A subset of ATM- and ATR-dependent phosphorylation events requires the BRCA1 protein

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BRCA1 is a central component of the DNA damage response mechanism and defects in BRCA1 confer sensitivity to a broad range of DNA damaging agents. BRCA1 is required for homologous recombination and DNA damage-induced S and G₂/M phase arrest. We show here that BRCA1 is required for ATM- and ATR-dependent phosphorylation of p53, c-Jun, Nbs1 and Chk2 following exposure to ionizing or ultraviolet radiation, respectively, and is also required for ATM phosphorylation of CtIP. In contrast, DNA damage-induced phosphorylation of the histone variant H2AX is independent of BRCA1. We also show that the presence of BRCA1 is dispensable for DNA damage-induced phosphorylation of Rad9, Hus1 and Rad17, and for the relocalization of Rad9 and Hus1. We propose that BRCA1 facilitates the ability of ATM and ATR to phosphorylate downstream substrates that directly influence cell cycle checkpoint arrest and apoptosis, but that BRCA1 is dispensable for the phosphorylation of DNA-associated ATM and ATR substrates.

Keywords: ATM/ATR/BRCA1/phosphorylation

Introduction

DNA damage induced by ionizing (IR) and ultraviolet (UV) irradiation or caused by abnormal structures, such as stalled replication forks, triggers a complex cascade of phosphorylation events that ultimately serve to influence or effect DNA repair, cell cycle delay and apoptosis with the overall purpose of maintaining genome stability. Two members of the phosphatidylinositol 3-kinase-related kinase (PIKK) family, ATM and ATR, play a central role in damage recognition and the initial phosphorylation events (for reviews, see Tibbetts *et al.*, 2000; Zhou and Elledge, 2000; Khanna *et al.*, 2001; Rouse and Jackson, 2002). These kinases are activated by different forms of DNA damage: ATM responds to DNA double-strand breaks (DSBs), whereas ATR functions following

exposure to other forms of DNA damage such as bulky lesions or stalled replication forks (O'Connell *et al.*, 2000; Khanna *et al.*, 2001; Shiloh, 2001).

Mutation of the *ATM* gene causes ataxia–telangiectasia (A-T), an autosomal recessive disorder associated with clinical radiosensitivity and cancer predisposition (Savitsky *et al.*, 1995). Cell lines derived from A-T patients display severe radiosensitivity and defects in G₁/S, S and G₂/M phase checkpoint arrest following exposure to IR (Lavin and Khanna, 1999; Khanna *et al.*, 2001). ATM phosphorylates multiple substrates including RPA, BRCA1, Nbs1, CtIP, Chk1, Chk2, c-Jun, p53, Mdm2, H2AX, Rad9 and Rad17 (for reviews, see Lavin and Khanna, 1999; Khanna *et al.*, 2001; Shiloh, 2001). Importantly, ATM is not activated in response to UV irradiation and A-T cell lines are neither UV sensitive nor impaired in UV-induced DNA damage checkpoints (Khanna *et al.*, 2001).

In contrast to *ATM*, *ATR* is an essential gene required for cell proliferation. When deleted in mice, it causes early embryonic lethality (Brown and Baltimore, 2000; de Klein *et al.*, 2000; Cortez *et al.*, 2001). Since *ATR* null cell lines are not available, the study of *ATR* function has relied on either the overexpression of a dominant-negative construct or *cre-lox*-mediated gene loss. These studies demonstrate that ATR-dependent phosphorylation events occur after exposure to various forms of DNA damage and replication blocks, but not following exposure to IR (Tibbetts *et al.*, 2000; Zhou and Elledge, 2000; Cortez *et al.*, 2001). The yeast homologues of ATM are known as *Tell* in both *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*. *ATR* is the sequence and functional homologue of *S.pombe Rad3* and *S.cerevisiae Mec1* (Carr, 2000). In both yeasts, *Tell* plays only a minor role in signalling the presence of DSBs, while *Mec1*- or *Rad3*-dependent pathways respond to all forms of DNA damage. Thus, *S.cerevisiae Mec1* or *S.pombe Rad3* mutants show sensitivity to IR, UV and hydroxyurea (HU) (Paciotti *et al.*, 2001). Notwithstanding the difference between yeasts and mammalian cells, studies with the lower organisms have been highly informative in investigations of signalling responses to DNA damage and have directed studies with mammalian cells. Using information from the yeasts as a model system, recent work has shown that *ATR* functions in cooperation with ATR-interacting protein (ATRIP) to bind to the sites of DNA damage (Cortez *et al.*, 2001). Furthermore, Rad17 (an RFC-like protein) binds chromatin independently at damage sites and forms a complex with the PCNA-like proteins Rad1, Rad9 and Hus1 (Rauen *et al.*, 2000; Zou *et al.*, 2002). ATR-dependent phosphorylation of H2AX, and most probably ATRIP (based on analogy with yeast), is not dependent on Hus1, whereas phosphorylation of the downstream ATR substrate Chk1 is dependent on this protein (Cortez *et al.*, 2001; Ward and Chen, 2001; Zou

Fig. 1. Adenovirus-infected HCC1937 cells express BRCA1 and are complemented for their radiosensitive phenotype. **(A)** Expression of BRCA1 protein in adenovirus-infected HCC1937 cells. Nuclear extracts from the indicated cell lines were subjected to immunoblotting using anti-BRCA1 antibody. The same extracts were also examined using anti-ATM antibody as a loading control and to verify ATM expression in BRCA1-defective cells. 1BRneo and AT5BIVA are transformed fibroblasts derived from a normal and an A-T patient, respectively. 293T is a tumour cell line. HCCAdco and HCCAdBI are HCC1937 cells infected with empty adenovirus vector or adenovirus expressing full-length BRCA1 protein, respectively. **(B)** Clonogenic survival after exposure of the indicated cell lines to 2 Gy irradiation. The results shown were obtained from a single survival analysis carried out on the corrected cell lines. **(C)** Radiosensitivity monitored by the formation of micronuclei in the indicated cell lines. Either unirradiated cells (empty boxes) or cells irradiated with 6 Gy γ -rays (filled boxes) were scored for micronuclei. The results represent the mean of three experiments and the error bars represent the standard deviation of the mean between these experiments. Each experiment was carried out after a separate infection with adenovirus.

