The Mechanism of p53-independent Late Apoptosis Induced by Exposure to Ionizing Radiation

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ABSTRACT

Once double-strand breaks (DSBs) are generated in genome DNA by exposure to ionizing radiation (IR), the cell cycle checkpoint is activated to facilitate DNA repair. However, incompletion of DNA repair causes apoptosis in irradiated cells. IR-induced apoptosis occurs at either early or late time points after exposure to IR. The early apoptosis is mediated by p53, but the late apoptosis seems to be regulated by unknown mechanism independent on p53. In order to clarify the mechanism of IR-induced late apoptosis, IR-induced cellular responses, including late apoptosis were analyzed using AT and NBS cells. AT cells suffered more apoptosis than normal cells at late-stage after IR, and this apoptosis in NBS cells was induced at an intermediate level between those in AT and normal cells. Moreover, AT cells showed a defect in phosphorylation of Chk2 and SMC1, which are involved in regulation of IR-induced apoptosis and intra-S-phase checkpoint, whereas NBS cells showed a partial defect in these phosphorylation following IR. Further, AT and NBS cells showed more increase of chromosomal aberrations than in normal cells after irradiation at S phase, compared to that after irradiation at G1 phase. These AT and NBS cells also showed more induction of apoptosis than normal cells after irradiation at S phase. Therefore, these results suggest that the lack of intra-S-phase checkpoint after IR, due to the defect in ATM and/or NBS1 gene may lead to p53-independent late apoptosis through chromosomal aberrations.

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INTRODUCTION

When cells are exposed to ionizing radiation (IR), DNA double-strand breaks (DSBs) are generated within the genomic DNA. Since DSBs are genotoxic, the irradiated cells rapidly arrest their cell cycles to repair the damage. IR-induced cell cycle arrest occurs at the G1, S, and G2 phases and is mediated by the respective checkpoint mechanisms[1,2]. Of these, the mechanism of the G1 checkpoint has been elucidated, and is known to be mediated by the *p53* tumor suppressor gene[3]. The generation of DSBs induces the accumulation of p53[4], which promotes p21 expression[5]. Thereafter, p21 represses the activity of the cdk/cyclin complex, which is required for cell cycle progression, and G1 arrest results[5]. DNA repair, following the cell cycle checkpoints, is mainly regulated by two pathways: (i) homologous recombination (HR), which uses an undamaged sister-chromatid or homologous chromosome as a template for repair; and (ii) non-homologous end-joining (NHEJ), in which DNA ends with little or no homology are joined together[6,7]. The completion of DSB repair leads to the resumption of cell cycle progression. Although DSB damage is repaired in most cell types using the above mechanisms, lymphocytes, thymocytes, and epithelial cells of the small intestine proceed to cell death or apoptosis without DNA repair soon after exposure to IR[8-10]. On the other hand, most cells undergo apoptosis at late time-points after IR, especially after the generation of high numbers of DSBs in response to high doses of IR[11].

Although different cell types show IR-induced early apoptosis, the underlying mechanism has been clarified using lymphoblastoid cell lines (LCLs). LCLs show a rapid increase in p53, as well as induction of apoptosis[12]. Wild-type LCLs undergo rapid apoptosis after IR, whereas p53-defective LCLs infected with HPV16 virus

lack this response[8]. Moreover, thymocytes and intestinal cells from *p53*-knockout mice do not undergo apoptosis at an early stage after IR[9,10]. Furthermore, p53 regulates the expression of Bax and Bcl-2 following IR, and is required for the activation of caspase-3[8,13]. Since Bax, Bcl-2, and caspase-3 are mediators of apoptosis[8,13], p53 may regulate IR-induced apoptosis through these factors.

While IR-induced early apoptosis is regulated by p53, IR-induced late apoptosis seems to be induced in a p53-independent manner. Small intestinal epithelia from *p53*-knockout mice do not suffer p53-dependent early apoptosis, but undergo apoptosis at a comparatively later time (24 h) after exposure to IR[14]. Moreover, T lymphocytes derived from *p53*-knockout mice undergo apoptosis at 48 h after IR[15]. Furthermore, the human promyelocytic leukemia cell line HL-60, which is known to be deficient for p53, undergoes apoptosis at a late stage (72 h) after IR[16]. These findings indicate that IR-induced late apoptosis occurs *via* a p53-independent pathway.

To date, cells from radiation-hypersensitive diseases, particularly ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS), have been used to identify IR-induced cellular responses. AT and NBS are two distinct, but closely related rare autosomal recessive disorders that share some common clinical features, such as immunodeficiency, clinical radiosensitivity, ovarian dysgenesis, and cancer predisposition. The prominent external signs of AT are progressive cerebellar ataxia, and bulbar telangiectasia[17]. Unlike AT patients, NBS patients show microcephaly, mental retardation, and a characteristic facial appearance[17,18]. However, AT and NBS patients show similar cellular phenotypes, such as chromosomal instability, radiation hypersensitivity, and radioresistant DNA synthesis (RDS), which is due to a defect in the intra-S-phase checkpoint[17,18]. These facts suggest that the gene products that are lacking in the cells of AT and NBS subjects are involved in common IR-induced cellular responses, such as cell cycle checkpoints and DNA repair.

Although AT and NBS cells have similar phenotypes, two different genes have been implicated in these diseases. The gene responsible for AT is *ATM*[17,19], while that responsible for NBS is *NBS1*[20-22]. The ATM protein contains a PI-3 kinase catalytic domain at the C-terminus, and is a protein kinase with homologies to ATR (ATM and Rad-3 related) and the DNA-dependent protein kinase catalytic subunit (DNA-PKcs)[17,19]. ATM phosphorylates proteins that contain the SQ motif[23,24]. ATM is activated rapidly in response to IR or to treatment with other radiomimetic agents that generate DSBs[25,26]. Recently, it has been reported that this activation is regulated by the phosphorylation of ATM at serine 1981[27]. ATM is known to phosphorylate several cell cycle checkpoint-related proteins, such as p53, Chk2, and Chk1, in response to IR[25,26,28,29]. AT fibroblast cells lack p53 phosphorylation and do not arrest at the G1 checkpoint[3,30], which suggests that ATM regulates the G1 checkpoint through p53 phosphorylation. Moreover, LCLs from AT patients and thymocytes from *Atm*-knockout mice do not undergo early apoptosis in response to IR[31,32]. Thus, ATM may function in IR-induced early apoptosis by affecting the phosphorylation of p53. Furthermore, it has been reported that the phosphorylation of Chk2 and Chk1 by ATM is required for the regulation of the G1, intra-S-phase, and G2 checkpoints in response to IR[28,29,33]. Taken together, these findings indicate that ATM modulates both cell cycle checkpoints and early apoptosis in response to IR through phosphorylation. ATM may also function in DNA repair. The cells of AT patients show spontaneous chromosomal aberrations and have an increased frequency of chromosomal breaks after IR[17]. The technique of γ-H2AX foci analysis has been suggested as an approach for the measurement of DSB repair, and the kinetics of disappearance of γ-H2AX foci after exposure to IR closely resemble the kinetics of DSB repair[34]. AT cells show slightly delayed formation of γ-H2AX foci following IR, although the numbers of foci are similar to those in normal cells. However, AT cells show significantly more residual foci than normal cells at 4 h or later after IR[34], which is in agreement with the DSB repair defect seen in AT cells. Furthermore, ATM phosphorylates DNA repair-related proteins, such as Brca1, 53BP1, and NBS1[35-37]. Therefore, ATM may also play a critical role in DNA repair through the phosphorylation of several proteins.

The *NBS1* gene encodes a protein that contains a forkhead-associated (FHA) domain and a breast cancer C-terminal (BRCT) domain at the N-terminus[22]. The FHA and BRCT domains are often found in eukaryotic nuclear proteins, and are associated with regulation of

cell cycle checkpoints and DNA repair[38,39]. NBS1 forms a complex with hRAD50 and hMRE11[21,40]. This NBS1/hMRE11/hRAD50 (N/M/R) complex is recruited rapidly to DSB sites that are induced by IR, and forms nuclear foci at these sites[21,41,42]. Moreover, the N/M/R complex co-localizes with similar nuclear foci that are formed by DNA repair-related proteins, such as 53BP1, H2AX, MDC1, and Brca1, at DSB sites[36,43–45], which suggests that NBS1 functions in DNA repair. Furthermore, *NBS1*-deficient cells show an increased frequency of chromosomal breaks[46], and *Nbs1*-disrupted chicken cells show a dramatic reduction in mitomycin C-induced sister chromatid exchange[47]. This reduction in *NBS1*-deficient cells is comparable to that in *Rad54* and *Rad51* paralog knockout chicken cells, which are typical HR-deficient cells[48,49]. These findings indicate that NBS1 is essential for HR-mediated DNA repair. On the other hand, the function of NBS1 in regulating the IR-induced cell cycle checkpoint has also been suggested. NBS cells show defects in both the induction of p53 and the G1 checkpoint after IR, which suggests the involvement of NBS1 in regulating the IR-induced G1 checkpoint[50]. In addition, NBS1 is phosphorylated at several SQ-sites by ATM in response to IR[37,51]. Both NBS and AT cells show RDS, which results from a defect in the intra-S-phase checkpoint, and this checkpoint requires NBS1 phosphorylation by ATM. Therefore, the intra-S-phase checkpoint is regulated by co-operation between ATM and NBS1. Furthermore, it has been reported recently that NBS cells show defects in the binding of ATM to DSB damage sites and subsequent activation[52]. This finding suggests that NBS1 mediates the activation of ATM through recruitment to DSBs, and this functional link between ATM and NBS1 may explain the similarities in the phenotypes of their respective knockout strains.

