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Functional analysis of BARD1 missense variants in homology directed repair of DNA double strand breaks

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Abstract

Genes associated with hereditary breast and ovarian cancer (HBOC) are often sequenced in search of mutations that are predictive of susceptibility to these cancer types, but the sequence results are frequently ambiguous due to detection of missense substitutions for which the clinical impact is unknown. The BARD1 protein is the heterodimeric partner of BRCA1 and is included on clinical gene panels for testing for susceptibility to HBOC. Like BRCA1, it is required for homology-directed DNA repair (HDR). We measured the HDR function of 29 BARD1 missense variants, 27 culled from clinical test results and two synthetic variants. 23 of the assayed variants were functional for HDR; of these, four are known neutral variants. Three variants showed intermediate function, and three others were defective in HDR. When mapped to BARD1 domains, residues crucial for HDR were located in the N- and C- termini of BARD1. In the BARD1 RING domain, critical residues mapped to the zinc-coordinating amino acids and to the BRCA1-BARD1 binding interface, highlighting the importance of interaction between BRCA1 and BARD1 for HDR activity. Based on these results, we propose that the HDR assay is a useful complement to genetic analyses to classify *BARD1* variants of unknown clinical significance.

Keywords

BARD1; BRCA1; homology directed repair; breast cancer

Introduction

Mutations in the *BRCA1* and *BRCA2* genes are the most common causes of hereditary breast and ovarian cancer (HBOC)(MM#114480) and are associated with a lifetime risk of breast cancer of 50–85%, and of ovarian cancer of 15–40% (Antoniou et al., 2003; Antoniou

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Conflict of Interest

The authors report no conflicts of interest that might influence the interpretation of results of this study.

et al., 2010; Chen and Parmigiani, 2007; Levy-Lahad and Friedman, 2007; Tai et al., 2007). It is now apparent that mutations of several other genes, such as *BARD1*, *PALB2*, and *BRIP1* (Turnbull and Rahman, 2008), contribute to familial breast cancer. The *BRCA1* and *BARD1* (MIM# 601593) proteins function as obligate heterodimers (Wu et al., 1996; Wu et al., 2010), and *BARD1* function is critical to the *BRCA1* tumor suppressor activity (McCarthy et al., 2003; Shakya et al., 2008). *BRCA1* and *BARD1* heterodimerize via their N-terminal RING domains, and both *BRCA1* and *BARD1* have C-terminal tandem BRCT domains that are required for protein-protein interactions important for the DNA damage response (Edwards et al., 2008; Mirkin et al., 2008; Nelson and Holt, 2010).

Among sequence changes in *BRCA1* that have been observed in individuals with reported history of HBOC, mutations that truncate the protein are clearly pathogenic (John et al., 2007; Ludwig et al., 2001; Novakovic et al., 2012). However, the vast majority of reported missense substitutions in *BRCA1* have unknown clinical impact; such variants are referred to as variants of unknown significance (VUS). VUS pose a diagnostic conundrum for cancer genetics care providers, as missense substitutions are common in aggregate, but each one is typically too low in prevalence or frequency to assign clinical impact, and pedigrees with multiple tested individuals are typically not available for segregation analysis.

Just as in the case of *BRCA1*, germline mutations in *BARD1* have been associated with an increased risk of breast cancer (De Brakeleer et al., 2010; Ghimenti et al., 2002). Truncating *BARD1* mutations have also been observed in individuals with ovarian cancer (Walsh et al., 2011). Unlike *BRCA1* and *BRCA2*, the proportion of hereditary breast cancer thought to be due to pathogenic mutations in *BARD1* is low and is estimated to be less than 1%. As a result, lifetime cancer risks associated with *BARD1* are not well characterized and robust penetrance studies have not been completed. There is not sufficient data on *BARD1* in order to definitively classify it as a susceptibility gene for HBOC (Easton et al., 2015; Richards et al., 2015). Nevertheless, *BARD1* is a strong candidate gene for HBOC and is included on clinical gene panels for testing for susceptibility to HBOC, and as such many missense substitutions in *BARD1* have been identified. The knowledge gap remains in linking sequence variations to disease, however, and most of the missense substitutions in *BARD1* are classified by clinical testing laboratories as VUS. Determining whether a missense substitution would damage protein function versus a functionally neutral variant is critical for preventive cancer care strategies among women with a personal or family history of HBOC if *BARD1* is indeed a susceptibility gene.

In the last five years, the number of genes included on testing panels for susceptibility to HBOC has expanded significantly, and the number of individuals being tested has also increased. In addition to germline genetic testing, tumor testing has also become more commonplace. With the increased number of genetic tests and genes being evaluated, more sequence changes are being detected; the vast majority of missense substitutions are VUS. Determination of the consequences of missense substitutions is a challenge in general for all genes. A recent survey of sequence results from breast cancer susceptibility panels (including *BARD1* and the *BRCA* genes) found that 42% of all tests of a 25-gene panel have findings of a VUS in one or more of the genes (Tung et al., 2015).

One strategy to identify the consequences of VUS is to analyze the effects of a missense substitution on cellular function of the gene product. Ultimately, the gene sequence is translated to protein function, and the effects of mutations are only important in the context of the protein. In the case of the BRCA1 protein, multiple functional assays have been done that address cell growth and DNA damage response (Bouwman et al., 2013; Laufer et al., 2007; McCarthy et al., 2003). BRCA1 function in DNA double-strand break repair via homologous recombination has been found to correspond with mutants and variants of known cancer predisposition phenotype (Ransburgh et al., 2010; Starita et al., 2015; Towler et al., 2013). Because BRCA1 functions as an obligate heterodimeric protein complexed with BARD1, it is reasonable to test BARD1 in the same functional assays.

