Characterization of a negative transcriptional element in the \textit{BRCA1} promoter

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Abstract

Introduction Decreased transcription of the \textit{BRCA1} gene has previously been observed to occur in sporadic breast tumours, making elucidation of the mechanisms regulating the expression of this gene important for our understanding of the etiology of the disease.

Methods Transcriptional elements involved in the regulation of the \textit{BRCA1} promoter were analysed by co-transfection experiments into the human MCF-7 and T-47D breast cancer cell lines.

Results We have identified a repressor element, referred to as the UP site, within the proximal \textit{BRCA1} promoter whose inactivation results in increased promoter activity. An E2F recognition element, previously suggested to mediate repression via E2F-6, is adjacent to the UP site and its inactivation also leads to increased \textit{BRCA1} expression. These two elements appear to form a composite repressor element whose combined effect is additive. The UP element is composed of two sequences, one of which binds the ubiquitously expressed \textit{ets} family transcription factor GABP alpha/beta. This site is distinct from a previously identified GABP alpha/beta site, the RIBS element, though the RIBS site appears to be necessary for derepression of the promoter via mutations in the UP site. Knockdown of GABP alpha using an shRNA vector confirms that this protein is important for the function of both the RIBS and UP sites.

Conclusion The identification of a repressor element in the \textit{BRCA1} promoter brings a new level of complexity to the regulation of \textit{BRCA1} expression. The elements characterized here may play a normal role in the integration of a variety of signals, including two different growth related pathways, and it is possible that loss of the ability to derepress the \textit{BRCA1} promoter during critical periods may contribute to breast transformation.

ChIP = chromatin immunoprecipitation; shRNA = small hairpin RNA.
BRCA1 gene in the breast results in breast hyperplasia, blunted ductal development and tumour formation [8]. Low BRCA1 levels in human breast cancers are correlated with tumour progression, increased malignancy and poor prognosis [9-11]. This suggests that altered BRCA1 levels have an ongoing effect on cellular processes.

The transcriptional regulation of BRCA1 expression is complex, being modulated by a variety of hormones, developmental cues and other effectors (reviewed in [12]). The BRCA1 gene is transcribed divergently with the NBR2 gene, with only several hundred base-pairs between them [13,14]. A minimal bidirectional promoter element has been defined and is located some 200 base-pairs upstream of the BRCA1 transcriptional start site [15]. Within this region we have previously identified a critical element, referred to as the RIBS site (EcoRI Band Shift), which interacts with the ets transcription factor GABP alpha/beta [16]. Functional analysis of the BRCA1 promoter revealed that the RIBS site is important for promoter activity, and appears to be differentially regulated in the MCF-7 and T-47D cell lines, with this element being less active in T-47D cells [16].

GABP alpha/beta is a ubiquitous transcription factor that binds to GA-rich sequences [17,18]. The human complex exists as a heterodimer consisting of an ets family helix-loop-helix DNA-binding domain subunit (GABP alpha), and a Notch-Ankyrin repeat family subunit (GABP beta) that contains the activation domain as well as a domain required for the formation of tetrameric complexes. GABP alpha/beta has been implicated in the regulation of genes in response to cell growth, activation of respiration related genes [19] and as a downstream mediator of ErbB3 and ErbB4 signalling [20]. The interaction of the GABP complex subunits with each other and with numerous other transcription factors and co-activators defines its ability to regulate target gene transcription.

Here, an element in the BRCA1 proximal promoter, referred to as the UP (UPstream) site, is identified and characterized. This site appears to act as a repressor, as mutation of key residues in this element results in an increase in the transcriptional activity of the promoter. Mutation of a downstream E2F site appears to have the same effect on promoter activity. The UP site is shown to contain a GABP alpha/beta binding element that is required for repressor activity. Both deletion constructs and experiments using a small hairpin RNA (shRNA) vector against the GABP alpha subunit confirm that the RIBS element and the GABP complex are required for activation of the promoter as a result of UP mutations.

