

A Syngeneic Variance Library for Functional Annotation of Human Variation: Application to *BRCA2*

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Abstract

The enormous scope of natural human genetic variation is now becoming defined. To accurately annotate these variants, and to identify those with clinical importance, is often difficult to assess through functional assays. We explored systematic annotation by using homologous recombination to modify a native gene in hemizygous (wt/ Δ exon) human cancer cells, generating a novel syngeneic variance library (SyVaL). We created a SyVaL of *BRCA2* variants: nondeleterious, proposed deleterious, deleterious, and of uncertain significance. We found that the null states *BRCA2* ^{Δ ex11/ Δ ex11} and *BRCA2* ^{Δ ex11/Y3308X} were deleterious as assessed by a loss of RAD51 focus formation on genotoxic damage and by acquisition of toxic hypersensitivity to mitomycin C and etoposide, whereas *BRCA2* ^{Δ ex11/Y3308Y}, *BRCA2* ^{Δ ex11/P3292L}, and *BRCA2* ^{Δ ex11/P3280H} had wild-type function. A proposed phosphorylation site at codon 3291 affecting function was confirmed by substitution of an acidic residue (glutamate, *BRCA2* ^{Δ ex11/S3291E}) for the native serine, but in contrast to a prior report, phosphorylation was dispensable (alanine, *BRCA2* ^{Δ ex11/S3291A}) for *BRCA2*-governed cellular phenotypes. These results show that SyVaLs offer a means to comprehensively annotate gene function, facilitating numerical and unambiguous readouts. SyVaLs may be especially useful for genes in which functional assays using exogenous expression are toxic or otherwise unreliable. They also offer a stable, distributable cellular resource for further research. [Cancer Res 2008;68(13):5023–30]

Introduction

The scope of natural human genetic variation is now becoming evident but is enormous (1, 2). To accurately annotate these variants and to identify which of them have clinical importance through functional assays may be difficult for several reasons. Natural cell lines expressing the variants homozygously are often unavailable and do not allow isogenic experimental controls. Artificially introduced null states can be inviable. For some genes, such as the clinically important *BRCA2* gene, even the exogenous expression of wild-type (wt) gene fragments can cause interference with endogenous gene function (squenching) unless assays are performed with extreme technical care and multiple controls (3–6).

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The *BRCA2* gene exhibits allelic differences in its sequence among members of the human population. Some of the variants are deleterious, causing inherited susceptibility to breast, ovarian, and other cancer types (7, 8). Unfortunately, in many patients who are tested and have a *BRCA2* variant identified, the alteration is subtle, changing a single amino acid (9). For many of these variants, it is not possible to use epidemiologic data or laboratory studies of protein function to determine unambiguously nor efficiently whether one should classify the variant as either deleterious or benign.

The *BRCA2* gene on chromosome 13q12-13 encodes a 3,418-amino acid protein. Its sequence reveals only a few clues to its cellular function and thus variation in the sequence is not readily interpreted. The role of the protein in DNA repair specifically involves homologous recombination, mediated by its interaction with RAD51 (10–12). *BRCA2* interacts with RAD51 through highly conserved BRC repeats in exon 11 and an interaction domain mapped to exon 27 (12, 13). The interaction of RAD51 with exon 27 has been proposed to be down-regulated by cyclin-dependent kinase-dependent phosphorylation of S3291, which is reduced during S phase and after DNA damage (4). However, most functional studies of *BRCA2* have been hampered by the lack of well-controlled human cancer cellular models (14). Such absence was in the past overcome by using methods of considerable technical difficulty (5).

To accurately annotate sequence variations of the *BRCA2* gene in an unambiguous and technically facile model, we performed targeted disruption of *BRCA2* exon 11 by homologous recombination, yielding the first available syngeneic human cancer *BRCA2* knockout cell line. Using hemizygous *BRCA2* cells, we constructed a library of syngeneic exon 27 genetic variant lines (a SyVaL). By functional evaluation of multiple clones harboring individual mutations, we were able to classify sequence variants as deleterious, hypomorphic, or neutral and to create a pseudophosphorylated *BRCA2* variant replacing the normal gene. Our data support the importance of exon 27 in recombination-mediated DNA repair and the role of S3291 in phosphorylation-regulated *BRCA2*-RAD51 interaction.

Materials and Methods

Cell lines and cell culture. DLD1 cells were obtained from the American Type Culture Collection and cultured in conventional medium supplemented with 10% FCS, L-glutamine, and penicillin/streptomycin.

Targeted disruption by homologous recombination. We first disrupted exon 11 of *BRCA2* by homologous recombination using the technique of Kohli and colleagues (15). The targeting construct was designed to excise a part of exon 11, containing a known mutation and including BRC repeat 6, thus causing a premature stop codon after BRC repeat 5. The construct, which consisted of a selection cassette flanked on

both sides by loxP sites and ~1 kb of genomic sequences adjoining the targeted part of exon 11, was ligated into pAAV (Stratagene). It was then cotransfected with pRC and pHelper into HEK 293 cells using Lipofectamine (Invitrogen). Two days later, virus was harvested from HEK 293 cells and used for gene targeting of DLD1 cells. Infected cells were split into 96-well plates, and 3 wk later, hygromycin-resistant clones having homologous integration of the targeting construct were identified by PCR using a primer inside the resistance gene and a primer outside of the homology arms (primer pairs SF/SR and SF2/SR2; Fig. 1). The selection cassette was removed by transiently expressing exogenous Cre recombinase. A recombinant hemizygous clone, identified by PCR using primers flanking the deletion (primer pair OF/OR; Fig. 1), was used for second allele targeting. Reusing the same exon 11 targeting construct, clones acquiring biallelic disruption of *BRCA2* were identified by PCR screening using a combination of primers described above.

