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RNF8 Regulates Assembly of RAD51 at DNA Double-Strand Breaks in the Absence of BRCA1 and 53BP1

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Abstract

The tumor suppressor protein BRCA1 localizes to sites of DNA double-strand breaks (DSB), promoting repair by homologous recombination through the recruitment of DNA damage repair proteins. In normal cells, homologous recombination largely depends on BRCA1. However, assembly of the pivotal homologous recombination regulator RAD51 can occur independently of BRCA1 in the absence of 53BP1, another DNA damage response protein. How this assembly process proceeds is unclear, but important to understand in tumor cell settings where BRCA1 is disabled. Here we report that RNF8 regulates BRCA1-independent homologous recombination in 53BP1-depleted cells. RNF8 depletion suppressed the recruitment of RAD51 to DSB sites without affecting assembly or phosphorylation of the replication protein RPA in neocarzinostatin-treated or X-ray-irradiated BRCA1/53BP1-depleted cells. Furthermore, RNF8/BRCA1/53BP1-depleted cells exhibited less efficient homologous recombination than BRCA1/53BP1-depleted cells. Intriguingly, neither RNF8 nor its relative RNF168 were required for RAD51 assembly at DSB sites in 53BP1-expressing cells. Moreover, RNF8-independent RAD51 assembly was found to be regulated by BRCA1. Together, our findings indicate a tripartite regulation of homologous recombination by RNF8, BRCA1, and 53BP1. In addition, our results predict that RNF8 inhibition may be a useful treatment of BRCA1-mutated/53BP1^{low} cancers, which are considered resistant to treatment by PARP1 inhibitors and of marked current clinical interest. *Cancer Res; 72(19); 4974–83. ©2012 AACR.*

Introduction

DNA double-strand breaks (DSB) are repaired by 2 major systems: nonhomologous end joining (NHEJ) and homologous recombination. NHEJ is an intrinsically error prone repair system that operates throughout the cell cycle. Homologous recombination is an error-free repair system, but it is limited to the late S and G_2 phases because the replicated DNA strand is used as a template (1). Homologous recombination is initiated by nucleolytic degradation of the DSB ends to generate a 3′ single-stranded DNA (ssDNA) overhang (2, 3). The resultant ssDNA is immediately coated by replication protein A (RPA). Breast cancer, early onset 2 (BRCA2) then promotes the displacement of RPA from the ssDNA by RAD51 to form a RAD51-ssDNA filament. RAD51 searches for homologous DNA sequences in a sister chromatid and promotes DNA strand invasion. The homologous DNA sequence then serves as template for the synthesis of new DNA. During homologous recombination, BRCA1 is also recruited to DSBs, where it promotes efficient RAD51 alignment (2). In addition to DSB repair, homologous recombination is also required for the repair of spontaneous DNA breaks, and defective homologous recombination induces severe genomic instability. Germline mutations in the *BRCA1* or *BRCA2* genes increase the risk of early-onset breast and ovarian cancers (2, 4). Because patient tumor cells often possess 2 mutant alleles of *BRCA1* or *BRCA2* and show defective homologous recombination, inactivation of either BRCA1- or BRCA2-dependent homologous recombination is thought to trigger tumorigenesis.

Upon DNA DSB introduction, the following processes occur: the histone H2AX is phosphorylated by ataxia telangiectasia mutated (ATM); the mediator of DNA damage checkpoint 1 (MDC1) binds to the phosphorylated H2AX (γH2AX); and ATM phosphorylates MDC1 at the region surrounding the DSB. The E3 ubiquitin ligase really interesting new gene (RING) finger protein 8 (RNF8) binds to phosphorylated MDC1 at DSB sites and promotes the recruitment of another E3 ubiquitin ligase, RNF168. RNF8 and RNF168 conjugate Lys 63-linked ubiquitin chains onto histone H2A with their cognate E2 ubiquitin-conjugating enzyme UBC13 and induce chromatin remodeling (5–11). Recent studies showed that RNF8 and RNF168 also ubiquitinate non-histone substrates, which are also important for DSB signaling (12–15). UBC13-RNF8/RNF168-dependent ubiquitination promotes the recruitment of BRCA1 and p53-binding protein 1 (53BP1), another DNA damage response factor that is recruited to DSBs (6–14, 16; Supplementary Fig S1). A large proportion of the BRCA1 that localizes to DSB sites is a component of the BRCA1-A complex, consisting of a

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BRCA1/BARD1 heterodimer, the ubiquitin interacting motif (UIM)-containing protein RAP80, deubiquitinating enzyme BRCC36, an adaptor protein ABRAXAS, BRCC45, and MERIT40 (2, 5–11, 17–19). RAP80 interacts with the Lys 63-linked chain that is generated by UBC13-RNF8/RNF168 and brings BRCA1 to DSB sites (2, 5–11, 17–20; Supplementary Fig S1A). This complex does not directly interact with the DSB ends and does not promote homologous recombination (21, 22). A small subset of BRCA1 forms protein complexes with homologous recombination factors such as BACH1, TopBP1, CtIP, MRE11, RAD50, NBS1, and RAD51, localizes to DSB sites independently of RAP80 and promotes DNA end resection, RAD51 assembly, and homologous recombination (2, 21, 22; Supplementary Fig. S1A and S1B). Chicken UBC13-knockout DT40 cells show defective RAD51 assembly at DSB sites and inefficient homologous recombination (5). Moreover, the overexpression of the deubiquitinating enzyme OTU domain, ubiquitin aldehyde binding 1 (OTUB1), which suppresses DNA damage-dependent chromatin ubiquitination through noncatalytic inhibition of UBC13 activity, suppresses homologous recombination (23). These data strongly suggest that DNA damage-dependent ubiquitination is crucial for homologous recombination. However, it is not known whether UBC13-RNF8/RNF168-dependent ubiquitination is required for homologous recombination and the RAP80-independent recruitment of BRCA1.

