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13. ABSTRACT (Maximum 200 Words)

The DNA-dependent protein kinase (DNA-PK) plays a very important role in the repair of DNA double-strand breaks generated by ionizing radiation (IR). The activation of DNA-PK in response to IR involves multiple autophosphorylations at S/TQ residues of the catalytic subunit, DNA-PKcs. We find that the activation of DNA-PKcs is attenuated during the S/G2 phases of the cell cycle, phases during which the tumor suppressor protein Brca1 is expressed. We found that DNA-PKcs interacts with Brca1 and have mapped the DNA-PKcs-interaction domain of Brca1. In order to investigate if the interaction of Brca1 with DNA-PKcs might attenuate DNA-Pkcs activation, we examined DNA-PKcs autophosphorylation in Brca1-deficient HCC1937 cells ectopically expressing Brca1. Although we did not observe any significant differences in DNA-PKcs autophosphorylation in the presence or absence of Brca1, the lack of observable differences could also be due to the low levels of Brca1 expression in these cells. We are currently attempting to overexpress the DNA-PK-interaction domain of Brca1 in human cells to see if this region of Brca1 has any effect on DNA-PK activation. We are also examining if the interaction of DNA-PK with Brca1 has any modulatory effect on Brca1 phosphorylation in response to IR.

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INTRODUCTION

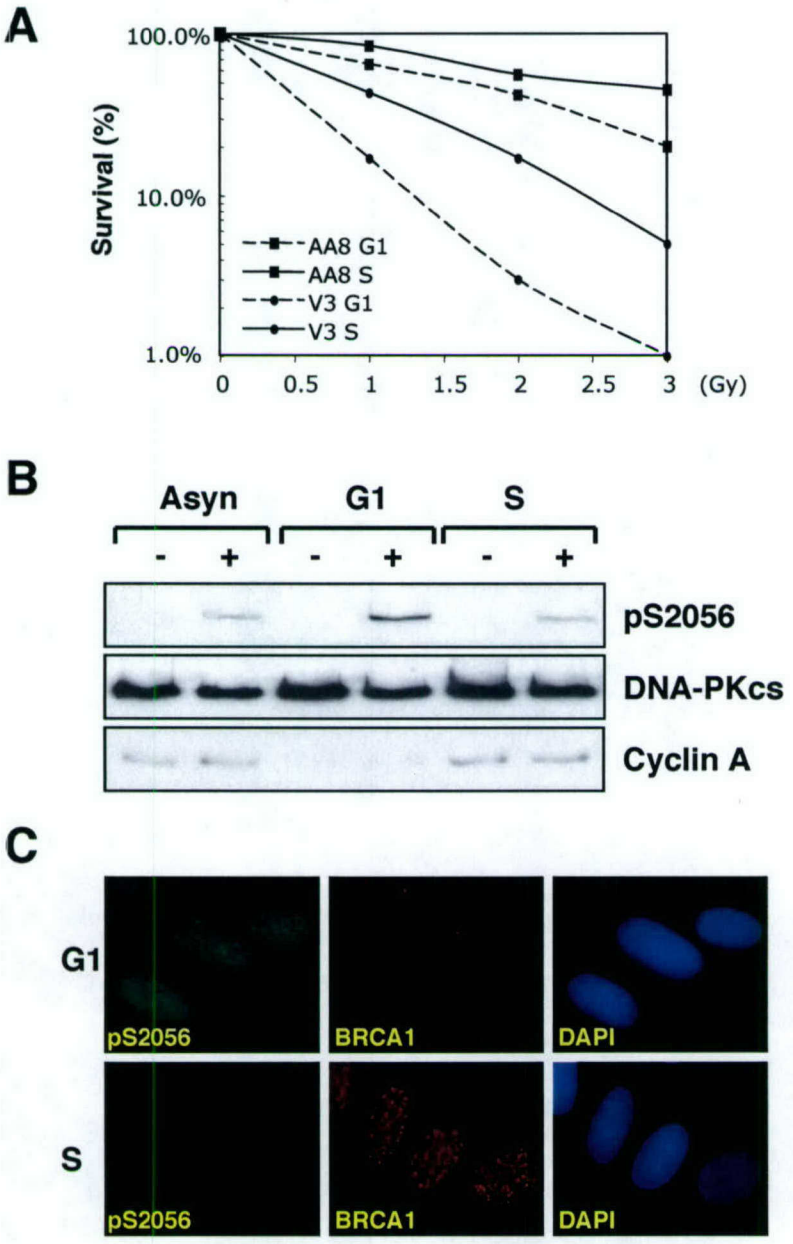
The DNA-dependent protein kinase (DNA-PK), consisting of a catalytic subunit DNA-PKcs and a DNA binding component Ku, plays a critical role in DNA double-strand break repair and in V(D)J recombination. DNA-PK also plays a very important role in triggering apoptosis in response to severe DNA damage or critically shortened telomeres. Components of the DNA-PK complex are also present at the mammalian telomere where they function in capping chromosome ends. In addition, DNA-PK appears to be involved in mounting an innate immune response to bacterial DNA and to viral infection. For a detailed review see (1).

The most important role of DNA-PK is in the repair of DNA double-strand breaks (DSBs) by the non-homologous end rejoining (NHEJ) pathway. Of the various types of DNA damage that arise within the cell, DNA double-strand breaks (DSBs) are particularly dangerous as they can lead to cell death or cancer if improperly repaired. Recently, our group, among others, discovered that that the activation of DNA-PKcs in response to DNA damage involves its autophosphorylation at several S/TQ residues (1). Phospho-specific antibodies recognizing these autophosphorylation sites can thus be used to accurately monitor the activation of DNA-PK within the cell as well as its localization. Using phospho-specific DNA-PKcs antibodies we found that the activation of DNA-PKcs in response to ionizing radiation (IR) is suppressed in the S/G2 phase of the cell division cycle. We also found that DNA-PKcs interacted with the tumor suppressor Brca1 whose expression is high during the S/G2 phases (2). We are interested in further dissecting this interaction and determining if Brca1 might modulate the activaton of DNA-PK in response to IR.

BODY

1) The activation of DNA-PK and the repair of DNA breaks by the NHEJ pathway is regulated in a cell cycle-dependent manner. It has been proposed that NHEJ and HR may be differentially regulated throughout the cell cycle with NHEJ playing a major role in G1/early S phases and HR playing a major role in late S/G2 phases when a sister chromatid is available (3). Indeed, DNA-PKcs-deficient V3 cells are less sensitive to IR in the S phase of the cell cycle as compared to G1 indicating that HR may partly compensate for NHEJ-deficiency in S phase cells (Fig. 1a). In order to directly monitor the phosphorylation of DNA-PK in different phases of the cell cycle, normal human skin fibroblasts (HSF) synchronized in G1 or S phases were irradiated and DNA-PKcs phosphorylation was analyzed. As shown in Fig. 1b, the protein level of DNA-PKcs remains constant throughout the cell cycle, whereas IR-induced S2056 autophosphorylation decreases from G1 phase to S phase. The decrease of IR-induced DNA-PKcs phosphorylation in S phase was also evident by fluorescent immunostaining with anti-S2056 antibody (Fig. 1c) and with anti-T2609 antibody (Fig. 1d) suggesting an overall reduction of IR-induced DNA-PKcs phosphorylation in the S phase of the cell cycle. Please note that the G1 cells stain poorly for Brca1 while the S phase cells stain strongly for Brca1 thereby confirming their cell cycle distribution (4). These results indicate that phosphorylation of DNA-PK in response to IR is regulated in a cell cycle-dependent manner. In HSF cells synchronized in G1 phase, greater than 90% of the HSF cells were positive for S2056 foci upon IR. In contrast, less than 15% of the HSF cells synchronized in S phase were positive for S2056 foci (Fig. 1e).

