Interaction of BARD1 and HP1 Is Required for BRCA1 Retention at Sites of DNA Damage

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Abstract

Stable retention of BRCA1/BARD1 complexes at sites of DNA damage is required for the proper response to DNA double-strand breaks (DSB). Here, we demonstrate that the BRCT domain of BARD1 is crucial for its retention through interaction with HP1. In response to DNA damage, BARD1 interacts with Lys9-dimethylated histone H3 (H3K9me2) in an ATMedependent but RNF168-independent manner. This interaction is mediated primarily by HP1γ. A conserved HP1-binding motif in the BARD1 BRCT domain directly interacted with the chromoshadow domain of HP1 in vitro. Mutations in this motif (or simultaneous depletion of all three HP1 isoforms) disrupted retention of BARD1, BRCA1, and CtIP at DSB sites and allowed ectopic accumulation of RIF1, an effector of nonhomologous end-joining, at damaged loci in S-phase. UNC0638, a small-molecule inhibitor of histone lysine methyltransferase (HKMT), abolished retention and cooperated with the PARP inhibitor olaparib to block cancer cell growth. Taken together, our findings show how BARD1 promotes retention of the BRCA1/BARD1 complex at damaged DNA sites and suggest the use of HKMT inhibitors to leverage the application of PARP inhibitors to treat breast cancer. Cancer Res; 75(7); 1311–21. ©2015 AACR.

Introduction

BRCA1, a breast and ovarian cancer suppressor, is a scaffold protein that maintains genome integrity through diverse molecular functions centered on the repair of DNA double-strand breaks (DSB) through homologous recombination (HR) and cell-cycle checkpoint activation (1). Loss of BRCA1 function causes basal-like breast cancer or serous ovarian cancer (2); both exhibit aggressive features with poor prognosis.

To carry out its tasks in HR and checkpoint activation, BRCA1 must be recruited and retained at sites of DNA damage (3). The well-characterized pathway for the retention of BRCA1 is triggered by ATM activation and is dependent on the BRCT domains of BRCA1 (BRCA1–BRCT; ref. 4). BRCA1–BRCT interacts with phosphorylated Abraxas, promoting a BRCA1/ Abraxas (FAM117A)/RAP80 (BRCA1-A) complex that is retained at the damaged chromatin via the RNF8/RNF168-mediated polyubiquitin chains (5–14). However, the role of the BRCA1–Abraxas–RAP80 complex is to suppress HR by inhibiting excess DNA resection (15, 16), and how the BRCA1 complex that promotes HR is retained at the DSB sites remains enigmatic.

BRCA1 forms a stable core complex with BARD1 (17). The N-terminus of BARD1 forms a RING heterodimer E3 ligase with BRCA1 (18, 19); however, the function of the BRCT domains of BARD1 has long been unclear. A recent study indicated that the BARD1–BRCT domains recognize PAR (20). Interaction of BARD1–BRCT with PAR is required for rapid recruitment of BRCA1/BARD1 to the damaged sites. PAR is synthesized within 20 seconds and disappears within 10 minutes after DNA damage. PARP inhibitors or a K619A mutation of BARD1, which disrupts the PAR interaction, abolishes the early recruitment of BRCA1/BARD1 to DSBs, whereas accumulation of the complex at later time points is not impaired (20). Thus, it appears that both PAR-mediated early recruitment via the BARD1–BRCT domains and ATM-mediated stable retention via the BRCA1–BRCT domains of the BRCA1/BARD1 complex are important for a fully functional BRCA1 at the damaged site, although their separate roles in HR are not entirely understood. The transient nature of PAR at the site of DNA damage suggests that BARD1–BRCT may be free for the remainder of the DNA damage response; alternatively, it could interact with another binding partner.