Alternately, exon 27 was targeted in the hemizygous cells using a construct whose right homology arm included intact exon 27 and individual sequence variants (Fig. 4). Homologous integration of the construct, the presence of desired sequence variants, and allelic phase of the construct (see below) were determined by allele-specific PCR and sequencing. An analogous vector design was used by Hurley and colleagues (16) to introduce an ATR mutation into alleles of a human cell and by Konishi and colleagues (17) to convert a wt *KRAS* gene to a mutant allele in a nontumorigenic human cell line.

Distinguishing homologous recombination events affecting the active or inactive allele ("phase" determination). The hemizygous

BRCA2^{wt/ Δ ex11} cells, having one active and one inactive allele, were used for subsequent experiments (Fig. 4). In preparation to target exon 27, residing at a large genomic distance from exon 11, we devised a means to readily distinguish long-distance *cis* relations of each allele. We first performed automated sequencing of the heterozygous cell line to identify natural, preexisting heterozygous nucleotide polymorphisms within intron 25. We next distinguished whether the intronic polymorphisms were on the active or inactive allele (termed the phase) by determining whether they were in *cis* or in *trans* to the deleted exon using limiting dilution of the template DNA and digital duplex PCR (18). Primers were specific for the exon 11 deletion sequence (primer pair OF/IR; Fig. 4) or flanked the identified polymorphisms (primer pair INF/INR; Fig. 4). Sequencing was performed to identify the allele amplified in each well.

Subsequently, after exon 27 targeting and clone isolation, the presence and the phase of the introduced exon 27 variant sequence were in turn assessed in relation to the known phase of the natural intron 25 polymorphic site by sequencing a PCR product incorporating both sites (primer pair SF, upstream of the intron 25 polymorphism, and SR, specific for the targeting construct; Fig. 4).

DNA and RNA isolation, PCR, and sequencing. Genomic DNA was isolated from cells using Lyse-N-Go PCR Reagent (Pierce Biotechnology). RNA was isolated using RNeasy Mini kit (Qiagen). Automated sequencing was performed by a core facility. PCR conditions and primer sequences are available on request.

Allele-specific PCR. The presence of the targeting construct and its subsequent Cre-mediated removal enabled selective amplification from one

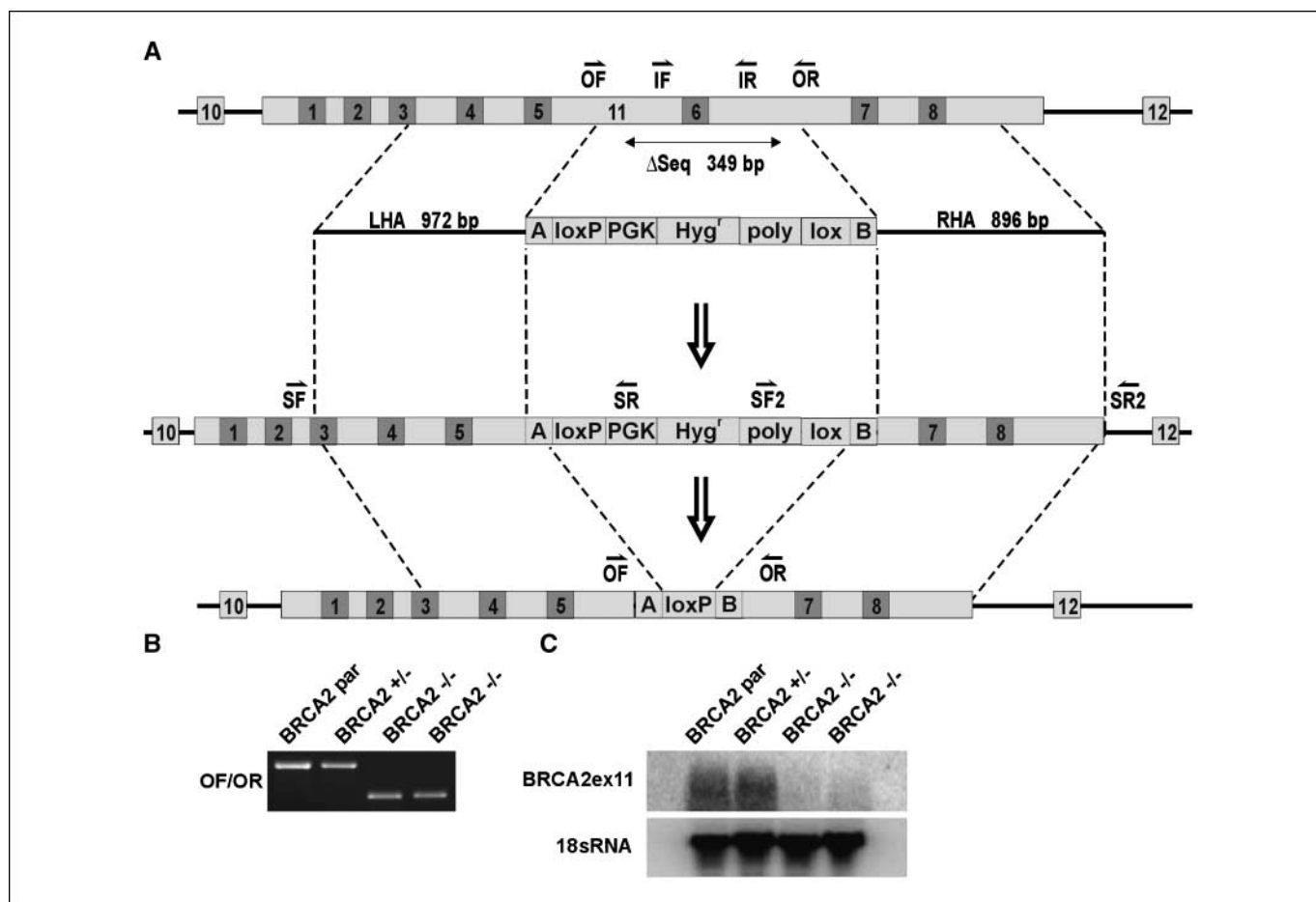


Figure 1. Structural and functional demonstration of *BRCA2* gene disruption. *A*, targeting scheme. *OF* and *OR*, primers outside the deleted sequence; *IF* and *IR*, primers inside the deleted sequence; *SF*, *SR*, *SF2*, and *SR2*, screening primer pairs; *LHA* and *RHA*, left and right homology arms. Numbers on light background indicate exons, and numbers on dark background indicate BRC repeats. *B*, PCR detecting the wt and targeted alleles using genomic DNA as template. *C*, Northern blot detecting the targeted exon 11 mRNA transcript.