Figure 1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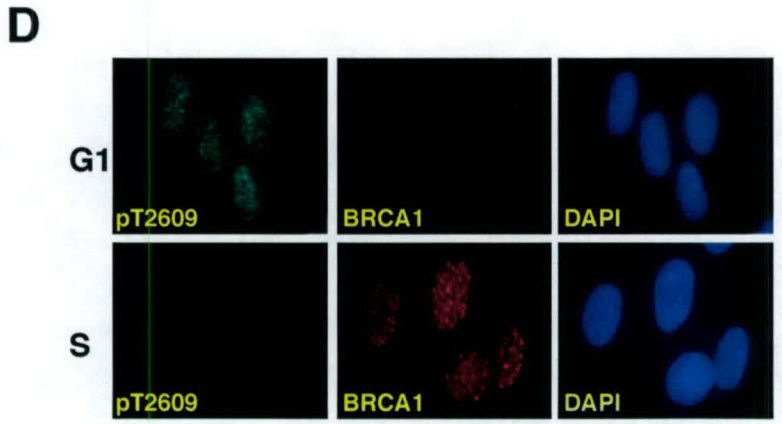


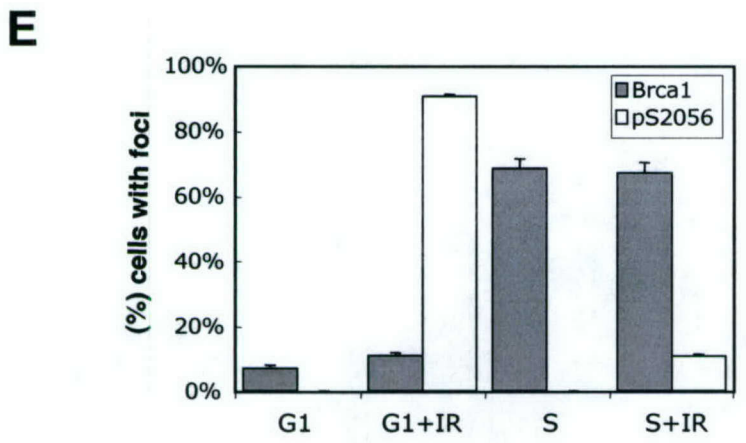


Fig. 1. Decrease of IR-induced S2056 and T2609 phosphorylation in the S phase. (a) V3 and the parental AA8 cells, synchronized in G1phase or in S phase, were irradiated at the indicated doses, and were plated out for analyzing their colony-forming abilities. (b) Asynchronized and synchronized HSFs were irradiated (10 Gy, 30 min recovery). Nuclear extracts were western blotted with anti-pS2056 antibody, anti-DNA-PKcs antibody, or anti-Cyclin A antibody. (c, d) HSFs synchronized in G1 or S phases were irradiated (10 Gy, 30 min recovery) and were co-immunostained with anti-pS2056/anti-Brca1 antibodies (c), or anti-pT2609/anti-Brca1 antibodies (d). (e) HSFs were scored for positive staining with anti-pS2056 or anti-Brca1 antibodies. More than 200 nuclei were counted in each experiment and the result is the average of two independent experiments.

2. DNA-PKcs constitutively interacts with Brca1. As DNA-PKcs activation is attenuated in the S/G2 phase of the cell cycle (see above), and because Brca1 is expressed in the S/G2 phases, we wanted to see if any regulatory interaction might exist between these two proteins. Towards this end, nuclear extracts were prepared from mock-irradiated and irradiated (10 Gy, 30 min) HeLa cells and the extracts were immunoprecipitated (ip) with anti-Brca1 or anti-DNA-PKcs antibodies and the immunoprecipitates were analyzed by Western blotting. Interestingly, we found that DNA-PKcs co-immunoprecipitated in the Brca1 ip (Fig. 2a). Conversely, we also found that Brca1 co-immunoprecipitated in a DNA-PKcs ip (Fig. 2b). The interaction between DNA-PKcs and Brca1 appeared to be constitutive and did not change upon irradiation of the cells.

Figure 2

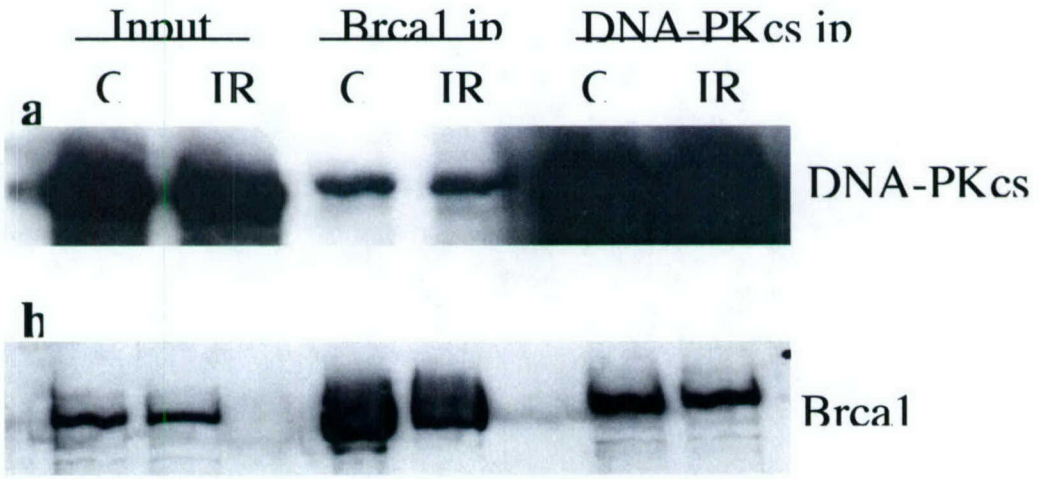
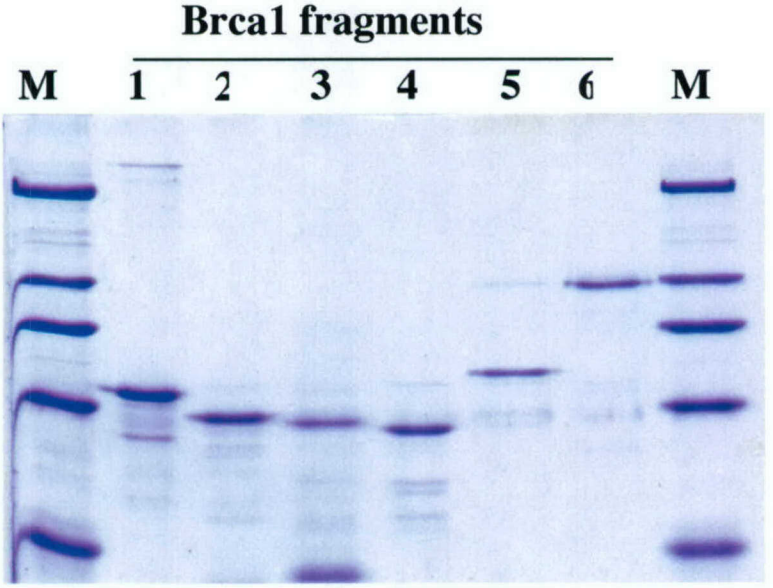


Fig. 2. DNA-PKcs co-immunoprecipitates with Brca1. Mock-irradiated (C) or irradiated (IR) HeLa nuclear extracts were immunoprecipitated with anti-Brca1 antibody or anti-DNA-PKcs antibody as indicated and the immunoprecipitates were analyzed by Western blotting with anti-DNA-PKcs antibody (a) or anti-Brca1 antibody (b).