In this study, we assayed the effects of 29 different missense substitutions on the function of BARD1. In the same homology-directed repair assay used previously for BRCA1, we assayed BARD1 variants identified in individuals undergoing clinical testing for HBOC. We infer that those BARD1 missense variants that are defective in the DNA repair assay could contribute to HBOC susceptibility, and such data could augment segregation studies and other information required for VUS classification.

Materials and Methods

Plasmids and siRNA

Wild-type and missense substituted BARD1 (NCBI Reference Sequence: NM_000465.3) were cloned into a pcDNA3 vector backbone containing a rabbit β -globin intron upstream of the BARD1 translation initiation site to drive expression of the 777 amino acid human BARD1 transgene. Missense substitutions were generated using oligonucleotides from IDT and the QuikChange Lightning Site-Directed Mutagenesis kit from Agilent. All amino acid residues were numbered with codon 1 as the initiation met codon. All plasmids were sequenced verified for mutations by Sanger sequencing. The BARD1 expression plasmid encoded a neutral change at residue 406 changing an arginine to a glutamine. All site-directed point mutations were made on this backbone containing a glutamine at residue 406, with codon 1 as the initiation met codon. This residue was changed back to arginine in the wild-type BARD1 construct used as a control for homology directed repair assays.

The siRNA with top strand sequence of AGCUGAAUAUUAUACCAGAdTdT is specific for the BARD1 3'-UTR.

Cell Culture

The homology-directed repair assay was performed in a cell line, HeLa-DR-13-9, containing a mutant GFP gene interrupted by the I-SceI restriction endonuclease site. Expression of I-SceI results in a double-strand DNA break that can be repaired by gene conversion with a second defective GFP gene, resulting in active GFP expression (Pierce et al., 2001; Ransburgh et al., 2010). Cells were cultured in DMEM containing 1% Penicillin/Streptomycin, 1% GlutaMAX, 10% bovine serum, and 1.5 μ g/mL puromycin. Transfections were done using Lipofectamine according to the manufacturer's recommended protocol. In the standard HDR assay, HeLa-DR cells were transfected with the BARD1 3'UTR specific

siRNA (5 pmole) plus BARD1 expression plasmid (300 ng) per well in a 24 well plate. One day following the first transfection, cells were transferred to 6 well plates, and 48 h after the first transfection, cells were transfected with siRNA (25 pmole), BARD1 expression plasmid (750 ng), and I-SceI expression plasmid (750 ng). Three days following the second transfection, cells were lifted off monolayers in trypsin and analyzed by flow cytometry using a FACSCalibur in the Analytical Cytometry Shared Resource at the Ohio State University Comprehensive Cancer Center. 10,000 cells were counted. Flow cytometry typically revealed 10–20% of the cells had converted to GFP-positive following expression of the I-SceI enzyme. Within a transfection set, results were normalized relative to the wild-type BARD1 plasmid in the same transfection set. Results were expressed as percentage of the wild-type response.

Immunoblots

Protein samples from cells leftover from flow cytometry analysis were extracted in 300 mM NaCl, 50 mM Tris pH 7.9, 1 mM EDTA, 5% glycerol, and 0.5% NP-40. Protein samples were resolved using 8% SDS-PAGE and transferred to PVDF membrane. Antibodies from Santa Cruz Biotechnology were used to detect BARD1 (1:500 dilution) and α -tubulin (1:1,000). Membranes were incubated in 1:20,000 rabbit and mouse fluorescent antibodies from LI-COR.

Immunoprecipitation

BARD1 expression plasmids were transfected into HeLa-DR cells as for the HDR assay with the exception that 10^7 cells were analyzed per sample. Protein extracts were subjected to immunoprecipitation analysis using antibody specific to BRCA1 (Sankaran et al., 2006) pre-bound to protein-A beads. Proteins bound to the antibody beads were washed thoroughly with PBS and denatured on the beads using SDS-PAGE loading buffer at 100°C prior to immunoblot analysis.

3-D structures

BARD1 missense substitutions were mapped to 3-D protein structures using PDB files in Jmol. The PDB ID for the BRCA1-BARD1 RING domain heterodimer is 1JM7 (Brzovic et al., 2001). The PDB ID for the BARD1 BRCT domain homodimer is 3FA2 (Fox 3rd, D., Le Trong, I., Stenkamp, R.E., Klevit, R.E. Crystal Structure of the BRCA1 Associated Ring Domain (BARD1) Tandem BRCT Domains, <http://www.rcsb.org/pdb/explore/explore.do?structureId=3FA2>).

