shorter DNA segments as described (*8*). Sequencing reactions were pipetted by a Beckman Biomek 2000 robot. Cycling conditions as well as reagent concentrations were as described (*8*). The sequencing products (0.5 μl) were run on LongReadIR4200 sequencers (LI-COR) with 3.75% RapidGel XL Sol (Amersham Pharmacia) gels. Running conditions were as recommended by the supplier. All sequences, including the orangutan (Fig. 1), have been submitted to the European Bioinformatics Institute database (accession numbers: AJ270061 to AJ270095).

32. Most samples were collected from chimpanzee and bonobo individuals for which clear records allowed them to be associated with a particular location in Africa. Central African chimpanzees ($n = 12$) were from the International Center for Medical Research, Gabon. Western chimpanzees were from Sierra Leone ($n = 12$), zoos, and primate research institutes ($n = 5$). The eastern chimpanzee was from Gombe, Tanzania. Bonobos, as well as the gorilla and orangutan samples, were from different zoos and primate research institutes. Male sex of all DNA samples was confirmed as described [J. F. Wilson and R. Erlandsson, *Biol. Chem.* **379**, 1287 (1998)].
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Requirement of ATM-Dependent Phosphorylation of Brca1 in the DNA Damage Response to Double-Strand Breaks

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The Brca1 (breast cancer gene 1) tumor suppressor protein is phosphorylated in response to DNA damage. Results from this study indicate that the checkpoint protein kinase ATM (mutated in ataxia telangiectasia) was required for phosphorylation of Brca1 in response to ionizing radiation. ATM resides in a complex with Brca1 and phosphorylated Brca1 in vivo and in vitro in a region that contains clusters of serine-glutamine residues. Phosphorylation of this domain appears to be functionally important because a mutated Brca1 protein lacking two phosphorylation sites failed to rescue the radiation hypersensitivity of a Brca1-deficient cell line. Thus, phosphorylation of Brca1 by the checkpoint kinase ATM may be critical for proper responses to DNA double-strand breaks and may provide a molecular explanation for the role of ATM in breast cancer.

Maintenance of genomic integrity is crucial for the development and health of organisms. Cell cycle checkpoints and DNA repair mechanisms help ensure the distribution of an intact genome to all cells and progeny. The inactivation of many of the genes involved in these activities has been linked to syndromes that cause a predisposition to cancer in humans. The *ATM*, *Brca1*, and *Brca2* genes are three such tumor suppressors involved in preventing genetic damage (*1*). Mutations in *ATM* cause ataxia telengiectasia (A-T), a disorder characterized by atrophy of the cerebellum and thymus, immunodeficiency, premature aging, predisposition to cancer, and sensitivity to ionizing radiation (*2*). Furthermore, heterozygous carriers of a dysfunctional *ATM* gene have been reported to be predisposed to breast cancer (*3*). Mutations in *Brca1* and *Brca2* are linked to inherited, early-onset breast cancer (*4*). Mutations in *Brca1*, *Brca2*, or *ATM* cause defects in cellular proliferation, genomic instability, and sensitivity to DNA damage (*5–7*).

ATM is a member of a protein family related to phosphoinositide kinases that includes ATR, Mec1, Tel1, and Rad53. These proteins are essential for signaling the presence of DNA damage and activating cell cycle checkpoints (*8*). ATM is activated in response to DNA damage and is required for efficient DNA double-strand break repair and optimal phosphorylation and activation of the p53, c-Abl, and Chk2 proteins that promote apoptosis or cell cycle arrest (*9–14*).

The Brca1 and Brca2 proteins form a complex with Rad51, a RecA homolog required for homologous recombinational repair of DNA double-strand breaks (*6*, *15–17*). These three proteins localize to discrete nuclear foci during S phase of the cell cycle, share developmental expression patterns, and are maximally expressed at the G_1-S transition (*16–19*). Brca1 mutations in mice result in genetic instability, defective G_2/M checkpoint control, and reduced homologous recombination (*7*). Exposure of cells to ionizing radiation or hydroxyurea causes dispersal of Brca1 foci and relocalization to sites of DNA synthesis where DNA repair may occur (*18*). Brca1 is phosphorylated during S phase and is also phosphorylated in response to DNA damage (*18*, *20*).

In the course of identifying Brca1-associated proteins by mass spectrometry, we identified ATM and confirmed this association by reciprocal coimmunoprecipitation (Fig. 1A). Given this physical association, we tested whether ATM was required for phosphorylation of Brca1 in response to DNA damage. Brca1 from γ-irradiated wild-type cells migrated more slowly than the Brca1 from untreated cells on SDS–polyacrylamide gel electrophoresis (PAGE) gels, indicating phosphorylation (*18*, *20*) (Fig. 1B). Brca1 in ATM-deficient fibroblast and lymphoblast cells derived from A-T patients was not hyperphosphorylated after exposure to γ irradiation (Fig. 1B).

