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demonstrates that GST-BARD1-BRCT with the L570E/V571E (PEELI) or L570A/V571A (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-E). 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 non-overlapping regions comprising BRCA1 residues 260-553 (33) or 219-758, 758-1057 and 1443-1649 (34). To further parse out the interaction between the HP1 family members and the BRCA1/BARD1 complex, we purified recombinant GST-BRCA1 fragments (Supplementary Fig. S4A) and tested their association with HP1s by SPR. In our hands, BRCA1 fragments 262-552 and 504-803 interacted detectably with all three isoforms of HP1, but with much weaker affinities than between BARD1-BRCT and HP1s (Supplementary Fig. S4B-D).

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

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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 BARD1-myc demonstrated the formation of IRIF, which co-localized with γH2AX (Fig. 3A and C). Notably, the PEELI and PAALI mutation dramatically inhibited IRIF formation. The same results were observed for BARD1 fragments 1-424 and 1-555. The IRIF formations four hours after IR demonstrated similar results (Fig. 3B). The results were recapitulated with laser-microirradiation of U2OS and HeLa cells that stably express wild-type or mutant BARD1-EGFP (Fig. 3D).

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

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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 Dox-inducible BARD1-specific shRNA together with the Dox-inducible wild-type or mutant BARD1-EGFP (r-BARD1 cells). The endogenous BARD1 was effectively replaced with exogenous BARD1 in a Dox-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 three members of HP1 with siRNA can independently suppress BRCA1-IRIF formation and the suppression was only at mild levels (Supplementary Fig. S5A and B) compared to that induced by PxVxL mutants of BARD1. HP1γ-null mouse embryonic fibroblasts also exhibited only minimal effect (Supplementary Fig. S5C and

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D). Because BARD1 is capable of interacting with all three 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 co-immunoprecipitated with BARD1 was increased by depletion of HP1γ (Supplementary Fig. S6). To overcome the redundancy, we established stable U2OS cells integrating three Dox-inducible constructs each expressing the shRNA specific to HP1α, β or γ (tnHP1), in addition to either single- or α/γ-double-negative cells (dnHP1). The targeted member(s) of HP1 were effectively inhibited in a Dox-inducible manner whereas BRCA1 and BARD1 were not affected by the Dox 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 BRCA1/BARD1 complex at the DSB sites.

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

After an initial dispersion step, HP1s accumulate at sites of DNA damage within 5 minutes after the damage occurs (36-39). This HP1 recruitment requires its chromoshadow domain (37) and BARD1 may be involved in the recruitment. However, Dox-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.

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Increase of the BARD1/HP1γ/H3K9me2 interaction was detectable after 30 min, peaked at one to two 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 KAP1 (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/Abrasas/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

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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 tnHP1 cells (Supplementary Fig. S9A and B). 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 BrdU to label the cells in S-phase, and exposed to IR. Whereas the RIF1-IRIF-positive fraction in the BrdU-positive cells was only 60.1% for wild-type cells, it was at the same high level (98.5%) as that in the BrdU-negative cells for the PEELI mutant cells (Fig. 6F and G). The same results were observed for Dox-induced tnHP1 cells (Supplementary Fig. S9C and D). 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

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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, 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 function(s) 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

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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, 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 HP1γ, which mainly acts in the facultative heterochromain (25), over other HP1s is currently unknown. Because BARD1-BRCT interacts with HP1α and β *in vitro* in the same manner as with

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HP1γ, 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 HP1α and β. 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 HP1γ knockdown was not enough to reduce the BRCA1/BARD1-IRIF formation and additional knockdown of HP1α and β 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 HP1γ 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 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

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complex (Supplementary Fig. S13). Disruption of BARD1/HP1 interaction or inhibition of H3K9me2 therefore profoundly reduces the retention of BRCA1 including that in 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.

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AUTHOR CONTRIBUTIONS

T.O. and W.W. designed the research. W.W., H.N., T.F., and T.O. conducted the experiments. VV. and R.E.K. analyzed the structures. M.A. and Y.M. contributed reagents. T.O. wrote the manuscript, with help from all authors.

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FIGURE LEGENDS

Figure 1. BARD1 interacts with histone H3K9me2 via HP1γ in response to DNA damage.

(A) BARD1 immunocomplex precipitated from the solubilized chromatin fraction of HeLa cells after IR or mock treatment was immunoblotted with the indicated antibodies. Inputs (1.5%) were also loaded. (B and C) The BARD1 immunocomplex was precipitated as in (A) with or without competing antigen peptide (pep) (B) or from indicated cells (C). (D) HeLa cells were treated with 5 μM CPT-11 or vehicle, and the BARD1 immunocomplex was precipitated as in (B). (E and F) The BARD1 immunocomplex was subjected to immunoblotting with the indicated HP1 antibodies (E) with or without competing antigen peptide (F). (G) HeLa cells transfected with two different (#1 and #2) HP1γ-specific or control (CTR) siRNAs were harvested after IR. The BARD1 immunocomplex was subjected to immunoblotting as in (A). (H) HEK-293T cells were co-transfected with BARD1-Venus (C) and non-fused Venus (N) or Venus (N)-HP1γ, as indicated. Cells were laser-microirradiated and subjected to γ-H2AX immunostaining to examine Venus fluorescence and its colocalization with γ-H2AX.

Figure 2. The chromoshadow domain of HP1 interacts with the PxVxL motif in the BRCT domain of BARD1.