In silico analyses

We used three functional prediction programs, SIFT (Sorting Intolerant from Tolerant) (sift.jcvi.org), Polyphen-2 version 2.2.2 (Polymorphism Phenotyping) (genetics.bwh.harvard.edu/pph2) (Adzhubei et al., 2010; Kumar et al., 2009; Ng and Henikoff, 2002) and PROVEAN version 1.1. (Choi et al., 2012) (provean.jcvi.org) to evaluate if the positions of BARD1 variants in our study were conserved. The underlying concept for these algorithms is that amino acid locations that are highly conserved across evolution are theorized to be important for protein function. SIFT, Polyphen-2 and

PROVEAN algorithms use slightly different means to assess whether a particular amino acid residue is more likely to be important for protein function using weighted conservation scores as well as type of amino acid substitution. For Polyphen-2 and SIFT programs we used protein identifier GI 32949402 for BARD1 and for PROVEAN, we input the FASTA protein sequence for BARD1 (NP_000456). For SIFT analysis we used SIFT Blink and used the estimates for each substitution that were generated from the scaled probabilities for the entire protein. The SIFT algorithm yields a score from 0 to 1, in which 0 indicates that the substitution is predicted to impact protein function and > 0.05 indicates that the substitution is predicted to be tolerated in the protein. For PROVEAN we utilized PROVEAN HUMAN PROTEIN BATCH and batched all variants for analysis. As this also provided SIFT scores, we confirmed that these were similar to the analysis using SIFT Blink.

To evaluate the potential effects of missense variants on splicing we used Human Splicing Finder (HSF) and Maximum Entropy Model (MaxEnt) using the Human Splicing Finder (HSF) version 3.0 website (www.umd.be/HSF3/HSF.html) (Desmet et al., 2009). We used the “analyze mutations” analysis. For each reference and variant allele being assessed for differences, we included the 500 basepairs of sequence surrounding the variant, which included intron and denoted the first and last positions of the exons. The published threshold for defining a splice acceptor or donor site for HSF is 65 and for MaxEnt is 3. When a variant introduced a difference in score variations of greater than 10% for HSF and 30% for MaxEnt, it was considered to either break or introduce a splice site.

Statistical analysis

All BARD1 variants were tested in triplicate, and results were normalized within an experimental set to the fraction of GFP-positive cells in the rescue with the wild-type BARD1 expression plasmid. The student's t-test was applied to determine whether results for a BARD1 variant/mutant significantly differed ($p < 0.05$) from wild-type rescue or differed from rescue with the empty plasmid.

Ethical compliance

The Ohio State University Institutional Review Board prospectively reviewed and approved this study.

Results

Homology-Directed Repair

The homology-directed repair (HDR) assay employs a HeLa-derived cell line with a reporter construct containing a recognition sequence for the I-SceI restriction endonuclease flanked by non-functioning GFP. Expression of I-SceI via a plasmid induces a DNA double strand break that is repaired via homology present in a second GFP sequence, producing functional GFP (Pierce et al., 2001). HeLa cells are competent for HDR and without inhibition, 10–20% of the cells are converted to GFP-positive by transfection of I-SceI (outlined in Figure 1A). The standard assay included two transfections, one to deplete endogenous BARD1 using siRNA targeting the 3' UTR and simultaneously express the plasmid-encoded BARD1 that is resistant to the siRNA. Two days later, the siRNA and the plasmid are transfected

again plus a second plasmid that expresses the I-SceI endonuclease. Since both BARD1 and BRCA1 are stable proteins during S, G2, and M phases and degraded following mitosis (Choudhury et al., 2004), a protocol that used a single transfection would not deplete the endogenous BARD1 before the I-SceI was expressed. In the time frame of the experiment, there was no evident toxicity due to the depletion of functional BARD1. In earlier studies, the effects of missense substitutions in BRCA1 were measured for HDR function using this protocol, and it was found that missense variants associated with predisposition to HBOC were deficient in HDR, whereas missense variants that were classified as neutral were functional in HDR (Ransburgh et al., 2010; Starita et al., 2015; Towler et al., 2013).

The 29 missense substitutions we selected for testing were spaced throughout the BARD1 gene, covering the three characterized domains: RING, ankyrin repeats, and BRCT (Figure 1B). We focused on multiple missense substitutions across the BARD1 RING, the dimerization domain for binding BRCA1. We also tested multiple substitutions in the BRCT domain. One variant, p.L44R, which had previously been shown to interfere with BARD1 binding to BRCA1, was included as it was expected to be deficient in HDR (Morris et al., 2002; Xia et al., 2003). We included a few missense variants that were classified as neutral by allele frequency, which we predicted would have no effect on HDR. Additional variants for study included those of uncertain significance but had been observed in women undergoing clinical testing (Table 1).

In this study, we applied the HeLa cell based HDR assay to the BARD1 protein. BARD1 depletion via transfection of siRNA specific for the 3'-UTR of the mRNA decreased homology-directed repair to approximately one twentieth of the control sample (Figure 1C, compare lanes 1 and 2). Transfection of the wild-type BARD1 in an expression plasmid rescued to 68% of control levels (Figure 1C, lane 3). All homology-directed repair results were therefore normalized to wild-type BARD1. Interestingly, our original BARD1 expression construct had a novel p.R406Q substitution, and this version of BARD1 fully restored HDR activity to the same level as seen in cells without BARD1 inhibition (Figure 1C, lane 19). The p.R406Q missense variant has not been described in an individual and likely was the result of a change that occurred during plasmid manipulations. All site-directed mutagenesis plasmids used in Figure 1C were made from the construct containing the p.R406Q variant.