Cells have multiple DNA repair systems other than homologous recombination. These systems work redundantly, each operating to repair DNA in the event that other repair systems are ineffective. PARP1 is required for efficient single-strand break repair and has a role at stalled replication forks (4). When PARP1 is pharmacologically inhibited, DNA damage is repaired through homologous recombination in normal cells. In contrast, homologous recombination-defective cells, such as BRCA1- or BRCA2-negative breast cancer cells, are intrinsically sensitive to PARP1 inhibitors. Therefore, PARP1 inhibition induces synthetic lethality in BRCA1- or BRCA2- negative cancer cells with mild side effects, and this synthetic lethality is exploited in the clinical setting (4). However, a recent study predicts that some of BRCA1-negative cancer cells are resistant to PARP1 inhibition. This study revealed that *BRCA1* mutations and low-53BP1 expression are correlated with poor patient prognosis (24). In an experimental setting, loss of *53BP1* restores homologous recombination in BRCA1-deficient murine cells and renders them insensitive to PARP1 inhibitors. (24, 25; Supplementary Fig. S1C). These data suggest the existence of a molecule that regulates BRCA1-independent homologous recombination in the absence of 53BP1, and if such a model exists, the molecule regulating BRCA1-independent homologous recombination may be a therapeutic target for the treatment of cancer cells with *BRCA1* mutation and low-53BP1 expression.

In this study, we analyzed the role of RNF8 in RAD51 assembly at DSB sites and the subsequent homologous recombination. Depletion of RNF8 or RNF168 did not affect the RAD51 assembly at DSB sites after X-ray irradiation. This RNF8-independent RAD51 assembly was abolished by BRCA1 depletion. In contrast, depletion of either RNF8 or RNF168 strongly suppressed RAD51 assembly in BRCA1/53BP1-deplet-

ed cells. Furthermore, homologous recombination, as measured by a direct repeat GFP (DR-GFP) reporter (26) was also significantly suppressed in RNF8/BRCA1/53BP1-depleted cells, although BRCA1/53BP1-depleted cells showed a normal level of homologous recombination efficiency. Our data indicate that RAD51-dependent homologous recombination is regulated by RNF8, RNF168, BRCA1, and 53BP1. Our findings also suggest that inhibition of the activity of RNF8 or RNF168 can suppress BRCA1-independent homologous recombination in 53BP1^{low} tumor cells.

Materials and Methods

Cell culture
HCT116 human colon carcinoma cells, HeLa cells, RNF8$^{-/-}$ mouse embryonic fibroblasts (MEF) and wild type (WT) MEFs were grown in Dulbecco's Modified Eagle's Medium with 10% FBS. U2OS human osteosarcoma cells were grown in McCoy's 5A medium with 10% FBS. HCT116 and *RAD18*$^{-/-}$ HCT116 cells were a gift form Drs. N. Shiomi and H. Shiomi (27). HeLa cells were obtained from the Health Science Research Resources Bank. U2OS cells were obtained from the American Type Culture Collection (HTB-96).

RNA interference
We used the following siRNAs: siBRCA1 #1: GGAACCUGU-CUCCACAAAG-dTdT (28); siBRCA1 #2: UCACAGUGUCCUU-UAUGUA-dTdT (29); si53BP1: GAAGGACGGAGUACUAAUA-dTdT; siUBC13: Dharmacon siGENOME SMARTpool; siRNF8 #D: Dharmacon siGENOME SMARTpool; siRNF8 #2: GGA-GAUAGCCCAAGGAGAA-dTdT (22; this siRNA sequence was not included in siRNF8 #D); siRNF168 #5: GACACUUUCUC-CACAGAUA-UU; siRNF168 #C: GGCGAAGAGCGAUGGAAGA-dTdT (12); and siRAP80: CCAGUUGGAGGUUUAUCAA-dTdT (22). Nontargeting control siRNA (siCTRL) was purchased from Sigma-Aldrich (Mission SIC-002). For the simultaneous depletion of RNF8, RNF168, BRCA1, and 53BP1, siRNF8 (#D or #2), siRNF168 (#5 or #C), siBRCA1 (#1 or #2), and si53BP1 were mixed to final concentrations of 18 nmol/L, 18 nmol/L, 36 nmol/L, and 4.5 nmol/L, respectively. The total siRNA amount was adjusted to be the same in each sample by adding siCTRL. All RNAi transfections were carried out using Lipofectamine RNAiMAX (Invitrogen).

Generation of a siRNA-resistant RNF8-expressing vector
To generate RNF8 constructs resistant to siRNF8#2, we introduced the following underlined silent mutations in RNF8: GGAGATAGCCCA GGGC GAG.

Immunofluorescence microscopy
HCT116 and 293T cells were grown on MAS-coated No. 1 glass coverslips (Matsunami). HeLa and U2OS cells were grown on No. 1 glass coverslips (Fisher Scientific). The cells were fixed by incubation in 3% paraformaldehyde and 2% sucrose in PBS for 15 minutes at room temperature. They were permeabilized by incubation in 0.5% Triton X-100 in PBS for 15 minutes at room temperature. For BRCA1 immunofluorescence assays, the cells were preextracted with 0.2% Triton X-100 in PBS for

5 minutes on ice and fixed using 3% paraformaldehyde and 2% sucrose in PBS. After fixation, the cells were washed with PBS 3 times and blocked with 2% bovine serum albumin (BSA) in PBS for 1 hour. The cells were then stained with the indicated antibodies, which were diluted in 2% BSA in PBS. For RPA immunofluorescence assays, the cells were preextracted with 0.5% Triton X-100 in CSK buffer [20 mmol/L Hepes (pH7.4), 50 mmol/L NaCl, 3 mmol/L MgCl$_2$, 300 mmol/L sucrose] for 5 minutes on ice and fixed using 3% paraformaldehyde and 2% sucrose in PBS. After fixation, cells were washed with PBS 3 times and blocked with 3% skim milk in 0.05% Tween-20 in PBS for 1 hour. The cells were then stained with anti-RPA antibodies, which were diluted in 1% BSA in PBS. The cells were washed with PBS twice and stained with Alexa Fluor 488 goat anti-mouse immunoglobulin G (IgG; Invitrogen), Alexa Fluor 555 goat anti-rabbit IgG (Invitrogen), or CF555 goat anti-chicken IgY (Biotium). The cells were washed with 2% BSA in PBS and PBS alone 2 times each. DNA was counterstained with 4′, 6-diamidino-2-phenylindole (0.2 μg/mL) in PBS, and samples were mounted with Prolong Gold mounting reagent (Invitrogen). Confocal images were captured using an inverted microscope (TCS SP5, Leica) equipped with a 63x oil immersion lens. A minimum of 300 cells or 100 53BP1 focus-negative cells (RNF168-knockdown) were analyzed per experiment. Images were acquired in LAS AF (Leica) format. Images were adjusted and combined using Photoshop (Adobe).