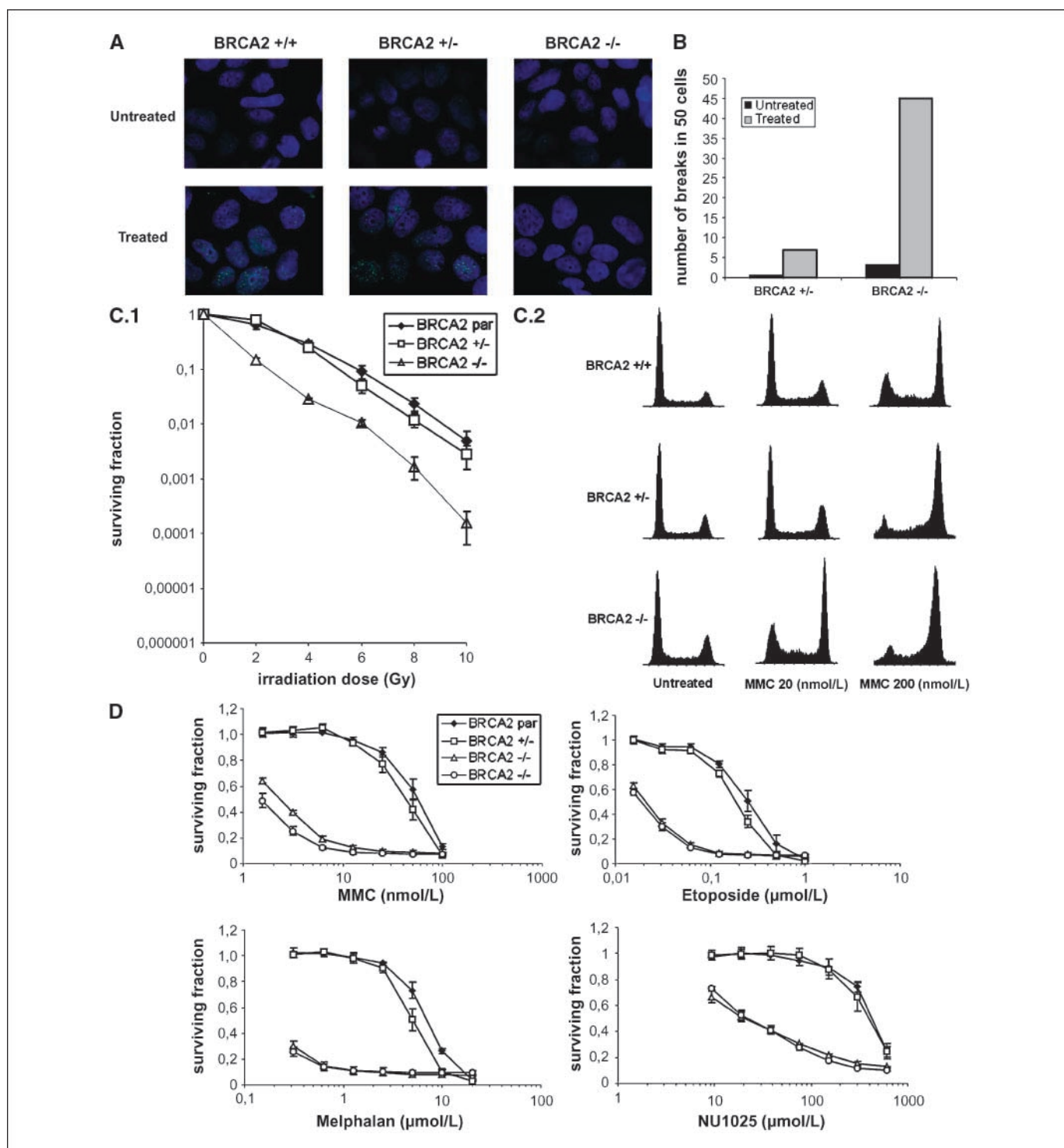


Figure 2. Characterization of BRCA2-null cells. **A**, immunofluorescence of RAD51 nuclear foci in untreated and treated cells (2.4 µg/mL MMC). *BRCA2* +/+, *BRCA2*^{wt/ Δ ex11}, *BRCA2* -/-, *BRCA2* ^{Δ ex11/ Δ ex11}. **B**, analysis of 50 metaphases for chromosomal aberrations in untreated and treated (equitoxic doses of MMC) heterozygous and knockout clones. **C.1**, colony formation assays on irradiation. *BRCA2* par, parental *BRCA2*^{wt/mut x 2}. Points, mean of three independent experiments; bars, SE. **C.2**, cell cycle analysis 48 h after MMC treatment. Representative cell cycle profiles obtained after treatment with indicated concentrations of MMC. **D**, cell proliferation following treatment with selected drugs. Two *BRCA2* -/- (*BRCA2* ^{Δ ex11/ Δ ex11}) subclones were analyzed. Points, mean of three independent experiments; bars, SE.

of two alleles using primers specific for either the construct or the deleted sequence.

