Analysis of Tumor Suppressor Gene Using Molecular Imaging for Personalized Medicine

Natsuko Chiba

Associate Professor
Department of Molecular Immunology, Institute of Development, Aging and Cancer
E-mail: nchiba@idac.tohoku.ac.jp

Abstract

BRCA1 is the first susceptibility gene to be linked to breast and ovarian cancer. Although mounting evidence has indicated that BRCA1 participates in the cellular responses to repair DNA double-strand breaks (DSBs), its precise mechanism is still unclear. To elucidate the molecular function of BRCA1 as a tumor suppressor, we have analyzed the various functions of BRCA1, such as apoptosis, DNA repair, and cell cycle regulation.

Recently, we are analyzing the cellular response of BRCA1 to various DNA damage using molecular imaging technique. We examined in situ response of BRCA1 accumulation at DSBs produced by laser micro-irradiation. The amino (N) and the carboxyl (C)-terminal fragments of BRCA1 accumulated independently at DSBs with distinct kinetics. The N-terminal BRCA1 fragment accumulated immediately after laser-irradiation at DSBs. In contrast, the C-terminal fragment of BRCA1 accumulated more slowly at DSBs. Interestingly, rapid accumulation of the BRCA1 N-terminus, but not the C-terminus, at DSBs depended on the non-homologous end-joining (NHEJ) factor, Ku80, independently BARD1. Two small regions in the N-terminus of BRCA1 independently accumulated at DSBs and interacted with Ku80. Missense mutations found within the N-terminus of BRCA1 in cancers significantly reduced the accumulation at DSBs. P142H mutant failed to associate with Ku80 and restore resistance to irradiation in *BRCA1*-deficient cells. These results suggest important roles of the N-terminus of BRCA1 in the early response to DSBs and carcinogenesis and that different interaction of BRCA1 with other repair factors at damaged sites influence its multifunctional roles in DNA repair.

On the other hand, BRCA1-deficient cells are sensitive to UV irradiation and BRCA1 localizes at nuclear foci after UV irradiation, although UV irradiation does not seem to induce DSBs directly. To analyze further the response of BRCA1 to UV irradiation, we generated local UV irradiation in cell nuclei using an isopore membrane filter. BRCA1 immediately accumulated at the site of local UV irradiation and the accumulation was abrogated by treatment cells with transcription inhibitors. Interestingly, the BRCA1 accumulation was dependent on the presence of the protein, which is involved in the nucleotide excision repair (NER) pathway. Furthermore, we found that BRCA1 is associated with this protein. These results suggest that BRCA1 is involved in TCR process for the DNA damage induced by UV irradiation.

Furthermore, we have identified new BRCA1 associated proteins by proteomic study and are examining the functions of those proteins.

These precise analyses of BRCA1 and its related proteins will contribute to the development of novel molecular diagnosis and therapies for personalizhed medicine for breast and ovarian cancer.

1. Intoroduction

Approximately 5% of breast cancers show a familial pattern of occurrence. In 1990, genetic studies provided initial evidence that the risk of breast cancer in some families is liked to chromosome 17q21. In 1994, *the breast-cancer susceptibility gene 1, BRCA1* was identified by positional cloning [1]. Our research has been focused on BRCA1. This gene encodes1863 amino acids, and more than 200 different germline mutations associated with cancer susceptibility have been identified. Mutation carriers of BRCA1 also have increased susceptibility to ovarian cancers. The risk of breast cancer developing is about 50-80% and the risk of ovarian cancer is 12-60% in carrier of *BRCA1* mutations. *BRCA1*-related breast cancers are characterized by early onset, a high frequency of bilateral involvement, and poor prognosis.

Somatic *BRCA1* mutations are rarely observed in sporadic breast cancer. However, both *BRCA1* mRNA and protein expression are down regulated in approximately 30% of sporadic breast cancer and 70% of ovarian cancers. It is believed that this may be due to non-mutational mechanism such as acquired methylation of the promotor or malfunctions in the upstream pathway that regulate BRCA1 expression. Furthermore, gene expression analysis revealed that basal-like subtype, which is characterized as resistance to hormonal therapy and trastuzumab and poor prognosis, show similar profile with BRCA1-related cancers. Therefore, lower expression or function of BRCA1 is thought be an important contributing factor in sporadic cancers.

BRCA1 contains a RING domain in the amino (N)-terminus and two tandem BRCT domains in the carboxy (C)-terminus. BRCT domain is frequently found in DNA repair proteins and function as a binding module for phospho-serine peptides. BRCA1 has also nuclear export signal (NES), nuclear

localization signal (NLS), and DNA binding region. After DNA damage BRCA1 is phosphorylated by Chk2 or ATM (Fig. 1).

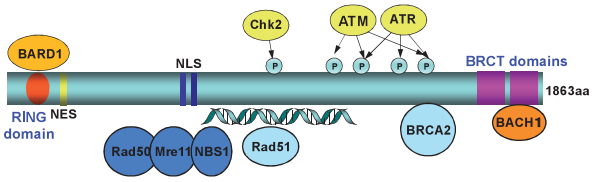

Fig. 1. Structure of BRCA1 proteins. BRCA1 contains the N-terminal RING domain, nuclear export signal (NES), nuclear localization signal (NLS), DNA binding region, and two C-terminal BRCT domains. Sites phosphorylated by Chk2 or ATM are indicated (red arrows).

The N-terminal region of BRCA1 directly interacts with BARD1 and the association with BARD1 enhances the ubiquitin polymerase activity of BRCA1. Like BRCA1, BARD1 has a RING domain at its N-terminus and two BRCT domains at its C-terminus.

BRCA1 has been implicated in a number of cellular processes, including the regulation of DNA damage repair, transcription, cell cycle, chromatin remodeling, and apoptosis.

To elucidate the molecular mechanisms of tumor suppressive function of BRCA1, we have been analyzed the various functions of BRCA1.

(1) Using biochemical technique, we resolved four distinct BRCA1-containing complexes. BRCA1 content of these complexes is altered following DNA damage and that one of these complexes, the HUIC (hydroxyurea induced complex), is associated with DNA damage. These suggest that BRCA1 participates in multiple cellular prosesses by forming multiple protein complexes [2].
(2) Furthermore, we found that BARD1, heterodimer partner of BRCA1, is an integral component of RNA polymerase II holoenzyme (holo-pol) and that the N-terminus of BRCA1 is important for the association with the holo-pol and the localization at nuclear foci in the S-phase of cell cycle [3].
(3) Expression of the amino terminus deleted BRCA1 revealed a powerful growth suppressive effect. Its growth suppression is associated with an increase in apoptosis [4].
(4) In addition, we identified RNA polymerase II (RNAPII) as a substrate of ubiquitnation activity of BRCA1. After UV irradiation, subpopulation of RNAPII is phosphorylated and recognized by the C-terminus of BRCA1 as a substrate after UV irradiation. BRCA1/BARD1 ubiquitinate the phosphorylated RNAPII, which is subsequently degradated at the proteasome. Then, BRCA1/BARD1 may recruit other DNA repair factor to the site of DNA damage. We presume that BRCA1 is involved in transcription coupled repair process by the mechanism shown in Fig. 2 [5].
(5) Furthermore, we have developed the functional assay for the molecular diagnosis of BRCA1 by ubiquitination activity and subcellular localization in S-phase and after DNA damage.

