DNA damage induces p53-dependent BRCA1 nuclear export

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**SUMMARY**

The tumor suppressor gene BRCA1 plays an important role in the response to DNA damage. BRCA1 function is regulated by a variety of mechanisms including transcriptional control, phosphorylation and protein-protein interactions. Recent studies have shown that BRCA1 is a nuclear-cytoplasmic shuttle protein. Its subcellular localization is controlled by a nuclear localization signal (NLS)-mediated nuclear import via the importin receptor pathway, and a nuclear export signal (NES)-facilitated nuclear export through CRM1-dependent pathway. Using the human breast cancer cell line, MCF7, the subcellular distribution of BRCA1 was assessed by immunohistochemical staining (IHC) and western blotting analyses of fractionated subcellular extracts. Ionizing radiation (IR) stimulated BRCA1 nuclear export in a dose-dependent manner. This DNA damage-induced BRCA1 nuclear export utilized a CRM1-dependent mechanism and also required wild-type p53, whose function was abrogated by the E6 protein in MCF7 cells. In addition, the dependence on p53 was confirmed using a second cell type operating a tetracycline inducible system. The effect of IR on BRCA1 export was observed in every phase of the cell cycle, even though BRCA1 localization did vary between the G1, S and G2/M phases. These results imply that, in addition to ATM-, ATR- and chk2-dependent phosphorylations, cytoplasmic relocalization of BRCA1 protein is a mechanism whereby BRCA1 function is regulated in response to DNA damage.

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1 The abbreviation used are: NLS, nuclear localization signal; NES, nuclear export signal; IHC, immunohistochemistry; IR, ionizing radiation; CRM1, chromosome maintenance region 1; LMB, Leptomycin B; Tet, tetracycline; PCNA, proliferating cell nuclear antigen; DAPI, 4’,6-diamidino-2-phenylindole; P-H3, phospho-Histone H3; Synchro, Synchronization.
INTRODUCTION

Carriers of BRCA1 mutations have an 85% risk of developing breast cancer by age 70, which is about 20-fold higher than the general population. Markedly reduced BRCA1 expression is also observed in high-grade sporadic breast and ovarian cancers, implying its role in sporadic breast and ovarian cancer as well (1). Despite accumulated evidence suggesting that BRCA1 is necessary for many cellular processes ranging from DNA replication, cell cycle checkpoint control, DNA repair, regulation of transcription, protein ubiquitination, apoptosis, to chromatin remodeling (2-5), the mechanisms for its tumor suppressive function are still not fully understood. It is possible that dysfunction of many or all of these BRCA1 properties may result in cancer development.

BRCA1 function is regulated by a variety of mechanisms including transcription (6), phosphorylation (7,8), and protein-protein interaction(9-11), and nuclear foci formation(12,13). It is known that wild-type BRCA1 is predominantly located within the nucleus (14-16). Given the critical role of BRCA1 in nuclear processes, such as DNA repair, transcription and chromosomal remodeling, the sub-cellular localization of the BRCA1 protein could be an important mechanism in regulating its function. Consistent with this notion, recent evidence has shown that BRCA1 is a shuttle protein, which is actively transported between the nucleus and cytoplasm. Two nuclear localization signals (NLS) assist the targeting of the BRCA1 protein to the nucleus through the importin system (17-19). In addition, one functional nuclear export sequence (NES) located at its N-terminal, which facilitates the export of BRCA1 from the nucleus to the cytoplasm through the CRM1/exportin pathway (20). However, little is known about how BRCA1 shuttling between the nucleus and cytoplasm is controlled.
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BARD1, the BRCA1-associated RING domain protein, binds to BRCA1 at its ring-domain and chaperones BRCA1 nuclear import via a mechanism independent of NLS/importin system. BARD1 also inhibits BRCA1 nuclear export (NE) by masking the NES located in the ring domain (21). Over-expression of BARD1 resulted in enhancement of DNA damage-induced BRCA1 nuclear foci formation. P53 has been reported to bind directly to BRCA1 at its N-terminus ring domain and its C-terminus BRCT domain (22,23). BRCA1 enhances p53-mediated transcriptional activation (22,24,25). However, p53 down-regulates BRCA1 expression by transcriptional repression after exposure to genotoxic stress (26). It is not known whether a change in the subcellular location of BRCA1 is an alternative mechanism for p53-mediated regulation of BRCA1 function in response to DNA damage.

Several lines of evidence suggest interplay between BRCA1 function and cell cycle regulation. The BRCA1 protein plays important role in controlling DNA damage induced G2/M checkpoint. A mutation in an ATM-dependent phosphorylation site in BRCA1, serine 1423, abolishes the ability of BRCA1 to mediate the G2/M checkpoint (27). On the other hand, the cell cycle determines the level of BRCA1 expression (28), with the highest levels found in S-phase (29). In addition, rapidly proliferating cells generally have a higher expression level of BRCA1. Cells in G0/G1 express BRCA1 at low level; the BRCA1 protein level increases during progression through G1, reaching a peak during S phase and remaining elevated in G2/M as compared with cells in G0/G1 (30,31). In parallel with the cell cycle dependent expression level, BRCA1 undergoes hyper-phosphorylation during late G1 and S phases of the cell cycle and is transiently de-phosphorylated early after M phase (16,32). Several studies showed that IR and chemotherapy agents produce decrease of BRCA1 expression at both mRNA and protein levels.
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(26,33). However, little is known about the regulation of BRCA1 subcellular distribution, or how it relates to p53-dependent functions such as G1 arrest in response to DNA damage.

Given the critical roles of BRCA1 in several DNA damage response pathways, we investigated the effect of ionizing radiation (IR)-induced DNA damage on BRCA1 localization in human cells. We found that IR promoted BRCA1 NE in human breast cancer cells through a CRM1-dependent mechanism. We further demonstrated that DNA damage-induced NE of BRCA1 critically depends upon the function of wild-type p53, and occurred irrespective of the phase of the cell cycle. Our results imply that p53-dependent BRCA1 NE might be an alternative mechanism to regulate BRCA1 function in response to DNA damage. Abnormal BRCA1 shuttling in breast cancer cells lacking functional p53 may result in aberrant DNA repair and increased genetic instability in surviving cells.
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EXPERIMENTAL PROCEDURES

Cell Culture

The human breast cancer cell line MCF7 and Hela cells were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 20 mM Hepes, 100 µg/ml streptomycin and 100 U/ml penicillin (all purchased from Sigma Co.). When required for MCF7 cells, Leptomycin B (LMB, purchased from Sigma Co.) was added to the culture medium at a final concentration of 6ng/ml for the indicated period time. Capan-1 cells were cultured in Iscove’s Modified Dulbecco’s Medium (Sigma Co.) with 15% FBS, 100 µg/ml streptomycin and 100 U/ml penicillin. The EJ-p53 cell line (a gift from Stuart A. Aaronson at Mount Sinai School of Medicine New York, NY 10029) was cultured in complete DMEM medium (as described) also containing 1µg/ml tetracycline (Tet), 75 µg/ml Hygromycin B, and 500 µg/ml G418. (Tet and Hygromycin B purchased from Sigma Co.; G418 from Fisher Scientific). The removal of Tet from the medium resulted in p53 induction (34). For transient overexpression of CRM-1 in MCF7 cells, 0.4µg of purified expression vector carrying YFP-CRM1 cDNA (provided by Dr. Beric R. Henderson, Australia) was transfected into cells using lipofectamine™ reagent (Invitrogen) according to the manufacturer’s instructions. The cells were irradiated at 24 hours after transfection, and BRCA1 IHC staining was performed a further 24 hours after irradiation.