**Materials and methods**

**Methylation interference assay**

This protocol was modified from Siebenlist and Gilbert [21]. One hundred nanograms of the individual strands of the UP oligonucleotide (sequence in Figure 1) were labelled using T4 polynucleotide kinase and gamma $^{32}$P-ATP. The reaction was heat inactivated and an excess of cold oligonucleotide was annealed to the labelled strand. The single-stranded ends were filled in using Klenow DNA polymerase. The labelled oligonucleotide was purified by exclusion chromatography on a Sephadel G-50 column in DMS buffer (50 mM Na-cacodylate pH 8.0, 1 mM EDTA, 50 mM NaCl). Methylation of the labelled oligonucleotide was carried out in a 200 μl reaction with 2 μg of poly dIdC and 1 μl of dimethyl sulphate at 37°C for 20 minutes. The reaction was terminated with 50 μl DMS stop buffer (1.5 M NaOAc pH 7.0, 100 μg/ml tRNA, 1.0 M beta-mercaptoethanol) with 10 μg of poly dIdC also added. The product was precipitated with ethanol and resuspended in TEN50. This probe was used in bandshift assays as described and both DNA:protein and free DNA was isolated from the wet gel using electrophoresion. The DNA was precipitated and resuspended in 90 μl of water. Piperidine (10 μl) was added and incubated at 90°C for 30 minutes. Piperidine was removed by lyophilization with several rounds of water addition. The fragmented DNA was then eletrophoresed on a 20% urea-polyacrylamide gel, dried and autoradiographed.

**Cloning**

Creation of the L6-pGL2 and L6DR-pGL2 BRCA1 promoter constructs has been described previously [16]. For these experiments these promoter constructs were cut with the restriction enzymes SmaI and HindIII and re-cloned into the pRL reporter vector (Promega, Madison, WI, USA), which had been cut with XhoI, blunted using Klenow, and then cut with HindIII. Creation of the L6-mUP-pGL2 construct was achieved using nested mutagenic primers and L6-pGL2 as the template. The products of these reactions were gel purified, annealed and a third PCR reaction was then performed to amplify the full-length mutated L6 promoter. The insert was cloned into the pGL2 vector using the restriction enzymes NheI and HindIII, and then re-cloned into the pRL vector as described above. The L6-mE2F-pRL construct was created in a similar manner, using nested primers and the L6-pRL construct as the template, and the NheI and HindIII restriction sites. The L6DR-mUP-pRL and L6DR-mE2F-pRL constructs were created by cutting the L6-mUP-pRL and L6-mE2F-pRL constructs, respectively, with the restriction enzymes Mscl and HindIII. The mutant fragments were then cloned into the L6DR-pRL vector using these same sites. The RIBS multimer in the GF-TATA-luc vector has been described previously [16]. The UP multimer was cloned upstream of the TATA box in a similar manner.

The H1 vector primers were derived from [22] and used along with human genomic DNA to amplify the proximal promoter from the histone H1 gene. This fragment was cloned into pBS+ using EcoRI and HindIII. Oligonucleotides corresponding to sequences in GABP alpha and luciferase were synthesized and annealed and the resulting fragments were cloned
Methylation interference assay of the UP site. (a) Nuclear proteins were used in a bandshift assay with a UP probe that had been chemically methylated. The various complexes indicated in (c) were extracted, chemically cleaved, individually separated on a denaturing gel and autoradiographed. The G residues whose methylation blocks binding to the upper and lower complexes are shown in bold in the sequence of the UP site. Only the non-coding strand is shown. (b) Location of G residues sensitive to methylation. Methylation of six G residues, indicated by the arrows, block binding to the upper and lower complexes. A UP oligonucleotide (UPmut) with mutations at these residues (circled) was created. (c) Binding of nuclear proteins to wild-type and mutant UP probes. A bandshift assay was performed with nuclear extracts using the wild-type UP oligonucleotide as a probe. The indicated amounts of cold wild-type UP or mutant UP oligonucleotides (oligo) were added into the reaction. The complexes correspond to Upper, Lower, non-specific (NS) and unbound (U).