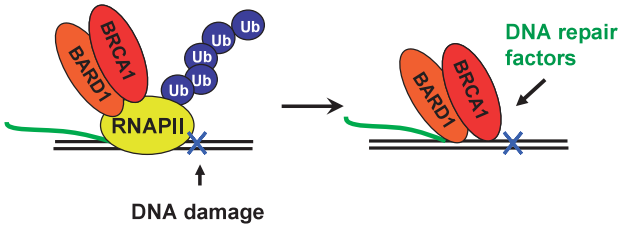

Fig. 2. After DNA damage, subpopulation of RNAPII is phosphorylated. BRCA1/BARD1 ubiquitinates the phosphorylated RNAPII, which is subsequently degradated at the proteasome. Then, BRCA1/BARD1 recruits other DNA repair factor to the site of DNA damage.

Recently, our research has been focused on the DNA repair function of BRCA1. Among various function of BRCA1, the tumor suppressor activity of BRCA1 is to be primarily attributed to its involvement in DNA repair. Clinically observed missense mutations often result in non-functional BRCA1 proteins that lose the ability to repair DNA damage. However, the precise mechanism of what role BRCA1 plays in DNA repair pathway is not fully understood. Now, we are collaborating with Dr. Akira Yasui, Department of Molecular Genetics, Institute of Development, Aging and cancer, Tohoku University, and are analyzing the cellular response of BRCA1 to various DNA damage using molecular imaging technique.

2. Results and Discussion

2.1. BRCA1 response to various DNA damage induced by laser micro-irradiation

2.1.1. Laser micro-irradiation system

To elucidate the molecular mechanisms of DNA repair processes for various DNA damage of BRCA1, we are analyzing the BRCA1 accumulation at the DNA damaged site induced by laser micro-irradiation in living cells [6].

Our experimental system is a laser micro-irradiation apparatus combined with a confocal microscopy. We use a 365nm pulse laser system for the irradiation of cells in the epi-fluorescence path of the microscope system. A lower dose or higher dose of irradiation was obtained by passing lasers through distinct filters in front of the lens. A 405nm pulse laser system is also used. The power of laser scan can be controlled by the number of scan (Fig. 3). By using these system, various types of DNA damage, such as single-strand breaks (SSBs), double-strand breaks (DSBs) and oxidative

base damage, were produced at restricted nuclear regions of cells [6,7].

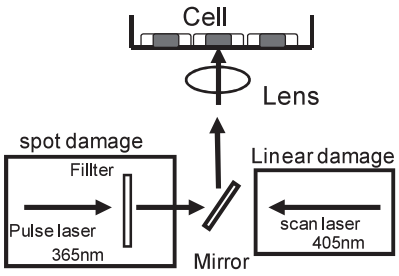

Fig. 3. Laser micro-irradiation systems. The left column shows the 365 nm pulse laser irradiation system, producing the lower dose and the higher dose irradiation, which are regulated by the filter in front of the mirror. The right column shows the 405 nm laser system. Fluorescent images were obtained and processed using an FV-500 confocal scanning laser microscopy system. A 405 nm scan laser system for irradiation of cells in the epifluorescence path of the microscope system was used. One scan of the laser light at full power delivers approximately 1600 nW. To primarily induce DSBs, we scanned cells 500 times using the 405 nm laser at full power, which has been shown The 405 nm laser light was focused through a 40× objective lens.

2.1.2. Endogenous BRCA1 and γH2AX accumulate at the laser micro-irradiated sites with different kinetics

We recently reported that micro-irradiation of living cells by scanning 500 times with a 405 nm laser-light through a confocal microscope can generate DSBs, and that GFP-tagged BRCA1 visibly accumulates at the laser-irradiated sites. The present study first examined whether endogenous BRCA1 accumulates at the irradiated sites, similar to the GFP-BRCA1. BRCA1 co-localizes with phosphorylated H2AX (γH2AX), a protein that is rapidly phosphorylated at the sites of DSBs after DNA damage. The localization of γH2AX was also examined.

Human Saos-2 cells were fixed and processed for double immunofluorescence at 2, 5, 8, 10, 60, 300, or 600 minutes after DSBs were introduced into the nuclei of the cells by laser micro-irradiation (Fig. 4). γH2AX was detected as bright fluorescence along the line of irradiation as early as two minutes after irradiation and reached its maximum level at 10 min. The accumulation of γH2AX persisted up to 60 min, but had decreased by 300 min and was not observed at 600 min. In contrast, the accumulation of BRCA1 along the line of irradiation was rather gradual and was not observed at two min. The fluorescent lines of BRCA1 were detected at five min and reached maximum intensity at 60 min. In striking contrast to the accumulation of γH2AX, the accumulation of BRCA1 persisted for at least 600 min. The time course of accumulation of fluorescence intensity for each protein is shown in Fig. 5. These quantitative analyses confirmed the qualitative description of the kinetics of accumulation of the proteins along the line of irradiation. Thus, both the accumulation and clearance of BRCA1 were slower than that of γH2AX, suggesting that distinct mechanisms mediate the mobilization of these two proteins.