We confirmed that the lack of phosphorylation of Brca1 in A-T cells is dependent on the ATM deficiency by examining A-T cells that had been complemented with an ATM cDNA. In the parental A-T cells or cells containing an empty vector, there was only a slight shift of Brca1 protein after γ irradiation (Fig. 1C), but addition of the ATM expression vector increased the shift. Therefore, functional ATM is required for maximal γ irradiation–induced phosphorylation of Brca1. In contrast, Brca2 was not required for γ irradiation–induced phosphorylation of Brca1 (Fig. 1C).

To determine whether ATM could phosphorylate Brca1, we produced several overlapping fragments of Brca1 fused to glutathione *S*-transferase (GST) in *Escherichia coli* and used these as substrates in an ATM–protein kinase assay. Fusion proteins containing Brca1 amino acids 1021 to 1552 and 1501 to 1861 were phosphorylated by wild-type ATM but not by a catalytically inactive mutant of ATM (Fig. 2). Most phosphorylation occurred between amino acids 1351 and 1552. Brca1 segments containing amino acids 1021 to 1211 and 1211 to 1351 were also phosphorylated to a lesser degree, suggesting that more than one residue may be targeted.

Analysis of the Brca1 sequence within this region revealed a cluster of serines (S)

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and threonines (T) followed by glutamine (Q) (SQ or TQ). SQ or TQ sequences are the preferred sites of ATM phosphorylation on p53 and c-Abl (*9*, *10*). The 244 amino acids between 1280 and 1524 contain 10 SQ/TQ sites, whereas the remaining 1619 amino acids of Brca1 contain only eight SQ/TQ sites.

We mapped the in vitro ATM phosphorylation sites on GST-Brca1 1021-1552 by mass spectrometry. Two phosphopeptides were identified by analyzing proteins before and after treatment with calf-intestinal phosphatase (CIP) (Fig. 3A). Peptide sequencing revealed that Ser^{1423} and Ser^{1524} were phosphorylated (Fig. 3, B and C). Five additional serines can be phosphorylated within amino acids 1021 to 1552 when the kinase reaction is allowed to proceed longer with higher concentrations of adenosine triphosphate and ATM (Table 1).

To determine if the ATM-dependent phosphorylation of Ser^{1423} or Ser^{1524} also occurred in vivo, we expressed Brca1 amino acids 1351 to 1552 fused to the flag epitope and the SV40 large T antigen nuclear localization signal (NLS). This flag- and NLS-tagged Brca1 segment localized to the nucleus of transfected cells. Protein immunoblotting showed that this segment migrated as several distinct bands on SDS-PAGE (Fig. 3D). This result may reflect phosphorylation on multiple sites given that the CDK2 phosphorylation site on Brca1 also maps to this domain (*21*). The mobility of a proportion of this protein was reduced after exposure of these cells to γ irradiation (Fig. 3D). Coexpression of wild-type ATM but not catalytically inactive ATM increased the number and intensity of slower migrating bands observed after γ irradiation. Phosphatase treatment increased the mobility of the shifted proteins, demonstrating that the altered migration does reflect phosphorylation (Fig. 3E). The 1351 to 1552 Brca1 fragment was also phosphorylated after γ irradiation in wild-type but not ATM-deficient fibroblasts, further supporting the hypothesis that this Brca1 fragment contains ATM-dependent, in vivo phosphorylation sites (Fig. 3F).

We introduced $Ser^{1423} \rightarrow$ Ala (S1423A) and $Ser^{1524} \rightarrow$ Ala (S1524A) mutations in Brca1 1351 to 1552. Phosphorylation of the mutant flag- and NLS-tagged protein was reduced compared with that of the wild-type protein (Fig. 3D). Phosphorylation in the absence of DNA damage was also reduced by expression of catalytically inactive ATM or by the Ser^{1423} and Ser^{1524} mutations. The mutated Brca1 segment was shifted slightly in response to γ irradiation, but this effect was not increased by wild-type ATM expression.

