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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 ATM-dependent 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 non-homologous end joining, at damaged loci in S phase. UNC0638, a small molecule inhibitor of histone lysine methyltransferase (HKMT), abolished retention and cooperated with the poly(ADP-ribose) polymerase 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.

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 (DSBs) 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) (4). BRCA1-BRCT interacts with phosphorylated Abraxas, promoting a BRCA1/Abraxas (FAM175A)/RAP80 (BRCA1-A) complex that is retained at the damaged chromatin via the RNF8/RNF168-mediated polyubiquitin chains (5-14). However, the role of 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 poly(ADP-ribose) (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, that disrupts the PAR interaction, abolish 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 anti-cancer 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 shRNA in a doxycycline (Dox)-inducible manner were established by the lentiviral infection of CS-RfA-ETBsd-shBARD1, CS-RfA-ETBsd-shHP1γ (CBX3),

CS-RfA-ETPuro-shHP1α (CBX5), or CS-RfA-ETHygro-shHP1β (CBX1) (See Supplementary Materials and Methods) followed by selection with blasticidin, puromycin, or hygromycin, respectively. Cells expressing multiple Dox-inducible shRNAs (dnHP1 and tnHP1) were established by the multiple lentivirus infections and multiple antibiotic selections. For the Dox-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 Dox 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 one hour before analysis unless otherwise indicated. For 5'-bromo-2-deoxyuridine (BrdU) staining of the S-phase cells, the cells were incubated with 30 μM BrdU 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, 10^7 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 mM 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 (SPR) 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 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 BrdU, cells were treated with 2N HCl for 10 minutes and neutralized with 50mM Tris-HCl [pH 9.0] twice for 5min. For staining of CtIP (RBBP8), cells were treated with PBS containing 0.7% Triton X100, 3% paraformaldehyde and 2% sucrose for 30min followed by 5min incubation with PBS containing 0.2% Triton X100. 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 μM BrdU 12 hours prior to irradiation. Laser microirradiation was performed one hour before analysis using a PALM UV-A pulsed

nitrogen laser (100 Hz, λ=355 nm; P.A.L.M. Microlaser, Bernried, Germany) mounted on an AxioObserver Z1 microscope (Zeiss) on a custom-designed granite plate.

Plasmids, lentivirus, RNA interference, antibodies, purified proteins, chemical agents, and clonogenic survival assay are described in the 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 co-precipitation 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 three members: HP1α, β and γ. Strikingly, HP1γ readily co-precipitated 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 co-expressed, suggesting a direct interaction between BARD1 and HP1γ (Supplementary Fig. S1A). Additionally, we co-transfected 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 γH2AX, 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) (26, 27). While the chromodomain interacts 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 co-transfected with BARD1-myc and wild-type StrepII-HP1γ or its W164A mutant. Whereas BARD1-myc was strongly detected in wild-type HP1γ pull-downs, it was only detected at the back ground 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 BARD1$^{1-424}$ nor BARD1$^{1-555}$ was detectable, suggesting that BARD1 interacts with HP1γ via its BRCT domains (Fig. 2C). Consistent with the *in vivo* interaction, *in vitro* pulldown 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 B) confirm the direct protein-protein interaction, which was disrupted by the W164A mutation in HP1γ (Fig. 2E). This was recapitulated using choromoshadow domain of HP1γ (Supplementary Fig. S3A and B).

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