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Mouse ES-cell-based functional assay to evaluate mutations in *BRCA2*

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Abstract

Individuals with mutations in breast cancer susceptibility genes, *BRCA1* and *BRCA2* have up to 80% risk of developing breast cancer by the age of 70. Sequencing based genetic tests are now available to identify mutation carriers in effort to reduce mortality through prevention and early diagnosis. However, lack of a suitable functional assay hinders risk assessment of more than 1900 *BRCA1* and *BRCA2* variants in the Breast Cancer Information Core database that do not clearly disrupt the gene product. We have established a simple, versatile and reliable assay to test for functional significance of mutations in *BRCA2* using mouse embryonic stem cells (ES-cells) and bacterial artificial chromosomes (BACs) and have used it to classify 17 sequence variants. The assay is based on the ability of human *BRCA2* to complement the loss of endogenous *Brca2* in mouse ES-cells. This technique may also serve as a paradigm for functional analysis of mutations found in other human disease genes.

Keywords

BRCA1; BRCA2; DNA repair; conditional knockout; teratoma; unclassified variants; missense mutation; Mouse ES-cells; *HPRT1* minigene

Segregation analysis in cancer-afflicted families provides the most reliable information to distinguish between deleterious and neutral alterations identified in *BRCA1* or *BRCA2*¹. However, there is an enormous need to have a functional assay to classify variants for which such information is not available because most mutations are rare and familial data are often insufficient. A few functional assays have been designed that either utilize a transcriptional regulation activity or rely on functional complementation tests using *BRCA1*-deficient human tumor cells or *BRCA2*-deficient hamster cell lines (see reference ² for review and references). Although these can distinguish between neutral and deleterious variants, such complementation assays have limited application because they rely on using cDNA expression vectors to deliver mutant proteins in cells that are inherently prone to genomic instability.

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Author contribution

S.G.K. conducted all the experiments and wrote the manuscript. P.L. helped with initial concept and generated selection marker cassettes. S.K.S. conceived the idea, generated the conditional ES cell line, supervised the study and wrote the manuscript.

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RESULTS

Design of an ES-cell-based functional assay

We have used mouse ES-cells and BACs to design a functional assay to classify BRCA2 variants based on the observation that *Brca2* is essential for ES-cell viability³. The ability of BRCA2 variants to rescue the lethality of *Brca2*-deficient ES-cells is used to evaluate the functional significance of individual variants (Fig. 1a). Sequence variants that fail to rescue *Brca2*-deficient ES-cells are considered deleterious. The non-lethal variants are screened for a defect in the known function of BRCA2 in DNA repair as described below.

We generated a conditional allele of *Brca2* by flanking the entire gene locus with two *loxP* sites along with two halves of the human *HPRT1* mini gene in AB2.2 ES-cells that lack functional *Hprt* gene and are sensitive to HAT selection (*HAT^s*)⁴. (Supplementary Figures 1 and 2 online) and then disrupted the second copy of *Brca2* by targeting a blasticidin resistance gene into exon 11 of the gene (Supplementary Figure 3 online). When Cre was transiently expressed in one of the resulting ES-cell clones (P12F7), we obtained only 5–10 *HAT^r* colonies (background colonies that retained a wild-type copy after plating 10⁵ cells). Control cells carrying a conditional and a wild-type allele of *Brca2* yielded 4,000–6,000 colonies (data not shown).

Next, we rescued the lethality of *Brca2*-deficient ES-cells by electroporating a human *BRCA2*-containing BAC retrofitted with a *neomycin* resistance gene into P12F7 cells. We tested three independent *Neo^r* clones showing presence of 5' and 3' portions of *BRCA2* (Fig. 1b) and expressing different levels of human BRCA2 protein (Fig. 1c). All three clones yielded thousands of *HAT^r* colonies that lacked endogenous *Brca2* (Fig. 1d,e).