To determine the functional importance of ATM-dependent phosphorylation of Brca1, we attempted to complement the radiation hypersensitivity of Brca1-deficient cells with wild-type or mutant Brca1 proteins. We produced recombinant retrovirus encoding hemagglutinin (HA)–tagged, full-length, wild-type Brca1 and a S1423A, S1524A mutant (SQ2). We infected the Brca1 mutant HCC1937 cell line and selected for stable, polyclonal cell populations. Analysis of the expression of exogenous Brca1 in these cells by protein immunoblotting with antibodies to HA revealed equal expression of the mutant and wild-type proteins (Fig. 4A). HCC1937 cells have a truncated Brca1 protein expressed at low levels compared with wild-type cells (Fig. 1C). Immunoblotting with antibodies specific to Brca1 revealed that the expression of the exogenous HA-tagged

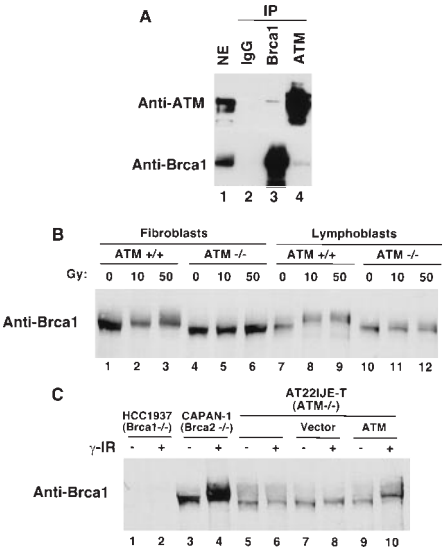

Fig. 1. Dependence of DNA damage–induced phosphorylation of Brca1 on ATM and their physical association. (**A**) HeLa cell nuclear extracts (NE) were incubated with antibody to Brca1 (affinity-purified polyclonal antibody raised against Gst-Brca1 1501-1861) or ATM (Ab-3, Oncogene Science; H-248, Santa Cruz). Immunoprecipitates were fractionated by SDS-PAGE and immunoblotted with antibody to Brca1 (anti-Brca1) (Ab-1, Oncogene Science) or ATM (anti-ATM) (Novus). IgG, immunoglobulin G. (**B**) Wild-type (GM00637G) or A-T fibroblasts (GM05849B) and wild-type (GM03323) or A-T lymphoblasts (GM3189C) were treated with 10 or 50 Gy of γ irradiation. Cell lysates were prepared 1 hour after irradiation, fractionated on SDS-PAGE, and immunoblotted with antibody to Brca1. (**C**) HCC1937 (homozygous Brca1 mutant cells (*31*), CAPAN-1 (homozygous Brca2 mutant cells) (*32*), AT22IJE-T (homozygous ATM mutant cells; lanes 5 and 6), AT22IJE-T cells containing the vector alone (lanes 7 and 8), or AT22IJE-T cells containing a vector encoding ATM (lanes 9 and 10) (*33*) were left untreated or treated with 25 Gy of γ irradiation (γ-IR) and harvested after 1 hour. Protein from these cells was fractionated by SDS-PAGE and immunoblotted with antibodies to Brca1.