3. Mapping of the DNA-PKcs-interaction domain of Brca1. In order to dissect the interaction of Brca1 with DNA-PKcs, we wanted to first determine the DNA-PKcs-interaction region of Brca1. We expressed overlapping fragments of Brca1 (5) as GST-fusion proteins in E.coli. (Fig. 3a). These fragments were used in GST pull down assays of purified DNA-PK complexes in the presence or absence of DNA. We found that Brca1 fragment 2 (aa 260-553) and fragment 3 (aa 502-802) interacted with DNA-PKcs while the remaining fragments did not (Fig. 3b). The pull down of DNA-PK by these two fragments was confirmed by Western blotting with anti-Ku70/80 antibodies. The addition of sheared salmon sperm DNA had no effect on the interaction.

Figure 3

a



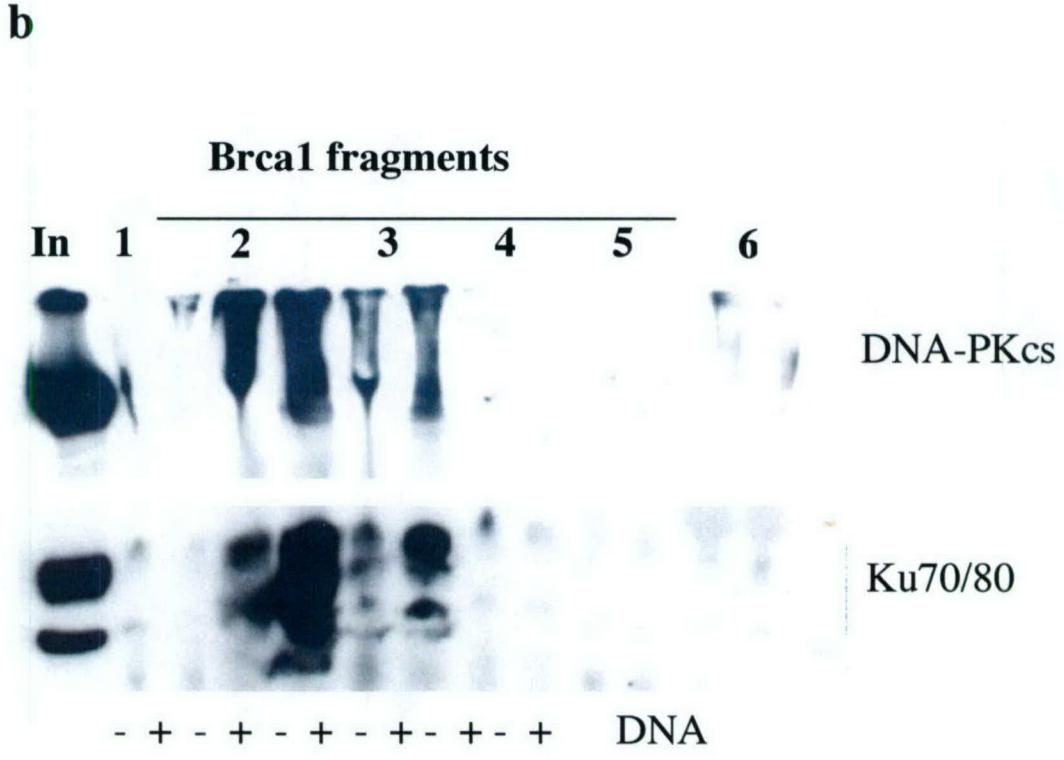


Fig. 3 (a) Expression of Brca1 fragments 1-6 (4) as GST-fusion proteins. **(b)** GST pulldown of DNA-PK components (DNA-PKcs and Ku70/80) by Brca1 fragments 2 and 3.

4. Effect of ectopic Brca1 expression on DNA-PKcs autophosphorylation. We have carried out preliminary experiments to investigate if the interaction of Brca1 with DNA-PKcs might modulate the activation of DNA-PKcs upon DNA damage. Brca1-deficient HCC1937 cells and HCC1937 cells ectopically expressing Brca1 (6) were irradiated with X-rays (10 Gy, 30 min) and the activation of DNA-PK examined by immunofluorescence staining with phospho-specific DNA-PKcs antibodies. We found that DNA-PKcs was autophosphorylated (at S2056 and T2609) and capable of forming radiation induced foci even in cells expressing Brca1 (Fig. 4). Therefore, it appears that DNA-PKcs activation may not be significantly suppressed by Brca1 alone. Alternately, it is also possible that the levels of ectopic Brca1 might not be sufficiently high to regulate DNA-PKcs which is

highly abundant in human cells. We are currently in the process of generating cells overexpressing fragments 2 and 3 of Brca1 to see if these DNA-PKcs-interacting regions might have a modulatory effect on DNA-PK activation.

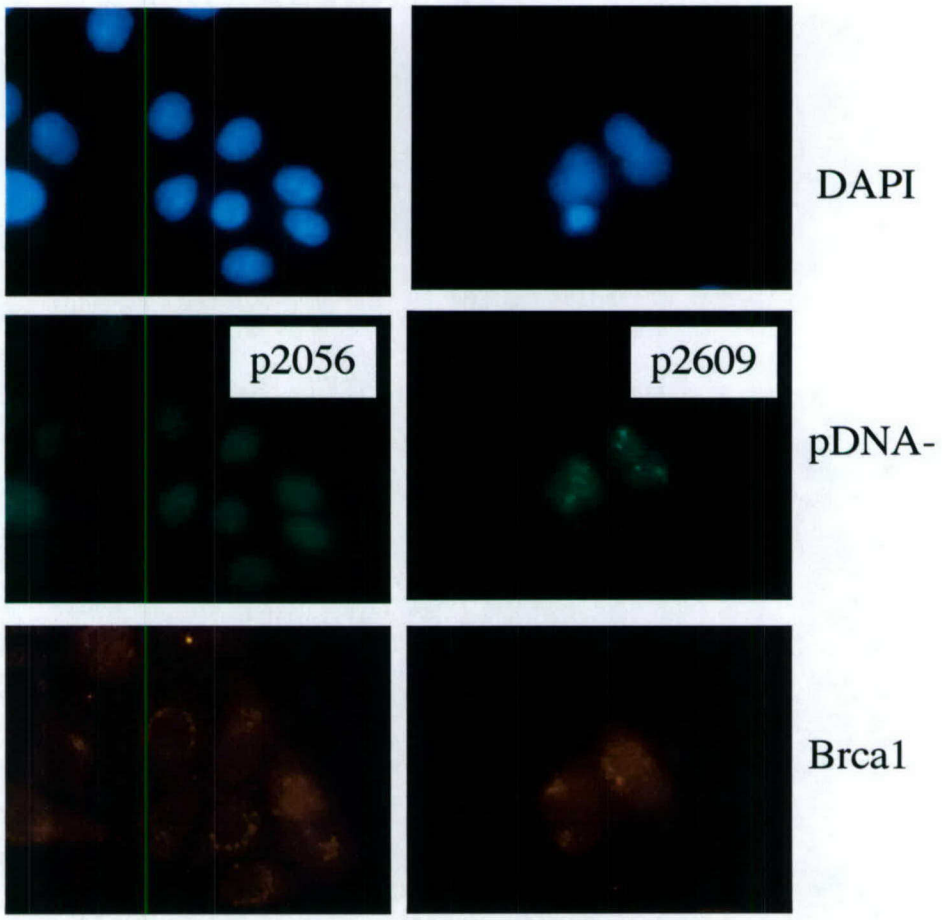


Fig. 4. DNA-PKcs autophosphorylation (at S2056 and T2609) and focus formation in reponse to IR in HCC1937 cells ectopically expressing Brca1.