Production of recombinant proteins
PCR was used to amplify the coding regions of the human GABP alpha and GABP beta genes, which were the kind gift of J-I Sawada and H Hanada, using the cDNAs in pCAGGs as the templates and the primer pairs GABP alpha-(ATG) GGG TCT AGA ATG ACT AAA AGA GAA GC, GABP alpha-(TERM) GGG AAG CTT TCA AT T ATC CTT TTC CG and GABP beta-(ATG) GGG TCT AGA ATG TCC CTG GTA GAT TTG G, GABP beta-(TERM) GGG GTC GAC GTT CAT TTC AAT TAA ACA GC, respectively. The products were then cloned into the pMAL-C2 vector using _Xba_I/ _HindIII_ restriction sites.

All restriction enzymes were obtained from NEB (Pickering, ON, Canada). Primer sequences are available from the authors upon request. All constructs were verified by sequencing.

Cell culture
The cell lines MCF-7 and T-47D were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 g/ml streptomycin. HeLa cell lines were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin. All lines were obtained from ATCC (ATCC, Manassas, VA, USA) and maintained at 37°C with 5% CO₂.

Transient transfections and luciferase assays
For all transfections for which luciferase activities were measured, cells were seeded in 12-well plates at a density of 1 x 10⁵ cells/ml, 24 hours before transfection. All transfections were carried out using 0.75 µl per well FuGene6 transfection reagent (Roche Applied Science, Laval, QC, Canada), according to the manufacturer’s instructions. To examine the relative activity of each promoter, 225 ng of each _BRCA1_ reporter construct was transfected along with 25 ng CMV-Luc internal control for a total of 250 ng DNA per well. For the over-expression studies, each condition consisted of 25 ng CMV-Luc internal control, 25 ng of each of the GABP expression vectors or their corresponding empty vector controls, and 175 ng of the specified renilla luciferase reporter vector, for a total of 250 ng of DNA per well. For the knock-down studies, 50 ng of the shRNA construct or its empty vector were used in place of the expression vectors. Each condition was performed in triplicate. The cells were lysed 48 hours post-transfection using passive lysis buffer, and assayed using the Dual-Luciferase Assay System (Promega) as per the manufacturer’s instructions. In order to test the effectiveness of the shRNA constructs, HeLa cells were plated on 12-well plates at a density of 4 x 10⁴ cells/ml, 24 hours prior to transfection. Transfections were performed using 3 µl of FuGene6 transfection reagent and 2 µg of shRNA plasmid, as per the manufacturer’s instructions. Seventy-two hours post-transfection, the cells were scraped and lysed using 50 µl of modified RIPA buffer (50 mM Tris-HCL pH 7.4, 1% Igepal C630, 0.25% Na-deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM Phenyl-Methyl-Sulfonyl-Floride, 1 µg/ml each of aprotinin, leupeptin and pepstatin, 1 mM Na3VO₄, 1 mM NaF) for 15 minutes at 4°C. An equal amount of 2× SDS-PAGE loading buffer was added to each lysate.

Western blotting
In order to detect GABP alpha, proteins were resolved by SDS-PAGE, blotted onto a nitrocellulose membrane and probed with an antibody directed against human GABP alpha. Secondary antibody detection was achieved by chemiluminescence (Pierce, Rockford, IL, USA). To confirm equal loading, the blots were then washed with PBS and re-probed with an
antibody directed against Sp1 (Santa Cruz Biotechnology, #sc59 Santa Cruz, CA, USA). Secondary antibody detection was achieved as described above.