Distinguishing between deleterious and neutral variants

To assess the ability to distinguish between a deleterious and a neutral variant using this experimental system, we generated four mutations (279delAC, 6174delT N991D and D2723H) with an indisputable functional effect^{5–7} in the BAC. First, we examined two known deleterious mutations: 279delAC deletion that results in a stop signal at codon 29 (stop29) and 6174delT, that truncates the protein at codon 2003⁵ (Fig. 1f). As expected, these mutations could not rescue the ES-cell lethality supporting their deleterious nature.

To further assess the validity of our assay, we tested two missense variants, N991D, a known polymorphism⁶, and D2723H, a known deleterious missense mutation⁷. ES-cells expressing the N991D variant survived after the deletion of the endogenous *Brca2* and exhibited no difference in sensitivity to various DNA damaging agents compared to control cells (Supplementary Table 1 online). We found D2723H mutant protein to be present exclusively in the cytoplasmic fraction (Fig. 2a) as reported previously⁷. In our assay, it failed to rescue the ES-cells after Cre expression. Our results confirm that D2723H is deleterious and N991D is a neutral BRCA2 variant demonstrating our ability to successfully distinguish between deleterious and neutral alterations.

Functional evaluation of additional variants

We then selected a panel of sequence variants (see Table 1) to further validate the system and to demonstrate its applicability to test variants with different degrees of evolutionary conservation, alterations found in different functional domains, those with conflicting or unclear functional data as well as one splicing variant. In addition we have generated a hypomorphic allele and examined its phenotype in detail using various functional assays.

We first tested 7 BRCA2 variants (see Table 1), five of them (Y42C, D1420Y, V2728I, E2856A, and S2988G) with a limited evolutionary conservation and two (E2663V, G2901D) conserved across the animal kingdom and plants. All less-conserved variants sustained the ES-cell viability after the endogenous *Brca2* was deleted and did not show hypersensitivity to any DNA-damaging agents. Four of these mutations, with the exception of S2988G that was found only once, have been frequently recorded in the BIC database⁸ and epidemiological data (Table 1) strongly suggested that these were neutral variants. Despite these data, Y42C was shown by *in vitro* studies to disrupt an interaction between the N-terminus of BRCA2 and the replication protein A (RPA)⁹, a protein essential for DNA repair, which directly contradicts the epidemiological studies¹⁰. Lack of any deleterious effect of Y42C in ES cells further validates our experimental system and reveals limitations of experiments in which an isolated portion of the protein is used to draw conclusions about causality.

The pathogenic potential of two evolutionary conserved variants, G2901D and E2663V, was unknown (see Table 1). Our assay shows that G2901D has no effect on BRCA2 function (Supplementary Table 1 online), suggesting that this mutation is neutral. The other variant, E2663V, did not rescue the cells after the Cre expression and, thus, should be classified as deleterious. Interestingly, it has recently been reported as a variant with high probability to be classified as deleterious¹¹.

We have previously demonstrated the reliability of using human BACs in mouse ES-cells to study BRCA1 splicing mutations¹². Here, we tested a BRCA2 missense variant T2722R that was recently reported to cause aberrant splicing and results in deletion of exon 18 in the transcript¹³. Indeed, in mutant ES-cells we found transcripts lacking exon 18 ($\Delta 18$, Fig. 3a,b). However, using RT-PCR and Western blot analysis we found that the major product of this allele was the full-length protein (Fig. 3a,c). We believe that unlike in ES-cells used in our studies, in tissue samples from individuals (who are heterozygous), such products may have escaped detection due to the presence of wild-type *BRCA2*. Nevertheless, the full-length T2722R BRCA2 did not rescue ES-cell viability corroborating the conclusion that the mutation is deleterious.