5. Effect of DNA-PKcs on the phosphorylation and activation of Brca1. We are also interested in examining if the interaction of DNA-PKcs with Brca1 might serve to

modulate IR-induced Brca1 phosphorylation and functions. We propose to first examine if Brca1 phosphorylation in response to IR is affected in cells deficient in DNA-PKcs. We have raised and purified phospho-specific antibodies against the phosphorylation sites on Brca1 that are modified in response to IR (serines 1387, 1423, 1524 (7)) (Fig. 5a). These antibodies can be used to visualize the phosphorylation of Brca1 in response to IR (Fig. 5b). We propose to use these antibodies to analyze if DNA-PKcs might modulate the modification of Brca1upon IR.

Figure 5

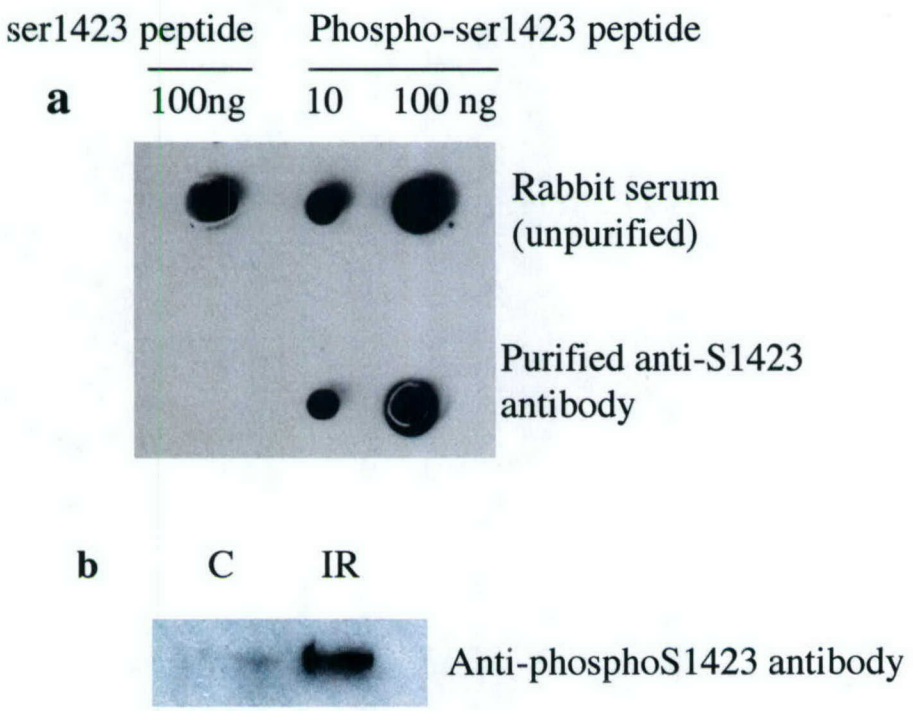


Fig. 5. a. Rabbit polyclonal antibodies were raised against peptides with phospho-S1423 site of Brca1, purified over a phospho-peptide column, and tested by dot blotting against unphosphorylated and phosphorylated peptides. **B.** The pS1423 antibody was used to visualize the phosphorylation of Brca1 upon irradiation of HeLa cells by Western blotting.

KEY RESEARCH ACCOMPLISHMENTS

1. The activation of DNA-PK is attenuated in the S/G2 phases of the cell cycle.
2. DNA-PK interacts with Brca1 and the interaction is not affected upon ionizing radiation.
3. The DNA-PK-interaction region of Brca1 lies between aa 260-553.
4. The phosphorylation and DNA damage-site localization of DNA-PKcs in cells ectopically expressing Brca1 was investigated. Experiments are underway to investigate the effect of overexpressing Brca1 fragments on DNA-PK activation.
5. Phospho-specific Brca1 antibodies were raised and will be used to investigate the influence of DNA-PKcs on Brca1 phosphorylation and functions upon irradiation.

REPORTABLE OUTCOMES

Submitted Manuscript **(Appendix 1)**

1] Cell cycle dependence of DNA-PK phosphorylation in response to double-strand breaks. B. Chen, D.W. Chan, J. Kobayashi, S. Burma, A. Asaithamby, K. Morotomi-Yano, J. Qin, and D.J. Chen Journal of Biological Chemistry (re-submitted after revision)

Meeting Abstracts;

1] Phosphorylation of DNA-PK and DNA-double strand break repair.
B. Chen, J. Kobayashi, S. Burma, D. Chan, A. Asaithamby, J. Qin, and D.J. Chen
In abstract of the AACR Special Conference on Radiation Biology and Cancer (2004)

2] Differential requirement of DNA-PKcs in S phase for double-strand break repair.
B. Chen, J. Kobayashi, S. Burma, D. Chan, A. Asaithamby, J. Qin, and D.J. Chen
In abstract of the Radiation Research Society Annual Meeting (2004)

3] Autophosphorylation of the DNA-dependent protein kinase is required for rejoining of DNA double-strand breaks.
B. Chen, J. Kobayashi, S. Burma, D. Chan, K. Yano, J. Qin, and D.J. Chen
In abstract of the International Workshop on Ataxia-Telangiectasia (2003)

CONCLUSIONS

We were interested in investigating whether the interaction of DNA-PKcs and Brca1, as observed by us, had any functional consequences. We were especially interested in determining if Brca1 might be responsible for the attenuated DNA-PKcs activation in S/G2 phases that we observed. We found that the ectopic expression of Brca1did not significantly affect DNA-PK activation and localization to damage sites. However, this could also be because the levels of ectopic Brca1 in the cell lines used might not have been sufficiently high. We are currently trying to overexpress the DNA-PKcs-interaction domain of Brca1 to see if DNA-PK activation is affected. We have also raised antibodies recognizing Brca1 phosphorylated at specific sites and will use these to investigate if the interaction of DNA-PKcs and Brca1 might modulate Brca1 phosphorylation upon DNA damage induction.

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Appendix 1

Resubmitted to **Journal of Biological Chemistry**

Cell Cycle Dependence of DNA-PK Phosphorylation in Response to DNA Double Strand Breaks

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Running Title: DNA-PKcs autophosphorylation

Key words: nonhomologous end-joining; DNA-PKcs autophosphorylation; ionizing radiation; cell cycle; DNA replication

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Abstract

DNA-dependent protein kinase (DNA-PK), consisting of Ku and DNA-PKcs subunits, is the key component of the non-homologous end joining (NHEJ) pathway of DNA double strand breaks (DSBs) repair. Though the kinase activity of DNA-PKcs is essential for NHEJ, thus far, no *in vivo* substrate has been conclusively identified except for an autophosphorylation site on DNA-PKcs itself (threonine 2609). Here we report the IR-induced autophosphorylation of DNA-PKcs at a novel site, serine 2056, and phosphorylation at this site is required for the repair of DSBs by NHEJ. Interestingly, IR-induced DNA-PKcs autophosphorylation is regulated in a cell cycle-dependent manner with attenuated phosphorylation in the S phase. In contrast, DNA replication-associated DSBs result in DNA-PKcs autophosphorylation and localization to DNA damage sites. These results indicate that, while IR-induced DNA-PKcs phosphorylation is attenuated in S phase, DNA-PKcs is preferentially activated by the physiologically relevant DNA replication-associated DSBs at the sites of DNA synthesis.

