) and an anti-rabbit Alexa 488 antibody (Molecular Probes). Nuclei were stained with 4′,6-diamidino-2-phenyindole (DAPI). Cells were then covered with 80% glycerol and fluorescence was observed under a Zeiss LSM 510 NLO confocal microscope (Zeiss). The photographs shown are representative for each experiment.

Coimmunoprecipitation analysis. MCF-7 cells were treated with ionizing radiation and, 4 h later, harvested and lysed for 1 h in radioimmunoprecipitation assay (RIPA) buffer (Upstate Biotech.) containing protease inhibitor and phosphatase cocktails I and II (Sigma). The cell lysates were centrifuged at 14,000 × *g* at 4°C for 10 min. The supernatants were then incubated with anti-RAP80 antibody (Bethyl) and protein G-Sepharose beads (Sigma) overnight to isolate RAP80 protein complexes. The beads were then washed thrice with RIPA buffer. The bound protein complexes were then solubilized in sample buffer and analyzed by Western blot analysis with anti-BRCA1 (Calbiochem) and anti-RAP80 antibodies.

ATM kinase assay. Phosphorylation of glutathione *S*-transferase (GST)-RAP80 proteins by ATM kinase was examined following a procedure described by Ziv et al. (21). For details, see Supplementary data.

Colony-forming assay. MCF-7 parental, MCF-7-RAP80, or MCF-7 cells transfected with RAP80 siRNA were trypsinized and resuspended at 5×10^4/mL. Cells were then irradiated at different doses, plated in triplicate dishes, and grown for 14 days at 37°C. Cells were then stained with 1% Coomassie brilliant blue, 5% acetic acid, and 30% methanol and the number of colonies was counted.

HDR assay. HDR assay was done as previously described (22). HEK293 cells with a chromosomally integrated single copy of the repair substrate pDR-GFP (23) were used to test the role of RAP80 in HDR. Chromosomal

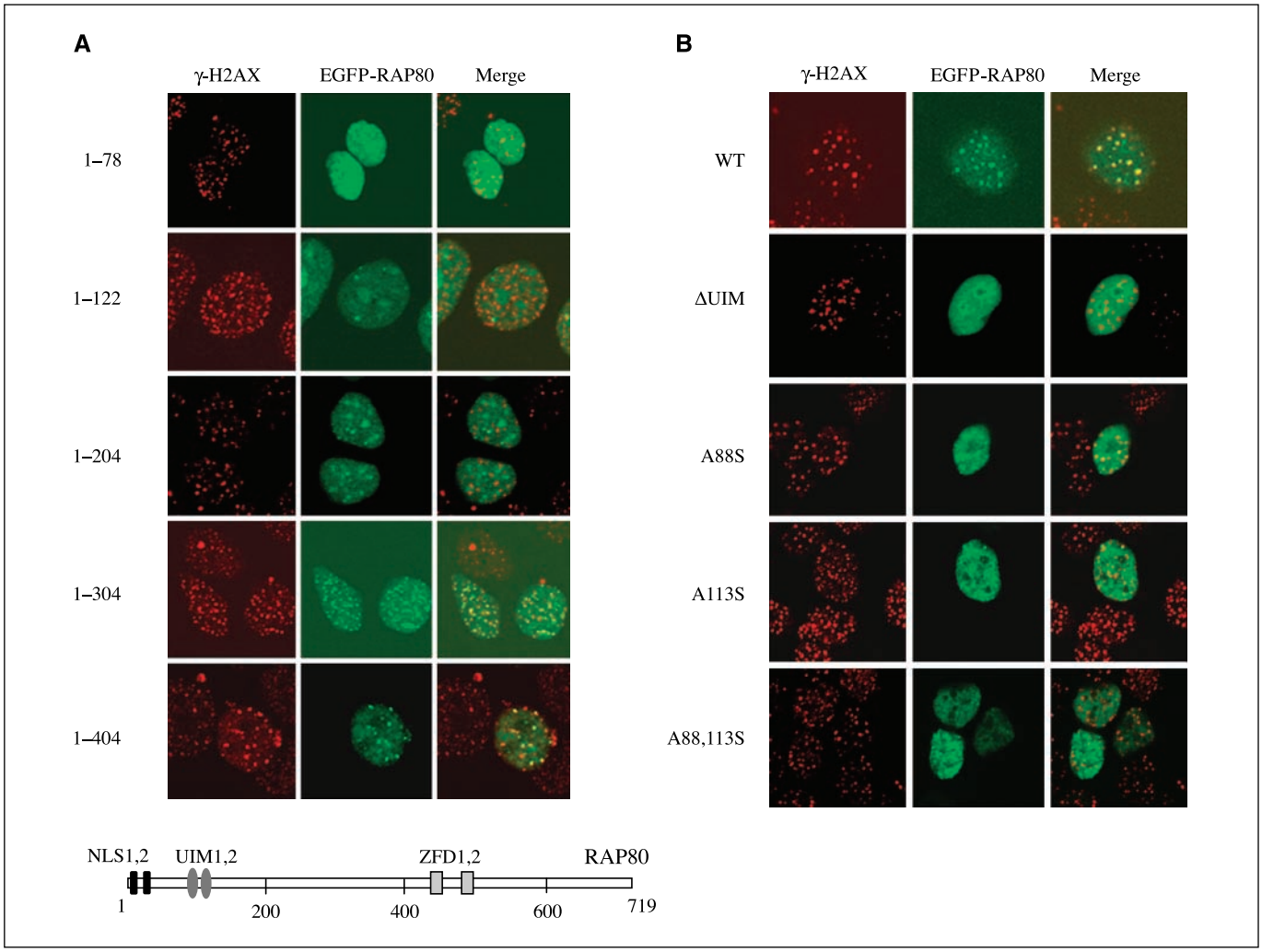

Figure 2. The region of amino acids 204 to 304 and the UIMs are required for the relocalization of RAP80 to IRIF. MCF-7 cells were transfected with several pEGFP-RAP80 mutants containing various COOH-terminal deletions as indicated (*A*) and pEGFP-RAP80 or pEGFP-RAP80ΔUIM in which the two UIMs are deleted or pEGFP-RAP80 mutants containing point mutations within the UIMs as indicated (*B*). After 48 h, cells were γ-irradiated (10 Gy) and, 3 h later, the subcellular distribution of EGFP-RAP80 and γ-H2AX was examined by confocal microscopy. Schematic view of RAP80 is shown in (*A*). *NLS,* nuclear localization signal; *ZFD,* zinc finger domain.