In the present study, we provide evidence that BARD1 interacts with K9-dimethylated histone H3 (H3K9me2) in response to DNA damage. The interaction is mediated by heterochromatin protein 1 (HP1) through direct binding of the chromoshadow domain of HP1 to a conserved PxVxL motif in BARD1–BRCT. The BRCA1 complex retained by this mechanism executes HR by inhibiting the non-homologous end-joining (NHEJ) pathway and is a target for an HKMT inhibitor UNC0638, a putative anticancer agent.
Materials and Methods

Cell lines and culture conditions
HeLa, HEK293T, T47D, MCF7, ZR-75-1, and U2OS cells were cultured as described (21). Transfections were performed using the standard calcium phosphate precipitation method for HEK293T cells. Cells stably expressing BARD1- or HP1-specific shRNAs in a doxycycline-inducible manner were established by the lentiviral infection of CS-RfA-ETBsd-shBARD1, CS-RfA-ETBsd-shHP1γ (CBX3), CS-RfA-ETBsd-shHP1α (CBX5), or CS-RfA-ETBsd-shHP1β (CBX1). See Supplementary Materials and Methods) followed by selection with blasticidin, puromycin, or hygromycin, respectively. Cells expressing multiple doxycycline-inducible shRNAs (dnHP1 and tshHP1) were established by the multiple lentivirus infections and multiple antibiotic selections. For the doxycycline-inducible replacement of endogenous BARD1 with exogenous BARD1 (r-BARD1 cells), HeLa cells were first infected with CSIV-TRE-RfA-UbC-KT-BARD1 and selected with puromycin, followed by CS-RfA-ETBsd-shBARD1 infection and double-selection with blasticidin and puromycin. The cells were treated with 1 μg/mL doxycycline for 48 hours and subjected to individual experiments. HeLa and U2OS cells stably expressing wild-type or mutant BARD1-EGFP were isolated by lentiviral infection with BARD1-EGFP. For ionizing irradiation (IR), the cells were exposed to X-irradiation (10 Gy) and cultured for 1 hour before further treatment unless otherwise indicated. For bromodeoxyuridine (BrdUrd) staining of the S-phase cells, the cells were incubated with 30 μmol/L BrdUrd for 30 minutes before IR.

Cell extracts, immunoprecipitation, and Western blotting
Immunoprecipitation and immunoblotting of the soluble fraction of transfected 293T cells were performed as described (21). For precipitation of StrepII-tagged proteins, StrepTactin resin was used according to the manufacturer’s instructions (IBA). For immunoprecipitation of chromatin extract, 107 cells were incubated with 1 mL of 0.5% NP-40 buffer (21) supplemented with 125 U/mL benzonase nuclease (Novagen) at 4°C for 120 minutes, and the reaction was stopped with 5 mmol/L EDTA. The extract was centrifuged to isolate the chromatin-bound proteins in the soluble fraction, filtered through a 0.45-μm pore size filter, and used for immunoprecipitations.

Surface plasmon resonance analysis
Purified His-FLAG-HP1 peptides or GST-BARD1-BRCT peptides were immobilized on a CM5 sensor chip using an amine coupling kit or a GST capture kit, respectively, and the surface plasmon resonance (SPR) with analyte was analyzed as described (22).

Immunofluorescence microscopy
Indirect immunofluorescence labeling of cells and fluorescence detection was performed as previously described (22). For staining of BRCA1 and BARD1, cells were preextracted with CSK buffer containing RNase A as described elsewhere (23). For staining of BrdUrd, cells were treated with 2N HCl for 120 minutes and neutralized with 50 mmol/L Tris-HCl (pH 9.0) twice for 5 minutes. For staining of Cdt1 (RBBP8), cells were treated with PBS containing 0.7% Triton X-100, 3% paraformaldehyde, and 2% sucrose for 30 minutes followed by 5-minute incubation with PBS containing 0.2% Triton X-100. Nuclear foci were mechanically counted using the Cellomics Image Analyzer (Thermo Fisher) for BRCA1, endogenous BARD1, and RAD51.