Western blotting

Cells were lysed in SDS sample buffer. Whole-cell lysates were separated using SDS-PAGE (5–20% e-PAGEL, ATTO) and transferred to nitrocellulose membranes using the iBlot Gel Transfer System (Invitrogen). Nitrocellulose membranes were blocked with 5% Difco skim milk (BD Biosciences) in TBST, blotted with the indicated primary antibodies and blotted with horseradish peroxidase (HRP)-conjugated secondary antibodies. The Western Lightning ECL Pro Reagent Kit (Perkin-Elmer) was used to detect chemiluminescent HRP-conjugated antibodies. Chemiluminescent signals were detected using a LAS4000 (Fuji Film) or ChemiDoc XRS$^+$ (BioRad).

Antibodies

We used the following primary antibodies: RAD51 (70-001 lot 1, Bio Academia, 1:20,000 for immunofluorescence and 1:2,000 for Western blotting; a gift from Dr. Kurumizaka, 1:4,000 for immunofluorescence; 70-005, Bio Academia, 1:2,000 for immunofluorescence), 53BP1 (clone 19, BD Biosciences, 1:5,000 for immunofluorescence and 1:2,000 for Western blotting), γ-H2AX (clone JBW301, Upstate, 1:5,000 for immunofluorescence), conjugated ubiquitin (clone FK2, Nippon Bio-Test Laboratories, 1:20,000 for immunofluorescence), RNF8 (B-2, Santa Cruz, 1:100 for Western blotting), BRCA1 (D-9, Santa Cruz, 1:200 for Western blotting; C-20, Santa Cruz, 1:100 for immunofluorescence), α-tubulin (T6074, Sigma-Aldrich, 1:1,000 for Western blotting), phospho-RPA32 Ser4/8 (A300-245A, Bethyl Laboratories, 1:1,000 for Western blotting), RPA32 (RPA34-19, Calbiochem, 5 μg/mL for immunofluorescence and Western blotting), UBC13 (ab25885, abcam,

1:1000), RAP80 (EPR5315, Epitomics, 1:10,000 for Western blotting), and RNF168 [RNF168-C (9), a gift from Dr. Durocher].

DR-GFP assay

A HeLa clone carrying the DR-GFP homologous recombination reporter was used for the analysis of homologous recombination. For analyses with siRNA, cells were transfected with the indicated siRNA and cultured in 6-well dishes for 48 hours. The cells were then transfected with 2 μg of pCBASce, the I-SceI expression plasmid using FuGene HD (Roche). The cells were collected 48 hours post-pCBASce transfection via trypsinization, washed twice with PBS, resuspended in 0.1% FBS/PBS, and filtered. The proportion of GFP-positive cells was determined using flow cytometry with a FACSCalibur flow cytometer (BD Biosciences).

Pharmacologic inhibition

Cells were incubated with 10 μmol/L KU55933 (Calbiochem) and 10 μmol/L NU7026 (Sigma) or 10 μmol/L MG132 (Sigma) for 1 hour before irradiation.

DNA damage

Cells were exposed to X-rays using an MBR-1520RX-ray irradiator (Hitachi Medico) or were treated with neocarzinostatin (Sigma-Aldrich).

Results

UBC13 is required for RAD51 assembly at DSB sites, but RNF8 and RNF168 are not

UBC13 is an essential E2 ubiquitin-conjugating enzyme for RAD51 assembly at DSBs (5). Upon DSBs formations, UBC13 cooperates with E3 ubiquitin ligases (RNF8 and RNF168) to generate Lys 63-linked ubiquitin chains on damaged chromatin (2, 6–11). Therefore, we predicted that not only UBC13 but also RNF8 and RNF168 would be required for RAD51-dependent homologous recombination. To test this hypothesis, UBC13, RNF8, or RNF168 was depleted from U2OS cells by using siRNA transfection (Supplementary Fig. S2) and RAD51 accumulation at DSB sites was analyzed by immunofluorescence. Cells transfected with UBC13-, RNF8-, or RNF168-specific siRNAs showed a complete deficiency of 53BP1 focus formation at DSB sites, indicating effective knockdown of these genes (Fig. 1A). RAD51 focus formation was also defective in UBC13-depleted cells (Fig. 1A and B). Nevertheless, RAD51 was recruited to DSB sites in RNF8- or RNF168-depleted cells (Fig. 1A and B). To further confirm the RNF8-independent recruitment of RAD51 to DSB sites, RNF8$^{-/-}$ MEFs and matched WT MEFs were treated with neocarzinostatin, a compound that specifically induces DSBs, and stained with anti-RAD51 antibody. RAD51 foci were observed in RNF8$^{-/-}$ MEFs, but these foci were smaller than those observed in WT MEFs (Supplementary Fig. S3). Thus, UBC13 is essential for RAD51 assembly at DSB sites, but RNF8 and RNF168 are not.

BRCA1 regulates RAD51 assembly in RNF8-depleted cells

BRCA1 forms several independent protein complexes. The BRCA1-A complex binds through the 2 UIMs of RAP80 to the
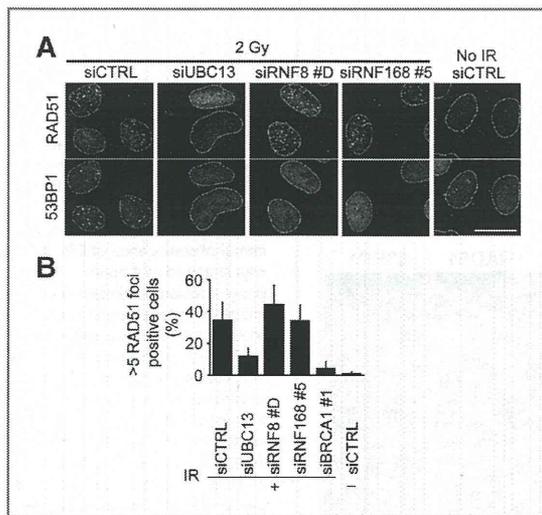
Figure 1. UBC13, but not RNF8 or RNF168, is required for RAD51 assembly at DSB sites. A and B, U2OS cells transfected with the indicated siRNAs were irradiated with 2 Gy and the immunofluorescence of RAD51 and 53BP1 was analyzed at 6 hours postirradiation. A, representative images. Nuclei are indicated by lines. Scale bar, 25 μm. B, the quantification of cells with more than 5 RAD51 foci (mean ± SD; $N = 3$). IR, irradiation.