Antibodies used for western blotting
Rabbit antibodies were prepared by Chemicon (Temecula, CA, USA), and were raised against a peptide (ASQEQQMQN-
NEIC) that corresponds to a region between the pointed and
ets domain of human GABP alpha, which is conserved
between mouse, rat and human sequences. A peptide (MQN-
QINTNPEC) corresponding to a region to the amino-terminal
side of the ankyrin repeats and also conserved between
mouse, rat and human was used to create antibodies against
human GABP beta.

Bandshift reactions
Bandshift conditions used were the same as outlined in [16].
Supershift assays were performed as described in [23], using
the Santa Cruz antibodies GABP alpha (H-2 X), CREB-1 (C-
21) and Ets2 (C-20).

Oligonucleotides
Specific oligonucleotides used are as indicated in the Figures
and the sequences are available on request.

Chromatin immunoprecipitation assays
Chromatin immunoprecipitation (ChIP) assays were carried
out with MCF-7 cells using the ChIP-It Express Enzymatic kit
(Active Motif, Carlsbad, CA, USA) as per the manufacturer’s
instructions. Each reaction was performed using chromatin
from 2 x 10^6 cells and 2 μg of affinity-purified antibody or 5 μl
of whole sera. Affinity-purified antibodies used include: GABP
alpha (Santa Cruz, (H-180 X)), haemagglutinin (Santa-Cruz,
(Y-11)), and acetylated-histone H3 (Upstate Biotechnology
(Lake Placid, NY, USA). Whole serum antibodies used include
GABP beta (Chemicon) and pre-immune serum (Chemicon).

Results
Identification of the UP binding site in the BRCA1 proximal promoter
Footprinting analysis of the BRCA1 promoter had identified an
element, referred to here as the UP site, located near the tran-
scriptional start site (data not shown). Bandshift analysis of a
variety of nuclear extracts derived from human breast cancer
cell lines indicated that two slowly migrating complexes were
formed with a UP probe. Self competition experiments con-
irmed that this interaction was specific. In order to further
characterize the interaction between the transcription factor
complexes and the UP site, individual nucleotide contact
points were identified using methylation interference assays
with the UP oligonucleotide (Figure 1a). These results indi-
cated that a series of G residues through the 5’ end of the site
were necessary for interaction with the protein complex. To
categorize the specificity of the transcription factor-DNA
complexes formed, gel shift assays were preformed. Mutation
of these nucleotides in the context of a double-stranded oligo-
nucleotide corresponding to the UP site (Figure 1b) eliminates
specific binding of factors to this site (data not shown), while
competition assays with the wild-type or mutant probes con-
firn that the mutant oligonucleotide no longer binds (Figure
1c).

The UP site acts as a repressor
To assess the functional significance of this site for BRCA1
promoter activity, a reporter construct was created with muta-
tions of all six of the nucleotides identified as being critical for
the binding of the complex to the UP site. These mutations
were made in the context of the BRCA1 L6 promoter, which
extends from nucleotide -208 to +27 and which we have pre-
viously determined to have optimal promoter activity in human
breast tumour lines (Figure 2a). This point mutant, referred to
as L6-mUP, was transfected into MCF-7 and T-47D cell lines.
In both cell lines the L6-mUP construct exhibited a three- to
five-fold increase in promoter activity compared to the wild-
type L6 promoter (Figure 2b). This suggests that the UP site
functions as a repressor element in these lines.

The UP and E2F sites form a composite repressor element
The presence of an E2F site in the BRCA1 promoter has been
previously reported and was thought to act as an element
mediating E2F-6 repression [24]. This element is immediately
downstream of the UP site (Figures 2a and 3a). Mutation of
the E2F site in the context of the L6 promoter (L6-mE2F)
resulted in increased expression in both the MCF-7 and T-47D
cell lines, with the activity being comparable to that of the L6-
mUP construct (Figure 2b). Mutation of both sites together
produces higher expression than the single mutants, with the
effect being approximately additive (Figure 2b, L6-mUP-
-mE2F). These two elements appear to be part of a composite
repressor element where mutation of either site results in loss
of repression of the promoter.

