The N-terminus of the Breast Cancer-1 predisposition protein (BRCA1) associates with the BRCA1-associated RING domain-1 protein (BARD1) to form a heterodimer, which exhibits ubiquitin ligase activity that is abrogated by known cancer-associated BRCA1 missense mutations. The majority of missense substitutions identified in patients with a personal or a family history of disease have not been followed in pedigrees, nor there is a functional understanding of their impact. We have examined, by extensive missense substitution, the interaction of BRCA1 with components that contribute to its ubiquitin ligase activity, BARD1 and the E2 ubiquitin-conjugating enzyme, UbcH5a. Selection from a randomly generated library of BRCA1 missense mutations for variants that inhibit the interaction with these components identified substitutions in residues found altered in patient DNA, indicating a correlation between loss of component-binding and propensity to disease development. We further show that the BRCA1:E2 interaction is sensitive to substitutions in all structural elements of the BRCA1 N-terminus, whereas the BARD1 interaction is sensitive to a subset of BRCA1 substitutions, which also inhibit E2-binding. Patient variants that inhibit the BRCA1:E2 interaction show loss of ubiquitin ligase activity and correlate with disease susceptibility and theoretical predictions of pathogenicity. These data link the loss of ubiquitin ligase activity, through loss of E2-binding, to the majority of non-polymorphic patient variants described within the N-terminus of BRCA1 and illustrate the likely significant role of BRCA1 ubiquitin ligase activity in tumour suppression.

INTRODUCTION

Inheritance of mutations in BRCA1 resulting in protein truncation predispose individuals to breast and ovarian cancer, whereas determining the significance of missense variants presents a major clinical problem (1). More than a quarter of entries recorded in the Breast cancer Information Core (BIC) dataset of BRCA1 sequence variants from patients are missense alterations (http://research.nhgri.nih.gov/bic/).

This study examines the 100 N-terminal amino acids (aa) of BRCA1 that includes the Zn2⁺-binding RING domain flanked by two α-helices (2). Forty-four different non-polymorphic missense variants have been reported in patient DNA encoding this region and the most frequently occurring are located within it. The pathogenicity of only a small number of these is known, and the rest remain unclassified. This is because it is not possible to follow the majority of variants by co-segregation analysis and the number of appropriate controls required to be content of a variant’s absence in unaffected individuals is prohibitive. For either approach, incomplete penetrance of the mutation may also be confounding. Thus far theoretical predictions based on evolutionary conservation...
of the region and chemical difference of the substitution have been used to postulate the pathogenicity of many missense variants (3,4) and there is a need for functional understanding to validate such analysis. Currently, without a clinical assay for BRCA1 activity, the discovery of the majority of missense variants leaves both carriers and their families in an ambiguous position.

Single exonic nucleotide mutations not associated with transcript stability, splicing or translational efficiency presumably confer loss of BRCA1 tumour suppressor ability through the loss of an activity brought about by protein conformational change. The BRCA1 N-terminal region has been reported to interact with seven protein partners and therefore potentially participates in several cellular activities (5–10). Hence, in common with the study of many disease-associated proteins, a key challenge is to determine which interaction and activities are relevant to the disease process and which are not.

An approach frequently taken in classifying a particular protein:protein interaction as significant has been to test the interaction with a disease-associated mutation in one of the protein partners. This approach may result in ambiguity when the disease-associated mutations tested have significant structural consequences (predicting an apparent plethora of significant interactions). The early discovery of BRCA1 and the subsequent reporting of variants from a population likely to be enriched for disease in a public database, the BIC, has provided a resource with which to more thoroughly test potentially important interactions. In the current study, we have made extensive use of, and reference to these variants to examine the likely relevance of the BRCA1 E3 ubiquitin ligase activity to its tumour suppressor function.