The rescue activity of the 29 missense substitutions fell into three categories: functional, intermediate, defective (Figure 1C). These categories were determined using the student's t-test for comparing the observed HDR activity to the activity detected with rescue of the wild-type BARD1 (lane 3) or to rescue with the vector (lane 2). Defective mutants of BARD1 had results for which the t-test was significantly different from the wild-type but not significantly different from the empty vector. Variants judged as functional were not significantly different from the wild-type but significantly different from the empty vector. Those mutants judged as intermediate had t-test results indicating significant differences from both wild-type and empty vector. It is important to emphasize that these statistical tests should not be used for clinical classification, but reflect on whether the biological function of the variant BARD1 protein is different from wild-type and different from the null mutant. On the graph in Figure 1C the dividing lines were approximately at 60% of wild-type and

14% of wild-type dividing functional, intermediate and defective, respectively. The p.W34R was classified as intermediate since its HDR activity (lane 6) was statistically different from both the wild-type and the vector control. Of the 29 missense variants tested, four were defective in HDR (p.L44R, p.C53W, p.C71Y, p.G623E), one had intermediate function (p.W34R), and 24 were fully functional (Figure 1 and Table 1). In subsequent experiments (see Figure 3), two BARD1 missense variants were reclassified as intermediate (p.G623E and p.A724V).

Expression levels of BARD1 substitution variants

We analyzed protein expression of the BARD1 in transfected cells. Clearly, the phenotype of HDR function was greatly diminished in cells that were BARD1 depleted, and this phenotype was rescued with the BARD1 expression plasmid, proving that the measured phenotype was due to the BARD1 protein. The importance of analyzing BARD1 expression was to determine whether low HDR activity of any BARD1 variant was due to poor protein expression from the transfected plasmid. Using the remaining cells following flow cytometry for the HDR assay, we extracted soluble protein and extracts were analyzed for protein content by immunoblot analysis. The detected endogenous BARD1 band, migrating at 97 kDa, is a triple band with a major cross-reacting protein at the leading edge of this band. The exogenously expressed epitope-tagged BARD1 is slightly shifted from the cross-reacting species, facilitating its detection. The level of depletion of BARD1 was evident in the extracts from cells transfected with the vector only (Figure 2A, lanes 2, 16, 30, and 37; to aid in interpretation, the BARD1 band was indicated with an arrowhead). Certainly, the cross-reacting band made identification of the depleted BARD1 difficult, but in all repeats, the effect on the HDR assay was about a 20-fold decrease in repair, indicating that this level of protein depletion was sufficient to block DNA repair. This decrease was rescued by the wild-type BARD1-expressing plasmid, stringently indicating that these results were not due to off-target effects of the siRNA. Expression of the BARD1 protein from the transfected plasmid was variable, depending on the sequence variant. Most variants expressed BARD1 protein at about 2- to 5-fold the levels of the endogenous protein (Figure 2A) although some, such as p.V507M was present at concentrations as high as ten times the level of the endogenously expressed BARD1 protein (lane 18). It is possible that over-expression of a protein can result in masking of a partial defect. In the case of p.V507M, which had the highest level of expression, we think such an effect was unlikely since its repair activity was higher than that of the wild-type. The p.W34R mutant/variant, the substitution that had intermediate activity in HDR (Figure 1), was expressed at very close to endogenous levels of BARD1 (Figure 2A, lane 5), suggesting that its intermediate function was not an artifact of protein expression level. The HDR results for several variants were decreased relative to the wild-type but were still within the functional range: p.N356I, p.M621I, p.A721T, and p.A724V (Figure 1, lanes 17, 22, 29, 30). The levels of expression for these four variants were about the same as the wild-type BARD1 (compare Figure 2A lanes 3, 13, and lanes 17, 20, 26, 27). We interpret from these expression results that the normal HDR activities of these BARD1 variants was not due to very high expression of the variant protein masking a true defect.

Conversely, some BARD1 proteins were not detectable; p.C53W, p.C71Y, and p.P84S were present at very low abundance (lanes 8–10 and 34). Although present at very low concentration, p.P84S was fully functional in the HDR assay (Figure 1), indicating that the little BARD1-P84S present was sufficient for binding to BRCA1 and stimulating HDR. The p.C53W and p.C71Y proteins were absent from soluble extracts (Figure 2A) but were readily detected at concentrations higher than the endogenous BARD1 protein from cells that were lysed directly into 1% SDS containing buffer (Figure 2B, lanes 4 and 11). We thus interpret that these two variants expressed well but were insoluble and unavailable for binding to BRCA1 and unavailable to catalyze DNA repair. We note that the non-functional p.L44R and p.G623E mutants were present in soluble extracts at higher levels than the wild-type protein (Figure 2A, lanes 7 and 21), and we suggest that these mutants have appropriate conformation but disrupt a protein-protein interaction critical for the DNA repair function. The RING domain variant, p.L44R, is known to be defective for binding BRCA1 (Morris et al., 2002; Xia et al., 2003).

It has been shown that in *Xenopus laevis* when the BRCA1 or BARD1 homologous protein was deleted, the partner protein was not expressed (Joukov et al., 2001). We thus tested whether depletion of endogenous BARD1 and rescue expression of a defective mutant affected soluble BRCA1 content in the cell. Using the same soluble extracts for analyzing BARD1 expression, BRCA1 abundance was evaluated by immunoblot. BRCA1 protein was observed as a doublet or triplet of bands that migrated on SDS-PAGE consistent with a mass of about 250 kDa. BRCA1 protein showed little variation when comparing samples that expressed endogenous, empty vector, wild-type, or even insoluble p.C71Y BARD1 proteins (Figure 2A, middle panel, lanes 1–3 and 9). There was a slight decrease in BRCA1 content in lane 34 in which a second p.C71Y sample was analyzed, but this lane was under loaded. We interpret this result with stable concentration of BRCA1 to indicate that within the time frame of the experiment, the defective BARD1 proteins did not affect BRCA1 abundance, and we interpret that changes in HDR due to BARD1 variants were due to BARD1 function and not an indirect readout of BRCA1 protein content.