Functional assays to evaluate BRCA2 function

In an effort to demonstrate the ability to perform various functional assays on *Brca2*-deficient ES-cells, we generated a known deleterious mutation that results in a protein truncated at codon 3308 (Y3308X)⁸. We expected this mutation to be a hypomorphic allele based on the phenotype of mice that have a similar truncating mutation in *Brca2* (exon 27-deletion)^{14,15}. Evaluation of the Y3308X variant in our assay was also relevant to a similar truncation involving the next codon, E3309X, found in a patient with ovarian cancer (personal communication, Dr. William Foulkes). Based on the deleterious nature of the Y3308X variant, E3309X was expected to be deleterious. However, existence of a known polymorphism a few amino acids downstream, K3326X¹⁶, added ambiguity to the interpretation. We, therefore, tested these three truncations in our assay.

We obtained viable ES-cell clones expressing an Y3308X transgene as confirmed by RT-PCR (Fig. 1f) but observed 5–10 fold reduction in the number of *HAT^r* colonies. The mutant cells grew significantly slower *in vitro* as well as *in vivo* (Supplementary Figure 4 a,b online). These cells had a reduced plating efficiency, and were hypersensitive to crosslinking and methylating agents as well as ionizing radiation (Supplementary Table 1 online and Supplementary Figure 5 online), which is consistent with drug sensitivities known for *Brca2*-deficient cell lines^{17–20}.

Because of the known role of BRCA2 in homologous recombination¹⁵, we tested the efficiency of homologous recombination in Y3308X mutant ES-cells using two gene-targeting vectors.

One was designed to target a genomic locus located 150 kb upstream of *Brcal* (Supplementary Figure 6 a,b online) and contained a negative selection marker (diphtheria toxin gene, *DT*). The second construct, which targeted the *Rosa26* locus, lacked a negative selection marker (Supplementary Figure 6 c,d online). Although the control cells exhibited a targeting efficiency within the range of normal experimental variation, we did not observe a single correctly targeted clone in cells expressing Y3308X BRCA2 (Fig. 2b), suggesting a defect in the homologous recombination process. BRCA2 plays a key role in the recruitment of RAD51 to the sites of DNA repair²¹, therefore, we examined the effect of the Y3308X mutation on radiation-induced RAD51 foci formation. We observed on average 32 RAD51 foci per cell after irradiation (10 Gy) in control cells whereas none or occasionally only one or two RAD51 foci could be found in the mutant cells (Fig. 2 c–e). These results provide additional evidence demonstrating a defect in RAD51-mediated DNA repair in Y3308X cells.

One of the hallmarks of BRCA2-deficient cells is a marked increase in genomic instability predisposing them to tumorigenesis²². Karyotyping revealed on average 68 chromosomal aberrations such as chromatid gaps and breaks, radial structures and fragmented chromosomes per 100 mutant cells versus 10.5 aberrations per 100 control cells (Fig. 2 f–h). The extent of genomic instability in Y3308X mutant cells strongly suggests that this variant is likely to predispose a cell to tumorigenesis. Interestingly, we found elevated levels of p53 and p21 proteins in unirradiated mutant cells (Supplementary Figure 4c online). This elevated DNA damage response may explain the defects in cell proliferation and plating efficiency of the Y3308X mutant cells.

We also tested the K3326X truncation in our assay and found no effect on BRCA2 function (Supplementary Table 1 online and Supplementary Figure 5 online), which corroborates its classification as a neutral variant. In contrast, E3309X mutant ES-cells exhibited reduced viability and were hypersensitive to various DNA damaging agents (Supplementary Table 1 online and Supplementary Figure 5 online). We, therefore, concluded that, like Y3308X, E3309X was a deleterious variant.