Laser microirradiation
Cells were sensitized with 30 μmol/L BrdUrd 12 hours before irradiation. Laser microirradiation was performed 1 hour before analysis using a PALM UV-A pulsed nitrogen laser (100 Hz, λ = 355 nm; P.A.L.M. Microlaser) mounted on an Axio Observer Z1 microscope (Zeiss) on a custom-designed granite plate.

Plasmids, antibodies, proteins, and agents
Plasmids, lentivirus, RNA interference, antibodies, purified proteins, chemical agents, and clonogenic survival assay are described in Supplementary Materials and Methods.

Results

BARD1 interacts with histone H3K9me2
We first screened modifications of histones in a BARD1 immunocomplex. HeLa cells were harvested after IR or mock treatment and lysed with benzonase nuclease to solubilize the chromatin proteins. The BARD1 immunocomplex was precipitated and immunoblotted with antibodies specific to histone modifications that occur in response to DNA damage (24) or are correlated with heterochromatin formation. H3K9me2 was readily detected and the amount precipitated was increased by IR (Fig. 1A). Although other modifications, including H3K9me3, were detected with longer exposure, we focused on H3K9me2 because its relative amount compared to its input was significantly high. A competing antigen peptide completely blocked the BARD1 precipitation and coprecipitation of H3K9me2, indicating a specific interaction of BARD1 with H3K9me2 (Fig. 1B). The IR-enhanced interaction was also observed in T47D, MCF7, and ZR-75-1 cells (Fig. 1C). Treatment with the topoisomerase I inhibitor CPT-11 also enhanced the interaction (Fig. 1D).

BARD1 interacts with HP1γ
Because H3K9me2 is recognized by HP1 proteins during heterochromatin formation, (25), we asked whether the BARD1–H3K9me2 interaction is mediated by HP1. Human HP1 comprises 3 members: HP1α, β, and γ. Strikingly, HP1γ readily coprecipitated with BARD1, and the interaction was enhanced by IR and inhibited by competing antigen peptide (Fig. 1E and F). HP1α and β were also faintly detected in the precipitates with increase of HP1α after IR (Fig. 1E).

To clarify the configuration, we inhibited HP1γ expression by siRNA and reexamined the interaction between BARD1 and H3K9me2. Depletion of HP1γ dramatically inhibited the BARD1–H3K9me2 association (Fig. 1G), consistent with HP1γ mediating the BARD1–H3K9me2 interaction. We verified the BARD1–HP1γ interaction with exogenously overexpressed proteins in 293T cells. FLAG-HP1γ was pulled down with StrepTactin when BARD1–StrepII was coexpressed, suggesting a direct interaction between BARD1 and HP1γ (Supplementary Fig. S1A). In addition, we cotransfected plasmids encoding BARD1 fused to the C-terminus of truncated Venus fluorescent protein (BARD1-VC) and HP1γ fused to the N-terminus of Venus (VN-HP1γ). Successful assembly of the Venus protein, as detected by fluorescence, would indicate a specific interaction between BARD1 and HP1γ. Cells transfected with BARD1-VC and VN-HP1γ demonstrated laser microirradiation–induced Venus fluorescence signals colocalized with h2AX, suggesting that BARD1 interacts with HP1γ at sites of DNA damage (Fig. 1H).
The chromoshadow domain of HP1s interacts with the PxVxL motif in the BRCT domain of BARD1

