RAP80 Responds to DNA Damage Induced by Both Ionizing Radiation and UV Irradiation and Is Phosphorylated at Ser^{205}

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Abstract

Receptor-associated protein (RAP80), a nuclear protein containing two ubiquitin-interacting motifs (UIM), was recently found to be associated with breast cancer-1 (BRCA1) and to translocate to ionizing radiation–induced foci (IRIF). In this study, we show that the BRCT mutant of BRCA1, R1699W, which is associated with increased risk of breast cancer, is unable to interact with RAP80. Previously, we showed that ataxia-telangiectasia mutated protein kinase (ATM) can phosphorylate RAP80 *in vitro* at Ser^{205}, but whether this site is a target of ATM in whole cells was not established. To address this question, we generated an anti-RAP80Ser205^{P} antibody that specifically recognizes RAP80 phosphorylated at Ser^{205}. Our data show that RAP80 becomes phosphorylated at Ser^{205} in cells exposed to ionizing irradiation and that RAP80Ser205^{P} translocates to IRIF. We show that this phosphorylation is mediated by ATM and does not require a functional BRCA1. The phosphorylation occurs within 5 minutes after irradiation, long before the translocation of RAP80 to IRIF. In addition, we show that UV irradiation induces translocation of RAP80 to DNA damage foci that colocalize with γ-H2AX. We further show that this translocation is also dependent on the UIMs of RAP80 and that the UV-induced phosphorylation of RAP80 at Ser^{205} is mediated by ATM- and RAD3-related kinase, not ATM. These findings suggest that RAP80 has a more general role in different types of DNA damage responses. [Cancer Res 2008;68(11):4269–76]

Introduction

Genotoxic stress can induce different types of DNA damage, among which double strand breaks (DSB) are the most detrimental (1). To maintain their genomic integrity, organisms have developed a sophisticated system to regulate DNA repair and cell cycle checkpoints. Receptor-associated protein 80 (RAP80) or ubiquitin-interacting motif containing 1 (UIMC1) is a nuclear protein containing two functional UIMs at its amino terminus (2, 3). Recently, we and others showed that RAP80 plays a critical role in DNA damage response signaling (4–7). These studies showed that RAP80 translocates to ionizing radiation (IR)–induced foci (IRIF) after IR and that the UIMs are essential for this relocalization. It was further shown that RAP80 forms a complex with BRCA1 and that this association is dependent on the BRCA1 COOH-terminal (BRCT) repeats of BRCA1. BRCA1 plays a critical role in DNA repair and activation of cell cycle checkpoints, and genetic alterations in the *BRCA1* gene have been implicated in several cancers (8–10). RAP80 depletion disrupts the translocation of BRCA1 to IRIF and causes defects in G_2-M checkpoint activation after IR (5–7). In addition, knockdown of RAP80 expression by small interfering RNA (siRNA) reduces DSB-induced homology-directed recombination (HDR) and increases the sensitivity of cells to IR-induced cytotoxicity (4, 5).

The ataxia-telangiectasia mutated (ATM) and ATM- and RAD3-related (ATR) kinases, members of phosphatidyl inositol 3-kinase–like kinase (PIKK) family, play a key role in several DNA damage repair response pathways (11). ATM is primarily activated by DSBs induced by IR and various chemicals. Once activated, ATM phosphorylates a variety of proteins with different roles in damage response signaling pathways, including proteins involved in the control of cell cycle checkpoints (12–15). Deletion of, or mutations in, *ATM* causes defective activation of cell cycle checkpoints and less efficient DSB repair (16). ATR responds to stalled replication forks or other forms of DNA damage, such as UV photoproducts (17, 18). *ATR* null mice are embryonic lethal due to loss of genomic integrity, suggesting a critical role for ATR in embryonic development (19), whereas deletion of ATR in cells causes loss of DNA damage checkpoint responses and cell death (20).

RAP80 was reported to be a target of ATM phosphorylation (4–7). We showed that ATM phosphorylates RAP80 at Ser^{205} and Ser^{402} *in vitro* (4), but whether these sites are phosphorylated by ATM in whole cells and are also targets of phosphorylation by ATR was not established. In this study, we report that RAP80 becomes phosphorylated at Ser^{205} in IR-treated cells. This phosphorylation was dependent on ATM and independent of BRCA1 and occurred at a time that preceded the translocation of RAP80 to IRIF by >60 min. Activation of ATR by UV treatment can catalyze the phosphorylation of some of the same substrates as IR-activated ATM (21–23). We show that after UV irradiation, RAP80 translocates to damage foci and colocalizes with γ-H2AX. We further show that UV treatment also induces phosphorylation of RAP80 at Ser^{205} and provide evidence indicating that this phosphorylation is mediated by ATR. These findings suggest that RAP80 plays a more general role and is important in several types of DNA damage responses.

Materials and Methods

Plasmids. pLXIN and pEGFP were purchased from BD Biosciences. pLXIN-3×FLAG-RAP80, pEGFP-RAP80, the mutant pEGFP-RAP80(S205G), and pEGFP-RAP80ΔUIM were described previously (2, 4). pLXIN-3×FLAG-RAP80 mutant, S205G, was generated by subcloning the RAP80(S205G) coding region of pEGFP-RAP80(S205G) into the *Eco*RI/*Bam*HI sites of pLXIN-3×FLAG vector. The pcDNA3-Myc-BRCA1 (24) was kindly provided by Dr. Jane E. Visvader (Walter and Eliza Hall Institute of Medical Research and Bone Marrow Research Laboratories, Melbourne, Australia). The

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

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pcDNA3-Myc-BRCA1 mutants R1699W and pLXIN-3×FLAG-RAP80 mutants T373S, T373A, and F376W were generated using a Quickchange site-directed mutagenesis kit (Stratagene). The sequence of each insert was verified by DNA sequencing.