Cell cycle synchronization and flowcytometry sorting and analysis
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To synchronize MCF7 cells, cells were seeded at low density and the serum was withdrawn from the culture medium. Cells were arrested at G0/G1 phase by growth factor deprivation (32). Cells were synchronized for 24 hours before they were irradiated; serum was then added to the cultured cells immediately after exposure to IR. BRCA1 IHC staining was carried out 24 hours after IR treatment. For cell cycle profile analysis using flow cytometry, cells with or without IR were washed in PBS three times, fixed in cold-ice 70% ethanol 15ml tubes on ice for at least 60min. Before proceeding to flowcytometry analysis, cells were incubated with freshly mixed buffer, composed of 50µg/ml Propidium Iodide (from Sigma), 0.5mg/ml RNase, 0.1% NP40 in PBS, for 30min on ice. Samples were analyzed by FACSCalibur (BD PharMingen). To separate and collect MCF7 in each phase of the cell cycle, cells were incubated with 10µg/ml Bisbenzimide H 33342 (Calbiochem) for 30min. Cells were then harvested, washed in PBS, and subjected to sorting using FACS (Cytomation MoFlo), with gate settings based on DNA content for G1, S and G2/M phases. After sorting, approximately 1 x 10^4 cells in each phase were transferred onto culture slides (Fisher Scientific) using Cytospin 2 (SHANDON) at 700rpm/8min, and fixed by ice-cold methanol for immunohistochemical staining. Sorted cells were also used for whole cell extracts and subsequent western blot analysis.

**Retrovirus Infection**

Retrovirus infections were carried out using 60mm dishes with MCF7 cells at 60% confluence. For infection, cell monolayers were washed two times in DMEM without serum or antibiotics, followed by addition of 2 ml DMEM containing 4 µg/ml polybrene (Sigma Co.) and 0.5 ml of virus stocks of E6 and LXSN, respectively (virus stocks were obtained from Carl Q. Maki at Harvard University, School of Public Health, MA 02115). After incubation for two hours at
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37°C, four ml of DMEM without serum was added and cells were incubated for four more hours. Infection solution was removed from cells, followed by addition of DMEM with 10% FBS serum and incubation for 24 hours. Then cells were split into T25 tissue culture flasks at a seeding density of 1000 cells/flask and grown another 24 hours. Forty-eight hours post-infection, selection media (DMEM with 10% FBS) containing 750 µg/ml G418 was added to cells and selection was continued 7–10 days to obtain MCF7/E6 and MCF7/LXSN-containing cell populations. To maintain selection, cells were cultured in complete DMEM containing 500 µg/ml G418.

**Immunohistochemistry Studies**

MCF7, MCF7/E6, MCF7/LXSN, and EJ-p53 cells were seeded into 4-chamber tissue culture slides (Fisher Scientific), incubated overnight. Adherent cells were treated with ionizing radiation (IR) using a Siemens Stabilipan 2 X-ray generator at 280 kVp, 12mA, and a dose-rate of 2.08Gy/min. Cells were then cultured for different period of time as indicated in the result, and were subjected to immunohistochemical staining. To assess dose effects, cells were analyzed 24 hours after irradiation with 0, 2.5, 5.0, and 10.0 Gy. To document the time course of response, cells were irradiated with 2.5 Gy and analyzed 0, 8, 24, and 48 hours post-IR.

Cells were fixed in 100% methanol at –20°C for 20 min, followed by 10 sec permeabilization with ice-cold acetone or were fixed in 4% Paraformaldehyde (in phosphate buffered saline [PBS]) for 15 min, followed by 10 min permeabilization in PBS containing 0.5% Triton X-100). All cells were washed in PBS and blocked in PBS with 10% FBS. BRCA1 was detected by immunofluorescence using mouse anti-BRCA1 monoclonal antibody Ab-1 (Oncogene Science) at a 1:50 dilution, which recognizes an amino-terminal epitope of BRCA1. p53 was detected using sheep anti-human p53 polyclonal antibody Ab-7 (Oncogene Science) at a 1:400 dilution,
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PCNA was detected using mouse anti-human PCNA monoclonal antibody Ab-1 (Oncogene Science) at a 1:100 dilution, and phospho-Histone H3 (P-H3) was detected using rat anti-P-H3 monoclonal antibody (Sigma) at 1:500 dilution. The secondary antibodies used were Alexa Fluor594-labeled goat anti-mouse IgG (Molecular Probes), Alexa Fluor488-labeled donkey anti-sheep IgG (Molecular Probes), and Alexa Fluor488-labeled chicken anti-rat IgG (Molecular Probes) -each at a 1:500 dilution. Images were collected by Olympus Microscope (BX51) and processed using Adobe PhotoShop software.