Thus, both ATM and NBS1 are key regulators for several IR-induced cellular responses, which include cell cycle checkpoints, DNA repair, and early apoptosis. However, it is still unclear whether ATM and/or NBS1 function in IR-induced late apoptosis. Moreover, the mechanism of this apoptosis has not been clarified, although this type of apoptosis is an important element in the radiation sensitivity of many p53-defective cancers. Therefore, the purpose of this study was to analyze the cellular responses after IR of p53-defective normal, AT and NBS cells, to clarify the mechanism of p53-independent late apoptosis.

MATERIALS AND METHODS

Cell lines

HeLa and MRC5SV cells were used as control cells. The HeLa cells form epitheloid cells in monolayers, are derived from a human cervix uteri carcinoma, and are defective for p53 function. MRC5SV is an immortalized cell line that was derived from primary human fibroblast MRC5 cells by infection with SV40 virus. The three AT (AT-T55, AT-28, AT-5BIVA), and three NBS (GM24SV, WGSV7, 1022QSV) cell lines were derived from the primary fibroblasts of ataxia telangiectasia (AT) and Nijmegen breakage syndrome (NBS) patients, by infection with SV40 virus. These AT and NBS cells were kindly provided by Dr. K. Komatsu (Kyoto University, Japan). The SV40-transformed cells were defective for p53 function. All the cell lines were cultured in Dulbecco's Modified Eagle's Medium (Sigma) that was supplemented with 10% fetal calf serum, and incubated in a 5% CO_2 incubator at 37°C.

Irradiation of cells

Sub-confluent cells in culture dishes were irradiated with 2, 5, or 10 Gy of ^{60}Co-γ-rays at room temperature, and used in the subsequent experiments.

Dye exclusion assay

The cells were collected by trypsinization and centrifugation, resuspended in phosphate-buffered saline [PBS; 20 mM phosphate (pH 7.4), 150 mM NaCl], and then diluted 1:1 with trypan blue (Life Technologies). Unstained (living cells) and stained cells (dead cells) were counted separately under the light microscope, and the percentage of cell death was calculated[53].

DNA fragmentation assay

The fragmentation of nucleosomal DNA is a widely recognized hallmark of apoptotic cell death[54]. The cells were collected by trypsinization and centrifugation, and then lysed in lysis buffer [10 mM Tris-HCl (pH 8.0), 10 mM EDTA, 0.5% Triton X-100] for 30 min. The cell supernatant solutions were then centrifuged at 15,000 rpm for 15 min at 4°C, to separate the supernatant-containing fragmented DNA from the intact chro-

matin (pellet). The supernatants were incubated with RNase A (50 μg/ml; Sigma) for 1 h at 37°C. The supernatants were then incubated with proteinase K (200 μg/ml; Sigma) plus 0.5% sodium dodecyl sulfate (SDS; Sigma) for 2 h at 50°C. The samples were mixed with a phenol:chloroform:isoamyl alcohol (25:24:1) solution that was saturated with TE buffer [10 mM Tris-HCl (pH 8.0), 1 mM EDTA]. The fragmented DNA solution was precipitated with cold 100% ethanol and 5 M NaCl, and subsequently air-dried. The DNA samples were dissolved in TE buffer, mixed with six volumes of DNA loading buffer (40% sucrose, 50 mM EDTA, 0.25% bromophenol blue), and loaded onto a 1.8% agarose gel that contained ethidium bromide (final concentration 0.2 μg/ml). Electrophoresis was conducted in TBE buffer [90 mM Tris (pH 8.0), 90 mM boric acid, 2 mM EDTA] at 5 V/cm. Finally, the DNA bands were visualized by UV illumination.

Western blot analysis

Cells were collected by trypsinization and centrifugation. The collected cells were lysed on ice for 10 min with RIPA buffer [50 mM Tris-HCl (pH 7.5), 5% Nonidet P-40, 0.5% SDS, 750 mM NaCl, 5 mM EDTA] that contained phenylmethylsulfonylfluoride (100 μg/ml), leupeptin (10 μg/ml), Na_3VO_4 (1 mM), and NaF (25 mM). The samples were then centrifuged at 14,000 rpm for 10 min at 4°C. Aliquots of 20 μl of the supernatant proteins were mixed with 5 μl of Laemmli loading buffer [50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue, 10% glycerol], and incubated for 5 min at 95°C. These samples were electrophoresed on a 6–12% SDS-polyacrylamide gel. After electrophoresis, the samples were electroblotted onto PVDF membranes, which were then treated with 5% skim milk in TBS-T buffer [20 mM Tris–HCl (pH 7.5), 500 mM NaCl, 0.5% Tween]. The membranes were incubated overnight in TBS-T with 1% bovine serum albumin (BSA), together with the anti-Bax (Santa Cruz Biotechnology), anti-Bcl-2 (Santa Cruz Biotechnology), anti-Chk2 (Santa Cruz Biotechnology), anti-SMC1 phosphoserine 957 and 966, and anti-SMC1 (Bethyl) primary antibodies. The membranes were washed with TBS-T, and incubated in TBS-T with 1% BSA and HRP-conjugated anti-rabbit IgG antibody for 1 h. The membranes were then washed with TBS-T, and the immunoreactive proteins were visualized using chemiluminescence reagents (Perkin Elmer Life Science) and high-performance chemiluminescence film (Hyperfilm-ECL; Amersham Biosciences).

Detection of chromosomes by Giemsa staining

Treatment with colcemid (final concentration 0.2 μg/ml; Sigma) at 37°C for 1 h was used to collect metaphase cells. These cells were resuspended in 0.075 M KCl and incubated for 10 min. The chromosomes were fixed in a methanol-acetic acid solution (methanol: acetic acid; 3:1) and spread on glass slides. The chromosomes were stained with a 5% Giemsa solution in PBS, and observed under the light microscope.

Apoptosis analysis after irradiation of cells at the S phase

Sub-confluent cells were treated for 24 h with thymidine (final concentration 2 mM; Amersham Biosciences) to stop cellular proliferation at the S phase. The cells were then washed three times with PBS, fresh medium was added, and the cells were re-incubated for 2 h, to re-start the S phase. The S phase cells and asynchronous cells were irradiated with 10 Gy of γ-rays, and apoptosis was monitored 48 h later by the dye exclusion assay.

RESULTS

Induction of late apoptosis in both AT and NBS cells following IR

To investigate the relationships between ATM and NBS1 and IR-induced p53-independent late apoptosis, the induction of apoptosis after exposure to IR was analyzed in p53-defective AT, NBS, and normal cells (HeLa, MRC5SV) using the dye exclusion assay. Figure 1A shows that approximately 30% of the normal cells (HeLa, MRC5SV) underwent apoptotic death at 72 h after exposure to 10 Gy of γ-rays. However, >50% of the AT cells (AT-T55, AT-28, AT-5BIVA) showed the apoptotic phenotype at the same time-point. The levels of cell death for the NBS cells (GM24SV, WGSV7, 1022QSV) at 72 h post-irradiation were intermediate to the levels detected for the AT and normal cells (Fig. 1A). This difference in apoptosis induction level between the AT and normal cells was statistically significant ($p < 0.01$), as was that between the NBS and normal cells ($p = 0.0271$). However, in the case of the earlier time-point (24 h post-irradiation), the level of

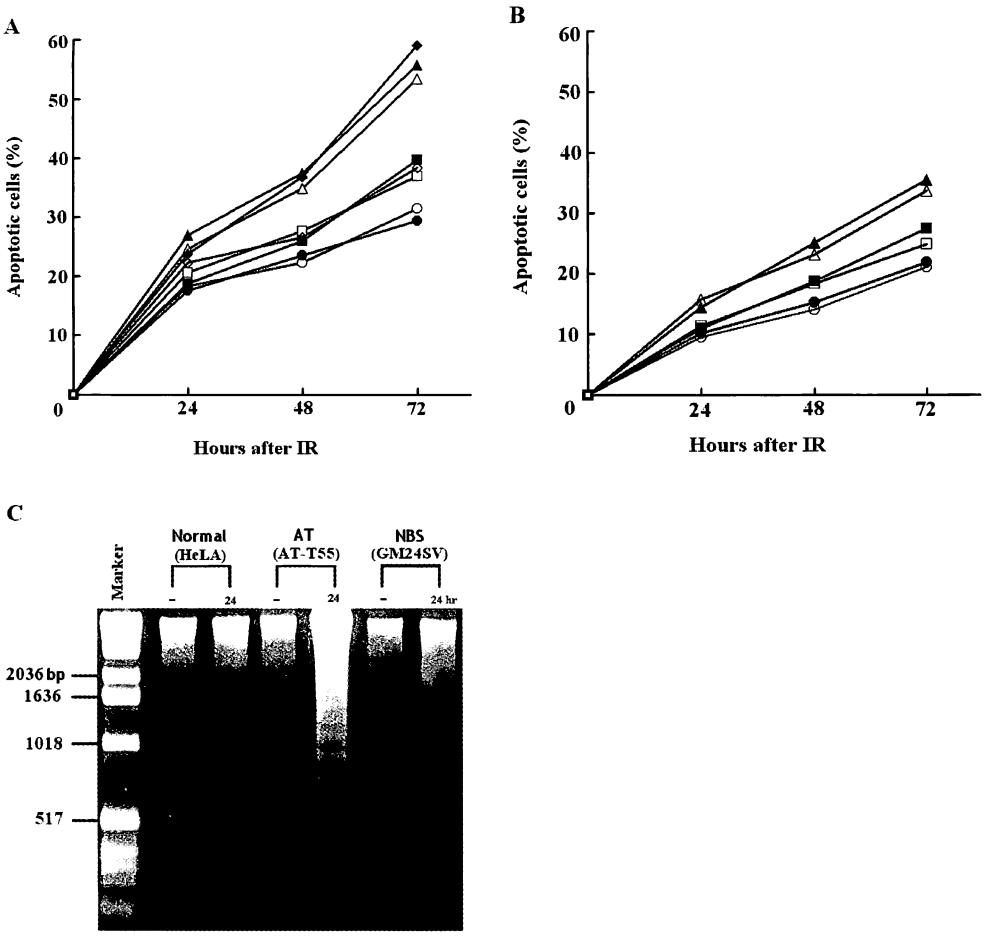

Fig. 1A, B. Induction of p53-independent apoptosis after exposure to γ-rays.
Normal cells (HeLa: open circles; MRC5SV: closed circles), AT cells (AT-T55: open triangles; AT-28: closed triangles; AT-5BIVA: closed rhombuses), and NBS cells (GM24SV: open squares; WGSV7: closed squares; 1022QSV: open rhombuses) were irradiated with 10 Gy (A) or 5 Gy (B) of γ-rays. Apoptosis induction was monitored by the dye exclusion assay at 24, 48, and 72 h post-irradiation. The differences in apoptosis levels between AT and normal cells, and between NBS and normal cells 72 h after 10 Gy of γ-rays were analyzed by two factor ANOVA statistical analysis for repeated measures. A value of $P < 0.05$ was considered to be statistically significant.