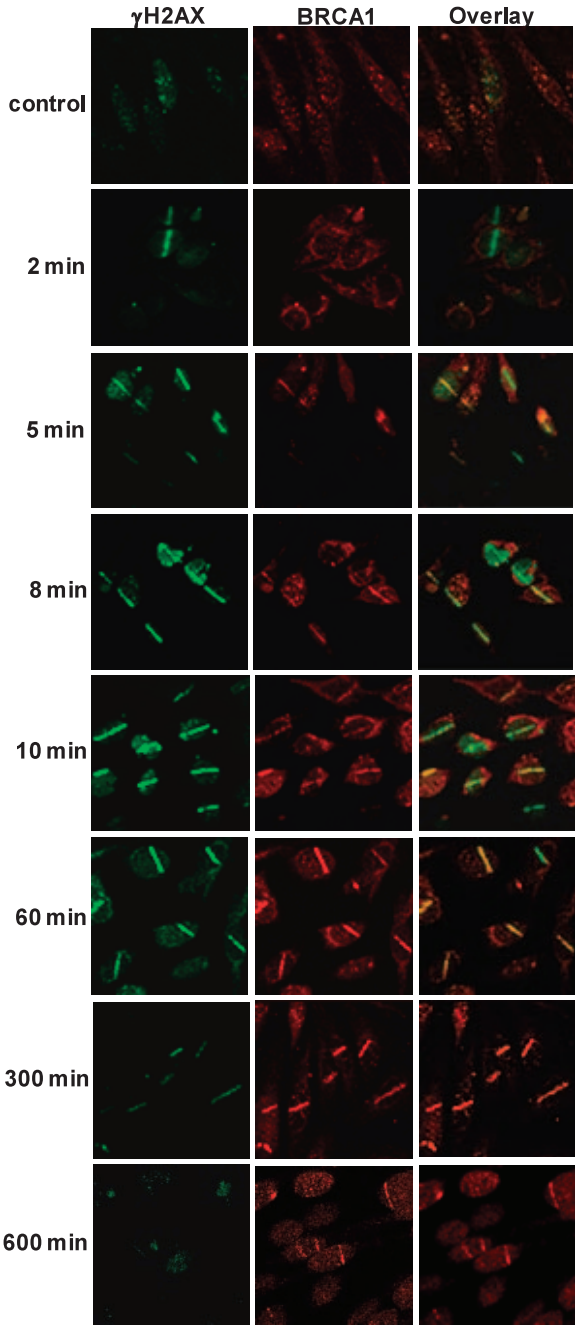

Fig. 4. A Immunochemical detection of γH2AX and BRCA1 in Saos-2 cells after 500 scans of 405 nm laser irradiation. Cells were fixed at the indicated time points after laser irradiation and stained with antibodies against γH2AX and BRCA1.

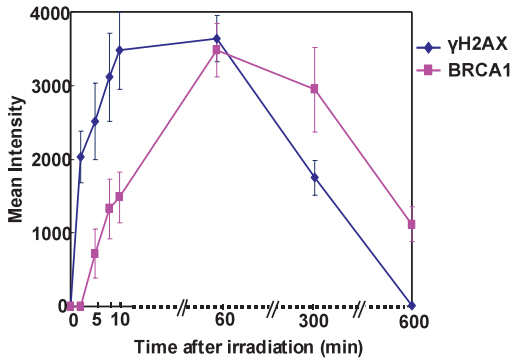

Fig. 5. Comparison of the fluorescence intensity of γH2AX and BRCA1 at the DSB sites. Standard deviations were derived from at least three independent experiments.

2.1.3. GFP-BRCA1 accumulats at DNA DSBs

We next examined the real time localization of GFP-tagged BRCA1 in living cells after laser-irradiation. GFP-BRCA1 was transfected into Saos-2 cells and the cells were then laser-irradiated. As shown in Figure 6, GFP-BRCA1 clearly accumulated at the irradiated sites. The mean intensity of GFP-BRCA1 at the accumulation site was quantified. Accumulation of BRCA1 at the irradiated sites was gradual.

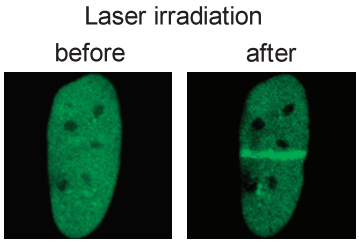

Fig. 6. GFP-tagged BRCA1 accumulates at DSB sites in Saos-2 cells.

2.1.4. BRCA1 localizes at DSBs via its N- and C-terminal regions

We next identified the regions in BRCA1 that mediate this accumulation. Several GFP-tagged deletion mutants of BRCA1 were constructed. The full-length BRCA1, as well as the deletion mutants, Δ305-770, Δ775-1292, Δ1-302, and Δ1527-1863, were transfected into Saos-2 cells (Fig.7). Protein expression was confirmed by Western blots using antibodies against BRCA1. Because the RING domain is located in a.a.1-302 and the BRCT domains are located in a.a.1528-1863, we hypothesized that deletion of either of these two regions would suppress BRCA1 accumulation at DSBs. However, each of the four deletion mutant BRCA1 proteins accumulated at DSBs. Therefore, the a.a.303-1526 BRCA1 fragment was constructed, which contained only the central region of BRCA1 and lacked both the N- and C-terminal regions. This BRCA1 fragment did not accumulate at the irradiated sites. These data suggest that either the N- or C-terminal region alone is sufficient for BRCA1 accumulation at DSBs. Subsequently, a.a.1-304 and a.a.1527-1863 fragments were constructed and each fragment was shown to accumulate at laser-induced linear DSBs.

The mean intensity of GFP-BRCA1 at the accumulation site was quantified and the kinetics of accumulation of full-length and these BRCA1 fragments were examined for 600 seconds after irradiation (Fig. 8). Interestingly, the N-terminal (a.a.1-304) fragment rapidly and maximally accumulated at the DSBs within 20 seconds, whereas the C-terminal (a.a.1527-1863) fragment slowly and gradually accumulated, reaching a plateau at 360 seconds. Full-length BRCA1 exhibited a pattern of accumulation that reflected the combination of N- and C-terminal fragment patterns.

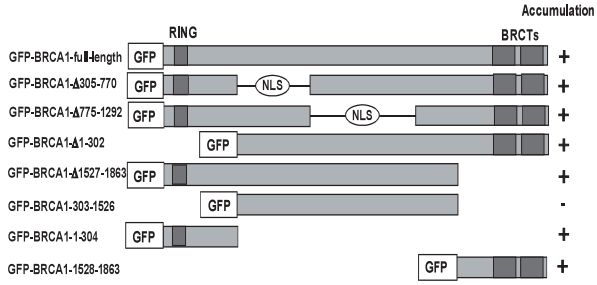

Fig. 7. Diagram of GFP-tagged BRCA1 full-length and deletion mutants and accumulation at DSBs showing the locations of the GFP tag (GFP) and the inserted nuclear localization signal (NLS)

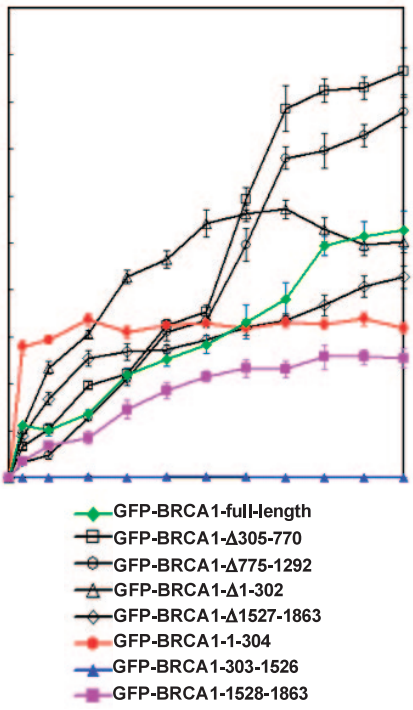

Fig. 8. Kinetics of accumulation of full-length and the a.a.1-304, a.a.303-1526, and a.a.1527-1863 fragments of BRCA1 after laser irradiation in Saos-2 cells.

