Arrest of the cell cycle by the tumour-suppressor BRCA1 requires the CDK-inhibitor $p21^{WAF1/CiPl}$

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Much of the predisposition to hereditary breast and ovarian cancer has been attributed to inherited defects in the *BRCA1* tumour-suppressor gene[1–3]. The nuclear protein BRCA1 has the properties of a transcription factor[4–7], and can interact with the recombination and repair protein RAD51 (ref. 8). Young women with germline alterations in *BRCA1* develop breast cancer at rates 100-fold higher than the general population[3], and *BRCA1*-null mice die before day 8 of development[9,10]. However, the mechanisms of *BRCA1*-mediated growth regulation and tumour suppression remain unknown. Here we show that BRCA1 transactivates expression of the cyclin-dependent kinase inhibitor $p21^{WAF1/CIP1}$ in a p53-independent manner, and that BRCA1 inhibits cell-cycle progression into the S-phase following its transfection into human cancer cells. BRCA1 does not inhibit S-phase progression in $p21^{-/-}$ cells, unlike $p21^{+/+}$ cells, and tumour-associated, transactivation-deficient mutants of BRCA1 are defective in both transactivation of p21 and cell-cycle inhibition. These data suggest that one mechanism by which BRCA1 contributes to cell-cycle arrest and growth suppression is through the induction of p21.

Because several known tumour-suppressor genes interact with or negatively regulate the cell-cycle machinery[11], we investigated the effect of BRCA1 on cell-cycle progression (Fig. 1, Table 1). By using green fluorescent protein (GFP) to mark specific transfected cells[12], we examined the effects of *BRCA1* transfection on new DNA synthesis in SW480 and HCT116 human colon cancer cells (Figs 1, 4 and Table 1). We found fewer BrdU(+)/GFP(+) SW480 cells following transfection of either BRCA1 or p53 than with their control vectors (Fig. 1). A quantitative summary of the percentage of BrdU(+)/GFP(+) cells from three independent experiments in SW480 cells is shown in Fig. 1 and Table 1. BRCA1 inhibited new DNA synthesis in SW480 cells by approximately 50% compared with the pCR3 vector. The extent of inhibition of BrdU incorporation following BRCA1 transfection was similar to p53 transfection (Fig. 1i). BRCA1 also inhibited S-phase progression in HCT116 cells (Fig. 4). These results suggest that BRCA1 can inhibit S-phase progression and thus negatively regulate the cell cycle in human cancer cells.

We investigated cyclin-dependent kinase (CDK) inhibition of cell-cycle progression as a potential mechanism by which BRCA1 may control cell proliferation. Induction of $p21^{WAF1/CIP1}$ expression has been linked to growth inhibition by p53 (ref. 13), and p21 expression also has been found to signal growth arrest, independent of p53, in cells undergoing differentiation[14]. The protein p21 is a universal cell-cycle inhibitor that specifically binds cyclin–CDK complexes and proliferating cell nuclear antigen, thereby serving as a potent growth inhibitor and effector of cell-cycle checkpoints[11]. As

Table 1 Cell cycle effects of tumour-derived BRCA1 mutants

	GFP(+) cells	BrdU(+) cells	BrdU(+)/GFP(+) (%)
pCR3	43	27	62.8
BRCA1	55	19	34.6
P1749R	59	31	52.5
Y1853insA	56	32	57.1

These BRCA1 mutants are defective at inhibiting DNA synthesis. GFP was used as a marker for transfection of SW480 cells. GFP(+) cells were examined for BrdU incorporation (BrdU(+)) by anti-BrdU staining. BrdU(+)/GFP(+) cells represents the fraction of GFP(+) cells with active DNA synthesis.