Comparing the effects of R3051Q and R3052W variants

Finally, we tested the impact of different amino acid substitutions at the same position. We examined arginine at position 3052 located at the interface between oligonucleotide/oligosaccharide-binding folds 2 and 3 (OB2 and OB3, respectively) of the human BRCA2²³, which is found to be changed either to glutamine (R3052Q) or to tryptophan (R3052W) in the human population (see Table 1). A recent study shows R3052Q to have relatively low odds in favor of neutrality (354:1)¹¹. Arginine at this position makes hydrogen bonds with four neighboring amino acid residues, two from each of the OB folds, thus linking them together (Fig. 3d, left panel). Based on the computer simulation, we predicted R3052Q variant to have an activity either similar or partially reduced compared to the wild-type BRCA2 whereas the R3052W alteration would severely disrupt the protein function (Fig 3d, middle and right panels). In our assay, ES cells expressing R3052W variant did not survive (Fig. 3e, right) suggesting it to be deleterious. On the other hand, viable R3052Q-expressing ES cells were obtained and there was no difference in the number of colonies compared to control cells (Fig. 3e, left). When challenged with DNA damaging drugs, R3052Q ES-cells displayed no sensitivity to γ -irradiation and UV but a moderate sensitivity to Mitomycin C, MMS, MNNG and Cisplatin (Fig. 3f and Supplementary Table 1 online), which suggests only a partial loss of BRCA2 function. The moderate defect in BRCA2 function associated with R3052Q variant may potentially contribute to developing cancer, albeit a low risk. Future studies may provide unequivocal proof for the pathogenicity of R3052Q variant. An alternative approach to evaluate the role of such variants in tumorigenesis is to study them in mouse models. We have generated D2723H variant in mice and found it to result in embryonic lethality, which validates the

deleterious nature of this variant (Kuznetsov and Sharan, unpublished data). Such deleterious mutations can be studied using conditional knockout allele of *Brca2* to evaluate their role in tumor development.

DISCUSSION

We have provided here a preliminary validation of our ES-cell-based functional assay for evaluating mutations in human *BRCA2* by testing 17 variants. Although, the ultimate validation of this assay in a true epidemiological sense is not possible at present, for 13 variants (four truncating variants excluding E3309X, plus six missense variants Y42C, N991D, D1420Y, E2663V, D2723H, V2728H, E2856A, R3052Q and one missense mutation, T2722R, which also affects splicing) the results obtained in our assay completely agreed with predictions based on epidemiological studies and other available functional data^{6,7,13,24}. The functional significance of 4 other mutations (E3309X, G2901D, S2988G, and R3052W) was unknown and reported here for the first time. Future studies will show how the range of phenotypes revealed for different mutations, i.e. cell lethality, degree of sensitivity to DNA damaging agents and, possibly, specific sensitivity to only a subset of toxic compounds correlates with their penetrance and disease presentation in humans.

We have established that this assay is accurate and one of the most comprehensive among those available to date, allowing analysis of virtually any type of mutation and may also serve as a model for investigating other human disease genes that result in a phenotype detectable in ES-cells. We have streamlined the process of mutation analysis. Three to five such sequence variants can be analyzed within 2–3 months (time for each procedure is specified in Fig. 4). This makes our assay a useful, tractable and viable tool for genetic counselors. However, until the assay is fully validated, caution must be exercised when using these data to make clinical decisions.

METHODS

Generation of mutations in *BRCA2* in a BAC clone

BAC RP11-777I19, containing full-length human *BRCA2* gene²⁵ was used to generate mutations. The mini-lambda-based recombineering system was introduced into BAC containing DH10B cells as described previously²⁶. The *loxP* site was deleted and *loxP*511 was replaced with a neomycin resistance gene under the control of *Pgk* promoter to enable a positive selection of transgenic ES-cells. Mutations were introduced using the oligonucleotide-based “hit and fix” BAC recombineering method as described previously²⁷. All mutations were confirmed by sequencing.