Digital PCR. Digital PCR was used to readily distinguish the active and inactive alleles at a distance from exon 11 (in intron 25) in the

hemizygous *BRCA2*^{wt/ Δ ex11} cells. It was performed essentially as described previously (18). In a duplex reaction, primers specific for deletion-specific sequences at exon 11 and spanning intron 25 were combined (Fig. 4).

Site-directed mutagenesis. Mutated targeting constructs were generated using the QuikChange Site-Directed Mutagenesis kit (Stratagene) using the manufacturer's protocol.

Northern blotting. To confirm absence of exon 11 transcript, Northern blotting was performed. Random primer-labeled cDNA was used to detect BRCA2 exon 11 mRNA, and end-labeled oligomers were used to detect 18S rRNA (loading control; ref. 19).

Cell cycle analysis. Cells were treated with mitomycin C (MMC) for 48 h, processed as described previously (20), and analyzed by flow cytometry.

Chromosome aberration assay. Cells were treated with equitoxic doses of MMC (80 nmol/L for BRCA2^{wt/Δex11} and 6 nmol/L for BRCA2^{Δex11/Δex11}) for 24 h. For each sample, 50 metaphases were analyzed by the cytogenetics core facility at Johns Hopkins according to standard protocols.

Cell proliferation assay. In each well of 96-well plates, 1,000 cells were plated, allowed to adhere, and treated with various drugs. The drugs were obtained from Sigma except for SJG-136 (NSC 694501), which was generously provided by Ipsen, Inc. and the National Cancer Institute, NIH. The cells were washed after 6 d and lysed in 100 μL of water. Fluorescence was measured after addition of 0.5% PicoGreen (Molecular Probes) using a fluorometer (Fusion, Perkin-Elmer). Three independent experiments were performed per drug.

Colony formation assay. Cells were plated in six-well plates, allowed to adhere, and exposed to ¹³⁷Cs γ-rays. After 14 d, cells were washed and stained with crystal violet and colonies were counted. For each dose,

cells were plated at three different concentrations in duplicate. Three independent experiments were performed.

Immunofluorescence. Cells were treated with MMC (2.4 μg/mL) or γ-irradiation and fixed in PBS/2% paraformaldehyde after 24 h of incubation. Following permeabilization in PBS/0.5% Triton X-100 and blocking for 30 min in PBS/1% bovine serum albumin/0.15% glycine, the cells were incubated with an anti-RAD51 antibody (1:200; Calbiochem) for 2 h. Cells were then washed and incubated with a rabbit IgG secondary antibody (1:200, Alexa Fluor 488; Molecular Probes) for 1 h. After washing, slides were mounted and analyzed by a fluorescent microscope (Zeiss Axiovert 135) and MetaMorph 4.6 software (Universal Imaging). Pictures were acquired, keeping exposure time and software settings constant for all samples.

Baseline or induced RAD51 focus formation was considered "negative" when less than 5% or 20%, respectively, of cells had more than five nuclear foci and "positive" when more than 5% or 30%, respectively, of cells had more than five nuclear foci. Intermediate phenotypes were not observed.

Results

Targeted disruption of BRCA2. We first attempted to disrupt the remaining wt allele in a BRCA2 heterozygous, p53 wt cell line RKO (BRCA2^{wt/535delA}), but we could not obtain viable BRCA2-null clones (21). We then tried the diploid p53-deficient cancer cell line,