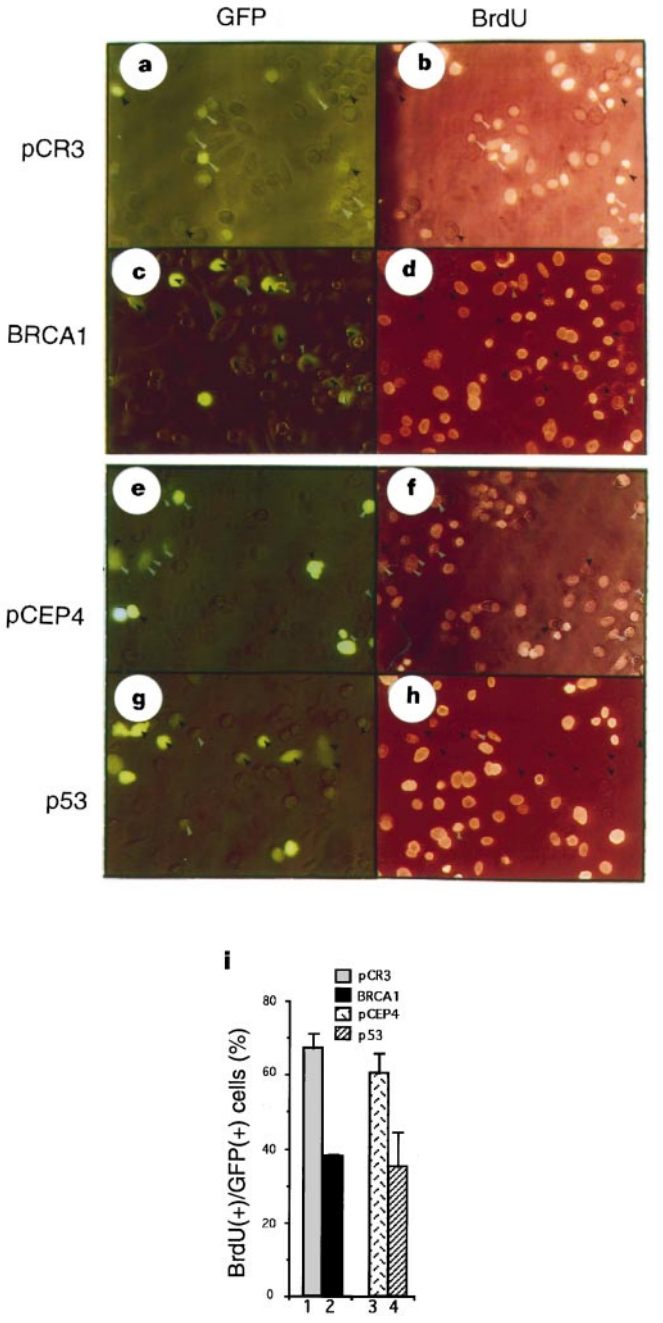


Figure 1 BRCA1 transfection inhibits DNA synthesis in human cancer cells. SW480 cells were co-transfected with pCR3 (**a**, **b**), pCR3–BRCA1 (**c**, **d**), pCEP4 (**e**, **f**), or pCEP4–p53 (**g**, **h**) and pGreen Lantern-1. The same fields were examined for GFP expression (**a**, **c**, **e**, **g**) and BrdU incorporation (**b**, **d**, **f**, **h**). White arrows indicate the GFP(+) cells that are positive for BrdU incorporation; black arrows indicate the GFP(+) cells that are negative for BrdU incorporation. **i**, The proportion of BrdU(+)/GFP(+) cells was determined by analysing at least 75 GFP(+) cells for each transfection in three fields, in two independent experiments.

BRCA1 contains a carboxy-terminal transactivation domain[6], we hypothesized that BRCA1 may transcriptionally induce p21 expression and thus negatively regulate cell-cycle progression. We examined the effect of BRCA1 on p21-promoter reporter gene expression following transfection into SW480, HCT116, COS-7, HeLa and CV1 cells (Figs 2, 3, and data not shown). BRCA1 activated the human p21 promoter luciferase-reporter by 5- to greater than 20-fold in SW480 (Fig. 2A), HCT116 (Fig. 2A), HeLa (data not shown) and COS-7 (Fig. 3B) cells, as compared to transfection of the pCR3 vector. BRCA1 also transactivated the mouse p21-promoter by more than 10-fold in CV1 cells (Fig. 2B).