Generation of RAP80Ser205^{P}-specific antibody. An antibody against the RAP80 phosphopeptide ^{199}CGSWDQSpSQPVFEN was raised in rabbits injected with keyhole-limpet hemocyanin–conjugated phosphopeptide.

Cell culture and transfection. Normal human fibroblasts (GM05757), ataxia-telangiectasia (A-T) cells (GM05823), and Seckel cells (GM18366; Coriell) were routinely maintained in MEM containing 15% fetal calf serum (FBS) and antibiotics. HEK293T cells were grown in DMEM supplemented with 10% FBS and antibiotics. HEK293T cells were transfected with Polyfect reagent (Qiagen) as indicated by the manufacturer. The breast cancer cell lines MCF-7 and HCC1937 were maintained in RPMI 1640 containing 10% FBS and antibiotics. For RAP80 knockdown, MCF-7 cells were transfected with control or RAP80 siRNA (Invitrogen) following the manufacturer's suggestions. MCF-7 cells were seeded at 10^5/mL in antibiotics-free RPMI 1640 containing 10% FBS. On the 2nd day, siRNAs were mixed with Lipofectamine 2000 (Invitrogen) in antibiotics- and serum-free medium for 20 min and then added to the cells at a final concentration of 20 nmol/L. After 72 h of incubation, cells were irradiated and then fixed for immunofluorescence or harvested for Western blot analysis.

Confocal microscopy. MCF-7 cells were transiently transfected with wild-type pEGFP-RAP80 plasmid DNA and 48 h later were treated with IR or UV 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 Biotechnology), and finally for 40 min with anti-mouse Alexa 595 (Molecular Probes). Endogenous RAP80Ser205^{P} was detected with the RAP80Ser205^{P} antibody and an anti-rabbit Alexa 488 antibody (Molecular Probes). FLAG-RAP80 in MCF-7-RAP80 cells (4) were detected with an anti-FLAG M2 antibody (Sigma). Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI). Cells were then covered with 80% glycerol and fluorescence was observed in a Zeiss LSM 510 NLO confocal microscope (Zeiss).

Coimmunoprecipitation assay. HEK293T cells were transfected with wild-type pLXIN-3×FLAG-RAP80, pcDNA3-Myc-BRCA1, or their mutants as indicated, and 48 h later were harvested and lysed for 1 h in radioimmunoprecipitation assay buffer (RIPA; Upstate Biotechnology) containing protease and phosphatase inhibitor 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-FLAG resin (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 using anti-FLAG M2 (Sigma) and anti-Myc (gift from Dr. Yue Xiong, University of North Carolina, Chapel Hill, NC) antibodies. In the case of phosphatase treatment, immunoprecipitated proteins were treated with λ-phosphatase (400 units/50 μL; New England Biolabs) for 30 min at 30°C before they were examined by Western blot analysis.

Results

ATM phosphorylates RAP80 at Ser^{205}. Previously, we found that ATM phosphorylates RAP80 at Ser^{205} and Ser^{402} *in vitro* (4). To investigate whether RAP80 is phosphorylated by ATM in whole cells, we generated a rabbit polyclonal antibody against a RAP80 phosphopeptide (from C^{199} to N^{212}) phosphorylated at Ser^{205} (hereafter referred to as anti-RAP80Ser205^{P}). To test the specificity of the antibody, HEK293T cells were transfected with wild-type pLXIN-3×FLAG-RAP80 plasmid or its mutant, S205G, and subsequently treated with IR. Protein lysates were then examined by Western blot analysis with the anti-RAP80Ser205^{P} antibody. As shown in Fig. 1*A* (*left*), the antibody did not recognize any protein in nonirradiated cells (*lane 1*) and reacted weakly with a 100 kDa protein, presumably endogenous RAP80, in IR-treated nontransfected cells (*lane 2*). The anti-RAP80Ser205^{P} antibody recognized low levels of phosphorylated FLAG-RAP80 in nonirradiated HEK293T cells transfected with pLXIN-3×FLAG-RAP80 (*lane 3*). This level was greatly enhanced after IR treatment (*lane 4*). The antibody recognized another lower band in lane 4, representing a COOH-terminal truncated FLAG-RAP80. The signal was specific for RAP80 phosphorylated on Ser^{205}, because like mock-transfected cells, the antibody recognized only one weak band, representing endogenous phosphorylated RAP80, in IR-treated HEK293T cells expressing the RAP80 S205G mutant (*lanes 2* and *6*). To further confirm the specificity of the antibody, proteins from irradiated U2OS cells were immunoprecipitated with anti-RAP80 antibody, subsequently treated with or without λ-phosphatase, and then examined by Western blot analysis with anti-RAP80Ser205^{P}. As shown in Fig. 1*A* (*right*), the recognition of RAP80Ser205^{P} by the anti-RAP80Ser205^{P} antibody was lost after phosphatase treatment. These results indicate that the anti-RAP80Ser205^{P} antibody specifically recognizes RAP80 phosphorylated at Ser^{205} and show that RAP80 becomes phosphorylated on Ser^{205} *in vivo* in an IR-dependent manner.

To determine whether RAP80Ser205^{P} translocated to DNA damage foci after IR, MCF-7 cells were treated with control or RAP80 siRNA and were irradiated 3 days later. Subsequently, cells were stained with anti-RAP80Ser205^{P} and anti–γ-H2AX antibodies. Figure 1*B* shows that RAP80 expression was down-regulated by >90% in cells transfected with RAP80 siRNA. Confocal microscopy showed that 3 hours after 10-Gy irradiation, 100% of the control cells (n > 300) contained foci identified either by staining with the anti–γ-H2AX or the anti-RAP80Ser205^{P} antibody. Most of the RAP80Ser205^{P} foci colocalized with those of γ-H2AX. The induction of RAP80Ser205^{P} foci was greatly abolished in cells in which RAP80 expression was knocked down by siRNAs whereas it did not affect the formation of γ-H2AX foci (Fig. 1*B*). These data are consistent with our conclusion that the antibody specifically recognizes RAP80Ser205^{P} and that after IR RAP80Ser205^{P} localizes to DNA damage foci.