The BRCA1 ubiquitin ligase is capable of forming covalently linked chains of ubiquitin on a substrate. Endogenous BRCA1-dependent ubiquitin conjugates occur at sites of double-strand DNA breaks and DNA repair in cells suggesting that BRCA1 ubiquitin ligase activity is linked to the DNA damage repair role of BRCA1 (11). The activity has also been linked to the control of centrosome duplication and microtubule nucleation, transcription and chromosome deacetylation (12–15). Although classically thought of as a pathway for protein degradation, it is now becoming clear that the post-translational addition of ubiquitin may act as a signal for other pathways (reviewed in (16)). In vitro, the BRCA1 ubiquitin ligase activity requires interaction with two proteins. The N-terminus of BRCA1 interacts with BARD1 to form a heterodimer. The heterodimer has substantial E3 ubiquitin ligase activity in vitro with the UbcH5 class of E2 ubiquitin conjugating enzymes, particularly UbcH5a (17,18). Importantly, loss of the activity is likely to occur in some forms of BRCA1-mediated cancer, as disease-associated mutations that substitute Zn$^{2+}$-binding residues within the BRCA1 RING affects BRCA1 ubiquitin ligase activity in vitro (19,20). However, as these mutations are structurally deleterious, it is unclear whether loss of ubiquitin ligase activity is causally linked to loss of tumour suppressor activity or an activity lost in conjunction with another, more relevant, interaction.

Based on the significant body of structural work that describes the BRCA1 N-terminal region, a proportion of the 44 patient missense variants would be expected to inhibit the BARD1 interaction, and a proportion inhibit the E2 enzyme interaction, while the impact of the remainder cannot be easily predicted. The BRCA1:BARD1 interface is formed of the two helices that flank the BRCA1 RING and mutational analysis has shown that the hydrophobic core formed at the interface is required for this interaction (21,22). The interface BRCA1 makes with the E2 has been predicted to be formed of residues in the two Zn$^{2+}$-binding loops and central helix of the RING domain. A missense mutation in a residue within this region prevented the E2 interaction and also ubiquitin ligase activity (20). Thus, patient mutations located close to the predicted E2-binding face are also expected to inhibit E2-binding and ubiquitin ligase activity. Missense alterations that cause a loss of interaction with either BARD1 or the E2 ubiquitin conjugating enzyme would be expected to inhibit significant BRCA1 ubiquitin ligase activity in vitro (17,20).

This study employs a comprehensive examination of missense variants derived from patient DNA to explore the relevance of BRCA1:protein interactions required for the E3 ubiquitin ligase activity of BRCA1. Our data show that loss of binding with the E2 component of the cascade correlates with variants found in a population enriched for a personal and family history of breast and ovarian cancer, ubiquitin ligase activity and theoretical predictions of pathogenicity. The data reveals the particular sensitivity of the BRCA1:E2 interaction to missense substitution within and beyond the predicted E2 interaction face and has implications for the clinical interpretation of missense variants within the N-terminus of BRCA1.

RESULTS

Selection of mutants from a randomly mutagenized BRCA1 library for loss of E2- or BARD1-binding identifies disease-associated mutations

If BRCA1-mediated tumourigenesis is promoted through the loss of binding to components of the ubiquitin ligase cascade we predicted that a screen for substitutions that inhibit E2 or BARD1 protein interactions from a randomly generated series of BRCA1 variants should identify substitutions similar to non-polymorphic variants reported in DNA from individuals with a personal or family history of breast cancer. We therefore screened a BRCA1 library generated through random mutagenic PCR by positive selection in yeast for loss of the BRCA1:BARD1 interaction and the BRCA1:E2 interaction (as illustrated in Supplementary Material, Fig. S1). These screens identified residues within BRCA1 that are required for interaction with each protein.

In a screen of the BRCA1 mutant library for substitutions that inhibit interaction with the E2 enzyme, UbcH5a, we identified 22 missense variants in 19 residues (Fig. 1, line 1). In the first screen against BARD1, we identified only truncating and frame-shift alterations (the truncation that deleted fewest residues removed amino acids from the C-terminal region of the peptide leaving aa 2–90), indicating that few or no single amino acid substitutions prevent BRCA1:BARD1 heterodimer formation in this system. We therefore conducted a third screen in which we selected mutations that inhibit the
interaction with BARD1 using a strain optimized for high-affinity interactions (23). From this experiment eight missense variants in seven residues were identified (Fig. 1, line 2).