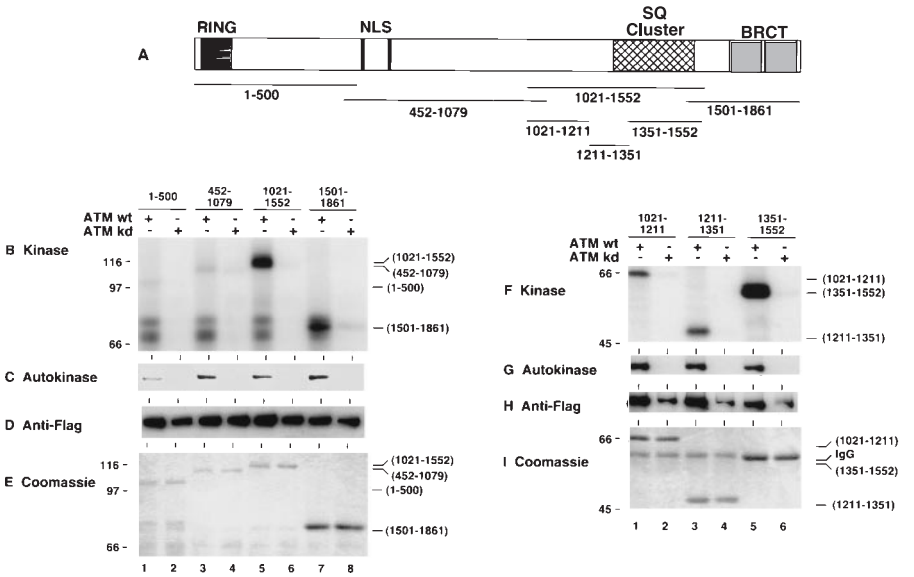

Fig. 2. In vitro phosphorylation of Brca1 by ATM. (**A**) Schematic diagram of Brca1 showing the location of the RING finger, NLS, BRCT repeats, and SQ cluster domain. The size and location of GST-fusion proteins used in the kinase reactions are shown below Brca1. GST-fusion proteins containing Brca1 amino acids 1 to 500 (B to E, lanes 1 and 2), 452 to 1079 (B to E, lanes 3 and 4), 1021 to 1552 (B to E, lanes 5 and 6), 1501 to 1861 (B to E, lanes 7 and 8), 1021 to 1211 (F to I, lanes 1 and 2), 1211 to 1351 (F to I, lanes 3 and 4), or 1351 to 1552 (F to I, lanes 5 and 6) were used as substrates in an in vitro kinase assay with immunoprecipitated flag-tagged, wild-type, or catalytically inactive ATM prepared from transfected 293T cells (*9*, *34*). (**C** and **G**) Autoradiograms showing the ability of wild-type but not mutant ATM to phosphorylate itself. (**B** and **F**) Autoradiograms showing the ability of wild-type but not mutant ATM to phosphorylate Brca1 fragments. The two phosphorylated proteins observed in all of the odd numbered lanes are unidentified proteins that coimmunoprecipitate with ATM from 293T cells. (**D** and **H**) Anti-flag (M5, Sigma) immunoblot showing the amounts of flag-tagged, wild-type and catalytically inactive ATM added to the reaction. (**E** and **I**) Coomassie staining showing the levels of the Brca1 substrates added to the reactions. wt, wild type; kd, kinase-inactive.