Next, we determined whether the IR-induced phosphorylation of RAP80 at Ser^{205} was dependent on ATM. Normal human fibroblasts (GM05757) and A-T cells (GM05823), which are deficient in ATM kinase activity, were treated with IR and protein lysates subsequently examined by Western blot analysis with anti-RAP80Ser205^{P} antibody. The results showed that RAP80Ser205^{P} was only detected in IR-treated normal fibroblasts (Fig. 1*C*) in agreement with the conclusion that phosphorylation of RAP80 at Ser^{205} is mediated by ATM. This conclusion was supported by confocal microscopy. RAP80Ser205^{P} was detected in foci, which colocalized with those of γ-H2AX, in IR-treated normal fibroblasts but not in A-T cells (Fig. 1*D*).

RAP80 phosphorylation by ATM occurs before its translocation. To study the time course of RAP80 phosphorylation by ATM, MCF-7 and U2OS cells were irradiated; at different time intervals, protein lysates were isolated and examined by Western blot analysis with the anti-RAP80Ser205^{P} antibody. Our data showed that in both cell lines RAP80 became phosphorylated as early as 5 min after IR treatment (Fig. 2*A*). The upper band of RAP80 signal represents a nonspecific signal, because it is not affected by RAP80 siRNA treatment (Supplementary Fig. S1). Previously, we reported that after IR treatment RAP80 translocates to DNA damage foci at times later than 60 min (4). Thus, phosphorylation of RAP80 by ATM occurs much earlier than its