2.1.5. Accumulation of the N-terminal region of BRCA1 at DSBs requires the presence of Ku80

Next, we examined whether BRCA1 accumulation at DSBs is dependent on specific DNA repair factors, using factor-deficient cells (Fig. 9). BRCA1 is phosphorylated by the Ataxia-telangiectasia mutated (ATM) kinase after ionizing radiation. GFP-BRCA1 N- and C-terminal fragments were transfected into human *ATM*-deficient AT1KY/T-n cells. Both fragments accumulated at laser-induced DSBs in these cells. Thus, phosphorylation of BRCA1 by ATM is not required for BRCA1 accumulation at irradiated sites. In *H2AX*-/- MEF cells, both N- and C-terminal fragments of BRCA1 accumulated at DSBs. Thus, accumulation of BRCA1 is not likely to be dependent on H2AX, even though γH2AX was detected at the irradiated sites before BRCA1.

NHEJ and homologous recombination (HR) are the major cellular mechanisms to repair DSBs. In the NHEJ pathway, the DNA-dependent protein kinase catalytic subunit (DNA-PKcs) and a Ku heterodimer of Ku80 and Ku70 are recruited to the sites of DNA DSBs, followed by subsequent recruitment of the XRCC4-ligase IV. In CHO-derived CHO9 XR-C1 (*DNA-PKcs* -/-), and XRCC4 (-/-) the N- and C-terminal fragments of BRCA1 accumulated at the laser-irradiated sites. However, in XRV15B (*Ku80* -/-) cells, the N-terminal fragment of BRCA1 failed to accumulate at DSB sites, whereas the C-terminal fragment did accumulate. Therefore, accumulation of the N-terminal fragment, but not the C-terminal fragment, of BRCA1 at DSBs is Ku80 dependent.

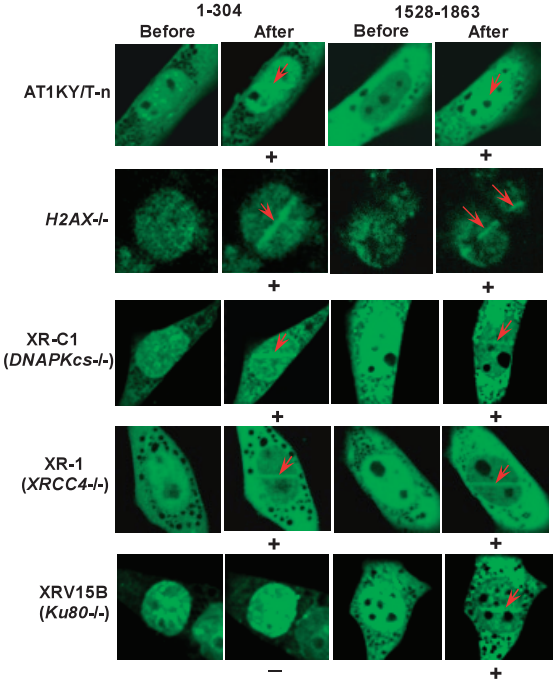

Fig. 9. Accumulation of the GFP-tagged N-terminus or C-terminus of BRCA1 at DSBs in cell lines deficient in *ATM, H2AX*, *DNA-PKcs, XRCC4,* or *Ku80* in cells. Arrows indicate the sites of irradiation.

To confirm that the accumulation of the N-terminal BRCA1 at DSB is dependent on the presence of Ku80 protein, we examined whether GFP-tagged N-terminus of BRCA1 accumulates at the laser-irradiated sites in XRV15B cells reconstituted with HA-tagged Ku80 (Fig. 9). XRV15B cells were transfected with vectors to express GFP-tagged N-terminus of BRCA1 and HA-tagged Ku80 or control vector. Ten minutes after laser irradiation, cells were fixed and stained with anti-HA. As shown in Fig. 4C, the N-terminus of BRCA1 accumulated at the laser-irradiated sites in cells that express Ku80 protein, but not in that transfected with control vector. Taken together, the N-terminus of BRCA1 accumulates at DSBs dependent on the presence of Ku80 protein.

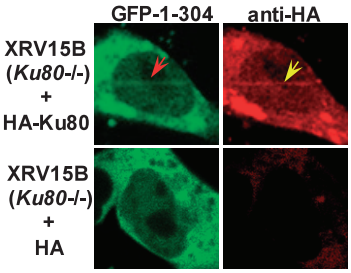

Fig. 10. Accumulation of GFP-tagged N-terminus of BRCA1 and HA-tagged Ku80 at DSBs in Ku80-deficient XRV15B cells, reconstituted with HA-tagged Ku80.

Since the accumulation of the a.a.1-304 fragment of BRCA1 at DSBs required Ku80, we analyzed the Ku80 the accumulation kinetics of GFP-Ku80. Ku80 was rapidly recruited to DSBs after irradiation, with kinetics similar to the a.a.1-304 fragment of BRCA1 (Fig. 11). We examined the localization of BARD1 in our experimental system. GFP-BARD1 accumulated at laser-irradiated sites in Saos-2 cells. The accumulation kinetics of BARD1 was, however, quite distinct from that of the a.a.1-304 fragment of BRCA1. The fluorescence intensity of the a.a.1-304 BRCA1 fragment reached a maximum level within 40 seconds, whereas the fluorescence intensity of BARD1 was only one-third of its maximum level after two minutes.

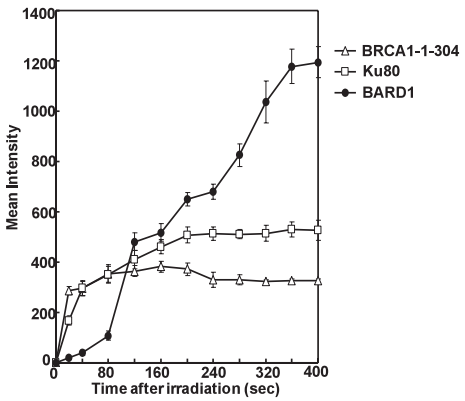

Fig. 11. Comparison of the accumulation kinetics of GFP-tagged Ku80, BARD1, and the N-terminus of BRCA1 (a.a.1-304) after laser irradiation at DSBs.