We sought sequence information of patient variants from the literature (24,25) and from the BIC dataset. This carries BRCA1 sequence information from over 10 000 individuals, most with a personal or a family history of breast or ovarian cancer, hence it is a database that is enriched with deleterious mutations in BRCA1 (although the pathogenicity of the majority of individual variants is not known) (3).

Comparison of the BRCA1 variants selected from the screens against the E2 and BARD1 with non-polymorphic variants encoded by patient DNA show that both interactions are inhibited by substitutions in residues that because of their co-ordination with structurally integral Zn\(^{2+}\) have been strongly predicted, or confirmed, to be involved in disease (Fig. 1, lines 1–3). A further four selected variants from the E2 screen result in the substitution of residues altered in patient DNA in non-Zn\(^{2+}\)-binding residues (Fig. 1), including the substitution of M18. The M18T/K missense variant has been identified in several independent families with breast and ovarian cancer (24,25). No variant identified in either screen is a known polymorphism. Hence, selection against either ubiquitin ligase cascade component, although interacting with BRCA1 at different affinities, identified substitutions of residues found altered in patient DNA, but only the E2 screen selected patient variants outside the structurally integral Zn\(^{2+}\)-binding residues.

The E2 component is more sensitive to BRCA1 patient missense variants

As experimental selection against the BARD1 and the E2 interactions identified variants that correlated with patient variants, we next wished to test directly the relative sensitivities of each protein interaction to non-polymorphic missense substitutions in BRCA1 encoded by patient DNA. We therefore generated constructs of all variants encoded within the first 100 amino acids of BRCA1 logged in the BIC database; both those known to be disease-associated and those that are unclassified. (Where multiple substitutions of Zn\(^{2+}\)-ligating cysteines have been reported, one variant was chosen; in addition M18K was generated (25).) These 35 variants were then tested for interaction with BARD1 and the E2 enzyme, UbcH5a, in a two-hybrid assay.

Figure 2 shows that the wild-type BRCA1:BARD1 interaction was stronger than that of the BRCA1:E2 interaction as indicated by growth at high levels of the HIS3 competitor 3-amino-1,2,4-triazole (3AT) (Fig. 2A). A subset of BRCA1 missense substitutions inhibited growth with BARD1 at high 3AT concentrations (100 mM). These were mutations of Zn\(^{2+}\)-binding residues, either previously confirmed or strongly expected to be pathogenic (C24R, C44F, C61G and to a lesser extent H41R and C39R).

The E2:BRCA1 interaction was more sensitive to missense substitution, 16 variants showed no interaction with the E2 at the lowest concentration of 3AT (1 mM), with 21 BRCA1 variants failed to grow at 3 mM 3AT (Fig. 2A). All Zn\(^{2+}\)-binding residue mutations inhibited the E2 interaction at 3 mM 3AT threshold and all patient variants that reduced the BARD1 interaction at a high threshold (100 mM 3AT), inhibited the E2 interaction at a lower one (1 mM 3AT). This was also the case for a non-patient mutation (L82D) selected for disruption of the BRCA1:BARD1 interaction (data not shown). Hence, we were unable to identify a missense mutation of BRCA1 that inhibited the BARD1 interaction, but that did not disrupt the interaction with the E2 enzyme.

In a recent study, a combination of sequence alignment of BRCA1 orthologs and chemical difference of substitution was used to classify patient variants pathogenically (3). In the current study, seven of nine patient variants predicted to be deleterious using this approach inhibited the E2 interaction and all (five) variants predicted to be neutral, or of little significance, interacted with the E2 enzyme (Fig. 2C). (Experiments designed to test BRCA1 N-terminal homodimerization failed to reveal an interaction and we saw no interaction between BARD1 and UbcH5a (data not shown).)