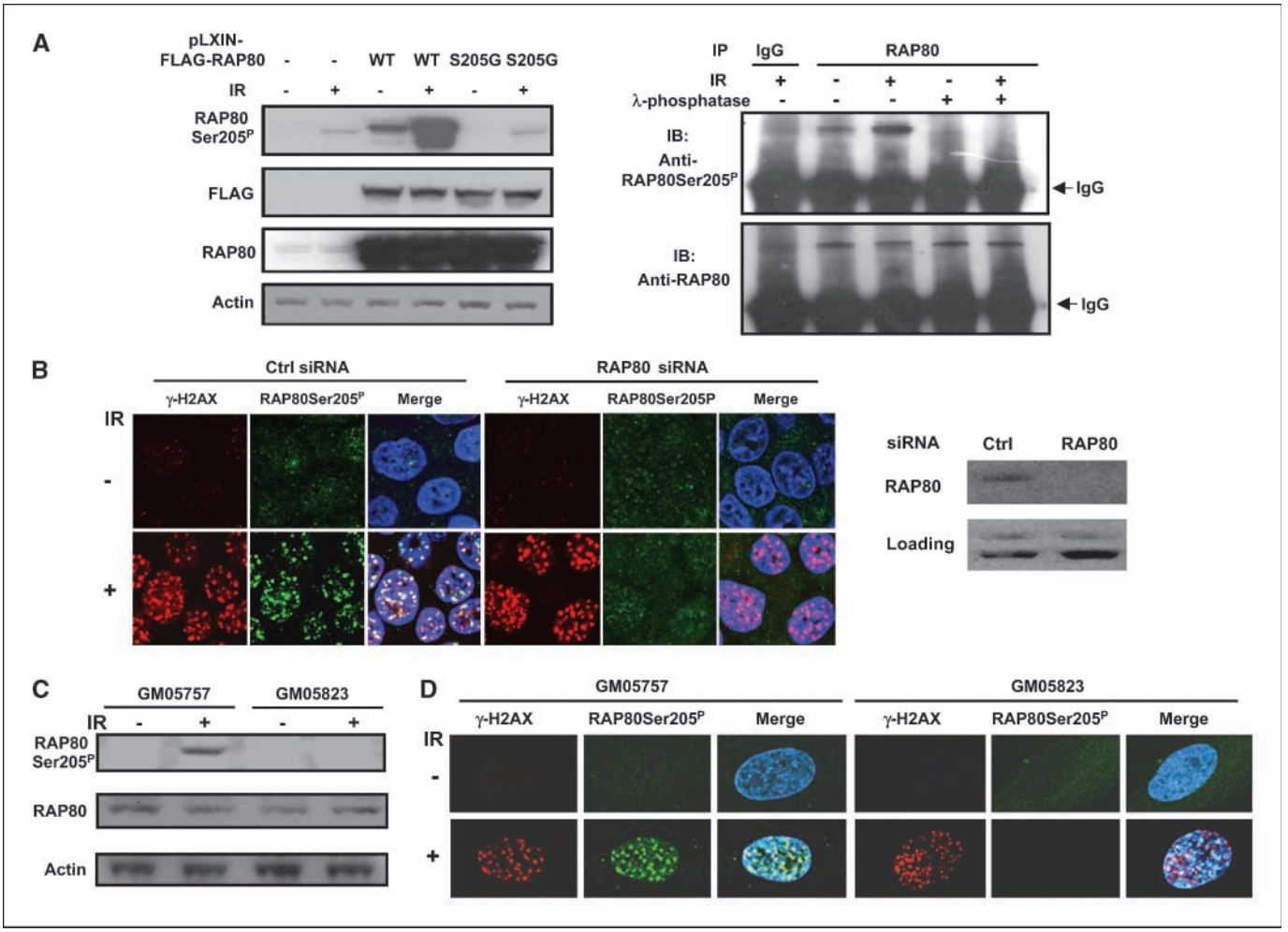

Figure 1. ATM phosphorylates RAP80 at Ser^{205} *in vivo. A, left,* HEK293T cells were transfected with wild-type pLXIN-3×FLAG-RAP80 or its mutant, S205G, and were γ-irradiated (10 Gy) 48 h later. Cells were collected 3 h after irradiation and lysates were examined by Western blot analysis with antibodies against RAP80Ser205^{P}, FLAG, RAP80, and actin. *Right,* U2OS cells were γ-irradiated (10 Gy); 30 min later, cell lysates were prepared. RAP80 protein complexes were immunoprecipitated (*IP*) with an anti-RAP80 antibody and subsequently treated with and without λ-phosphatase. Complexes were then examined by Western blot analysis with antibodies against RAP80Ser205^{P} and RAP80. *B,* MCF-7 cells were treated with control or RAP80 siRNA, and 3 d later were irradiated (10 Gy). Three hours later, cells were fixed and stained with anti-RAP80Ser205^{P} and anti–γ-H2AX antibodies. Nuclei were identified by DAPI staining. Localization of RAP80Ser205^{P} and γ-H2AX was examined by confocal microscopy. Protein lysates from a parallel set of cells were examined by Western blot analysis with anti-RAP80 antibody to analyze the efficiency of the RAP80 knockdown (*right*). *C,* GM05757 and GM05823 cells were exposed to IR (10 Gy). Three hours later, cell lysates were prepared and proteins were examined by Western blot analysis with anti-RAP80Ser205^{P} and anti-RAP80 antibodies. *D,* GM05757 and GM05823 cells were exposed to IR (10 Gy). Cells were fixed and stained 3 h after irradiation as described in *B*.

translocation to IRIF. To further examine this, MCF-7-RAP80 cells, which stably express FLAG-RAP80 (4), were irradiated and at different time intervals examined by confocal microscopy with anti-FLAG and anti-RAP80Ser205^{P} antibodies. Consistent with Western blot analysis, RAP80 became phosphorylated at Ser^{205} within 10 minutes after IR. Initially, RAP80Ser^{205} was distributed within the nucleus in a rather homogeneous pattern but became localized to nuclear foci 60 minutes after IR (Fig. 2*B*). As expected, at 120 minutes after IR, the patterns of foci formation obtained after anti-FLAG and anti-RAP80Ser205^{P} staining largely overlapped. Similar results were obtained when the phosphorylation of endogenous RAP80 was examined in MCF-7 cells with the anti-RAP80Ser205^{P} antibody. As shown in Fig. 2*C*, RAP80 was clearly phosphorylated at Ser^{205} within 15 minutes after IR; however, very few cells formed RAP80Ser205^{P} foci that colocalize with γ-H2AX (2%, $n > 300$). After 3 hours, RAP80Ser205^{P} colocalized with γ-H2AX to IRIF in all cells (100%, $n > 300$). These results show that RAP80 phosphorylation by ATM happens very quickly and long before its translocation to IRIF.

RAP80Ser^{205} phosphorylation by ATM is BRCA1 independent. Previous studies showed that RAP80 is in a protein complex with BRCA1 (4–7). The phosphorylation of some proteins have been reported to depend on the presence of a functional BRCA1, whereas that of others occur in a BRCA1-independent manner (25). To determine whether a functional BRCA1 was required for RAP80 phosphorylation by ATM, we analyzed RAP80Ser^{205} phosphorylation in HCC1937 cells, which contain a mutant BRCA1. Figure 3 shows that as in MCF-7 cells, RAP80 was phosphorylated in IR-treated HCC1937 cells, indicating that a functional BRCA1 is not required for RAP80 phosphorylation by ATM.

The R1699W BRCT mutation disrupts the interaction of BRCA1 with RAP80. Recent studies reported that RAP80 is required for the translocation of BRCA1 to DNA damage foci and that the BRCT motifs of BRCA1 are required for the interaction of