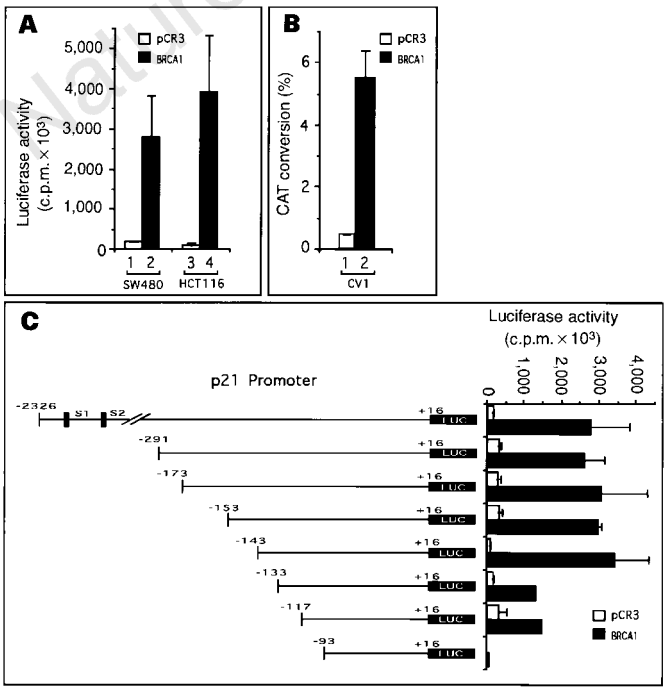
Deletion mapping within the human p21 promoter identified a control region of 50 base pairs (between −143 and −93) within the proximal promoter that seems to mediate activation of p21 by BRCA1 (Fig. 2C). The two p53-binding sites are not required for BRCA1 transactivation of p21. Whether p21 activation by BRCA1 is a direct consequence of BRCA1 binding to the p21 promoter or is an indirect effect is not known. We also investigated whether BRCA1 could activate endogenous p21 mRNA and protein expression. Figure 3D shows that p21 mRNA levels were elevated in HeLa cells after BRCA1 transfection. By using immunochemical methods, we also found increased levels of endogenous p21 protein in SW480 cells transfected with BRCA1, compared with cells transfected with vector alone (Fig. 2D). These results strengthen the hypothesis that transcriptional activation of p21 by BRCA1 is functionally relevant.

To further investigate the biological importance of p21 regulation by BRCA1, we studied the effect of various synthetic and tumour-associated mutant BRCA1 proteins on p21 expression and cell-cycle progression (Figs 3, 4 and Table 1). Mutants of BRCA1 lacking a functional nuclear localization signal, the C-terminal transactivation domain, the RAD51-interacting domain or all three domains were deficient in activating p21 expression (Fig. 3A, B, D). Similarly, three different tumour-associated transactivation-deficient[6,7] BRCA1 mutants were defective in activating the human p21-promoter luciferase-reporter gene (Fig. 3C). The expression of the synthetic deletion and tumour-derived point mutant BRCA1 proteins is shown in Fig. 3E, F. The two tumour-associated transactivation-deficient BRCA1 mutants tested for cell-cycle inhibition were also found to be deficient in cell-cycle inhibition in SW480 cells (Table 1). These results suggest that transactivation by BRCA1 may be required for its cell-cycle inhibitory effect, and that tumour-derived BRCA1 mutants may be defective in cell-cycle inhibition.

To determine whether p21 is required for the cell-cycle inhibitory effect of BRCA1, we examined the extent of new DNA synthesis following BRCA1 transfection into $p21^{+/+}$ and $p21^{-/-}$ HCT116 cells. BRCA1 inhibited new DNA synthesis in $p21^{+/+}$ HCT116 cells, but there was no evidence of DNA synthesis inhibition resulting from BRCA1 in the $p21^{-/-}$ cells (Fig. 4). These observations suggest that p21 induction may be required for cell-cycle inhibition by BRCA1 in HCT116 cells. Expression of p21 has previously been shown to be required for cell-cycle arrest following γ-irradiation of these cells[15].