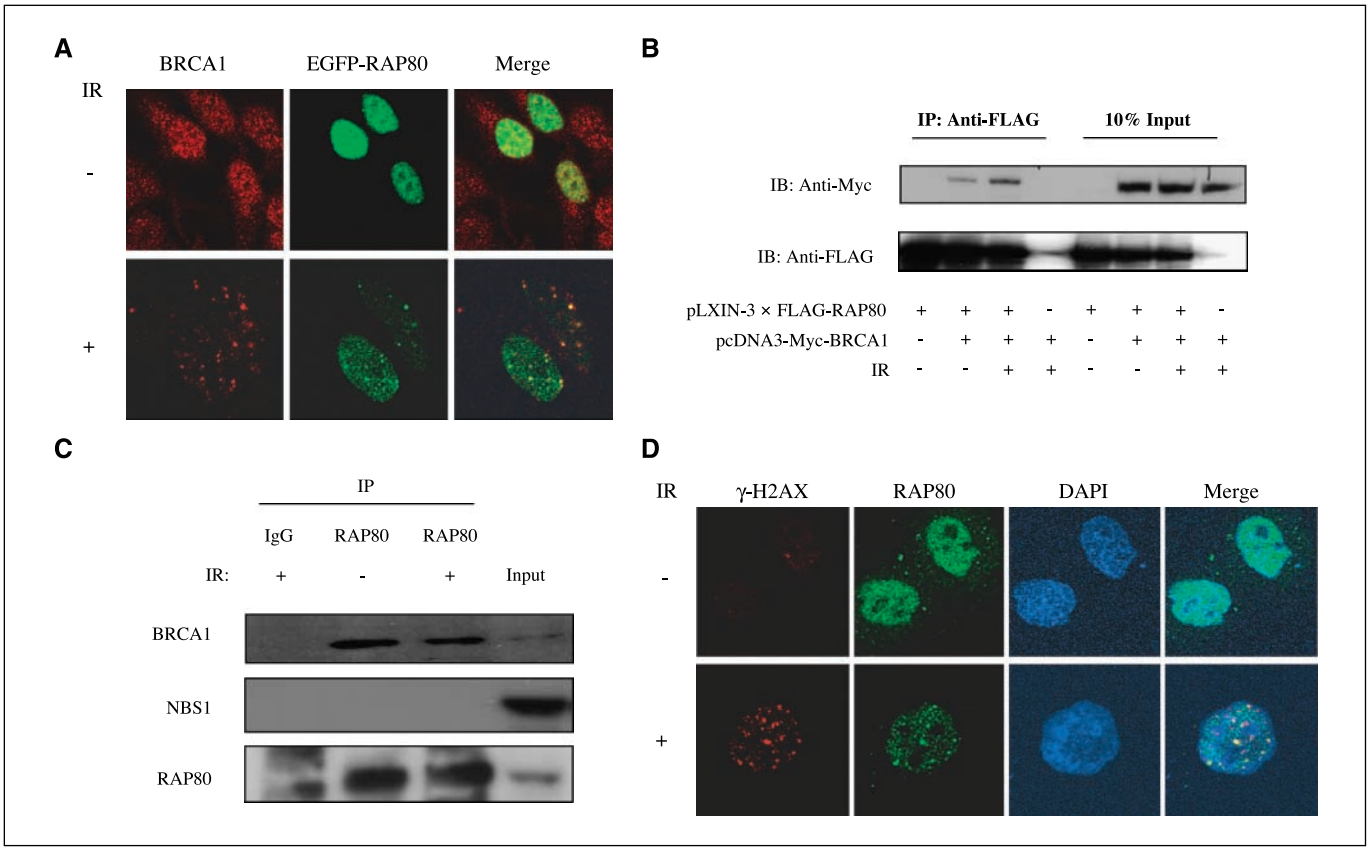

Figure 3. RAP80 and BRCA1 are part of the same protein complex. *A,* HeLa cells were transfected with pEGFP-RAP80 and, 48 h later, treated with and without 15-Gy γ-irradiation. Four hours later, cells were stained with anti-BRCA1 antibody. Subsequently, the subcellular distribution of EGFP-RAP80 and BRCA1 was examined by confocal microscopy. *B,* HEK293T cells were transfected with pLXIN-3×FLAG-RAP80 and pcDNA3-Myc-BRCA1 and, 48 h later, treated with or without γ-irradiation. After 4-h incubation, protein lysates were prepared and FLAG-RAP80 protein complexes were isolated with anti-FLAG resin and examined by Western blot analysis with antibodies against Myc and FLAG. *C,* MCF-7 cells were treated with or without γ-irradiation. After 4-h incubation, protein lysates were isolated and RAP80 protein complexes immunoprecipitated with an anti-RAP80 antibody or immunoglobulin G (control). The immunoprecipitated protein complexes were then examined by Western blot analysis with antibodies against RAP80, BRCA1, or NBS1. *D,* HCC1937 cells containing mutant BRCA1 were γ-irradiated (10 Gy) and, 3 h later, the subcellular distribution of RAP80 and γ-H2AX was examined.

DSBs were induced with the expression of I-SceI. DSB-induced HDR resulted in restoration and expression of green fluorescent protein (GFP) and was quantified by fluorescence-activated cell sorting.

Results

RAP80 relocalizes to nuclear foci after DNA damage. Many proteins involved in DNA damage responses translocate to nuclear foci after DNA damage (5, 24). To study the potential role of RAP80 in DNA damage responses, we first examined the effect of DNA damage on the potential relocalization of RAP80. MCF-7 cells were transiently transfected with pEGFP-RAP80 and the effect of γ-irradiation on the cellular distribution of RAP80 was examined by confocal microscopy. As shown in Fig. 1*A*, RAP80 was rather homogeneously expressed in the nucleus of nonirradiated cells, whereas after ionizing radiation, RAP80 was localized in discrete nuclear foci that colocalized with DSB-associated foci of γ-H2AX, which served as an indicator for the sites of DNA damage. Treatment of the cells with bleomycin, a DNA DSB–inducing agent, induced a similar relocalization of RAP80 (Fig. 1*A*). These observations indicated that RAP80 translocates to DNA damage sites after ionizing radiation or treatment with DSB-inducing agents. Previous studies have shown that after DNA damage, proteins locate to foci at different time intervals. For example, γ-H2AX, 53BP1, and activating transcription factor 2 (ATF2) localize to damage foci very rapidly in response to ionizing radiation, whereas BRCA1 translocates to foci several hours after ionizing radiation (25, 26). Examination of the time course of the translocation of RAP80 showed that RAP80 did not rapidly relocate to DNA damage foci; however, translocation of RAP80 to the foci was clearly observed at 90 min after ionizing radiation (Fig. 1*B*).

To examine the translocation of endogenous RAP80 in response to DNA damage, MCF-7 cells were treated with ionizing radiation and the localization of RAP80 was examined by immunohistochemistry with anti-RAP80 antibody. Figure 1*C* shows that endogenous RAP80 translocated to DNA repair foci after ionizing radiation, in agreement with the observations obtained with exogenous RAP80.