HP1 proteins contain an N-terminal chromodomain and a C-terminal chromoshadow domain (Fig. 2A; refs. 26, 27). While the chromodomains interact with H3K9me2/3, the chromoshadow domain recognizes conserved PxVxL motifs (28–31). Therefore, we tested whether mutation of the W164 residue of HP1γ, a key residue required for the chromoshadow structure (27, 31, 32), would inhibit the interaction of BARD1 with HP1γ. HEK293T cells were cotransfected with BARD1-myc and wild-type StreptII-HP1γ or its W164A mutant. Whereas BARD1-myc was strongly detected in wild-type HP1γ pull-downs, it was only detected at the background level in W164A pull-down (Fig. 2B). Next, we mapped the HP1γ interaction sites in BARD1. Whereas full-length BARD1-myc was precipitated with HP1γ, neither BARD11–424 nor BARD11–1,649 was detectable, suggesting that BARD1 interacts with HP1γ via its BRCT domains (Fig. 2C). Consistent with the in vivo interaction, in vitro pull-down assays using purified proteins (Supplementary Fig. S2A) revealed that the BRCT domain interacts significantly with HP1γ, whereas ankyrin repeats and GST alone do not (Fig. 2D). SPR analyses with the purified BARD1–BRCT and HP1γ (Supplementary Fig. S2A and S2B) confirm the direct protein–protein interaction, which was disrupted by the W164A mutation in HP1γ (Fig. 2E). This was recapitulated using chromoshadow domain of HP1γ (Supplementary Fig. S3A and S3B).

Because the chromoshadow domain of HP1 recognizes PxVxL motifs, we searched for this motif in the BARD1 sequence and found that the BRCT domain contains PLVLI, which resembles PxVxL (Fig. 2A). Importantly, SPR analysis demonstrates that GST–BARD1–BRCT with the L570E/V571E (PEELI) or L570A/V571I (PAALI) mutation (Supplementary Fig. S2C) dramatically inhibited the interaction of BARD1–BRCT with HP1γ (Fig. 2F). Furthermore, the mutations effectively disrupted the interaction in vivo (Fig. 2G), whereas neither mutation affected BRCA1–BARD1 interaction (Supplementary Fig. S1B).

Because recognition of the PxVxL motif by the chromoshadow domain is conserved in the HP1 protein family, we tested other HP1s and found that HP1α and β were capable of interacting with BARD1–BRCT in a manner dependent on the PxVxL motif (Supplementary Fig. S3C–S3E). This indicates that the observed specificity of the interaction between endogenous HP1γ and BARD1 in vivo is not due to differences in its binding site compared with those of other HP1 family members. The results imply that HP1α and β may have redundant role for BARD1 interaction in vivo.

It has been reported that BRCA1 also physically interacts with HP1γ through multiple nonoverlapping regions comprising BRCA1 residues 260–553 (33) or 219–758, 758–1,057, and 1,443–1,649 (34). To further parse out the interaction between the HP1 family members and the BRCA1/BARD1 complex, we purified recombinant GST–BRCA1 fragments (Supplementary...
In our hands, BRCA1 fragments 262–552 and 504–803 interacted detectably with all 3 isoforms of HP1 but with much weaker affinities than between BARD1–BRCT and HP1s (Supplementary Fig. S4B–S4D).

PxVxL is critical for the IR-induced nuclear focus formation of BARD1

The identification of missense mutations of BARD1 that disrupt its binding to HP1 allowed us to test whether defective interaction would affect the cellular localization of BARD1 after IR. HEK293T cells expressing wild-type or mutants of Strepti-HP1γ and BARD1-Myc, as indicated, and subjected to StrepTactin pull-down followed by immunoblotting with the indicated antibodies. *, nonspecific products. Arrow, BARD1-Myc1-424. D, recombinant GST-tagged BARD1 fragments comprising ankyrin repeats (424–555) and BRCT (555–777) or GST alone were incubated with His-FLAG-HP1γ peptide and precipitated with anti-FLAG M2 Sepharose. Precipitates and input were immunoblotted using the indicated antibodies. *, nonspecific peptides. E, SPR analysis with wild-type or the W64A mutant of His-FLAG-HP1γ peptides (200 nmol/L) injected over immobilized GST–BARD1–BRCT peptides. F, SPR analysis with wild-type or the mutants of GST–BARD1–BRCT peptides (200 nmol/L) injected over immobilized His-FLAG-HP1γ peptides. G, HEK293T cells were transfected with the indicated plasmids and subjected to StrepTactin pull-down followed by immunoblotting with the indicated antibodies.