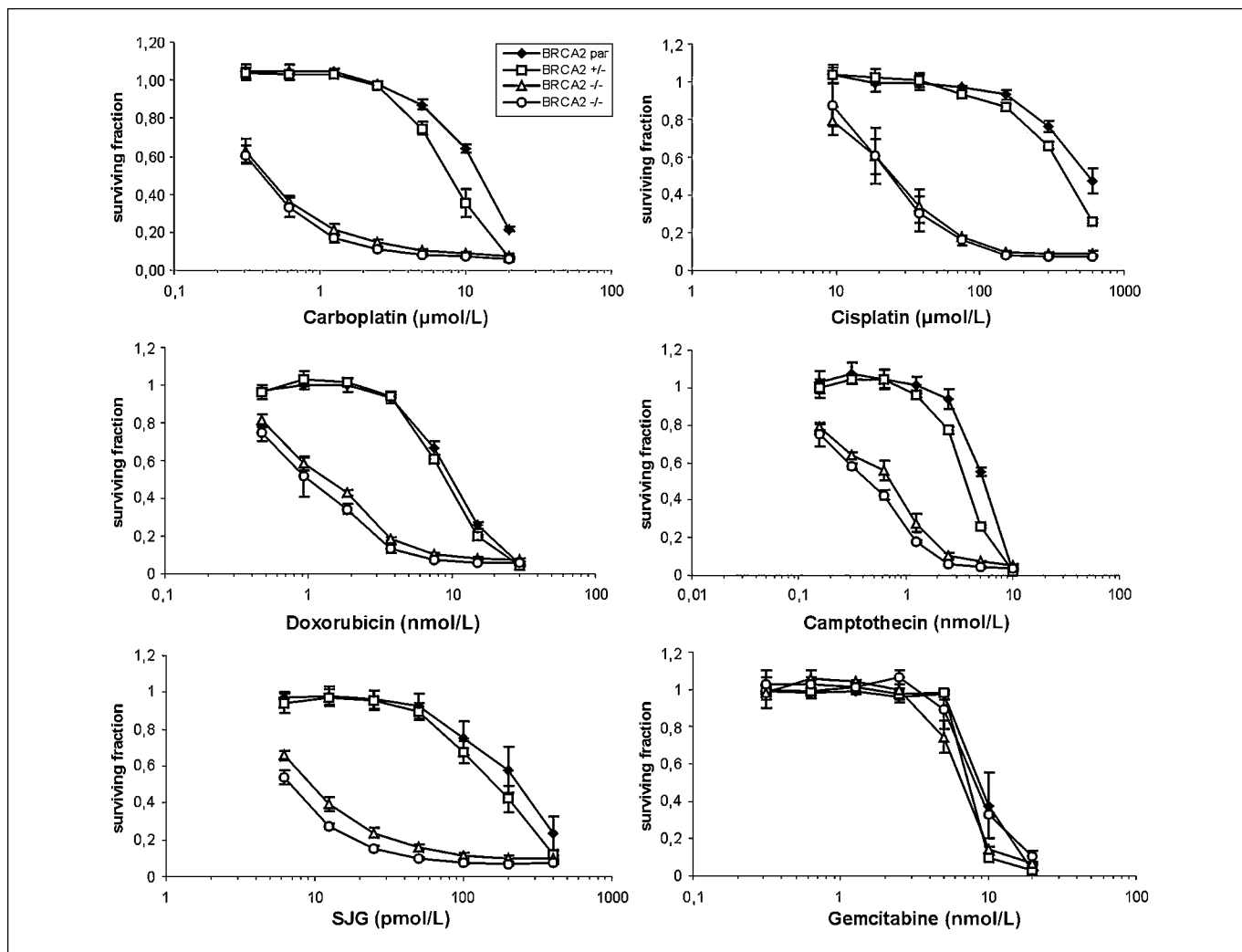


Figure 3. Drug sensitivity studies. Cell proliferation on treatment with various drugs at the indicated concentrations compared with untreated cells. Points, mean of three independent experiments; bars, SE.

Table 1. Characterization and proposed classification of *BRCA2* SyVaL clones

Clone	RAD51	MMC	Etoposide	Chromosomal instability	Classification
<i>BRCA2</i> ^{wt} / <i>Δex11</i>	Pos (16.7 ± 1.5/59.0 ± 0.7)	No Δ	No Δ	Neg	Neutral
<i>BRCA2</i> ^{<i>Δex11/Δex11</i>}	Neg (2.5 ± 0.8/3.3 ± 2.8)	20-fold Δ	10-fold	Pos	Deleterious*
<i>BRCA2</i> ^{<i>Δex11/Y3308X</i>}	Neg (1.8 ± 0.5/1.6 ± 0.3)	20-fold Δ	10-fold	—	Deleterious
<i>BRCA2</i> ^{<i>Δex11/Y3308Y</i>}	Pos (12.4 ± 0.6/34.1 ± 1.1)	No Δ	No Δ	—	Neutral
<i>BRCA2</i> ^{<i>Δex11/P3292L</i>}	Pos (13.6 ± 2.4/41.6 ± 4.7)	No Δ	No Δ	—	Neutral
<i>BRCA2</i> ^{<i>Δex11/P3280H</i>}	Pos (12.2 ± 1.3/41.7 ± 4.5)	No Δ	No Δ	—	Neutral
<i>BRCA2</i> ^{<i>Δex11/S3291E</i>}	Neg (3.6 ± 0.6/12.4 ± 0.9)	3-fold Δ	3-fold Δ	—	Hypomorphic
<i>BRCA2</i> ^{<i>Δex11/S3291A</i>}	Pos (23.8 ± 4.1/47.6 ± 9.7)	No Δ	No Δ	—	Neutral

NOTE: Numbers in parentheses represent mean percentage of untreated/treated cells with more than five RAD51 foci ± SE. Fold Δ refers to the ratio of the IC₅₀ of cells having the hemizygous wt allele to the IC₅₀ of cells having the mutated allele.

Abbreviations: Pos, positive; Neg, negative; —, not done; Δ, change.

*The extreme rarity of knockout clones may indicate an especially severe deleterious effect (21).

DLD1. DLD1 originally harbored two nonsense mutations of *BRCA2* (22). Both mutations are present on one allele (data not shown). In the first round of *BRCA2* targeting, we deleted exon 11 of this allele, ensuring a permanent null state without a possibility of the reversion of the mutations (23). The other allele in the hemizygous clone remained wt. In the second round of *BRCA2* targeting, 81 viable clones having homologous integration of the targeting construct were obtained in several independent targeting attempts, of which only one clone had integrated the construct in the wt allele, producing a homozygous *BRCA2*^{*Δex11/Δex11*} knockout. The other 80 clones had reintegrated the construct into the already-knocked-out allele.

PCR and Northern blot analyses confirmed the heterozygous and homozygous gene disruption (Fig. 1). Northern blot revealed the expression of exon 11 transcript in the parental and heterozygous populations. There was no detectable transcript in the homozygous cells.

Functional analysis of *BRCA2*^{*Δex11/Δex11*} cells. Both engineered and natural cells harboring truncated BRCA2 exhibit a proliferative impediment that may worsen with successive passages (24, 25). As expected, we also confirmed the proliferation rate in our *BRCA2* *Δex11/Δex11* cells usually to be slower than syngeneic controls using daily direct cell counting and confirmation by assaying DNA content of the culture (data not shown). For example, in three replicate cultures of each cell type (parental, heterozygote, and biallelic knockout) counted on each day of day 0 to day 5, the following calculated doubling times for each 5-day period were obtained: for parental *BRCA2* wt/mut x 2, 0.64, 1.06, and 1.07 days; for heterozygous knockout *BRCA2* wt/*Δex11*, 0.59, 0.83, and 1.6 days; and for biallelic knockout *BRCA2* *Δex11/Δex11*, 1.61, 1.22, and 1.58 days. This modest proliferation defect seemed stable with successive passage in culture.