Since our original “wild-type” BARD1 expression plasmid was found to have a missense change encoding p.R406Q, all of the variants we have tested were in the presence of this change. In the HDR assay, the p.R406Q was found to be functional and at 150% of the level of the wild-type (Figure 1C). We tested whether the p.R406Q change co-occurring with a second missense variant could have affected results. We selected five BARD1 constructs to correct this p.R406Q sequence and compare to the results in Figure 1C. We chose p.G32V, p.N356I, and p.A724V since these variants were judged functional but had lower HDR values than the wild-type and could potentially have results skewed by a hyperactive p.R406Q. In addition, we corrected the p.W34R intermediate mutant and the p.G623E defective to test if their HDR assay results were affected by the p.R406Q in the protein. Results in Figure 3 were similar to those in Figure 1C. The depletion of BARD1 and rescue with empty vector resulted in a 20-fold reduction in HDR activity. Interestingly, rescue with the wild-type BARD1 plasmid fully restored HDR levels, suggesting that the functional BARD1 proteins could vary to some extent from 68% of endogenous, as was the case of the wild-type BARD1 in Figure 1C, to 100%. The p.G32V and p.N356I variants were clearly in the functional range. The p.A724V was 66% of wild-type in our original studies (Figure 1C)

and in our subsequent studies using the corrected version (Figure 3) it was 59% of wild-type. Interestingly, that slight shift in outcome and low variation in the replicate experiments made it significantly different from both wild-type (functional) and empty plasmid (non-functional). Thus, we reclassified BARD1-A724V as intermediate. The defective mutant p.G623E changed from 19% to 14%, and even though results with this p.G623E substitution had a lower value on the HDR assay, the statistical t-test put it in the intermediate range as it was significantly different from the empty vector. The intermediate mutant p.W34R changed from 40% to 31% and remained statistically intermediate in phenotype. The BARD1 proteins expressed well (Figure 3, lower panel). From the results in Figure 3, we interpret that the p.R406Q change in the backbone of the BARD1 construct caused a minimal increase of the HDR levels. The consistency in results among replicate experiments (with five independent transfection assays) in Figure 3 indicated statistically significant changes that were not apparent in the first experiment, thus changing classification to the intermediate phenotype for two variants.

Effect of BARD1 mutants on binding to BRCA1

Using truncated BRCA1 and BARD1, it is well-established that these two proteins interact via the RING domains of each protein (Wu et al., 1996). Using full length BRCA1 and BARD1, it had been found that truncation of the C-termini of either protein decreased the amount of the partner protein found in complex, suggesting that the C-termini also interact and contribute to the stability of the heterodimers (Simons et al., 2006). We thus tested whether the BARD1 mutants that had HDR activity below 50% could bind to BRCA1. We excluded the p.C53W and p.C71Y mutants since these did not express soluble BARD1 protein, and we included two functional variants for comparison. Cells were transfected with the appropriate BARD1 expression plasmid and soluble extracts were incubated with antibody specific for BRCA1 and immunopurified and detected on immunoblots via the HA epitope on the plasmid encoded BARD1. The BRCA1 antibody bound to protein residues 400–1100 (Sankaran et al., 2006), which would be unlikely to impact BRCA1-BARD1 binding. We found that the wild-type, p.W146C, and p.N356I variants all bound to BRCA1 (Figure 4, lanes 7, 10, 11), and these all were active in HDR. By contrast, the p.W34R, p.L44R, and p.G623E mutants of BARD1 did not bind to BRCA1. p.L44R was known to block binding to BRCA1 (Morris et al., 2002), and p.W34R also did not bind (L. Starita, unpublished observations). The lack of binding of the p.G623E mutant confirms that the C-terminus of BARD1 contributes to BRCA1 binding.

Effects of nucleotide changes on accurate splicing of *BARD1* mRNA

We evaluated 26 variants in our study for *in silico* predictions on splice junctions using Human Splicing Finder (HSF) and Maximum Entropy Models using the HSF website (Desmet et al. 2009). Eleven of the 26 variants were predicted to affect splicing by HSF and six of the 26 variants were predicted to affect splicing by MaxEnt. However, only four variants, p.W146C, p.V695I, p.A724V, and p.S761N, were predicted to affect splicing by both algorithms using the recommended cutoffs for differences. Splicing changes may lead to protein truncation in aberrantly spliced mRNA copies. Whether these splicing changes occur *in vivo* or whether a predicted effect in splicing only affects a fraction of the total pool of BARD1 encoding mRNA is unclear. Since the HDR assay used cDNA encoding BARD1,

the results could only be interpreted to define protein function, and those variants found to be functional at the protein level in this assay may have defects at the level of RNA splicing.