2.1.6. The N -terminal region of BRCA1 is associated with Ku80

BRCA1 functions in the NHEJ pathway as well as the HR pathway, and yet interaction of BRCA1 with proteins involved in NHEJ has not been reported. To examine directly whether BRCA1 interacts with Ku80, we performed co-immunoprecipitation assays (Fig. 12). HEK-293T cells were co-transfected with HA-tagged wild-type BRCA1 and FLAG-tagged Ku80. After exposure to ionizing radiation, cell extracts were prepared and immunoprecipitated with an anti-FLAG antibody and the precipitates were probed with an anti-HA antibody. A substantial amount of BRCA1 co-precipitated with Ku80 from the irradiated cells, whereas only a small amount of BRCA1 co-precipitated with Ku80 from cells that were not irradiated, suggesting that the BRCA1 association with Ku80 is significantly enhanced by irradiation. To identify the region of BRCA1 responsible for the Ku80 interaction, N- and C-terminal deletion mutants were transfected into HEK-293T cells. The C-terminal deletion mutant, but not the N-terminal deletion mutant, co-precipitated with Ku80.

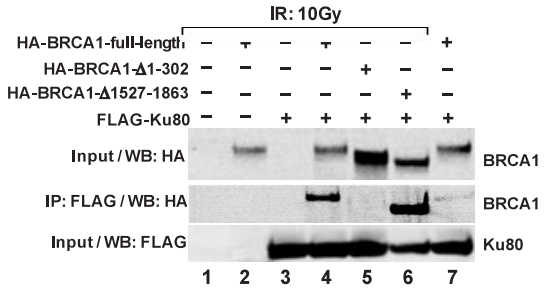

Fig. 12. N-terminal deleted BRCA1 does not associate with Ku80. HEK-293T cells were transfected with plasmids for the expression of HA-BRCA1-full-length, HA-BRCA1-Δ1-302, HA-BRCA1-Δ1527-1863, and FLAG-Ku80 and treated with 10 Gy ionizing radiation.

2.1.7. The a.a.1-100 and a.a.101-200 regions of BRCA1 accumulate at DSBs

We next investigated which motifs in the N-terminus of BRCA1 mediate Ku80-dependent accumulation at DSBs. To examine the role of the RING domain, which is located at a.a.24-64, we constructed several GFP-tagged fragments of BRCA1 including a.a.1-100, 1-200, 1-304, 101-200, 101-304, and 201-304 (Fig. 13).

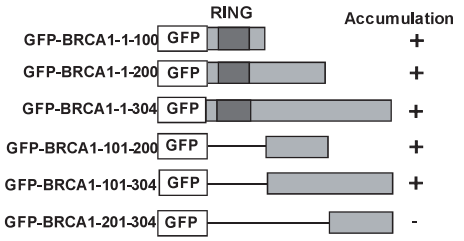

Fig. 13. Diagram of GFP-tagged BRCA1 fragments and results of accumulation at laser irradiated sites.

The a.a.1-100, 1-200, and 1-304 fragments, each of which contains the RING domain, accumulated at the laser-induced DSBs. However, the a.a.101-200 and 101-304 fragments, which lack the RING domain, also accumulated at DSBs. The a.a.201-304 fragment was the only one that did not accumulate at the DSBs. These results indicate that, in addition to the a.a.1-100 region where the RING domain is located, the a.a.101-200 region alone can also accumulate at DSBs. The accumulation kinetics were quantified for each of these N-terminal fragments of BRCA1 (Fig. 14). Interestingly, all of the fragments, with the exception of the a.a.201-304 fragment, showed similar kinetics, with a rapid (within 20 seconds) accumulation at DSBs after laser irradiation. These data suggest that the a.a.1-100 and 100-200 regions of BRCA1 use similar mechanisms to accumulate at DSBs.

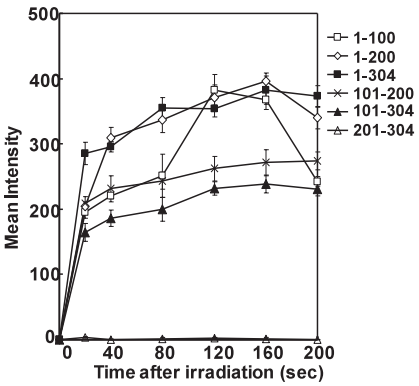

Fig. 14. Kinetics of GFP-tagged BRCA1 fragment accumulation after laser irradiation.

2.1.8. Missense mutations in a.a.101-200 change the accumulation kinetics of BRCA1 at DSBs and abolished the association with Ku80.

Although the a.a.101-200 fragment of BRCA1 alone accumulated at laser-irradiated sites, there do not appear to be any known domains or motifs in this region responsible for the accumulation. Thus, we examined whether missense mutations would affect the accumulation of BRCA1 at DSBs. Four missense mutations, Y105C, P142H, E143K, and Y179C were selected, on the basis of their relatively frequent occurrence in the Breast Cancer Information Core database (www.nhgri.nih.gov). However, the pathological significance of these mutations has not been verified. GFP-BRCA1 constructs (a.a. 101-200), each harboring one of the missense mutations, were generated. Each of the four mutants accumulated at the irradiated sites, but the fluorescence intensity, especially that of P142H, was markedly diminished relative to that of the wild-type BRCA1 fragments (Fig. 15).

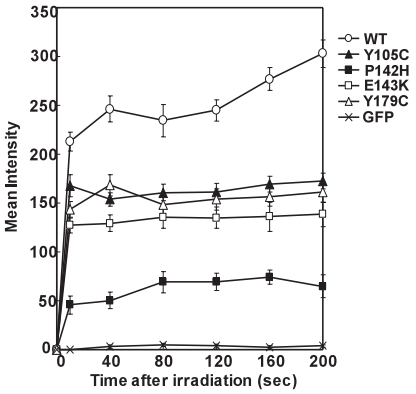

Fig. 15. Comparison of the accumulation kinetics of GFP-tagged BRCA1 fragments containing a missense mutation in Saos-2 cells.

Next, we analyzed the accumulation kinetics of GFP-tagged full-length BRCA1 containing these missense mutations. As shown in Fig. 6B, the four mutants of BRCA1 accumulated at the irradiated sites, but the accumulation kinetics of these mutants were distinct from that of the wild-type BRCA1. These mutants did not show the rapid accumulation observed in that of wild-type BRCA1. The patterns of the accumulation of these mutants were similar with that of the C-terminus of BRCA1 (Fig. 16). Especially, P142H mutant, which showed the most significant reduction of the accumulation analyzed by a.a.101-200 fragment, completely lost the rapid accumulation and slowly accumulated at the laser-irradiated sites. These results also suggest that the N-terminal fragment of BRCA1 is responsible for the rapid accumulation of BRCA1.