Together, the interaction with HP1γ is essential for the retention of BARD1 at DSB sites during later times of the DNA damage response. However, the BRCT domain of BARD1 recognizes PAR, and disruption of this interaction by a K619A mutation abolishes the rapid PAR-dependent recruitment of BARD1 to the DSB sites (20). In the crystal structure of the BARD1 tandem BRCTs, Ile573 of the PLVLI motif is within 3 Å of K619 in the putative PAR/phosphopeptide-binding site. However, only the PLV residues of the motif are exposed on the protein surface and they are on the opposite side of the BRCT structure from the phosphopeptide/PAR-binding surface (Fig. 3E). Nevertheless, we sought to assess whether the observed inhibition of BARD1 retention due to the PEELI and PAALI mutations resulted from the failure to interact with PAR by asking whether K619A–BARD1 inhibits IRIF formation. Importantly, K619A–BARD1 accumulated at the sites of DNA damage to the same extent as wild-type BARD1 (Fig. 3A, C and D), indicating that the PAR interaction is not required for BARD1 retention at later times after IR. Hence, the observed inhibition of BARD1 retention due to the PEELI and PAALI mutations cannot be attributed to a failure to interact with PAR.
PxVxL of BARD1 is critical for the IRIF formation of BRCA1.

Because isolated BRCT domains of BRCA1 accumulate at DSB sites (20, 35), we tested the possibility that the interaction between BARD1 and HP1 is dispensable for BRCA1 retention at DSB sites. To avoid the effect of endogenous BARD1, we established stable cells integrating doxycycline-inducible BARD1-specific shRNA together with the doxycycline-inducible wild-type or mutant BARD1-EGFP (r-BARD1 cells). The endogenous BARD1 was effectively replaced with exogenous BARD1 in a doxycycline-inducible manner (Fig. 4A). Endogenous BRCA1 accumulated at the DSB sites after laser microirradiation or IR in wild-type and K619A mutant of r-BARD1 cells, but the accumulation was dramatically reduced in PEELI- and PAALI-mutant cells (Fig. 4B–D). These results suggest that the BARD1/HP1 interaction is required for the stable retention of the BRCA1/BARD1 complexes at DSB sites.

The suppression of HP1 reduces BRCA1/BARD1 retention.

Because BARD1 interacts primarily with HP1γ among HP1 family members following DNA damage, inhibition of HP1γ may specifically affect BRCA1/BARD1 retention. However, inhibition of all 3 members of HP1 with siRNA can independently suppress BRCA1–IRIF formation and the suppression was only at mild levels (Supplementary Fig. S5A and S5B) compared with that induced by PxVxL mutants of BARD1. HP1γ-null mouse embryonic fibroblasts also exhibited only minimal effect (Supplementary Fig. S5C and S5D). Because BARD1 is capable of interacting with all 3 HP1 isoforms in a similar fashion in vitro, the discrepancy between the BARD1 mutants and HP1γ depletion for the level of foci reduction could be due to partial redundancy of the HP1 members. Indeed HP1α coimmunoprecipitated with BARD1 was increased by depletion of HP1γ (Supplementary Fig. S6). To overcome the redundancy, we established stable U2OS cells integrating three doxycycline-inducible constructs each expressing the shRNA specific to HP1a, b, or g (tnHP1), in addition to either single- or a/g double-negative cells (dnHP1). The targeted members of HP1 were effectively inhibited in a doxycycline-inducible manner, whereas BRCA1 and BARD1 were not affected by the doxycycline induction (Fig. 5A). Whereas the inhibition of each single HP1 exhibited a small or no effect consistent with the siRNA experiments, the IRIF formation of BRCA1/BARD1 was dramatically reduced in dnHP1 and tnHP1 cells to the level comparable with that induced by PxVxL mutations of BARD1 (Fig. 5B–E). These results confirm that the interaction of BARD1 and HP1 is crucial for the retention of the BRCA1/BARD1 complex at the DSB sites.

ATM, but not RNF168, pathway-dependent BARD1 retention by HP1γ.