**Cell Fractionation and Western Blot Analyses**

For western blot analyses, either whole-cell extract or cytoplasmic and nuclear fractions were used. Cells were irradiated with 2.5Gy and incubated for various periods of time as indicated. Whole-cell extracts were prepared by lysis of the monolayer culture in RIPA buffer (150 mM NaCl, 50 mM Tris pH 8.0, 5 mm EDTA, 0.5% sodium deoxycholate, 0.1% SDS, 1.0% NP40, and protease/phosphatase inhibitors—2.0 mM PMSF, 1.0 mM Na₃VO₄, 1.0 mM Leupeptin). Nuclear and cytoplasmic fractions were isolated by first incubating cells in buffer A (10mM Hapes, 2.5mM MgCl₂, 10 mM KCl and protease/phosphatase inhibitors). Cells were then allowed to swell on ice for 15 minutes to allow rupture of the cell membrane, and nuclei were pelleted by centrifugation. The cytoplasmic fractions were located in the supernatants, which were collected. The nuclear pellets were then incubated for 20 minutes in buffer C (20mM Hapes, 2.5M MgCl₂, 20M EDTA, 420mM NaCl, 25% glycerol, protease/phosphatase inhibitors), on ice (mixing lysates every 5 mins). The soluble nuclear protein fraction was collected after centrifugation at 20,000G for 20 mins. Total protein concentration in each extraction was measured using BCA protein assay reagent system from PIERCE.
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Protein samples were loaded and subjected to SDS-PAGE electrophoresis. Equal loading of the cytoplasmic, nuclear or total protein samples after transfer to the Immobilon™-P membrane was confirmed by staining the membrane with Ponceau Red. The subsequent immunoblots would not be conducted if we detected that there was any difference between lanes due to loading or transfer efficiency. To detect the protein of interest, the membranes were incubated respectively with mouse anti-human p53 monoclonal antibody (Ab-6, Oncogene Science [diluted 1:100 in PBS/3% milk]), mouse anti-human p21 monoclonal antibody (Clone SXM30, BD Biosciences [1-2 µg/ml in PBS/3% milk]), mouse anti-human BRCA1 monoclonal antibody (Ab-1, Oncogene Science [diluted 1:100 in PBS/3% milk]), mouse anti-human Exportin-1/CRM1 monoclonal antibody (BD Biosciences, diluted 1:100 in PBS/3% milk), or mouse anti-human β-actin (Sigma, diluted 1:2,000 in PBS/3% milk). Bands were detected using the ECL chemiluminescence detection method (Amersham) and exposed on X-Ray film.
RESULTS

IR induces BRCA1 nuclear export

We examined the subcellular distribution of the BRCA1 protein in the nuclear and cytoplasmic compartments in MCF7 cells by subcellular fractionation and western blot analysis. Our results showed that before treatment with IR, the BRCA1 protein is present predominantly in the nuclear compartment with only a small amount of BRCA1 protein being detected in the cytoplasmic fraction. However, a notably increased amount of BRCA1 was detected in the cytoplasmic fraction at four and eight hours after exposure of MCF7 cells to 5 Gy of IR (Figure 1), suggesting that IR causes BRCA1 to translocate to the cytoplasm.

To confirm this initial observation and further quantitatively analyze BRCA1 distribution, we examined subcellular localization of BRCA1 before and after IR by immunohistochemistry (IHC) staining using anti-BRCA1 antibody (Ab-1). As reported previously (20) by others, we observed that three subcellular patterns of BRCA1 distribution in untreated MCF7 cells: nuclear only (N), cytoplasmic only (C), and both nuclear and cytoplasmic (N/C) (Figure 2a). The distribution of BRCA1, scored by their subcellular localization pattern, is nuclear-dominant, with 45.3% (±1.31%) being nuclear only, 44.5% (±1.68%) being cytoplasmic and nuclear, and only 11.00% (±1.9%) being cytoplasm only. However, after IR, the pattern of BRCA1 subcellular distribution in MCF7 cells shifts to cytoplasmic-dominant (Figure 2b). At 24 hours after 2.5 Gy of IR, the proportion of cells with N-pattern staining decreased from 45.3% (±1.31%) to 27.9% (±2.6%) (p<0.01), while cells with the C-pattern of staining increased from 11.00% (±1.9%) to 26.9% (±2.46%) (p<0.05). By 48 hours after IR, cells with N and C-patterns were 14.45% (±0.40%) and 38.46% (±2.23%) (p<0.01), respectively, suggesting that BRCA1 protein...
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continues to be exported from the nucleus to cytoplasm over a prolonged period after initial DNA damage.

Next, we examined DNA damage-induced BRCA1 NE as a function of the IR dose given to MCF7 cells. We found that 2.5 Gy of IR was sufficient for inducing significant BRCA1 NE (Figure 2c). The degree of IR-induced BRCA1 NE reached a plateau after 5 Gy with the N-pattern reducing to 22.7% (±0.55%), the C-pattern rising to 32.2% (±2.80%), and N/C-pattern remaining unchanged as a proportion. The data demonstrate that IR leads to nuclear export of BRCA1 in a dose-dependent manner up to a maximum effect at 5 Gy.

To exclude the possibility that the increase in the levels of cytoplasmic BRCA1 protein after IR is due to nuclear membrane leakage, we examined the subcellular distribution of the proliferating cell nuclear antigen (PCNA). Similar to BRCA1, PCNA is a nuclear protein associated with the nuclear matrix, based on the proportion of the protein that is resistant to detergent extractions (data not shown). PCNA is directly involved in DNA replication and commonly localized at the sites of ongoing DNA synthesis (35). In addition, PCNA also plays a role as an accessory factor in many DNA repair pathways, such as nucleotide excision repair, base excision repair, and possibly in homologous recombination (36-38). Using immunohistochemical staining with anti-PCNA antibody, we found that prior to IR PCNA was evenly distributed throughout the nucleus in a majority of cells, and discrete PCNA nuclear foci were observed in very few MCF7 cells. Twenty-four hours after doses up to 10 Gy of IR, PCNA remained in the nucleus and forms numerous large, brightly stained foci (Figure 2d). No cytoplasmic signal of PCNA was detected.

These results are consistent with findings from previous studies, suggesting that PCNA foci form in the nucleus in response to IR (35). More importantly, this result confirmed that there was no significant cytoplasmic contamination from nuclear protein leakage as a consequence of nuclear...
DNA damage induces p53-dependent BRCA1 nuclear export membrane injury under the experimental conditions used for studying BRCA1 NE, further suggesting that BRCA1 shuttling from the nuclear to the cytoplasmic compartment is a regulated process in response to DNA damage.

**Effect of the cell cycle on BRCA1 subcellular distribution**

BRCA1 protein is highly expressed in the nucleus of rapidly proliferating cells in a cell-cycle dependent manner, with low levels in G1 phase, peak levels at the G1/S border, and high levels maintained through S phase (30,39-42). To explore the mechanisms involved in BRCA1 shuttling, we examined if BRCA1 subcellular distribution is associated with the cells being in various phase of the cell cycle. Untreated MCF7 cells were separated into G0/G1-, S-, or G2/M-phases according to their DNA content and collected using flow cytometric sorting technique (Figure 3a). M-phase cells were recognized by their positive IHC staining of phosphorylated Histone-3 (p-H3) in G2/M population (Figure 3b). Using IHC staining with BRCA1 antibody, we found that the patterns of BRCA1 distribution were considerably different in each cell cycle phase. As shown in Figure 3c and 3d, during S-phase, BRCA1 accumulated predominantly in the nucleus [e.g. 79.19% (±2.23%) S phase cells being N pattern]. As cells entered the G2-phase, BRCA1 started to be transported to cytoplasm [e.g. N-pattern decrease to 56.73% (±2.17%) (p<0.05) and N/C-pattern increase to 36.73% (±2.82%) (p<0.05) in G2-phase cells compared to 79.19% (±2.23%) and 17.34% (±2.74%) in S phase cells respectively]. BRCA1 NE reached to peak level in M-phase with almost 100% cells demonstrated C-pattern. After mitosis, BRCA1 began to re-enter nuclear again. In G1/G0 phase, 14.21% (±0.71%) cells already re-accumulated BRCA1 in the nuclear, 11.94% (±2.61%) cells still are C-pattern, and the majority of cells [76.5% (±0.66%) (p<0.01)] exhibited the N/C pattern that may represent a transition stage of
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BRCA1. The dynamic changes of BRCA1 subcellular distribution with progress through the cell cycle correlated well with the cell cycle dependent BRCA1 protein expression. For example, when peak level of protein is reached at S-phase (29,31), the BRCA1 was predominantly in the nucleus, where BRCA1 may be required for DNA replication.