Introduction

DSB repair is critical for the maintenance of genome integrity, cell survival, and prevention of tumorigenesis (Khanna and Jackson, 2001). NHEJ and Homologous recombination (HR) are the two major pathways for DSB repair in eukaryotes. While HR requires the presence of a homologous chromosome or sister chromatid, NHEJ does not depend on the presence of homologous DNA sequences and is the predominant pathway for DSB repair in mammalian cells (Critchlow and Jackson, 1998). DNA-PK, the key component of the NHEJ pathway, is composed of the Ku70/80 heterodimer and the catalytic subunit (DNA-PKcs) (Smith and Jackson, 1999). Although the biochemical properties of DNA-PK have been extensively studied *in vitro*, it is still not clear how it functions *in vivo* in the context of NHEJ. Wild type DNA-PKcs, but not a kinase-dead mutant, is able to rescue the radiation sensitivity and DSB-repair defect of DNA-PKcs-defective V3 cells demonstrating that the kinase activity of DNA-PKcs is essential for the NHEJ pathway (Kurimasa et al., 1999). To further elucidate the molecular mechanism(s) underlying NHEJ, it is critical to identify the *in vivo* substrates of DNA-PK and to understand exactly how its kinase activity is regulated. Towards this end, we have previously reported that ionizing radiation (IR) induces DNA-PKcs autophosphorylation at threonine 2609 (T2609) and demonstrated that T2609 phosphorylation is required for DSB repair (Chan et al., 2002). In addition to T2609, several putative phosphorylation sites have been identified (Douglas et al., 2002; Soubeyrand et al., 2003); however, none of these sites have been demonstrated to be phosphorylated *in vivo* in response to DSBs. Here we report that the IR-induced phosphorylation of DNA-PKcs at a novel site, serine 2056 (S2056) is required for NHEJ and that the phosphorylation of DNA-PKcs is regulated in a cell cycle and DNA replication-dependent manner.

Results and Discussion

DNA-PKcs purified from IR treated HeLa cells was separated on SDS-PAGE, digested with Asp_N. The extracted peptides before and after CIP treatment were analyzed with MALDI-TOF (Zhang et al., 1998). A putative phosphopeptide encompassing a.a. 2044-2072 was identified. The phosphopeptide was too large to be sequenced by LC/MS/MS to find the precise phosphorylation site. To identify the site, we generated a phospho-specific antibody (Fig. 1b) and confirmed that S2056 is indeed phosphorylated *in vivo* in response to IR. Similar to T2609, S2056 is also conserved in all known DNA-PKcs homologues (Fig. 1a) suggesting that phosphorylation of DNA-PKcs at S2056 upon IR maybe be an evolutionary conserved response. S2056 phosphorylation could be detected as early as 10 minutes post-irradiation in HeLa cells after 10 Gy of irradiation. At this dose, the phosphorylation of S2056 reaches a maximum at 30 min and remains detectable even at 8 hrs after irradiation (Fig.1c). Phosphorylation at S2056 increases in a dosage-dependent manner; phosphorylation is detectable after 2 Gy of irradiation and the level of phosphorylation continues to increase with the dose (up to 50 Gy) without reaching saturation (Fig. 1c).

IR-induced S2056 phosphorylation is abolished by low doses of wortmannin (Supplementary Fig. 2) that inhibit DNA-PKcs and ATM but not ATR (Sarkaria et al., 1998). To determine if DNA-PKcs itself or ATM is responsible for S2056 phosphorylation, we examined IR-induced S2056 phosphorylation in V3 cell lines stably expressing either wild type DNA-PKcs (V3-WT) or kinase-dead mutant DNA-PKcs (V3-KD). IR-induced S2056 phosphorylation is markedly reduced in V3-KD cells as compared to that of V3-WT cells, whereas DNA-PKcs protein levels in both cell lines are comparable (Fig. 1d). Moreover, S2056 phosphorylation is unaffected in a panel of cell lines deficient in ATM (data not shown). Thus, IR-induced modification of S2056 is clearly an autophosphorylation event. In addition, IR-induced S2056 phosphorylation is not affected in a V3 cell line expressing a mutant DNA-PKcs with an alanine substitution at T2609 indicating that IR-induced S2056 phosphorylation is independent of T2609 phosphorylation (Fig. 1d). S2056 phosphorylation is specifically in response to DSBs. In addition to IR, other DSB-inducing agents such as bleomycin and etoposide can also induce S2056 phosphorylation, whereas hydroxyurea, methyl methanesulfonate, and UV

irradiation induce minimal phosphorylation at S2056 (Fig. 1e). Fluorescent immunostaining further revealed that DNA-PKcs phosphorylated at S2056 is present at discrete nuclear foci that co-localize with γ-H2AX foci (Fig. 1f and Supplementary Fig. 3), presumably at the DNA-damage sites (Paull et al., 2000).

To investigate the biological significance of DNA-PKcs phosphorylation at S2056, we examined the radiation sensitivities and DSB rejoining capabilities of V3 cell lines stably expressing either wild type DNA-PKcs or DNA-PKcs harboring a S2056 to alanine substitution. As shown in Fig. 2b, V3 cells complemented with S2056A or S2056A/T2609A mutant DNA-PKcs were significantly radio-sensitive compared to V3 cells complemented with wild type DNA-PKcs suggesting that phosphorylation at S2056 is required for resistance to ionizing radiation. To analyze the role of S2056 phosphorylation in DSB rejoining, we utilized a previously established plasmid based end-joining assay for NHEJ (Verkaik et al., 2002) that measures direct end-joining *versus* alternative microhomology-directed end-joining activity. In cells defective in NHEJ, a dramatic increase in microhomology-directed end-joining has been observed. We also observed elevated microhomology-directed end-joining in V3 cells, whereas the parental AA8 CHO cells and V3-WT cells displayed only minimal levels of microhomology-directed end-joining thereby establishing the validity of this assay (Fig. 2c). In contrast to V3-WT, V3 cells complemented with S2056A, T2609A, S2056A/T2609A mutants showed a dramatic increase in microhomology-directed end-joining suggesting that these cells are significantly defective in NHEJ. Taken together, these results provide compelling evidence indicating that the phosphorylation of DNA-PKcs at S2056 is critical for the repair of DSBs by the NHEJ pathway.

It has been proposed that NHEJ and HR may be differentially regulated throughout the cell cycle with NHEJ playing a major role in G1/early S phases and HR playing a major role in late S/G2 phases when a sister chromatid is available (Hendrickson, 1997; Takata et al., 1998). Indeed, DNA-PKcs-deficient V3 cells are less sensitive to IR in the S phase of the cell cycle as compared to G1 indicating that HR may partly compensate for NHEJ-deficiency in S phase cells (Fig. 3a). In order to directly monitor the phosphorylation of DNA-PK in different phases of the cell cycle, normal human skin fibroblasts (HSF) synchronized in G1 or S phases were irradiated and DNA-

PKcs phosphorylation was analyzed. As shown in Fig. 3b, the protein level of DNA-PKcs remains constant throughout the cell cycle, whereas IR-induced S2056 phosphorylation decreases from G1 phase to S phase. The decrease of IR-induced DNA-PKcs phosphorylation in S phase was also evident by fluorescent immunostaining with anti-S2056 antibody (Fig. 3c) and with anti-T2609 antibody (Fig. 3d) suggesting an overall reduction of IR-induced DNA-PKcs phosphorylation in the S phase of the cell cycle. Please note that the G1 cells stain poorly for Brca1 while the S phase cells stain strongly for Brca1 thereby confirming their cell cycle distribution (Vaughn et al., 1996). These results indicate that phosphorylation of DNA-PK in response to IR is regulated in a cell cycle-dependent manner. In HSF cells synchronized in G1 phase, greater than 90% of the HSF cells were positive for S2056 foci upon IR. In contrast, less than 15% of the HSF cells synchronized in S phase were positive for S2056 foci (Fig. 3e).