Derepression of a BRCA1 promoter construct is
dependent upon the RIBS site
We have previously identified a GABP alpha/beta site, which
we refer to as the RIBS element, upstream of the UP site in the
BRCA1 proximal promoter [16]. The RIBS site is required for
optimal promoter activity and is part of the minimal bidirec-
tional transcription element that is involved in the expression
of both the BRCA1 gene and the divergently expressed NBR2
gene [15]. Deletion of this element in the context of the L6 pro-
moter construct decreases its expression significantly and has
a similar effect on the activity of the UP and E2F single-site
mutants in both the MCF-7 and T-47D cell lines (Figure 2b,
L6DR). In MCF-7 cells the construct with both the RIBS dele-
tion and the UP mutation (L6DR-mUP) has comparable activity
to the L6DR mutant, which lacks only the RIBS site. This sug-
gests that the derepression resulting from mutation of the UP
site may be dependent on the function of the RIBS element. A
similar effect is observed with the double RIBS and E2F mutant (L6DR-mE2F) and with the triple RIBS, UP and E2F mutant (L6DR-mUP-mE2F). These results are generally comparable in T-47D cells.

Multiple complexes assemble on the UP site
The possible presence of a composite complex with repressor activity occurring on the UP and E2F sites led us to investigate the effect of additional mutations in the context of the UPFR6 probe, which includes both the UP and E2F sites (Figure 3b). A five base-pair region downstream of the original UP mutations was mutated (UPFR6mUS) and when tested in bandshift assays with nuclear extracts from both MCF-7 and T-47D cells resulted in the loss of the upper complex (UPPER) seen with the wild-type probe but retained the lower (LOWER) and non-specific (NS) complexes (Figure 3c). Similarly, the original UP mutation (UPFR6mUP) or a two base-pair mutation (UPFR6mGA) corresponding to the sequence of the middle UP mutation also resulted in the loss of the upper complex. The double GA and US mutant (UPFR6mGA-mUS) resulted in loss of binding to the middle complex but appears to produce an even lower novel complex. These results suggest that the upper complex is composed of at least two proteins, one binding to the sequence defined by the GA mutant and another factor binding to the US region. Disruption of binding of either of these proteins results in an intermediate complex, and there is the suggestion that a third protein may bind as the double mutant still interacts with a faster migrating complex. This factor could be interacting with the E2F site, although mutation of this element alone does not result in a change of protein complexes. Given the large size of the upper complex, the effect of the E2F mutation might not be visible, and may be revealed only when the other sites are mutated.

GABP alpha/beta binds directly to the UP site
Inspection of the sequence of the UP element reveals several different potential recognition elements for previously characterized transcription factors. These include two general ets factor binding sites (GGAA) that are also preferential binding sites for the ets factor GABP alpha/beta (CGGAA), one on each of the coding and non-coding strands (Figure 3a,b, arrows) that correspond to both the GA and US binding sites for nuclear proteins. GABP alpha/beta sites are often found as direct repeats, as the protein can form heterotetramers on two such elements [25], but in the UP site they are inverted repeats. In order to determine if the UP element, or any other element in the promoter, could bind GABP alpha/beta, a series of overlapping double-stranded oligonucleotide bandshift probes were generated spanning the promoter. This comprehensive approach, which we refer to as bandshift scanning, allows for the specific identification of all binding elements within the proximal promoter. We then used recombinant GABP alpha/beta dimers in bandshift assays with these probes. As expected, a strong complex was seen with the BRIBS probe as well as with a slightly larger overlapping probe, FRAG1, as both contain the previously characterized GABP alpha/beta binding RIBS element [16] (Figure 4a). In addition, a complex was seen with three other probes, UP, UPFR6 and UP/PR. The minimal UP element appears to be sufficient to bind recombinant GABP alpha/beta. The ability of
this site to bind GABP alpha/beta was confirmed by the use of a supershift assay with an antibody to the GABP alpha subunit. A distinct supershift is seen when nuclear extracts from MCF-7 (Figure 4b) or T-47D cells (data not shown) were used.