Identification of domains important for the relocalization of RAP80. Previously, we reported that RAP80 contains two putative zinc finger motifs between amino acids 505 and 582 and two functional UIMs between amino acids 80 and 124 (15, 16). To determine what regions within RAP80 are important for its translocation to IRIF, we examined the effect of a series of COOH-terminal deletions on its relocalization. This analysis showed that, after ionizing radiation, the deletion mutants RAP80(1–404) and RAP80(1–304) still colocalized with γ-H2AX–positive foci whereas

RAP80(1–78), RAP80(1–122), and RAP80(1–204) did not (Fig. 2*A*). These observations suggest that the region of amino acids 204 to 304 is essential for the migration of RAP80 to IRIF and that the zinc finger domain in the COOH-terminal half of RAP80 is not required. The effect of several NH_2-terminal deletions in RAP80 could not be analyzed because they were rapidly degraded by the proteasome system.

Previous studies have shown that the UIMs are important for the modulation of ERα-mediated transcriptional activation by RAP80 (16). We therefore examined the effect of a UIM deletion on the ionizing radiation–induced translocation of RAP80. In contrast to EGFP-RAP80, which colocalized with γ-H2AX after ionizing radiation, EGFP-RAP80ΔUIM did not translocate to IRIF as efficiently as wild-type RAP80 but remained rather diffusely distributed in the nucleus (Fig. 2*B*). Previous studies showed that Ala^{88} and Ala^{113} within the UIMs of RAP80 are highly conserved among UIMs and critical for the functions of UIMs (16, 27). Figure 2*C* shows that the single-point mutations Ala^{88}Ser and Ala^{113}Ser greatly impaired the migration of RAP80 to IRIF, whereas the Ala^{88}Ser/Ala^{113}Ser double mutation totally abolished its translocation. These observations support the conclusion that the UIMs are of critical importance in the ionizing radiation–induced relocalization RAP80 to IRIF.

RAP80 is associated with a BRCA1 protein complex. Proteins recruited to IRIF are part of large multiprotein complexes. These proteins migrate to IRIF at different time intervals (26, 28, 29). ATF2 is recruited very early after ionizing radiation–induced damage, whereas RAP80, as reported for BRCA1, migrates to IRIF at a later time. RAP80 likely forms a complex with several other DNA damage response proteins. We therefore examined the association of RAP80 with several DNA damage repair proteins. To examine its association with BRCA1, HeLa cells were transfected with pEGFP-RAP80, irradiated, and, 4 h later, the localization of RAP80 and BRCA1 was examined by confocal microscopy. As shown in Fig. 3*A*, in nonirradiated cells, both RAP80 and BRCA1 exhibited a rather diffuse pattern of nuclear distribution, whereas after irradiation both proteins colocalized to IRIF. The possible association of RAP80 and BRCA1 was further examined by coimmunoprecipitation analysis. Our data showed that 3×FLAG-RAP80 could coimmunoprecipitate Myc-BRCA1 from lysates isolated from both irradiated and nonirradiated cells (Fig. 3*B*). Moreover, in coimmunoprecipitation analysis using an anti-RAP80 antibody, endogenous RAP80 was able to pull down endogenous BRCA1 (Fig. 3*C*). These observations suggest that RAP80 and BRCA1 are associated with the same protein complex and that their association was independent of ionizing radiation.

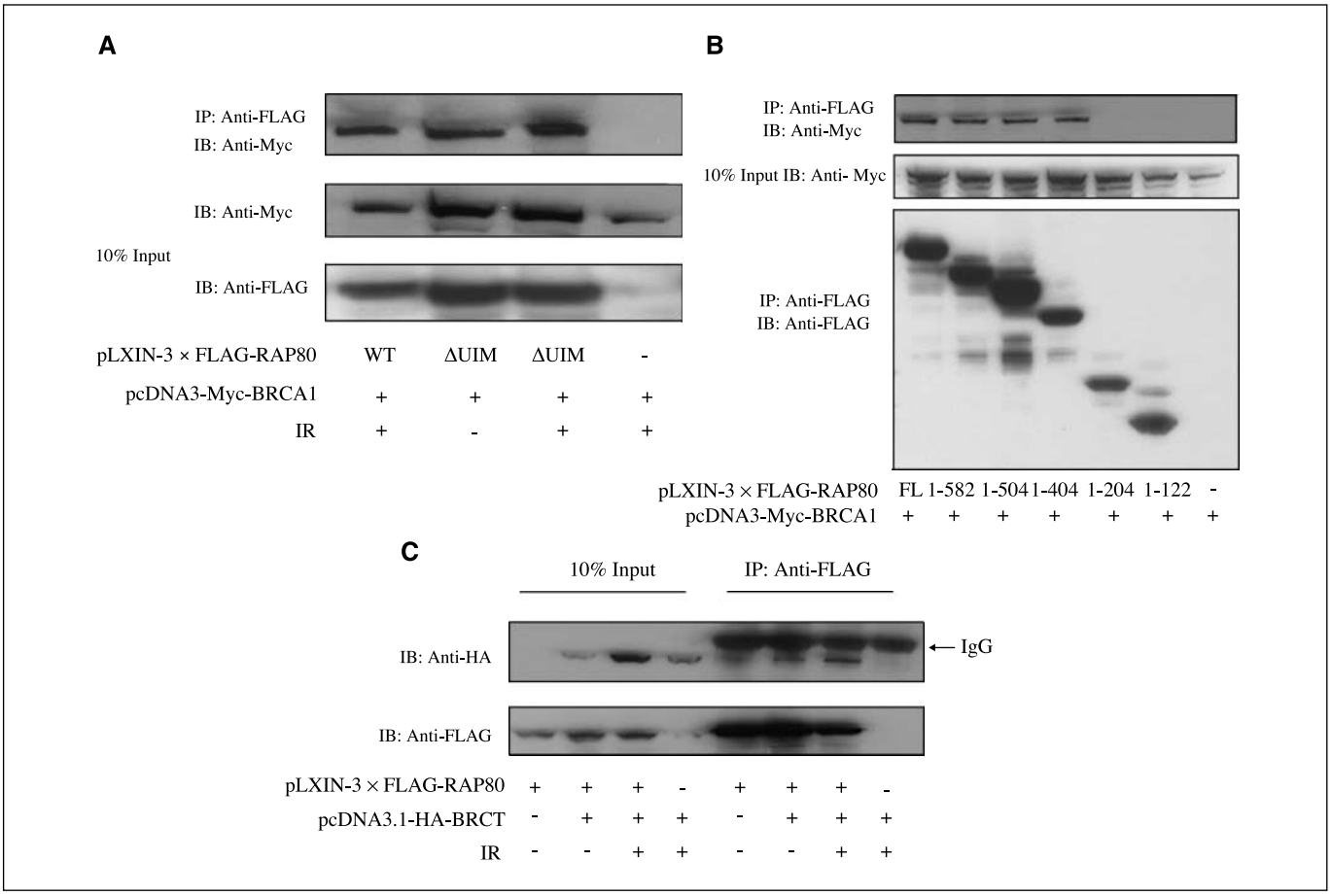

Figure 4. Identification of regions important for the association of RAP80 and BRCA1. HEK293T cells were transfected with pcDNA3-Myc-BRCA1, pcDNA3.1-HA-BRCT, and pLXIN-3×FLAG encoding wild-type (*WT*) RAP80, RAP80ΔUIM, or a COOH-terminal deletion mutant of RAP80 as indicated. Forty eight hours later, cells were γ-irradiated and, after an additional 4-h incubation, FLAG-RAP80 protein complexes were isolated with anti-FLAG resin and examined by Western blot analysis with antibodies against FLAG, Myc or HA. *A,* effect of the UIM deletion on the association of RAP80 and BRCA1. *B,* effect of COOH-terminal deletions on the interaction of RAP80 with BRCA1. *C,* the BRCT domain is sufficient for the association of BRCA1 with RAP80.