It is well known that mammalian cells undergo cell cycle arrest in response to IR-induced DNA damage. Based on the cycle-dependent BRCA1 subcellular distribution results, we reasoned that BRCA1 NE after DNA damage might be dependent on the phase of the cell cycle. To test this theory, we initially compared the effect of IR on BRCA1 NE in synchronized and asynchronized MCF7 cells. We used serum starvation to synchronize MCF7 cells. In exponentially growing MCF7 cells, G0/G1 cells consisted of 50.88% (±3.87%) of the asynchronized cell population. Serum deprivation resulted in cells arrested in G1/G0 phase. After 24 hours of serum withdrawal G0/G1 phase cells can be enriched to approximately 81.33% (±0.82%) (p<0.01), consistent with previously report by others (32) (Figure 4a). After release from serum starvation, cells started to re-enter the cell cycle; after 24 hours MCF7 cells returned to an asynchronized cell cycle profile (Figure 4a). Prior to IR treatment, BRCA1 distribution patterns were significantly different between the synchronized and asynchronized populations. In asynchronized MCF7 population, BRCA1 exhibited nuclear dominant pattern [e.g. 45.3% (±1.31%) N pattern, 44.5% (±1.68%) N/C pattern and 11.00% (±1.9%) C pattern]; while in synchronized cells, the distribution of BRCA1 became predominantly a nucleus/cytoplasm pattern [e.g., N/C pattern increased to 55.78% (±1.81%) (p<0.05) and the N-pattern decrease to 25.61% (±3.64%) (p<0.05), Figure 4b]. The shift in BRCA1 distribution with synchronized cells correlated with the high percentage of cells accumulated in G0/G1 phase (81.33%), and small number of cells remained in S phase (11.09 %) (Figure 4a). We found that DNA damage
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induced BRCA1 NE in both populations: the difference in BRCA1 distribution observed in synchronized compared with asynchronized cells prior to IR, was no longer present after IR (Figure 4b) since there was a significant shift to C and N/C pattern in both cell populations. This implies that the effect of IR on BRCA1 export is maintained independently of cell cycle distribution.

To confirm whether the effect of IR on BRCA1 localization was seen independently of cell cycle, we further looked at the effect of IR on sorted populations of cells. We compared BRCA1 distribution pattern prior and after IR in each individual phase of the cell cycle. Using flow cytometric sorting techniques as described above, MCF7 cells were separated to G0/G1, S and G2/M phases, exposed to IR and analyzed separately at 24 hours after IR. In M-phase cells that were separated from G2-phase cells by their positive p-H3 IHC staining, BRCA1 is completely excluded from nucleus with or without IR (Figure 4c). For cells in G0/G1, S, and G2 phases, BRCA1 IHC staining revealed that IR-induced BRCA1 NE occurred in each of the three cell cycles phases studied. We found the highest level of DNA damage-induced NE in S-phase cells, which characteristically accumulate BRCA1 in the nucleus in the absence of IR. As shown in Figure 4c, IR resulted in approximately 3-, 7-fold increase of cytoplasm only pattern in G0/G1, S-phase cells, with p values of 0.05 and 0.01 respectively. Accordingly, the nucleus only pattern decreased from 79.19% (±2.23%) to 53.72% (±7.39%) in S phase (p<0.05), from 56.73% (±2.16%) to 43.34% (±2.88%) in G2 phase (p<0.05), and in G1 cells the N/C pattern decreased from 76.5% (±0.66%) to 58.34% (±2.71%) (p<0.01). Thus, although the cell cycle does determine the sub-cellular location of BRCA1, the effect of DNA damage upon BRCA1 NE is seen independently of the cell cycle.
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**DNA damage-induced BRCA1 protein nuclear export uses a CRM1-receptor pathway**

To continue our investigation on the mechanisms involved in DNA damage-induced BRCA1 NE, we next studied CRM1 (Chromosome region maintenance 1), a nuclear export receptor that specifically forms a complex with proteins in the nucleus via their leucine-rich nuclear export signal (NES) to translocate nuclear proteins through the nuclear pore to the cytoplasm. In a recent study (20), a NES was identified in BRCA1 at its N-terminal region, and mutational inactivation of the NES in BRCA1 resulted in nuclear accumulation of wild-type BRCA1. To determine whether DNA damage-induced BRCA1 NE employs the CRM1-receptor pathway, we examined the effect of Leptomycin B (LMB), an antifungal compound that prevents the CRM1-NES interaction and specifically inhibits CRM1-mediated protein NE, on the subcellular localization of BRCA1 in MCF7 cells. As demonstrated in Figure 5a and 5b, LMB treatment of MCF7 cells alone caused a significant accumulation of BRCA1 in the nucleus, consistent with the previous report (20). The proportion of cells with BRCA1 in the nucleus (N-pattern) increased significantly from 45.6% (±1.12%) to 64.3% (±1.42%) (p<0.01), and cells with the N/C-pattern of BRCA1 decreased from 39.9% (±0.65%) to 14.4% (±0.88%) (p<0.01). Furthermore, when we pre-incubated MCF7 cells with LMB, IR no longer induced BRCA1 translocation from the nucleus to the cytoplasm (Figure 5, a and b). Instead, nuclear accumulation of BRCA1 was found, and the proportion of cells with BRCA1 retained in the nucleus was 73.5% (±1.41%). This compares to 23.6% (±1.62%) (p<0.01) with IR only or 45.6% (±1.12%) (p<0.01) with untreated control cells. The proportion of cells with BRCA1 located in the cytoplasm or nucleus/cytoplasm was significantly decreased. The retention of BRCA1 in the nucleus after IR in LMB pretreated MCF7 cells, together with the PCNA control experiments, demonstrate that there was no detectable non-specific nuclear membrane leakage.
DNA damage induces p53-dependent BRCA1 nuclear export up to 24 hours after IR treatment. Furthermore, these results demonstrate a direct inhibitory effect of LMB on IR-induced BRCA1 NE, suggesting that this process uses the CRM1-mediated pathway.