Fig. 1C. Induction of p53-independent apoptosis after exposure to γ-rays.
Normal cells (HeLa), AT cells (AT-T55), and NBS cells (GM24SV) were irradiated with 10 Gy of γ-rays. The DNA fragmentation assay was performed after the extraction of DNA from these cells 24 h after IR. (–): non-irradiated cells.

apoptosis induction in normal cells was similar to those found in the AT and NBS cells (approximately 20%; Fig. 1A). These results suggest that a defect in ATM or NBS1 promotes p53-independent apoptosis at late time-points after IR, and that another type of p53-independent apoptosis, which is independent of ATM and NBS1, operates at the early time-points. In addition, apoptosis induction after exposure to 5 Gy of γ-rays was investigated in these cells. Although the levels of apoptosis induction in all the cell lines were lower after 5 Gy of γ-rays than after 10 Gy of γ-rays, the AT cells showed higher levels of apoptosis induction than the normal cells at 72 h after IR (Fig. 1B). At that time-point, the level of apoptosis in the NBS cells was higher than in the normal cells, and at a level that was intermediate to the levels in AT and normal cells (Fig. 1B).

Thus, the patterns of apoptosis induced by 5 Gy and 10 Gy of IR are similar.

The induction of apoptosis following IR was confirmed by the DNA fragmentation assay, since the fragmentation of nucleosomal DNA is a widely recognized hallmark of apoptotic cell death[54]. DNA fragmentation was clearly observed in AT cells, but not in normal cells (Fig. 1C). A low level of DNA fragmentation was detected in the NBS cells after IR (Fig. 1C). Therefore, these results suggest that defects in the ATM and/or NBS1 genes may promote p53-independent apoptosis at late stages (>72 h) after IR.

Expression of Bax and Bcl-2 in the p53-independent apoptotic response following IR

Recent studies have implicated several proteins in the regulation of apoptosis. Among those, Bax and Bcl-2 are known to be critical regulators of different apoptosis pathways. Normal thymocytes undergo apoptosis following IR by increasing Bax and decreasing Bcl-2 expression. The cells from *p53*-knockout mice are defective in the regulation of Bax and Bcl-2 expression, and do not undergo IR-induced early apoptosis[13]. These lines of evidence suggest that p53 mediates IR-induced early apoptosis through Bax and Bcl-2 expression[13]. Moreover, tumor necrosis factor-alpha (TNF-α) and Fas ligand (FasL) induce the apoptosis of breast cancer cells (MCF7), whereas the overexpression of Bcl-2 in these cells protects against apoptosis induction by TNF-α or FasL[55]. Furthermore, the activation of JNK following UV irradiation increases apoptosis in fibroblasts that express wild-type Bax, whereas Bax-deficient fibroblasts do not show this phenomenon[56]. These observations suggest that both Bax and Bcl-2 function in the regulation of apoptosis induced by different stimuli, such as TNF-α /FasL and UV[55-57]. Therefore, Bax and Bcl-2 may also function in p53-independent late apoptosis following IR. The expression levels of Bax and Bcl-2 were measured by Western blot analysis in normal, AT, and NBS fibroblast cells after exposure to 10 Gy of γ-rays. Figure 2A shows that the expression of Bax increased in normal cells (HeLa, MRC5SV) at both 8 h and 24 h after IR. Bax expression also increased in the AT (AT-T55, AT-28, AT-5BIVA) and NBS fibroblast cell lines (GM24SV, WGSV7, 1022QSV) after IR (Fig. 2A). Although Figure 1A and B shows that the apoptosis of

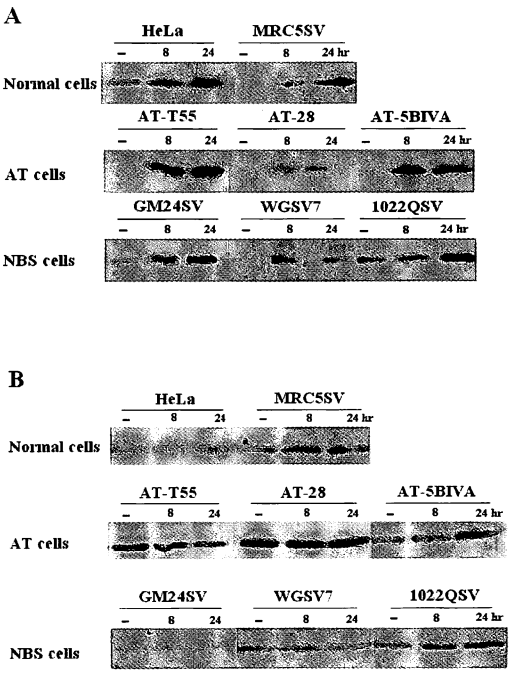

Fig. 2. Expression of Bax and Bcl-2 after exposure to γ-rays.
Normal cells (HeLa, MRC5SV), AT cells (AT-T55, AT-28, AT-5BIVA), and NBS cells (GM24SV, WGSV7, 1022QSV) were irradiated with 10 Gy of γ-rays. The cells were harvested at the indicated time-points, and Western blot analyses were performed using the anti-Bax (A) and anti-Bcl-2 (B) antibodies. (–): non-irradiated cells.

AT cells was induced at a higher level than in normal cells, the levels of Bax expression by these cells were similar, which suggests a partial contribution of Bax to late apoptosis. Figure 2B shows that the expression of Bcl-2 was unchanged in normal cells (HeLa, MRC5SV) after IR, although Bcl-2 was expressed without IR. Bcl-2 expression was also unchanged in the AT and NBS cells both with and without IR (Fig. 2B). Thus, Bcl-2 expression may be dispensable for this type of late apoptosis.

Defective Chk2 phosphorylation in AT and NBS cells after IR

Recently, it has been suggested that Chk2 functions in the regulation of apoptosis following IR[58,59]. Thymocytes from *Chk2*-knockout mice were shown to be defective in IR-induced apoptosis, whereas IR-induced apoptosis was restored in these cells by the

re-introduction of the wild-type *Chk2* gene[58,59]. Moreover, *Chk2* mutations are found more frequently in patients with variant Li-Fraumeni syndrome (LFS), and cells with *Chk2* mutations are reported to be more defective in IR-induced apoptosis than *Chk2* wild-type LFS cells[60]. These lines of evidence suggest an important role for Chk2 in the regulation of IR-induced apoptosis. Chk2 is known to be activated by ATM-mediated phosphorylation, and activated Chk2 phosphorylates several proteins that are associated with IR-induced cellular responses, including apoptosis[28,61]. Therefore, the phosphorylation of Chk2 was investigated by Western blot analysis of normal, AT, and NBS cells that were exposed to 10 Gy of γ-rays, to clarify the relationship with p53-independent late apoptosis. Since Chk2 phosphorylation retards the mobility of the Chk2 protein in SDS-PAGE gels[62], Chk2 phosphorylation could be extrapolated from the electrophoresis of cell protein extracts. Figure 3A shows that Chk2 was phosphorylated in normal cells (HeLa, MRC5SV) at 8 h and 24 h after IR. Chk2 phosphorylation did not occur in AT (AT-T55, AT-28, AT-5BIVA) and NBS (GM24SV, WGSV7, 1022QSV) cells at these time-points after IR. Although the AT and NBS cells showed different levels of apoptosis induction (Fig. 1A and B), similar defects in Chk2 phosphorylation at 8 h and 24 h after IR were observed for these two cell lines. Therefore, the phosphorylation of Chk2 was investigated at earlier time-points after exposure to 10 Gy of γ-rays. Figure 3B shows that Chk2 was phosphorylated in normal cells (HeLa, MRC5SV) at 1 h and 3 h after IR. In the AT cells (AT-T55, AT-28), Chk2 was not phosphorylated at these time-points after IR. On the other hand, Chk2 showed phosphorylation at 1 h and 3 h after IR in NBS cells (GM24SV, WGSV7), which may be responsible for the induction of apoptosis in NBS cells at a level that is intermediate to the levels induced in AT and normal cells. Therefore, defective Chk2 phosphorylation may lead to p53-independent late apoptosis following IR.

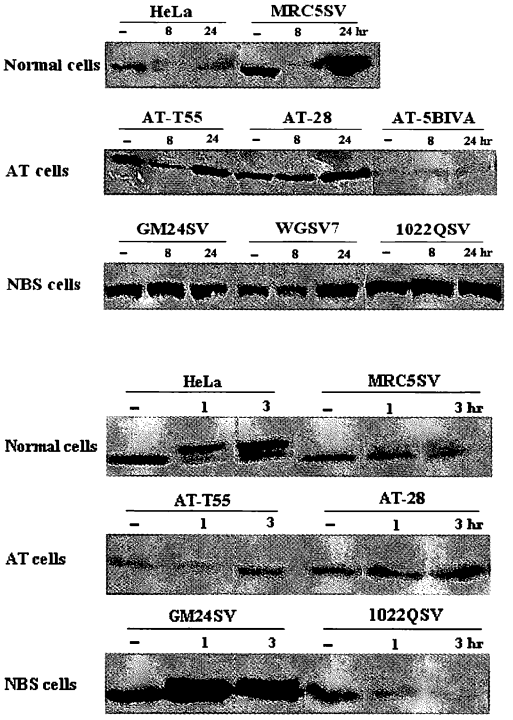

Fig. 3. Phosphorylation of Chk2 after exposure to γ-rays
Normal cells (HeLa, MRC5SV), AT cells (AT-T55, AT-28, AT-5BIVA), and NBS cells (GM24SV, WGSV7, 1022QSV) were irradiated with 10 Gy of γ-rays. The cells were harvested at the indicated time-points, and Western blotting was performed using the anti-Chk2 antibody. (–): non-irradiated cells.