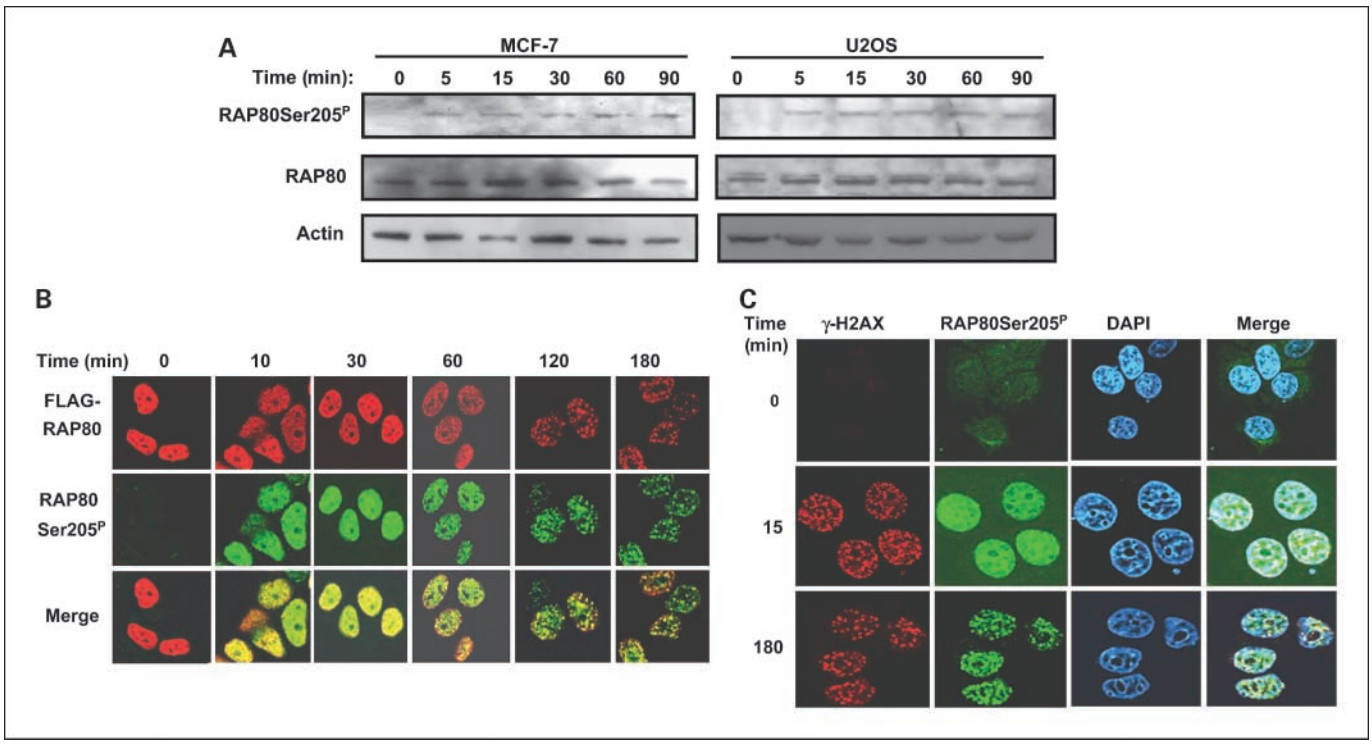

Figure 2. Phosphorylation of RAP80 by ATM occurs long before its translocation to IRIF. *A,* MCF-7 and U2OS cells were exposed to IR (10 Gy), and, at time points indicated, cells were collected and protein lysates were prepared. The lysates were examined by Western blot analysis with anti-RAP80Ser205^{P} and anti-RAP80 antibodies. *B,* MCF-7-RAP80 cells were exposed to 10 Gy γ-irradiation and fixed at the indicated time points. Cells were then stained with anti-FLAG and anti-RAP80Ser205^{P} antibodies, and the localization of FLAG-RAP80 and RAP80Ser205^{P} was examined by confocal microscopy. *C,* MCF-7 cells were exposed to IR (10 Gy), fixed at the time points indicated, and the subcellular distribution of RAP80Ser205^{P} and γ-H2AX was examined by staining with DAPI, and anti-RAP80Ser205^{P} and anti–γ-H2AX antibodies.

RAP80 with BRCA1 (4–7). The structural integrity of the BRCT tandem repeats is essential for the interaction of BRCA1 with phosphorylated protein targets (26, 27). The missense mutation R1699W abolishes the binding of phosphopeptides and is associated with an elevated risk for hereditary breast/ovarian cancer (28–30). Coimmunoprecipitation analysis showed that this mutation abolished the interaction of BRCA1 with RAP80 (Fig. 4*A*) in agreement with the observation that the BRCT is essential for this association.

BRCT was identified as a phosphopeptide binding motif that specifically recognizes a pSer(Thr)-X-X-Phe motif (31, 32). Sequence analysis showed that RAP80 has one potential pSer(Thr)-X-X-Phe motif (^{373}TKDF^{376}) in a region (between amino acids 204–404) previously identified as being important for the association of RAP80 with BRCA1 (4). To determine whether the ^{373}TKDF^{376} motif plays a role in this interaction, we made several point mutations, T373A, T373S, and F376W, in 3×FLAG-RAP80 and examined whether these mutations affected the interaction of RAP80 with BRCA1. The data showed that none of the mutations had an effect on this interaction (Fig. 4*B*), suggesting that this motif is not essential.

RAP80 translocates to the DNA damage foci after UV treatment and is phosphorylated by ATR. To determine whether RAP80 might be involved in DNA damage responses induced by UV treatment, we transfected MCF-7 cells with pEGFP-RAP80 and examined its subcellular localization after UV irradiation. Cells were treated with a moderate dose of UV (10 J/m^2) that affects cell survival to a similar extent as 4 Gy IR (5, 33). Our data showed that after UV treatment RAP80 translocated to DNA damage foci that colocalized with those of γ-H2AX (Fig. 5*A*; ref. 22). In addition, we found that UV treatment induced phosphorylation of RAP80 at Ser^{205} and translocation of RAP80Ser205^{P} to DNA damage foci (Fig. 5*B*). It has been suggested that UV-induced phosphorylation of H2AX and γ-H2AX foci formation are triggered by DSBs induced by blocked replication forks in S-phase cells (34). However, subsequent studies showed phosphorylation of H2AX in non–S-phase cells triggered by ssDNA intermediates (33). Our observations showing that more than 70% of the cells contained foci that were positive for both γ-H2AX and RAP80, a percentage that is much higher than that of the S-phase cell population, are consistent with this. These data suggest that RAP80 is involved in DNA damage responses other than those triggered by DSBs.

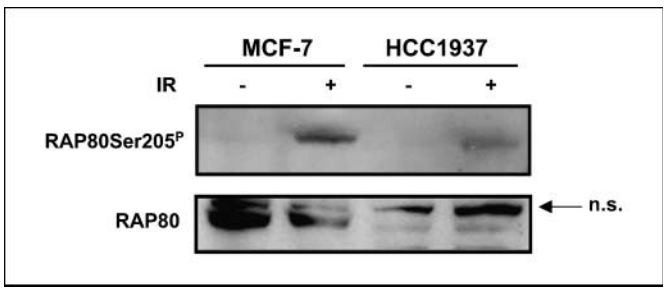

Figure 3. RAP80 phosphorylation by ATM is independent of a functional BRCA1. MCF-7 and HCC1937 were treated with 10 Gy γ-irradiation. Three hours later, cells were collected and examined by Western blot analysis using anti-RAP80 and anti-RAP80Ser205^{P} antibodies. n.s., nonspecific.