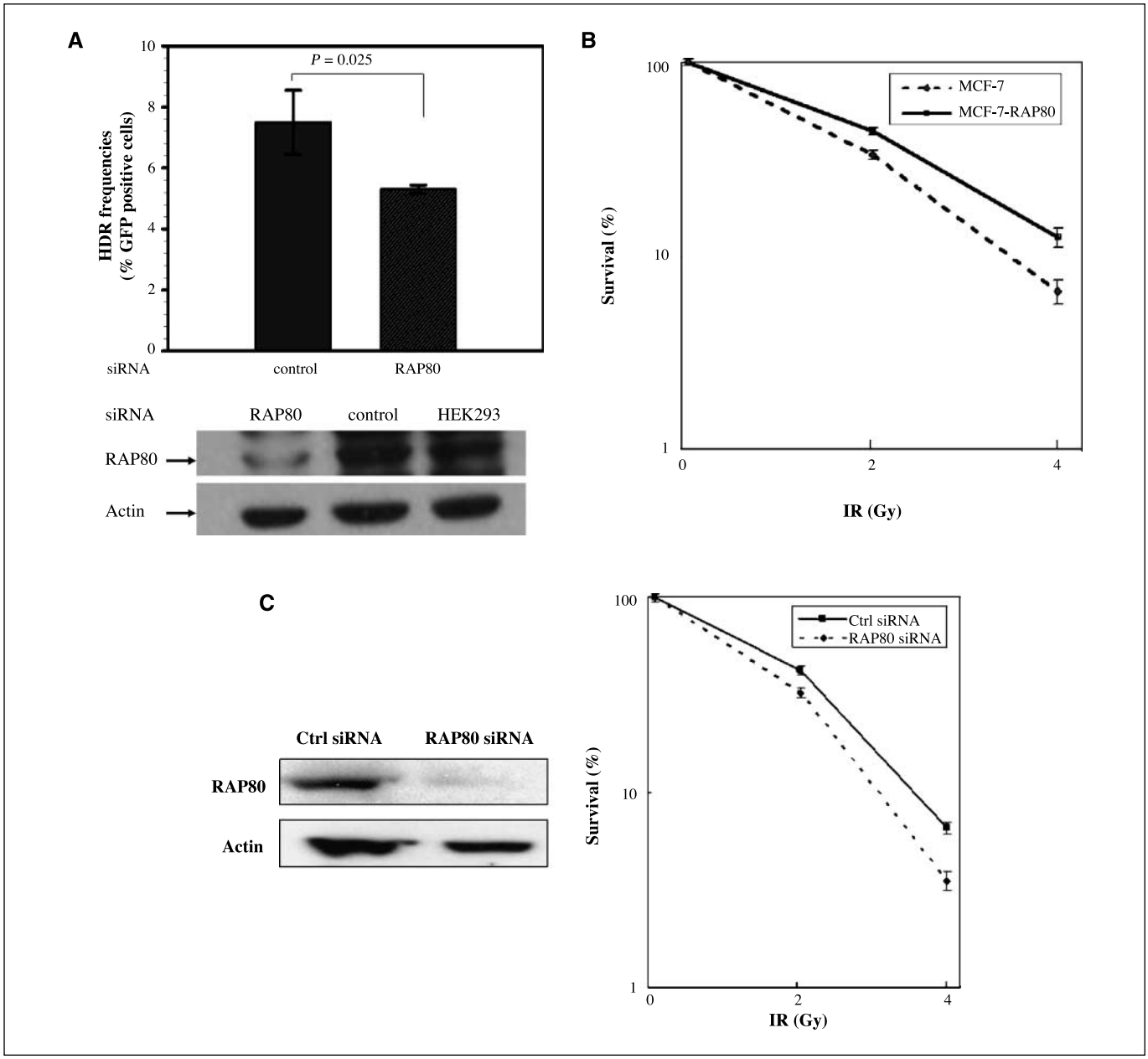

Figure 5. RAP80 expression affects DSB-induced HDR and sensitivity of MCF-7 to ionizing radiation. *A,* DSB-induced HDR in HEK293 cells transfected with scrambled or RAP80 siRNAs was assessed by flow cytometry as described in Materials and Methods. *Columns,* mean of three independent experiments; *bars,* SD. *, $P < 0.025$. Knockdown of RAP80 expression was examined by Western blot analysis with an anti-RAP80 antibody. *B,* MCF-7 and MCF-7-RAP80 cells were seeded at low density and irradiated with the indicated doses of ionizing radiation. Cells were incubated for 14 d at 37°C to allow colonies to form. Colonies were then stained and counted. *C,* MCF-7 cells were transfected with scrambled or RAP80 siRNAs and then were treated as described in (*B*). *Right,* decreased survival of cells treated with RAP80 siRNA. *Left,* part of the cells was used in Western blot analysis to examine the down-regulation of RAP80 protein expression.

In contrast, NBS1 was not immunoprecipitated by RAP80 antibody, suggesting that it does not seem to be part of a RAP80 protein complex (Fig. 3*C*).

To study whether the translocation of RAP80 to IRIF was dependent on BRCA1, the migration of endogenous RAP80 was examined in HCC1937 cells, which express a truncated form of BRCA1 that does not migrate to IRIF after DNA damage (30). Confocal microscopy showed that RAP80 still translocated to IRIF after ionizing radiation (Fig. 3*D*), suggesting that this function of BRCA1 was dispensable for this relocalization.

Mapping regions important for the association of RAP80 with BRCA1. First, we examined whether the UIMs were required for the interaction of RAP80 with BRCA1. Coimmunoprecipitation analysis showed that, like RAP80, RAP80ΔUIM was able to pull down BRCA1 in an ionizing radiation–independent manner (Fig. 4*A*). Next, we examined the effect of a series of RAP80 COOH-terminal deletion mutants for their ability to coimmunoprecipitate BRCA1. Coimmunoprecipitation analysis showed that the region of amino acids 204 to 404 was required for the interaction (Fig. 4*B*). Because the BRCT domain (amino acids 1,528–1,863) functions as a docking module for

several BRCA1-interacting proteins (31–33), we determined its role in the interaction with RAP80. Figure 4*C* shows that the BRCT domain was sufficient to mediate the interaction with RAP80.