Comparison of clinical knowledge to HDR activity for BARD1 missense substitutions

In this study, we measure the impact of a missense variant of BARD1 on HDR activity. It is not known whether the HDR function of BARD1 is linked to tumor suppressor activity or whether the HDR assay can be used to predict clinical predisposition to HBOC. As more clinical data of *BARD1* missense variants become available, assay systems such as the one used in this study may associate a measurable activity with tumor suppressor function. Of the 29 variants tested in this study, four can be considered likely neutral based on their high frequencies in the general population. Our findings confirm that the four BARD1 variants described as neutral/benign in ClinVar (p.V507M, p.R658C, p.I692T, and p.R731G) are functional in DNA repair (Figure 1B–C). The p.L44R mutant has been shown to be defective in vitro, and for this reason it served as a positive control, but the clinical impact of p.L44R is unknown. Of the 23 missense variants of uncertain clinical significance that were tested, 19 were functional in the DNA repair assay. None of the three variants that were defective for HDR have been reported in dbSNP or in the Exome Variant Server (EVS), suggesting that they are rare. It is as yet unclear whether defective mutants of BARD1 predispose to cancer since they have not been detected in cancer cases at this time, but we suggest that a BARD1 mutant that causes a defect in HDR is a strong candidate for predisposing for HBOC, just as is the case for BRCA1.

Evolutionary conservation of the positions of BARD1 variant residues

We determined if the positions of BARD1 variants in our study were conserved through evolution using three functional prediction programs, SIFT (Sorting Intolerant from Tolerant), Polyphen-2 (Polymorphism Phenotyping) (Adzhubei et al., 2010; Kumar et al., 2009; Ng and Henikoff, 2002) and PROVEAN (Choi et al., 2012). The four variants that were defective for HDR by our assay were predicted to be damaging by SIFT, to probably affect protein function by Polyphen-2 and to be deleterious by PROVEAN (Table 2). The p.W34R, which had an intermediate HDR activity, was also predicted to affect function by all algorithms. A number of additional variants that were functional for HDR in our assay were also predicted to affect protein function (Table 2). One of the limitations of the prediction algorithms is that they depend on sequence diversity based on a calculated conservation value across selected sequences near the variant, and all of the calls predicted to affect protein function by SIFT (scores <0.05) were low confidence calls due to not enough sequence diversity within the aligned sequences. This limitation may explain the discrepancy between the functional data and the SIFT predictions. Of the three algorithms tested, PROVEAN performed best, with PROVEAN predictions having 90% concordance (26 of 29 variants) between the prediction and the functional data.

Analysis of BARD1 variants in the context of the 3-D structure

We analyzed the substituted residues on the 3-D structures available for BARD1. In the BARD1 RING domain, residues that coordinate the Zn atoms are required for maintaining its structure (Figure 5A) (Brzovic et al., 2001). In concordance, the variants with substitutions at these residues, p.C53W and p.C71Y were not soluble (Figure 2) and did not

support HDR. The RING domains of BARD1 and BRCA1 are held together by a 4-helix bundle with each protein providing two helices. Variants with substitutions in the 4-helix bundle p.W34R and p.L44R residues were partially defective or non-functional for HDR reiterating the importance of this structure for dimerization. The p.T54A substitution also is present on the BRCA1 binding interface in the 4-helix bundle, but the p.T54A variant was fully functional for HDR. Given the large protein-protein interface, substitutions might have to be severe or at specific amino acids in the bundle in order to cause this for the interaction to be disrupted. Only upon further analysis of more substitution variants will that be determined.

Several residues of the BARD1 BRCT domain were also analyzed, and two were found to be intermediate, p.G623E and p.A724V. The p.G623 residue appears to be buried in the BRCT domain and the p.A724 residue is partially buried (Figure 5B). It is possible that a substitution at either residue would have a strong effect on the structure of the BRCT domain. Since these mutant proteins were soluble, we do not interpret these mutants to impact the overall BARD1 structure. It will be important in the future to test more mutants/variants in the BARD1 BRCT domain to map the critical residues more fully.

Discussion

The tumor suppressor BRCA1 is essential for HDR of double stranded DNA breaks (Moynahan et al., 1999; Snouwaert et al., 1999). Similarly, the BRCA1-associated BARD1 protein is also required for HDR (Westermarck et al., 2003). Mutation of *BRCA1* and the consequent loss of functional HDR predispose to cancer, in particular breast and ovarian cancer (Cerbinskaite et al., 2012; Starita and Parvin, 2003; Stratton and Rahman, 2008). Similarly, *BARD1* mutations have been associated with loss of tumor suppressor activity and susceptibility to HBOC cancer (Baer and Ludwig, 2002; McCarthy et al., 2003; Thai et al., 1998). While missense substitutions in *BARD1* have been observed in DNA samples from individuals who report familial breast or ovarian cancer, just as is the case for *BRCA1*, it is often difficult to determine from these small families and rare variants which missense substitutions predispose to cancer and which are neutral variants.

In this study, we established a tissue culture based method for analyzing BARD1 missense substitutions for effects in DNA repair. This high throughput method analyzes the human BARD1 protein in a human cell line that is competent for HDR. By analyzing 29 different missense substitutions, we identified three mutants, p.L44R, p.C53W, and p.C71Y that were defective in DNA repair. Two of the non-functional mutants, p.C53W and p.C71Y, were likely nonfunctional by virtue of making the BARD1 protein insoluble. The p.L44R variant served as our “positive” control as it was shown experimentally to disrupt binding of BARD1 to BRCA1 (Morris et al., 2002; Xia et al., 2003). Three variants were identified in the intermediate activity, p.W34R, p.G623E, and p.A724V. We further determined that 23 variants from clinical data were functional in DNA repair. By mapping the functional and non-functional residues to 3-D structures, we were able to identify several epitopes that are critical for BARD1 activity in HDR. Results indicate that sites along the BRCA1-BARD1 protein-protein interface are critical for HDR activity. Though it had been known that BARD1 was important for HDR (Westermarck et al., 2003), and though it was reasonable to

hypothesize that BARD1 functioned in HDR as a heterodimer with BRCA1, this is the first study to show that BARD1 does not function independently in HDR but rather as a heterodimer with BRCA1.

