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ORIGINAL ARTICLE

NF-κB is a critical mediator of BRCA1-induced chemoresistance

MT Harte[1,4], JJ Gorski[1,4], KI Savage[1], JW Purcell[2], EM Barros[1], PM Burn[1], C McFarlane[1], PB Mullan[1], RD Kennedy[1], ND Perkins[3] and DP Harkin[1]

BRCA1 mediates resistance to apoptosis in response to DNA-damaging agents, causing BRCA1 wild-type tumours to be significantly more resistant to DNA damage than their mutant counterparts. In this study, we demonstrate that following treatment with the DNA-damaging agents, etoposide or camptothecin, BRCA1 is required for the activation of nuclear factor-κB (NF-κB), and that BRCA1 and NF-κB cooperate to regulate the expression of the NF-κB antiapoptotic targets *BCL2* and *XIAP*. We show that BRCA1 and the NF-κB subunit p65/RelA associate constitutively, whereas the p50 NF-κB subunit associates with BRCA1 only upon DNA damage treatment. Consistent with this BRCA1 and p65 are present constitutively on the promoters of *BCL2* and *XIAP*, whereas p50 is recruited to these promoters only in damage treated cells. Importantly, we demonstrate that the recruitment of p50 onto the promoters of *BCL2* and *XIAP* is dependent upon BRCA1, but independent of its NF-κB partner subunit p65. The functional relevance of NF-κB activation by BRCA1 in response to etoposide and camptothecin is demonstrated by the significantly reduced survival of BRCA1 wild-type cells upon NF-κB inhibition. This study identifies a novel BRCA1–p50 complex, and demonstrates for the first time that NF-κB is required for BRCA1-mediated resistance to DNA damage. It reveals a functional interdependence between BRCA1 and NF-κB, further elucidating the role played by NF-κB in mediating cellular resistance of BRCA1 wild-type tumours to DNA-damaging agents.

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INTRODUCTION

Germline mutations within BRCA1 confer a genetic predisposition to breast and ovarian cancer. In response to DNA damage, cells activate a complex DNA damage response, including DNA repair, cell cycle checkpoint activation, apoptosis and the transcriptional regulation of genes associated with all these pathways. BRCA1 has been implicated in all of these functions, and is critical to the mounting of an effective DNA damage response.[1,2] Upon DNA damage, BRCA1 localizes to damage-induced foci, which are the site of DNA double-stranded breaks, where it has been shown to be required for the repair of double-stranded breaks by homologous recombination. BRCA1 is also critical to initiating both S and G2/M phase checkpoint arrest, in response to DNA damage.[1,3] Consistent with the role of BRCA1 in the DNA damage response, multiple studies have demonstrated that BRCA1-mutant or -deficient cells are hypersensitive to DNA-damaging agents such as topoisomerase inhibitors and crosslinking agents.[4–6] In addition, BRCA1-deficient cells are sensitive to inhibitors of DNA damage repair enzymes such as poly(ADP-ribose) polymerase-1 (PARP-1).[7] The resistance to DNA damage[5,8] or PARP inhibition[7] mediated by BRCA1 correlates with a suppression of apoptosis.

A role for BRCA1 in transcriptional regulation is well established, with a variety of activated and repressed transcriptional targets having been identified. These include proteins involved in DNA repair such as DDB2 and XPC;[9] cell cycle regulators such as p21 and GADD45;[10] and regulators of growth and apoptosis such as oestrogen receptor-α,[11] psoriasin[12] and 2,5 oligoadenylate synthetase.[13] BRCA1 interacts with various proteins having different roles in transcription. It associates via RNA helicase with RNA polymerase II, a component of the core transcriptional complex. BRCA1 also associates with a variety of sequence-specific DNA-binding transcription factors, such as p53, c-Myc, STAT1 and Oct-1.[2] Recently, BRCA1 has been shown to be recruited to the promoters of many genes via interaction with various sequence-specific DNA-binding transcription factors, where it is then poised to regulate transcription in response to external stimuli such as DNA damage.[14] Of interest to this study, BRCA1 has been shown to associate with the p65 subunit of nuclear factor-κB (NF-κB) in a tumour necrosis factor (TNF)-α-dependent manner.[15] BRCA1 also associates with a range of chromatin-modifying proteins, such as the histone acetyltransferases p300/CBP and hGCN5/TRRAP; the histone deacetyases HDAC1 and HDAC2;[2] and with two subunits of the chromatin remodelling Swi/Snf complex, BRG1,[16] and BRD7.[17]

NF-κB is an anti-apoptotic transcription factor, which is activated in response to DNA damage. In mammalian cells, there are five NF-κB family members RelA (p65), RelB, c-Rel, p50/p105 and p52/p100, which combine to form different NF-κB homo- and heterodimers. In most unstimulated cells, NF-κB complexes are held in the cytoplasm by a family of inhibitory proteins known as the inhibitors of NF-κB (IκB).[18,19] Stimulation of NF-κB activity by DNA damage results in movement of the adaptor protein NF-κB essential modifier (NEMO) into the nucleus where it is sumoylated by the SUMO E3 ligase PIASγ. Other proteins, such as RIP1 (receptor interacting protein 1), PIDD (p53-induced death domain protein) and PARP-1, have been shown to modulate NEMO SUMOylation.[20,21] After its SUMOylation, NEMO is then phosphorylated by ataxia telangiectasia mutated

[1]Centre for Cancer Research and Cell Biology, Queen's University Belfast, Belfast, Northern Ireland, UK; [2]Biologics Technologies, Global Pharmaceutical Research and Development, Abbott Biotherapeutics, Redwood City, CA, USA and [3]Institute for Cell and Molecular Biosciences, Medical School, Newcastle University, Framlington Place, Newcastle Upon Tyne, UK. Correspondence: Dr MT Harte or Professor DP Harkin, Centre for Cancer Research and Cell Biology, Queens University Belfast, Lisburn Road, Belfast BT9 7BL, Northern Ireland, UK.
E-mail: m.harte@qub.ac.uk (MTH) or d.harkin@qub.ac.uk (DPH)
[4]These authors contributed equally to this work.
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(ATM).[22] Phosphorylation by ATM results in NEMO mono-ubiquitination. The ubiquitinated ATM–NEMO complex then translocates to the cytoplasm, where it stimulates the activation of the IκB kinase (IKK) complex composed of IKK-α and -β. ATM has also been demonstrated to be required for the activation of another kinase, transforming growth factor-β-activated kinase 1, which is in turn necessary for the activation of IKK in response to DNA damage. Phosphorylation of IκB by the IKK complex results in the ubiquitination and degradation of IκB allowing the NF-κB dimer free to translocate to the nucleus.[19,21,22] The activation of NF-κB in response to DNA damage results in the upregulation of specific anti-apoptotic target genes, including members of the *BCL* and *IAP* families.[23] In addition to its well-characterized role in transcription, recent work has uncovered a novel function for p65 in the regulation of genomic stability, and suggests that p65/RelA has a direct role in DNA damage repair specifically through homologous recombination.[24,25]

In this study, we investigated the role of NF-κB in mediating BRCA1-dependent resistance to DNA damage. We demonstrate that BRCA1 is able to activate NF-κB, and this activation of NF-κB is partially responsible for the chemoresistance to DNA-damaging agents mediated by BRCA1.