To verify that DNA damage-induced BRCA1 NE is mediated through CRM1 pathway, we evaluated the effect of overexpressing CRM-1 on DNA damage induced BRCA1 NE. The expression vectors carrying either CRM1-YFP or YFP alone were transiently transfected into MCF7 cells. As shown in Figure 5, c and d, overexpression of CRM1 in MCF7 cells caused a significant shift of BRCA1 from the nucleus to the cytosol, consistent with the previous reported by others (20). As shown in Figure 5d, the proportion of cells with BRCA1 in the nucleus (N-pattern) decreased significantly from 47.7% (±1.07%) to 26.89% (±1.65%) (p<0.01), the N/C-pattern increased from 45.68% (±0.60%) to 60.79% (±0.16%) (p<0.01), and the C-pattern increased from 6.63% (±1.67%) to 12.33% (±1.85%). Addition of IR treatment to cells overexpressing CRM1-YFP fusion protein further induced BRCA1 translocation from the nucleus to the cytoplasm. The substantial inhibitory effect of LMB (Figure 5a, 5b) suggested that CRM1 pathway is an essential mechanism for BRCA1 NE both during normal cell cycling and in response to DNA damage. The moderate enhancement of radiation induced BRCA1 NE by overexpression of CRM-1 indicates that the basal level of CRM-1 receptor is sufficient to carry out most of the required nuclear export function.

Given the data that CRM1 is an important factor for determining BRCA1 distribution, we next examined whether cell cycle regulated CRM-1 expression. MCF7 cells in G1, S and G2/M were collected through flow cytometric sorting according to their DNA content, as shown previously. Whole cell extracts were obtained from cells in different cell cycle phases and were subjected to SDS-PAGE gel separation. CRM-1 expression levels were determined using western blot using
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an anti-CRM1 antibody. As shown in Figure 5e, there was no difference in CRM-1 protein levels between the different cell cycle phases. Thus, CRM-1 was expressed at a constant level throughout the cell cycle, suggesting that control of CRM-1 protein expression is not a mechanism for regulating BRCA1 NE.

**DNA damage-induced BRCA1 Nuclear Export and p53 status**

We sought to determine whether DNA damage-induced BRCA1 cytoplasmic relocation was part of the cellular response to DNA damage involving the p53 protein. Several human cancer cell lines were examined for BRCA1 protein subcellular distribution with and without exposure to IR. A considerable increase in BRCA1 protein in the cytoplasm after IR was found in some cell lines, including MCF7 and primary human fibroblasts, but not in others, such as Hela cells, Capan-1 cells, and Saos2 cells, derived from human cervical cancer, pancreatic carcinoma, and osteosarcoma, respectively (Figure 6). This suggested a correlation between DNA damage-induced BRCA1 NE and their p53 status: both MCF7 and primary fibroblasts have wild-type p53, while Hela, Capan-1, and Saos2 cells have inactivated p53. It appeared that DNA damage-induced BRCA1 NE only occurred in cells that contain wild-type p53.

The p53 protein binds to BRCA1 (22-24,26). In response to genotoxic stress, p53 suppresses BRCA1 expression by down-regulating BRCA1 transcription (26). We reasoned that p53 might regulate BRCA1 function via several mechanisms. To examine the role of p53 in DNA damage-induced BRCA1 NE, we established a pair of isogenic cell lines differing only in p53 status, by infection of MCF7 cells, which contain wild-type p53, with retrovirus carrying either an empty control vector (MCF7/control) or HPV16-E6 (MCF7/E6). Expression of HPV16-E6 protein targets p53 protein for ubiquitination and degradation, thereby inactivating p53 function. Using
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western blot analysis, the functional status of p53 was verified by DNA damage-induced p53 expression and p53-dependent p21 induction as well as p53-dependent G1 arrest in parental MCF7 and MCF7/LXSN control. MCF7/E6 cells that stably express HPV16-E6 protein have lost p53 function (Figure 7d). Using co-immunostaining of BRCA1 and p53, we found that BRCA1 NE in response to DNA damage occurred only in MCF7 and MCF7/LXSN cells that have functional p53 (Figure 7a). Loss of functional p53 correlated with nuclear accumulation of BRCA1 in the nucleus of MCF7/E6 cells (Figures 7a-c) even after DNA damage. With IHC staining using anti-p53 antibody, we also found that approximately 30% of the MCF7/E6 cells still expressed p53 protein (Figure 7a). Therefore, BRCA1 compartmentalization in MCF7/E6 cells was scored separately in accordance to their p53 status. The proportion of cells with BRCA1 restricted to the nucleus was significantly increased in cells without functional p53 regardless of IR treatment [more than 71.09% (±4.05%)], compared to cells with functional p53 [approximately 49.71% (±4.35%) (p<0.05) in un-irradiated cells and 29.12% (±3.29%) (p<0.01) after IR]. Therefore, inactivation of endogenous p53 function in MCF7 cells by expression of HPV16-E6 protein abrogates DNA damage-induced BRCA1 NE.

We recognize that the use of the E6 protein to inactivate p53 may not be specific. In order to confirm that BRCA1 NE was dependent on wt-p53 function, we tested another pair of isogenic cell lines, the human bladder cancer cell line (EJ-p53) in which the endogenous p53 is mutated and nonfunctional, but is not dominant-negative, and restoration of p53 function is enabled using a tetracycline-regulated inducible expression system(34). The EJ-p53 cell has been demonstrated in numerous papers to be a valid p53+ / p53− system. Exogenous wild-type p53 expression can be induced in EJ-P53 cells within 6h by removal of tetracycline (Tet) from the culture media. Wild-type p53 function was confirmed by ionizing radiation induction of the downstream target
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gene, p21 (Figure 8d). BRCA1 subcellular distribution and p53 expression were determined by IHC co-staining with anti-BRCA1 and anti-p53 antibodies. In the presence of Tet (Tet-on), p53 expression is absent (Figure 8a) and the majority of cells have BRCA1 protein retained in the nucleus, with the proportion of N-pattern distribution being 83.48% (±2.06) (Figure 8b). IR treatment did not alter BRCA1 localization (Figure 8c). In the absence of Tet (Tet-off), 74.39% (±2.72%) EJ-p53 now were positive for p53, determined by IHC staining (Figure 8a), while 25.51% (±2.70%) of the cells still had no detectable p53. In this latter group of cells, BRCA1 protein was retained in the nuclei regardless of IR treatment, similar to that observed in EJ-p53 with Tet-on. However, in cells with Tet-off-induced p53 expression, we found that the proportion of EJ-p53 cells with BRCA1 in the nucleus decreased significantly to less than 41.29% (±2.86) (p<0.01), compared with more than 83.48% (±2.06%) in p53-negative EJ-p53 cells. Restoration of p53 expression in EJ-p53 cells resulted in a significant increase in the proportion of cells with BRCA1 N/C distribution pattern [more than 51.63% (±3.43%) (p<0.01)], compared with that of p53-negative cells [less than 15.01% (±1.51%) (p<0.01)]. We noted that the degree of BRCA1 cytoplasmic re-localization in p53-positive EJ-p53 cells was not enhanced further by IR treatment (Figure 8a,c). The explanation may be that the expression level of p53 induced by Tet-off reached a maximum level, equivalent to that induced by IR, which suggests that p53 is the key determinant for subcellular distribution of BRCA1 in response to IR. Taken together, these data demonstrate that loss of p53 leads to BRCA1 nuclear accumulation, and DNA damage-induced BRCA1 NE requires p53.