RAP80 expression affects DSB-induced HDR and sensitivity of MCF-7 to ionizing radiation. Because RAP80 is in a protein complex with BRCA1, which plays a critical role in DSB-induced HDR, we examined the effect of RAP80 knockdown on DSB-induced HDR (22). HEK293 cells, stably expressing the HDR target vector pMJ-GFP, were transfected with control or RAP80 siRNA and pCMV-I-SceI, which encodes a product that induces DSB. Cells were subsequently

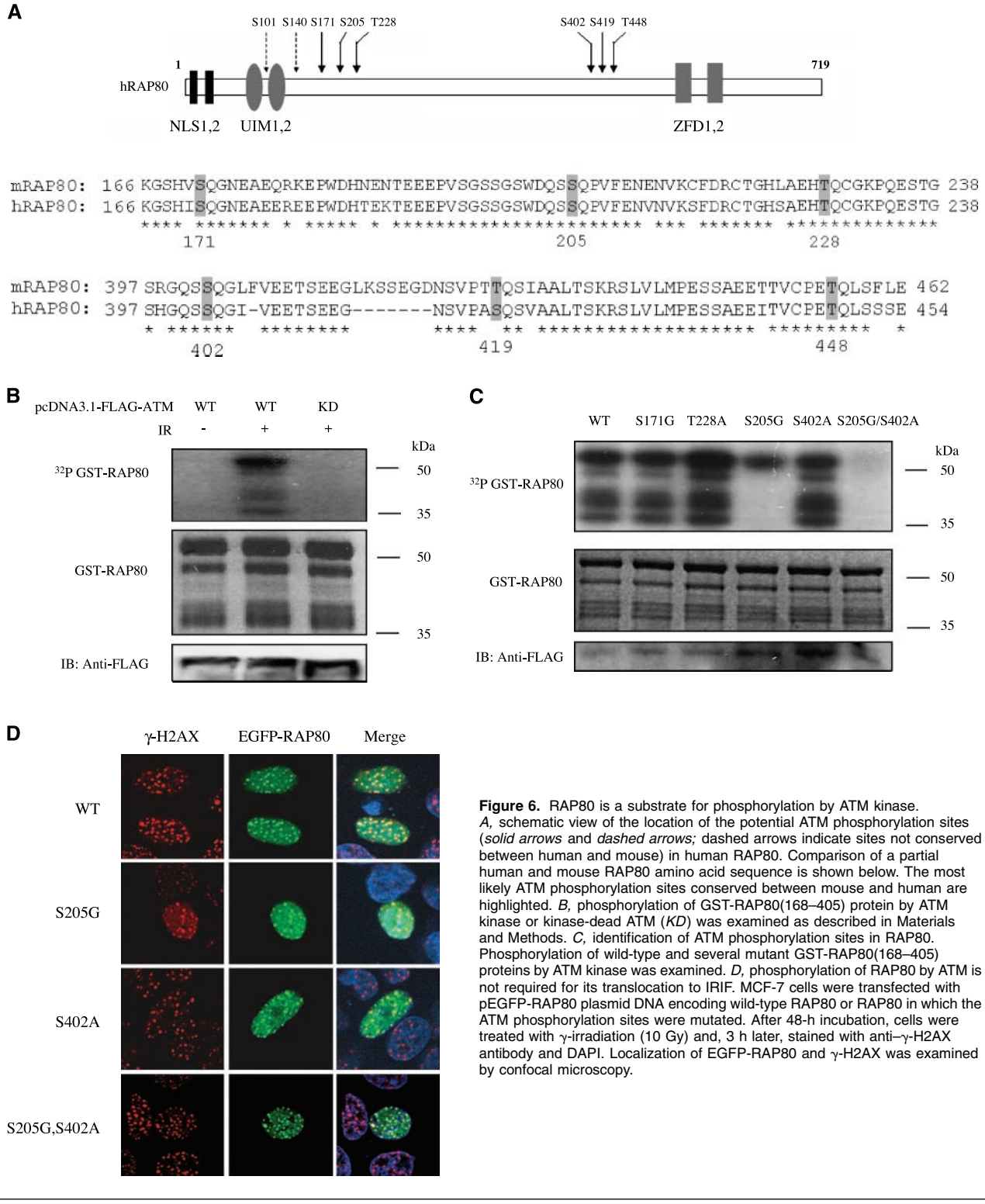

Figure 6. RAP80 is a substrate for phosphorylation by ATM kinase. *A,* schematic view of the location of the potential ATM phosphorylation sites (*solid arrows* and *dashed arrows;* dashed arrows indicate sites not conserved between human and mouse) in human RAP80. Comparison of a partial human and mouse RAP80 amino acid sequence is shown below. The most likely ATM phosphorylation sites conserved between mouse and human are highlighted. *B,* phosphorylation of GST-RAP80(168–405) protein by ATM kinase or kinase-dead ATM (*KD*) was examined as described in Materials and Methods. *C,* identification of ATM phosphorylation sites in RAP80. Phosphorylation of wild-type and several mutant GST-RAP80(168–405) proteins by ATM kinase was examined. *D,* phosphorylation of RAP80 by ATM is not required for its translocation to IRIF. MCF-7 cells were transfected with pEGFP-RAP80 plasmid DNA encoding wild-type RAP80 or RAP80 in which the ATM phosphorylation sites were mutated. After 48-h incubation, cells were treated with γ-irradiation (10 Gy) and, 3 h later, stained with anti–γ-H2AX antibody and DAPI. Localization of EGFP-RAP80 and γ-H2AX was examined by confocal microscopy.

examined by a flow cytometric analysis for HDR. As shown in Fig. 5*A*, cells in which RAP80 was depleted showed a significantly reduced HDR compared with cells transfected with control siRNA, suggesting that RAP80 affects DSB repair by regulating HDR.

To investigate the potential role of RAP80 in DNA damage repair, we generated an MCF-7 cell line that stably expressed FLAG-RAP80. Confocal microscopy showed that FLAG-RAP80 colocalized with γ-H2AX after ionizing radiation (Supplementary Fig. S1). To examine the effects of FLAG-RAP80 on DNA damage response, we examined the effect of various doses of ionizing radiation on the colony-forming ability of MCF-7 and MCF-7-RAP80 cells. As shown in Fig. 5*B*, MCF-7-RAP80 cells exhibited a decreased sensitivity to ionizing radiation, suggesting that overexpression of RAP80 enhances cell viability. We next examined the effect of RAP80 knockdown on cell survival. MCF-7 cells were transfected with control or RAP80 siRNA before the effect of various doses of ionizing radiation on the colony-forming ability was examined. As shown in Fig. 5*C*, MCF-7 cells in which RAP80 expression was reduced formed fewer colonies than parental cells, suggesting that RAP80 expression regulates cell survival after DNA damage. Although these effects are modest, they are in agreement with a regulatory role in DNA damage response signaling.