RESULTS

BRCA1 mediates resistance to DNA damage and activates NF-κB

We and others have previously demonstrated that BRCA1 mediates resistance to a range of DNA-damaging agents.[5,6] We re-examined this in HCC-1937 cells, a breast cell line containing a transcriptionally inactive COOH-terminal truncated version of BRCA1, which was reconstituted with either empty vector (HCC-EV) or BRCA1 (HCC-BR).[5] HCC-BR cells were significantly more resistant than their mutant counterparts, to treatment with either of the DNA damagers etoposide (topoisomerase II inhibitor) or camptothecin (topoisomerase I inhibitor; Figure 1a). The chemoresistance mediated by BRCA1 to etoposide treatment was reflected in a suppression of apoptosis in HCC-BR cells, as shown either by a decrease in caspase-3 cleavage (Figure 1b), or a decrease in the number of annexin-V-positive cells (Figure 1c). In a reciprocal model, depletion of BRCA1 in T47D cells led to an increase in apoptosis in response to etoposide treatment (Figures 1d and e).

BRCA1 activates NF-κB

BRCA1 has a well-characterized role in transcriptional regulation, and the transcription factor NF-κB is known to mediate resistance to apoptosis in the presence of DNA-damaging agents by the transcriptional upregulation of anti-apoptotic molecules. We investigated if NF-κB may have any role in the resistance mediated by BRCA1 to DNA-damaging agents. The effect of BRCA1 depletion on the activation of NF-κB was examined using MDA-MB-435 cells, which stably express an NF-κB-regulated green fluorescent protein (GFP) reporter (Figures 2a and b). MDA-MB-435 cells were depleted of BRCA1 and treated with the topoisomerase inhibitors etoposide and camptothecin, or the DNA crosslinking agents mitomycin C or cisplatin. Depletion of BRCA1 blocked the expression of GFP in response to etoposide and camptothecin, but had no effect on the activation of NF-κB by either mitomycin C or cisplatin (Figure 2). In addition, BRCA1 depletion had no effect on the TNF-α-mediated induction of NF-κB-regulated GFP. These results indicate that BRCA1 has a role in NF-κB activation by a subset of DNA-damaging agents.

BRCA1 regulates the transcription of anti-apoptotic NF-κB targets in response to DNA-damaging agents

To further investigate the role of BRCA1 in NF-κB activation, we examined the effect of the DNA damagers etoposide, camptothecin, mitomycin C and cisplatin on the regulation of the NF-κB anti-apoptotic targets Bcl2 and XIAP, and the NF-κB target and negative regulator IκBα. We had previously identified XIAP as a potential BRCA1-regulated transcript on an messenger RNA (mRNA) microarray from either scrambled or BRCA1 short interfering RNA (siRNA)-treated T47D cells.[26] Analysis of this microarray data indicated that expression of XIAP was downregulated (2.3-fold) in the absence of BRCA1. BRCA1 mutant tumours have a decreased expression of Bcl2 compared with sporadic control tumours as shown by immunostaining.[27] That this loss of Bcl2 may be controlled at the transcriptional level was suggested by a study in which overexpression of BRCA1 was able to transactivate a Bcl2-driven promoter.[28]

T47D cells were depleted for either BRCA1 or the subunits of the most abundant NF-κB heterodimer p65 or p50, and the cells treated for 8 h with either etoposide, camptothecin, cisplatin or mitomycin C. All of the drugs stimulated expression of all three targets relative to untreated cells. However, depletion of BRCA1, p50 and p65 were only able to significantly reduce the expression of Bcl2, XIAP and IκBα mRNA stimulated upon treatment with etoposide or camptothecin. In contrast, the expression of Bcl2, XIAP and IκBα mRNA stimulated by cisplatin or mitomycin C treatment were unaffected by depletion of either p65, p50 or BRCA1 (Figure 3a), indicating that activation by these damagers in our system is independent of p65/p50, the most abundant NF-κB complex. To further confirm this result a time course of either etoposide or camptothecin treatment was carried out and the expression of all three target mRNAs monitored (Figure 3b). Over the time course, the expression of Bcl2, XIAP and IκBα were significantly reduced in cells depleted of BRCA1, p65 or p50 relative to Scr-transfected cells upon treatment by both drugs. These results indicate that BRCA1 is required for the activation of the most abundant NF-κB complex, p65/p50.

BRCA1 is on the promoter of anti-apoptotic genes along with p65 and p50

We have previously shown that BRCA1 is present on the promoters of genes which it transcriptionally regulates.[11,12,14,26] To determine the mechanism by which BRCA1 may regulate NF-κB activation and further investigate which NF-κB subunits are involved in the BRCA1-dependent regulation of NF-κB activity, we examined if BRCA1 or any of the NF-κB family members p65 (RelA), RelB, RelC, p50 or p52 were present on the promoters of *BCL2* or *XIAP* upon etoposide treatment. Chromatin immunoprecipitation (ChIP) analysis revealed that BRCA1 and the NF-κB subunit p65/RelA were constitutively present on the *BCL2* and *XIAP* promoters (Figure 4a). In contrast, p50 was recruited onto the promoters only upon etoposide treatment (Figure 4a). None of the other NF-κB subunits, RelB, RelC or p52 were detected on either promoter, confirming that p50/p65 heterodimers make up the active NF-κB DNA-binding complex.

There are three NF-κB binding sites on the *BCL2* gene.[29] To ensure binding to the κ1-site examined in Figure 4a was reflective of binding at the other sites, the recruitment of BRCA1, p65 and p50 to each of these sites was examined using a time course ChIP (Figure 4b). BRCA1 and p65 bound constitutively at the κ1 and to a lesser extent at the P1 site, and p50 was recruited to both sites at 4 h post etoposide treatment. There was no detectable binding of either p65, p50 or BRCA1 to the κB-binding site within the P2 promoter.

BRCA1 recruits p50 onto the *BCL2* promoter

BRCA1 has previously been shown to interact with the p65 subunit of NF-κB upon treatment with TNF-α.[15] Therefore, we examined if BRCA1 was able to interact with either p50 or p65 in response to DNA damage treatment. BRCA1 associated constitutively with p65 as shown by coimmunoprecipitation, in both T47D and 293T cells, whereas BRCA1 and p50 formed a complex only in response to etoposide treatment (Figure 5a).