Although phosphorylation of DNA-PKcs in response to IR is attenuated in S phase, V3 cells are reported to be sensitive to agents (camptothecin and hydroxyurea) that disrupt the progression of the replication fork and induce replication-associated DSBs (Arnaudeau et al., 2001; Lundin et al., 2002). This suggests that, though DNA-PKcs is less responsive to IR in the S phase, it may still play an important role in the resolution of DNA replication-associated DSBs in this phase of the cell cycle. We, therefore, examined DNA-PKcs phosphorylation in response to camptothecin which inhibits topoisomerase I and induces replication-associated DSBs(Liu et al., 2000). Camptothecin treatment of HSF cells indeed induced DNA-PKcs phosphorylation at S2056 (Fig. 4a). It was reported that a DNA polymerase activity is required to convert camptothecin-induced lesions into DSBs and pretreatment of cells with aphidicolin (DNA polymerase α & δ inhibitor) blocks the induction of DSBs by camptothecin (Furuta et al., 2003). Camptothecin-induced S2056 phosphorylation was significantly attenuated in the presence of aphidicolin (Fig. 4a) indicating that the observed phosphorylation of DNA-PKcs in response to camptothecin is associated with replication fork progression. In addition to the increase in S2056 phosphorylation, fluorescent immunostaining revealed that camptothecin also induces phospho-S2056 (Fig. 4b) and phospho-T2609 (Fig. 4c) foci only in HSF cells synchronized in S phase but not in G1 phase.

To confirm that camptothecin-induced phospho-DNA-PKcs is associated with the sites of DNA replication, HSFs was pulse-labeled with BrdU for 10 min followed by camptothecin treatment for 30 min. Fluorescent immunostaining demonstrated that camptothecin-induced S2056, T2609, and γ-H2AX foci co-localize with the sites of BrdU incorporation, and camptothecin-induced S2056 foci also co-localize with p34 subunit of replication protein A /RPA2 (Fig. 4d) (Vassin et al., 2004). The observed co-localizations strongly suggest that DSBs generated at the sites of replication forks selectively activate DNA-PK. Thus, while IR-induced DSBs do not induce significant DNA-PKcs phosphorylation in the S phase, the DNA replication-associated DSBs selectively activate DNA-PKcs.

Although DNA-PKcs promiscuously phosphorylates a plethora of substrates *in vitro* including its own self, *bona fide* targets of DNA-PK have largely eluded identification. Recently, the locations of seven putative phosphorylation sites on DNA-PKcs were reported based upon analyses of DNA-PKcs autophosphorylated *in vitro* (Chan et al., 2002; Douglas et al., 2002; Soubeyrand et al., 2003). Among the seven identified sites, only T2609 has been conclusively demonstrated to be phosphorylated *in vivo* in response to IR (Chan et al., 2002). In order to directly identify sites that are important in the cellular response to IR, we analyzed DNA-PKcs from irradiated HeLa cells and successfully identified an *in vivo* autophosphorylation site, S2056. The significance of S2056 phosphorylation in DSBs repair is demonstrated by its localization at the sites of DSBs, and by the fact that serine to alanine mutation at this site compromise both radiation resistance and NHEJ mediated DSBs repair. IR-induced S2056 phosphorylation, however, might be distinctly regulated compared to that of T2609. First, the physical location of S2056 on the DNA-PKcs molecule is far removed from T2609 which is part of a cluster of six putative S/TQ phosphorylation sites (Chan et al., 2002; Douglas et al., 2002; Soubeyrand et al., 2003). Second, the level of S2056 phosphorylation continues to increase with the dose (up to 50 Gy) without reaching saturation, whereas IR-induced T2609 phosphorylation saturates at 10 Gy (Chan et al., 2002). Third, IR-induced S2056 phosphorylation is unaffected in a panel of paired ATM-deficient and -proficient cell lines (B.P.C. and D. J. C. unpublished results), whereas IR-induced T2609 phosphorylation is reduced in ATM-deficient cells (Chan et al., 2002;

B.P.C. and D. J. C. unpublished results). We speculate, therefore, that while S2056 phosphorylation is purely an autophosphorylation event, additional kinases might contribute to T2609 phosphorylation. It is not yet clear whether the differences between S2056 and T2609 phosphorylations reflect different roles of DNA-PKcs in NHEJ and this aspect of DSB repair certainly merits further investigation.

Another important finding from this study is that DNA-PKcs phosphorylation and focus formation in response to IR, while quite robust in G1 cells, is significantly attenuated in S phase cells. This lends credence to the notion that DNA-PK functions primarily in G1 while HR takes over during S/G2 (Hendrickson, 1997; Takata et al., 1998). DNA-PKcs and Ku appear to have the ability to suppress HR in S/G2 phases(Allen et al., 2002). It is still not clear if there is a passive competition between the two pathways or whether an active mechanism exists that prevents DNA-PKcs phosphorylation and activation during S/G2 phases thereby shunting DSBs to the competing HR pathway. However, DSBs generated when a DNA replication fork encounters a damaged template may be more physiologically relevant in S phase cells. Interestingly, we find that such breaks result in robust DNA-PKcs phosphorylation and focus formation. Therefore, while DSBs generated by IR do not elicit a DNA-PK-activation response in S phase cells, the more physiologically relevant replication-associated breaks may perhaps be preferentially repaired by DNA-PK-dependent mechanisms. It is tempting to speculate that the response of DNA-PK to such breaks may be due to an intimate association with the replication machinery mediated, perhaps, by Replication Protein A (RPA) that DNA-PK is known to associate with and phosphorylate (Shao et al., 1999).

Methods

Cell culture and drug treatments

HeLa and normal human skin fibroblasts (HSFs) were maintained in a humidified atmosphere with 5% CO_2 in α-MEM medium with 10% fetal calf serum, 100 units ml^{-1} penicillin, and 100 μg ml^{-1} streptomycin. HeLa cells were treated with DNA-damaging agents for 2hrs at the indicated concentrations: bleomycin (50 μg ml^{-1}), etoposide (30 μg ml^{-1}), methyl methanesulfonate (50 μg ml^{-1}), hydroxyurea (1 mM). UV irradiation of HeLa cells was carried out at the rate of 0.15 J m^{-2} sec^{-1} to achieve a cumulative dose of 10 J m^{-2} followed by a 2hrs recovery period. DNA replication-associated DSBs were induced in HSFs by camptothecin (1 μM) for 1hr, or aphidicolin (2μg ml^{-1}) for 1hr, or aphidicolin pretreatment for 30min followed by camptothecin for 1hr.

Site-directed mutagenesis and generation of V3 stable cell lines

Site-directed mutagenesis and isolation of the mutant cell lines was performed as previously described (Chan et al., 2002). V3 cells complemented with wild type DNA-PKcs or kinase dead mutant DNA-PKcs were described previously (Kurimasa et al., 1999). V3 cell lines stably expressing S2056A or S2056A/T2609A mutant DNA-PKcs were generated by co-transfection of S2056A or S2056A/T2609ADNA-PKcs expression plasmids together with pSV2neo plasmid into the V3 cells.

Cell cycle synchronization and colony formation assay

HSFs were subjected to serum starvation (0.5% FCS) for 48 hrs followed by 1 hr release (10% FCS) to synchronize in the G1 phase (Chen et al., 1997). Cells synchronized in G1 were treated with aphidicolin overnight followed by 4 hrs release to synchronize cell cycle in the mid-S phase. V3 and AA8 cells were synchronized in G1 by isoleucine-deprivation followed by release in complete medium (Nagasawa et al., 1991). Colony formation assay was performed as previously described (Kurimasa et al., 1999).

Fluorescent immunostaining and antibodies

Fluorescent immunostaining was performed as previously described (Burma et al., 2001). Anti-pS2056 polyclonal antibodies were prepared by immunizing New Zealand white rabbits with KLH-conjugated phospho-peptide QSYSYSS[PO3]QDRKPTC. Anti-pT2609 mouse monoclonal antibody was prepared by immunizing bulb/c mice with KLH-conjugated phospho-peptide TPMFVET[PO3]QASQGT according to standard procedures (Harlow and Lane, 1988). Anti-pT2609 and anti-γH2AX polyclonal antibodies were prepared as previously described (Burma et al., 2001; Chan et al., 2002). Anti-DNA-PKcs 25-4 mAb (NeoMarkers), anti-Cyclin A mAb (Upstate), anti-Brca1 mAb (Oncogene), anti-BrdU mAb (Roche), and anti-RPA2 mAb (Oncogene) are commercially available.