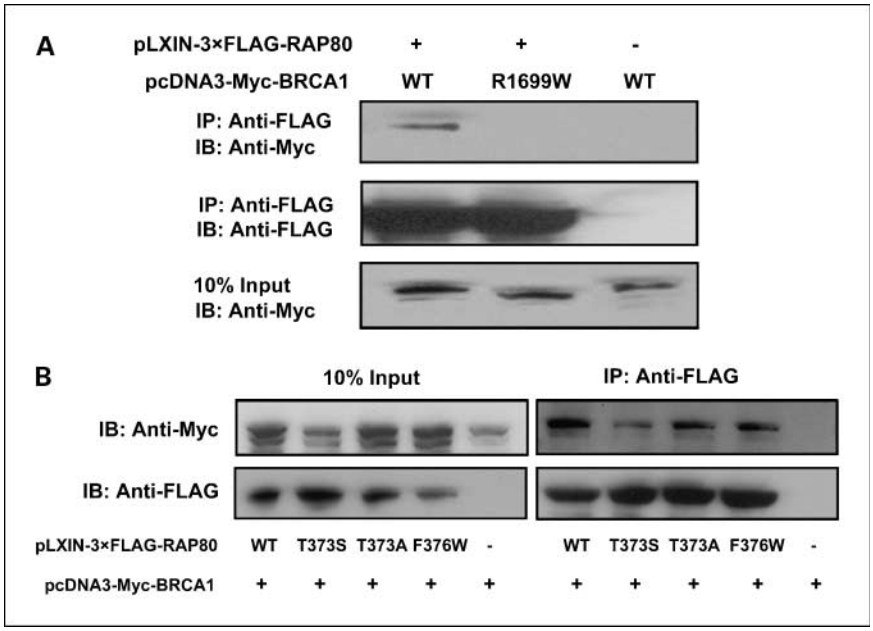

Figure 4. The cancer-predisposing mutation R1699W disrupts the association of BRCA1 with RAP80. *A,* HEK293T cells were transfected with pLXIN-3×FLAG-RAP80 and pcDNA3-Myc-BRCA1 or mutant pcDNA3-Myc-BRCA1 (R1699W) as indicated. *B,* cells were transfected with pcDNA3-Myc-BRCA1 and pLXIN-3×FLAG-RAP80 or one of the RAP80 mutants, pLXIN-3×FLAG-RAP80(T373S), (T373A), or (F376W) as indicated. Forty-eight hours after transfection, cell lysates were prepared and FLAG-RAP80 protein complexes were isolated with an anti-FLAG resin. Complexes were then examined by Western blot analysis with antibodies against Myc and FLAG.

Because UV treatment activates ATR instead of ATM, it is likely that UV-induced RAP80 phosphorylation is mediated by ATR. However, a recent study reported that ATR can phosphorylate and activate ATM after UV treatment (21). To rule out the possibility that UV-induced phosphorylation of RAP80 is mediated by ATM rather than ATR, we treated A-T and control cells with UV irradiation and examined RAP80 phosphorylation by Western blot analysis with anti-RAP80Ser205^{P} antibody. The results in Fig. 5*C*