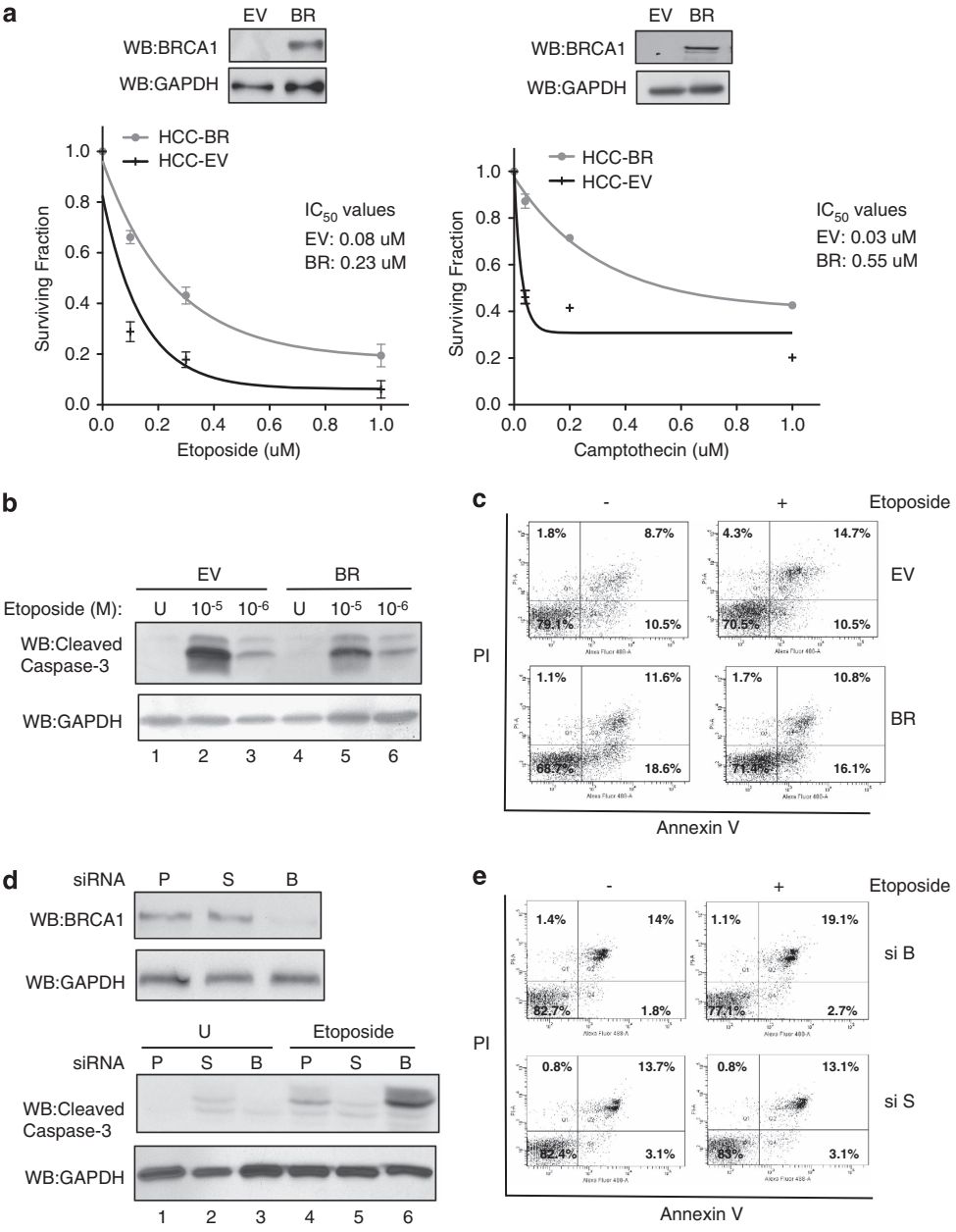

Figure 1. BRCA1 mediates resistance to DNA damage by inhibiting apoptosis. (**a**) HCC-1937 cells reconstituted either with BRCA1 (BR) or empty vector (EV) were seeded for colony counts and treated with the indicated concentrations of etoposide or camptothecin. Colonies were allowed to develop for 10 days and the surviving fraction calculated. The mean surviving fraction of three independent experiments was plotted (error bars represent 1 s.d. from the mean). Response curves were then fitted allowing calculation of the IC_{50} doses. The IC_{50} values were significantly different between HCC-EV and -BR cells for treatment with both etoposide and camptothecin at $P<0.001$. Alternatively, whole-cell extracts were prepared from HCC-EV or -BR cells and western blotted (WB) for BRCA1. Membranes were reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**b**) HCC-EV or -BR cells were treated with etoposide (1×10^{-5} M or 1×10^{-6} M) for 48 h. Whole-cell lysates were prepared and blotted for cleaved Caspase-3. Membranes were reprobed for GAPDH. (**c**) HCC-EV or –BR cells were treated with etoposide (25×10^{-6} M) for 24 h, stained with FITC-Annexin V and propidium iodide, and analysed by flow cytometry. (**d** and **e**) T47D cells (P) were transfected with BRCA1 siRNA (B) or a scr (S) control siRNA (**d**) The cells were treated with etoposide (5×10^{-6} M) for 48 h. Whole-cell extracts were prepared and blotted for cleaved Caspase-3 or for BRCA1. Membranes were reprobed for GAPDH. (**e**) The cells were treated with etoposide (25×10^{-6} M) for 24 h and stained with FITC-Annexin V and propidium iodide, and analysed by flow cytometry.

To investigate the complexes present on the *BCL2* promoter, re-ChIP analysis was carried out. In agreement with the co-immunoprecipitation results, re-ChIP revealed that BRCA1 and p65 were constitutively associated on the *BCL2* promoter, but p50 was only found associated with BRCA1 and p65 upon etoposide treatment (Figure 5b).

We have demonstrated that BRCA1 is present constitutively on the promoters examined, and associates with p50 in a damage-inducible manner. To further elucidate the mechanism by which BRCA1 may be regulating NF-κB activation, we wanted to determine whether BRCA1 has any role in the damage-dependent recruitment of p50 onto the *BCL2* promoter. In T47D cells depleted for BRCA1, we saw a loss of the damage-induced recruitment of p50 onto the *BCL2* and *XIAP* promoters (Figure 6a); however, there was no effect on the ability of p65 to be recruited in the presence of damage. We have already shown that once on the promoter,

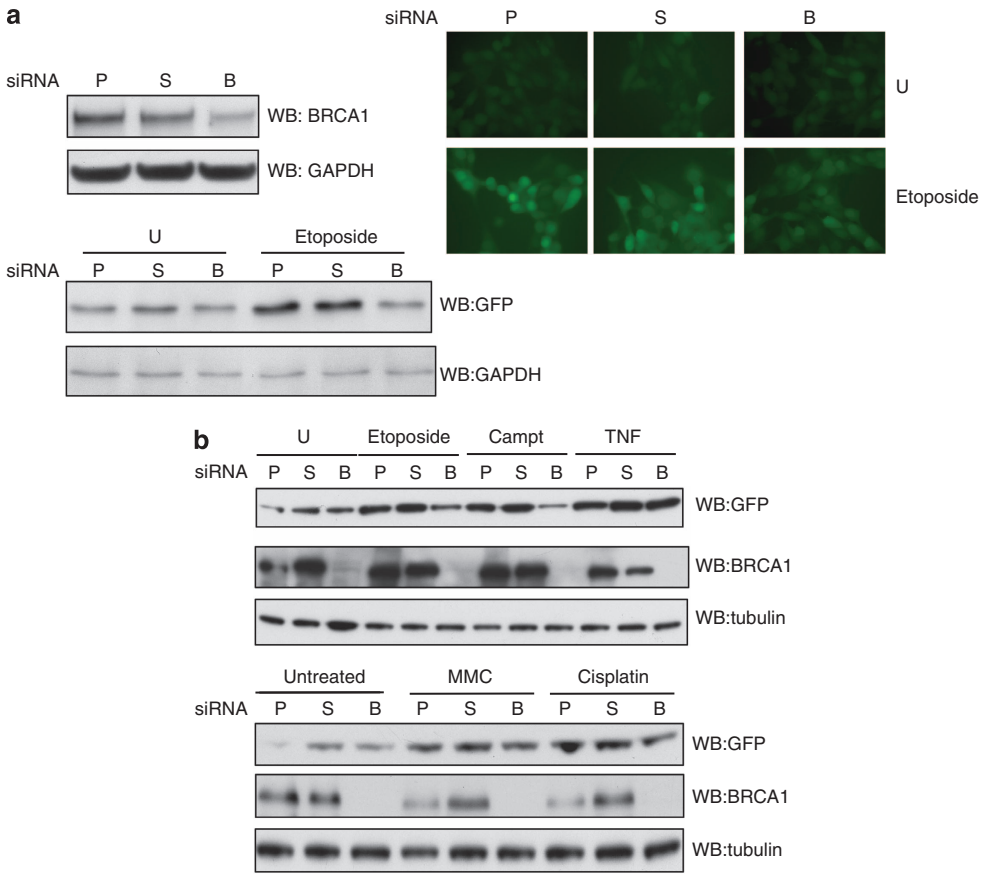

Figure 2. BRCA1 mediates the activation of NF-κB. (**a**) MDA-MB-435 cells stably expressing GFP under the control of an NF-κB-regulated promoter (P) were transfected with siRNA to BRCA1 (B) or a SCR control siRNA (S). The cells were left untreated or treated with 5×10^{-6} M etoposide for 20 h. The extent of GFP expression was determined either by immunofluoresence or by western blotting (WB) whole-cell lysates for GFP. Lysates were also blotted for BRCA1 to demonstrate depletion. Membranes were reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH). (**b**) MDA-MB-435 cells were transfected as above and treated with etoposide (5×10^{-6} M), camptothecin (Campt; 1×10^{-6} M), TNF-α (1×10^{-7} M) mitomycin C (MMC; 1×10^{-8} M), or cisplatin (1×10^{-5} M) for 8 h and the lysates blotted as above except the membranes were reprobed for tubulin to ensure equal loading.

p50 forms a complex with both BRCA1 and p65. To investigate if the BRCA1-dependent recruitment of p50 onto promoters was co-regulated by p65, we examined the recruitment of p50 to the *BCL2* and *XIAP* promoters in cells depleted for p65. In contrast to loss of BRCA1, depletion of p65 had no effect on the recruitment of p50 onto either promoters (Figure 6b). These results indicate that BRCA1 is required for the recruitment of p50 onto promoters in a p65-independent manner.