Generation of *Brca2*-null ES-cells carrying a mutant transgene

Mouse embryonic stem cells were cultured in M15 media (Knock-out DMEM (Gibco) and supplemented with 15% fetal bovine serum (HyClone), GPS (glutamate-penicillin-streptomycin, Gibco) and 0.1 mM β -mercaptoethanol with SNL feeder cells. 25 μ g BAC μ DNA was electroporated into 10⁷ P12F7 ES-cells (for details on generation of these cells from the AB2.2 cell line see Supplementary Figures 1–3 online) in 0.9 ml PBS at 230 V, 500 μ F. Transgenic colonies were selected with G418 (Gibco) and analyzed for the presence of *BRCA2* gene by Southern blot. Genomic DNA was hybridized with a 581 bp fragment corresponding to intron 1 of *BRCA2* as a 5' probe (nucleotides 13,869,785–13,870,365 of NT_024524.13) recognizing a 1,835 bp *EcoRI* fragment and a 356 bp fragment corresponding to exon 27 of *BRCA2* as a 3' probe (nucleotides 13,953,046–13,953,401 of NT_024524.13) recognizing a 4,275 bp *EcoRI* fragment (Fig. 1b). Double-positive clones were further tested for expression of *BRCA2* by RT-PCR and Western blot analysis. *BRCA2* expressing clones

were electroporated with *Pgk-Cre* plasmid⁴ and 10⁵ cells were seeded in a 100 mm dish with SNL-feeder cells. Recombinant colonies were selected in the HAT media (Gibco) for 5 days followed by 2 days in HT media (Gibco). Viable colonies were analyzed by Southern blot to confirm the loss of the conditional *Brca2* allele. A 1.5 kb probe specific to *Brca2* exon 11 (nucleotides 5208–6710 of NM 009765) has been used to detect a 2.2 kb *EcoRV* fragment corresponding to the mutant allele (ko) and a 4.8 kb fragment corresponding to the wild-type or conditional allele (cko) (Fig. 1e and Supplementary Figure 3 online). *HAT^r* colonies were also stained with methylene blue (2% methylene blue w/v in 70% ethanol for 15 minutes followed by washing with 70% ethanol) to facilitate their quantification.

Expression analysis

For Western blot analysis proteins were extracted in RIPA buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 0.25% sodium deoxycholate, 1 mM sodium fluoride, 1 mM orthovanadate). NE-PER buffer system (Pierce) was used to isolate nuclear and cytoplasmic protein fractions. Proteins were then separated on a precast NuPAGE 4–12% Bis-Tris gel (Invitrogen) in MOPS SDS running buffer (Invitrogen). The following antibodies have been used: BRCA2 (Ab-2, Calbiochem, 1:500), actin (Ab-5, NeoMarkers, 1:1,000), p53 (CM5p, Novocastra), p21 (F-5, Santa Cruz). Secondary antibodies were goat anti-rabbit IgG-HRP (sc-2004, Santa Cruz) at 1:5,000 dilution and goat anti-mouse IgG-HRP (sc-2005, Santa Cruz) at 1:2,000 dilution. ECL Plus Western Blotting Detection system (Amersham) was used for chemiluminescent detection.

Genotoxin sensitivity assay

8,000 ES-cells per well (16,000 cells per well for Y3308X and E3309X mutants to compensate for lower seeding and growth efficiency) were seeded in 96-well plates in 2–4 replicas. Drug treatment started 18 hours later (concentrations indicated in Supplementary Figure 5 online). For UV sensitivity testing 32,000 ES-cells (64,000 cells for Y3308X and E3309X) per well were seeded in triplicates in 24-well plates. Next day cells (without media) were irradiated at indicated doses in a UV-crosslinker (Stratagene) and then cultured in fresh media. For γ -irradiation plates were exposed to a ¹³⁷Cs source at 146.3 rad/minute without media change. 72 hours later the relative number of living cells was measured using XTT assay²⁸. Values were adjusted for “no cells” background.

RAD51 foci formation assay

40,000 cells per well were plated in gelatinized SonicSeal plastic slides (NUNC). 48 hours later slides were irradiated with 10 Gy. Six hours later cells were fixed with 4% paraformaldehyde for 5 minutes, washed twice with PBS and permeabilized in PBS-buffered 0.1% Triton X-100 for 10 minutes. After two additional washes with PBS, cells were blocked in a blocking solution (1% BSA, 0.05% Triton X100, 10% donkey serum in PBS). The antibody staining and imaging was performed as described previously²⁹.