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Figure Legends

Fig. 1. Autophosphorylation of DNA-PKcs at S2056 *in vivo* in response to DSBs. (a) Alignment of human DNA-PKcs S2056 with DNA-PKcs sequences available in the NCBI database. **(b)** Anti-pS2056 phospho-specific antibody recognizes S2056 phosphorylation specifically. **(c)** HeLa cells were irradiated (10 Gy) and allowed to recover for the indicated times, or irradiated at the indicated doses and allowed to recover for 30 min. Nuclear extracts were western blotted with anti-pS2056 or anti-DNA-PKcs antibodies. **(d)** V3 cells stably expressing wild type DNA-PKcs (V3-WT), kinase-dead mutant (V3-KD), or T2609A mutation (V3-T2609A) were either mock-treated or irradiated (10 Gy, 30 min recovery). DNA-PKcs was immuno-precipitated from nuclear extracts and western blotted. **(e)** HeLa cells were treated with the following DNA-damaging agents for 2hr: bleomycin (Ble, 50 $\mu g\ ml^{-1}$), etoposide (Eto, 30 $\mu g\ ml^{-1}$), methyl methanesulfonate (MMS, 50 $\mu g\ ml^{-1}$), hydroxyurea (HU, 1 mM), or subjected to UV irradiation (10 $J\ m^{-2}$) followed by a 2hr recovery. **(f)** Human skin fibroblasts (HSFs) were irradiated (10 Gy, 30 min) and immuno-stained with anti-pS2056 antibody alone or together with anti-γH2AX antibody.

Fig. 2. Phosphorylation at S2056 is required for NHEJ-mediated DSBs repair. (a) DNA-PKcs protein expression levels in stable V3 cell lines expressing wild type or mutant DNA-PKcs. **(b)** A colony formation assay was performed to compare radiation sensitivities of V3-JM (vector), V3-WT, V3-S2056A, and V3-S2056A/T2609A cell lines. **(c)** Microhomology-directed end-joining assay. The V3-JM, V3-WT, V3-S2056A, V3-T2609A, and V3-S2056A/T2609A cell lines and the parental AA8 CHO cell line were transfected with linearized pDVG9 plasmid DNA (Verkaik et al., 2002). Plasmid DNA was recovered 48 hrs after transfection and was PCR amplified across the joining region. Equal amounts of PCR products were digested with BstXI to determine NHEJ directed (uncut) and microhomology directed (cut) end-joining. Both assays **(b, c)** have been repeated at least twice and the error bars indicate the standard error derived from the independent experiments.

Fig. 3. Decrease of IR-induced S2056 and T2609 phosphorylation in the S phase. (a) V3 and the parental AA8 cells, synchronized in G1phase or in S phase, were irradiated at the indicated doses, and were plated out for analyzing their colony-forming abilities. **(b)** Asynchronized and synchronized HSFs were irradiated (10 Gy, 30 min recovery). Nuclear extracts were western blotted with anti-pS2056 antibody, anti-DNA-PKcs antibody, or anti-Cyclin A antibody. **(c, d)** HSFs synchronized in G1 or S phases were irradiated (10 Gy, 30 min recovery) and were co-immunostained with anti-pS2056/anti-Brca1 antibodies **(c)**, or anti-pT2609/anti-Brca1 antibodies **(d)**. **(e)** HSFs were scored for positive staining with anti-pS2056 or anti-Brca1 antibodies. More than 200 nuclei were counted in each experiment and the result is the average of two independent experiments.

Fig. 4. Induction of DNA-PKcs phosphorylation by DNA replication-associated DSBs. (a) Asynchronous HSFs were subjected to the following treatments: camptothecin (Cpt, 1 μM) or aphidicolin (Aph, 2μg ml^{-1}) for 1hr, Aph pretreatment for 30min followed by Cpt for 1hr (A/C), or IR (10 Gy, 30 min recovery). Nuclear extracts were western blotted with anti-pS2056 or anti-DNA-PKcs antibody. **(b, c)** HSFs synchronized in G1 or S phase were subjected to 1 μM Cpt treatment for 1hr and were co-immunostained with anti-pS2056/anti-Brca1 antibodies **(b)**, or anti-pT2609/anti-Brca1 antibodies **(c)**. **(d)** HSFs were pulse-labeled with BrdU for 10 min followed by Cpt treatment for 30 min. The cells were co-immunostained with anti-BrdU and anti-γH2AX, anti-pS2056, or anti-pT2609 antibodies as indicated. HSFs without BrdU labeling were subjected to Cpt treatment for 1hr and were immunostained with anti-pS2056 and anti-RPA2 antibodies.

Figure 1 Chen et al.

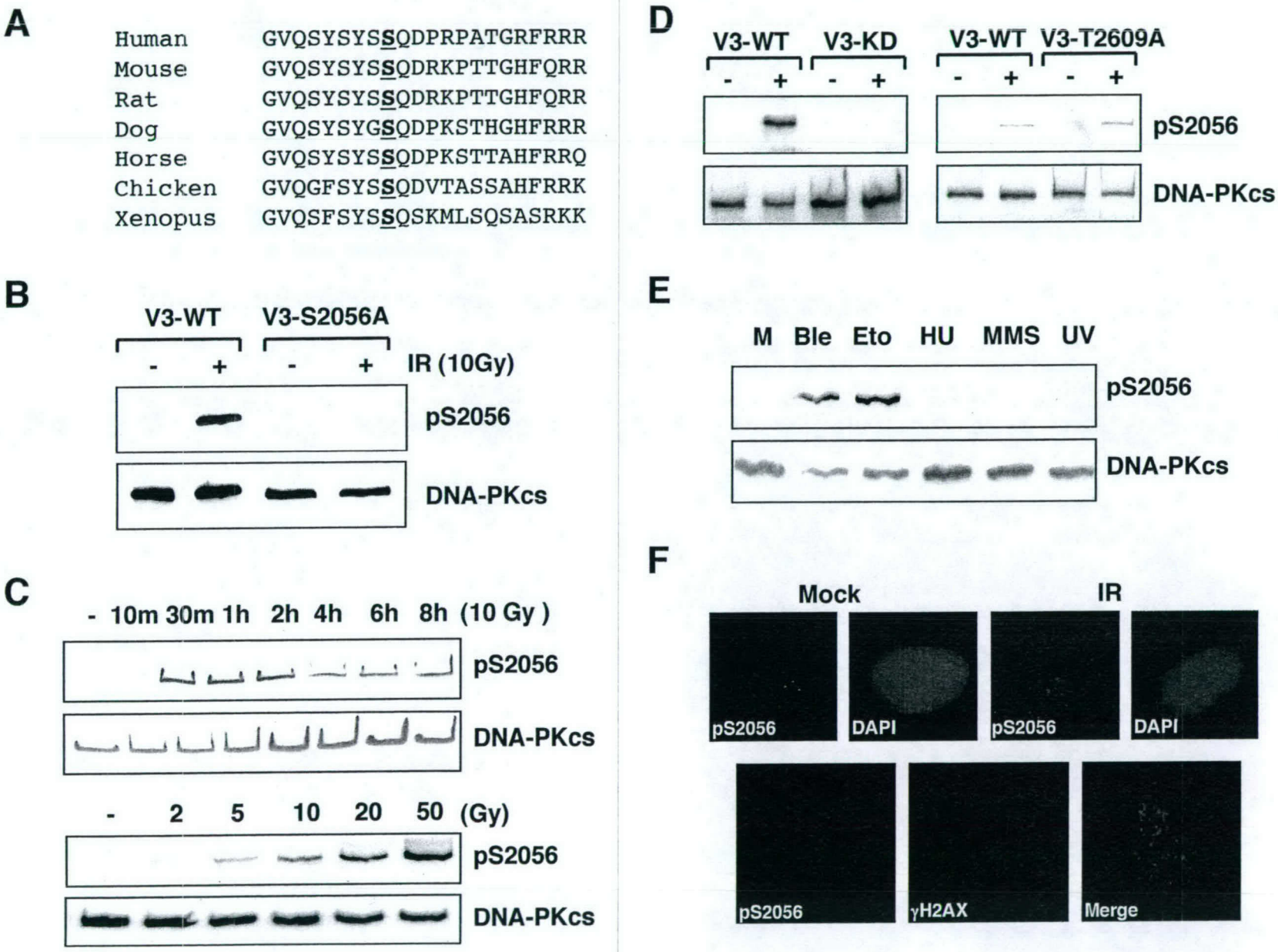
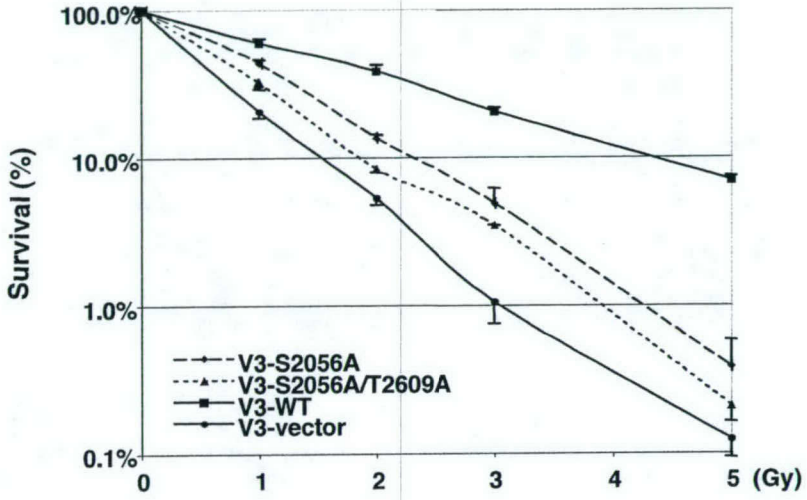