RNF8/RNF168-generated Lys 63-linked ubiquitin chain (2, 6–8, 11, 17–20). Therefore, the irradiation-induced foci (IRIF) of BRCA1 are significantly diminished in RNF8- and RNF168-depleted cells. However, other BRCA1 complexes are recruited to DSB sites in a RAP80-independent manner (21, 22), and these complexes promote RAD51 assembly at DSB sites. These results imply that a small subset of BRCA1 protein might be recruited to DSB sites in the absence of RNF8 (Supplementary Fig. S1D and S1E). To test this possibility, we conducted a preextraction before cell fixation to detect a small amount of chromatin-bound BRCA1 accumulation. RNF8- or RAP80-depleted cells contained BRCA1 foci, but these foci were smaller than the foci observed in control cells (Fig. 2A and Supplementary Fig. S2B). This result was consistent with the observation that BRCA1, but not 53BP1 or conjugated ubiquitin, weakly accumulates at microlaser-generated DSB tracks in RNF8-depleted cells (7) and RNF168-depleted cells (10). These data led us to hypothesize that the small amount of BRCA1 at DSB sites might promote RAD51 assembly in RNF8-depleted cells (Supplementary Fig. S1E). As a result, we analyzed the impact of BRCA1 depletion on RNF8-depleted cells. The effective and simultaneous knockdown of RNF8 and BRCA1 was confirmed using Western blotting (Fig. 2B). The depletion of BRCA1 significantly inhibited RAD51 assembly in HCT116 cells, indicating that BRCA1 knockdown was sufficient to inhibit BRCA1-dependent RAD51 assembly (Supplementary Fig. S1B). The depletion of BRCA1 significantly suppressed irradiation-induced RAD51 assembly in RNF8-depleted cells (Fig. 2C and D). As cell-cycle distribution was comparable among cells transfected with various combinations of nontargeting, BRCA1- and RNF8-specific siRNAs, it

is unlikely that the percentage of RAD51 focus-positive cells was affected by the ratio of cells in S-G_2 phase (Supplementary Fig. S4A). These results indicate that BRCA1 promotes RAD51 assembly independently of RNF8-dependent chromatin ubiquitination.

RAD51 is recruited to DSB sites in BRCA1/53BP1-depleted human cells

Loss of 53BP1 IRIF restores RAD51 assembly in cells derived from BRCA1 hypomorphic ($BRCA1^{\Delta11/\Delta11}$) mice (24, 25; see also Supplementary Fig. S1C). In contrast, depletion of RNF8 from BRCA1-depleted human HCT116 cells resulted in loss of 53BP1 IRIF, but this depletion did not restore RAD51 foci (Fig. 2C and Supplementary Fig. S1F). These data appear to be contradictory. This discrepancy may result from differences between murine and human cells. Therefore, we next examined whether the simultaneous silencing of 53BP1 and BRCA1 gene expression using siRNAs could rescue the loss of RAD51 foci in BRCA1-depleted human cells. To avoid off-target siRNA effects, 2 different BRCA1-specific siRNAs were used for the experiments. The silencing of 53BP1 in the BRCA1-depleted cells restored RAD51 assembly at DSB sites (Supplementary Fig. S5A and S5B). The efficiency of BRCA1 knockdown in cells concomitantly transfected with 53BP1- and BRCA1-specific siRNAs was equivalent to the knockdown efficiency in cells transfected with both the nontargeting siRNA and the identical BRCA1-specific siRNA (Supplementary Fig. S5C). Furthermore, the restored RAD51 focus formation was not due to a change in the cell-cycle distribution (Supplementary Fig. S4B). These data indicate that BRCA1 is dispensable for RAD51 assembly at DSB sites in 53BP1-depleted human cells, reproducing previous results found using $BRCA1^{\Delta11/\Delta11}$ murine cells (24, 25).

RNF8-dependent chromatin ubiquitination is required for RAD51 assembly in BRCA1/53BP1-depleted cells

No BRCA1 or 53BP1 focus formation was observed in either BRCA1/53BP1-depleted cells or RNF8/BRCA1-depleted cells. However, these cells showed a sharp contrast in the efficiency of RAD51 assembly at DSB sites: 53BP1/BRCA1-depleted cells displayed efficient RAD51 IRIF whereas RNF8/BRCA1-depleted cells did not (Supplementary Fig. S1C and S1F). These results suggest that RAD51 assembly in BRCA1/53BP1-depleted cells may depend on RNF8. To test this hypothesis, RNF8 was concomitantly depleted with BRCA1 and 53BP1 in HCT116 cells, and the impact of RNF8 depletion on RAD51 assembly at DSB sites in BRCA1/53BP1-depleted cells was monitored. The effective and simultaneous depletion of RNF8, BRCA1, and 53BP1 was confirmed using Western blotting (Fig. 3A), and the effective knockdown of RNF8 was also confirmed in terms of DNA damage response signaling by the observation of defective conjugated-ubiquitin IRIF, as visualized using the FK2 antibody (Fig. 3B). The depletion of RNF8 from BRCA1/53BP1-depleted cells did not reduce the amount of S-G_2 phase cells (Supplementary Fig. S4C) or affect the amount RAD51 protein (Fig. 3A), but it strongly suppressed RAD51 assembly at DSB sites (Fig. 3B and C). Two different RNF8-specific siRNAs showed a similar inhibitory effect on RAD51 IRIF in BRCA1/53BP1-depleted cells, excluding the possibility of off-target
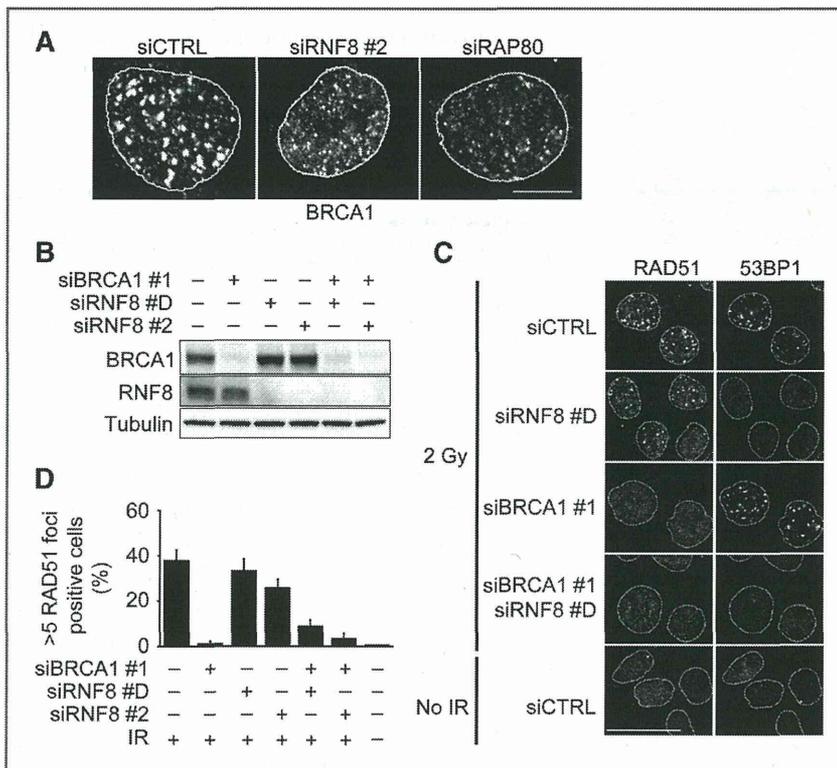
Figure 2. RNF8-independent RAD51 assembly is regulated by BRCA1. A, HeLa cells transfected with the indicated siRNAs were irradiated with 2 Gy, and the immunofluorescence of BRCA1 was analyzed at 4 hours postirradiation. Representative images. Nuclei are indicated by lines. Scale bar, 10 µm. B–D, HCT116 cells transfected with the indicated siRNAs were subjected to Western blotting for the indicated proteins (B) or irradiated with 2 Gy, and the immunofluorescence of RAD51 and 53BP1 was analyzed at 6 hours postirradiation. Representative images. Nuclei are indicated by lines. C, scale bar, 25 µm. D, the quantification of cells with more than 5 RAD51 foci (mean ± SD; N = 3). IR, irradiation.