Defective SMC1 phosphorylation in AT and NBS cells after IR

We also examined another apoptosis-related factor, SMC1, which is phosphorylated by ATM in response to IR[63,64]. ATM phosphorylates SMC1 at serine (Ser) 957 and Ser 966, and cells that express mutated SMC1, in which either Ser 957 or Ser 966 is changed to alanine, show increased radiation sensitivity[63,64]. Since radiation sensitivity correlated with late apoptosis, we hypothesized that SMC1 might function in late apoptosis. Therefore, the phosphorylation of SMC1 was investigated in normal, AT, and NBS cells after exposure to 10 Gy of γ-rays using Western blot analysis with antibodies against the phosphorylated residues (Ser 957 or Ser 966) in SMC1. Since the levels of Chk2 phosphorylation were different between AT and NBS cells at early time-points after exposure to IR (Fig. 3B), SMC1 phosphorylation was examined at early time-points after IR. Figure 4 shows that SMC1 phosphorylation at Ser 957 and Ser 966 was detected in normal cells (HeLa, MRC5SV) at 1 h and 3 h after exposure to 10 Gy of γ-rays, whereas phosphorylation at theses sites was not detected in AT cells (AT-T55, AT-28). On the other hand, the levels of Ser 957 phos-

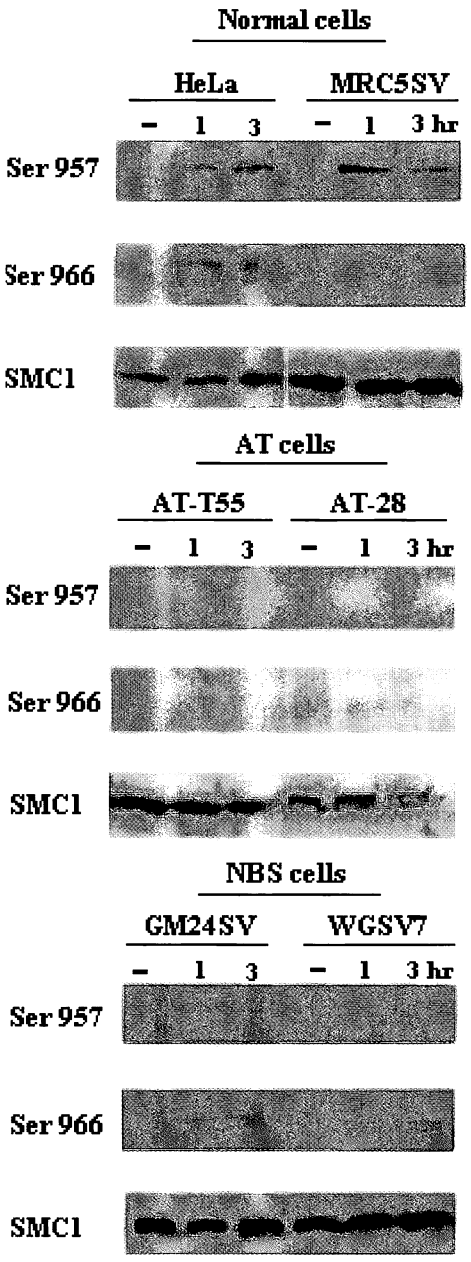

Fig. 4. Phosphorylation of SMC1 at Ser 957 and Ser 966 after exposure to γ-rays.
Normal cells (HeLa, MRC5SV), AT cells (AT-T55, AT-28) and NBS cells (GM24SV, WGSV7) were irradiated with 10 Gy of γ-rays. The cells were harvested at the indicated time-points, and Western blot analyses were performed using anti-SMC1 antibodies with specificity for the phosphorylation sites (Ser 957 or Ser 966), and with an anti-SMC1 polyclonal antibody. (–): non-irradiated cells.

phorylation at 1 h and 3 h post-irradiation were lower in NBS cells than in normal cells. Nevertheless, the levels of Ser 966 phosphorylation in NBS cells were similar to those in normal cells at both 1 h and 3 h post-irradiation (Fig. 4). Thus, SMC1 phosphorylation was partially defective in NBS cells, which may explain our finding that the level of apoptosis induced in NBS cells was intermediate to the levels induced in AT and normal cells. Moreover, the expression of SMC1 was examined using Western blot analysis with the anti-SMC1 polyclonal antibody in normal, AT, and NBS cells after exposure to 10 Gy of γ-rays. SMC1 expression was detected in these cells with and without IR, which suggests that the observed changes in SMC1 phosphorylation are not due to the induction of SMC1 expression. Taken together, these results suggest that defective SMC1 phosphorylation is involved in p53-independent late apoptosis.

Chromosomal aberrations in AT and NBS cells

Although the linkage of Chk2 and SMC1 with IR-induced late apoptosis has been demonstrated, these factors also function at the intra-S-phase checkpoint following IR[28,63,64]. This checkpoint is regulated by at least two pathways, namely, the ATM/Chk2/Cdc25A pathway and ATM/NBS1/SMC1 pathway[28,63,64]. ATM phosphorylates Chk2 and SMC1, and these phosphorylation events function at the intra-S-phase checkpoint *via* the above-mentioned pathways, to stop DNA synthesis following IR[28,63,64]. Figures 3 and 4 show defective phosphorylation of the intra-S-phase checkpoint regulatory factors Chk2 and SMC1 in AT cells, and phosphorylation of these proteins was partially defective in NBS cells. AT and NBS cells are known to be defective in terms of the intra-S-phase checkpoint, which results in radioresistant DNA synthesis (RDS)[17]. The RDS level of the AT cells was higher than that of normal cells, whereas the NBS cells showed RDS at a level that was intermediate to the levels in AT and normal cells[63-66]. The AT cells showed a more pronounced apoptotic phenotype than the normal cells, and the level of apoptosis induction in the NBS cells was intermediate to the levels in the AT and normal cells (Fig. 1A and B). Thus, the levels of RDS in the AT and NBS cells (relative to the levels in normal cells) reflect the induction levels of late apoptosis in these cells. These phenomena appear to be connected by

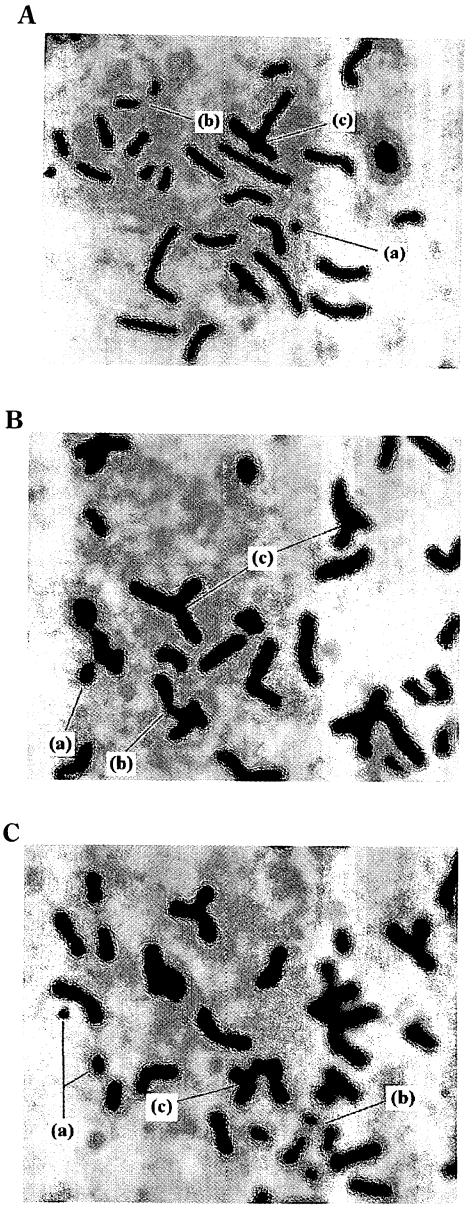

Fig. 5. Induction of chromosomal aberrations after exposure to γ-rays
Normal cells (HeLa) (A), AT cells (AT-T55) (B), and NBS cells (GM24SV) (C) were irradiated with 2 Gy of γ-rays. Metaphase chromosomes were collected from these cells 3 h and 10 h after IR. The samples were fixed in a methanol: acetic acid (3:1) and spread on glass slides. The chromosomes were stained with a 5% Giemsa solution in PBS, and chromosomal aberrations (fragmentation, a; gaps, b; and association, c) were detected under the light microscope.

the defective phosphorylation of Chk2 and SMC1. Thus, the defect in the intra-S-phase checkpoint may be related to p53-independent late apoptosis. Therefore, we speculate that AT and NBS cells show higher levels of apoptosis than normal cells after irradiation at the S phase. Since apoptosis induction at a late stage after IR has been correlated with chromosomal aberrations[67,68], the chromosomes of the normal, AT, and NBS cells were studied after irradiation with 2 Gy of *g*-rays at the G1 or S phase. Three types of aberrations were noted: (a) fragmentation; (b) gap; and (c) association (Fig. 5). Table 1 shows that after irradiation at the S phase, the percentage of normal cells that contained chromosomal aberrations increased by approximately 21%, compared to the non-irradiated control. As expected, the numbers of chromosomal aberrations in the AT and NBS cells after irradiation at the S phase increased by approximately 50% and 45%, respectively, compared to that in normal cells. However, after irradiation at the G1 phase, the increases in the numbers of chromosomal aberrations in AT and NBS cells were similar to those of the normal cells (Table 1). Moreover, the total number of aberrations per cell was higher in AT and NBS cells than in normal cells when the cells were irradiated at the S phase, although the increases seen in AT and NBS cells after irradiation at the G1 phase were similar to those seen in normal cells. The numbers of aberrations increased mainly as a result of gaps and associations, whereas the numbers of fragmentations were increased slightly in these cells, as compared to normal cells (Table 1). These results suggest that defects in the intra-S-phase checkpoints following IR may lead to chromosomal aberrations in AT and NBS cells.