To ensure that the effects of BRCA1 on NF-κB activation are restricted to its ability to recruit p50 onto promoters, we investigated the effect of BRCA1 depletion on upstream signalling in the NF-κB pathway by examining the degradation of IκB. The degradation of the IκB inhibitor is required to liberate the NF-κB p50–p65 complex, leaving it free to move into the nucleus. IκB was degraded to a similar extent upon DNA damage treatment in both BRCA1 wild-type and depleted T47D cells (Figure 6c, compare lanes 5 and 6), indicating the upstream signalling pathway required for IκBα degradation was equally active in the absence or presence of BRCA1. The resynthesis of IκBα induced at 8 and 16 h etoposide treatment was significantly attenuated upon depletion of BRCA1 (Figure 6c, lanes 8 and 10), mirroring the reduction of IκBα mRNA in BRCA1-depleted cells upon damage treatment (Figure 3). In agreement with the lack of effect of BRCA1 loss on IκBα degradation, BRCA1 depletion had no effect on the ability of p65 or p50 to move into the nucleus upon damage (Figure 6d). Collectively, our results reveal that the ability of BRCA1 to mediate NF-κB activation is due to the requirement for BRCA1 to facilitate the recruitment of p50 onto promoters.

Inhibition of NF-κB activation attenuates BRCA1-dependent survival in response to DNA damage

To determine whether the activation of NF-κB has any role in the chemoresistant phenotype mediated by BRCA1 (Figure 1), we inhibited the activation of NF-κB using the inhibitor Bay11-7082, which prevents the degradation of IκBα, and determined what effect this had on the extent of apoptosis and survival in BRCA1-null (EV) and reconstituted (BR) HCC-1937 cells upon etoposide treatment. Treatment with Bay11-7082 led to an increase in apoptosis in BRCA1 reconstituted cells (BR) but not in BRCA1-null cells (EV), when treated with etoposide, as shown by an increase in active caspase-3 (Figure 7a). Consistent with this, inhibition of NF-κB activation severely attenuated survival only of the BRCA1 wild-type cells following etoposide treatment ($P < 0.001$) (Figure 7b). As an alternative approach, we depleted p50 and p65 from HCC-EV or BR cells using siRNA and assessed the effect of etoposide or camptothecin treatment using a colony count assay. Depletion of either subunits severely attenuated the survival of BRCA1 wild-type cells, but had little effect on the survival of BRCA1-null cells in response to both drugs (Figure 7c and Supplementary Table 5). Together these results clearly demonstrate that the activation of NF-κB contributes towards

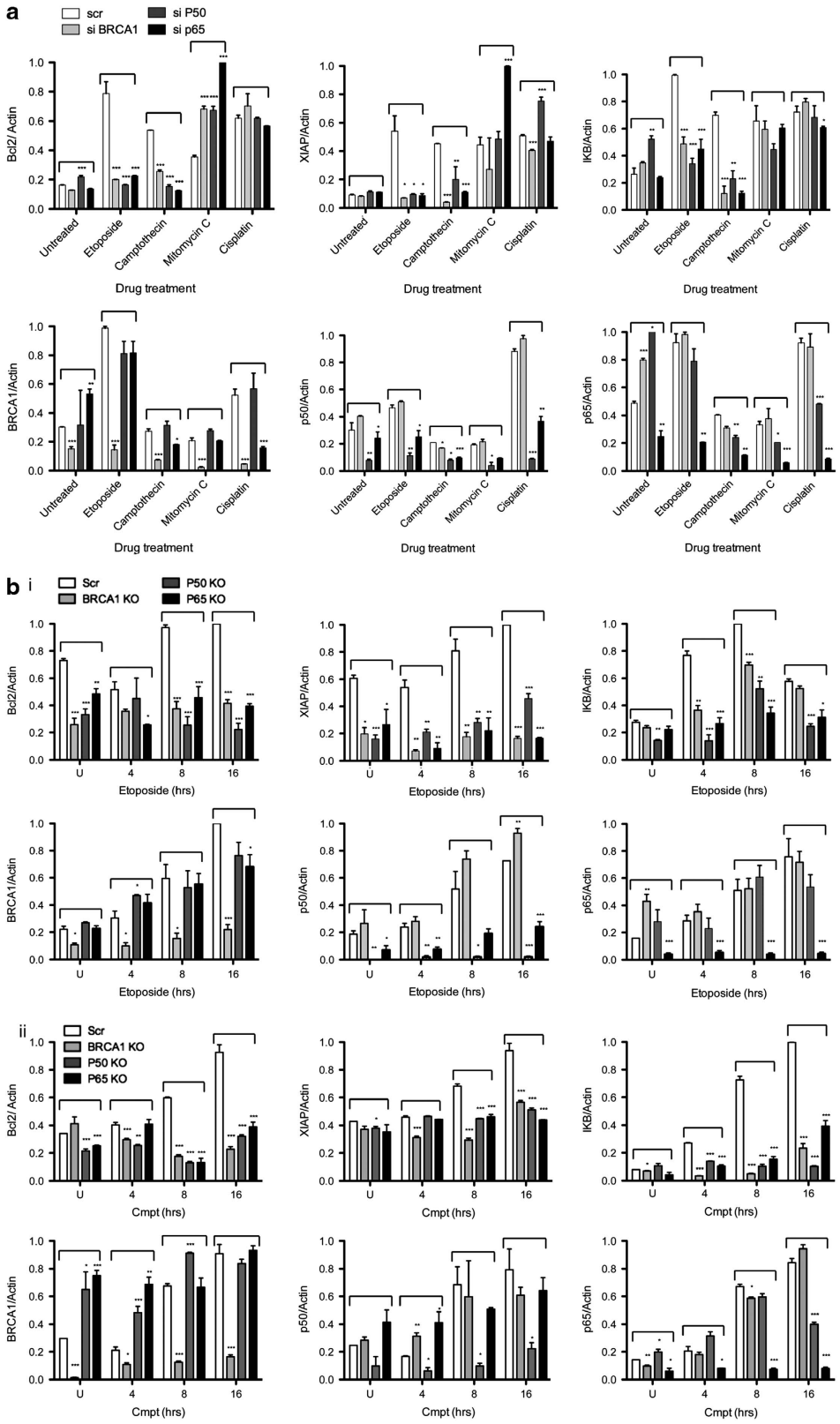

Figure 3. BRCA1 and NF-κB regulate the expression of Bcl2, XIAP and IκBα. (**a**) T47D cells were transfected with siRNA to BRCA1, p65, p50 or a Scr control siRNA and left untreated or treated for 8 h with etoposide (5×10^{-6} M), camptothecin (1×10^{-6} M), mitomycin C (1×10^{-8} M) or cisplatin (1×10^{-5} M). Quantitative reverse transcription PCR analysis was carried out to quantitate Bcl2, XIAP, IκBα, BRCA1, p65 and p50 mRNA levels. RNA was generated from three independent transfections and mean mRNA levels are shown ± s.e., normalized with respect to actin mRNA. For all treatments, within each group of four transfections each transfection was compared for statistical significance to the Scr control using the Student's paired two-tailed *t*-test; $*P<0.05$; $**P<0.01$; $***P<0.001$. (**b**) T47D cells were transfected as above and treated for 0, 4, 8 or 16 h with 5×10^{-6} M etoposide (i) or 1×10^{-6} M camptothecin (ii). RNA was harvested and analysed as above.