These results demonstrate that BRCA1 can negatively regulate the mammalian cell cycle, and suggest that this effect is at least partly mediated by the ability of BRCA1 to induce p21. Although previous studies have reported that expression of BRCA1 is cell-cycle dependent[16–18], our results demonstrate that BRCA1 can inhibit cell-cycle progression. The absence of this inhibition in $p21^{-/-}$ cells indicates that p21 expression may be essential for BRCA1 to inhibit new DNA synthesis. Decreased cell-cycle inhibition by transactivation-deficient tumour-derived BRCA1 mutants is consistent with the idea that regulation of p21 by BRCA1 may contribute to growth control. In support of this hypothesis, recent observations in the yeast *Saccharomyces cerevesiae* demonstrate that the C-terminal 303 amino acids of BRCA1 are sufficient to inhibit yeast colony formation, and that tumour-associated mutations in the context of the 303 amino-acid region fail to inhibit colony growth[19].

There is a fundamental discrepancy between our results and recent observations that cells from BRCA1-null mouse embryos have increased levels of p21 mRNA, which suggest that BRCA1 may suppress p21 expression during development to allow cell growth[10]. However, p21 protein level and its effect on the cell cycle have not been determined in BRCA1-null embryos, and the mechanism of increased p21 expression remains unclear[10]. It is conceivable that BRCA1 may serve different functions during development and adulthood. It also is possible that the absence of BRCA1 in these cells perturbs a feedback loop controlling expression of p21. Although our data do not provide a clear explanation for this



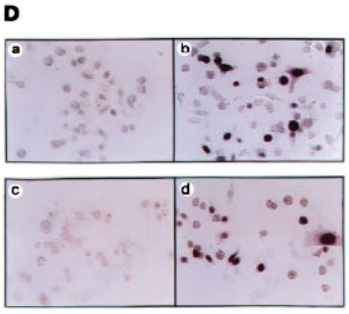


Figure 2 BRCA1 transactivates the human and mouse p21-promoter and upregulates endogenous p21 protein expression. **A**, SW480 or HCT116 cells were co-transfected with pWWP–Luc and either pCR3 or pCR3–BRCA1, and luciferase activity was measured 24 h later. **B**, CV1 cells were co-transfected with pCAT1 and pCR3 or pCR3–BRCA1, and CAT activity was measured 48 h later. **C**, Structure of the human p21-promoter luciferase reporter and several 5′-deletions are shown schematically (left). The 5′ end of each deletion is as indicated; the 3′ boundary is 16 bp downstream of the p21 transcription initiation site[13], fused to the luciferase reporter gene[21]. S1 and S2 indicate the relative locations of the two p53 DNA-binding sites within the 2.3-kb regulatory region upstream of the *WAF1/CIP1* gene[22]. pWWP-Luc or the 5′-deletion mutants were co-transfected with pCR3 or PCR3–BRCA1 into SW480 cells and luciferase activity was assayed as in **A**. **D**, Expression of endogenous p21 protein is increased in BRCA1- or p53-transfected SW480 cells. SW480 cells were transfected with pCEP4 (**a**), pCEP4–p53 (**b**), pCR3 (**c**), or pCR3–BRCA1 (**d**), and the cells were immunochemically probed for p21 expression.

difference, our results demonstrate that BRCA1 can transcriptionally induce p21 expression and negatively regulate the cell cycle. The identification of BRCA1 as an RNA polymerase II holoenzyme-associated protein provides additional evidence for the role of BRCA1 in transcriptional activation[20]. The importance of this role in tumour suppression is further supported by the fact that about 90% of the mutations in *BRCA1* result in C-terminal truncations that involve the transactivation domain. The loss of cell-cycle inhibition in $p21^{-/-}$ cancer cells and the deficiency in p21 activation and cell-cycle inhibition by tumour-derived BRCA1 mutants provides support for the hypothesis that p21 expression may lead to a quiescent or growth-inhibited state, which may contribute to BRCA1-dependent tumour suppression. □

Methods

Plasmid constructs. The pCEP4–p53 (ref. 13) and pWWP–Luc (ref. 13) plasmids were provided by B. Vogelstein. The construction of the p21-promoter deletions fused to the luciferase reporter gene has been described[21].