RAD51 focus formation. To investigate whether the *BRCA2*^{*Δex11/Δex11*} cells were defective in homologous recombination, we examined their ability to form RAD51 foci on DNA damage induction. Treatment with MMC induced RAD51 foci (more than five foci) in about 60% to 80% of *BRCA2*-proficient cells and, with irradiation, in about 50% to 60% of cells. *BRCA2*^{*Δex11/Δex11*} cells had reduced levels of foci in untreated cells, and no induction of foci was observed on DNA damage (Fig. 2A).

Chromosomal instability. *BRCA2*-deficient cells display increased chromosomal instability, which is further enhanced after

treatment with MMC (26). Treatment-induced breakage serves as a diagnostic test for the Fanconi anemia (FA) syndrome, including the FA-D1/*BRCA2* group (27). *BRCA2*^{*Δex11/Δex11*} displayed much higher rates of chromosomal aberrations than *BRCA2*^{wt/*Δex11*} cells, including breaks and radials, on MMC treatment at equitoxic doses (Fig. 2B).

Cell survival after irradiation. There has been conflicting data about the sensitivity of *BRCA2*-deficient cells to irradiation (24, 26). Therefore, we assessed the effect of *BRCA2* disruption on survival after treatment with X-rays using colony formation assays. We observed a 10-fold decrease in relative survival of *BRCA2*^{*Δex11/Δex11*} cells compared with their controls (Fig. 2C.1).

Cell proliferation on drug treatment. The role of *BRCA2* in DNA repair and replication fork maintenance is indicated by the hypersensitivity of *BRCA2*-defective cells to DNA-damaging agents. We assayed survival following exposure to several clinically relevant drugs in parental cells and heterozygous and homozygously deleted cells. *BRCA2*-deficient cells exhibited greatly increased sensitivity to various interstrand cross-linking (ICL) agents, including MMC (20-fold), melphalan (25-fold), cisplatin (16-fold), carboplatin (18-fold), oxaliplatin (10-fold), and SJG-136 (NSC 694501; 22-fold; Figs. 2D and 3; data not shown).

Topoisomerase inhibitors affect enzymes incising either one (topoisomerase I inhibitors) or two (topoisomerase II inhibitors) strands of DNA in the process of DNA replication. Our *BRCA2*-deficient cells displayed increased sensitivity to topoisomerase II inhibitors etoposide (10-fold) and doxorubicin (10-fold) and to a smaller extent to topoisomerase I inhibitor camptothecin (6-fold; Figs. 2D and 3). Etoposide hypersensitivity was additive to, but not synergistic with, other anticancer agents (data not shown; ref. 28).

Hypersensitivity of *BRCA2*-deficient cells to poly(ADP-ribose) polymerase (PARP) inhibitors has been reported (29, 30). Our cells having an acute disruption of *BRCA2* were more sensitive (20-fold) to NU1025 than control cells (Fig. 2D).

We observed no differences in sensitivities to gemcitabine and vinblastine as well as to agents that have various DNA-damaging potential, such as norethindrone (31), apigenin, curcumin, and hesperidin (data not shown).

The increased genotype-dependent toxicity to MMC was confirmed by flow cytometry, indicating a profound G₂-M arrest of the cell cycle (Fig. 2C.2).