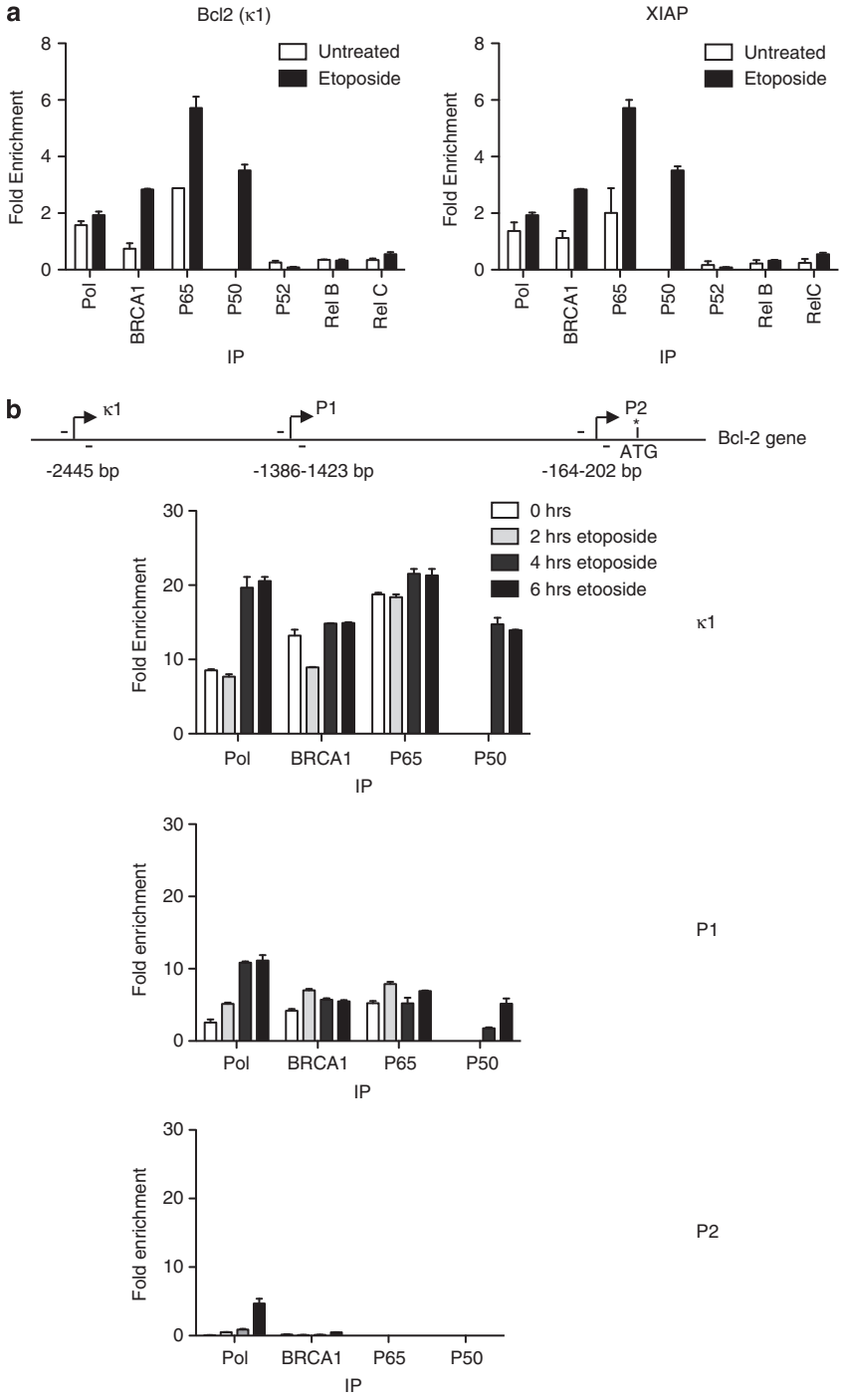

Figure 4. BRCA1 is on the promoter of anti-apoptotic genes along with p65 and p50. (**a**) T47D cells were left untreated or treated with etoposide (5×10^{-6} M) for 8 h. A ChIP assay was carried out to assess the recruitment of Pol II, BRCA1 or the NF-κB subunits p65, p50, p52, RelB and RelC onto the *Bcl2* or *XIAP* promoter. The extent of recruitment was assessed by quantitative PCR (qPCR) analysis using primers specific to the κ1 site (see schematic in panel **b**) on the *Bcl2* gene. The results are presented as fold enrichment from immunoprecipitated DNA versus input DNA, for the specific primer product relative to that of a nonspecific primer product. (**b**) T47D cells were treated with 5×10^{-6} M etoposide for 0, 2, 4 or 6 h. A ChIP assay was carried out to determine the time course of interaction of PolII, BRCA1, p50 and p65 with the NF-κB-binding sites at position κ1, P1 or P2 on the *Bcl2* gene. The schematic shows the position of the three NF-κB-binding sites on the *Bcl2* gene relative to the translation start site. The extent of recruitment was assessed by qPCR as described above.

the chemoresistance mediated by BRCA1 following treatment with DNA-damaging agents.

DISCUSSION

In this study, we identified BRCA1 as a novel regulator of NF-κB (p65/p50) activation in response to treatment with the DNA damagers etoposide and camptothecin. Our data support a mechanism whereby BRCA1 activates NF-κB by facilitating the p65-independent recruitment of the p50 subunit of NF-κB onto the promoter of responsive genes. We highlight the functional significance of this BRCA1-dependent NF-κB activation, by showing that activation of NF-κB is required for the repression

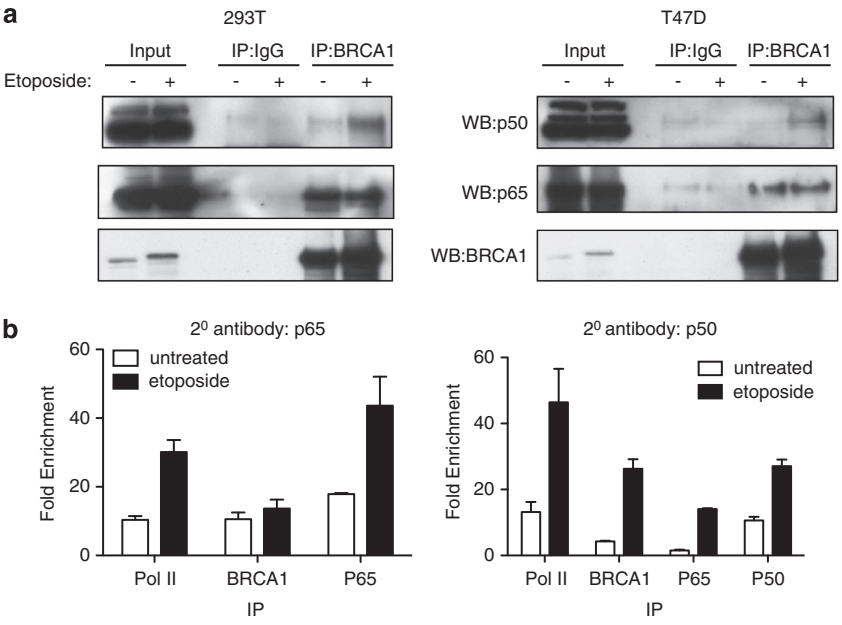

Figure 5. BRCA1 associates with p50 upon damage. (**a**) Either 293T or T47D cells were left untreated or treated with 5×10^{-6} M etoposide for 8 h. Whole-cell extracts were prepared and immunoprecipitated for BRCA1 (Ab-1) or IgG1. The immunoprecipitates were western blotting (WB) for either p50, p65 or BRCA1. A total of 5% (p65 and p50) or 10% (BRCA1) of the lysate used in the immunoprecipitate was loaded directly (input). (**b**) T47D cells were etoposide treated as above. A re-ChIP assay was carried out to assess the association of Pol II, BRCA1, p65 or p50 with either p65 or p50 on the *Bcl2* promoter. The results are presented as fold enrichment from immunoprecipitated DNA versus input DNA, for the specific primer product relative to that of a nonspecific primer product.