Loss of E2 interaction correlates with loss of BRCA1 ubiquitin ligase activity

The sensitivity of the E2:BRCA1 interaction to missense substitution of BRCA1 suggested that the majority of non-polymorphic missense variants reported in the population enriched for family or personal history of cancer might inhibit BRCA1 ubiquitin ligase activity, as confirmed breast cancer-associated missense variants do. In order to test this, we performed a biochemical assay which examined the ability of the BRCA1:BARD1 heterodimer to form ubiquitin
chains in the presence of the E2 ubiquitin conjugating enzyme, UbcH5a. In this assay, co-purified wild-type BRCA1:BARD1 N-terminal complex was able to generate poly-ubiquitin chains and underwent extensive auto-ubiquitination (Fig. 2B and Supplementary Material, Fig. S2). Nineteen of the 35 BRCA1 variants co-purified with BARD1 showed reduced activity in the assay. As expected all seven mutations in Zn$^{2+}$-ligating residues inhibited ubiquitin ligase activity and the activity of the remaining variants correlated with the BRCA1:E2 interaction (compare Fig. 2A with B, summarized in C). All variants that interacted with E2 at 10 mM 3AT had ubiquitin ligase activity and all but one that did not interact at this threshold showed no activity. The E2-binding activity of a single variant, with a helical substitution (I89T) correlates poorly with its ubiquitin ligase activity. It is possible that the E2 interaction is stabilized by the presence of BARD1 in the biochemical assay.

**The BRCA1:E2 interaction requires integrity of the BRCA1 N-terminus**

The BARD1 interaction was less sensitive to missense substitution of BRCA1 than the E2 interaction and restricted to the flanking helices that form the BRCA1:BARD1 interaction face (Q12Y, L82P) and the Zn$^{2+}$-ligating residues of the RING (C39S/L, H41R, C44R, C47R and C61L) (Fig. 3H). However, the impact of these variants was measurable only at high concentrations of 3AT, or in a high threshold assay, suggesting that these mutations weaken the high-affinity BARD1:BRCA1 interaction. Conversely, the BRCA1
interaction with E2 was disrupted by many missense substitutions and at low 3AT concentrations. Modelling the BRCA1:E2 interaction on the solved RING E3:E2 complex of c–Cbl (shown in Fig. 3) and comparing it with the NMR solution structure of BRCA1:BARD1 reveals that the face between the RING-E3 and E2 (UbcH7) is not as extensive as the BRCA1:BARD1 interaction. The BARD1:BRCA1 interface is bigger (1337 versus 869 Å²), flatter and has a smaller gap volume, indicating that it is a tighter interaction. Both interactions have three hydrogen bonds but the BRCA1:BARD1 interaction also has two more salt bridges. Hence, single amino acid substitutions may be tolerated by the BRCA1:BARD1 interaction. Some of the amino acid substitutions of BRCA1 that inhibit the E2 interaction are located in the predicted E2 interface; in residues that co-ordinate with Zn²⁺ and that are within the central helix (Fig. 3C, E and H). However, the BRCA1:E2 interaction was also sensitive to non-E2 interface substitutions present within loops forming the cross-brace structure of the RING (Fig. 3D), at the join between the RING domain and flanking helices (Fig. 3F), as well as within the flanking helices (Fig. 3G). Substitutions that did not inhibit the E2 interaction are conservative in their nature (V11A, I15L, I21V, I42V, L87V), away from the E2 interface (D67E/Y, R71G, K38N) or in the distal portions of the α-helices (S4P, R7C, I90T, D96N, G98R and also Y105C, I124V and P142H (data not shown)) (illustrated in Supplementary Material, Fig. S4). Thus the locations and type of the majority of E2 disruptive variants

Figure 3. Model of the BARD1:BRCA1:E2 (UbcH5b) complex. The RING domain of BRCA1 (PDB 1JM7 chain A (2)) was superimposed on the RING domain of Cbl-UbcH7 (PDB 1FBV (29)) and the structure of UbcH5b (PDB 1W4U (31)) was then superimposed on the structure of UbcH7 in the Cbl–UbcH7 complex. (UbcH5b is 88% identical to UbcH5a and both have activity with BRCA1:BARD1 (18).) BRCA1 is shown in green, BARD1 in orange and UbcH5b in blue. (A) Filled atom-model of BRCA1:BARD1 with UbcH5b. (B) Ribbon representation of BRCA1:BARD1 and UbcH5b, the Zn²⁺ are denoted by red spheres. (C–G) Structural classes of BRCA1:E2 disruptive variants are shown and listed beneath. (H) Summary of BARD1 and E2 disruptive missense variants. Line 1 shows secondary structure information based on the BRCA1 NMR structure (2). Line 2 shows residues in the BRCA1:BARD1 interface (blue) (2) and residues identified by Brzovic et al., (20) whose resonances are perturbed by the binding of the E2 (UbcH5c) and are therefore likely to make up the E2 interface (black). Line 3 summarizes all the E2 disruptive substitutions identified in the study, and Line 4 the BARD1 disruptive substitutions.
illustrate that the integrity of the BRCA1 RING domain, the flanking helices and the relationship between these elements are required for interaction with the E2 (Table 1).