et al., 2002). ATR-dependent substrates include Chk1, Chk2, p53 and BRCA1 (Tibbetts *et al.*, 1999, 2000; Xu *et al.*, 2002; Zhao and Piwnicka-Worms, 2002). Of these substrates, p53 is required to effect p21-dependent G₁/S checkpoint arrest. Chk1 and Chk2 kinases, which are regulated by their phosphorylation status, phosphorylate various targets including Cdc25A (which is degraded in response to DNA damage) and p53. Ultimately, the phosphorylation of cell cycle regulators prevents progression through G₁/S and G₂/M via the regulation of Cdk activities (Zeng *et al.*, 1998; Mailand *et al.*, 2000).

Heterozygous germ-line mutations in *BRCA1* are responsible for a subset of hereditary breast cancers, indicating that *BRCA1* encodes a tumour suppressor gene. Cell lines impaired in BRCA1 display hypersensitivity to a range of DNA damaging agents including IR and UV irradiation (Venkitaraman, 2002). They also display a failure to effect both S and G₂/M checkpoint arrest after DNA damage (Xu *et al.*, 2001). BRCA1 is phosphorylated by ATM and ATR following IR and UV irradiation, respectively (Tibbetts *et al.*, 2000; Gatei *et al.*, 2001). While evidence suggests that BRCA1 functions in both ATM- and ATR-dependent signalling pathways, its precise role remains unclear. BRCA1 has an N-terminal ring finger motif and a C-terminal tandem BRCT domain which is thought to mediate protein–protein interactions (Brzovic *et al.*, 2001; Williams *et al.*, 2001).

BRCA1 has been purified as part of a large protein complex known as BASC (BRCA1-associated genome surveillance complex), which contains a wide range of DNA repair and replication proteins (Wang *et al.*, 2000). Further interactions of BRCA1 with ATM, ATR, Rad51, Rad50–Mre11–Nbs1, BLM, p53, Chk1, Chk2 and FANCD2 have been identified by co-immunoprecipitation analysis, supporting the notion that BRCA1 functions in DNA repair and/or cell cycle checkpoint arrest (Scully *et al.*, 1997; Zhong *et al.*, 1999; Lee *et al.*, 2000; Tibbetts *et al.*, 2000; Wang *et al.*, 2000; Garcia-Higuera *et al.*, 2001; Yarden *et al.*, 2002). Additional interactions with hSNF1/SW1, BACH1, CtIP and COBRA1 suggest a possible additional role in the maintenance of chromatin topology (Deng and Brodie, 2000; Cantor *et al.*, 2001; Ye *et al.*, 2001).

Crb2, a *S.pombe* protein that shares sequence similarity with BRCA1, is phosphorylated in a Rad3-dependent manner after exposure to IR and is required for Rad3-dependent phosphorylation of Chk1 (Esashi and Yanagida, 1999). Furthermore, *S.cerevisiae* ScRad9p, the homologue of Crb2, is required for the phosphorylation of SpChk2 (Brondello *et al.*, 1999). Based on these yeast studies, and considering the fact that many of the proteins that bind to BRCA1 are ATM or ATR substrates, we investigated whether BRCA1 is required for the phosphorylation of ATM and ATR substrates after IR and UV treatment, respectively. In an intriguing similarity with the yeast models, our data indicate that BRCA1 is required for downstream ATM- and ATR-dependent phosphorylation events including the phosphorylation of p53, c-Jun, Nbs1, CtIP and Chk2. In contrast, BRCA1 is not required for activation of ATM kinase activity or for phosphorylation of the DNA-associated substrates H2AX, Rad9, Hus1 or Rad17. Our results suggest a model in which BRCA1 functions as a scaffold for the two PIKKs, ATM and ATR, to pass on their phosphorylation to downstream components required for apoptosis and checkpoint activation.

Results

BRCA1 is required for multiple IR-induced phosphorylation events

To determine whether BRCA1 is required for ATM-dependent phosphorylation events, we examined a BRCA1-mutated cell line, HCC1937, and derivatives

Fig. 2. Phosphorylation of p53, c-Jun, Nbs1, CtIP and Chk2 is impaired in HCC1937 cells following exposure to IR. (A) Phosphorylation of the indicated substrates was examined in the absence of irradiation and at 0.5, 1 and 4 h after exposure to 20 Gy γ -rays. Phosphospecific antibodies against p53, c-Jun, Nbs1 and Chk2 were employed. Below these samples are the non-phosphospecific antibodies used as expression controls. CtIP was examined by immunoblotting. Phosphorylation was also examined in HCC1937 cells expressing BRCA1 following adenovirus infection (HCCAdB1) and HCC1937 cells infected with empty adenovirus (HCCAdco). Phosphorylation was compared with 293T cells which express similar levels of BRCA1 to HCCAdB1 cells. The majority of immunoblots have been analysed from three independently prepared cellular extracts. (B) Phosphorylation of p53 at 8 h post-irradiation. At this later time point, marked phosphorylation was observed in AT5BIVA but not in HCC1937 cells. Similar results were also obtained 12 h post-irradiation (data not shown).