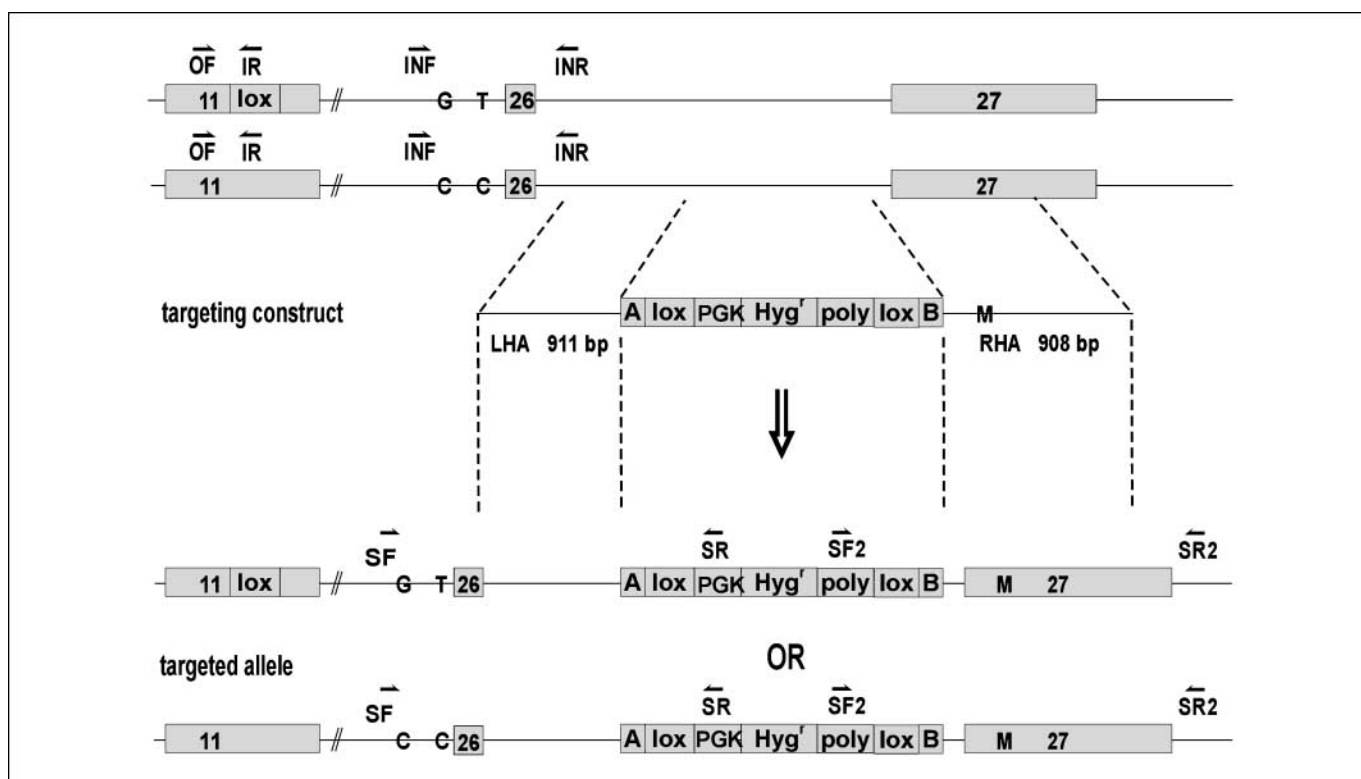


Figure 4. Structural demonstration of *BRCA2* exon 27 targeting. Targeting scheme. *OF*, primer outside the deleted sequence; *IR*, primer inside the deleted sequence; *SF*, *SR*, *SF2*, and *SR2*, screening primer pairs; *INF* and *INR*, intron 25 spanning primers; *LHA* and *RHA*, left and right homology arms.

Introducing sequence variants into exon 27. The heterozygous clone used for exon 27 targeting was verified to have intact *BRCA2* function by showing an absence of MMC hypersensitivity and the formation of RAD51 foci on DNA damage (Fig. 2*A* and *D*). A series of mutations was introduced by site mutagenesis into a targeting construct whose original right homology arm included wt exon 27 (Table 1; Fig. 4). As a positive control, a known deleterious nonsense mutation (10152C→G, Y3308X) was used. As a negative control, a synonymous mutation was introduced in the same codon (10152C→T, Y3308Y). Two naturally occurring missense human sequence variants (10067C→A, P3280H; 10103C→T, P3292L) reported from several cancer families and listed in the *BRCA2* mutation database⁴ as variants of uncertain significance were examined (Table 1).

The significance of the recently reported phosphorylation site in exon 27, S3291, was investigated by introducing a glutamate (mimicking constitutive phosphorylation of the protein) and alanine (to prevent phosphorylation) at codon 3291.

On average for each mutation, 20 clones having homologously integrated the targeting construct were identified by PCR; between 30% and 80% of these clones harbored the introduced mutation. The phase of the construct was subsequently determined in these clones by PCR and sequencing (data not shown). Clones having integrated the mutated construct into the inactive allele, as well as clones having integrated the targeting construct at an unrelated genomic site, were used as controls in the functional assays.

Functional evaluation of SyVal clones. The robust and reproducible differences between wt *BRCA2*^{wt/mut} × 2 and *BRCA2*^{Aex11/Aex11} cells in drug treatment assays and in RAD51 focus formation assays (Table 1; Fig. 2) suggested that these tests were well suited to evaluate the effect of introduced mutations on *BRCA2* function.

MMC and etoposide sensitivity in the SyVal. *BRCA2*^{Aex11/Y3308X} had profound sensitivity to MMC and etoposide (20- and 10-fold, respectively), whereas *BRCA2*^{Aex11/Y3308Y} did not differ from control cells. *BRCA2*^{Aex11/P3280H} and *BRCA2*^{Aex11/P3292L} were not significantly more sensitive than the corresponding controls. *BRCA2*^{Aex11/S3291E}, but not *BRCA2*^{Aex11/S3291A}, cells were 3-fold more sensitive than controls (Table 1).

RAD51 focus formation in the SyVal. RAD51 focus formation was drastically diminished in untreated and treated *BRCA2*^{Aex11/Y3308X} (a negative assay result) but robust in *BRCA2*^{Aex11/Y3308Y} cells (a positive, normal result). No significant reduction in focus formation was observed in *BRCA2*^{Aex11/P3280H} and *BRCA2*^{Aex11/P3292L} (each, positive). A significant decrease in focus formation on MMC treatment was seen in *BRCA2*^{Aex11/S3291E} (negative) but not *BRCA2*^{Aex11/S3291A} cells (positive; Table 1).

Discussion

We sought to develop a model in which human gene function could be evaluated based on comparative assessments of the functional effect of individual *in situ* sequence alterations of one allele. We believe that this has not been previously done. We constructed a syngeneic stable library of sequence variants of *BRCA2* exon 27. This enabled us to functionally classify *BRCA2* genomic sequence variants of previously unknown significance in a

⁴ <http://research.nhgri.nih.gov/bic/>

robust manner that may help to predict the cancer risk associated with presence of the same mutations in heterozygous patients. Furthermore, we interrogated the protein based on functional evaluation of subtle protein residue substitutions.

To show the utility of SyVaLs, we chose *BRCA2* as our initial target gene. First, as a positive control for functional evaluation of our SyVaL clones, we generated *BRCA2*-null cells. To date, no *BRCA2* engineered human gene knockout cells have been reported. *BRCA2* knockout models exist in mouse, rat, hamster, and chicken cells. The pancreatic cancer cell line CAPAN1, the only *BRCA2*-deficient human cancer cell line (7), is cumbersome for its low transfectability, poor clonogenicity, and lack of isogenic controls (14). Our *BRCA2^{Aex11/Aex11}* cells now provide a stable human cancer syngeneic *BRCA2* knockout model.

Our knockout cells had features of *BRCA2* deficiency, being defective in homologous recombination as assessed by absence of induction of RAD51 foci on DNA damage, having chromosomal instability after treatment with MMC, and being modestly more sensitive to irradiation.

To further confirm the *BRCA2* phenotype and explore potential treatment options for patients having *BRCA2*-deficient tumors, we tested an informative panel of drugs in our cells. We confirmed increased sensitivity to various ICL agents and the PARP inhibitor NU1025, producing pharmacogenetic windows between 10 and 25 (28).