A previous study had analyzed BARD1 mutants in HDR in the context of BARD1 null murine cells (Laufer et al., 2007). In that study, various deletion mutants were found to be defective for HDR, but none of the missense substitutions tested were defective for DNA repair. Three of the variants tested in the current study were also tested in the earlier study, and our results corroborate the previous findings that variants p.Q564H, p.V695L, and p.S761N were functional in DNA repair (Laufer et al., 2007). While the BRCT domain is necessary for DNA repair, missense substitutions for these three BRCT residues did not alter HDR. We were able to identify one BRCT residue that, when mutated, greatly reduced HDR activity; the mutant p.G623E was greatly reduced in DNA repair and it did not bind the BRCA1 protein. It is possible that other variants in the BRCT domain would be non-functional in HDR if they altered BARD1 solubility or, like p.G623E, had the potential to change the structure of the domain.

Truncating mutations in BARD1 are thought to confer an increased risk of developing breast and ovarian cancer (Pennington and Swisher, 2012) although the status of *BARD1* as a HBOC susceptibility gene is currently unclear (Easton et al., 2015; Richards et al., 2015). As only a few missense variants in *BARD1* have been classified, the vast majority are considered VUS. Indeed, of the 129 missense variants in *BARD1* reported in ClinVar, only eight are reported to be neutral and none are definitively classified as pathogenic (<http://www.ncbi.nlm.nih.gov/clinvar>).

Although DNA repair assay results alone are insufficient to make a clinical judgment about the potential pathogenicity of BARD1 sequence variations (MacArthur et al., 2014), our HDR results can be combined with available clinical information to inform individuals who have been tested and found to have these missense changes. Previous studies of BRCA1 variants elucidated the correlation between function in DNA repair and clinical outcomes (Ransburgh et al., 2010; Towler et al., 2013). Less is known about *BARD1* variations in individuals and only a few have been reported in more than one study. The p.C557S is the most-described *BARD1* variant because it was initially reported to contribute to breast cancer risk in Caucasian individuals and a lesser extent in South American individuals (Gonzalez-Hormazabal et al., 2012). However, p.C557S is functional in HDR (Laufer et al., 2007) and in subsequent studies has been shown to have similar allele frequencies in individuals from breast cancer families and controls (Ratajska et al., 2012; Vahteristo et al., 2006). Furthermore, case-control studies in sporadic breast cancer studies showed no evidence of increased risk (Ding et al., 2011). Thus, data suggests that this variant is likely neutral/benign.

It is important to more fully characterize the role of BARD1 in cancer risk by combining a moderately high-throughput method of analyzing missense changes with a more extensive clinical portfolio of family history and disease outcome. Combining DNA repair data, functional analysis, and patient outcomes will bring cohesion to cancer genetics and replace lists of VUS with informative predictions for patient prognosis and treatment. A deeper

understanding of variants across human DNA samples grows more important as predictive and diagnostic sequencing expands in sensitivity and accessibility in healthcare.

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Figure 2. Expression of BARD1 in HDR assay

A. Soluble proteins were extracted from cells left over from the flow cytometry (Figure 1) and subjected to immunoblot analysis. Endogenous BARD1 (top panel of each row) migrates at a position consistent with a 97 kD mass, and a cross-reacting protein migrates at the leading edge of the BARD1 band. To differentiate the BARD1-specific band from the closely migrating cross-reacting species, the BARD1 band was marked with an arrowhead in each lane. Soluble BRCA1 protein was analyzed from these lysates in immunoblots (middle panel of each row). Cells were transfected with control (lanes 1, 15, 29, 36) or BARD1-specific siRNA (lanes 2–14, 16–28, 30–35, and 37–42). Transfected plasmids were empty vector (lanes 2, 16, 30, 37) and the indicated BARD1 expression plasmid. Blots were also stained for α -tubulin as a loading control (bottom panel of each row).

B. Total BARD1 protein expression was determined by lysing cells in 1% SDS containing buffer, sonicating, and heat denaturing prior to immunoblot analysis. Cells were transfected with the indicated siRNAs and BARD1 expression plasmids as in panel A, and left over cells from flow cytometry analyses were used to analyze by immunoblots as in panel A.

Figure 3. Analysis of BARD1 missense variants with corrected R406 residue
A subset of the BARD1 expression plasmids had residue p.R406 corrected to the wild-type sequence and were analyzed in the HDR assay. Control siRNA (lane 1) or BARD1 3'-UTR specific siRNA (lanes 2–8) were transfected into HeLa-DR cells along with the indicated expression plasmid. The percentage of GFP-positive cells in each sample was normalized relative to the wild-type BARD1 expression construct. Results are taken from five independent transfection experiments. Color scheme is consistent with Figure 1 (lane 5, red; lanes 7–8, green; others as shown). Immunoblots for BARD1 and tubulin were done as in Figure 2A.

Figure 4. Association of BARD1 missense variants with BRCA1

The indicated BARD1 expression construct was transfected into cells and soluble extracts were subjected to immunoaffinity purification with an antibody specific for BRCA1 residues 400–1100. Input samples (5%, lanes 1–6) and immunopurified samples (lanes 7–12) were stained with antibody specific for the HA tag on the expressed BARD1 protein. The blot was re-stained using an antibody specific for the BRCA1 protein (bottom panel).