(A) Schematic representation of BARD1 and HP1γ. The residue(s) critical for BARD1/HP1γ interaction in each protein are shown. (B and C) HEK-293T cells were

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transfected with plasmids expressing wild-type or mutants of StrepII-HP1γ and BARD1-Myc, as indicated, and subjected to StrepTactin pulldown followed by immunoblotting with the indicated antibodies. * indicates non-specific products. The arrow indicates BARD1-Myc$^{1\text{-}424}$. (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. * indicates non-specific peptides. (E) SPR analysis with wild-type or the W164A mutant of His-FLAG-HP1γ peptides (200 nM) injected over immobilized GST-BARD1-BRCT peptides. (F) SPR analysis with wild-type or the mutants of GST-BARD1-BRCT peptides (200 nM) injected over immobilized His-FLAG-HP1γ peptides. (G) HEK293T cells were transfected with the indicated plasmids and subjected to StrepTactin pulldown followed by immunoblotting with the indicated antibodies.

Figure 3. HP1 interaction is required for the stable retention of BARD1 at sites of DNA damage.

(A and B) HEK293T cells were transfected with either wild-type or the indicated mutants of BARD1-myc, exposed or not to IR and immunostained with anti-γH2AX and anti-myc antibodies 1 hour (A) or 4 hours (B) after IR. (C) Quantification of the cells displaying more than five BARD1-myc foci per BARD1-myc-expressing cell in (A). Error bars, S.D. of three independent experiments, each based on more than 100 cells. (D) U2OS or HeLa cells stably expressing either wild-type or indicated mutants of BARD1-EGFP were laser-microirradiated. The retention of BARD1-EGFP at the sites of DNA damage was

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detected by fluorescence. (E) Crystal structure of BARD1-BRCT domains is shown in surface representation. Highlighted in red are residues of the PLVLI (putative PxVxL) motif of BARD1. K619 is highlighted in blue. Surface shown in magenta is the predicted phosphopeptide binding surface based on a structural alignment of the BARD1-BRCT structure (PDB #3fa2) and the BRCA1-BRCT with BACH1 phosphopeptide bound (PDB #1t29). Note that K619 is part of the phosphopeptide/PAR binding surface, which is on the opposite face of the protein structure from the PxVxL motif. *Right* The structural model rotated 45°.

Figure 4. BARD1 mutants that do not bind HP1 disrupt stable retention of BRCA1 at sites of DNA damage.

(A) HeLa cells conditionally expressing shRNA to BARD1 together with wild-type or mutants of shRNA-insensitive BARD1-EGFP (r-BARD1 cells) were induced or not with Dox for 48 hours and subjected to immunoblotting with the indicated antibodies. exo: exogenous BARD1-EGFP, endo: endogenous BARD1. (B and C) Cells from (A) were induced with Dox, and either microirradiated (B) or exposed to IR (C), and after 1 hour immunostained for BRCA1 with γH2AX. (D) Quantification of the cells displaying more than ten BRCA1 foci in (C). Error bars, S.D. of two independent experiments, each based on more than 50 cells. Note that relatively high percentage of BRCA1 foci positive fraction in wild-type and K619A cells were due to high sensitivity setting of the analyzer. PEELI and PAALI cells showed dramatically low level in such a setting.

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Figure 5. Inhibition of all three isoforms of HP1 disrupts stable retention of BRCA1/BARD1 at sites of DNA damage.

(A) U2OS cells conditionally expressing shRNA to either single (shHP1α, shHP1β and shHP1γ), double (dnHP1) or triple (tnHP1) isoform(s) of HP1 were induced or not with Dox and subjected to immunoblotting with the indicated antibodies. (B and C) The tnHP1 cells from (A) were induced with Dox, exposed to IR and immunostained with anti-γH2AX and anti-BARD1 (B) or anti-BRCA1 antibody (C). (D) Quantification of the cells displaying more than ten BARD1 or BRCA1 foci in (B) and (C). Error bars, S.D. of two independent experiments, each based on more than 70 cells. (E) The mean numbers of foci per cell were also shown, together with dhHP1 cells or cells with knockdown of each single member of shHP1. 1st, 2nd: Data from each independent experiment.

Figure 6. ATM- but not RNF168 pathway-dependent BARD1 retention by HP1γ

(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 μM 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

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mutant of r-BARD1 cells induced with Dox were fixed one or three hours after IR and immunostained for CtIP or RAD51, respectively. (E) Quantification of the cells displaying more than five CtIP foci or ten RAD51 foci is shown. Error bars, S.D. of two independent experiments, each based on more than 100 cells. (F) Wild-type or PEELI mutant of r-BARD1 cells induced with Dox were incubated with BrdU for 30 minutes, exposed to IR and immunostained for RIF1 and BrdU. Cells positive for the BrdU 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 BrdU-positive or -negative cell shown in (F). Error bars, S.D. of three independent experiments, each based on more than 100 cells.

Figure 7. H3K9-specific HKMT inhibitor UNC0638 disrupts BRCA1/BARD1 retention at sites of DNA damage.

(A and B) U2OS cells treated with DMSO (control: C) or 3 μM UNC0638 (U) for 24 hours were immunoblotted with indicated antibodies (A), or exposed to IR and immunostained with indicated antibodies (B). The yellow-framed squares show an enlarged view. Note that BARD1 and BRCA1 do not merge with γH2AX with UNC0638. (C) Quantification of the cells displaying more than ten BARD1 or BRCA1 foci. Error bars, S.D. of two independent experiments, each based on more than 70 cells. (D and E) MCF7 cells exposed to olaparib and UNC0638 for 24 hours (D), or r-BARD1-wild-type and -PEELI mutant cells exposed to olaparib for 24 hours (E), were analyzed for the clonogenic survival. The data are shown with the nonlinear regression fit curves of one phase decay (GraphPad Prism). Average ± S.E.M. normalized to cells without olaparib

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were derived from triplicate experiments. Statistical significances were calculated using the two-way ANOVA. * $P<0.0001$.

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