After an initial dispersion step, HP1γ accumulates at sites of DNA damage within 5 minutes after the damage occurs (36–39). This HP1γ recruitment requires its chromoshadow domain (37), and BARD1 may be involved in the recruitment. However,
doxycycline-induced depletion of BARD1 did not affect the HP1γ accumulation at laser-microirradiated DSB loci (Supplementary Fig. S7). Thus, the interaction is important for the accumulation of BRCA1/BARD1 but not of HP1. BARD1 could be retained by anchoring to the newly recruited HP1.

Increase of the BARD1–HP1γ–H3K9me2 interaction was detectable after 30 min, peaked at 1 to 2 hours after IR and decreased thereafter (Fig. 6A). Considering the timing, the interaction may be dependent on ATM and the RNF8–RNF168–RAP80–Abraxas pathway. To examine this possibility, the ATM inhibitor KU55933 was added to HeLa cells before IR (Fig. 6B). The efficacy of the inhibitor was monitored by IR-induced phosphorylation of Chk1 (S317), Chk2 (T68), and RAP1 (S824), which were successfully inhibited. Importantly, the interaction of BARD1 with HP1γ and H3K9me2 was dramatically inhibited by the inhibitor. The result could be reproduced with anti-GFP immunoprecipitation from HeLa cells that stably expressed BARD1–EGFP (Supplementary Fig. S8). To further clarify the role of the ATM–RNF8–RNF168–RAP80 cascade in the BARD1–HP1 interaction, we inhibited RNF168 using siRNA. Quite unexpectedly, the interaction of BARD1 with HP1γ or H3K9me2 was dramatically increased, rather than being decreased, by depletion of RNF168 (Fig. 6C), indicating that BARD1 interacts with HP1γ and H3K9me2 in RNF168-independent manner.

The BRCA1/Abras/RAP80 complex functions to suppress excess DNA resection, whereas the BRCA1/CtIP complex performs the resection that leads to HR after DSB (15, 16). Therefore, we examined how BARD1–HP1 interaction affects these pathways. Using the wild-type or PEELI mutant of r-BARD1 cells, we analyzed the IRIF formation of CtIP and RAD51, an effector of HR. The CtIP–IRIF formation was dramatically suppressed in the PEELI-mutant cells (Fig. 6D and E), suggesting that BARD1–HP1 interaction is required for CtIP retention at DSB sites. Although depletion of BRCA1 significantly suppresses IRIF formation of RAD51, it was reported that depletion of RAP80 does not affect or even increases the RAD51 accumulation at DSB sites (15, 16). In contrast, RAD51–IRIF formation was significantly suppressed in the PEELI-mutant cells (Fig. 6D and E) and tNP1 cells (Supplementary Fig. S9A and S9B). The PEELI-mutant cells in G2 phase exhibited prolonged dissolution kinetics of γH2AX in later time points after IR, consistent with HR defect (Supplementary Fig. S10). Cell-cycle analyses indicated that the observed effects were not due to cell-cycle alteration (Supplementary Fig. S11A). Together, these results suggest that the complex containing BARD1 and HP1 is important for promoting HR. The BRCA1/CtIP complex protects DSB from NHEJ by blocking 53BP1 and its effector RIF1 in S- and G2-phases (40–43). Because BARD1–HP1 is required for the CtIP function, it would be possible that inhibition of BARD1–HP1 interaction may allow RIF1 to accumulate at DSB sites in S-phase. The wild-type or PEELI mutant of r-BARD1 cells were incubated with BrdUrd to label the cells in S-phase and exposed to IR. Whereas the RIF1–IRIF–positive fraction in the BrdUrd-positive cells was only 60.1% for wild-type cells, it was at the same high level (98.5%) as that in the BrdUrd-negative cells for the PEELI-mutant cells (Fig. 6F and G). The same results were observed for doxycycline-induced tNP1 cells (Supplementary Fig. S9C and S9D). Hence, loss of BARD1–HP1 interaction leads to ectopic RIF1 recruitment that indicates suppression of HR.