obtained following infection with adenovirus alone (HCCAdco) or with adenovirus containing wild-type BRCA1 (HCCAdB1) for their ability to phosphorylate p53, c-Jun, Nbs1, CtIP and Chk2 following exposure to IR. The HCC1937 and HCCAdco cells express very low level of a mutant BRCA1 protein. The HCCAdB1 line expresses similar levels of BRCA1 to the tumour cell line 293T, which is ~5-fold higher than the levels seen in the transformed 1BRneo line (HeLa cells also show similar levels of BRCA1 to 293T cells) (Figure 1A). The HCCAdB1 cells are substantially corrected for the radio-sensitive phenotype of HCC1937 cells observed using a clonogenic survival assay (Figure 1B) and the analysis of radiation-induced micronuclei, consistent with previous findings (Foray *et al.*, 1999) (Figure 1C). Untreated HCC1937 cells also show enhanced levels of micronuclei relative to either 1BR3neo or 293T cells, consistent with their reported genomic instability, and this also appears to be reduced in HCCAdB1 cells (Figure 1C). To ascertain the requirement for BRCA1 in ATM-dependent phosphorylation events, we used phosphospecific antibodies to examine the phosphorylation status of p53 (anti-p53^{Ser15}), c-Jun (anti-c-jun^{Ser63}), Nbs1 (anti-Nbs1^{Ser343}) and Chk2 (anti-Chk2^{Thr68}), and standard immunoblotting to examine CtIP since the phosphorylated protein has an altered

mobility on SDS–PAGE. Following exposure to IR (20 Gy), the level of phosphorylated product was increased in control cells (1BRneo and 293T) for all the proteins examined. Phosphoprotein was normally evident 30 min post-irradiation and increased over a 4 h post-irradiation period (Figure 2A). However, phosphorylation of each protein was either decreased or absent in the A-T cell line AT5BIVA and in HCC1937 (*BRCA1*-mutated) cells. To demonstrate that these were *BRCA1*-dependent phosphorylation events, we examined the phosphorylation status of each protein in HCCAdco cells and in HCC1937 cells expressing ectopic *BRCA1* (HCCAdB1), and compared this with the level of phosphoprotein in 293T cells which express similar *BRCA1* protein levels. The phosphorylation of each substrate occurs to similar levels and with similar kinetics in 1BRneo, 293T and HCCAdB1 cells, demonstrating that the levels of *BRCA1* do not appreciably affect this aspect of the response to DNA damage (Figure 2A). It has previously been reported that p53^{Ser15} phosphorylation is observed at later times after irradiation in A-T-defective cell lines and that this represents an ATR-dependent event (Siliciano *et al.*, 1997). Consistent with these observations, phosphorylation of p53^{Ser15} was observed in AT5BIVA cells 8 h post-irradiation (Figure 2B). In striking contrast, no

Fig. 3. Phosphorylation of p53, c-Jun, Nbs1 and Chk2 is impaired in HCC1937 cells following exposure to UV. Phosphorylation of the indicated substrates was examined in the absence of irradiation and at 0.5, 1 and 4 h after exposure to UV (20 J/m²). Phosphorylation was also examined in HCC1937AdB1 and HCCAdco cells, and compared with that observed in 293T cells. Details of the antibodies used are given in Figure 2. CtIP is not phosphorylated after exposure to UV irradiation.

phosphorylation was observed in HCC1937 cells even up to 12 h post-irradiation (Figure 2B; data not shown), indicating that BRCA1 might also be required for the delayed ATR-dependent p53 phosphorylation after IR.

To confirm these observations, we also employed immunofluorescence to examine the IR- and UV-induced phosphorylation of three proteins for which phosphospecific antibodies were utilized (p53, c-Jun, Nbs1). We did not observe significant phosphorylation of c-Jun, p53 and Nbs1 by immunofluorescence in HCC1937 cells (data not shown). We conclude that both ATM and BRCA1 are required for the IR-induced phosphorylation of these DNA damage response substrates.

BRCA1 is also required for multiple UV-induced phosphorylation events

Most of the substrates phosphorylated by ATM in response to IR are phosphorylated by ATR following exposure to UV. Therefore we investigated whether these ATR-dependent phosphorylation events are also BRCA1 dependent. 1BRneo control cells showed phosphorylation of all these substrates, except CtIP, following exposure to UV (Figure 3). Previous studies have also reported that CtIP is not phosphorylated following exposure to UV (Li *et al.*, 2000). As expected, these ATR-dependent phosphorylation events were evident in AT5BIVA cells. With the exception of p53 and Chk2, none of the substrates was phosphorylated following exposure of HCC1937 or HCC1937Adco cells to UV, whereas phosphorylation was observed in control and HCC1937AdB1 cells (Figure 3A). Although phosphorylated p53 was substantially delayed in HCC1937 cells, marked phosphorylation was evident at 4 h post-irradiation. Similarly, phosphorylation of Chk2 was observed after longer times (4 h) in some experiments in HCC1937 cells (see Discussion). Immunofluorescence using the phosphospecific antibodies against p53^{ser15}, c-Jun^{ser63} and Nbs1^{ser343} was also performed at 4 h post-irradiation, and using all three

antibodies phosphoprotein was detected in 1BRneo and AT5BIVA but not in HCC1937 cells (data not shown).

Taken together, our results show that whilst phosphorylation of a range of damage response substrates is ATM or ATR dependent after exposure to IR or UV, respectively, all the phosphorylation events examined require BRCA1.

Activation of ATM kinase is not dependent upon BRCA1

A possible explanation for these findings is that BRCA1 is required for the activation of ATM kinase. We examined ATM activation *in vivo* using immunoprecipitation and kinase assays with 1BRneo, AT5BIVA, HCCAdco1 and HCCAdB1 cells (Figure 4A). Following immunoprecipitation with anti-ATM antibodies, a GST-p53₁₋₄₀ fusion peptide was used as a phosphorylation substrate. Phosphorylation was monitored using the anti-p53^{ser15} antibody, a technique that gives a low background in unirradiated cells (Girard *et al.*, 2002). Activation of ATM kinase activity is seen clearly in 1BRneo and HCC1937 cells whether or not they express BRCA1 protein. In contrast, no phosphorylation is seen using AT5BIVA cells (Figure 4A). We conclude that IR-induced activation of ATM does not require BRCA1.