ATM phosphorylates RAP80 *in vitro*. Many proteins involved in ionizing radiation–induced DNA damage responses are phosphorylated by phosphatidylinositol 3-kinase–like family member ATM (5, 10, 11). In several instances, the translocation of proteins to IRIF is dependent on their phosphorylation by ATM. We therefore examined whether the migration RAP80 to IRIF was dependent on its phosphorylation by ATM. Analysis of the RAP80 amino acid sequence identified several potential ATM phosphorylation sites (Fig. 6*A*). Based on their conservation between mouse and human RAP80 and their similarity to the consensus sequence of the ATM phosphorylation site ΦXΦS(T)Q (in which Φ is a hydrophobic amino acid, although there are some exceptions to this; ref. 34), Ser^{171}, Ser^{205}, and Thr^{228}, and Ser^{402}, Ser^{419}, and Thr^{448} were identified as the most likely ATM phosphorylation sites. To determine whether RAP80 could function as a substrate of ATM, we examined the phosphorylation of GST-RAP80(413–500) and GST-RAP80(168–405) that contain the six potential ATM phosphorylation sites. *In vitro* kinase assays were done using Flag-ATM immunoprecipitated from HEK293T cell lysates. In contrast to GST-RAP80(413–500) (data not shown), RAP80(168–405) was efficiently phosphorylated by wild-type ATM (Fig. 6*B*), suggesting that the ATM phosphorylation site(s) resides within the 168–405 region. We further show that this phosphorylation was dependent on the activation of ATM in HEK293 cells by ionizing radiation and that "kinase-dead" ATM did not phosphorylate RAP80 (Fig. 6*B*). These results indicated that RAP80 might be a target for ATM phosphorylation *in vivo*. To determine which of the site(s) was phosphorylated, we examined the phosphorylation of several RAP80(168–405) mutants containing point mutations at the four potential sites. This analysis showed that mutation of Ser^{205} reduced RAP80 phosphorylation by ATM. Particularly, the shorter GST-RAP80 fragments, which were generated by proteolytic cleavage of GST-RAP80(168–405) at its COOH terminus, were no longer phosphorylated. These data suggested that Ser^{402}, and not Ser^{171} and Thr^{228}, functions as an ATM phosphorylation site. This was confirmed by observations showing that mutation of Ser^{171} and Thr^{228} did not affect the phosphorylation of these shorter fragments. These data suggest that Ser^{205} and Ser^{402} function as ATM phosphorylation sites. This was confirmed by the data showing that the mutation of both Ser^{205} and Ser^{402} totally abolished the phosphorylation of RAP80(168–405) (Fig. 6*C*). These observations indicate that RAP80 functions as a substrate for ATM protein kinase *in vitro* and that ATM can phosphorylate RAP80 on both Ser^{205} and Ser^{402}.

To examine whether this phosphorylation could be involved in the translocation of RAP80 to DNA damage foci, we examined the relocation of pEGFP-RAP80 proteins with point mutations at the different ATM phosphorylation sites. Confocal analysis showed that neither the single nor the double mutations affected the translocation of RAP80 to DNA repair foci after ionizing radiation (Fig. 6*D*). The translocation of a pEGFP-RAP80 mutant (8MT) with all eight potential ATM phosphorylation sites mutated was also examined. This mutant also translocated to the IRIF (Supplementary Fig. S2*A*). That RAP80 migration was independent of phosphorylation by ATM was further confirmed by infecting normal and ataxia-telangiectasia cells with retrovirus encoding FLAG-RAP80. The data showed that RAP80 also translocated to IRIF in ataxia-telangiectasia cells (Supplementary Fig. S2*B*).

Discussion

DNA damage initiates a series of cellular events that control DNA repair, gene transcription, and cell cycle checkpoints. These DNA signaling responses function to maintain genomic integrity and determine whether a cell survives or undergoes apoptosis. DNA damage triggers the recruitment of a large number of DNA damage signaling and repair proteins to the sites of genomic damage. In this study, we provide evidence indicating that RAP80 plays an important role in DNA damage repair signaling. Our study identifies RAP80/UIMC1 as a protein that migrates to IRIF after DNA damage. In untreated cells, RAP80 is distributed rather diffusely within the nucleus, whereas after treatment with ionizing radiation or bleomycin, RAP80 translocates to nuclear foci (IRIF), where it colocalizes with γ-H2AX (Fig. 1*A*). The translocation of RAP80 to IRIF after DSB-induced damage suggests that RAP80 plays a role in DNA damage responses.

Proteins are recruited to IRIF at different times after genomic damage, reflecting different functions of these proteins in DNA repair responses (5, 24). The initial migration of proteins to IRIF occurs very quickly in response to ionizing radiation. For example, the appearance of ATF2 and 53BP1 occurs within minutes whereas other proteins, including BRCA1, are recruited to IRIF hours after DNA damage (25, 26, 29). Our results show that ionizing radiation–induced translocation of RAP80 to IRIF occurred after a substantial delay and was first observed 90 min after ionizing radiation (Fig. 1*B*), a time course more similar to that reported for BRCA1 (28). These observations suggest that RAP80 may not be an initial sensor of genomic damage but function as a downstream mediator or modulator of DNA damage signals.

We previously reported that RAP80 contains two putative Cys-X_2-Cys-X_{11}-His-X_3-Cys zinc finger–like motifs near its COOH terminus and two UIMs near its NH_2 terminus that are able to bind K63-linked polyubiquitin chains (15, 16). To determine what region of RAP80 is important for its translocation to IRIF, the effects of several deletion and point mutations on this migration were examined. Our data showed that deletion of the COOH terminus up to Gln^{304} had little effect on the translocation of RAP80, whereas RAP80(1–204) was unable to migrate to the foci. These results suggest that the zinc-finger domain is not required and the region between Ser^{204}-Gln^{304} is critical for the migration of RAP80 to IRIF. We previously showed that the UIMs in RAP80 are functional and

play a critical role in the function of RAP80 (16). UIMs consist of a short sequence motif of ~20 amino acids that can bind ubiquitin and/or specific ubiquitinated proteins (17). UIM-containing proteins have been reported to direct (multi)monoubiquitination thereby regulating a broad range of cellular functions including membrane protein trafficking, histone function, transcriptional regulation, DNA repair, replication, and (de)ubiquitination control (17, 18, 35, 36). Our results show that deletion of the UIMs (RAP80ΔUIM) abolished the ability of RAP80 to translocate to IRIF. In addition, point mutations $Ala^{88}Ser$ and $Ala^{113}Ser$ within the UIMs greatly impaired the migration of RAP80 to IRIF, whereas the double mutation totally abolished its translocation (Fig. 2). These data indicate that the UIMs play a critical role in the migration of RAP80 to IRIF. Because the UIMs in RAP80 bind K63-linked polyubiquitin, they might provide a binding site for a specific ubiquitinated protein(s) and a mechanism to recruit RAP80 to IRIF. Deletion of the UIMs would prevent such interaction and block the migration of RAP80 to IRIF. Alternatively, UIMs have been reported to promote monoubiquitination and induce changes in conformation and activity of certain UIM-containing proteins (17, 18). Such ubiquitination might be required for RAP80 to migrate to IRIF. Recently, ubiquitination of the BRCA1-interacting protein CtIP has been reported to be required for its association with chromatin and its participation in checkpoint control (37).