P53 is a well-known regulator of transcription, either activating or repressing transcription of its numerous downstream target genes. Given that both p53 and CRM-1 are involved in the BRCA1 NE process, we next measured whether p53 could regulate CRM1 expression. We
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found that CRM-1 expression is independent of p53 function (Figure 7d, 8d). Therefore, the data suggest that regulating the level of CRM-1 protein was not the mechanism by which p53 controls DNA damage-induced BRCA1 NE.
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DISCUSSION

The tumor suppressor gene BRCA1 plays multiple roles in the cellular response to DNA damage. Several aspects of BRCA1 function require its nuclear localization, such as DNA repair, transcriptional regulation, and cell cycle checkpoint control. Recent studies have shown that BRCA1 is a nuclear-cytoplasmic shuttling protein (20). It is targeted to the nuclear compartment through an NLS-mediated importin pathway (17,18), and exported to the cytoplasmic compartment via a NES-facilitated exportin/CRM1 mechanism (20).

In this paper, we studied the effect of DNA damage on BRCA1 nuclear localization and showed evidence that BRCA1 relocated to the cytoplasm in response to DNA damage. Although the cell cycle had an influence upon BRCA1 localization, the effect of DNA damage was found above and beyond cell cycle effects. BRCA1 nuclear export was dependent on CRM-1/exportin pathway. Furthermore, p53 controlled the IR-induced BRCA1 cytoplasmic relocation, and loss of p53 function results in BRCA1 nuclear accumulation. The control of BRCA1 localization between nuclear and cytoplasmic compartments may be an additional mechanism for regulating BRCA1 function in response to DNA damage. The concept is that p53 regulates a complex cascade of effects in response to DNA damage, which can extend to BRCA1 NE, implying that the presence of BRCA1 in the nucleus after DNA damage may create a conflict with other aspects of the p53-dependent DNA damage response. BRCA1 nuclear export occurs from 4 to >24 hours, consistent with a role in the G1 to S checkpoint and recovery, when the BRCA1 protein is not required. Indeed, BRCA1 binding to DNA damage could activate responses that are incompatible with activating the G1/S checkpoint.

Regulation of BRCA1 function by nuclear export
Multiple functions of the BRCA1 protein have been identified (43), but the critical roles of BRCA1 appear to occur in response to DNA damage. BRCA1 function is essential for suppression of tumorigenesis during cell proliferation and growth with genotoxic stress. Several mechanisms are involved in regulation of BRCA1 function. For example, BRCA1 is activated by transcription during cell cycle and in response to DNA damage (6, 26); the binding of BRCA1 to different cellular proteins to form a variety of functional complexes is based on the cell cycle stage or response to genotoxic stress (9-11); and phosphorylation of BRCA1 occurs in S-phase of the cell cycle and after DNA damage (7, 8, 32).

Subcellular localization is another potential way to regulate the function of BRCA1. The function of several cancer-related gene products is reported to be regulated by subcellular localization. For example, cytoplasmic retention of cyclin-dependent kinase inhibitor p27^{kip1}, as a consequence of PKB/Akt-mediated phosphorylation, compromises its function in G1-arrest, which may contribute to the process of cancer development. It is known that tumor suppressor proteins p53 and APC (adenomatous polypsis coli) (26, 44) are shuttled between the nucleus and cytoplasm for control of their function. Data from this investigation (and others) provide cellular and molecular evidence suggesting that BRCA1 is a shuttle protein and its function can be regulated by subcellular localization using both the NLS/importin and the NES/exportin/CRM1 pathways (17-20).

**Control of BRCA1 subcellular location**

BRCA1 function is closely associated with the cell cycle (28). In this study, we showed evidence that the cell cycle controls the subcellular location of BRCA1, which was predominantly in the nucleus during S phase, but was exclusively restricted to the cytoplasm in
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M phase. The transition between nuclear accumulation and exclusion take places in G1 and G2 phases. The cell cycle dependent expression of BRCA1, with increased expression found in S-phase, is consistent with BRCA1’s primary function in cell proliferation and DNA replication.

Genetic evidence suggests that BRCA1 is exported from the nucleus through the interaction of its NES with CRM-1 (20). This study revealed that DNA damage-induced BRCA1 NE is suppressed by LMB, the specific inhibitor of CRM-1, and enhanced by transient overexpression of exogenous BRCA1. The CRM-1 receptor pathway could be employed by both cell cycle dependent and DNA damage induced BRCA1 NE. However, since LMB can affect the export of other proteins, the possibility remains that the effect of LMB on DNA damage-induced BRCA1 NE is secondary to the effects on other proteins.

We observed that only the phosphorylated band of BRCA1 was exported in the fractionated western blot analysis (Figure 1), implying that phosphorylation facilitates the export process directly. It has been reported that BRCA1 is hyper-phosphorylated during S-phase and after IR through DNA damage-activated kinases such as ATM, ATR, and hCds1/Chk2. Several phosphorylation sites in BRCA1 have been identified, including Ser-988, -1387, -1423, -1457 and -1524 (7,45-48). The function of BRCA1 mutants including the ATM-phosphorylation sites, Ser-1423 and Ser-1524 was found to result in a defective G2 to M transition. Mutation at the Chk2-phosphorylation site, Ser-988, resulted in defects in DNA repair (7,27,46,49). Furthermore, the phosphorylation status of BRCA1 influences its sub-nucleus location (50). Chk2-mediated Ser-988-phosphorylation of BRCA1 leads its translocation to the perinuclear region in response to IR or UV radiation in S phase. Further investigations using specific genetic alterations and phosphorylation specific antibodies will be needed to clarify the role of BRCA1 phosphorylation in subcellular location.
Functional interplay between p53 and BRCA1

p53 and BRCA1 are tumor suppressors and both involved in many cellular processes ranging from DNA double strand repair (32,51-53), cell cycle arrest (27,49,54,55), apoptosis (56-59), and transcriptional regulation (32,60-62). A direct functional link between p53 and BRCA1 was initially suggested by the observation that loss of p53 can partially rescue embryonic lethality in BRCA1-knockout mice (63). BRCA1 has been reported to bind directly to p53 through its N-terminal ring domain and its C-terminal BRCT domain, thereby enhancing p53-mediated transcriptional activation (22-24,26). Interestingly, several studies have found that the BRCA1 protein level decreases in response to DNA damage (26,64), and that down-regulation of BRCA1 is dependent upon wild-type p53 function (26). We found that nuclear export of BRCA1 in response to DNA damage occurred only in cells with functional p53. In cells lacking wild-type p53, BRCA1 was retained in the nucleus. In addition, the induction of p53 results in BRCA1 NE in the absence of IR, suggesting that nuclear export of BRCA1 is a specific regulatory mechanism dependent on p53. The lack of association between CRM-1 expression p53 function excludes the possibility that p53 regulates BRCA1 NE through direct control CRM-1 protein level.