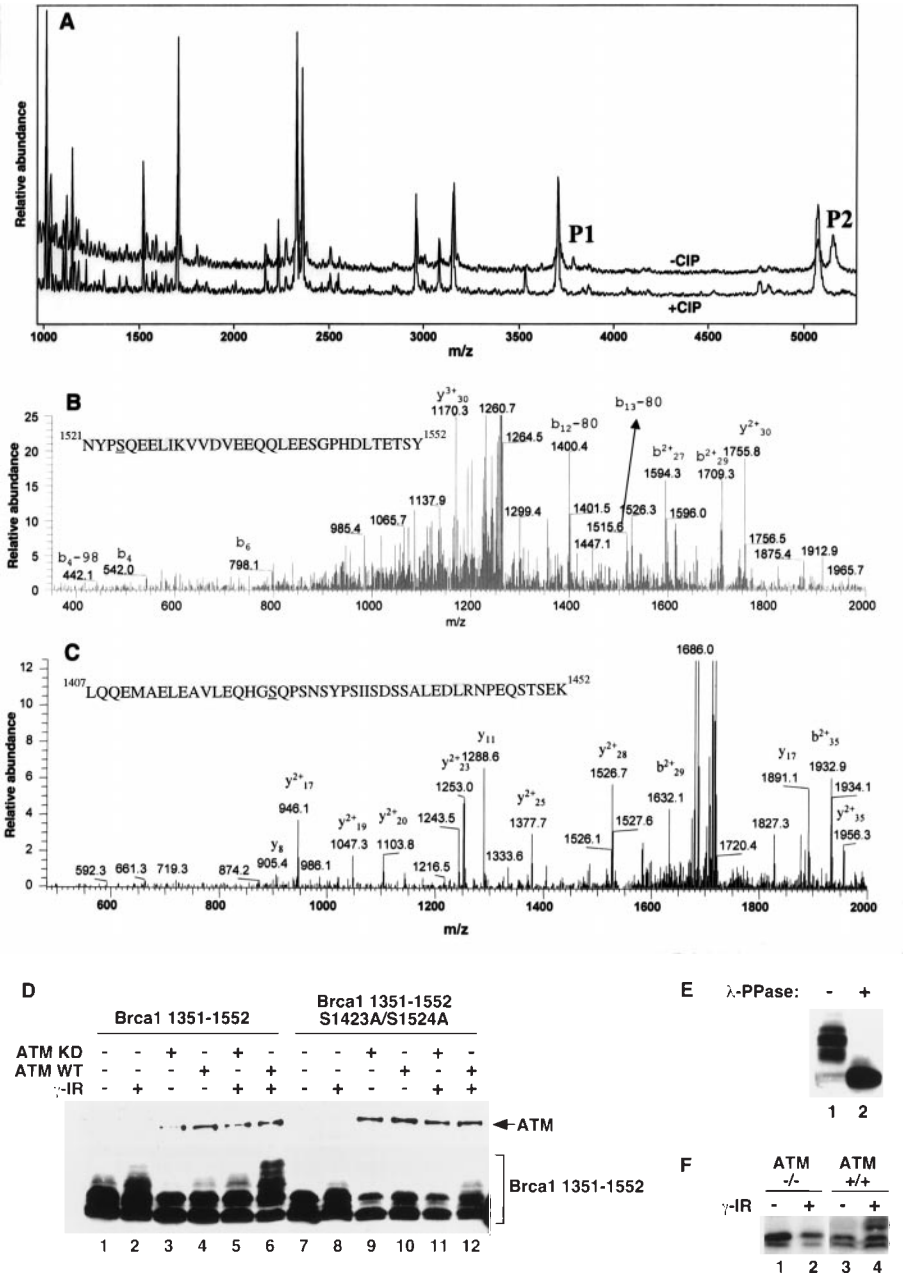

Fig. 3. Phosphorylation of Brca1 on Ser^{1423} and Ser^{1524} by ATM. After an in vitro kinase reaction with wild-type ATM and SDS-PAGE, a Coomassie blue–stained band of GST-Brca1 1021 to 1552 was digested in the gel with trypsin. The resulting peptides were extracted and measured with mass spectrometry as described (*35*, *36*). (**A**) A portion of the extracted peptide was measured with matrix-assisted laser desorption/ionization (MALDI)/time of flight mass spectrometry before (top trace) and after (bottom trace) treatment with CIP. Two phosphopeptides (P1 and P2) were identified. (**B** and **C**) Phosphopeptides P1 and P2 were fragmented with capillary liquid chromatography (LC)/electrospray ionization (ESI)/mass spectrometry (MS)/MS in an ion trap mass spectrometer. Ser^{1423} and Ser^{1524} (underlined) were unambiguously identified as the sites of phosphorylation (*37*). Labeling of fragment ions of y and b is according to Biemann (*36*). *m/z* is the mass-to-charge ratio. (**D**) A vector expressing a flag- and NLS-tagged Brca1 fragment containing amino acids 1351 to 1552 (wild-type) or containing the identical fragment with serines 1423 and 1524 mutated to alanine was transfected into 293T cells with or without flag-tagged, wild-type, or kinase-inactive ATM expression vectors as indicated (*38*). Cells were left untreated or exposed to 25 Gy of γ irradiation. One hour later, the cells were harvested, proteins from lysates were separated by SDS-PAGE, and the blot was probed with antibodies to the flag epitope to detect ATM and Brca1. (**E**) Flag- and NLS-tagged Brca1 1351 to 1552 was cotransfected with wild-type ATM into 293T cells. The cells were irradiated with 50 Gy of γ irradiation and harvested after 1 hour. Lysates were treated with or without lambda phosphatase (λ-PPase), separated by SDS-PAGE, and probed with antibodies to flag. (**F**) Flag- and NLS-tagged Brca1 1351 to 1552 was transfected into A-T fibroblasts (GM05849B, ATM$^{-/-}$) or wild-type fibroblasts (GM00637G, ATM$^{+/+}$) with Effectene (Qiagen). Cells were left untreated or irradiated with 50 Gy of γ irradiation and harvested after 1 hour. Lysates were separated by SDS-PAGE and probed with antibodies to flag.

Brca1 proteins was similar to that of the mutant endogenous protein (*22*). Expression of large amounts of Brca1 from heterologous promoters is not expected because it causes growth inhibition (*23*, *24*). Both the SQ2 mutant and wild-type Brca1 localized to discrete nuclear foci (Fig. 4, B and C).

We examined the sensitivity of these cells to γ irradiation by counting viable cells 3 days after damage. Little cell death occurred during this time period in any of the cell populations. Thus, differences in cell number are mainly caused by differences in the ability of the cells to recover and proliferate after the damage. Therefore, damage-induced inhibition of cell proliferation served as a measure of the sensitivity of these cells to irradiation. Expression of wild-type Brca1, but not the SQ2 Brca1 mutant, significantly decreased the growth inhibition caused by 1 gray (Gy) of irradiation compared with that in cells expressing an empty vector (analysis of variance, $P = 0.0006$) (Fig. 4D). Brca1 afforded less protection after 2 Gy of irradiation, but its effect was still significantly different from that of the SQ2 mutant. Doses greater than 2 Gy resulted in essentially no recovery or proliferation of any of the cell populations within the 3 days of this experiment (*22*).

We also analyzed radiation sensitivity by colony-forming ability after exposure of cells to various doses of γ irradiation. Expression of wild-type Brca1, but not the SQ2 mutant, produced a significant decrease in the sensitivity of HCC1937 cells to γ irradiation (Fig. 4E). Thus, phosphorylation of Brca1 on serines 1423 and 1524 is important for Brca1 function after exposure to γ irradiation.