The time course of the translocation of RAP80 to IRIF shows similarities with those of BRCA1 and several other DNA repair signaling proteins (5, 38). In addition, we showed that, after DNA damage, RAP80 colocalized with BRCA1. These observations suggested that RAP80 and BRCA1 might be part of the same protein complex. Coimmunoprecipitation analysis supported the conclusion that RAP80 is associated with BRCA1. This association seemed to be independent of the induction of DNA damage because RAP80 was also associated with a BRCA1 protein complex in nonirradiated cells (Fig. 3*B*). Deletion of the UIMs did not affect this association, suggesting that this interaction was not regulated through the UIMs of RAP80. In addition, RAP80 was able to translocate to IRIF in HCC1937 cells which express a truncated BRCA1 that is unable to migrate to nuclear foci (30). These observations suggest that, in contrast to the relocation of several other binding partners of BRCA1 (39), the translocation of RAP80 to IRIF is not dependent on the migration of BRCA1 to IRIF (Fig. 3*D*). Because the translocation of RAP80 is dependent on its UIMs, one might predict that the translocation of proteins associated with this RAP80 complex, including BRCA1, is also dependent on the UIMs of RAP80.

Many studies have shown that altered expression or activity of DNA damage response proteins, including BRCA1 (22), ATF2 (26), MDC1 (40), and BRIT1 (41), can affect the sensitivity of cells to ionizing radiation. Specific mutations in these genes or knockdown of their expression by siRNAs can result in aberrant control of DNA repair and cell cycle checkpoints and subsequently reduce the survival of cells after ionizing radiation. Our data show that knockdown of RAP80 expression by siRNAs caused a significant reduction in DSB-induced HDR (Fig. 4), which constitutes a major mechanism by which cells repair DSB (22, 42). In addition, our study shows that MCF-7 cells overexpressing RAP80 were more resistant to ionizing radiation–induced cytotoxicity, whereas knockdown of the expression of endogenous RAP80 increased the sensitivity of MCF-7 cells to ionizing radiation (Fig. 5). Although these effects were modest, they are in agreement with the concept that RAP80 functions as a facilitator of efficient DSB repair. Because HDR is regulated by BRCA1, the association of RAP80 with BRCA1 and its effect on HDR described in this study support our conclusion that RAP80 has a function in DNA damage repair signaling, possibly by modulating a specific activity/function of BRCA1.

Ionizing radiation–induced DNA damage results in the activation of ATM kinase, which in turn leads to the modulation of various signaling pathways through phosphorylation of many target proteins (5, 11). Some of these proteins seem to function as scaffolds to recruit additional phosphatidylinositol 3-kinase–like targets. Several of these target proteins are involved in the activation of cell cycle checkpoints. These include p53, murine double minute-2, and checkpoint kinase 2, which regulate the G_1 checkpoint, and BRCA1, which regulates the G_1 or G_2 checkpoint depending on its site of phosphorylation. Our results show that RAP80 is an *in vitro* target of the ATM protein kinase. This phosphorylation was specific because it was dependent on the ionizing radiation–induced activation of ATM whereas the kinase-dead ATM mutant did not phosphorylate RAP80. Analysis of the phosphorylation of several RAP80 mutants showed that Ser^{205} and Ser^{402} are ATM phosphorylation sites (Fig. 6).

The translocation of many DNA damage response proteins to IRIF has been shown to be dependent on ATM phosphorylation (5, 26). In contrast, the migration of other proteins, including BRCA1 and NBS1 (43), occurs independently of their phosphorylation by ATM. We showed that a RAP80 mutant, in which both ATM phosphorylation sites are mutated, was still able to translocate to IRIF. This suggests that, like BRCA1 and NBS1, the migration of RAP80 to IRIF occurs independently of its phosphorylation by ATM. Phosphorylation by ATM could affect other functions of RAP80, including its interaction with other proteins. Moreover, phosphorylation of RAP80 at different sites may regulate different functions of RAP80 as has been reported for BRCA1. Phosphorylation at Ser^{1423} of BRCA1 is important for the G_2-M checkpoint but not for the intra-S checkpoint, whereas the inverse is true for the phosphorylation at Ser^{1387} (44). Future studies have to determine the functional significance of *in vivo* phosphorylation of RAP80 by ATM.

In summary, in this study, we show that DSB induces the relocation of the UIM-containing protein RAP80 to DNA damage foci. We provide evidence showing that RAP80 is associated with a protein complex containing BRCA1, but its translocation does not depend on the relocalization of BRCA1. The UIMs and a region between amino acids 204 and 304 are critical for the translocation of RAP80 and proteins associated with this RAP80 complex, including BRCA1. We show that RAP80 knockdown significantly reduces DSB-induced HDR. In addition, overexpression or knockdown of RAP80 expression respectively reduces or increases ionizing radiation–induced cytotoxicity. Its translocation to IRIF, its association with the tumor suppressor BRCA1, and its effects on DSB-induced damage repair suggest that RAP80 has a critical function in DNA damage repair signal transduction and promotes DSB repair. Therefore, mutations in the RAP80 gene that result in aberrant expression or function of RAP80 might have an effect on genome integrity and cancer development.

Acknowledgments

Received 3/9/2007; revised 5/29/2007; accepted 5/31/2007.

Grant support: Intramural Research Program of the NIH, National Institute of Environmental Health Sciences.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

We thank Drs. Richard Paules and Daniel Menendez for their comments on the manuscript.