IR- and UV-induced phosphorylation of H2AX is not dependent upon BRCA1

One of the earliest substrates phosphorylated after DNA damage is a variant form of the histone H2A, known as H2AX (Burma *et al.*, 2001; Ward and Chen, 2001). H2AX phosphorylation (formation of p-H2AX foci) by ATR occurs in a Hus1-independent manner, in contrast to the phosphorylation of other ATR-dependent substrates (Ward and Chen, 2001). Therefore we investigated whether BRCA1 is required for phosphorylation of H2AX. We examined H2AX phosphorylation using phosphospecific anti-p-H2AX^{ser139} antibody by immunofluorescence

following exposure to IR (20 Gy) or UV (20 J/m²) in 1BRneo, 293T and HCC1937 cells. Phosphorylated H2AX was clearly observed in HCC1937 cells at 1 h post-exposure (Figure 4B). Untreated HCC1937 cells appear to represent a heterogeneous population for H2AX foci, with a small percentage of cells showing numerous foci, potentially a consequence of the high genomic instability of these cells. This heterogeneity is evident in the lower-scale image (Figure 4B). Detailed foci after damage are also shown in Figure 4B. We also examined the kinetics of appearance of foci after DNA damage. Although

HCC1937 cells had a higher level of p-H2AX foci after damage compared with 1BRneo and 293T cells, the kinetics of their appearance was similar in all three cell lines (Figure 4C). Thus, in contrast to the above substrates, the phosphorylation of H2AX is BRCA1 independent.

BRCA1 is dispensable for damage-induced phosphorylation of Hus1, Rad9 and Rad17

Rad17 binds to chromatin in the absence of DNA damage and is required for the recruitment of the Rad1–Rad9–Hus1 complex to the sites of DNA damage and its

Fig. 4. HCC1937 cells activate ATM normally and phosphorylate H2AX, Rad17, Rad9 and Hus1 after exposure to IR and UV irradiation. (A) ATM was immunoprecipitated from the indicated cell lines without exposure to IR and 4 h after exposure to 20 Gy. α -ATM indicates the level of immunoprecipitated ATM in each sample. The immunoprecipitated material was examined for ATM kinase activity using a p53 peptide. Phosphorylation of p53 was determined using the anti-p53^{Ser15} antibody. ATM kinase activity is activated normally independently of BRCA1 expression. (B) The phosphorylation of H2AX phosphospecific anti-p-H2AX^{Ser139} antibody was examined 1 h after exposure of HCC1937 cells to IR (20 Gy) or UV (20 J/m²). Results are only shown for HCC1937 cells, but similar results were observed with 1BRneo and 293T cells. Results are shown at low-power magnification to show the response of multiple cells and also in individual cells. (C) The kinetics of p-H2AX foci formation at varying times after exposure of 1BR3, 293T and HCC1937 to IR (20 Gy) or UV (20 J/m²). (D) The mobility shift observed after immunoblotting of Rad9 and Hus1 was examined 4 h after exposure of 1BRneo cells to 20 J/m² with and without treatment with λ -phosphatase (λ -PPase). (E) The phosphorylation of Rad17, Rad9 and Hus1 was examined in 1BRneo, AT5BIVA and HCC1937 cells following exposure to IR (20 Gy) and UV (20 J/m²). Phosphorylation of Rad17 was examined using anti-p-Rad17 antibody and anti-Rad17 antibodies were used as a control for Rad17 expression. Phosphorylation of Rad9 and Hus1 was examined by mobility shift after immunoblotting. Phosphorylation was observed to a similar extent in 1BRneo, AT5BIVA and HCC1937 cells. (F) Rad17 was phosphorylated efficiently in HCCAdB1 and HCCAdco cells after UV and IR. Phosphorylation of Rad17 was examined in 293T cells and cells infected with empty adenovirus or adenovirus expressing BRCA1. (G) Formation of Rad9, Hus1 and Rad17 foci occurs normally in HCC1937 cells following exposure to UV. Thirty minutes after exposure to UV (20 J/m²), cells were examined by immunofluorescence for foci using anti-Rad9, anti-Hus1 and anti-Rad17 antibodies. Foci formed to a similar level in 1BRneo and HCC1937 cells. Additionally, the Hus1 and Rad17 foci were shown to overlap in merged images. Only a single cell has been shown to enhance visualization of the foci, but foci were observed in ~40% of the cells 30 min after UV treatment (20 J/m²). A slightly higher level of foci was observed in untreated HCC1937 cells relative to untreated 1BRneo cells, which may reflect the elevated spontaneous instability reported in these cells (data not shown).

loading onto chromatin (Zou *et al.*, 2002). ATR and ATM phosphorylate Rad17 after damage, but the phosphorylation of Rad17 is not required for the loading of the Rad1–Rad9–Hus1 complex (Bao *et al.*, 2001). However, the phosphorylation of Rad17 does require Hus1, suggesting that the Rad1–Rad9–Hus1 complex recruited by Rad17 facilitates ATR-dependent phosphorylation of Rad17 (Zou *et al.*, 2002). Furthermore, Rad9 is also phosphorylated following DNA damage and appears to be required for checkpoint activation (Kostrub *et al.*, 1998; Chen *et al.*, 2001). Since BRCA1 is required for ATR- and ATM-dependent phosphorylation of downstream sub-

strates, but not for the phosphorylation of chromatin-associated H2AX, we investigated whether BRCA1 is required for the phosphorylation of Rad17, Rad9 and Hus1 following exposure to UV and IR.