The mobility of full-length Brca1 containing the S1423A and S1524A mutations on SDS-PAGE is still retarded after treatment with γ irradiation (*22*), indicating that other residues besides Ser^{1423} and Ser^{1524} may also be targets for damage-induced phosphorylation. To examine this, we mapped damage-induced phosphorylation sites on Brca1 in vivo using mass spectrometry. After irradiation of 293T cells that had been cotransfected with expression vectors for full-length Brca1 and ATM, Brca1 was phosphorylated on five serines (Table 1). Four of these sites are in the SQ domain, and three match sites phosphorylated by ATM in vitro.

Our data indicate that ATM may be one of the kinases responsible for DNA damage–induced phosphorylation of Brca1 after exposure of cells to γ irradiation. ATM appears to be especially critical in responding to DNA double-strand breaks that are produced by ionizing radiation. Phosphorylation of Brca1 in response to ultraviolet light, methylmethane sulfonate, and hydroxyurea appears to be independent of ATM (*18*, *22*). Optimal phosphorylation of other ATM substrates such as p53 and Chk2 also appears to be specific to DNA double-strand breaks because other types

of damage elicit ATM-independent phosphorylation of these substrates (*9*, *12*, *25*).

As yet, it is unclear how ATM-dependent phosphorylation regulates Brca1 function. We did not observe any differences in Brca1 localization in wild-type or A-T cells after exposure to γ irradiation (*26*). Thus, ATM may not be essential for regulating the intracellular localization of Brca1. Phosphorylation may regulate the binding of other proteins to Brca1. The Brca1 binding domain for Brca2 has been mapped to a region containing the SQ cluster; however, damage-dependent phosphorylation has not been shown to alter the amount of Brca2 complexed with Brca1 (*17*). The activity of Brca1 as a transcriptional regulator may be changed by phosphorylation because Brca1 has a transcriptional activation domain that maps to just downstream of the SQ cluster domain (*27*). Alternatively, the activity of Brca1 in modulating DNA repair may be altered by phosphorylation. Because DNA repair is defective in both ATM- and Brca1-deficient cells, it is tempting to speculate that the defect in repair and genetic instability found in ATM-deficient cells is at least partially caused by an inability to properly regulate Brca1.

The biochemical link between ATM and Brca1 may partially explain why heterozygous carriers of a dysfunctional ATM gene are at increased risk of breast cancer (*3*). It has been estimated that 6.6% of all breast cancer cases occur in the 1.4% of women who are A-T heterozygotes. Combined with the about 1 to 3% of breast cancers attributable to inherited Brca1 mutations, mutation of the ATM-Brca1 checkpoint–DNA repair pathway may account for nearly 10% of all breast cancer cases. Mice heterozygous for ATM mutations are more sensitive to sublethal doses of ionizing radiation than wild-type mice (*28*). Given the specific defect of ATM mutants in regulation of Brca1 in response to ionizing radiation, these results may have relevance to the issue of the relative benefits of broad x-ray–based mammog-