Figure 2 Chen et al.

A

WT S2056A S2056A/ T2609A

B



C

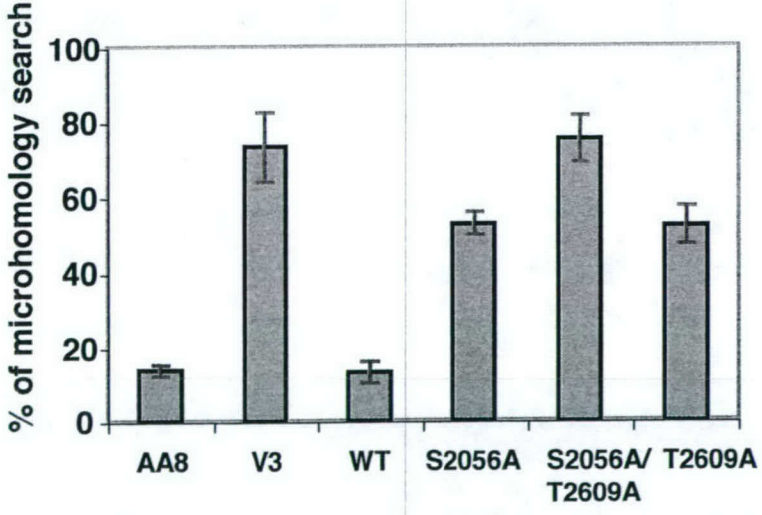
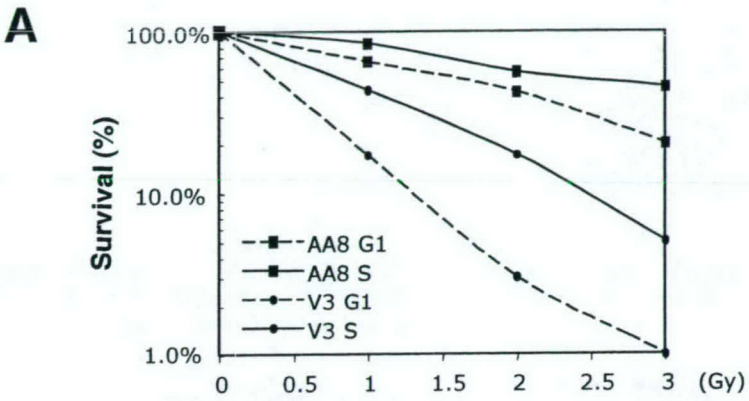
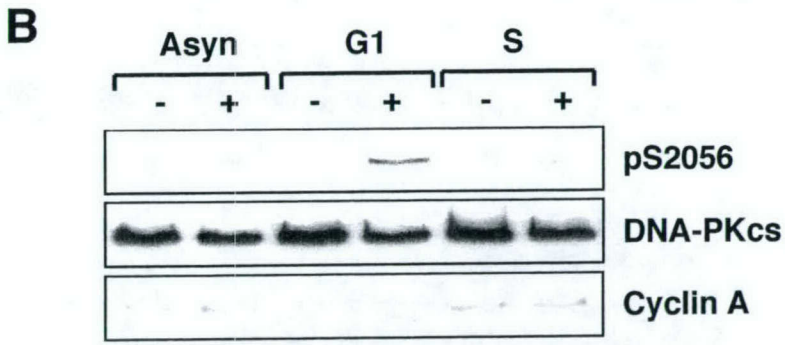
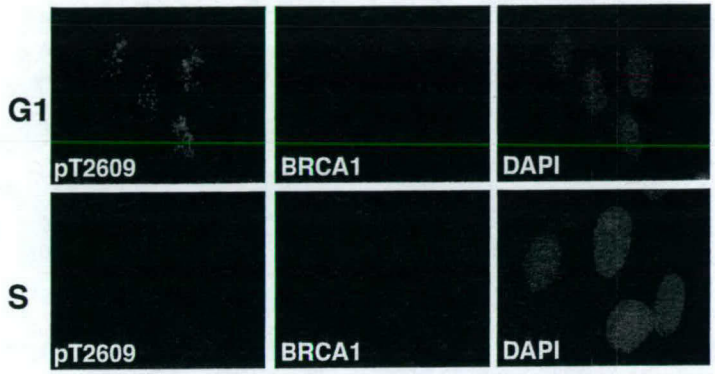


Figure 3 Chen et al.







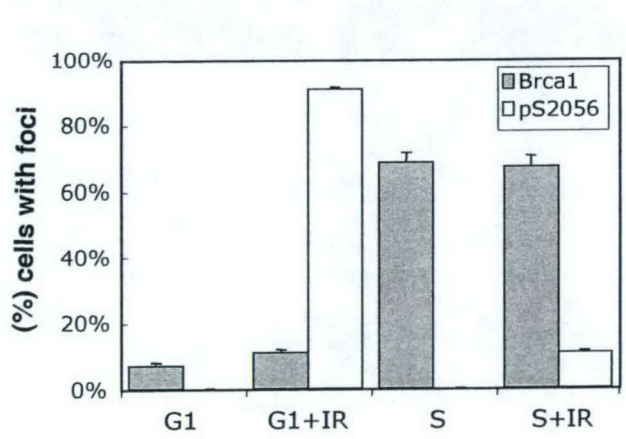


Figure 4 Chen et al.