DISCUSSION

The identification of several interacting proteins have suggested alternative functions for the BRCA1 N-terminal region (6–10). Although we cannot rule out the possibility that this region participates in a function unrelated to ubiquitin ligase activity, in our hands BRCA1 N-terminal interactions with full length ATF, BAP1 and reported interacting regions of ER-alpha, MSH2 and p300/CBP could not be confirmed (data not shown).

Data reported here correlate missense variants from individuals with a personal or family history of breast cancer with loss of E2 enzyme interaction and ubiquitin ligase activity. Selection from a randomly generated library of BRCA1 mutations for variants that inhibit the interaction with BRCA1 ubiquitin ligase components, BARD1 or the E2 enzyme, UbcH5a, identified substitutions in residues found altered in patient DNA. The majority of patient variants inhibited the E2 interaction, and mutations in Zn$^{2+}$-binding residues reduced the already low-affinity BRCA1:E2 interaction (and weakened the high-affinity BRCA1:BARD1 interaction). Causal confirmation linking the loss of the BRCA1:E2 interaction with tumour susceptibility awaits further experiments where the subtle disruption of the E2 interaction, perhaps in an animal model, is shown to be tumourigenic. However, concordance of loss of BRCA1:E2-binding with variants confirmed or theoretically predicted to be pathogenic suggests that the BRCA1:E2 interaction may be an indicator of variant pathogenicity and may therefore form the basis of a functional assay. Further validation through investigation of BRCA1 families bearing currently unclassified variants, and continued reporting in public databases such as the BIC will be needed to confirm this for the purposes of diagnosis, counselling and clinical intervention.

Our data are unable to dissect BARD1 from the E2 interaction as a cause of loss of ubiquitin ligase activity for the Zn$^{2+}$-ligating residue class of mutations as these variants cause loss of binding to BARD1 and the E2 enzyme. This is presumably because of the deleterious impact these variants have on the N-terminal structure as a whole, preventing both correct folding of the RING domain and proper folding of the half helical bundle for high-affinity interaction with BARD1. Importantly, the inhibition of ubiquitin ligase activity, through loss of the E2 interaction, was not restricted to structurally detrimental mutations in Zn$^{2+}$-ligating residues,