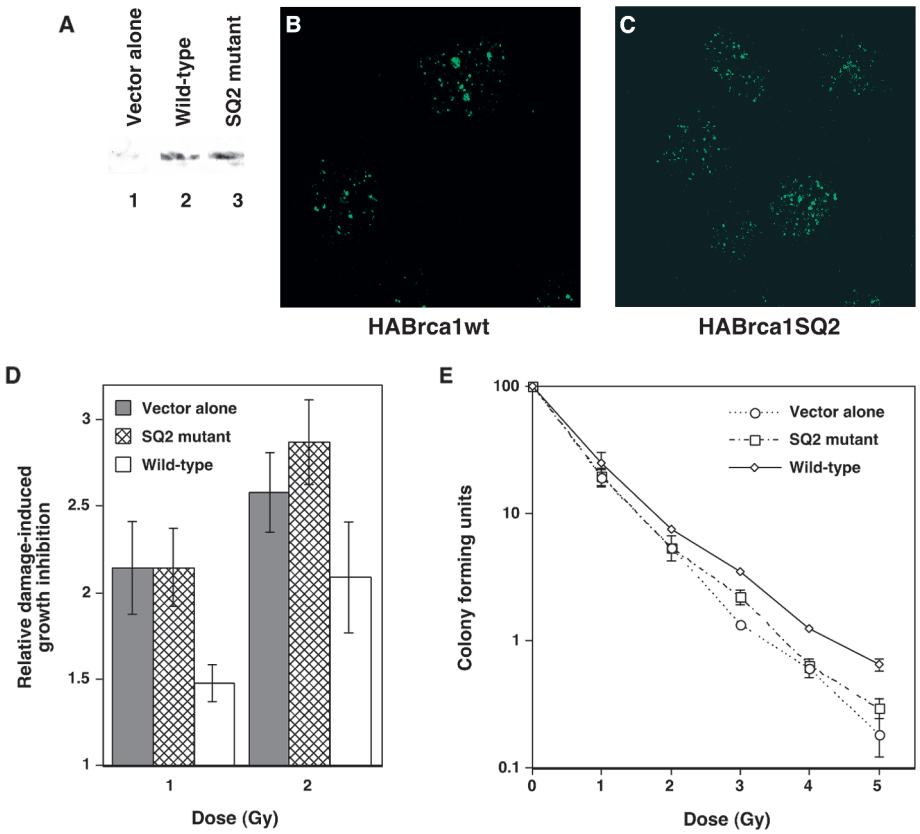

Fig. 4. Decreased sensitivity of Brca1-deficient cells to ionizing radiation after expression of wild-type Brca1 but not the SQ2 mutant. Recombinant retrovirus expressing full-length, HA-tagged wild-type Brca1 or S1423A, S1524A Brca1 (SQ2 mutant) was used to create stable, polyclonal populations of HCC1937 cells (*39*). Expression of the full-length proteins was detected by protein immunoblotting (**A**) or immunofluorescent staining with antibodies to HA (**B** and **C**). Magnification, ×1200. (**D**) Cell populations were treated with the indicated doses of γ irradiation, and viable cells were counted by trypan blue exclusion 3 days after irradiation (both floating and adherent cells were collected). Relative growth inhibition was calculated by dividing the number of cells recovered from the untreated population by the number of cells recovered from the treated population. By definition, the untreated samples have a growth inhibition of 1. The mean and standard deviation of five samples are shown. (**E**) Cells were stained with methylene blue 13 days after γ irradiation with the indicated doses, and the number of colonies (cell clusters with ≥10 cells) was scored. Numbers were normalized by setting the untreated sample at 100%. The mean of three samples is shown, and bars represent the standard deviation. Where no bars are present, the standard deviation is smaller than the symbol height. Both types of sensitivity experiments were repeated four times with two independently derived cell populations expressing each construct. Representative experiments are shown.

Table 1. Summary of in vitro and in vivo phosphorylated sites on Brca1 as detected by mass spectrometry.

Sites	Peptide*	Molecular weight† (measured/calculated)	Number of PO_3 groups
In vitro			
S1330	^{1326}HQSE**S**QGVGLSDKELVSDDEER^{1347}	2444/2444.5	1
S1423	^{1407}LQQEMAELEAVLEQHG**S**QPSNSYPSIISDSSALEDLRNPEQSTSEK^{1452}	5074/5074.4	1
S1466	^{1460}SSEYPI**S**QNPEGLSADKFEVSADSSTSK^{1487}	2961/2961.1	1
S1524	^{1521}NYP**S**QEELIKVVDVEEQQLEESGPHDLTETSY^{1552}	3706/3706.9	1
S1542	^{1531}VVDVEEQQLEE**S**GPHDLTETSY^{1552}	2504/2504.6	1
‡	^{1279}ASQEHHLSEETKCSASLFSSQCSELEDLTANTNTQDPFLIGSSK^{1322}	4772/4772.1	1,2§
In vivo			
S1189	^{1189}**S**PSPFTHTHLAQGYR^{1203}	1698/1698.9	1
S1457‡	^{1453}AVLT**S**QKSSEYPISQNPEGLSADKFEVSADSSTSK^{1487}	3689/3689.0	1,2§
S1542	^{1531}VVDVEEQQLEE**S**GPHDLTETSYLPR^{1555}	2871/2871.1	1
S1524, S1542	^{1521}NYP**S**QEELIKVVDVEEQQLEE**S**GPHDLTETSYLPR^{1555}	4073/4073.4	1,2§

*Amino acids in boldfaced type were unambiguously determined to be sites of phosphorylation by LC/MS/MS. †Average molecular weights of the dephosphorylated peptide are listed. ‡At least one phosphorylated amino acid within these peptides could not be determined unambiguously by LC/MS/MS. §Peptides with both 1 and 2 mol of phosphate were observed.

raphy screening for the early detection of breast cancer, a question to be resolved by epidemiological studies.