To confirm the \textit{in vivo} occupancy of the promoter by GABP alpha/beta, we also carried out ChIP assays using antibodies directed against GABP and a PCR assay targeted to the human \textit{BRCA1} promoter. MCF-7 chromatin was precipitated with various controls, including no antibody (Figure 4c, No Antibody), pre-immune serum from the rabbit used to raise antibodies against the GABP beta subunit (Figure 4c, Pre-Immune Serum), and an affinity purified anti-heamagglutinin tag antibody (Figure 4c, Anti-HA). All of these negative controls gave no or minimal PCR product. A general positive control using antibodies against acetylated histone H3 (Figure 4c, Anti-Acetylated H3) gave a robust product as expected. Antibodies against both GABP beta (Figure 4c, Anti-GABPβ) and GABP alpha (Figure 4c, Anti-GABPα) also gave a positive signal, confirming the presence of GABP alpha/beta on the \textit{BRCA1} promoter. Due to the lack of spacial resolution inherent in the ChIP assay it is impossible to determine if the
The binding of GABP alpha/beta corresponds to interaction with the RIBS, UP or both sites but indicates that it is able to interact with this promoter.

Both the GA and US mutants of the UP site result in \textit{BRCA1} promoter activation

The GA mutant clearly affects GABP alpha/beta binding and while the US mutant does not, it does affect the formation of the upper complex associated with the UP site. Both mutations, alone and in concert, were introduced into the L6 reporter construct and assayed for activity in MCF-7 and T-47D cells. In MCF-7 cells all three mutant constructs resulted
in greater promoter activity compared to the wild-type L6 construct (Figure 6b). Interestingly, the double GA/US mutant was less active than either of the single mutants alone. This may indicate that mutation of either site results in derepression of the UP element, thereby unmasking the effect of other proteins that then act as activators. Mutation of both sites therefore mediates derepression, but also abolishes some of this additional activation. This suggests then that GABP bound to the GA site, and some as yet unidentified protein associated with the US site, can both independently function to activate the promoter once repression has been lifted. Similar results were observed in T-47D cells, although the effect of the GA mutation was much less (Figure 6c). This finding implies that the factor associated with the US site in MCF-7 cells is absent or unable to mediate transactivation in this line. As with the UP and E2F mutants, removal of the RIBS site results in an overall loss of promoter activity (Figure 6b,c, deleted RIBS (DR)).

**shRNA knockdown of GABP alpha/beta inhibits BRCA1 promoter activity through the RIBS and UP sites**

In order to assess the effect of endogenous GABP alpha/beta levels on BRCA1 promoter activity, an shRNA vector was created that targeted the alpha subunit of GABP alpha/beta. This construct was able to efficiently down-regulate GABP alpha/beta protein levels by 60% to 80% when transfected into HeLa cells (Figure 6a) or MCF-7 cells (data not shown). The activity of the L6 promoter is dramatically reduced by the cotransfection of the shRNA vector in both MCF-7 and T-47D cell lines, indicating that GABP alpha/beta is an important regulator of the BRCA1 promoter in these lines (Figure 6b,c, +shGABP). The activities of the constructs containing the GA and US UP site mutations, both of which result in loss of repression of the promoter, are also greatly decreased by the GABP alpha shRNA in both the MCF-7 and T-47D lines, although complete loss of activity is not achieved. This may be the result of incomplete knock-down of the GABP complex. Removal of the RIBS site from these constructs, however, results in further decreases in activity as well as the abrogation of all mutation-specific activity (Figure 6b,c, deleted RIBS (DR) +shGABP).