| Table 1. Predicted impact of missense substitutions in BRCA1 that prevent interaction with the E2 ubiquitin conjugating enzyme UbcH5a |
|-----------------|-----------------|-----------------|
| Class          | Substitution    | Predicted effect of substitution (based on superimposition of BRCA1 (2) and UbcH5b (31) structures over RING E3:E2 structure of Cbl:UbcH7 (29)) |
| Zn$^{2+}$ ligand | C24R            | Loss of Zn$^{2+}$ ligand likely to weaken RING domain folding |
|                | C39S/R          | Hydrophobic to polar: expected to disrupt RING domain |
|                | H41L/Y/R        | Non-conservative substitution of a buried residue: likely to disrupt the RING domain |
|                | C44F/R          | Substitution disrupts salt bridge to BRCA1 Glu75: likely to weaken overall fold |
|                | C47G/T          | Introduction of more bulky or polar group to a buried residue: likely to weaken overall fold |
|                | C61G            | Charged substitution of largely buried hydrophobic group: likely to disrupt RING domain |
|                | C64G            | Charged substitution of buried hydrophobic group: likely to disrupt RING domain |
| RING domain    | I31T or N       | Introduction of a larger buried group likely to disrupt RING domain |
|                | T37R            | Disrupts salt bridge (to K4 in UbcH5b) and weaken binding to the E2 |
|                | K45E/T/N        | May disrupt salt bridge (to D59 of UbcH5b) and weaken binding to the E2 |
|                | L52F/H          | May disrupt H-bond (to S91 of UbcH5b) and to weaken binding to the E2 |
|                | S59R            | Lies close to E2 binding site; substitution likely to disrupt H-bonds or salt bridge |
|                | I68K            | Charged substitution of buried hydrophobic group: likely to disrupt RING domain |
|                | S72R            | Disrupts hydrogen bond to T69; predicted to weaken overall fold |
|                | T77M            | May form H-bond to S80 in some conformations; substitution to a hydrophobic surface residue predicted to weaken binding by removal of H-bond |
|                | L63F            | Introduction of a larger buried group likely to disrupt RING domain |
| Interface with UbcH5a | E29V          | Disrupts salt bridge (to K4 in UbcH5b) and weaken binding to the E2 |
|                | K50L/E          | May disrupt salt bridge (to D59 of UbcH5b) and weaken binding to the E2 |
|                | K56N            | May disrupt H-bond (to S91 of UbcH5b) and to weaken binding to the E2 |
|                | Q60L            | Charged substitution of buried hydrophobic group: likely to disrupt RING domain |
|                | K65M            | May form part of the E2 interface or may be a structural disruption (I26 forms packing interactions within the RING domain) |
|                | I26N            | Loss of hydrophobic contact with the E2 enzyme, proline substitution likely also to disrupt backbone |
|                | L28P            | Preventing folding |
|                | G57R            | BRCA1:Cbl backbones differ at this point, may alter packing with E2 or within RING |
| Orientation of helical bundle to RING | F79S            | F79 lies at interface between RING and helical domains. Mutation to a polar group is predicted to alter RING-domain to helical-domain packing |
| Helical bundle | I15T            | Polar substitution of buried hydrophobic group: likely to induce distortion to helices |
|                | M18K T          | Polar/charged substitution of buried hydrophobic group: likely to induce distortion to helices |
|                | I89T or N       | Polar substitution of buried hydrophobic group: likely to induce distortion to helices |
but was also caused by the majority of reported patient variants, whose structural impact is more subtle. The profile of BAR1 and E2 disruptive variants overlap; while E2 disruptive substitutions include Zn$^{2+}$ residue binding mutants, they are also located throughout the N-terminal region, as are patient variants (Figs 1 and 3H).

Our findings have revealed the fragility of the BRCA1:E2 interaction, which is sensitive to missense substitution in all elements of the BRCA1-N-terminus. The high density of missense variants in the BIC patient database in this region of BRCA1 may reflect a requirement for interaction with the E2 enzyme in tumour suppression. In cells, the formation of ubiquitin conjugates by exogenous BRCA1 is dependent both on BARD1 and on an intact BRCA1:E2-binding face, suggesting that loss of the E2 interaction alone is sufficient to inhibit BRCA1 ubiquitin ligase activity in cells (11).

However, as substitution of Zn$^{2+}$-binding residues are many times more frequent in patients than those elsewhere in the N-terminal region, it may be that the additional loss of interaction with BARD1, perhaps resulting in reduced BRCA1 protein stability (19,26), or loss of a further function, causes greater inhibition of BRCA1 tumour suppressor function, and more frequently results in tumourigenesis than loss of the E2 interaction alone.

We speculate that a weak interaction between the E3 RING and its E2 component may be a necessary part of enzyme turnover in the generation of ubiquitin chains. The E3 RING:E2 interaction is a common biological interface, with more than 300 RING-bearing proteins in the human database, many of which may function as E3 ligases for ubiquitin or ubiquitin like molecules (27). Hence, if the conclusions made in relation to the BRCA1 RING:E2 interaction can be extrapolated to other RING:E2s, then we would expect perturbations in this interactive face to occur in other diseases and syndromes.

MATERIALS AND METHODS

Plasmid construction

The yeast expression vectors used contained human BRCA1-N-terminal amino acids 2–101 expressed as a fusion with the VP16 and LacZ genes (pSHM.1.LacZ), and full length E2 ubiquitin conjugating enzyme, human UbcH5a, or human BARD1 amino acids 27–146 expressed as fusions with the DNA-binding protein LexA (pLexA). UbcH5a cDNA was a kind gift from Roger Everett, MRC Virology Unit, Glasgow. BRCA1 and BARD1 sequences were amplified from human cDNA. For bacterial expression of human BRCA1: BARD1 heterodimer a bi-cistronic expression vector encoding six histidine-tagged BARD1 amino acids 1–147 and six histidine-tagged BARD1 amino acids 26–142 was generated by amplification of human BRCA1 and BARD1 cDNA templates and cloned into pET15b. All constructs were verified by DNA sequencing.