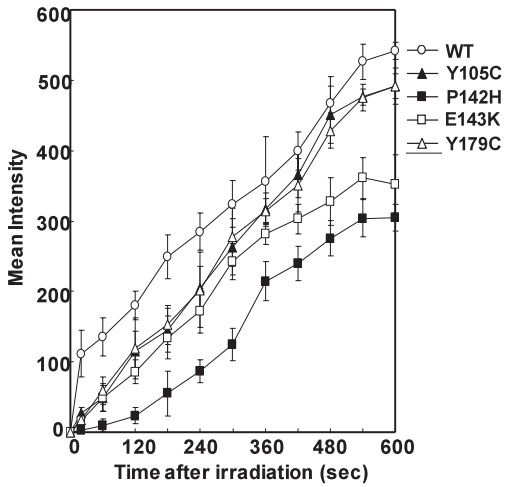

Fig. 16. Comparison of the accumulation kinetics of GFP-tagged full-length BRCA1 containing a missense mutation in Saos-2 cells.

Furthermore, we tested whether P142H mutant is associated with Ku80 protein. HEK-293T cells were co-transfected with GFP-tagged a.a.101-200 fragment of wild-type BRCA1 or P142H mutant together with FLAG-Ku80. After exposure to ionizing irradiation, cell lysates were immediately prepared and anti-FLAG immunoprecipitates were probed with anti-GFP and anti-FLAG antibodies. As shown in Fig. 17, a.a.101-200 fragment of BRCA1-P142H was not associated with Ku80 protein.

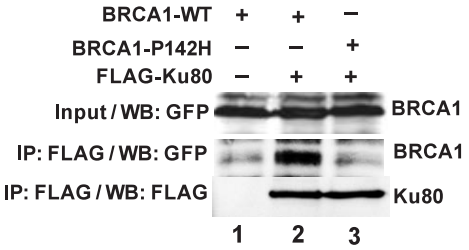

Fig. 17. The association of a.a.101-200 BRCA1 fragment containing P142H mutation with Ku80. HEK-293T cells were transfected with plasmids for the expression of GFP-BRCA1-101-200-WT (lanes 1, 2) or GFP-BRCA1-101-200-P142H mutant (lane 3) and FLAG-Ku80 (lanes 1-3) and treated with 10 Gy ionizing radiation (lanes 1-3).

HCC1937 is BRCA1-deficient breast cancer cell line and sensitive to DNA damage. Exogenous expression of BRCA1 restores resistance to DNA damage in these cells. We compared BRCA1-P142H mutant with BRCA1-wild-type for the ability to confer the resistance to ionizong irradiation. HCC1937 cells were transfected with HA-tagged BRCA1-wild-type or P142H. Cells were irradiated with varying doses of irradiation and their ability to form colonies was assessed (Fig. 18). Under conditions of BRCA1-wild-type expression, HCC1937 cells were more resistant to irradiation. In contrast, expression of BRCA1-P142H did not appear to effect the sensitivity to irradiation. These results suggest that BRCA1/Ku80 association is involved in the repair following ionizing irradiation, and mediates the resistance to DNA damage induced by irradiation. Therefore, a.a.101-200 fragment indeed is a functional protein interaction domain and especially, P142H mutation of BRCA1 is likely to be a pathological mutation.

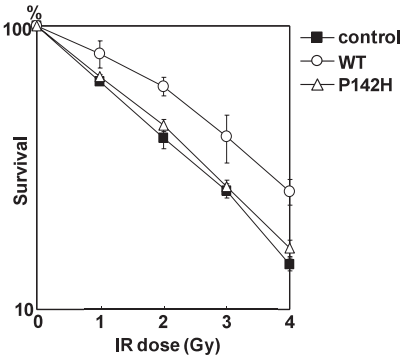

Fig. 18. Colony formation assay of HCC1937 cells expressing HA-BRCA1-WT and P142H. HCC1937 cells were transfected with vectors for control, HA-BRCA1-WT or BRCA1-P142H. Cells were replanted 48 hours after transfection, and then 8 hours later, cells were exposed to X-ray irradiation, as indicated. Ten days after irradiation, colonies were counted.

2.1.9. Discussion

In this study, we laser micro-irradiated living cells and demonstrated that endogenous as well as GFP-BRCA1 accumulated at DSBs. Using various deletion mutants and fragments of BRCA1, we found that both the N- (a.a.1-304) and C- (a.a.1528-1863) terminal regions of BRCA1 accumulate at DSBs. Au et al. have reported that the RING and BRCT domains of BRCA1 cooperate in the localization of BRCA1 at irradiation-induced nuclear foci [9]. In those studies, the RING domain (a.a.1-304) did not form nuclear foci and the BRCT domain only poorly localized at foci, whereas an artificial fragment composed of the RING and BRCT domains localized at nuclear foci as efficiently as full-length BRCA1. One explanation for the apparent inconsistency between the previous report and our present findings is that the N-terminus of BRCA1 may not accumulate at nuclear foci, but only at DSBs. Alternatively, if the N-terminus of BRCA1 accumulates at DSBs, but remains at the damaged site only transiently, its fluorescence intensity at nuclear foci may not be detectable.

In fact, our detailed kinetic analyses revealed distinct behaviors of the N- and C-terminus of BRCA1. The N-terminal region of BRCA1 accumulated at DSBs immediately and transiently after laser-irradiation, whereas the C-terminal region slowly and gradually accumulated and remained associated with the DSB for longer times. We propose that the N-terminal region may function to guide BRCA1 to the DSBs in the early phase of the repair process, and that the C-terminal region may be responsible for the accumulation in the later phase of repair and the sustained retention. Moreover, such a mechanism may apply to other DNA repair factors as well. Celeste et al. reported that in *H2AX-/-* cells, 53BP1 localized at irradiation-induced nuclear foci and laser-irradiated sites in the early phase, but not in the late phase of repair (in *H2AX+/+* cells, 53BP1 accumulated in both the early and late phases) [10].

We analyzed the possible contribution of H2AX to BRCA1 accumulation at DSBs. Both N- and C-terminal fragments of BRCA1 accumulated at DSBs, even in *H2AX-/-* cells. These observations are consistent with some reports that BRCA1 accumulates at laser-induced DSBs in *H2AX-/-* cells [10], but other studies reported that BRCA1 did not localize at nuclear foci in the same *H2AX-/-* cells [11,12]. The requirement for H2AX may differ for BRCA1 localization at DSBs versus nuclear foci. Alternatively, Bassing et al. and Celeste et al. may have been unable to detect nuclear foci (note that their observations were made at 0.75-6 hours after irradiation) because the accumulation of BRCA1 at DSBs may be transient in the absence of H2AX. H2AX could be involved in retention of the C-terminal fragment of BRCA1, analogous to H2AX mediated 53BP1 retention in the late phase.