effects of the RNF8-specific siRNAs. The depletion of RNF8 also suppressed RAD51 IRIF in BRCA1/53BP1-depleted HeLa and 293T cells (Supplementary Fig. S6).

RNF8 is recruited to DSB sites through binding to phospho-MDC1 with its forkhead-associated (FHA) domain; the protein then ubiquitinates the chromatin surrounding the DSBs through a mechanism that depends on its RING finger domain (6–8). To explore the roles of RNF8 in RAD51 assembly at DSB sites in the absence of BRCA1 and 53BP1, we simultaneously knocked down RNF8, BRCA1, and 53BP1 and transiently reintroduced the following siRNA-resistant forms of RNF8 into 293T cells: WT GFP-RNF8, FHA mutant (RNF8^{R42A}), and RING mutant (RNF8^{C403S}). The cells were irradiated and processed for RAD51 immunofluorescence. In this assay, we only analyzed cells with weak GFP-RNF8 expression because overexpression of GFP-RNF8 suppressed RAD51 assembly even in control cells. Neither the reintroduction of RNF8^{C403S} nor RNF8^{R42A} restored RAD51 assembly efficiency to the levels observed with WT RNF8 (Fig. 3D and E). These data strongly suggest that RNF8-dependent ubiquitination is required for BRCA1-independent RAD51 assembly in 53BP1-depleted cells.

The recruitment of RNF8 to DSB sites is dependent on ATM (6–8). This led us to examine whether the inhibition of ATM suppressed RAD51 assembly at DSB sites in BRCA1/53BP1-depleted cells. The activities of ATM and DNA-PK are redundant (30); therefore, we treated cells with a combination of an ATM-specific kinase inhibitor KU55933 and a DNA-PK-specific

inhibitor NU7026. The pharmacologic inhibition of the kinase activities of ATM and DNA-PK suppressed RAD51 focus formation in BRCA1/53BP1-depleted cells. The suppressive effect on RAD51 assembly observed in control cells was less efficient than the suppressive effect observed in BRCA1/53BP1-depleted cells (Fig. 4A and B). These data most likely indicate that the suppressive effect of RNF8 depletion on RAD51 IRIF formation is restricted to BRCA1-depleted cells (Figs. 1–3). In contrast, MG132, a protein that inhibits ubiquitin recycling and ubiquitination-dependent DSB signaling (31), completely inhibited RAD51 focus formation in both BRCA1/53BP1-depleted and control cells (Fig. 4A and B). This inhibition most likely occurred because the UBC13-dependent ubiquitination pathway is indispensable for RAD51 assembly (Fig. 1; refs. 5, 32).

We next monitored homologous recombination to obtain more direct evidence for the role of RNF8 in BRCA1-independent homologous recombination. To measure frequency of homologous recombination, we used DR-GPF assay (26). In this assay, a direct repeat of a full-length GFP mutated to contain a restriction enzyme (I-SceI) recognition site and a 5′- and 3′-truncated GFP were integrated into the genome of HeLa cells. A DSB was generated at the I-SceI site by transfection of I-SceI expression vectors. Cells that repaired the DSB by homologous recombination using 5′- and 3′-truncated GFP as a template expressed WT GFP and exhibited green fluorescence beyond levels of autofluorescence. Compared with control siRNA transfected cells, the depletion of BRCA1 led to a