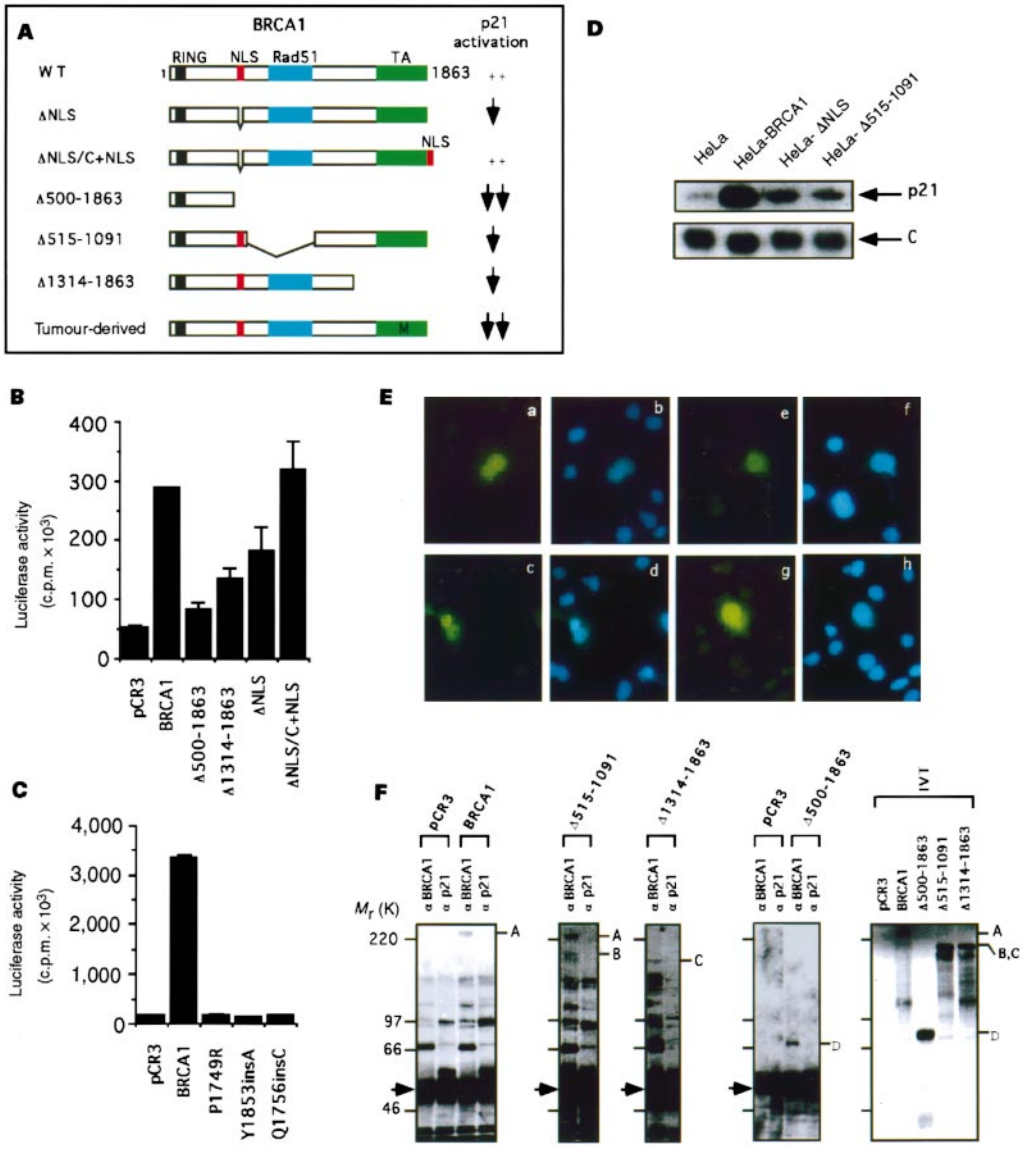


Figure 3 BRCA1 mutants are defective for activation of p21. **A**, Structure of wild-type BRCA1 (WT) and various mutants used is shown schematically. Synthetic mutants lacking the functional nuclear localization signal[23] (red, ΔNLS), a ΔNLS mutant with a C-terminal fused NLS (ΔNLS/C + NLS), mutants lacking the RAD51-interacting domain (blue, Δ515–1091), the C-terminal transactivation domain (green, Δ1314–1863) or both (Δ500–1863) are shown. Tumour-derived mutant BRCA1 is shown with an M within the green transactivation domain to signify the presence of point mutations as indicated in **C**. The corresponding extent of p21 induction (data from **B**–**D**) is shown (right; ++ indicates >5-fold induction; a single arrow, 1.5- to 2-fold reduction in p21 activation; double arrow, substantial decrease in p21 activation, corresponding to less than 1.5-fold induction). **B**, **C**, COS-7 (**B**) or SW480 (**C**) cells were co-transfected with pWWP-Luc and pCR3 or either WT or mutant BRCA1 expression plasmids as indicated, and luciferase activity was determined as in Fig. 2. **D**, Northern analysis of p21 expression in HeLa cells transfected with BRCA1 or various mutants as indicated. The same blot was reprobed with rpl32 (indicated by C). **E**, COS-7 cells (DAPI stain of permeabilized cells; **b**, **d**, **f**, **h**) transfected with WT BRCA1 (**a**, **b**) or the tumour-derived mutants P1749R (**c**, **d**), Y1853insA (**e**, **f**) and Q1756insC (**g** and **h**) were analysed by immunofluorescence (**a**, **c**, **e**, **g**) for BRCA1 expression. **F**, Expression of BRCA1 or various truncated mutant proteins (**A**). SW480 cells (left 4 panels) were transfected with the pCR3 control, BRCA1 or its mutant expression vectors, and lysates were analysed for BRCA1 expression at 24 h by immunoprecipitation and western blot analysis. A p21-specific monoclonal antibody was used as a negative control. Protein M_r (K) markers are shown on the left. A, position of (endogenous ± exogenous) WT BRCA1 protein; B, C and D indicate the position of the Δ515–1091, Δ1314–1863 and Δ500–1863 mutant BRCA1 proteins, respectively. Black arrows indicate the position of the immunoglobin heavy chain. *In vitro* translation of WT and truncated BRCA1 proteins without (right-hand panel, ^{35}S-labelled) or followed by western analysis (not shown) was performed to identify transfected BRCA1.

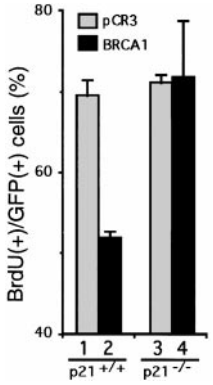