The N-terminus of BRCA1 accumulated at DSBs in a Ku80-dependent manner. Accordingly to Kim et al., other NHEJ factors, Ku70 and DNA-PKc, are only transiently localized to the laser-irradiated site [13]. In our analysis, the N-terminus of BRCA1 accumulated only transiently, suggesting that BRCA1 functions with NHEJ factors in the early phase of the repair process, and dissociates from the site of DSBs together with NHEJ factors.

Breast cancer risk has been correlated with single nucleotide polymorphisms in NHEJ factor genes. Furthermore, the BRCA1 genotype significantly affects the degree of this risk, if the NHEJ factors are a high-risk genotype. There have been conflicting reports on the role of BRCA1 in NHEJ, including promotion, suppression, or no effect. The existence of two subpathways have been recently proposed for NHEJ, namely error-free and error-prone NHEJ [14,15]. Previously reports have indicated that BRCA1 functions to promote error-free NHEJ, or to inhibit error-prone NHEJ [16,17]. It is therefore possible that the above facilitation and/or suppression of NHEJ might be achieved by the accumulating BRCA1 at DSBs.

The missense mutations that we analyzed significantly reduced BRCA1 accumulation at DSBs. The pathological significance of mutations found in the BRCA1 N-terminus have been evaluated only around the RING domain by examining BARD1 association and ubiquitination activity. In our analysis, those four mutants were able to interact with BARD1 and exhibited ubiquitination activity (unpublished data). We found for the first time that these four missense mutations could be pathological mutations since they disrupted BRCA1 accumulation at DSBs. These data suggest that rapid accumulation of BRCA1 at DSBs via its N-terminus is important its tumor suppressor activity.

Bekker-Jensen et al. reported that BRCA1 accumulates in two distinct nuclear compartments, the DSB-flanking chromatin marked by H2AX and ssDNA–microcompartments [18]. Down-regulation of the checkpoint mediator Mdc1/NFBD1 dissociates BRCA1 from the DSB-flanking chromatin, but not from ssDNA-microcompartments. This observation suggests that BRCA1 may form different types of protein complexes and that each complex may localize to distinct structures near sites of DNA damage. Our results indicate that BRCA1 forms distinct protein complexes using different intramolecular regions. Taken together, BRCA1 may contribute to multiple protein complexes, whose constitution is spatiotemporally and dynamically regulated, and which function in various phases of DNA DSB repair processes.

2.2. BRCA1 response to local UV irradiation

As mentioned above, in the field of DNA repair, mounting evidences have indicated that BRCA1 is

engaging in DNA DSBs repair pathways, HR and NHEJ. On the other hand, BRCA1 seems to be involved in the processes of transcription coupled repair (TCR) [19]. BRCA1-deficient cells are defective in a process in which oxidative base damage are removed preferentially from the transcribed DNA strand [20].

BRCA1 localizes in nuclear foci after UV irradiation. BRCA1 is phosphorylated on Ser1423 and Ser1524 by the ataxia telenguectasis mutated-related kinase (ATR) after UV damage. Previously we and other group reported that BRCA1 ubiquitinates RNAPII after UV irradiation [5, 21, 22].

The predominant DNA damage produced by UV irradiation is cyclobutane pyrimidine dimmers (CPD) and 6-4 photoproduct adducts. These lesions are removed by nucleotide excision repair (NER), which eliminates a wide variety of bulky helix-distorting DNA lesions. NER operate via two pathways: global genome repair (GGR) and TCR. GGR can repair DNA lesions at any location in the whole genome, while TCR selectively removes DNA lesions on the transcribed strands of expressed genes.

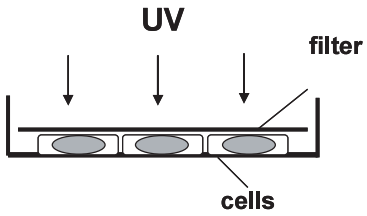

Fig. 19. A schema of exposing cells to the local UV irradiation using membrane filter.

To gain further insights into the regulation of BRCA1 upon TCR pathway, we induced UV damage in restricted small areas of the cell nucleus by an isopore membrane filter. Cells mono-layered in 35-mm glass-bottom dishes were treated with or without transcription inhibitor before being covered with a polycarbonate isopore membrane filter with pores of 3μm in diameter and 254nm UV irradiated with a dose of 60J/m^2 (Fig. 19). The polycarbonate blocked the 254nm UV-light, and cells were exposed only though the pore of the filter and immunostained with antibody against CPD and BRCA1. After local UV irradiation, CPD, the major types of DNA damage induced by UV irradiation, was observed as fine nuclear dots and BRCA1 colocalized with CPD in the cell nucleus. When cells were stained with another antibody against BRCA1, C-20 (Santa Cruz), we found similar images of BRCA1 accumulation. These suggest that BRCA1 immediately accumulates at the UV damaged sites (Fig. 20).

To examine whether the BRCA1 accumulation at the sites of local UV irradiation is associated with transcription, similarly as localization at nuclear foci, we treated the cells with actinomycin D or α-Amanitin before exposing to local UV irradiation. Treatment with these chemical completely abolished the accumulation of BRCA1

Furthermore, we found that the BRCA1 accumulation at the UV irradiated sites was dependent on the presence of the protein, which is involved in the NER pathway and that BRCA1 is associated with the protein. Our data will show the mechanism of involvement of BRCA1 in the TCR at the lesion induced by UV irradiation.

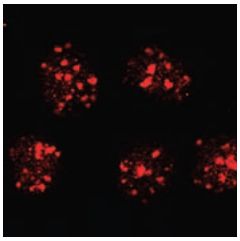

Fig. 20. BRCA1 accumulation at the sites of local UV irradiation. Saos-2 cells were fixed after local UV irradiation through membrane filters and stained with antibody against BRCA1.

2.3. Identification of BRCA1 associated molecules

We have already identified new some BRCA1 associated proteins by proteomic study. Now, we are examining their function. One of those molecules colocalized at centrosome and nuclear foci with BRCA1. This molecule might be involved in the tumor suppressor activity of BRCA1.

3. Conclusion

It was recently reported that the inhibition of poly(ADP-ribose) polymerase 1 (PARP1), a critical enzyme in the SSB repair pathway, leads to a severe, highly-selective toxicity in *BRCA1*-deficient cells [23]. This seems to be because PARP inhibition results in unrepaired SSBs, giving rise to DSBs. These provide new concepts for the development of potential cancer treatments. The reduced expression or function of BRCA1 is observed in sporadic cancers, suggesting that the precise analysis of BRCA1-related proteins will contribute to the discovery of novel molecular targets for cancer chemotherapy.