**RIBS and UP multimer sites act as GABP alpha/beta-dependent activator elements**

To confirm the activities of the individual GABP alpha/beta binding sites we cloned multimers of the RIBS and UP sites upstream of a TATA box-containing minimal promoter [16]. The RIBS multimer was transactivated by cotransfection of the GABP alpha/beta expression vectors in MCF-7 and T-47D lines (Figure 7). The shRNA vector dramatically decreased promoter activity in both cell lines. The UP multimer behaved in a similar manner to the RIBS multimer reporter, with GABP alpha/beta cotransfection increasing activity in both lines. The shRNA vector reduced the activity of the UP multimer in MCF-7 and T-47D lines but the degree of this decrease was not as great as for the RIBS element. In isolation, both the RIBS and
UP elements appear to act as GABP alpha/beta dependent activator elements in MCF-7 and T-47D cell lines.

**Discussion**

**A composite repressor element is present in the BRCA1 promoter**

The structure and regulation of the BRCA1 promoter has been of particular interest due to the association of decreased BRCA1 gene transcription with the development of sporadic breast cancer [12]. We have identified a new element in the BRCA1 promoter that functions as a transcriptional repressor. The sequence of this element is well conserved between human and mouse promoters (16/18 bases), emphasizing its importance for regulation of this gene. Inactivation of the UP element using point mutations results in a three- to five-fold increase in expression in two different human breast cancer cell lines. We also determined that a previously identified E2F binding site [26] immediately downstream of the UP site is also able to act as a repressor in breast tumour cells. The repressor element in the BRCA1 promoter appears to extend from the UP through to the E2F element, though these appear to have independent functions as mutation of both sites is additive. The E2F site was originally identified as a potential mediator of increased BRCA1 expression in response to the induction of growth, likely mediated through E2F-1 [26]. Subsequently, E2F-6 activity was associated with repression of the BRCA1 promoter using an shRNA approach and its binding was thought to occur in a reciprocal manner with E2F-1 to regulate the promoter [24]. Bindra and Glazer [27] independently characterized two E2F sites within the proximal BRCA1 promoter, their E2Fb site (Figure 3a,b) being coincident with the previously identified downstream E2F site, and a second E2F recognition element within the UP site that overlaps with our GA element. These sites appear to bind both E2F1 and E2F4 and may be regulated in turn by interaction with p130/p107. In agreement with our results, mutation of either site was shown to increase promoter activity; however, the E2Fα site (the UP site) was neither necessary nor sufficient to bind E2F proteins as judged by DNA capture assays [27]. Overall, these results emphasize that a composite repressor element encompasses both the UP and E2F sites, though the question of the composition and partners of the E2F proteins involved remains complex.

**The role of GABP alpha/beta in promoter regulation**

We have previously identified GABP alpha/beta as a critical regulator of the BRCA1 promoter acting through the RIBS element [16] and in this paper we have characterized a second GABP alpha/beta site within the UP element. The RIBS element is crucial in that it is required for basal BRCA1 promoter activity as well as being essential for the derepression of the BRCA1 promoter resulting from mutations in the UP site (Figure 6, wild-type RIBS verses DR mutants). In contrast to its interaction with the RIBS element, where it acts solely as an activator, the binding of GABP alpha/beta to the UP element appears to also have a repressor function as mutation of the GABP alpha/beta recognition element leads to loss of UP mediated repression. However, when the UP site is taken out of the context of the promoter and multimerised, GABP alpha/beta was shown to activate this site. This is in keeping with previous observations that GABP alpha/beta can act as either an activator or repressor depending on the specific context of...
the promoter it interacts with [19,28]. The function of the GABP complex is thought to be influenced by the composition of the heterodimerisation partner of the DNA-binding alpha subunit. Differential splicing of the GABP beta gene generates the gamma subunit, which interacts with GABP alpha/beta while does not allow for tetramerisation [25,29]. The GABP alpha/ gamma subunit is thus thought to function as a repressor, acting in opposition to the GABP alpha/beta complex. We have not been able, however, to detect the gamma subunit product in these breast cancer cell lines (data not shown), suggesting this is not the mechanism by which the GABP complex regulates the activity of the UP site.