Induction of p53-independent apoptosis in both AT and NBS cells after irradiation at the S phase

It has been proposed that chromosomal aberration induction following IR leads to cell death at a late stage after IR[11,67,68]. From the data shown in Table 1, increases in the frequency of late apoptosis of AT and NBS cells after irradiation at the S phase were expected. Therefore, the induction of apoptosis was monitored in normal, AT, and NBS cells at 48 h after irradiation with 10 Gy of γ-rays at the S phase. Figure 6 shows that apoptosis was induced in asynchronous

Table 1 Induction of chromosomal aberrations after irradiation at the G1 or S phase

	% of cells with chromosomal abberations	Number of Fragments	Number of Association	Number of Gaps	Total number of aberrations/cells with abnormal chromosomes
Without IR:					
Normal cells (MRC5SV)	14	25	4	5	2.4
AT cells (AT-T55)	21	10	41	14	3.1
NBS cells (GM24SV)	19	5	37	11	2.8
With IR at G1 phase:					
Normal Cells (MRC5SV)	27	29	21	31	3.0
AT cells (AT-T55)	36	17	75	37	3.6
NBS cells (GM24SV)	34	9	54	45	3.2
With IR at S phase:					
Normal cells (MRC5SV)	35	52	53	27	3.8
AT cells (AT-T55)	71	85	203	120	5.8
NBS cells (GM24SV)	64	89	153	77	5.0

Normal cells (HeLa), AT cells (AT-T55) and NBS cells (GM24SV) were irradiated with 2 Gy of γ-irradiation. Metaphase chromosomes were collected at 3 and 10 h after IR. The samples were fixed in methanol-acetic acid (3:1) solution, and spread on glass slides. The chromosomes were stained with 5% Giemsa solution in PBS, and chromosomal aberrations were detected under a light microscope.

AT and NBS cells with similar patterns to those shown in Figure 1A and B. However, approximately 30% of the normal cells underwent apoptosis after irradiation at the S phase, whereas approximately 23% of the normal asynchronous cells entered apoptosis. The levels of apoptosis after irradiation at the S phase were higher for the AT and NBS cells, at approximately 70% and 60%, respectively, while approximately 35% and 30% of the respective asynchronous cells underwent apoptosis (Fig. 6). These results show that apoptosis induction was higher in AT and NBS cells than in normal cells after irradiation at the S phase, as compared to the asynchronous cells. Therefore, defects in the intra-S-phase checkpoint may lead to p53-independent apoptosis in AT and NBS cells.

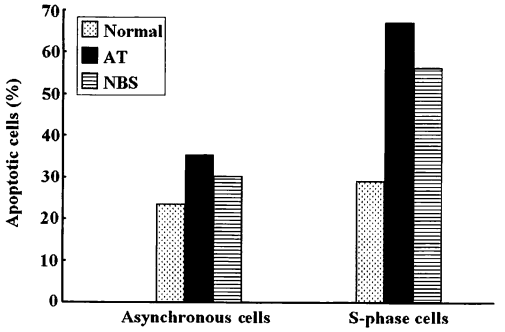

Fig. 6. Induction of p53-independent apoptosis after irradiation at the S-phase.
Sub-confluent normal cells (HeLa), AT cells (AT-T55), and NBS cells (GM24SV) were treated with thymidine for 24 h, to synchronize the cells at the S-phase. These S phase cells and the asynchronous cells were irradiated with 10 Gy of γ-rays, and apoptosis induction was monitored by the dye exclusion assay 48 h after IR.

DISCUSSION

In this study, we investigated IR-induced, p53-independent, late apoptosis using AT and NBS cells. AT and NBS are two radiation hypersensitive disorders, and the cells from AT and NBS patients show similar phenotypes, such as chromosomal instability, radiation sensitivity, and RDS[17,18]. The data presented here show that more AT cells undergo apoptosis than normal cells at late stages after IR, and NBS cells undergo apoptosis at a level that is intermediate to the apoptosis levels of AT and normal cells (Fig. 1A and B). Moreover, AT cells show defective phosphorylation of Chk2 and SMC1, which are involved in regulating apoptosis and the intra-S-phase checkpoint in response to IR[28,63,64], whereas the phosphorylation of these proteins is only partially defective in NBS cells (Figs. 3

and 4). Furthermore, AT and NBS cells show higher levels of chromosomal aberration than normal cells after irradiation at the S phase, as compared to the levels of chromosomal aberration after irradiation at the G1 phase (Table 1). AT and NBS cells also show higher levels of apoptosis induction than normal cells after irradiation at the S phase (Fig. 6). Therefore, these results suggest that the lack of the intra-S-phase checkpoint, which is due to defects in ATM and/or NBS1, leads to IR-induced late apoptosis through chromosomal aberrations.

Figure 1 shows a slight induction of apoptosis (approximately 20%) in p53-defective AT and NBS cells, which is similar to the level in normal cells at 24 h after IR. Although p53-independent apoptosis is known to occur at a late time-point after IR[14-16], some cell types show a slight induction of apoptosis at early time-points after IR. Proliferating lymphocytes derived from *p53*-knockout mice undergo a low level of apoptosis at 24 h after IR[15], and small intestinal epithelia from *p53*-knockout mice undergo apoptosis at 24 h after exposure to IR[14]. Furthermore, an adenoma cell line carrying a mutated p53 protein undergoes apoptosis at 24 h after IR[69]. Since both AT and NBS cells show apoptosis at early time-points after IR (Fig. 1), it seems likely that ATM and NBS1 do not participate in p53-independent early apoptosis.

Figure 1 also shows a difference in apoptosis induction level between AT and NBS cells at a late stage after IR. AT cells show more induction of apoptosis than normal cells at 72 h after IR, and this level of apoptosis in NBS cells is intermediate to the apoptosis levels in AT and normal cells. AT cells display higher radiation sensitivity than do normal cells, and the level of this radiation sensitivity in NBS cells is intermediate to those in AT and normal cells[70]. Moreover, AT cells show more chromosomal aberrations than normal cells after IR, whereas NBS cells show an induction level that is intermediate to those seen in AT and normal cells[65,66]. DNA synthesis in normal cells is inhibited markedly following IR, but AT cells display lower levels of inhibition of RDS, and NBS cells show intermediate levels of inhibition[63-66]. In general, NBS cells display abnormal IR-induced cellular responses at levels that are intermediate to those observed in AT and normal cells. This phenotype of IR-induced apoptosis may be correlated with the induction of chromosomal aberrations and radiation sensitivity.

Figures 3 and 4 show that the phosphorylation of both Chk2 and SMC1 is partially defective in NBS cells following IR. NBS cells are known to display deficient phosphorylation of several ATM substrates following IR. ATM phosphorylates p53 at Ser 15 following IR[25], and this phosphorylation of p53 on Ser 15 has been shown to be reduced in NBS cells[71]. ATM also phosphorylates Brca1 in response to IR[35]. The *Brca1* gene is a tumor suppressor gene that is mutated in a high percentage of hereditary breast and ovarian cancers[72]. Brca1 has been shown to interact and co-localize with the N/M/R complex[73], which is implicated in HR and NHEJ DNA damage responses[74]. However, Brca1 phosphorylation is reported to be defective in NBS cells following IR[63]. In addition, ATM has been shown to phosphorylate the checkpoint effector kinase Chk1 following IR[29], and Chk1 phosphorylation is known to be defective in NBS cells[29]. Moreover, ATM phosphorylates Ser 222 of Fancd2 in response to various DNA-damaging agents, which include mitomycin C and IR[75]. *Fancd2* is the mutated gene in the chromosomal instability syndrome of Fanconi's anemia[76]. The phosphorylation of Fancd2 is required for the intra-S-phase checkpoint and its localization to DNA damage foci[77,78]. NBS cells have also been reported to be deficient in Fancd2 phosphorylation[77]. Thus, NBS cells show partial defects in the phosphorylation of several ATM substrates following IR. NBS1 phosphorylation by ATM has been suggested to function as a promoter of ATM-mediated signals. Cells from NBS patients are reported to be defective in ATM phosphorylation of p53 and Fancd2 following IR[71,77]. These findings are in agreement with an NBS1 function downstream of ATM. However, the similarities of cellular phenotypes that result from mutations of the *ATM* and *NBS1* genes indicate that NBS1 is likely to be of central importance to the DSB-specific component of ATM activation[79,80]. Moreover, recent reports have suggested that the binding of ATM to DSB sites and subsequent activation are deficient in NBS cells following IR[52,79,80]. Thus, these findings show a functional link between ATM and NBS1, and suggest that NBS1 mediates the activation of ATM through recruitment to DSBs. These observations may explain why the phosphorylation of Chk2 and SMC1 is partially defective in NBS cells following IR.