of apoptosis mediated by BRCA1 in response to DNA damage, and thus contributes to the chemoresistance mediated by BRCA1 to DNA damage.

Previous work, which found that the activation of NF-κB by camptothecin was delayed in BRCA1 mutant HCC-1937 cells when compared with HeLa cells, hinted at a role for BRCA1 in the control of NF-κB activation.[30] A separate study demonstrated that upon TNF-α treatment, BRCA1 overexpression was able to enhance the activity of a variety of NF-κB-regulated promoters and increase the endogenous expression of NF-κB-regulated genes.[15] Other work found that overexpression of BRCA1 enhanced both basal and TNF-α-activated expression of a reporter under the control of an NF-κB-regulated promoter and enhanced binding of NF-κB in a gel-shift assay.[28] All of these studies provided hints that BRCA1 is involved in NF-κB activation, which our study consolidates. In this report, we demonstrate conclusively that NF-κB (p65/p50) is activated in a BRCA1-dependent manner upon treatment with the DNA-damaging agents etoposide and camptothecin.

We have identified a novel BRCA1–p50 complex, and demonstrate that BRCA1 is required for the recruitment of p50 onto the promoters of target genes in response to DNA damage. The BRCA1-dependent recruitment of p50 onto promoters occurs independently of p65, however, once on the promoter p50 is able to associate with p65 as shown by re-ChIP analysis. The transcription of Bcl2, XIAP and IκBα in response to DNA damage is dependent on p65, p50 and BRCA1, indicating that the BRCA1–p65–p50 complex identified by re-ChIP is transcriptionally active. We also see a variable damage-independent regulation of the expression of Bcl2 and XIAP but not IκBα by BRCA1, p65 and p50, despite the fact that in the absence of DNA damage p50 is not present on the promoter, indicating that the constitutive regulation is likely to be indirect. This damage-independent regulation may be a consequence of the genetic instability produced by downregulation of BRCA1, p65 and p50, causing the cell to respond by shifting the balance of apoptotic regulators. This is consistent with the observed downregulation of Bcl2 in BRCA1 mutant tumours.[27]

We show that the survival of BRCA1 wild-type cells in response to etoposide and camptothecin treatment is dependent on both the p50 and p65 subunits of NF-κB. Recent work using either p65 or IκBα-SR (the IκBα super-repressor is resistant to degradation) overexpression, has shown that NF-κB has a direct role in DNA repair by homologous recombination.[25] Previous work using either p65 or p50 $-/-$ mouse embryonic fibroblast (MEF) cells showed that only p65 $-/-$ MEFs were compromised in DNA repair and found no evidence of a role for p50 in DNA repair.[24] Depletion of both p50 and p65 conferred equivalent sensitivity to DNA damage in BRCA1 wild-type cells, suggesting that in this case it is not the role of p65 in DNA repair that is being affected, but rather its role in transcription, which requires a functionally active heterodimer of p50 and p65. It has previously been shown that p53 is able to recruit the NF-κB subunit p52 onto target promoters.[31] The results of our study show that BRCA1 is acting in an analogous manner on the p50 subunit, and identify a novel BRCA1-dependent mechanism for the recruitment of the p50 subunit. IκB degradation, as well as movement of p65 into the nucleus in response to DNA damage are unaffected by the loss of BRCA1, suggesting that the main effect of BRCA1 on NF-κB activation is at the level of recruitment of p50 onto target promoters.

It has been clearly shown that the presence of BRCA1 confers resistance to DNA-damaging agents,[4,5,8] and this has been reflected in retrospective clinical studies, where it has been shown that patients whose tumours express a high level of BRCA1 have a decreased survival following treatment with DNA-damaging chemotherapy.[8,32,33] In this study, we clearly demonstrate that part of the chemoresistance mediated by BRCA1 to DNA damage is due to the activation of NF-κB. Inhibition of NF-κB in the presence of wild-type BRCA1 following DNA damage treatment led to a significant increase in apoptosis and was reflected in sensitivity to etoposide and camptothecin. Inhibitors of NF-κB such as IKK-β inhibitors are in pharmaceutical development. In addition, the proteasome inhibitor bortezomib, whose efficacy is thought to be at least in part due to inhibition of NF-κB, is being used clinically in a range of cancers.[34–36] The