Homologous recombination assay

Exponentially growing ES-cells were electroporated with each of the targeting vectors described in Supplementary Figure 6 online. Clones were selected for resistance to hygromycin or puromycin. 96 colonies for each cell line were analyzed for homologous recombination by Southern blot analysis as shown in Supplementary Figure 6 online.

Karyotyping

ES-cells were treated with colcemid (Invitrogen) for 1.5 hours to arrest at metaphase. Cells were trypsinized, washed and resuspended in hypotonic solution at 37°C (0.075M KCL) for 15 minutes and fixed in a methanol/acetic acid mixture (3:1 v/v). Air-dried preparations were

stained in Giemsa solution (10% Sorensen's buffer and 2% Giemsa, J.T. Baker). Two hundred well-spread metaphases containing at least 40 chromosomes were examined blindly from each genotype for structural aberrations.

Crystal structure modeling

Crystal structure 1MIU for a C-terminal portion of mouse BRCA2 was retrieved from the protein data bank at <http://www.rcsb.org/> and analyzed using Sirius software available at <http://sirius.sdsc.edu> and DS ViewerPro from Accelrys Software Inc.

Statistical analysis

Results obtained from testing mutant ES-cells for sensitivity to genotoxins were further evaluated for statistical significance using ANOVA test. Because Y3308X and E3309X cells showed identical phenotype, they were later tested as one group (n=6) against the group of controls (two WT clones and K3326X polymorphism, n=9) using two-tailed t-test with unequal variance.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. *BRCA2* mutation analysis using mouse embryonic stem cells

(a) Scheme representing functional analysis of human *BRCA2* mutations. Boxes with HP and RT indicate two exons of *HPRT* minigene. *loxP* sites are shown by solid triangles. Star in the BAC construct indicates a mutation in *BRCA2*. (b) Southern blot analysis of *Neo^r* colonies to detect the presence of the 5' and 3' ends of the *BRCA2* gene (labeled as 5' and 3', respectively). Fragment size is shown in brackets). Numbers above the lanes indicate different ES-cell clones. (c) Western blot analysis of three ES-cell clones expressing human *BRCA2* (numbers correspond to clone numbers). Human H-1 ES-cells (H-1) and heterozygous mouse ES-cells (No BAC) were used as a positive and negative control, respectively. β-actin was used as a loading control. (d) Survival of the ES-cell colonies after HAT selection. Numbers in the upper

left corner indicate ES-cell clones shown in (c). (e) Southern blot analysis of the *HAT^r* clones for the loss of the conditional *Brca2* allele (cko), lanes 1–10. Lower band indicates the knockout allele (ko). Size of the fragments is shown in brackets. (f) Expression of the mutant *BRCA2* constructs as detected by Western blot (WB) and RT-PCR analysis. b-actin is used as a loading control. Some *BRCA2* variants were not detected by WB analysis due to deletion of the region recognized by the antibody.

Figure 2. Mutant phenotypes revealed by Y3308X and other BRCA2 variants in ES-cells
 (a) Western blot analysis of nuclear (N) and cytoplasmic (C) protein fractions showing abnormal subcellular localization of D2723H mutant BRCA2 protein. HSP90 and Oct1 were used as controls to show purity of cytoplasmic and nuclear fractions, respectively. b-actin is used as a loading control. (b) Y3308X mutant is defective in homologous recombination process as shown by two gene-targeting vectors (see Supplementary Fig. 6). Numbers on the bars indicate the actual number of colonies undergoing homologous recombination relative to the total number of colonies tested. (c, d) Radiation-induced RAD51 foci formation is severely compromised in Y3308X mutant ES-cells. RAD51 foci are shown in green, γ-H2AX foci marking DNA damage are shown in pink. Nuclei are stained with DAPI (in blue). (e)