To explore further the mechanisms of p53-mediated regulation of BRCA1 NE, we examined whether p53 expression in our experimental system resulted in apoptosis. A small percentage of MCF7 cells and even fewer EJ-p53 cells (after the induction of wt-p53) underwent apoptosis after IR treatment within the 24-48 hours of observation (data not shown). In addition, we found no correlation between BRCA1 NE and apoptosis. Therefore, the data argue against the idea that the observed BRCA1 NE occurs as a result of p53-induced apoptosis.
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P53 may regulate BRCA1 NE by several mechanisms. For example, p53 may enhance BRCA1 NE either directly through protein-protein interaction with BRCA1 or indirectly as a consequence of p53-induced G1 arrest. In fact, DNA damage-induced G1 arrest was compromised in both MCF7/E6 and EJ-p53/tet-off cell system compared to their wt-p53 counterparts, MCF7 and EJ-p53/tet-on (data not shown). It is well known that BRCA1 is required for cell proliferation and DNA replication. Eliminating BRCA1 from the nucleus may be required for the cell to arrest in the cell cycle in response to DNA damage without creating genomic instability. A failure to remove BRCA1 from the nucleus during cell cycle arrest may create conflicting signals and lead to an unstable cell.

BARD1 chaperones BRCA1 into the nucleus via a direct protein interaction, and is independent of the NLS/importin pathway. BARD1 also inhibits BRCA1 NE by directly masking the NES within BRCA1. Therefore, BARD1 can promote BRCA1 nuclear targeting and the formation of DNA repair foci after DNA damage (21). In this regard, p53 appears to counteract BARD1’s function in regulating BRCA1 subcellular localization, but whether BARD1 is critical for mediating the effect of p53 on BRCA1 NE is not yet established.

The biological significance of controlling BRCA1 subcellular location

Given the functions of BRCA1 in DNA repair, it seems at first sight paradoxical that a cell chooses to move BRCA1 to the cytoplasm after DNA damage. The kinetics of this process suggest that DNA damage-induced BRCA1 NE was not an immediate response to IR treatment in MCF7 cells, or to the removal of tetracycline in EJ-p53 cells. Significant BRCA1 NE was not detected until 4 hours after IR (Figures 1 and 2b), while DNA damage-induced BRCA1 hyper-phosphorylation occurs within 30 minutes. Proteolytic cleavage of key repair proteins can be an
DNA damage induces p53-dependent BRCA1 nuclear export early step in the apoptotic response, but cleavage of BRCA1 was not observed. Furthermore, BRCA1 was exported from the nucleus in a much greater percentage of living cells than the proportion of cells that die as a result of IR or p53 induction (data not shown).

In light the fact that BRCA1 is critical protein that promotes homologous recombination and replication, we favor a model in which specific export of BRCA1 is designed to remove a known DNA replication/repair factor from the active sites of an irradiated cell in the short term, to allow processing of these complex lesions to be coordinated with the activation of cell cycle checkpoints following IR. The presence of BRCA1 in the setting of complex double-stranded DNA lesions may activate aberrant homologous recombination at an improper time, which would be detrimental to cell. Recent studies showed that expression of a truncated BRCA1 mutant that contains the ring domain and the NES only, enhanced BRCA1 nuclear export activity, resulted in decreased endogenous BRCA1 nuclear staining and ionizing radiation induced foci (IRIF), and conferred increased radiosensitivity. Expression of the same mutant in transgenic mice caused delayed lactational mammary development (21,65). These findings, together with the data from this study, support the view that spatial and temporal control of the BRCA1 protein is an important mechanism for regulating its function.
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**Figure legends:**

**Figure 1. Nuclear export of BRCA1 protein after DNA damage:** MCF7 cells were irradiated to a dose of 5 Gy, the nuclear and cytoplasmic fractions were isolated at the indicated times after irradiation, and the proteins were separated by SDS-PAGE gel electrophoresis. The BRCA1 protein was detected by Western blotting with anti-BRCA1 antibody (Ab-1).

**Figure 2. Nuclear export of BRCA1 protein after IR in MCF7 cells:** The BRCA1 protein was identified by the Ab-1 antibody and the Alexa Fluor594-labeled anti-mouse IgG secondary antibody (red). The cell nuclei were stained with DAPI (blue). (a). The different subcellular distribution patterns of BRCA1 are shown. The N, NC and C patterns of staining for BRCA1 stand for nuclear, nuclear/cytoplasmic and cytoplasmic patterns respectively (see text). (b). The kinetics of BRCA1 translocation after exposure to 2.5 Gy IR. Each data point represents the results (mean ± S.E.) from three independent experiments. At least 500 cells were counted per experiment. The percentage of cells exhibiting N (black circles), NC (open circles), or C (black triangle) patterns of BRCA1 distribution are shown. (c). IR-induced nuclear export of BRCA1 as a function of dose. Cells were stained at 24 hours after IR at the doses indicated. The number of observations and symbols are as in (b). (d). The subcellular distribution of PCNA (red), in contrast to BRCA1. The distribution of the nuclear protein PCNA before and 24 hours after 10Gy of IR are displayed in the upper and lower panels respectively.

**Figure 3. The effect of cell cycle on BRCA1 subcellular distribution:** (a). MCF7 cells were separated by DNA content and sorted by flow cytometry into G0/G1, S, and G2/M phases, using narrow channel gating as shown to assure relative purity of the sorted samples. (b). To identify
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M phase cells within G2/M population, the phosphorylated form of histone H3, a specific marker of M phase, was stained with the anti-phospho-Histone 3 (p-H3) antibody and the Alexa Fluor488-labeled secondary antibody (green). (c). The subcellular localization of BRCA1 in cells sorted into G0/G1-, S-, G2-, or M-phases. The BRCA1 protein was identified with Ab-1 antibody (red) as in Figure 2. (d). The proportion of cells with N, N/C, and C pattern of BRCA1 staining in the different phases of the cell cycle. Bars represent the observed proportion +/- SEM from three independent experiments. P-values (see text) were estimated using the paired t-test.