Figure 3. RNF8 regulates RAD51 assembly in BRCA1/53BP1-depleted cells. A–C, HCT116 cells transfected with the indicated siRNAs were subjected to Western blotting for the indicated proteins (A) or irradiated with 2 Gy and analyzed for RAD51 and conjugated ubiquitin (FK2) or γH2AX immunofluorescence at 6 hours postirradiation. Representative images. Nuclei are indicated by lines. B, scale bar, 25 μm. C, the quantification of cells with more than 5 RAD51 foci (mean ± SD; N = 3). D and E, siRNA-resistant GFP-RNF8 WT, R42A, or C403S was reintroduced into 293 T cells transfected with siRNF8#2, siBRCA1#1, and si53BP1 or siCTRL. The cells were irradiated with 2 Gy and analyzed for RAD51 immunofluorescence at 4 hours postirradiation. Representative images. D, scale bar, 25 μm. E, the quantification of cells with more than 5 RAD51 foci and weak GFP-RNF8 expression (mean ± SD; N = 3). *, nonspecific bands. IR, irradiation.
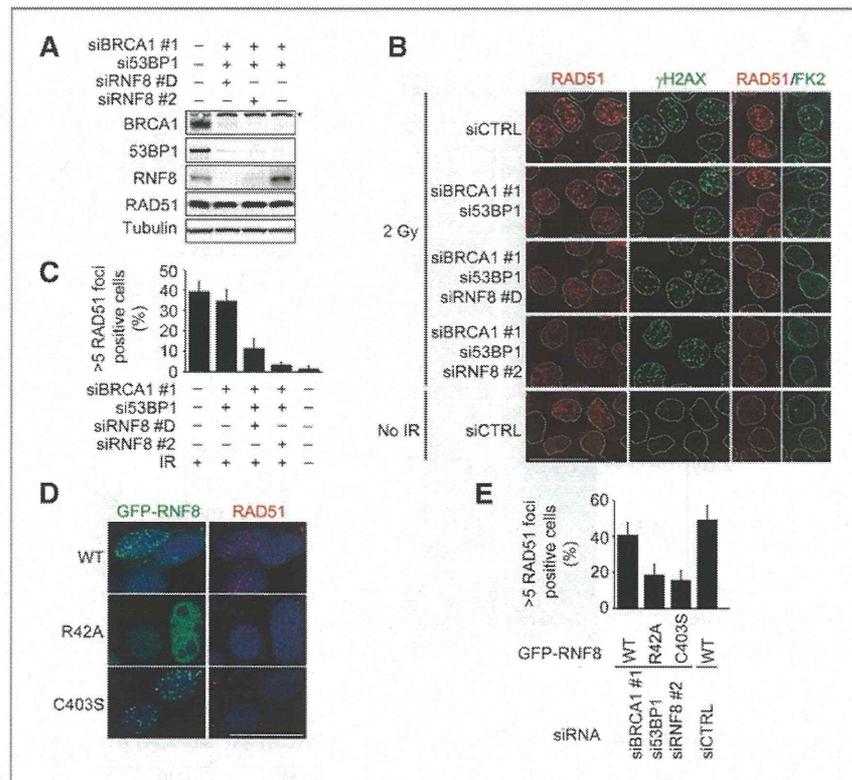
significant reduction in the frequency of GFP-positive cells and the concomitant depletion of 53BP1 and BRCA1 did not reduce the frequency of GFP-positive cells. However, when RNF8, BRCA1, and 53BP1were concomitantly depleted, the frequency of GFP positive cells was decreased as comparable to BRCA1-depleted cells (Fig. 4C and D). Thus, RNF8 regulates homologous recombination in BRCA1/53BP1-depleted cells.

RNF168 is required for RAD51 assembly in BRCA1/53BP1-depleted cells

The E3 ligase activity of RNF8 is a prerequisite for RNF168/UBC13-mediated Lys 63-linked ubiquitin chain formation on histone H2A (9, 10) and RNF168-dependent turnover of histone demethylase JMJD2A, JMJD2B (14), and a polycomb protein L3MBTL1 (13) at DSB sites. On the other hand, RNF8 but not RNF168 is required for Lys 48-linked ubiquitin chain formation, which promotes KU80 degradation (15). Therefore, we examined whether RNF168 was required for RAD51 IRIF formation in BRCA1/53BP1-depleted cells. The protein levels of RNF168 were significantly suppressed when cells were concomitantly transfected with RNF168-, BRCA1-, and 53BP1-specific siRNAs (Supplementary Fig. S2). However, this RNF168 reduction was not sufficient to completely inhibit the 53BP1 IRIF that is mediated by RNF168. Thus, we analyzed RAD51 IRIF in cells without 53BP1 foci. Two different RNF168-specific siRNAs (#5 and #C) were used for this experiment, and both siRNAs clearly

suppressed RAD51 IRIF in BRCA1/53BP1-depleted cells (Fig. 5A and B). These data indicate that both RNF8 and RNF168 promote RAD51 assembly in BRCA1/53BP1-depleted cells.

RNF8 is not required for DNA end resection in BRCA1/53BP1-depleted cells

RAD18 is an important E3 ubiquitin ligase for postreplication repair at stalled replication forks (11). In addition, RAD18 binds to RNF8-dependent ubiquitin chains at irradiation-induced DSBs, recruits RAD51C to DSB sites and promotes homologous recombination when cells are exposed to irradiation. The recruitment of RAD18 to DSB sites is independent of BRCA1 (33). Therefore, we tested whether RAD18 is required for RNF8-dependent RAD51 assembly in BRCA1/53BP1-depleted cells. $RAD18^{-/-}$ HCT116 cells (27) showed clear irradiation-induced RAD51 foci after the depletion of BRCA1 and 53BP1: this focus formation was comparable to $RAD18^{+/+}$ HCT116 cells (Supplementary Figs. S5 and S7), suggesting that RAD18 is dispensable for the BRCA1-independent assembly of RAD51.

During homologous recombination, DSB edges are resected to form a 3′ overhang. RPA is loaded onto the ssDNA, phosphorylated and displaced from single-stranded DNA by RAD51 (3). BRCA1 promotes induction of the ssDNA overhang (34), and 53BP1 inhibits the resection (25). Therefore, BRCA1-depleted cells show defective homologous recombination, and
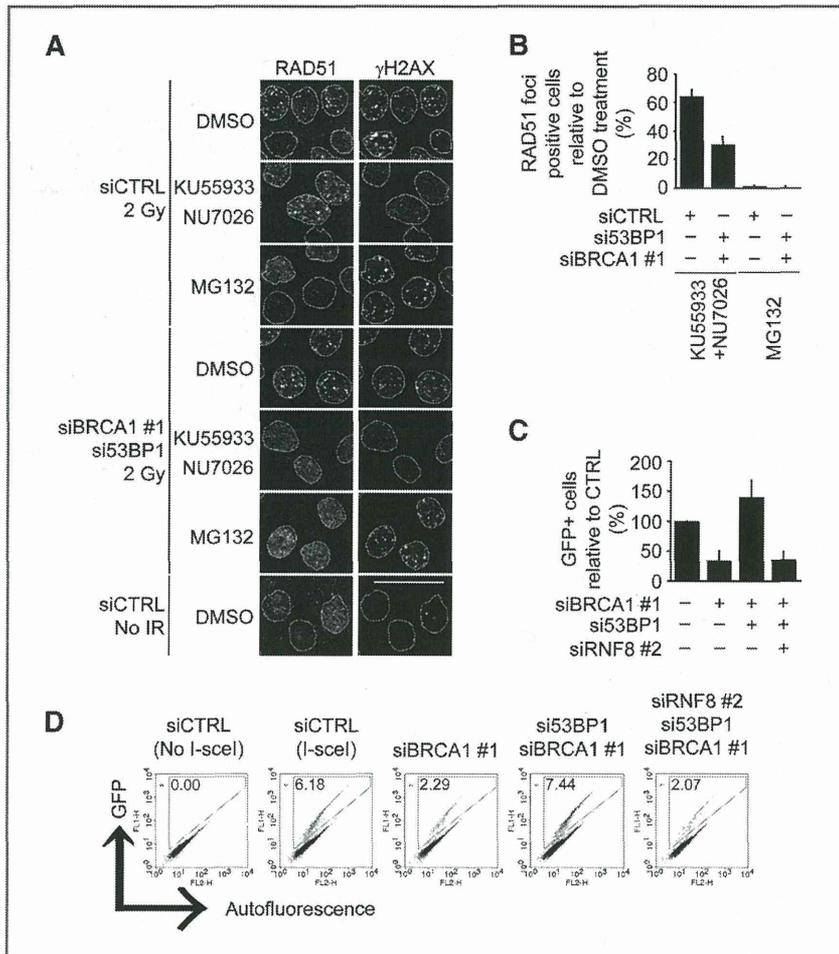
Figure 4. RNF8 regulates homologous recombination in BRCA1/53BP1-depleted cells. A and B, HCT116 cells transfected with the indicated siRNAs were treated with KU55933+NU7026, MG132, or dimethyl sulfoxide for 1 hour and then irradiated with 2 Gy and analyzed for RAD51 and γH2AX immunofluorescence at 4 hours postirradiation. Nuclei are indicated by lines. A, scale bar, 25 μm. B, the quantification of cells with more than 5 RAD51 foci (mean ± SD; N = 3). C and D, cells transfected with the indicated siRNAs were subjected to the DR-GFP assay. The quantification of cells with GFP expression (mean ± SD; N = 3). D, representative data from flow cytometry. DMSO, dimethyl sulfoxide; IR, irradiation