HKMT inhibitor disrupts the retention of BRCA1 and BARD1 at sites of DNA damage

The treatment of U2OS cells with UNC0638, an H3K9-specific HKMT inhibitor (44), reduced the H3K9me2 modification without affecting the steady-state levels of BARD1 and BRCA1 (Fig. 7A). Importantly, UNC0638 dramatically inhibited the IRIF formation of BRCA1 and BARD1, whereas it did not affect the IRIF
formation of 53BP1, RNF168, or RAP80 (Fig. 7B and C and Supplementary Fig. S12). Cell-cycle analyses showed that this effect was not due to G1 arrest of cells (Supplementary Fig. S11B). These results prompted us to test whether UNC0638 increases cytotoxic effect of a PARP inhibitor. The MCF7 cells were exposed to a range of doses of olaparib for 24 hours in the presence or absence of UNC0638. Clonogenic survival was synergistically reduced by the combined olaparib and UNC0638 at all doses (Fig. 7D). We also tested the effect of olaparib on r-BARD1–PEELI cells. The PEELI-mutant cells were more sensitive to olaparib than were the wild-type cells (Fig. 7E).

Discussion

In this study, we demonstrate that BARD1–BRCT interacts with H3K9me2 through HP1. This complex plays a critical role in retention of a BRCA1/BARD1 complex at the site of DNA damage. Together with another recently identified role (20), the BARD1–BRCT is required for both early recruitment and stable retention of the protein at DSB sites via its interaction with PAR and HP1, respectively. We showed that the K619A–BARD1 PAR-binding mutant was intact for the stable retention of BRCA1/BARD1. It has been reported that BARD1-null cells reconstituted with K619A–BARD1 exhibit proficient HR and some as yet unknown functions of BRCT domains are required for HR because BARD1-null cells reconstituted with BARD1 lacking BRCT domains (Δ602–777) fail to compensate for the loss of HR function (45). Although BARD1 Δ602–777 still contains the PxVxL motif it lacks its native structural context, which could well affect the HP1 interaction. Therefore, the interaction with HP1 may be the previously suggested "unknown function." An association of BRCA1 with HP1 has recently been reported (33, 34, 46). Depletion of HP1s reduces the accumulation of BRCA1 at DSBs and causes defects in HR (46). Loss of Wip1, a phosphatase that inhibits ATM signaling pathways, induces ATM activation and subsequent BRCA1–HP1γ interaction (34). However, the mechanism underlying BRCA1 recruitment to chromatin through HP1 is not yet understood. Because the HP1-binding fragments of BRCA1 previously determined do not contain PxVxL motifs, the interaction is likely to function differently from that mediated between BARD1 and HP1. In any case, it is clear from our results that a BRCA1–HP1 interaction is incapable of compensating for the BARD1–HP1 interaction in the context of BRCA1/BARD1 retention at the DSB sites.

It is notable that BRCA1 retention is so dramatically abrogated by mutants of BARD1 with disrupted HP1 binding, as isolated BRCA1–BRCT domains have been reported to accumulate at the DSB sites (20, 35). This apparent discrepancy suggests that the inhibition of BRCA1 retention at the DSB sites could be caused via its interaction with BARD1, which, when incapable of binding to...
HP1\textgreek{a}, may dissociate the BRCA1/BARD1 complex from the DSB site. Alternatively, BARD1–HP1 and Abraxas–RAP80 may mediate sequential retention of endogenous BRCA1 that can be overcome by overexpressed BRCA1–BRCT fragment.

The mechanism underlying the binding preference of BARD1 for HP1\textgreek{a}, which mainly acts in the facultative heterochromatin (25), over other HP1s is currently unknown. Because BARD1–BRCT interacts with HP1\textgreek{a} and \textgreek{b} in vitro in the same manner as with HP1\textgreek{g}, the observed specificity in vivo could be due to the different localization patterns of the HP1 proteins. The constitutive heterochromatin structure may reduce the accessibility of BARD1 to HP1\textgreek{a} and \textgreek{b}. We suggest the possibility that the BARD1–HP1 interaction, which occurs in response to DNA damage, plays a role in regions involving other HP1 proteins and H3K9me1/3. Because single HP1\textgreek{g} knockdown was not enough to reduce the BRCA1–BARD1–IRIF formation and additional knockdown of HP1\textgreek{a} and \textgreek{b} reduced the foci dramatically, some redundancy among the isoforms is implied for BARD1 binding in vivo.