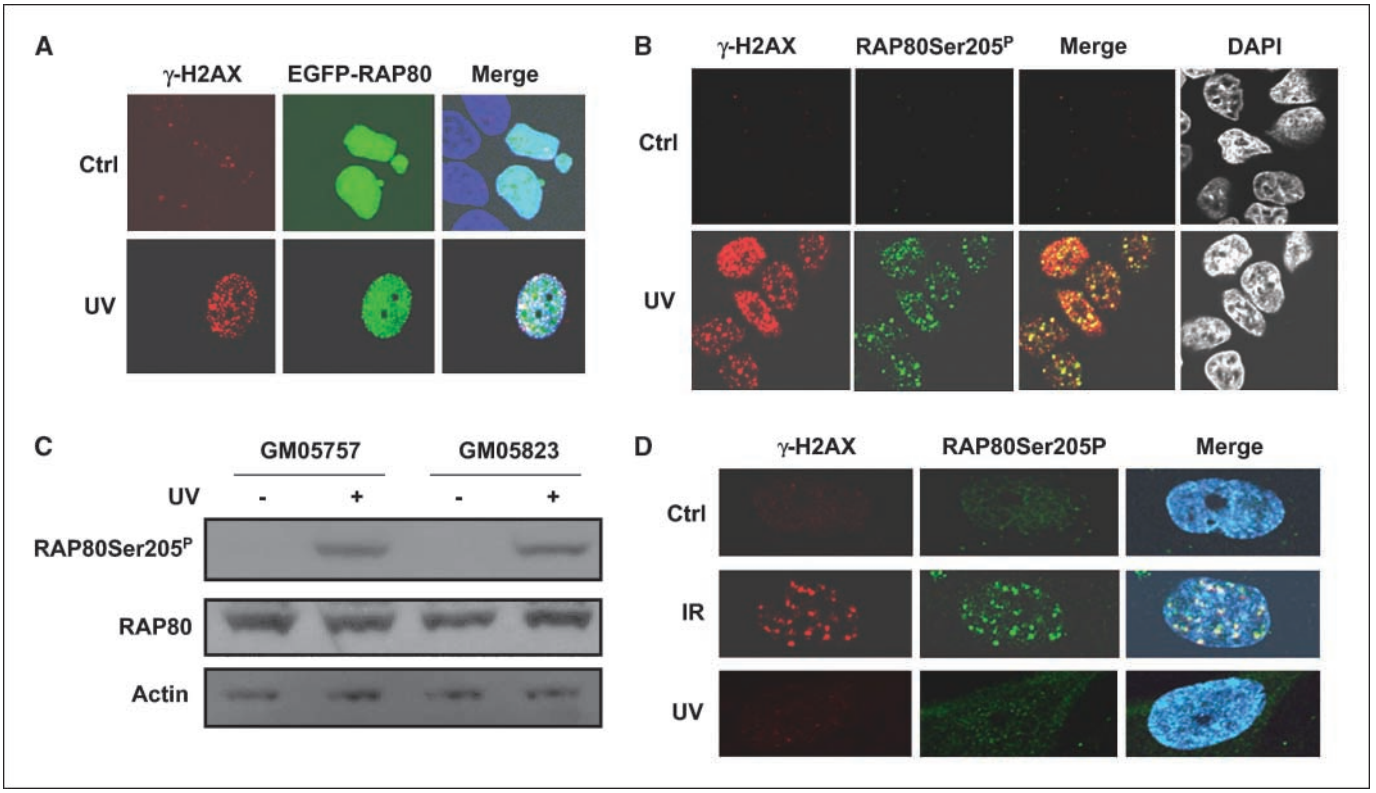

Figure 5. UV irradiation induces translocation and phosphorylation of RAP80. *A,* MCF-7 cells were transfected with pEGFP-RAP80 and were treated with UV (10 J/m^2) 48 h later. After 3-h incubation, cells were fixed and subsequently stained with anti–γ-H2AX antibody and DAPI. Localization of EGFP-RAP80 and γ-H2AX was examined by confocal microscopy. *B,* MCF-7 cells were UV-irradiated (10 J/m^2); 2 h later, cells were fixed and stained with DAPI, and anti-RAP80Ser205^{P} and anti–γ-H2AX antibodies. *C,* GM05757 and GM05823 cells were exposed to UV irradiation (10 J/m^2). Three hours later, cell lysates were prepared and examined by Western blot analysis using anti-RAP80 and anti-RAP80Ser205^{P} antibodies. *D,* Seckel cells were exposed to IR (10 Gy) or UV (10 J/m^2), and 3 h later were stained with anti-RAP80Ser205^{P} and anti–γ-H2AX antibodies. Nuclei were identified by DAPI staining. Localization of RAP80Ser205^{P} and γ-H2AX was examined by confocal microscopy.

show that RAP80 was phosphorylated at Ser^{205} in both control and A-T cells consistent with the conclusion that ATM is not required for RAP80 phosphorylation after UV treatment. To further establish that the phosphorylation of RAP80 after UV treatment was mediated by ATR, we examined the effect of UV or IR on RAP80Ser205 phosphorylation in Seckel cells that have an impaired ATR function (22). The results in Fig. 5*D* show that IR exposure of these cells induces phosphorylation of both H2AX and RAP80 whereas UV treatment does not. These results suggest that after UV treatment, ATR is responsible for the phosphorylation of H2AX and RAP80. As shown for IR (4), EGFP-RAP80ΔUIM lacking the UIMs failed to translocate to damage foci after UV treatment (Fig. 6*A*), indicating that the UIMs are also critical for translocation of RAP80 in response to UV-induced DNA breaks. Moreover, UV treatment did not affect the association of RAP80 with BRCA1 (Fig. 6*B*).

Discussion

The human genome is under constant attack by radiation and different genotoxic chemicals. These exposures induce various DNA lesions that subsequently generate DNA damage responses to maintain genome integrity (11, 16, 35, 36). This involves a large number of proteins that are implicated in DNA damage repair, the regulation of cell cycle checkpoints and transcription, and apoptosis when damage is not properly repaired. We, and others, recently reported that RAP80 translocates to IRIF after IR treatment and plays an important role in DNA damage responses (4–7). RAP80 interacts with BRCA1 and is critical for efficient repair through DNA damage–induced HDR and in cell cycle checkpoint control.

ATM plays a key role in the activation of cell cycle checkpoints after IR-induced DSBs. Loss of ATM, as in A-T cells, results in defects in the control of all checkpoints (16). In addition to ATM itself, ATM phosphorylates many DNA damage response proteins, including BRCA1 and H2AX. RAP80 was recently identified as a novel substrate of ATM and after IR became phosphorylated at several sites (4–7). Ser^{205} was identified as one of the ATM phosphorylation sites *in vitro* (4). In the current study, we show that exposure of cells to IR induces phosphorylation of RAP80 at Ser^{205} and that, subsequently, phosphorylated $RAP80Ser205^{P}$ translocates to DNA damage foci where it colocalizes with γ-H2AX (Fig. 1). This phosphorylation was shown to depend on the activation of ATM. Both ATM and DNAPK have been reported to be able to phosphorylate H2AX, and in A-T cells, it is DNAPK that phosphorylates H2AX after IR (37). Our results show that RAP80 is not phosphorylated at Ser^{205} in A-T cells after IR (Fig. 1*C* and *D*), indicating RAP80Ser205 is not a target of DNAPK.

We further show that the phosphorylation of RAP80 at Ser^{205} occurs rapidly and long before $RAP80Ser205^{P}$ translocates to DNA damage foci. The delayed translocation to IRIF suggests a role for RAP80 at a later stage of DNA repair, whereas its rapid phosphorylation by ATM suggests that RAP80 might have a role in the regulation of an early event in the DNA damage response as well, such as control of cell cycle checkpoints. The latter hypothesis is consistent with findings showing that depletion of RAP80 causes defects in the control of the G_2-M checkpoint after IR (5–7). Besides Ser^{205} and Ser^{402}, several other ATM phosphorylation sites were identified in RAP80 (5–7). Interestingly, none of the sites is highly conserved across species (5). This raises several interesting questions: What is the function of RAP80 phosphorylation? Are