Phosphospecific anti-*Rad17*^{Ser635} antibody was used to examine DNA damage-induced Rad17 phosphorylation. Rad9 and Hus1 phosphorylation was examined by a change in migration following immunoblotting and SDS–PAGE analysis. Since Hus1 phosphorylation has not been reported previously in human cells and the shift in migration for Rad9 is small, we verified that these represent phosphorylated proteins by showing that the

band disappears after λ -phosphatase treatment (Figure 4D). Following exposure to IR, phosphorylation of Rad17, Rad9 and Hus1 was observed in control 1BRneo cells (Figure 4E). The phosphorylation of Rad17 and Hus1 was delayed in AT5BIVA cells, consistent with previous findings (for Rad17) and the notion that ATR is activated in a delayed fashion by IR (Siliciano *et al.*, 1997; Zou *et al.*, 2002). In contrast, the kinetics of Rad17 phosphorylation in HCC1937 cells was similar to that observed in 1BRneo control cells (Figure 4E). The mobility shift of the Rad9 protein was small, as observed previously, but is ATM dependent after IR, as expected (Chen *et al.*, 2001) (Figure 4C). Rad9 phosphorylation is efficient in HCC1937 cells. Similar results were obtained following exposure to UV, although in this case phosphorylation was efficient in AT5BIVA cells, consistent with the fact that this phosphorylation event is ATR dependent (Figure 4E). Hus1 phosphorylation was less marked after UV treatment in all cell lines, possibly because Hus1 does not harbour a consensus 'S-TQ motif' normally required for ATM/ATR-directed phosphorylation. Thus, Hus1 phosphorylation may be mediated via other serine-containing sites that are numerous in the Hus1 sequence or via an indirect ATM- and ATR-dependent phosphorylation event. Notwithstanding this, the phosphorylation of all three proteins occurred normally in HCC1937 cells following UV treatment, as well as after IR. Finally, Rad17 phosphorylation was shown to occur to similar extents in HCCAdB1 and HCCAdco cells following exposure to both IR and UV. Taken together, these results demonstrate that the phosphorylation of Rad17, Rad9 and Hus1, like the phosphorylation of H2AX, does not require BRCA1, whereas the phosphorylation of many other substrates (p53, c-Jun, Nbs1, CtIP and Chk2) is BRCA1 dependent.

Following exposure to UV, Rad9 and Hus1 are relocalized to the sites of DNA damage in a Rad17-dependent but ATR-independent manner (Zou *et al.*, 2002). We employed immunofluorescence to examine the relocalization of Rad9, Hus1 and Rad17 following UV treatment. After UV irradiation, these three proteins could be seen to relocalize to discrete foci in control and HCC1937 cells (Figure 4F). In merged images, the Hus1 and Rad17 foci were shown to overlap. Thus, we conclude that BRCA1 does not play a major role in either the relocalization or phosphorylation of Rad17 or its ability to recruit Rad9 and Hus1 to the sites of DNA damage.

***Brcal* mutant embryonic stem cells are also defective in the phosphorylation of a subset of ATM/ATR targets**

Since HCC1937 is a tumour cell line, we sought to verify our findings using an independent system and exploited an embryonic stem (ES) cell line (*Brcal*^{-/-}) with one targeted mutant *Brcal* allele and one conditional allele (A.Gabriel and A.Ashworth, unpublished data). Transfection of Cre recombinase generates a cell line homozygous for a deletion of *Brcal* exons 22–24, resulting in a truncated protein of 1739 amino acids compared with the wild-type protein of 1812 amino acids, which will be designated *Brcal*^{-/-}. Wild-type and *Brcal*^{-/-} ES cells were cotransfected with Cre-expressing and puromycin-expressing plasmids, and the cells were examined for Brcal expression by immunoblotting. Pools of cells that

had lost Brcal expression were characterized further (Figure 5A). The antibodies used to examine Brcal expression were raised to the N-terminus of the protein, thus verifying that even a truncated protein is not expressed in these cells. The *Brcal*^{-/-} cells were observed to have an increased number of cells with micronuclei, consistent with the elevated spontaneous instability observed in HCC1937 cells (data not shown) (Foray *et al.*, 1999). The phosphorylation of c-Jun, p53 and Chk2 was examined by immunofluorescence in the *Brcal*^{+/+} and *Brcal*^{-/-} cells 1 h and 4 h following exposure to UV and IR. Whereas a marked increase in cells expressing these three phosphorylated proteins was observed in the *Brcal*^{+/+} cells at 4 h post-treatment, this was not observed in the *Brcal*^{-/-} cells (Figure 5B). The phosphorylation of c-Jun was also examined by immunoblotting following exposure to UV and IR. Whilst *Brcal*^{+/+} cells showed the anticipated increase in phosphorylated c-Jun, this was not seen in the *Brcal*^{-/-} cells (Figure 5C). The phosphorylation of Rad17 was also examined by immunofluorescence in a parallel manner. In this case, a similar increase in cells expressing phosphorylated Rad17 occurred independently of Brcal expression (Figure 5D). Taken together, these results provide confirmation of those obtained with the HCC1937 cells and verify that our findings can be attributed to impaired Brcal expression rather than being specific to a single cell type.

Discussion

BRCA1 is a tumour suppressor protein with a central role in the DNA damage responses of mammalian cells. Understanding the role of BRCA1 and its contribution to preventing tumorigenesis has been a major task in basic cancer research. One way of understanding the role of BRCA1 is to determine the position that it occupies in the hierarchy of DNA damage responses regulated by the PIKKs ATM and ATR. We show here that BRCA1 is dispensable for the activation of ATM kinase following exposure to IR. Consistent with this, BRCA1 is also dispensable for ATM-dependent phosphorylation of Rad17, Rad9 and Hus1, as well as for the relocalization of these proteins following exposure to IR. However, the foci involving these proteins were more prominent in HCC1937 cells than in those observed in the control cell line (Figure 4G). Thus, whilst relocalization clearly can occur in the absence of BRCA1, we cannot exclude the possibility of a subtle influence of BRCA1 on this process. Surprisingly, in separate studies we have observed efficient phosphorylation of H2AX in A-T cell lines, in contrast to a published report of a dependence of pH2AX foci formation on ATM (Burma *et al.*, 2001; T.Stiff, unpublished findings). Thus, even though BRCA1 is dispensable for p-H2AX formation, we do not necessarily conclude that it is dispensable for ATM-dependent formation of p-H2AX foci, although this may be the case. We also show that BRCA1 is dispensable for the phosphorylation of these same substrates after UV irradiation, implying that it is not required for ATR kinase activity. Since the formation of pH2AX foci after UV irradiation is ATR dependent (Ward and Chen, 2001; O'Driscoll *et al.*, 2003), we can conclude that p-H2AX