Both the GA and US mutants of the UP site result in the loss of the large molecular weight complex (Figure 3) and induce similar levels of derepression (Figure 6). This may be due to cooperative interactions between these proteins in which loss of either protein results in decreased complex formation and failure to form a repressor complex (Figure 8). The observation in MCF-7 cells that the GA/US double mutant promoter construct has lower activity than either of the single mutants (Figure 6), suggests that once unmasked by loss of the repressor complex, the factors bound to these sites are individually able to mediate activation of the BRCA1 promoter. In T-47D cells the US mutant exhibits an increase in activity similar to what was seen in the MCF-7 cell line. In contrast, the level of activity seen with the GA mutant is comparable to that of the GA/US mutant in this line and both constructs are significantly less active than in the MCF-7 line. As the complexes obtained by Electrophoretic Mobility Shift Assay (EMSA) using the various UP site probes with either MCF-7 or T-47D nuclear extracts are similar (Figure 3c), it would appear that the factor associated with the US site in T-47D cells is present on the promoter, but is either inactive or missing a functional co-activator (Figure 8).

### Implications for promoter function

A recent report has identified 53BP1 as a positive regulator of the BRCA1 promoter that acts through sequences in the UP element [30]. 53BP1 contains a BRCA1 carboxy-terminal domain, localizes to sites of double-strand breaks, and activates the Ataxia Telangiectasia Mutant (ATM) pathway [31]. A small interfering RNA directed against 53BP1 represses BRCA1 expression, while an expression vector activates the promoter. It is suggested that induction of BRCA1 expression by DNA damage could be mediated by 53BP1. However, a mutant within the UP site, which abrogates 53BP1 binding based on bandshift and ChIP assays, still responds to 53BP1 overexpression, suggesting its effect may be indirect. These results were obtained primarily in U2OS cells, which are a human osteosarcoma derived cell line, rather than in breast cells. 53BP1 does not bind to DNA in a sequence specific manner, suggesting that its effect is likely mediated through its recruitment by other transcription factors. It is possible that 53BP1 may modulate the repressor and or activator functions of the UP site during periods of DNA damage to bring about an increase in BRCA1 levels. However, its role in constitutive expression of BRCA1 is not clear.

The E2F family is directly involved in mediating cell cycle regulation and it is known that BRCA1 expression increases in response to growth [26,32]. Similarly, GABP alpha/beta has been implicated in the cell cycle regulation of genes such as Skp2 [33] and indeed appears to regulate a growth mediated pathway distinct from that of the D-type cyclins [34]. By incorporating both of these factors into a composite regulatory element, the UP/E2F site may be critical for integrating signals coming from different growth activated pathways that determine the nature and level of BRCA1 expression.

The BRCA1 promoter is part of a bidirectional transcription unit that also directs expression of the NBR2 gene [15]. The UP element is outside of the minimal bidirectional transcription unit that is able to direct transcription in both directions. Its location near the start site for BRCA1 expression may mean that it plays a role exclusively in regulating BRCA1 expression, while elements that regulate NBR2/BRCA1 directionality actually lie farther up and downstream of this region [35].
Conclusion
The identification of a repressor element in the BRCA1 promoter brings a new level of complexity to the regulation of BRCA1 expression. Given the critical role that decreased BRCA1 expression has in the development of sporadic breast cancer, the study of mechanisms that can down-regulate this key tumour suppressor are of particular importance. The elements characterized here may play a normal role in the integration of a variety of signals, including two different growth related pathways, and it is possible that loss of the ability to derepress the BRCA1 promoter during critical periods may contribute to breast transformation.

Competing interests
The authors declare that they have no competing interests.

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