Generation of BRCA1 mutant library by PCR mutagenesis

Mutagenic PCR exploited the 1 in 500 bp error rate of Taq polymerase (Promega). The VP16-BRCA1 aa 2–101-LacZ construct was used as template and reactions contained standard PCR buffer and dNTP concentrations as described previously (22). The primers used annealed to elements in the vector; the forward primer (5′–3′) annealed to the VP16 sequence, TATATATGTTTTTCTAATGTGTTAAAGT, and reverse primer (5′–3′) to the LacZ, TGTGCTGACTTACCAGATGGGACACTCTAA. The product from 30 cycles of 95°C for 30 s, 50°C for 30 s and 68°C for 2 min was purified and combined at a ratio of 2:1 of product to digested (BamHI/ BgII) pVP16-LacZ vector in yeast Y1671 or Y1584 cells (transformed together with pLexA-BARD1 amino acids 27–146, or pLexA-UbcH5a). Yeast gap-repair in situ ligated the randomly mutated library.

Site-directed mutagenesis

Single base changes were introduced by 21 bp forward and reverse primers, perfectly homologous to the DNA template, 10 bp either side of the substitution. Pfu-turbo proof-reading polymerase (Stratagene) was used to generate product from 30 cycles of 95°C for 30 s, 50°C for 1 min and 68°C for 24 min. Transformed bacteria were selected on X-gal before sequencing.

Yeast assays, library screens, β-galactosidase assay and plasmid rescue

The yeast two-hybrid strain, L40 (a kind gift of Richard Goodman, Vollum Institute, OR, USA), and yeast split-hybrid strains, Y1671 and Y1584 (QBIogene, Hatfield, UK and John Crispino, University of Chicago), were used as described previously (22,23). In the split-hybrid screens 75 000 transformants per pair (BRCA1 versus BARD1, BRCA1 versus E2-UbcH5a) were screened, of which ~10 000 grew on selective media; 100 clones were picked and re-streaked on selective media and β-galactosidase assays performed to eliminate frameshift, nonsense mutations or clones with poor protein expression. DNA from these was then transformed into bacteria and pVP16-BRCA1-LacZ containing bacteria discriminated from pLexA-BARD1 or pLexA-UbcH5a plasmid transformants by selection on X-gal. These were sequenced and those with two or more amino acid substitutions or mutations in the VP16 transactivation domain were discarded.

Protein production and ubiquitin ligase assays

BRCA1:BARD1 heterodimer constructs were expressed in Escherichia coli BL21-CodonPlus (DE3) cells (Stratagene). And purified using nickel resin as described previously (24). Co-purification was confirmed by resolution on 10–20% Tis-Tricine gel followed by Coomassie stain. Ubiquitin ligase activity assays were performed as described by Boutilier et al. (28).

Interrogation of protein structure

The RING domain of BRCA1 (PDB 1JM7 chain A) was superimposed on the RING domain of Cbl-UbcH7 (PDB 1FBV (29)) using Swiss PDB-viewer (30). The structure of UbcH5b (PDB 1W4U (31)) was then superimposed on the
structure of Ubch7 in the Cbl–Ubch7 complex. The reported mutants in BRCA1 were then assessed using this model for the likely outcome of the mutation by visual inspection.

SUPPLEMENTARY MATERIAL
Supplementary Material is available at HMG Online.

ACKNOWLEDGEMENTS
Thanks to Dr Roger Everett for reagents, critical comment and support, Sheila Fisher for statistical analysis and Drs Caroline Whitehouse and David Grimwade for critical reading of the manuscript. E.S. and J.R.M. are supported by MRC Programme Grant G6900577, L.P. is supported by the Generation Trust.

Conflict of Interest statement. None declared.

REFERENCES