It is particularly interesting that the interaction of BARD1 with HP1\textgreek{g} and H3K9me2 is dependent on ATM, but not on RNF168. Disruption of the BARD1–HP1 interaction resulted in ectopic RIF1–IRIF in S-phase cells, suggesting that the BRCA1 that accumulates at DSB sites via BARD1–HP1 is distinct from that in the BRCA1/Abraxas/RAP80 complex, as the latter is recruited via ATM–RNF8–RNF168-mediated polyubiquitin chains and suppresses excessive DNA end processing (15, 16). Instead, BARD1–HP1 interaction is critical for the retention of CtIP, which promotes DNA-end resection and RIF1 inhibition. An important candidate for an ATM substrate that may play a role in the BARD1–HP1 interaction is KAP1, a PxVxL motif containing transcriptional repressor, which is required for recruitment of RAD51 at DSB sites (47, 48). Interestingly, dynamic depletion and re-accumulation of KAP1 and H3K9me2 at DSB sites was recently reported (49, 50). The BARD1–HP1 interaction may be involved in this process. A possible model is that the HP1-interacting BRCA1/BARD1 complex reinforces DNA end resection and HR, and BRCA1/Abraxas/RAP80 complex sequesters the resection thereafter by recruiting BRCA1 from the HP1-interacting complex (Supplementary Fig. S13). Disruption of BARD1–HP1 interaction or inhibition of H3K9me2 therefore profoundly

Figure 6. ATM, but not RNF168, pathway-dependent BARD1 retention by HP1. A, solubilized chromatin fractions prepared from HeLa cells harvested at the indicated time points after IR were immunoprecipitated with anti-BARD1 antibody and subjected to immunoblotting with the indicated antibodies. Inputs (1.5%) were also loaded. B, HeLa cells treated with 10 μmol/L of ATM inhibitor KU55933 or DMSO for 30 minutes were exposed to IR or left unexposed, and the solubilized chromatin fractions were immunoprecipitated with anti-BARD1 antibody and subjected to immunoblotting with the indicated antibodies. C, HeLa cells stably expressing BARD1–EGFP were transfected with RNF168-specific (+) or control (−) siRNA. Solubilized chromatin fractions were prepared after IR, immunoprecipitated with anti-GFP antibody, and subjected to immunoblotting with the indicated antibodies. D, wild-type and PEELI mutant of r-BARD1 cells induced with doxycycline were fixed 1 or 3 hours after IR and immunostained for CtIP or RAD51, respectively. E, quantification of the cells displaying more than 5 CtIP foci or 10 RAD51 foci is shown. Error bars, SD of two independent experiments, each based on more than 100 cells. F, wild-type or PEELI mutant of r-BARD1 cells induced with doxycycline were incubated with BrdUrd for 30 minutes, exposed to IR, and immunostained for RIF1 and BrdUrd. Cells positive for the BrdUrd stain are highlighted with dashed lines in the RIF1 stain of the wild-type cells. G, quantification of the cells displaying more than five RIF1 foci per BrdUrd-positive or -negative cell shown in F. Error bars, SD of three independent experiments, each based on more than 100 cells.
reduces the retention of BRCA1, including that in the BRCA1/Abraxas/RAP80 complex. Supporting the model HKMT inhibitor UNC0638 exerts a synergistic effect with a PARP inhibitor to arrest cellular growth. In conclusion, we have defined the mechanism for BRCA1/BARD1 retention at DSB sites through H3K9 methylation. This complex may provide a new target for cancer therapy.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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