In this well-controlled human cancer model, we confirmed the etoposide hypersensitivity of *BRCA2*-deficient cells, first reported by Wiegant and colleagues (23) and Abbott and colleagues (32) in CAPAN1 cells and syngeneic hamster cells, and showed that the difference in sensitivity may be even greater (10-fold) than recently reported by Treszezamsky (2.75-fold; ref. 33) in fibroblasts from a Fanconi D1 patient. In yeast, the requirement of homologous recombination for reducing topoisomerase-associated toxicity was shown by Flores and colleagues (34). Not all models have been in agreement. For example, no increased etoposide sensitivity was observed in *BRCA2*-deficient mouse thymocytes (35) or small intestine (36). The findings thus distinguish cells having defects of the distal (*BRCA2*, etoposide hypersensitive) and proximal (no etoposide hypersensitivity) FA pathway (37). We extended our experiments to additional topoisomerase inhibitors. Given the rather low side effect profile of some of these drugs and our measured pharmacogenetic window of up to 10 (28), we concur that they represent candidate agents for rational treatment of patients with *BRCA2*-deficient tumors.

The *BRCA2^{Aex11/Aex11}* cells together with various SyVaL clones thus also represent an optimal model suitable for high-throughput drug screening of compound libraries to identify novel agents having selective toxicity in cells containing pathogenic mutations. We have not seen any intermediate phenotype of our heterozygous clones, suggesting that use of these drugs may be permissible in cancer patients who are carriers of heterozygous germ-line *BRCA2* mutations.

The lack of understanding of the functional importance and associated cancer risk of sequence variants in the *BRCA2* gene represents a serious clinical problem. The strongest interpretive evidence tends to come from segregation analyses and observed co-occurrence with known pathogenic mutations, both of which require information that is often not available (9). Functional studies of human *BRCA2* have proven very difficult. Prior cell-based *in vitro* assays depended on expression of either partial or complete wt or mutant proteins. Testing of partial gene and protein

sequences may not fully reveal the influence of the variant on the activity of the intact protein, and findings obtained by their use were not always confirmed using independent types of assay (38). The use of full-length wt or mutant constructs has been mostly limited to transient expression because it has proven difficult and at times impossible to generate stably expressing lines (4). Expression of exogenous *BRCA2*, including the BRC repeats, may result in *BRCA2* deficiency, perhaps due to squelching (titrating out) of free RAD51 (3, 4, 6). Studies using cells having endogenous wt *BRCA2* alleles depend on the presumption that the codominant or dominant-negative effects of exogenous mutated constructs will govern the assay results. Thus, evaluating *BRCA2* function using exogenous expression constructs is of extreme technical difficulty.

We present here a new approach to evaluate genomic variants by applying a simple and fast homologous recombination replacement technique to produce syngeneic clones. This syngeneic replacement strategy was approximately as rapid as the stable transfection of routine expression plasmids, a technique with which it shares many invariant steps. Such approach allows an unambiguous readout by generating multiple clones of stable cell lines having the endogenous gene altered.

We applied a panel of functional assays to our syngeneic knockout cell pair and selected two robust assays that in combination provided a clear and unambiguous readout of *BRCA2* function that we applied to our library of variants: RAD51 focus formation and MMC sensitivity. By applying these two assays, we observed our positive control to have a detrimental phenotype, our negative control to have a neutral phenotype (same as proficient cells), and the two variants of uncertain significance to have a neutral phenotype.

The high ratio, relating the number of clones having integrated a construct into the already targeted allele and the clones having integrated a construct in the wt allele, observed for the *BRCA2^{Aex11/Aex11}* cells (80:1) was not observed for any of the exon 27 variants. We believe that the ratio is influenced by selection against emergence of clones with a detrimental phenotype (21). In addition, the ratio may be potentially influenced by differences in the targeting construct as well as sequence variation or regional differences of chromatin at the targeted locus. This interesting aspect of the technique deserves further investigation.

SyVaLs can also be used to test the function of individual *in situ* residues of a protein or regulatory code, unrestricted by the limited number of known natural variants. For such a purpose, we selected the 3291 serine residue recently evaluated for its phosphorylation. Cyclin-dependent phosphorylation of this site was shown to inhibit both the interaction of *BRCA2* with RAD51 and with recombination repair. This site was discovered and initially evaluated using expression of partial protein sequences (4). By mimicking constitutive phosphorylation by substituting serine with glutamate, we observed a hypomorphic phenotype characterized by a decrease in induced RAD51 focus formation and a slight increase in sensitivity to MMC. When we replaced serine with alanine to prevent phosphorylation, neither a decrease in RAD51 focus formation nor increased sensitivity to MMC was observed.

Our findings of inhibition of RAD51 focus formation by the glutamate, and no effect of the alanine, do support the hypothesis of phosphorylation-dependent inhibition of RAD51 interaction. Yet, the use of variant endogenous *BRCA2* genes provided a nuanced relation that was not previously determinable. We found that this inhibition is partial, not complete. In addition, where the prior authors saw a surprising inhibitory effect on RAD51 binding also of

alanine 3291 when partial peptides were tested, our studies suggest differently. We find that phosphorylation of S3291 is dispensable for BRCA2-governed cellular phenotype as judged by the intact function of the alanine 3291 variant, as assayed by a set of robust tests used here. Our syngeneic lines thus serve as a starting point in addressing the teleologic purpose, if any, of cell cycle regulation of the RAD51-BRCA2 interaction.

In conclusion, we propose the creation of SyVaVs as a technically facile, ethically acceptable, means to compare the effects of subtly altering human genomes. It could be applied to any other clinically relevant genes, thus permitting high-throughput annotation of natural human genetic variation. SyVaVs also enable the interrogation of individual codons and regulatory codes in human chromosomes while avoiding many common artifacts of introducing exogenous genes and gene fragments.

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Disclosure of Potential Conflicts of Interest

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A Syngeneic Variance Library for Functional Annotation of Human Variation: Application to *BRCA2*

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