In cancer treatment, it is important to select a treatment method and predict its effectiveness in individual cancer patients. Molecular targeting therapies are focus of investigation with a view to developing novel approaches to cancer control. New biomarkers are similarly essential for improved cancer diagnostics. Our research has focused on the identification and functional analysis of critical proteins mediating carcinogenesis and on the development of diagnostic biomarkers and molecular targets for therapies for personalized medicine. Molecular imaging techniques will be useful tools for these aims.

Acknowledgements

This study was supported by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan. N. Chiba also acknowledge the support of Tohoku University Global COE Program "Global Nano-Biomedical Engineering Education and Research Network Centre".

References

[1] Miki Y, J. Swensen D, Shattuck-Eidens PA, Futreal K, Harshman S, Tavtigian Q, Liu C, Cochran LM, and et al. A strong candidate for the breast and ovarian cancer susceptibility gene BRCA1. *Science* **266**, 66-71, 1994.

[2] Chiba N and Parvin JD. Redistribution of BRCA1 among four different protein complexes following replication blockage. *J Biol Chem* **276**, 38549-38554, 2001.

[3] Chiba N and Parvin JD. The BRCA1 and BARD1 association with the RNA polymerase II holoenzyme. *Cancer Res* **62**, 4222-4228, 2002.

[4] You F, Chiba N, Ishioka C, and Parvin JD. Expresssion of an amino-terminal BRCA1 deletion mutant causes a dominant growth inhibition in MCF10A cells. *Oncogene* **23**, 5792-5798, 2004.

[5] Starita LM, Howitz AA, Keogh M-C, Ishioka C, Parvin JD, and Chiba N. BRCA1/BARD1 ubiquitinate phosphorylated RNA polymerase II. *J Biol Chem* **280**, 24498-24505, 2005.

[6] Lan L, Nakajima S, Oohata Y, Takao M, Okano S, Masutani M, Wilson SH, and Yasui A. In situ analysis of repair processes for oxidative DNA damage in mammalian cells. *Proc Natl Acad Sci USA* **101**, 13738-13743, 2004.

[7] Lan L, Nakajima S, Komatsu K, Nussenzweig A, Shimamoto A, Oshima J, and Yasui A. Accumulation of Werner protein at DNA double-strand breaks in human cells. *J Cell Sci* **118**, 4153-4162, 2005.

[8] Wei L, Lan L, Hong Z, Yasui A, Ishioka C, and Chiba N. Rapid recruitment of BRCA1 to DNA double-strand breaks is dependent on its association with Ku80. *Mol Cell Biol* (in press).

[9] Au WW and Henderson BR. The BRCA1 RING and BRCT domains cooperate in targeting BRCA1 to ionizing radiation-induced nuclear foci. *J Biol Chem* **280**, 6993-7001, 2005.

[10]Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, Bonner RF, Bonner WM, and Nussenzweig A. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol* **5**, 675-679, 2003.

[11]Bassing CH, Chua KF, Sekiguchi J, Suh H, Whitlow SR, Fleming JC, Monroe BC, Ciccone DN, Yan C, Vlasakova K, Livingston DM, Ferguson DO, Scully R, and Alt FW. Increased ionizing radiation sensitivity and genomic instability in the absence of histone H2AX. *Proc Natl Acad Sci USA* **99**, 8173-8178, 2002.

[12]Celeste A, Petersen S, Romanienko PJ, Fernandez-Capetillo O, Chen HT, Sedelnikova OA, Reina-San-Martin B, Coppola V, Meffre E, Difilippantonio MJ, Redon C, Pilch DR, Olaru A, Eckhaus M, Camerini-Otero RD, Tessarollo L, Livak F, Manova K, Bonner WM, Nussenzweig MC, and Nussenzweig A. Genomic instability in mice lacking histone H2AX. *Science* **296**, 922-927, 2002.

[13]Kim JS, Krasieva TB, Kurumizaka H, Chen DJ, Taylor AM, and Yokomori K. Independent and sequential recruitment of NHEJ and HR factors to DNA damage sites in mammalian cells. *J Cell Biol* **170**, 341-347, 2005.

[14]Bau DT, Mau YC, and Shen CY. The role of BRCA1 in non-homologous end-joining. *Cancer Lett* **240**, 1-8, 2006.

[15]Zhang J and Powell SN. The role of the BRCA1 tumor suppressor in DNA double-strand break repair. *Mol Cancer Res* **3**, 531-539, 2005.

[16]Wang HC, Chou WC, Shieh SY, and Shen CY. Ataxia telangiectasia mutated and checkpoint kinase 2 regulate BRCA1 to promote the fidelity of DNA end-joining. *Cancer Res* **66**, 1391-1400, 2006.

[17]Zhuang J, Zhang J, Willers H, Wang H, Chung JH, van Gent DC, Hallahan DE, Powell SN, and Xia F. Checkpoint kinase 2-mediated phosphorylation of BRCA1 regulates the fidelity of nonhomologous end-joining. *Cancer Res* **66**, 1401-8, 2006.

[18]Bekker-Jensen S, Lukas C, Kitagawa R, Melander F, Kastan MB, Bartek J, and Lukas J. Spatial organization of the mammalian genome surveillance machinery in response to DNA strand breaks. *J Cell Biol* **173**, 195-206, 2006.

[19]Abbott DW, Thomson ME, Robinson-Benion C, Tomlinson G, Jensen RA, and Holt JT. BRCA1 expression restores radiation resistance in BRCA1-defective cancer cells through enhancement of transcription-coupled DNA repair. *J Biol Chem* **274**, 18808-18812, 1999.

[20]Le Page F, Randrianarison V, Marot D, Cabannes J, Feunteun J, and Sarasin A. BRCA1 and BRCA2 are necessary for the transcription-coupled repair of the Oxidative 8-Oxoguanine lesion in human cells. *Cancer Res* **60**, 5548-5552, 2002.

[21]Kleiman FE, Wu-Baer F, Fonseca D, Kaneko S, Baer R, and Manley JL. BRCA1/BARD1 inhibition of mRNA 3' processing involves targeted degradation of RNA polymerase II. *Genes & Development* **19**, 1227-1237, 2005.

[22]Wu W, Nishikawa H, Hayami R, Sato K, Honda A, Aratani S, Nakajima T, Fukuda M, and Ohta T. BRCA1 ubiquitinates RPB8 in response to DNA damage. *Cancer Res* **67**, 951-958, 2007.

[23]Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, Santarosa M, Dillon KJ, Hickson I, Knights C, Martin NM, Jackson SP, Smith GC, and Ashworth A. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* **434**, 917-921, 2005.