knockdown of 53BP1 restores homologous recombination in BRCA1-depleted cells. Intriguingly, RPA formed foci in neocarzinostatin-treated RNF8/BRCA1/53BP1-depleted cells (Fig. 6A). Furthermore, substantial levels of phosphorylated RPA were detected not only in control or BRCA1/53BP1-depleted cells but also in RNF8/BRCA1/53BP1-depleted cells after irradiation (Fig. 6B). These data indicate that RNF8 is required for RAD51 assembly but not for DNA end resection and RPA assembly at DSB sites in BRCA1/53BP1-depleted cells.

Discussion

DNA damage-dependent chromatin ubiquitination has an important role as a signal transducer in the DNA DSB response. The depletion of RNF8 or RNF168 suppresses the accumulation of 53BP1 and BRCA1 at DSB sites and induces an aberrant G_2–M checkpoint and hyper-radiosensitivity (6–10). Furthermore, biallelic mutations in RNF168 have been found in radiosensitive primary immunodeficiency (RIDDLE syndrome or RNF168 deficiency) patients (9, 35, 36), and RNF8-knockout

mice display decreased levels of serum IgG, chromosomal aberration, radiosensitivity, impaired spermatogenesis, and increased cancer predisposition (37–39). Owing to their physiologic importance, the roles of RNF8 and RNF168 in DNA repair are being extensively studied. One important role of RNF8-/RNF168-dependent chromatin ubiquitination in DNA repair is the fine-tuning of DNA end resection. The K63-linked ubiquitin chain generated by RNF8 and RNF168 promotes the recruitment of the BRCA1-A complex through the tandem UIM of RAP80 by specific binding to the Lys 63-linked ubiquitin chain (6–10, 17–20); this chain retains excess BRCA1 at a position slightly distant from the edges of DSBs and suppresses excessive DSB end processing (21, 22). In contrast, BRCA1 also forms complexes with the homologous recombination factors and accumulates at DSB sites in a RAP80-independent manner (2). In addition to the generation of Lys 63-linked ubiquitin chains at DSB sites, RNF8 plays other roles in DNA repair. RNF8 and RNF168 promote ubiquitination-dependent turnover of JMJD2A, JMJD2B (14), and L3MBTL1 (13) at DSB sites. The loss of chromatin-bound JMJD2A, JMJD2B, and L3MBTL1 exposes

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Figure 5. RNF168 regulates RAD51 assembly in BRCA1/53BP1-depleted cells. A and B, HCT116 cells transfected with the indicated siRNAs were irradiated with 2 Gy and analyzed for RAD51 and 53BP1 immunofluorescence at 6 hours postirradiation. Representative images. Nuclei are indicated by lines. A, scale bar, 25 μm. The quantification of cells with more than 5 RAD51 foci (mean ± SD; N = 3). B, cells with small 53BP1 foci (transfected with siCTRL, si53BP1, and siBRCA1) or cells without 53BP1 foci (transfected with siRNF168, si53BP1 and siBRCA1) were analyzed.
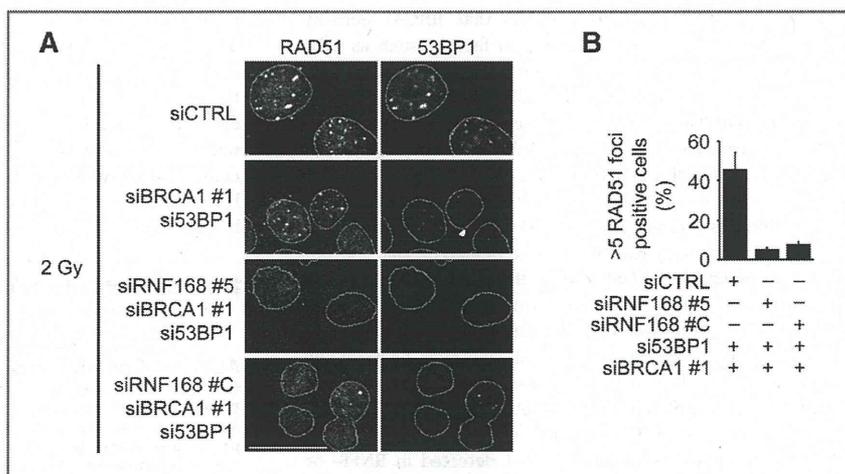
Lys-20 di-methylated histone H4 and promotes the binding of 53BP1 to the Lys-20 di-methylated histone H4 at DSB sites. RNF8 also promotes the degradation of KU80 at DSB sites (15) and RNF4-dependent/proteasome-mediated turnover of MDC1 and RPA at DSB sites (40). The degradation of KU80 at DSB sites facilitates NHEJ (15). The depletion of RNF4 results

in decreased efficiency of homologous recombination and NHEJ. Furthermore, RNF8 recruits a chromatin remodeling factor, chromodomain helicase DNA-binding protein 4 (CHD4), independently of its E3 ubiquitin ligase activity and the canonical phosphoprotein-binding property of the FHA domain and thereby promotes chromatin decondensation (16).