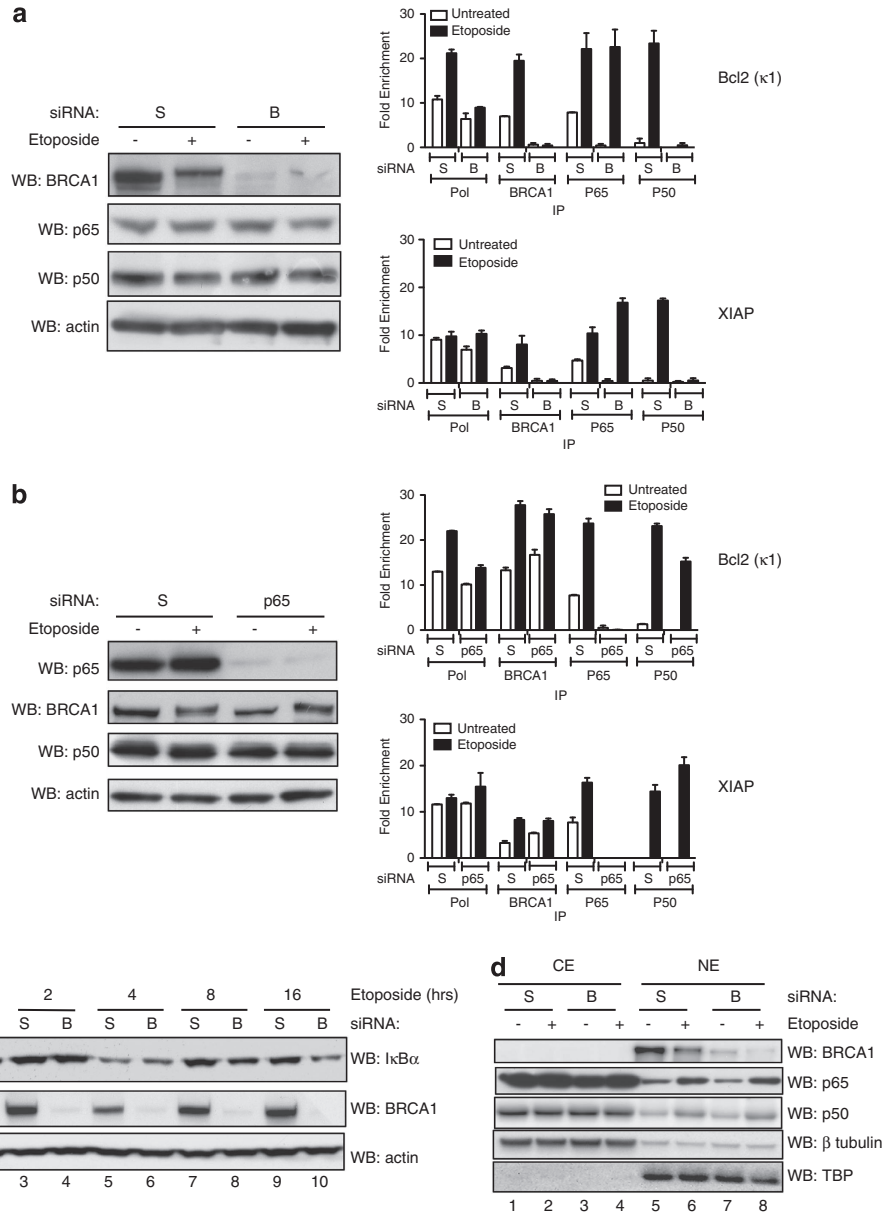

Figure 6. BRCA1 recruits p50 onto the Bcl2 promoter. (**a**) T47D cells were transfected with siRNA to BRCA1 (B) or Scr control siRNA (S). Forty-eight hours post transfection, cells were left untreated or treated with 5×10^{-6} M etoposide for 8 h. A ChIP assay was carried out to assess the recruitment of Pol II, BRCA1, p65 or p50 to the Bcl2 or XIAP promoter. The extent of recruitment was assesssed by quantitative PCR and the results are presented as fold enrichment for the specific primer product from immunoprecipitated DNA versus input DNA relative to that of a nonspecific primer product. Left panel: From the same transfections WCLs were prepared and western blotted (WB) for BRCA1, p65 or p50. Membranes were reprobed with actin. (**b**) T47D cells were transfected with siRNA for p65 or a Scr control siRNA (S) and processed for ChIP assays or western blotting as described above. (**c**) T47D cells were transfected with siRNA to BRCA1 (B) or a Scr control siRNA (S), and at 48 h post transfection they were left untreated (U) or treated with 5×10^{-6} M etoposide for 2, 4, 8 or 16 h. WCLs were prepared and western blotted for IκBα or BRCA1. Membranes were reprobed with actin. (**d**) T47D cells were transfected and treated with 5×10^{-6} M etoposide for 8 h as described above. Cytoplasmic (CE) or nuclear (NE) extracts were prepared, and blotted for BRCA1, p65 or p50. Membranes were reprobed with β-tubulin or TATA binding protein (TBP) as marker proteins of the CE and NE, respectively.

results of our studies suggest that NF-κB would be a promising target to sensitize BRCA1 wild-type tumours to DNA-damaging chemotherapy, and conversely suggest that patients with BRCA1-null or -mutated tumours may obtain little benefit.

MATERIALS AND METHODS

Cells and drug treatment

The HCC1937-EV and BR were maintained as previously described.[5]

MDA-MB-435 cells stably expressing GFP under the control of the HIV-LTR NF-κB promoter were derived by stable transfection of the pSHL-2EG plasmid (this encodes d2EGFP1 under the control of the HIV-LTR NF-κB promoter subcloned into the pSIR plasmid backbone (Takara Bio Europe/Clontech, Saint-Germaine-en Laye, France), a gift from Richard Clarkson, Cambridge University. The NG25 cells were maintained in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 1 mM sodium pyruvate and 450 μg/ml G418.

T47D and 293T cells were obtained from the American Type Culture Collection and have been passaged for <6 months since receipt.

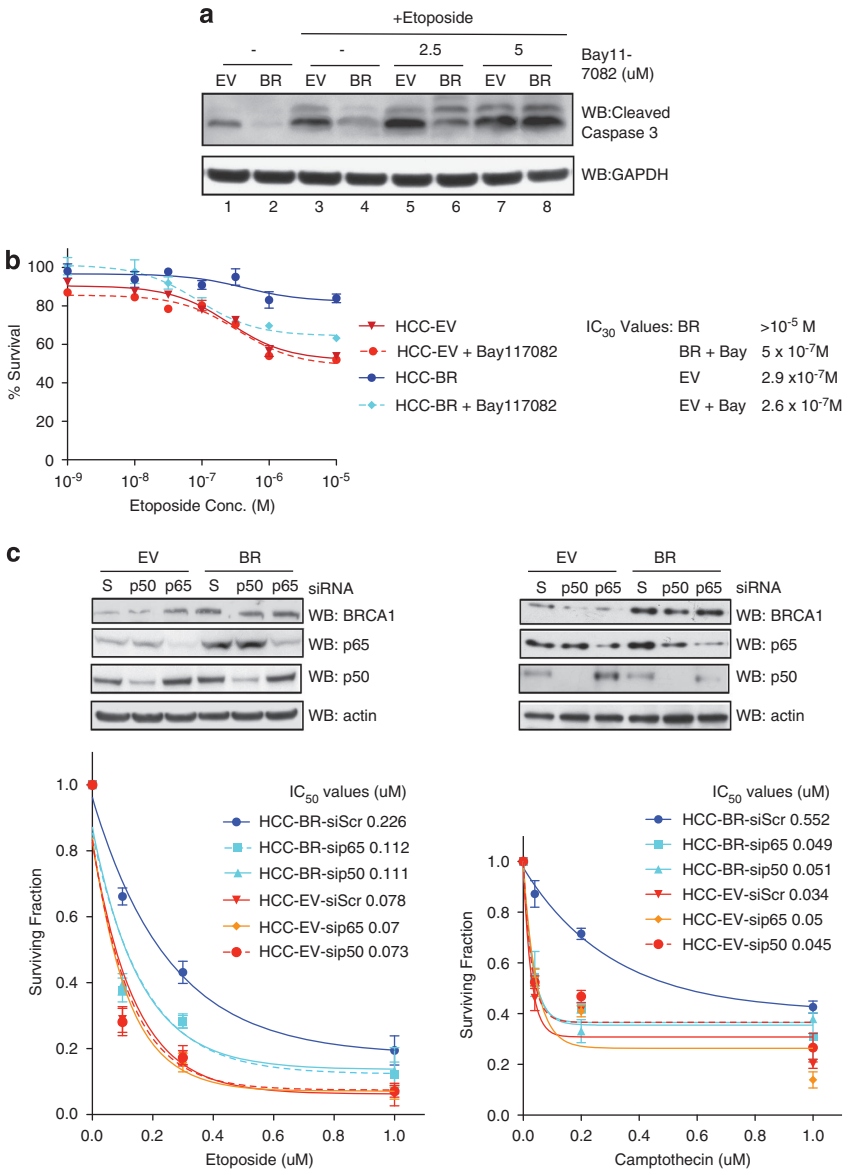

Figure 7. NF-κB activation is required for BRCA1-dependent resistance to DNA damage. (**a**) HCC-EV or BR cells were pre-treated for 1 h with Bay11-7082 (5×10^{-6} M), and treated for a further 16 h with etoposide (5×10^{-6} M). Whole-cell extracts were prepared and western blotted (WB) for cleaved caspase-3 or reprobed for glyceraldehyde 3-phosphate dehydrogenase (GAPDH) to confirm equal loading. (**b**) HCC-EV or BR were pretreated for 1 h with BAY11-7082 (2.5×10^{-6} M). Cells were then treated with increasing doses of etoposide for 72 h. Cell counts were carried out and the mean % survival of three independent experiments was plotted (error bars represent 1 s.d. from the mean). Response curves were then fitted allowing calculation of the IC_{30} doses. The *P*-values for significant difference between IC_{30} values are: HCC-EV versus BR $P = 0.01$; HCC-BR versus HCC-BR + BAY11-7082, $P < 0.001$; HCC-EV versus HCC-EV + BAY11-7082, $P = 0.96$. (**c**) HCC-EV or BR cells were transfected with siRNA against p65, p50 or a Scr control. At 48 h post transfection, the cells were seeded for colony counts and treated with the indicated concentrations of etoposide or camptothecin. Colonies were allowed to develop for a further 10 days and the surviving fraction calculated. The mean survival fraction of three independent experiments was plotted (error bars represent 1 s.d. from the mean). Response curves were then fitted allowing calculation of the IC_{50} doses. The *P*-values for significance of changes in IC_{50} values are given in Supplementary Table 5. Alternatively, at 72 h post transfection WCLs were prepared and probed for BRCA1, p65 or p50, and reprobed for actin. BR, BRCA1; EV, empty vector.