Chk2 and SMC1 phosphorylation are known to function in the regulation of the intra-S-phase checkpoint following IR[28,63,64]. However, the results presented here show that defects in the phosphorylation of both Chk2 and SMC1 correlate with the induction of late apoptosis following IR (Figs. 1, 3, and 4). In addition, these cells show more late apoptosis after irradiation at the S phase (Fig. 6). These results suggest that defects in the intra-S-phase checkpoints may lead to IR-induced apoptosis. Besides Chk2 and SMC1, several genes are known to function at the intra-S-phase checkpoints. Of these, the *Fancd2* gene has been shown to regulate the intra-S-phase checkpoint following IR[77], and cells from Fanconi's anemia patients, in which Fancd2 is mutated, exhibit RDS and radiation hypersensitivity[77,78,81], which is related to late apoptosis following IR. Brca1 is another regulator of the intra-S-phase checkpoint following IR[82]. Cells from *Brca1*-deficient mice show defects in the intra-S-phase checkpoint[82] and are radiosensitive[83]. Therefore, the previous findings and those presented here suggest that the defects in the intra-S-phase checkpoint lead to late apoptosis following IR. Moreover, checkpoint defects at either the G1 or G2 phase following IR may result in radiation hypersensitivity that is related to late apoptosis. The G1 checkpoint is regulated by p53[3], and cells from Li-Fraumeni patients, which have germline mutations of the *p53* gene, show defects in the G1 checkpoint and are hypersensitive to IR[84]. Furthermore, the human promyelocytic leukemia cell line HL-60, which is known to be deficient in p53, displays lack of arrest at the G1 checkpoint and hypersensitivity to IR[16]. The p53 protein regulates the G1 checkpoint through p21[3,5]. The cells from *p21*-knockout mice show defects in the G1 checkpoint and hypersensitivity to IR[85]. These findings suggest a correlation between defects in the G1 checkpoint and cellular radiation hypersensitivity. On the other hand, a defect in the G2 checkpoint seems to result in radiation hypersensitivity. Chk1 is one of the regulators of the G2 checkpoint[29], and *Chk1*-deficient DT40 cells show a defect in the G2 checkpoint and hypersensitivity to IR[86]. Moreover, Brca1 is known to function in regulating the G2 checkpoint following IR[82]. *Brca1*-deficient cells display a defect in the G2 checkpoint[80] and hypersensitivity following IR[82,83]. Thus, defects in the G1 and G2 checkpoints, as well as in the intra-S-phase checkpoints may result in IR-induced late apoptosis. Furthermore, IR-induced late apoptosis seems to be correlated with chromosomal aberrations. Fibroblast cells from *p53*-knockout mice are defective in the G1 checkpoint and show remarkable chromosomal aberrations and late apoptosis following IR[67]. Likewise, fibroblasts from *Brca1*-knockout mice exhibit defects in the G2 checkpoint, and show more chromosomal aberrations and late apoptosis following IR[83]. Therefore, the defect in the intra-S-phase checkpoint may result in chromosomal aberrations, which lead to apoptosis at a late stage after IR.

The results of this study of p53-independent late apoptosis may be useful in cancer therapy. The induction of p53-dependent apoptosis by IR is one of the methods used to kill cancer cells. Since *p53* gene mutations are common in several cancers, such as oral squamous cell carcinoma, soft tissue sarcoma, osteosarcoma, breast cancer, brain tumors, leukemias, and adrenocortical carcinoma, the p53-dependent pathway cannot be activated by IR in these cancers. In this study, we have shown that a defect in the intra-S-phase checkpoint may promote apoptosis *via* the p53-independent pathway following IR. This apoptosis pathway may be useful for therapy against p53-mutated cancers. The generation of a defective intra-S-phase checkpoint in these cancer cells by depression of checkpoint genes, by for example, the transfection of small interfering RNA (siRNA), may lead to late apoptosis following IR. Other intra-S-phase checkpoint genes, such as *Brca1* and *Fancd2*, and G1 and G2 checkpoint genes may also be useful for this hypothetical therapy. However, the detailed mechanisms by which defects in checkpoints result in IR-induced late apoptosis remain unclear. In order to use this pathway for cancer therapy, we need to fully clarify these mechanisms.

CONCLUSIONS

This study of IR-induced p53-independent late apoptosis shows that defects in Chk2 and SMC1 phosphorylation in AT and NBS cells correlate with the induction level of p53-independent late apoptosis following IR. In addition, AT and NBS cells show more chromosomal aberrations than normal cells after irradiation at the S phase, as compared to the corresponding levels after irradiation at the G1 phase. These cells also show more induction of late apoptosis after

irradiation at the S phase. Therefore, the defect in the intra-S-phase checkpoint, which is due to impaired phosphorylation of Chk2 and SMC1, results in p53-independent late apoptosis in response to IR through chromosomal aberrations. Moreover, previous reports have suggested a relationship between p53-independent late apoptosis and defects in the G1 or G2 checkpoint, as well as the intra-S-phase checkpoint. The previous findings and the results presented here indicate that the defects in cell cycle checkpoints lead to p53-independent late apoptosis following IR.

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REFERENCES

1) Iliakis, G.: Cell cycle regulation in irradiated and non-irradiated cells. *Semin. Oncol.*, **24**, 602–615, 1997.
2) Elledge, S.J.: Cell cycle checkpoints: preventing an identity crisis. *Science*, **274**, 1664–1672, 1996.
3) Kastan, M.B., Zhan, Q., el-Deiry, W.S., Carrier, F., Jacks, T., Walsh, W.V., Plunkett, B.S., Vogelstein, B., and Fornace, A.J. Jr.: A mammalian cell cycle checkpoint pathway utilizing p53 and GADD45 is defective in ataxia-telangiectasia. *Cell*, **71**, 587–597, 1992.
4) Khanna, K.K., and Jackson, S.P.: DNA double-strand breaks: signaling, repair and the cancer connection. *Nat. Genet.*, **27**, 247–254, 2001.
5) Dulic, V., Kaufmann, W.K., Wilson, S.J., Tlsty, T.D., Lees, E., Harper, J.W., Elledge, S.J., and Reed, S.I.: p53-dependent inhibition of cyclin-dependent kinase activities in human fibroblasts during radiation-induced G1 arrest. *Cell*, **76**, 1013–1023, 1994.
6) Haber, J.E.: DNA recombination: the replication connection. *Trends Biochem. Sci.*, **24**, 271–275, 1999.
7) Tsukamoto, Y., and Ikeda, H.: Double-strand break repair mediated by DNA end-joining. *Genes Cells*, **3**, 135–144, 1998.
8) Yu, Y., and Little, J.B.: P53 is involved in but not required for ionizing radiation-induced caspase-3 activation and apoptosis in human lymphoblast cell lines. *Cancer Res.*, **58**, 4277–4281, 1998.
9) Lowe, S.W., Schmitt, E.M., Smith, S.W., Osborne, B.A., and Jacks, T.: P53 is required for radiation-induced apoptosis in mouse thymocytes. *Nature*, **362**, 847–849, 1993.
10) Clarke, A.R., Gledhill, S., Hooper, M.L., Bird, C.C., and Wyllie, A.H.: P53 dependence of early apoptotic and proliferative responses within the mouse intestinal epithelium following gamma-irradiation. *Oncogene*, **9**, 1767–1773, 1994.
11) Olive, P.L.: The role of DNA single-and double-strand breaks in cell killing by ionizing radiation. *Radiat. Res.*, **150**, S42–S51, 1998.
12) Siliciano, J.D., Canman, C.E., Taya, Y., Sakaguchi,

K., Appella, E., and Kastan, M.B.: DNA damage induces phosphorylation of the amino terminus of p53. *Genes Dev.*, **11**, 3471–3481, 1997.

13) Miyashita, T., Krajewski, S., Krajewska, M., Wang, H.G., Lin, H.K., Liebermann, D.A., Hoffman, B., and Reed, J.C.: Tumor suppressor p53 is a regulator of bcl-2 and Bax gene expression in vitro and in vivo. *Oncogene*, **9**, 1799–1805, 1994.

14) Merrit, A.J., Allen, T.D., Potten, C.S., and Hickman, J.A.: Apoptosis in small intestinal epithelia from p53-null mice: evidence for a delayed, p53-independent G2/M-associated cell death after gamma-irradiation. *Oncogene*, **14**, 2759–2766, 1997.

15) Strasser, A., Harris A.W., Jacks T., and Cory, S.: DNA damage can induce apoptosis in proliferating lymphoid cells via p53-independent mechanisms inhibitable by Bcl-2. *Cell*, **79**, 329–339, 1994.

16) Han, Z., Chatterjee, D., He, D.M., Early, J., Pantazis, P., Wyche, J.H., and Hendrickson, E.A.: Evidence for a G2 checkpoint in p53-independent apoptosis induction by X-irradiation. *Mol. Cell. Biol.*, **15**, 5849–5857, 1995.

17) Shiloh, Y.: Ataxia-telangiectasia and the Nijmegen breakage syndrome: Related disorders but genes apart. *Annu. Rev. Genet.*, **31**, 635–662, 1997.

18) Tauchi, H., Matsuura, S., Kobayashi, J., Sakamoto, S., and Komatsu, K.: Nijmegen breakage syndrome gene, NBS1, and molecular links to factors for genomic stability. *Oncogene*, **21**, 8967–8980, 2002.

19) Savisty, K., Bar-Shira, A., Giald, S., Rotman, G., Ziv, Y., Vanagaite, L., Tagle, D.A., Smith, S., Uziel, T., Sfez, S., Ashkenazi, M., Pecker, I., Frydman, M., Harnik, R., Patanjali, S.R., Simmons, A., Clines, G.A., Sartiel, A., Gatti, R.A., Chessa, L., Sanal, O., Lavin, M.F., Jaspers, N.G.J., Taylor, A.M.R., Arlett, C.F., Miki, T., Weismann, S.M., Lovett, M., Collins, F.S., and Shiloh, Y.: A single ataxia-telangiectasia gene with a product similar to PI-3 kinase. *Science*, **268**, 1749–1753, 1995.

20) Matsuura, S., Tauchi, H., Nakamura, A., Kondo, N., Sakamoto, S., Endo, S., Smeets, D., Solder, B., Belohradsky, B.H., Der Kaloustian, V.M., Oshimura, M., Isomura, M., Nakamura, Y., and Komatsu, K.: Positional cloning of the gene for Nijmegen breakage syndrome. *Nat. Genet.*, **19**, 179–181, 1998.