References

1. Mills KD, Ferguson DO, Alt FW. The role of DNA breaks in genomic instability and tumorigenesis. Immunol Rev 2003;194:77–95.
2. Nyberg KA, Michelson RJ, Putnam CW, Weinert TA. Toward maintaining the genome: DNA damage and replication checkpoints. Annu Rev Genet 2002;36:617–56.
3. Hopfner KP, Putnam CD, Tainer JA. DNA double-strand break repair from head to tail. Curr Opin Struct Biol 2002;12:115–22.
4. Deng CX. BRCA1: cell cycle checkpoint, genetic instability, DNA damage response and cancer evolution. Nucleic Acids Res 2006;34:1416–26.
5. Houtgraaf JH, Versmissen J, van der Giessen WJ. A concise review of DNA damage checkpoints and repair in mammalian cells. Cardiovasc Revasc Med 2006;7:165–72.
6. Sancar A, Lindsey-Boltz LA, Unsal-Kacmaz K, Linn S. Molecular mechanisms of mammalian DNA repair and the DNA damage checkpoints. Annu Rev Biochem 2004; 73:39–85.
7. Rouse J, Jackson SP. Interfaces between the detection, signaling, and repair of DNA damage. Science (New York) 2002;297:547–51.
8. Melo J, Toczyski D. A unified view of the DNA-damage checkpoint. Curr Opin Cell Biol 2002;14:237–45.
9. Assenmacher N, Hopfner KP. MRE11/RAD50/NBS1: complex activities. Chromosoma 2004;113:157–66.
10. Kurz EU, Lees-Miller SP. DNA damage-induced activation of ATM and ATM-dependent signaling pathways. DNA Repair (Amst) 2004;3:889–900.
11. Kitagawa R, Kastan MB. The ATM-dependent DNA damage signaling pathway. Cold Spring Harb Symp Quant Biol 2005;70:99–109.
12. Bakkenist CJ, Kastan MB. DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. Nature 2003;421:499–506.
13. Banin S, Moyal L, Shieh S, et al. Enhanced phosphorylation of p53 by ATM in response to DNA damage. Science (New York) 1998;281:1674–7.
14. Cortez D, Wang Y, Qin J, Elledge SJ. Requirement of ATM-dependent phosphorylation of brca1 in the DNA damage response to double-strand breaks. Science (New York) 1999;286:1162–6.
15. Yan Z, Kim YS, Jetten AM. RAP80, a novel nuclear protein that interacts with the retinoid-related testis-associated receptor. J Biol Chem 2002;277:32379–88.
16. Yan J, Kim S-K, Yang X-P, Albers M, Koegl M, Jetten AM. Ubiquitin interacting motifs of RAP80 are critical in its regulation of estrogen receptor α. Nucl Acids Res 2007;35:1673–86.
17. Di Fiore PP, Polo S, Hofmann K. When ubiquitin meets ubiquitin receptors: a signalling connection. Nat Rev 2003;4:491–7.
18. Hoeller D, Crosetto N, Blagoev B, et al. Regulation of ubiquitin-binding proteins by monoubiquitination. Nat Cell Biol 2006;8:163–9.
19. Wang H, Zhai L, Xu J, et al. Histone H3 and H4 ubiquitylation by the CUL4-DDB-ROC1 ubiquitin ligase facilitates cellular response to DNA damage. Mol Cell 2006;22:383–94.
20. Huang TT, Nijman SM, Mirchandani KD, et al. Regulation of monoubiquitinated PCNA by DUB autocleavage. Nat Cell Biol 2006;8:339–47.
21. Ziv Y, Banin S, Lim DS, Canman CE, Kastan MB, Shiloh Y. Expression and assay of recombinant ATM. Methods Mol Biol 2000;99:99–108.
22. Zhang J, Willers H, Feng Z, et al. Chk2 phosphorylation of BRCA1 regulates DNA double-strand break repair. Mol Cell Biol 2004;24:708–18.
23. Nakanishi K, Yang YG, Pierce AJ, et al. Human Fanconi anemia monoubiquitination pathway promotes homologous DNA repair. Proc Natl Acad Sci U S A 2005; 102:1110–5.
24. Lisby M, Rothstein R. Localization of checkpoint and repair proteins in eukaryotes. Biochimie 2005;87:579–89.
25. Paull TT, Rogakou EP, Yamazaki V, Kirchgessner CU, Gellert M, Bonner WM. A critical role for histone H2AX in recruitment of repair factors to nuclear foci after DNA damage. Curr Biol 2000;10:886–95.
26. Bhoumik A, Takahashi S, Breitweiser W, Shiloh Y, Jones N, Ronai Z. ATM-dependent phosphorylation of ATF2 is required for the DNA damage response. Mol Cell 2005;18:577–87.
27. Polo S, Sigismund S, Faretta M, et al. A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins. Nature 2002;416:451–5.
28. Celeste A, Fernandez-Capetillo O, Kruhlak MJ, et al. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. Nat Cell Biol 2003;5: 675–9.
29. Rai R, Dai H, Multani AS, et al. BRIT1 regulates early DNA damage response, chromosomal integrity, and cancer. Cancer Cell 2006;10:145–57.
30. Scully R, Ganesan S, Vlasakova K, Chen J, Socolovsky M, Livingston DM. Genetic analysis of BRCA1 function in a defined tumor cell line. Mol Cell 1999;4:1093–9.
31. Glover JN, Williams RS, Lee MS. Interactions between BRCT repeats and phosphoproteins: tangled up in two. Trends Biochem Sci 2004;29:579–85.
32. Manke IA, Lowery DM, Nguyen A, Yaffe MB. BRCT repeats as phosphopeptide-binding modules involved in protein targeting. Science (New York) 2003;302:636–9.
33. Yu X, Chini CC, He M, Mer G, Chen J. The BRCT domain is a phospho-protein binding domain. Science (New York) 2003;302:639–42.
34. Kim ST, Lim DS, Canman CE, Kastan MB. Substrate specificities and identification of putative substrates of ATM kinase family members. J Biol Chem 1999;274: 37538–43.
35. Fujiwara K, Tenno T, Sugasawa K, et al. Structure of the ubiquitin-interacting motif of S5a bound to the ubiquitin-like domain of HR23B. J Biol Chem 2004;279: 4760–7.
36. Huang TT, D'Andrea AD. Regulation of DNA repair by ubiquitylation. Nat Rev 2006;7:323–34.
37. Yu X, Fu S, Lai M, Baer R, Chen J. BRCA1 ubiquitinates its phosphorylation-dependent binding partner CtIP. Genes Dev 2006;20:1721–6.
38. Scully R, Xie A, Nagaraju G. Molecular functions of BRCA1 in the DNA damage response. Cancer Biol Ther 2004;3:521–7.
39. Greenberg RA, Sobhian B, Pathania S, Cantor SB, Nakatani Y, Livingston DM. Multifactorial contributions to an acute DNA damage response by BRCA1/BARD1-containing complexes. Genes Dev 2006;20:34–46.
40. Stewart GS, Wang B, Bignell CR, Taylor AM, Elledge SJ. MDC1 is a mediator of the mammalian DNA damage checkpoint. Nature 2003;421:961–6.
41. Lin SY, Rai R, Li K, Xu ZX, Elledge SJ. BRIT1/MCPH1 is a DNA damage responsive protein that regulates the Brca1-1 pathway, implicating checkpoint dysfunction in microcephaly. Proc Natl Acad Sci U S A 2005;102:15105–9.
42. Zhuang J, Zhang J, Willers H, et al. Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining. Cancer Res 2006; 66:1401–8.
43. Kitagawa R, Bakkenist CJ, McKinnon PJ, Kastan MB. Phosphorylation of SMC1 is a critical downstream event in the ATM-NBS1-1 pathway. Genes Dev 2004;18: 1423–38.
44. Xu B, O'Donnell AH, Kim ST, Kastan MB. Phosphorylation of serine 1387 in Brca1 is specifically required for the Atm-mediated S-phase checkpoint after ionizing irradiation. Cancer Res 2002;62:4588–91.