Etoposide, camptothecin, cisplatin and mitomycin C were all obtained from Sigma-Aldrich Corp. (St Louis, MO, USA).

Annexin V flow cytometry

Cells were collected post drug treatment by trypsinization. The cells were resuspended to 1×10^6 cells per ml of annexin-binding buffer. A volume of 5 μl of FITC-Annexin V (Invitrogen/Life Technologies, Paisley, UK) and 2.5 μl of 50 μg/ml propidium iodide were incubated for 15 min at room temperature with 100 μl of cells. Post incubation, 400 μl of binding buffer was added per 100 μl of cells and the samples were acquired using a FACS LSRII (BD Biosciences, Oxford, UK) and analysed using FACSDiva Software (BD Biosciences).

Preparation of whole-cell lysates (WCL) and western blot analysis

Cells were washed with 1 × phosphate-buffered saline and resuspended in ELB buffer (50 mM HEPES pH 7.2, 250 mM NaCl, 2 mM EDTA, 0.1% NP40 and 1 mM dithiothreitol) containing protease and phosphatase inhibitors (Roche Diagnostics, Mannheim, Germany), passed × 10 through a 21-G syringe and allowed to incubate for 30 min on ice. The supernatant

was collected after centrifugation for 15 min at maximum speed, and quantified using the Bio-Rad assay. A measure of 60 μg of WCL was resolved by SDS–PAGE and western blotted for: BRCA1; GFP; cleaved Caspase-3; Bcl2; XIAP; p65; or p50. To show equal loading, membranes were reprobed for glyceraldehyde 3-phosphate dehydrogenase or actin. Details of the antibodies are in Supplementary Table 1.

Real-time PCR

RNA isolation, reverse transcription, real-time PCR, primer design and statistical analysis were carried out as previously described.[17] RNA was generated from three independent transfections. The primer sequences are listed in Supplementary Table 2.

ChIP assay

Chromatin immunoprecipitation assays were performed as previously described.[11] A measure of 2 μg of antibody was used per immunoprecipitation (IP). The purified DNA was amplified either by semi-quantitative PCR or quantitative PCR using primers specific to the Bcl2 and XIAP promoters. Quantitative PCR was carried out from IP-derived DNA versus input DNA, using both a promoter-specific primer set and a nonspecific primer set >2000 bp upstream. The amount of PCR product in immunoprecipitated DNA was normalized to input and reported relative to the level of product from a nonspecific control region. Isotype-matched IgG were used as internal controls. Details of the antibodies and ChIP primer sequences are listed in Supplementary Tables 3 and 4.

Re-ChIP assay

The ChIP protocol was followed up to the point of the initial IPs. The DNA–protein complexes were extracted from the IPs $\times 2$ with 25 μl of 10 mM dithiothreitol at 37 °C for 20 min, diluted $\times 20$ with IP buffer, and then subjected to a second IP with antibodies against either p50 or p65. The re-ChIPs were processed as described above.

GFP fluorescence detection

NG-25 cells were seeded at 50% confluency on slide chamber flasks (Nunc). Twenty hours post etoposide treatment, the cells were washed with $1\times$ phosphate-buffered saline, and fixed in 4% paraformaldhyde for 15 min. Cells were washed again in $1\times$ phosphate-buffered saline, and mounted for viewing using a Zeiss Axiovert 200M fluorescent microscope (Zeiss, Gottingen, Germany).

siRNA transfection

siRNA transfection of T47D, HCC1937 or NG-25 cells was carried out using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. Each siRNA was used at a concentration of 15 nM, and etoposide treatment commenced at 48 h post transfection. The siRNA sequences used were: BRCA1, 5′-GCGUGCAGCUGAGAGGCAU-3′; Scrambled siRNA, 5′-AGCAGCACGACTTCTTCAAG-3′; p65, 5′-GCCCUAUCCC UUUACGUCA-3′. Prevalidated FlexiTube siRNA directed against p50 was obtained from Qiagen (Manchester, UK).

Dose-response curves

Dose inhibition assays with etoposide were carried out in triplicate as previously described.[5] Bay11-7082 was obtained from Calbiochem-Merck Millipore (Billercia, MA, USA). The representative means of triplicate independent dose responses ± 1 s.d. were plotted and curves describing drug response were fitted by Graphpad Prism (version 5.0C, Graphpad Software, San Diego, CA, USA) using sigmoidal dose-response curves. IC_{30} values were then calculated for each dose-response curve, with uncertainties calculated by propagation of error from the fitted parameters. The significance of changes in IC_{30} values was tested by applying the *Z*-test to the differences in IC_{30} values and calculating the corresponding *P*-value.

Colony count assay

At 48 h post transfection with siRNA, the HCC1937 EV or BR cells were split onto six-well dishes in triplicate at a concentration of 2000, 6000 and 9000 cells per well and left untreated or treated with 0.1 or 0.3 μM etoposide; or plated in triplicate at a concentration of 6000, 9000, or 12 000 cells per well and treated with 1 μM etoposide. Colonies were allowed to form for 2 weeks, counted manually and the survival fraction calculated according to the method of Franken *et al*.[37] For camptothecin treatment, HCC1937 EV or BR cells were transfected and split onto six-well dishes in triplicate at a concentration of 10 000, 40 000 and 100 000 cells per well and left untreated or treated with 0.04, 0.2 or 1 μM camptothecin. Colonies were allowed to develop for 2 weeks and stained with crystal violet. The representative means of triplicate independent colony counts ± 1 s.d. were plotted and curves describing etoposide or camptothecin response were fitted by Graphpad Prism (version 5.0C) using exponential dose-response curves. IC_{50} values were then calculated for each dose-response curve, with uncertainties calculated by propagation of error from the fitted parameters. The significance of changes in IC_{50} values was tested by applying the *Z*-test to the differences in IC_{50} values and calculating the corresponding *P*-value.

Co-immunoprecipitation

Co-immunoprecipitations of BRCA1 with p50 and p65 were carried out from WCL of HEK-293 and T47D cells. A measure of 2 μg of anti-BRCA1 (Ab-1, Calbiochem) or control IgG1 antibody, (DAKO, Ely, Cambridgeshire, UK) were precomplexed overnight at 4 °C with 50 μl of anti-mouse IgG Dynabeads (Invitrogen) in ELB buffer. A measure of 2 mg of WCL was made up to 500 μl with ELB buffer, the precomplexed antibodies were added and incubated overnight at 4 °C. The pellet was washed $\times 4$ in ELB buffer. The samples were western blotted for p65, p50 and BRCA1. Details of the antibodies are in Supplementary Materials and methods.

Subcellular fractionation

Cytoplasmic or nuclear extracts were prepared using the Subcellular protein fractionation kit (Thermo Scientific, Rockford, IL, USA) according to the manufacturer's instructions.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information accompanies the paper on the Oncogene website (http://www.nature.com/onc)