Fig. 5. *Brcal*^{-/-} ES cells are impaired in phosphorylation of c-Jun, p-53 and Chk2. (A) Wild-type and *Brcal*^{-/-} ES cells were transfected with a Cre-expressing plasmid. The result shows western blot analysis using a pool of cells selected in puromycin. (B) Cells from the same batch of cells used in (A) were examined for the expression of p-c-jun, p-p53, p-Chk2 and p-Rad17 by immunofluorescence. Filled symbols represent *Brcal*^{+/+} positive cells and closed symbols are *Brcal*^{-/-} cells. The percentage of positive cells represents the percentage of cells with one or more foci. Whilst the phosphorylation of the first three substrates decreased in *Brcal*^{-/-} cells, Rad17 phosphorylation was seen to occur independently of *Brcal* expression. (C) Cell extracts from the pool of cells used in (A) were also examined by immunoblotting using anti-p-c-jun^{ser63}. Cells were exposed to IR (20 Gy) or UV (20 J/m²) and were examined 1 and 4 h after irradiation. The *Brcal*^{-/-} cells showed no detectable c-Jun phosphorylation.

foci are dispensable for this ATR-dependent event. In contrast to these findings, we show that BRCA1 is required for ATM- and ATR-dependent phosphorylation of five other substrates, namely p53, c-Jun, Nbs1, CtIP and Chk2 (however, CtIP is not phosphorylated after UV irradi-

ation). Recent studies have shown that BRCA1 is also necessary for ATM-dependent phosphorylation of Smc1, an event required for the IR-induced S phase checkpoint (Kim *et al.*, 2002) and for IR-induced Chk1 phosphorylation (Yarden *et al.*, 2002). We show that phosphorylation of the above substrates is regained in *BRCA1*-complemented HCC1937 cells, demonstrating that these substrates are intact and capable of being phosphorylated in HCC1937 cells. Since HCC1937 is a tumour cell line, this control shows that the lack of p53^{ser15} phosphorylation is a direct consequence of the loss of BRCA1 rather than an indirect effect due to a mutation in p53. This finding indirectly suggests that BRCA1 will be required to effect the G₁/S checkpoint after IR since this checkpoint depends upon p53^{ser15} phosphorylation. Since BRCA1 has already been shown to be required for the S and G₂/M phase checkpoints, our findings imply that BRCA1 will be required for the same range of checkpoint responses that are controlled by ATM and ATR (Xu *et al.*, 2001). Surprisingly, although the phosphorylation of c-Jun, Nbs1, Chk1 and Chk2, as well as the phosphorylation of p53, is ablated in HCC1937 cells following IR treatment, marked phosphorylation of p53 is observed 4 h after UV treatment in HCC1937 cells. We also observed delayed UV-induced phosphorylation of Chk2 in some, although not all, experiments. Confirmation of delayed p53 phosphorylation in HCC1937 cells was shown by immunofluorescence, where we observed no phosphorylation 2 h after UV treatment (data not shown). The late phosphorylation could represent some residual BRCA1 function in HCC1937 cells or some ability of ATR to phosphorylate p53 and Chk2 in a BRCA1-independent manner (see below). p53 also interacts with several proteins and could potentially be presented to ATR without the requirement for BRCA1. Finally, using a subset of these substrates, we have confirmed these observations in an independent mouse ES cell system, demonstrating that the findings can be attributed to defective BRCA1 expression.

The proteins that require BRCA1 for their DNA damage-induced phosphorylation include downstream proteins required for checkpoint activation and/or apoptosis. In all cases, these proteins have been shown to associate with BRCA1 constitutively and, for CtIP and Chk2, the interaction is disrupted following exposure to DNA damage (Li *et al.*, 2000; Lee *et al.*, 2001). In contrast, the proteins that are phosphorylated by ATM and ATR in a BRCA1-independent manner are associated with the chromatin following genotoxic damage. Rad17 has been shown to be chromatin associated and to recruit the Rad9–Rad1–Hus1 complex after DNA damage in an ATR-independent manner (Rauen *et al.*, 2000; Zou *et al.*, 2002). In another study, H2AX phosphorylation by ATR has been shown to be distinct from Chk1 phosphorylation in its genetic requirements since it is independent of Hus1 (Ward and Chen, 2001). Several reports have recently described 53BP1 as another protein involved in the DNA damage checkpoint signalling response (Abraham, 2002; DiTullio *et al.*, 2002; Fernandez-Capetillo *et al.*, 2002; Wang *et al.*, 2002). Like BRCA1, 53BP1 has C-terminal BRCT repeat domains and is required for the S and G₂ checkpoints after IR treatment. In a striking similarity to our findings here, 53BP1 appears to facilitate the phosphorylation of certain ATM-dependent substrates and has