21) Carney, J.P., Maser, R.S., Olivares, H., Davis, E.M., Le Beau, M., Yates J.R.III, Hays, L., Morgan, W.F., and Petrini. J.H.: The hMre11/hRad50 protein complex and Nijmegen breakage syndrome: linkage of double-strand break repair in the cellular response to DNA damage. *Cell*, **93**, 477–486, 1998.

22) Varon, R., Vissinga, C., Platzer, M., Cerosaletti, K.M., Chrzanowska, K.H., Saar, K., Beckmann, G., Seemanova, E., Cooper, P.R., Nowak, N.J., Stumm, M., Weemaes, C.M., Gatti, R.A., Wilson, R.K., Digweed, M., Rosenthal, A., Sperling, K., Concannon, P., and Reis, A.: Nibrin, a novel DNA double-strand break repair protein, is mutated in Nijmegen breakage syndrome. *Cell*, **93**, 467–476, 1998.

23) Kim, S.T., Lim, D.S., Canman, C.E., and Kastan, M.B.: Substrate specification and identification of putative substrates of ATM kinase family members. *J. Biol. Chem.*, **274**, 37538–37543, 1999.

24) O'Neill, T., Dwyer, A.J., Ziv, Y., Chan, D.W., Lees-Millar, S.P., Abraham, R.H., Lai, J.H., Hill, D., Shiloh, Y., Cantley, L.C., and Rathbun, G.A.: Utilization of oriented peptide libraries to identify substrate motifs selected by ATM. *J. Biol. Chem.*, **275**, 22719–22727, 2000.

25) Canman, C.E., Lim D.S., Cimprich K.A., Taya, Y., Tamai, K., Sakaguchi, K., Appella, E., Kastan, M.B., and Siliciano, J.D.: Activation of the ATM kinase by ionizing radiation and phosphorylation of p53. *Science*, **281**, 1677–1679, 1998.

26) Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C.W., Chessa, L., Smorodinsky, N.I., Prives, C., Reiss, Y., Shiloh, Y., and Ziv, Y.: Enhanced phosphorylation of p53 by ATM in response to DNA damage. *Science*, **281**, 1674–1677, 1998.

27) Bakkenist C.J., and Kastan M.B.: DNA damage activates ATM through intermolecular autophosphorylation and dimer dissociation. *Nature*, **421**, 499–506, 2003.

28) Flack, J., Mailand, N., Syljuasen, R.G., Bartek, J., and Lukas, J.: The ATM-Chk2-Cdc25A checkpoint pathway guards against radioresistant DNA synthesis. *Nature*, **410**, 842–847, 2001.

29) Gatei, M., Sloper, K., Sorensen, C., Syljuasen, R., Falck, J., Hobson, K., Savage, K., Lukas, J., Zhou, B.B., Bartek, J., and Khanna, K.K.: Ataxia-telangiectasia-mutated (ATM) and NBS1-dependent phosphorylation of Chk1 on Ser-317 in response to ionizing radiation. *J. Biol. Chem.*, **278**, 14806–14811, 2003.

30) Guofeng, X., Robert, C.H., Yiwen, J., Scott, R.P., Bruce, E.L., Morton, E.B., and Joseph, A.D.A.: Requirements for p53 and the ATM gene product in the regulation of G1/S and S phase checkpoints. *Oncogene*, **16**, 721–736, 1998.

31) Takagi, M., Delia, D., Chessa, L., Iwata, S., Shigeta, T., Kanke, Y., Goi, K., Asada, M., Eguchi, M., Kodama, C., and Mizutani, S.: Defective control of apoptosis, radiosensitivity, and spindle

checkpoint in ataxia-telangiectasia. *Cancer Res.*, **58**, 4923–4929, 1998.

32) Xu, Y., and Baltimore, D.: Dual roles of ATM in the cellular response to radiation and in cell growth control. *Genes Dev.*, **10**, 2401–2410, 1996.

33) Nabil, H.C., Asra, M., Michael, A., and Thanos, D.H.: Chk2/hCds1 functions as a DNA damage checkpoint in G_1 by stabilizing p53. *Genes Dev.*, **14**, 278–288, 2000.

34) Kuhne, M., Riballo, E., Rief, N., Rothkamm, K., Jeggo, P.A., and Lobrich, M.: A double-strand break repair defect in ATM-deficient cells contributes to radiosensitivity. *Cancer Res.*, **64**, 500–508, 2004.

35) Cortez, D., Wang, Y., Qin, J., and Elledge, S.J.: Requirement of ATM- dependent phosphorylation of BRCA1 in the DNA damage response to double-strand breaks. *Science*, **286**, 1162–1166, 1999.

36) Lindsay, A., Catherine, H., and Yasuhisa, A.: Phosphorylation and rapid relocalization of 53BP1 to nuclear foci upon DNA damage. *Mol. Cell. Biol.*, **21**, 1719–1729, 2001.

37) Lim, D.S., Kim, S.T., Xu, B., Maser, R.S., Lin, J., Petrini, J.H., and Kastan, M.B.: ATM phosphorylates p95/nbs1 in an S phase checkpoint pathway. *Nature*, **404**, 613–617, 2000.

38) Durocher, D. and Jackson, S.P.: The FHA domain. *FEBS Lett.*, **513**, 58–66, 2002.

39) Xiaochun, Y., Claudia Christiano, C., Miao, H., Georges, M., and Junjie, C.: The BRCT domain is a phospho-protein binding domain. *Science*, **302**, 639–642, 2003.

40) Tauchi, H., Kobayashi, J., Morishima, K. I., Matsuura, S., Nakamura, A., Shirashi, T., Ito, E., Masnada, D., Delia, D., and Komatsu, K.: The forkhead- association domain of NBS1 is essential for nuclear foci formation after irradiation but not essential for hRAD50-hMRE11-NBS1 complex DNA repair activity. *J. Biol. Chem.*, **278**, 12–15, 2001.

41) Lucas, C., Flack, J., Bartkova, J., Bartek, J. and Lucas, J.: Distinct spatiotemporal dynamic of mammalian checkpoint regulators induced by DNA damage. *Nat. Cell. Biol.*, **5**, 255–260, 2003.

42) Celeste, A., Fernandez-Capetillo, O., Kruhlak, M.J., Pilch, D.R., Staudt, D.W., Lee, A., Bonner, R.F., Bonner, W.M., and Nussenzweig, A.: Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat. Cell. Biol.*, **5**, 675–679, 2003.

43) Kobayashi, J., Tauchi, H., Sakamoto, S., Nakamura, A., Morishima, K., Matsuura, S., Kobayashi, T., Tamai, K., Tanimoto, K., and Komatsu, K.: NBS1 localizes to γ-H2AX foci through interaction with the FHA/BRCT domain. *Curr. Biol.*, **12**, 1846–1851, 2002.

44) Goldberg, M., Stucki, M., Falck, J., D'Amours, D., Rahman, D., Pappin, D., Bartek, J., and Jackson, S.P.: MDC1 is required for the intra-S-phase DNA damage checkpoint. *Nature*, **421**, 952–956, 2003.

45) Wang, Y., Cortez, D., Yazdi P.T., Neff, N., Elledge, S.J., and Qin, J.: BASC, a super complex of BRCA1-associated proteins involved in the recognition and repair of aberrant DNA structures. *Genes Dev.*, **14**, 927–939, 2000.

46) Weemaes, C.M., Hustinx, T.W., Scheres, J.M., van Munster, P.J., Bakkeren, J.A., and Taalman, R.D.: New chromosomal instability disorder: the Nijmegen breakage syndrome. *Acta. Paediatr. Scand.*, **70**, 557–564, 1981.

47) Tauchi, H., Kobayashi, J., Morishima, K., Van Gent, D.C., Shirashi, T., Verkaik, N.S., VanHeems, D., Ito, E., Nakamura, A., Sonoda, E., Takata, M., Takeda, S., Matsuura, S., and Komatsu, K.: Nbs1 is essential for DNA repair by homologous recombination in higher vertebrate cells. *Nature*, **420**, 93–98, 2002.

48) Bezzubova, O., Silbergleit, A., Yamaguchi-Iwai, Y., Takeda, S., and Buerstedde, J.M.: Reduced X-ray resistance and homologous recombination frequencies in RAD54–/– mutant of the chicken DT40 cell line. *Cell*, **89**, 185–193, 1997.

49) Takata, M., Sasaki, M.S., Tachiiri, S., Fukushima, T., Sonoda, E., Schild, D., Thompson, L.H., and Takeda, S.: Chromosome instability and defective recombinational repair in knockout mutants of the five Rad51 paralogs. *Mol. Cell. Biol.*, **21**, 2858–2866, 2001.

50) Jongmans, W., Vuillaume, M., Chrzanowska, K., Smeets, D., Sperling, K., and Hall, J.: Nijmegen breakage syndrome cells fail to induce the p53-mediated DNA damage response following exposure to ionizing radiation. *Mol. Cell. Biol.*, **17**, 5016–5022, 1997.

51) Gatei, M., Young, D., Cerosaletti, K.M., Desai-Mehta, A., Spring, K., Kozlov, S., Lavin, M.F., Gatti, R.A., Concannon, P., and Khanna, K.K.: ATM-dependent phosphorylation of nibrin in response to radiation exposure. *Nat. Genet.*, **25**, 115–119, 2000.

52) Uziel, T., Lerenthal, Y., Moyal, L., Andegeko, Y., Mittelman, L., and Shiloh, Y.: Requirement of the MRN complex for ATM activation by DNA damage. *EMBO J.*, **22**, 5612–5621, 2003.

53) Altman, S.A., Randers, L., and Rao, G.: Comparison of trypan blue dye exclusion and flurometric assays for mammalian cell viability determinations. *Biotechnol. Prog.*, **9**, 671–674, 1993.