**Figure 4. The effect of IR on BRCA1 distribution in relation to cell cycle stages:** (a). Cell cycle profile (percentage of cells in G1 vs S vs G2/M by flow cytometric analysis) of MCF7 cells in exponential growth; 24 hours after serum deprivation; and, 24 hours after releasing from synchrony. The effect of exposure to IR with or without synchrony is shown. (b). The effect of IR on BRCA1 distribution in asynchronized and synchronized cells before and after IR. BRCA1 location was determined using the same categories as in Figure 2. (c). The effect of IR on BRCA1 distribution in MCF7 cells from different phases of the cell cycle. Bars in panel a, b, and c represent the observed proportion +/- SEM from three independent experiments. P-values (see text) were estimated using the paired t-test. *Synchro represents synchronization.

**Figure 5. CRM1 mediates IR-induced BRCA1 NE in MCF7 cells:** (a). The redistribution of BRCA1 in response to DNA damage was compared in cells with (+) or without (−) LMB treatment 16 hours prior to exposure to 10 Gy of IR (lower panel) and to the control unirradiated conditions (upper panel). BRCA1 was stained with antibody (Ab-1) 24 hours after IR. (b). The proportion of cells, out of 500 cells evaluated per experiment, showing the N, NC and C patterns
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of BRCA1 localization were scored for the same conditions as shown in (a). (c). Overexpression of CRM-1 increases DNA damage induced BRCA1 NE. BRCA1 was stained with antibody Ab-1, shown in red. The expression of YFP-tagged CRM-1 is visualized as green. The presence of CRM-1 results in more BRCA1 shifted to the cytoplasm. (d). The effect of IR on proportion of cells with N, N/C, and C pattern of BRCA1 staining, with or without CRM-1 expression. Bars in panel b, and panel c represent the observed proportion +/- SEM from three independent experiments. P-values (see text) were estimated using the paired t-test. (e). The effect of cell cycle on CRM-1 expression. The whole cell extracts from cells in G0/G1, S and G2/M phases were separated on SDS-PAGE gels, and CRM-1 was detected by Western blotting using anti-human Exportin-1/CRM1 monoclonal antibody (BD, Biosciences).

Figure 6. DNA damage-induced nuclear export of BRCA1 protein is associated with functional p53: The BRCA1 protein was visualized in the cytoplasmic and nuclear fractions of cells by western blotting at 0, 4 and 8 hours after exposure to 5 Gy of IR. Cells with (MCF7) and without (MCF7/E6, HeLa, Capan-1) functional p53 are shown. In cells with inactivated p53, BRCA1 was retained in nucleus and no detectable BRCA1 was found in the cytoplasmic fraction after DNA damage. BRCA1-P represents the lower mobility phosphorylated form of BRCA1.

Figure 7. Abolishing wild-type p53 by expression of HPV16-E6 in MCF7 cells inhibits nuclear export of BRCA1: (a). The effect of p53 on the localization of BRCA1, with or without IR treatment. The loss of p53 in MCF7/E6 cells results in nuclear retention of BRCA1, regardless of IR-induced DNA damage. In cells with p53, BRCA1 is detected in the cytoplasm before IR, and becomes more prominently cytoplasmic after IR. (b). The percentage of cells
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showing N, NC or C pattern of BRCA1 staining before IR. Each data point represents the results (mean ± S.E.) from three independent experiments, with at least 500 cells were counted per experiment. P-values (see text) were estimated using the paired t-test. MCF7/LXSN denotes MCF7 cells transfected with the empty retroviral vector. MCF7/E6 is a pooled population of MCF7 cells transfected with HPV16-E6. A proportion of the cells in this population still maintains a normal amount of p53 expression, and was scored separately (MCF7/E6-p). Those cells in which p53 was degraded by E6 and was no longer detectable were also scored separately (MCF7/E6-n). (c). Same as (b), but 24 hours after exposure to IR (2.5 Gy). (d). The functional status of p53 was verified by the amount of p53 before and after IR, together with p21 induction after IR in MCF7, MCF7/LXSN and MCF7/E6 cells. The levels of p53 and p53-dependent p21 induction were decreased both before and after IR in MCF7/E6 cells.

Figure 8. Restoration of p53 function in EJ cells leads to recovery of IR-induced BRCA1 nuclear export: (a). Localization of BRCA1 and p53 visualized with immunofluorescence. The upper panels denote unirradiated cells and the lower panels show cells 24 hours after exposure to 2.5 Gy. The removal of tetracycline from the tissue culture medium (Tet-off) results in expression of p53, as shown in the second column. BRCA1 is found in the cytoplasm when p53 is induced. The yellow arrows point to the cells without p53 expression; white arrowheads point to the cells with wt-p53 expression. (b). The proportion of cells with N, NC and C patterns of BRCA1 staining. With Tet-on, p53 staining is not seen. After Tet-off, some cells are p53 (+) and others are p53 negative (−), which were scored separately. Each data point represents the results (mean ± S.E.) from three independent experiments, with at least 500 cells were counted per experiment. P-values (see text) were estimated using the paired t-test. (c). Same as (b) with
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the addition of 2.5 Gy IR as indicated. (d). The levels of p53, p21 and CRM1 were verified by western blotting, as in Figure 7. With Tet-on, p53 levels are almost undetectable. Tet-off results in the induction of significant levels of p53 and p21, implies that the p53-p21 pathway is functionally intact.
Figure 1. Nuclear export of BRCA1 protein after DNA damage
DNA damage induces p53-dependent BRCA1 nuclear export

Figure 2. Nuclear export of BRCA1 protein after IR in MCF7 cell
DNA damage induces p53-dependent BRCA1 nuclear export

Figure 3. The effect of cell cycle on BRCA1 subcellular distribution
DNA damage induces p53-dependent BRCA1 nuclear export

Figure 4. The effect of IR on BRCA1 distribution in relation to cell cycle stages
Figure 5. CRM1 mediates IR-induced BRCA1 NE in MCF7 cell
DNA damage induces p53-dependent BRCA1 nuclear export

| IR (Hrs) | cytoplasm | nucleus |
|----------|-----------|---------|
| 0        | 1         | 6       |
| 0        | 1         | 6       |

- MCF7
  - BRCA1-p
  - BRCA1
- MCF7/E6
  - BRCA1-p
  - BRCA1
- Hela
  - BRCA1-p
  - BRCA1
- Capan-1
  - BRCA1-p
  - BRCA1

Figure 6. DNA damage-induced nuclear export of BRCA1 protein is associated with functional p53
DNA damage induces p53-dependent BRCA1 nuclear export

Figure 7. Abolishing wild-type p53 by expression of HPV16-E6 in MCF7 cells inhibits nuclear export of BRCA1
Figure 8. Restoration of p53 function in EJ cells leads to recovery of IR-induced BRCA1 nuclear export.