Fig. 6. Model of the role of BRCA1 in damage response signalling. ATM and ATR respond to DNA damage induced by IR or UV, respectively. ATR is associated with ATRIP at the damage sites (not shown for simplicity). Rad17 is recruited to the damage sites independently of ATR, and recruits Rad9, Rad1 and Hus1. BRCA1 is also recruited to the damage sites, in part by an interaction with Rad9 and Hus1 that occurs in undamaged cells. A Rad17–Rad1–Rad9–Hus1 complex also forms at the damage sites after IR treatment but the requirements for its assembly are not well understood. ATR phosphorylates H2AX, Rad17, Rad9 and Hus1 in the absence of BRCA1, possibly because these substrates are directly attached to the DNA. ATM or ATR also phosphorylates a range of additional substrates including Chk1, Chk2, p53, c-Jun, Nbs1 and CtIP, which are required to effect damage-response processes including checkpoint delay and apoptosis. Although the figure shows symmetrical pathways for ATM and ATR phosphorylation events, there are likely to be differences in their requirements. For ATR, the downstream phosphorylation events require BRCA1 and, most likely, the assembly of the Rad17–Rad9–Rad1–Hus1 complex. For ATM, phosphorylation of these substrates also requires the presence of BRCA1, but any requirement for the Rad17 complex is currently unknown. ATM may also require Nbs1 for phosphorylating these substrates (Girard *et al.*, 2002). A requirement of BRCA1 for the phosphorylation of Smc1 and Chk1 by ATM has also recently been demonstrated (Kim *et al.*, 2002; Yarden *et al.*, 2002). We propose that BRCA1 acts as a scaffold to facilitate the downstream phosphorylation of ATM and ATR substrates. BRCA1-dependent phosphorylation events are shown as solid lines, and BRCA1-independent phosphorylation events are represented by broken lines.

been proposed to be involved in the presentation of substrates to ATM/ATR. Thus, like BRCA1, 53BP1 also appears to have overlapping function with the yeast BRCT-containing proteins ScRad9 and SpCrb2. This raises the possibility that the yeast Rad9 and Crb2 functions are shared between BRCA1 and 53BP1 in higher organisms, and that there may be some overlapping function between these proteins. The delayed phosphorylation of some substrates (p53 and Chk2) under certain conditions in HCC1937 cells could potentially be explained by some redundancy in function.

Taken together with published findings on the phosphorylation requirements of ATR substrates, our findings suggest that three classes of ATR substrates can be identified: (i) H2AX, which requires only ATR and most probably ATRIP; (ii) Rad17, Rad9, Hus1 and Rad1, which require ATR, ATRIP and the loading of the Rad9–Hus1–Rad1 complex onto chromatin; (iii) the downstream substrates such as p53, which require the above proteins plus BRCA1.

Based on our data and other published findings, including those based on studies in *S.pombe*, we propose the following model for BRCA1 involvement in damage response (Figure 6). In response to DNA damage, ATR/ATRIP or ATM, depending on the nature of the damage, is recruited to the site of damage and its kinase activity is activated. Phosphorylation of chromatin-associated H2AX ensues without the requirement for additional proteins. Rad17 is localized at the site of the damage independently of ATM or ATR and recruits Rad1–Rad9–Hus1 (Zou *et al.*,

2002). ATM and ATR are both associated with BRCA1, and the recruitment of Rad1–Rad9–Hus1 to the damage sites may serve to bring BRCA1 to the separately assembled Rad17 complex on the DNA, although other interactions may also serve to bring it to the complex. Phosphorylation of Rad17, Rad9 and Hus1 ensues, but since these proteins are directly accessible by ATM or ATR on the chromatin, their phosphorylation does not require BRCA1. However, BRCA1 acts as a scaffold to enable ATM or ATR to phosphorylate the non-DNA-associated downstream substrates, which, when phosphorylated, are released from the BRCA1 complex (Lee *et al.*, 2000; Li *et al.*, 2000) (Figure 6). This model provides strong support for the notion that BRCA1 acts as a scaffold or bridging protein and demonstrates that this scaffold is required for ATM and ATR to phosphorylate their downstream non-DNA-associated substrates. Therefore, it is significant that HCC1937 cells have defects that overlap with those observed in A-T cells, including S-phase arrest, G₂/M arrest and marked sensitivity to IR (Foray *et al.*, 1999; Xu *et al.*, 2001). We propose that BRCA1-defective primary cells would also display a G₁/S checkpoint defect. However, it should be noted that HCC1937 cells have defects that are distinct from those of A-T cells. Most notably, BRCA1 has been shown to be required for homologous recombination (Moynahan *et al.*, 1999). Therefore, there are certainly additional functions for BRCA1 in addition to its requirement for ATM- and ATR-dependent phosphorylation events, as has recently been demonstrated for

S.pombe Crb2, which functions to control Top3 and the BLM/WRN-like Rqh1 DNA helicase in DSB repair (Caspari *et al.*, 2002).

In summary, we have provided evidence that BRCA1 is required for many of the downstream ATM- and ATR-dependent phosphorylation events. In contrast, however, we have shown that phosphorylation of H2AX, Rad17, Hus1 and Rad9 occurs independently of BRCA1.

Materials and methods

Cell culture

The HCC1937 (*BRCA1*-mutated) tumour cell line is derived from a human ductal carcinoma. AT5BIVA (*ATM*^{−/−}) and 1BRNeo (control) are SV40-transformed human fibroblasts. 293T is a human embryonic kidney carcinoma cell line transformed using SV40 T-antigen (Lewis and Manley, 1985). Cells were routinely cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with antibiotics and 20% fetal calf serum (FCS).

Embryonic stem cell lines

ES cell lines carrying one truncated *Brcal* allele (*Brcal*tr1379) and the other allele with exons 22–24 flanked by loxP sites were derived by standard techniques and their derivation will be described elsewhere (A.Gabriel and A.Ashworth, unpublished work). ES cells were cotransfected with a Cre-expressing plasmid and plasmids expressing the puromycin resistance gene, and selected in puromycin (Tutt *et al.*, 2002). Transfections were performed by using GeneJuice solution (Novagen, Madison, WI). Experiments described here were performed on transiently transfected cells as pools, re-transfected with Cre-Pac vector every 3 days. ES cells were cultured routinely in 0.1% gelatin coat plasticware in DMEM medium supplemented with 10% FCS, non-essential amino acids, nucleosides, neomycin, hygromycin, puromycin and 10⁵ U/ml LiF to prevent differentiation.