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34. GST-Brca1 expression vectors were constructed in pGEX2Tkcs or pGEX5X3 by polymerase chain reaction (PCR) with the following primers: pDC78 (Brca1 1 to 500), GAAAGCATATGATGGATTTATCTGCTCTTCGC and GAAAGCTCGAGTTAATTTGTGAGGGGACGCTC; pDC79 (Brca1 452 to 1079), GAAAGCATATGGTAGAGAGTAATATTGAAGAC and GAAAGCTCGAGCTATTTTGGCCCTCTGTTTCT; pDC80 (Brca1 1021 to 1552), GAAAGCATATGAGTACAGTGAGCACAATTAGC and GAAAGCTCGAGCTAGTAAGATGTTTCCGTCAA; pDC81 (Brca1 1501 to 1861), GAAAGCATATGTGCCCATCATTAGATGATAGG and GAAAGCTCGAGTCAGTAGTGGCTGTGGGGGAT; pDC113 (Brca1 1021 to 1211), GAAAGGATCCCAAGTACAGTGAGCACAATTAGCCG and GAAAGGTCGACGGACTCTAATTTCTTGGCCCCTC; pDC114 (Brca1 1211 to 1351), GAAAGGACCAGTCCTCAGAAGAGAACTTATCTAG and GAAAGGTCGACCAAGCCCGTTCCTCTTTCTTCATC; and pDC115 (Brca1 1351 to 1552), GAAAGGATCCGCTTGGAAGAAAATAATCAAGAAGA and GAAAGGTCGACGTAAGATGTTTCCGTCAAATCGTG. In vitro kinase assays were performed essentially as described (*9*). The ATM expression constructs were a kind gift of M. Kastan.
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37. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
38. Flag-tagged NLS-Brca1 1351 to 1552 was constructed with the univector plasmid fusion system (*29*). The host vector is based on pCMV2-Flag (Sigma). The SV40 large T antigen NLS was inserted between the Hind III and Not I sites with the following primers: AGCTTCCCAAGAAGAAGAGGAAGGC and GGCCGCCTTCCTCTTCTTCTTGGGA. pUNI15 Brca1 1351 to 1552 was made with the same primers as those for pDC115 described above. The serine to alanine mutations were made by single-stranded mutagenesis with the following primers: S1423A, ACAGCATGGGGCCCAGCCTTCTAACAG; and S1524A, AAACTACCCAGCTCAAGAGGAACTCATTAAGGTTGTT. All mutations and PCR products were sequenced. Transfections were performed by standard calcium phosphate technique (*30*).
39. pBABEpuro HABrca1 was made by digesting pCDNA3βHABrca1 (*16*) with Sal I and Xho I and inserting it into the Sal I site of pBABEpuro. The serine to alanine mutations were inserted into wild-type Brca1 in pBABEpuro by exchanging a Pfl MI to Apa I Brca1 fragment from the mutated gene into pBABEpuro HABrca wild type. This deletes a portion of Brca1 because an Apa I site had been created by the introduction of the S1423A mutation. This deleted Brca1 segment was reintroduced as an Apa I to Apa I fragment derived from the full-length S1423A/S1524A mutant in pBSKII(−) reconstituting full-length HABrca1 S1423A/S1524A. Retrovirus was made by cotransfection of the pBABEpuro vectors with amphotrophic packaging DNA into 293T cells essentially as described (*30*). Viral supernatants were used to infect the HCC1937 cells. Two days after infection, cells were selected for puromycin resistance with puromycin (1 μg/ml; Sigma).

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Control of the DNA Damage Checkpoint by Chk1 and Rad53 Protein Kinases Through Distinct Mechanisms

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In response to DNA damage, cells activate checkpoint pathways that prevent cell cycle progression. In fission yeast and mammals, mitotic arrest in response to DNA damage requires inhibitory Cdk phosphorylation regulated by Chk1. This study indicates that Chk1 is required for function of the DNA damage checkpoint in *Saccharomyces cerevisiae* but acts through a distinct mechanism maintaining the abundance of Pds1, an anaphase inhibitor. Unlike other checkpoint mutants, *chk1* mutants were only mildly sensitive to DNA damage, indicating that checkpoint functions besides cell cycle arrest influence damage sensitivity. Another kinase, Rad53, was required to both maintain active cyclin-dependent kinase 1, Cdk1(Cdc28), and prevent anaphase entry after checkpoint activation. Evidence suggests that Rad53 exerts its role in checkpoint control through regulation of the Polo kinase Cdc5. These results support a model in which Chk1 and Rad53 function in parallel through Pds1 and Cdc5, respectively, to prevent anaphase entry and mitotic exit after DNA damage. This model provides a possible explanation for the role of Cdc5 in DNA damage checkpoint adaptation.

Arrest of the cell cycle in response to the DNA damage checkpoint in *Saccharomyces cerevisiae* does not require inhibitory phosphorylation of Cdk1 (*1, 2*), and cells arrest in metaphase with active Clb/Cdk1 (*3*). Thus, given the role of Chk1 in Cdk phosphorylation in other systems, it seemed unlikely that *S. cerevisiae* Chk1 (YBR274w) would be required for checkpoint control. Disruption of *CHK1* revealed that it is not essential, and *chk1* mutants grown asynchronously were not sensitive to γ- or UV-radiation (*4*). How-