Figure 4 BRCA1 fails to inhibit DNA synthesis in $p21^{-/-}$ human cancer cells. The proportion of BrdU(+)/GFP(+) cells was determined following transfection of $p21^{+/+}$ (lanes 1, 2) or $p21^{-/-}$ (lanes 3, 4) HCT116 cells with pCR3 (lanes 1, 3) or pCR3-BRCA1 (lanes 2, 4), as described in Fig. 1.

The human p21-promoter luciferase-reporters with 5′-truncations at −153, −143 and −133 were constructed and sequenced as described[21]. The murine p21 promoter-CAT reporter, pCAT1, has been described[22]. The GFP expression vector pGreen Lantern-1 was obtained from Gibco. The pCR3–BRCA1 expression plasmid has been previously described[23]. pCR3 vectors encoding synthetic and tumour-associated BRCA1 mutants (Fig. 3A) were constructed as follows. The RAD51-interaction-deficient mutant plasmid (Δ515-1091) was constructed by digestion of pCR3–BRCA1 using *Bsu36*I to delete nucleotides 1661–3392 (ref. 1), followed by in-frame intramolecular ligation. The transactivation domain deletion mutant plasmid (Δ1314–1863) was prepared by digestion of pCR3–BRCA1 with *Bam*H1 (nucleotide 4058) and *Not*1 (nucleotide 5833), Klenow fill-in and subsequent intramolecular blunt-end ligation at 37 °C. The double mutant (Δ500–1863) was cloned by polymerase chain reaction (PCR) amplification of nucleotides 1–1616 using pCR3-BRCA1 as a template and the following primers: 5′-GCAAGCTTGC-CACCATGGATTT ATCTGCTCTTCGC-3′ and 5′-TTGTGAGGGGACGCTCTT-GTA-3′. The 1.6-kilobase PCR product was ligated into the pCR3 vector, and a clone expressing the N-terminal region of BRCA1 in the sense orientation downstream of the CMV-promoter was isolated. The ΔNLS/C + NLS vector was prepared as follows. A 488-bp DNA fragment containing a fusion between the extreme C terminus of BRCA1 and the NLS region (amino acids 499–510) was generated by PCR amplification of pCR3–BRCA1 using the following primers: 5′-AGGAGATGTGGTCAATGGAAG-3′ and 5′-TATCATGATGTAGGTCTCCTT TTACGCTTTAATTTATTGTAGTGGCTGTG-3′. This PCR product was sub-cloned into pCR3 and released as an *Apa*1 fragment which was cloned in-frame into an *Apa*1-digested ΔNLS plasmid. pCR3 vectors encoding the transactivation-domain tumour-derived BRCA1 mutants[6,7] (P1749R, Y1853insA, Q1756insC) were constructed by amplification of a 1.8-kb C-terminal region containing the specific mutations and subcloning into pCR3–BRCA1 digested with *Bam*H1 and *Not*1. All mutant BRCA1 expression plasmids used were sequenced and shown to express protein (Fig. 3E, F, and data not shown). Deletion of the nuclear localization signal[23] (ΔNLS mutant) resulted in cytoplasmic staining, which reverted back to the nucleus upon addition of a C-terminal NLS (ΔNLS/C + NLS; unpublished data).