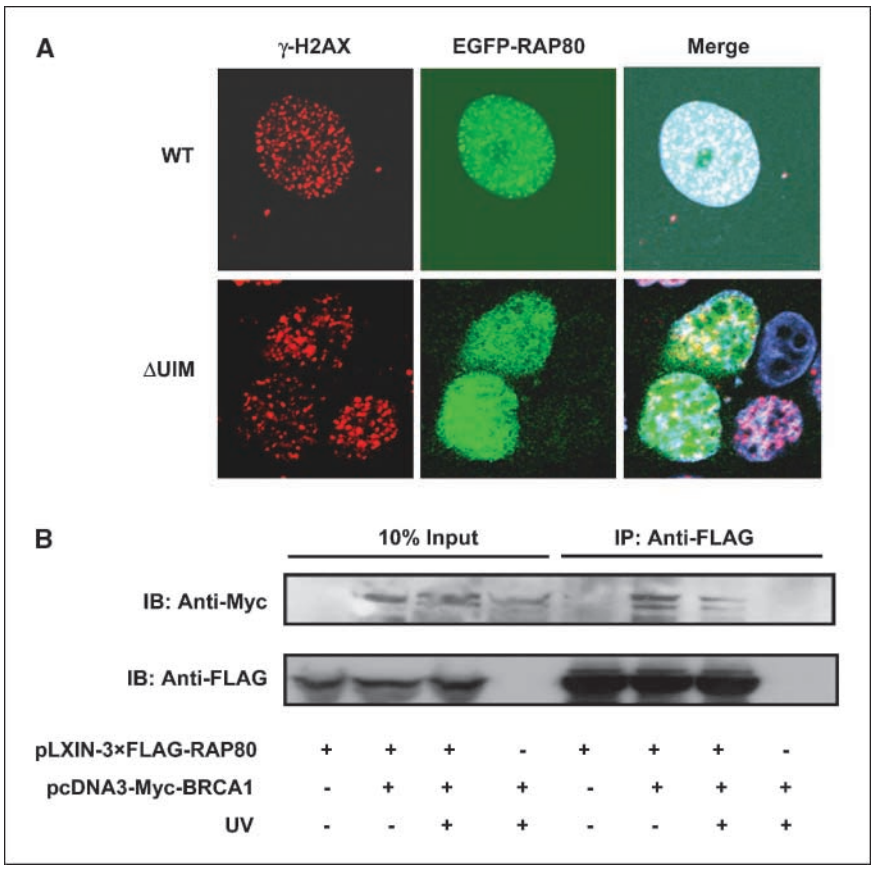

Figure 6. UIMs of RAP80 are required for its translocation to DNA damage foci after UV irradiation. *A,* MCF-7 cells were transfected with pEGFP-RAP80 or pEGFP-RAP80ΔUIM and then treated as described in Fig. 5*A*. B, UV irradiation does not affect the association of RAP80 and BRCA1. HEK293T cells were transfected with pLXIN-3×FLAG-RAP80 and pcDNA3-Myc-BRCA1 and 48 h later were exposed to UV irradiation (10 J/m^2). Three hours later, protein lysates were prepared and FLAG-RAP80 protein complexes were isolated with an anti-FLAG resin. The complexes were then examined by Western blot analysis with antibodies against Myc and FLAG.

all phosphorylation sites important for G_2-M checkpoint control or is phosphorylation of RAP80 at different sites associated with different functions? Although RAP80 phosphorylation by ATM does not affect its association with BRCA1 or its translocation to IRIF (4), the phosphorylation might change the conformation of RAP80 and affect the interaction of RAP80 or RAP80/BRCA1 complex with other proteins. Subsequently, this might affect the activity of the complex and the roles it plays in DNA damage response signaling. In support of this hypothesis, phosphorylation of BRCA1 at distinct sites has been reported to be linked to the regulation of different checkpoints (38). Whether phosphorylation of RAP80 at distinct sites is linked to different functions needs further investigation.

Previous studies reported that a functional BRCA1 is required for ATM- and ATR-dependent phosphorylation of several proteins, including p53, c-Jun, NBS1, CtIP, and Chk2 (25). These proteins are required for checkpoint activation and/or apoptosis, and, as observed for RAP80, seem to be part of a BRCA1 complex even before the induction of DNA damage. However, we show that the phosphorylation of RAP80 is independent of a functional BRCA1 (Fig. 3).

In this study, we further show that RAP80 translocates to DNA damage foci after UV irradiation and that this migration is dependent on the UIMs of RAP80 (Fig. 4). We show that RAP80 is phosphorylated at Ser^{205} and provide evidence that this phosphorylation is mediated by ATR and not ATM. Unlike IR, which directly induces DSBs, UV treatment mainly induces cyclobutane pyrimidine dimers and pyrimidine (6-4) pyrimidone photoproducts (39). Although UV-induced phosphorylation of H2AX and γ-H2AX foci formation can be triggered by DSBs induced by blocked replication forks in S-phase cells (34), recent studies showed a cell cycle–independent induction of H2AX phosphorylation and γ-H2AX foci formation after UV triggered by ssDNA repair intermediates (21, 22, 33). Our observations showing that, after UV, most cells contained foci that were positive for both γ-H2AX and RAP80 are consistent with this and suggest a role for RAP80 in DNA damage responses triggered by other types of DNA lesions, in addition to those induced by DSBs. This was supported by studies demonstrating that RAP80 depletion caused increased sensitivity to UV irradiation (5). Thus, RAP80 seems to play a role in several types of DNA damage responses.

BRCT motifs are found in a number of proteins with functions in DNA repair responses (29). Mutations in the BRCT motifs of BRCA1 have been linked to elevated risk for breast and ovarian cancer (29, 30). Structural analysis showed that many of the cancer-related mutations in the BRCT repeats disrupt the structure of the pSer(Thr)-X-X-Phe binding pocket, abolish the interaction of BRCT with its partners, and prevents BRCA1 from translocating to DNA damage foci (26–29). The BRCT motifs of BRCA1 were shown to be essential for its association with RAP80 and this interaction is required for the translocation of BRCA1 to DNA damage foci (4–7). In agreement with these findings, we showed that the BRCT missense mutation R1699W abolished the interaction of BRCA1 with RAP80 (Fig. 4*A*). Although RAP80 has one potential pSer(Thr)-X-X-Phe motif within the region essential for its association with BRCA1 (4), this motif is not required for this interaction (Fig. 4*B*), suggesting that RAP80 and BRCA1 may interact indirectly by binding an intermediary protein. This concept was supported by recent studies showing that this interaction is mediated by CCDC98 (40, 41).

In summary, our study extends previous observations and shows that RAP80 becomes phosphorylated at Ser^{205} in an ATM-dependent manner in IR-treated cells. This phosphorylation occurs long before RAP80 translocates to IRIF and is independent of BRCA1. UV irradiation also induces phosphorylation of RAP80 at Ser^{205} and its translocation to DNA damage foci. This phosphorylation requires ATR, not ATM. We further show that the BRCT mutant R1699W does not interact with RAP80. Future studies have to determine what the functions are of the different phosphorylation sites of RAP80.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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