54) MacManus, J.P., Fliss, H., Preston, E., Rasquinha, I., and Tuor, U.: Cerebral ischemia produces laddered DNA fragments distinct from cardiac ischemia and archetypal apoptosis. *J. Cerb. Blood Flow Metab.*, **19**, 502–510, 1999.

55) Jaattela, M., Benedict, M., Tewari, M., Shayman, J.A., and Dixit, V.M.: Bcl-x and Bcl-2 inhibit TNF and Fas-induced apoptosis and activation of phospholipase A2 in breast carcinoma cells. *Oncogene*, **10**, 2297–2305, 1995.

56) Kui, L., Anjaruwe, N., Wei-Xing, Z., Norman, J.K., Richard A.F., Craig, B.T., Dafna, B-S., and Roger J.D.: The Bax subfamily of Bcl2-related proteins is essential for apoptotic signal transduction by c-Jun NH_2-terminal kinase. *Mol. Cell. Biol.*, **22**, 4929–4942, 2002.

57) Lei, K., and Davis, R.J.: JNK phosphorylation of Bim-related members of the Bcl2 family induces Bax-dependent apoptosis. *Proc. Natl. Acad. Sci. USA*, **100**, 2432–2437, 2003.

58) Hirao, A., Cheung, A., Duncan, G., Girard, P.M., Elia, A.J., Wakeham, A., Okada, H., Sarkissian, T., Wong, J.A., Sakai, T., De Stanchina, E., Bristow, R.G., Suda, T., Lowe, S.W., Jeggo, P.A., Elledge, S.J., and Mak, T.W.: Chk2 is a tumor suppressor that regulates apoptosis in both an ataxia telangiectasia mutated (ATM)-dependent and an ATM-independent manner. *Mol. Cell. Biol.*, **22**, 6521–6532, 2002.

59) Takai, H., Naka, K., Okada, Y., Watanabe, M., Harada, N., Saito, S., Anderson, C.W., Appella, E., Nakanishi, M., Suzuki, H., Nagashima, K., Sawa, H., Ikeda, K., and Motoyama, N.: Chk2-deficient mice exhibit radioresistance and defective p53-mediated transcription. *EMBO J.*, **21**, 5195–5205, 2002.

60) Bell, D.W., Varley, J.M., Szydlo, T.E., Kang, D.H., Wahrer D.C., Shannon, K.E., Lubratovich, M., Verselis, S.J., Isselbacher, K.J., Fraumeni, J.F., Birch, J.M., Li, F.P., Garber, J.E., and Haber, D.A.: Heterozygous germ line hCHK2 mutations in Li-Fraumeni syndrome. *Science*, **286**, 2528–2531, 1999.

61) Hirao, A., Kong, Y. Y., Matsuoka, S., Wakeham, A., Ruland, J., Yoshida, H., Liu, D., Elledge, S.J., and Mak, T.W.: DNA damage-induced activation of p53 by the checkpoint kinase Chk2. *Science*, **287**, 1824–1827, 2000.

62) Matsuoka, S., Huang, M., and Elledge, S.J.: Linkage of ATM to cell cycle regulation by the Chk2 protein kinase. *Science*, **282**, 1893–1897, 1998.

63) Yazdi, P.T., Wang, Y., Zhao, S., Patel, N., Lee, E.Y., and Qin, J.: SMC1 is a downstream effector in the ATM/NBS1 branch of the human S-phase checkpoint. *Genes Dev.*, **16**, 571–582, 2002.

64) Kim, S.T., Xu, B., and Kastan, M.B.: Involvement of the cohesin protein, Smc1, in Atm-dependent and independent responses to DNA damage. *Genes Dev.*, **16**, 560–570, 2002.

65) Antoccia, A., Stumm, M., Saar, K., Ricordy, R., Maraschio, P., and Tanzarella, C.: Impaired p53-mediated DNA damage response, cell-cycle disturbance and chromosome aberrations in Nijmegen breakage syndrome lymphoblastoid cell lines. *Int. J. Radiat. Biol.*, **75**, 583–591, 1999.

66) Girard, P.M., Foray, N., Stumm, M., Waugh, A., Riballo, E., Maser, R.S., Phillips, W. P., Petrini, J., Arlett, C. F., and Jeggo, P.A.: Radiosensitivity in Nijmegen Breakage Syndrome cells is attributable to a repair defect and not cell cycle checkpoint defects. *Cancer Res.*, **60**, 4881–4888, 2000.

67) Jochen, L., and Bernd, K.: DNA double-strand breaks trigger apoptosis in p53-deficient fibroblasts. *Carcinogenesis*, **22**, 579–585, 2001.

68) Lackinger, D., Eichhorn, U., and Kaina, B.: Effect of ultraviolet light, methyl methanesulfonate and ionizing radiation on the genotoxic response and apoptosis of mouse fibroblasts lacking c-Fos, p53 or both. *Mutagenesis*, **16**, 233–241, 2001.

69) Bracey, T.S., Williams, A.C., and Paraskeva, C.: Inhibition of radiation-induced G2 delay potentiates cell death apoptosis and/or the induction of giant cells in colorectal tumor cells with disrupted p53 function. *Clin. Cancer Res.*, **3**, 1371–1381, 1997.

70) Matsuura, K., Balmukhanov, T., Tauchi, H., Weemaes, C., Smeets, D., Chrzanowska, K., Endou, S., Matsuura, S., and Komatsu, K.: Radiation induction of p53 in cells from Nijmegen breakage syndrome is defective but not similar to ataxia-telangiectasia. *Biochem. Biophys. Res. Commun.*, **242**, 602–607, 1998.

71) Girard, P.M., Riballo, E., Begg, A.C., Waugh, A., and Jeggo, P.A.: Nbs1 promotes ATM dependent phosphorylation events including those required for G1/S arrest. *Oncogene*, **21**, 4191–4199, 2000.

72) Futreal, P.A., Liu, Q., Shattuck-Eidens, D., Cochran, C., Harshman, K., Tavtigian, S., Bennett, L.M., Haugen-Strano, A., Swensen, J., Miki, Y., McClure, M., Frye, C., Weaver-Feldhaus, J., Ding, W., Gholami, Z., Soderkvist, P., Terry L., Jhanwar, S., Berchuck, A., and Iglehart, J.D.: BRCA1 mutations in primary breast and ovarian carcinomas. *Science*, **266**, 120–122, 1994.

73) Zhong, Q., Chen, C.F., Li, S., Chen, Y., Wang, C.C., Xiao, J., Chen, P. L., Sharp, Z.D. and Lee, W.H.: Association of BRCA1 with the hRad50-hMre11-p95 complex and the DNA damage

response. *Science,* **285**, 747–750, 1999.

74) Haber, J.E.: The many interfaces of Mre11. *Cell,* **95**, 583–586, 1998.

75) Taniguchi, T., Garcia-Higuera, I., Xu, B., Andreassen, P.R., Gregory, R.C., Kim, S.T., Lane, W.S., Kastan, M.B., and D'Andrea, A.D.: Convergence of the Fanconi anemia and ataxia telangiectasia signaling pathways. *Cell,* **109**, 459–472, 2002.

76) Timmers, C., Taniguchi, T., Hejna, J., Reifsteck, C., Lucas, L., Bruun, D., Thayer M., Cox, B., Olson, S., D'Andrea, A.D., Moses, R., and Grompe, M.: Positional cloning of a novel Fanconi anemia gene, FANCD2. *Mol. Cell,* **7**, 241–248, 2001.

77) Nakanishi, K., Taniguchi, T., Ranganathan, V., New, H.V., Moreau, L.A., Stotsky, M., Mathew, C.G., Kastan, M.B., Weaver, D.T., and D'Andrea, A.D.: Interaction of FANCD2 and NBS1 in the DNA damage response. *Nat. Cell. Biol.,* **4**, 913–920, 2002.

78) Garcia-Higuera, I., Taniguchi, T., Ganesan, S., Meyn, M.S., Timmers, C., Hejna, J., Grompe, M., and D'Andrea, A.D.: Interaction of the Fanconi anemia proteins and BRCA1 in a common pathway. *Mol. Cell,* **7**, 249–262, 2001.

79) van den Bosch, M., Bree, R.T., and Lowndes, N.F.: The MRN complex: coordinating and mediating the response to broken chromosomes. *EMBO Rep.,* **4**, 844–849, 2003.

80) Lee, J.H., and Paull, T.T.: Direct activation of the ATM protein kinase by the Mre11/Rad50/Nbs1 complex. *Science,* **304**, 93–96, 2004.

81) Wang, X., and D'Andrea, A.D.: The interplay of Fanconi anemia proteins in the DNA damage response. *DNA Repair (Amst),* **3**, 1063–1069, 2004.

82) Xu, B., Kim, S.T., and Kastan, M.B.: Involvement of Brca1 in S phase and G(2)-phase checkpoints after ionizing irradiation. *Mol. Cell. Biol.,* **21**, 3445–3450, 2001.

83) Xiaoling, X., Olga, A., Peter, M., Chu, X.D., and Priscilla, A.F.: Impaired meiotic DNA-damage repair and lack of crossing-over during spermatogenesis in BRCA1 full-length isoform deficient mice. *Development,* **130**, 2001–2012, 2003.

84) Boyle, J.M., Spreadborough, A.R., Greaves, M.J., Birch, J.M., Varley, J.M. and Scott, D.: Delayed chromosome changes in gamma-irradiated normal and Li-Fraumeni fibroblasts. *Radiat. Res.,* **157**, 158–165, 2002.

85) Jackson, R.J., Engelman, R.W., Coppola, D., Cantor, A.B., Wharton, W., and Pledger, W.J.: $p21^{Cip1}$ nullizygosity increases tumor metastasis in irradiated mice. *Cancer Res.* **63**, 3021–3025, 2003.

86) Zachos, G., Rainey, M.D., and Gillespie, D.A.: Chk1-deficient tumour cells are viable but exhibit multiple checkpoint and survival defects. *EMBO J.,* **22**, 713–723, 2003.