Cells, transfections and luciferase assays. The $p21^{+/+}$ parental and $p21^{-/-}$ HCT116 human colon cancer cells[15] were provided by T. Waldman and B. Vogelstein. SW480, HeLa, COS-7 and CV1 cells (ATCC) were transfected as described[13]. Luciferase and CAT assays were performed as described[13,22]. HeLa cells expressing BRCA1 or mutants were obtained following transfection of HeLa cells with BRCA1, ΔNLS or Δ515–1091 mutants (Fig. 3D) and continuous growth in 0.4 mg ml^{-1} G418.

Analysis of BrdU incorporation in transfected GFP(+) cells. SW480 or HCT116 cells were co-transfected with pGreen Lantern-1 and mammalian expression vectors (as indicated in Figs 1, 4 and Table 1) at a ratio of 1 : 3. At 12 h after transfection, BrdU (Sigma) was added at a final concentration of 20 μM and the cells incubated for 20 h at 37 °C. Cells were examined by fluorescence microscopy to identify GFP(+) cells. Cells were treated with a mouse anti-BrdU monoclonal antibody (BM 9318, Boehringer Mannheim) and Rhodamine-conjugated goat anti-mouse IgG (Pierce) as described[12]. The number of BrdU(+) cells was determined for all of the GFP(+) cells in three high-power fields, as described in Fig. 1.

Immunocytochemistry and immunofluorescence. SW480 cells, transfected with expression plasmids (as indicated in Fig. 2D), were stained 24 h later with an anti-human-WAF1 monoclonal antibody (Ab1; Calbiochem) as described[22]. Immunofluorescence analysis of BRCA1 expression was performed as described[23].

Northern blot analysis. Total RNA was isolated and northern blot analysis was performed as described[13], and p21 mRNA expression was detected using a 2.1-kb human p21 cDNA probe[13]. Equivalent loading of various RNA samples was demonstrated using a probe for rpl32, which encodes a ribosomal protein[24].

Immunoprecipitation and western blot analysis. Cells were lysed in a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM EDTA, pH 8.0, 1 mM phenylmethylsulphonyl fluoride (Gibco), 1% antipain (Sigma), 1% leupeptin (Sigma), 1% pepstatin A (Sigma), 1% chymostatin (Sigma) and 1% AEBSF (Calbiochem). Immunoprecipitations were carried out in the lysis buffer using 2 μg of anti-BRCA1 monoclonal antibody (Ab1; Calbiochem) for 2 h at 4 °C, followed by the addition of 50% protein A-agarose beads (Sigma) and incubation for 1 h. After 3 washes with lysis buffer the immunoprecipitated proteins were analysed by western blotting as described[25] using a 1 : 250 dilution of the anti-BRCA1 monoclonal antibody, which was raised using the N-terminal portion of recombinant human BRCA1 (amino acids 1–304) as the immunogen (Calbiochem).

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