Recombinant adenovirus and viral infection

AdB1 and AdCo are non-replicative E1/E3-defective recombinant adenoviruses carrying a wild-type *BRCA1* cDNA and an empty cassette, respectively. The construction of the adenoviruses and the infection procedures have been described previously (Randrianarison *et al.*, 2001). Viral infection was monitored by viruses carrying a LacZ transgene by scoring LacZ-positive blue cells, as described elsewhere (Randrianarison *et al.*, 2001).

Irradiation

Irradiations were performed using a γ -ray ¹³⁷Cs source (20 Gy at 4 Gy/min) or a UVC source (20 J/m²). Post-irradiation incubations were carried out at 37°C.

Analysis of radiosensitivity

Clonogenic cell survival after exposure to IR was performed using standard protocols described previously (Foray *et al.*, 1999). The number of micronuclei in binucleated cells was counted by microscopy 3 days post-infection, as described previously (Foray *et al.*, 1999). For experiments requiring IR, micronuclei were analysed 72 h post-irradiation.

Immunoblotting and antibodies

Preparation of nuclear extracts, immunoprecipitation and immunoblotting were performed using standard protocols published elsewhere (Foray *et al.*, 2002). Aliquots of nuclear extracts were stored at −70°C and protein concentrations were measured by the Bio-Rad Bradford assay (Bio-Rad, Hercules, CA). Treatment of extracts with λ -phosphatase was carried out at 37°C as recommended by the manufacturer (Pharmacia, Buckinghamshire, UK). Immunoprecipitations were performed with protein A-Sepharose beads (Pharmacia), 300 μ g of precleared extracts and specific antibody for 4 h at 4°C (see below). Preimmune rabbit IgG (Dako, Glostrup, Denmark) and mouse IgG (Jackson ImmunoResearch, West Grove, PA) were used as controls. Immunoblottings were performed with 100 μ g of cell extracts boiled in loading buffer and separated by standard SDS-PAGE. Semidry transfer system (BioRad, Hercules, CA) was used for transferring proteins to nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany) as detailed elsewhere. Membranes were incubated with primary antibody for 6 h and

with secondary antibody for 40 min. Antibody binding was revealed using an ECL kit (Amersham, Buckinghamshire, UK). Anti-*BRCA1* (Ab-1), anti-*ATM* (Ab-3), anti-*Rad9* (Ab-1) and anti-*c-jun* (Ab-3) antibodies were purchased from Oncogene Research (Darmstadt, Germany). Anti-*ChIP* (T-16), anti-*Chk2* (H-300), anti-*Hus1* (M-281) and anti-*Rad17* (H-300) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-p-*Nbs1*^{Ser343}, p-*c-jun*^{Ser63}, p-*Chk2*^{Thr68} and anti-p-*53*^{Ser15} were purchased from Cell Signalling, New England Biolabs (Hitchin, UK). Anti-H2AX^{Ser139} antibodies were obtained from Upstate Technology (Buckingham, UK). Anti-p53 (DO-7) primary and anti-rabbit, anti-mouse and anti-goat secondary antibodies were purchased from Dako (Glostrup, Denmark). The anti-p-*53*^{Ser15} antibody was obtained from New England Biolabs (Hitchin, UK) and the anti-p-H2AX^{Ser139} was purchased from Upstate Technology (Buckingham, UK). The anti-*Chk2* (for immunoblotting) and anti-p-*Rad17*^{Ser635} antibodies were kindly provided by Dr Steve Elledge (Houston, TX). Note that detection of Chk2 protein was performed by immunoprecipitation with the anti-*Chk2* antibody purchased from Santa Cruz Biotechnology, followed by immunoblotting with the anti-*Chk2* antibody from Dr Elledge. The antibody used to detect *Brcal* in the mouse ES cells was a rabbit polyclonal antibody H-100 (Santa Cruz Biotechnology) raised to the first 100 amino acids of *BRCA1*.

ATM kinase activity assay

First, 300 μ g of nuclear extracts were subjected to immunoprecipitation with anti-ATM antibody (Ab-3; Oncogene Research) for 2 h at 4°C. Immunocomplexes were then washed in ATM kinase buffer (10 mM HEPES pH 7.5, 50 mM NaCl, 10 mM MgCl₂, 10 mM MnCl₂, 1 mM dithiothreitol, 5 μ M ATP), supplemented with 50 mM NaF, 2 mM sodium orthovanadate, 50 mM β -glycerophosphate and protease inhibitors, and incubated for 30 min at 30°C in kinase buffer with 10 μ g of GST-p53 (residues 1–40) as ATM substrate peptide (Girard *et al.*, 2002). Protein A-Sepharose beads bound to substrates were collected and ATM kinase activity was quantified by using the phosphospecific anti-p53^{Ser15} antibody. In all these experiments, the assay was validated using radioactive ATP (Girard *et al.*, 2002).

Immunofluorescence

Cells were fixed in 3% paraformaldehyde, 2% sucrose phosphate-buffered saline (PBS) for 10 min at room temperature and permeabilized in 20 mM HEPES pH 7.4, 50 mM NaCl, 3 mM MgCl₂, 300 mM sucrose and 0.5% Triton X-100 (Sigma-Aldrich, Poole, UK) for 5 min at 4°C. Thereafter, coverslips were washed in PBS prior to immunostaining. Primary antibody incubations were performed for 40 min at 37°C at 1:100 dilutions in PBS supplemented with 2% bovine serum fraction V albumin (BSA) (Sigma-Aldrich) and followed by washing in PBS. Incubations with anti-mouse TRITC and FITC or with anti-rabbit FITC secondary antibodies (Sigma) were performed at 37°C at 1:100 in 2% BSA for 20 min. Nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Sigma-Aldrich, Poole, UK) (data not shown) for 10 min at 4°C. Coverslips were mounted in Vectashield (Vector Laboratories, Burlingame, CA) and examined, and foci were counted using a fluorescence microscope.

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