In this study, we showed that RNF8 promotes RAD51 assembly at DSB sites in BRCA1/53BP1-depleted cells (Fig 3), although Lys 63-linked ubiquitination is dispensable for RAD51 assembly in the presence of BRCA1. The accumulation of RPA at neocarzinostatin-induced DSBs and the irradiation-induced phosphorylation of RPA (Fig. 6) suggest that RNF8 is not required for nuclease accessibility to DSBs in BRCA1/53BP1-depleted cells. These data indicate that RNF8 functions downstream of RPA and upstream of RAD51-ssDNA filament formation during homologous recombination signaling. RNF8 is likely to promote the displacement of RPA by RAD51. We propose 2 models that explain the phenomenon: (i) RNF8/RNF168-dependnet histone H2A ubiquitination induces local structural changes in chromatin and create a space allowing RPA displacement by RAD51 in BRCA1/53BP1-depleted cells. (ii) Some substrates, which restrict the displacement of RPA by RAD51, may be ubiquitinated by RNF8 and/or RNF168, and removed from DSB sites by VCP/p97, proteasome or others, as recent studies revealed that RNF8- and/or RNF168-dependent ubiquitination induces protein turnover at DSB sites (12–15, 40). Even in the case where RNF8 have such functions, their functions are not obvious in the presence of BRCA1. BRCA1 probably has ability to promote RAD51 assembly without the support of RNF8 and RNF168. The requirement of RNF8 E3 ubiquitin ligase activity for RAD51 assembly in BRCA1/53BP1-depleted cells suggests that RNF8/CHD4-dependent chromatin unfolding (16) is not sufficient for the displacement of RPA by RAD51.

BRCA1-independent DNA end resection is restricted by 53BP1, but end resection functions properly when the restriction imposed by 53BP1 is lifted. Therefore, in the absence of 53BP1, BRCA1 is not required for DNA end resection and
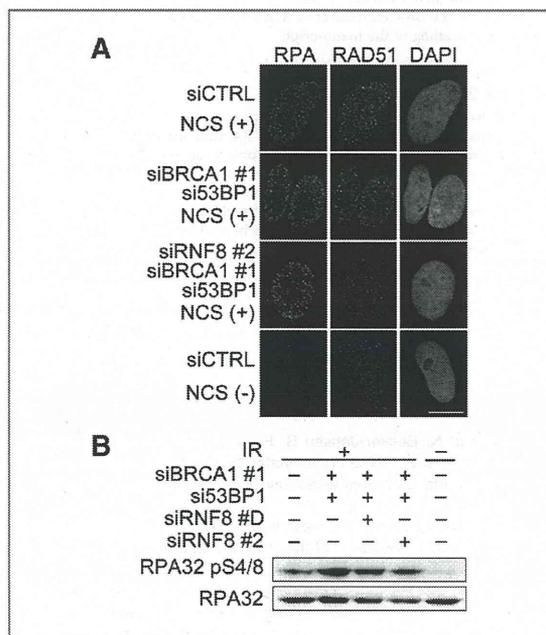

Figure 6. RNF8 promotes the displacement of RPA by RAD51 after DNA end resection in BRCA1/53BP1-depleted cells. A, HeLa cells transfected with the indicated siRNA were treated with 100 ng/mL NCS for 15 minutes and then subjected to immunofluorescence with the indicated antibodies at 5 hours post-neocarzinostatin treatment. Scale bar, 10 μm. B, HCT116 cells transfected with the indicated siRNAs were irradiated with 20 Gy and subjected to Western blotting for the indicated proteins at 4 hours postirradiation.

RAD51 assembly (25). This suggests that BRCA1 actively promotes the recruitment of resection factors, such as CtIP and MRE11, to overcome the jamming effect of 53BP1. In RNF8- or RNF168-depleted cells, 53BP1 is not recruited to DSB sites. Therefore, DNA end resection is not inhibited by 53BP1. Nevertheless, RAD51 assembly requires BRCA1 in RNF8-depleted cells. Furthermore, RNF8-/BRCA1-/53BP1-depleted cells do not show RAD51 assembly at DSB sites. These findings suggest that BRCA1 also actively promotes RPA displacement by RAD51 in RNF8-depleted cells.

RAD51 assembles at DSB sites in the absence of chromatin ubiquitination by RNF8 and RNF168 at DSB sites (Figs. 1 and 2). Nevertheless, ubiquitination is an essential posttranslational modification for RAD51 assembly at DSB sites because the depletion of UBC13 and the inhibition of ubiquitin recycling using MG132 completely suppress RAD51 IRIF (Fig. 4; ref. 32). These findings suggest that another E3 ligase may weakly (because ubiquitin conjugation is not detected in RNF8- or RNF168-depleted cells) ubiquitinate some substrates, whose ubiquitination is required for RAD51 assembly at the damaged chromatin in the absence of either RNF8 or RNF168. One of the candidate E3s is BRCA1. BRCA1 forms a ubiquitin chain at sites of DNA damage (41) and ubiquitinates histone H2A to maintain heterochromatin structure (42). Nevertheless, the E3 ligase activity of BRCA1 is not required for RAD51 assembly at DSB sites in ES cell (43). In addition, the E3 ubiquitin ligase activity of BRCA1 is not required for tumor suppression, but the BRCA1-BARD1 interaction through the RING domain is important for this activity (44, 45). These lines of evidence do not support the conclusion that BRCA1-dependent ubiquitination promotes homologous recombination in the presence of RNF8. However, we can speculate a model in which BRCA1 and RNF8 are redundant for the ubiquitination of some proteins and that BRCA1-dependent ubiquitination gains in importance in homologous recombination only when RNF8 is depleted.

Our data predict that the pharmacologic inhibition of RNF8 or RNF168 would suppress homologous recombination only in BRCA1-mutated/53BP1^{low} cancer cells but not in healthy cells. In addition, ATM/DNA-PK inhibition would suppress homologous recombination more effectively in BRCA1-mutated/53BP1^{low} cancer cells than in healthy cells. If these predictions are correct, the combination of DNA-damaging agents and the inhibition of RNF8, RNF168, or ATM may be useful as a cancer therapy. However, RNF8 is required for resistance not only to irradiation (6–8) but also to hydroxyurea and aphidicolin treatment (46). Therefore, a careful examination should be conducted to determine which DNA-damaging agents can be combined with RNF8 inhibition for cancer chemotherapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: S. Nakada
Development of methodology: S. Nakada
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): S. Nakada
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): S. Nakada
Writing, review, and/or revision of the manuscript: S. Nakada, K. Matsuo
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): S. Nakada, R.M. Yonamine

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