Figure 5. Mapping of variants/mutants on the BARD1/BRCA1 RING domain structure
A. BARD1 RING domain is shown in blue and the BRCA1 RING domain is shown in pink. Residues that were tested in HDR and the zinc ions are shown in space-filling format. Missense substitutions functional in HDR shown in green; intermediate shown in yellow; non-functional shown in red.
B. The BARD1 BRCT domain is shown in blue with tested residues indicated with space-filling. Green indicates functional in HDR and red indicates non-functional. The crystal structure of the BARD1 BRCT domain is of a homodimer, but for simplicity one chain is shown in white.

Table 1

Function of BARD1 variants in homology directed repair (HDR)

Missense Substitution	MAF	%HDR	HDR function	Clinical significance [^]
p.E27Q	N/A	89%	Functional	VUS
p.G32V	N/A	87%	Functional	VUS
p.W34R	N/A	40%	Intermediate	N/A *
p.A40V	N/A	113%	Functional	VUS
p.L44R	N/A	10%	Defective	N/A *
p.C53W	N/A	15%	Defective	VUS
p.T54A	0.1%	92%	Functional	VUS
p.C71Y	N/A	14%	Defective	VUS
p.P84S	0.01%	135%	Functional	VUS
p.R112Q	N/A	105%	Functional	VUS
p.H116Y	0.02%	107%	Functional	VUS
p.W146C	N/A	120%	Functional	VUS
p.S279P	N/A	88%	Functional	VUS
p.N356I	0.04%	65%	Functional	VUS
p.R406G	0.01%	100%	Functional	VUS
p.R406Q	N/A	149%	Functional	VUS
p.V507M	21–46%	120%	Functional	Neutral
p.Q564H	N/A	127%	Functional	VUS
p.M621I	N/A	70%	Functional	VUS
p.G623E	N/A	19%	Intermediate	VUS
p.N626S	N/A	90%	Functional	VUS
p.R658C	0.5–0.6%	123%	Functional	Neutral
p.I692T	N/A	120%	Functional	Neutral
p.V695I	0.01%	101%	Functional	VUS
p.V695L	N/A	111%	Functional	VUS
p.A721T	N/A	75%	Functional	VUS
p.A724V	N/A	66%	Intermediate	VUS
p.R731G	0.18–0.2%	99%	Functional	Neutral
p.S761N	0.02–0.4%	121%	Functional	VUS

N/A: No data in dbSNP or EVS;

* not found in patients, affects BRCA1 binding,

[^] classified as neutral based on frequency or as listed in ClinVar

Table 2

In silico prediction of BARD1 variant function

Missense Substitution	HDR function	SIFT	SIFT Score	PolyPhen-2	PROVEAN
p.E27Q	Functional	Tolerated	0.08	Benign	Neutral
p.G32V	Functional	Affect protein function *	0.02	Probably damaging	Neutral
p.W34R	Intermediate	Affect protein function *	0	Probably damaging	Deleterious
p.A40V	Functional	Affect protein function *	0	Probably damaging	Neutral
p.L44R	Defective	Affect protein function *	0	Probably damaging	Deleterious
p.C53W	Defective	Affect protein function *	0	Probably damaging	Deleterious
p.T54A	Functional	Tolerated	0.51	Probably damaging	Neutral
p.C71Y	Defective	Affect protein function *	0.01	Probably damaging	Deleterious
p.P84S	Functional	Affect protein function *	0	Possibly damaging	Deleterious
p.R112Q	Functional	Tolerated	0.18	Probably damaging	Neutral
p.H116Y	Functional	Tolerated	0.18	Benign	Neutral
p.W146C	Functional	Affect protein function *	0	Probably damaging	Deleterious
p.S279P	Functional	Tolerated	0.24	Possibly damaging	Neutral
p.N356I	Functional	Affect protein function *	0.01	Benign	Neutral
p.R406G	Functional	Tolerated	0.37	Benign	Neutral
p.R406Q	Functional	Tolerated	1	Benign	Neutral
p.V507M	Functional	Tolerated	0.15	Benign	Neutral
p.Q564H	Functional	Tolerated	0.07	Benign	Neutral
p.M621I	Functional	Affect protein function *	0.05	Possibly damaging	Neutral
p.G623E	Intermediate	Affect protein function *	0	Probably damaging	Deleterious
p.N626S	Functional	Tolerated	0.72	Benign	Neutral
p.R658C	Functional	Affect protein function *	0	Probably damaging	Deleterious
p.I692T	Functional	Tolerated	0.1	Benign	Neutral
p.V695I	Functional	Tolerated	0.15	Benign	Neutral

Missense Substitution	HDR function	SIFT	SIFT Score	PolyPhen-2	PROVEAN
p.V695L	Functional	Affect protein function *	0.01	Possibly damaging	Neutral
p.A721T	Functional	Affect protein function *	0	Probably damaging	Neutral
p.A724V	Intermediate	Affect protein function *	0	Probably damaging	Neutral
p.R731G	Functional	Affect protein function *	0.01	Probably damaging	Deleterious
p.S761N	Functional	Affect protein function *	0.03	Benign	Neutral

* Substitution may have been predicted to affect function because the sequences used were not diverse enough leading to low confidence in this prediction.