Quantification of RAD51 and γ -H2AX foci before (–IR) and after (+IR) ionizing radiation. 30 and 40 nuclei have been counted in each case for Y3308X mutant and the wild-type (WT) control, respectively. (f – h) Genomic instability revealed by karyotypic analysis of Y3308X cells. Shown are representative metaphases of the wild-type (WT) control cells (f) and Y3308X (g). Arrow shows a radial structure, arrowheads indicate chromatid gaps. (h) Histogram showing quantitative analysis of chromosomal aberrations in untreated WT and Y3308X ES-cells. 100 metaphases were counted twice and scored for different types of chromosomal aberrations. Error bars in (e) and (h) indicate standard deviation. Scale bar in (c, d, f, and g) represents 20 μ m.

Figure 3. Analysis of BRCA2 mutants affecting splicing or domain structure

(a) T2722R transcript reveals skipping of exon 18 (Δ18, lower band, lane T2722R) not detected for wild-type *BRCA2* (WT). Mutant ES-cells also express the full-length transcript (FL, upper band). PCR was performed on cDNA synthesized with a sequence specific primer 5'-CCTCCTGAATTTTGTGAATAAGGCTTCTAGTCTC-3'. PCR amplification was carried out with forward primer 5'-AGGGCTCTGTGTGACACTCC-3' and reverse primer 5'-GCAGGCCGAGTACTGTTAGC-3' residing in exons 17 and 19, respectively. M, 1kb plus (Invitrogen) molecular weight marker. (b) T2722R clones express FL isoform carrying a missense mutation (boxed in the upper panel) and a Δ18 transcript. (c) Independent clones with T2722R variant (lanes 2–5) show expression of the full-length protein. β-actin was used as

loading control. (d) Computer simulation of the mouse Q2971 (corresponding to human R3052Q, middle panel) and mouse W2971 (R3052W in humans, right panel) substitutions at the interface between OB2 and OB3 domains. Letter in the bottom left corner of each panel indicates a simulated residue. Numbers in pink indicate neighboring residues. Solid yellow lines signify stable hydrogen bonds and dashed yellow lines represent potential hydrogen bonds. Numbers in yellow show distances between interacting groups in angstroms. (e) Survival of R3052Q (left) and R3052W (right) variants after Cre expression. (f) ES-cells carrying R3052Q variant are moderately sensitive to MMC. R3052Q sensitivity is intermediate between wild type cells and Y3308X mutation carriers.

Figure 4. Evaluation of BRCA2 variants

Schematic representation of the assay designed to examine functional significance of mutations in the human *BRCA2* gene in mouse ES-cells as described in the text. Time required for each individual procedure is indicated in parenthesis below step description.

Table 1
Summary of the functional assay results and available epidemiological and sequence conservation data.

Nucleotide change	Predicted AA change	Entries in BIC database ^a	Evolutionary conservation ^b	Co-occurrence with additional deleterious mutations ^c	Co-segregation excluded in No. of families ^c	Cell viability	Sensitivity to DNA damaging drugs	Pathogenic effect
Truncations (5):								
279delAC	stop29	5	–	N/D	N/D	No	N/A	Deleterious
6174delIT	stop2003	1087	–	N/D	N/D	No	N/A	Deleterious
10152 C>G	Y3308X	13	++	N/D	N/D	Reduced	Sensitive	Deleterious
10153 G>T	E3309X	1	–	N/D	N/D	Reduced	Sensitive	Deleterious
10204 A>T	K3326X	293	–	N/D	N/D	Yes	No	Neutral
Splice Variant (1):								
8393 C >G	T2722R	4	++++	0	0/1	No	N/A	Deleterious
Point mutations (11):								
353 A>G	Y42C	170 ^c	+++	9/170	9/16	Yes	No	Neutral
3199 A>G	N991D	3357 ^c	–	65/3357	N/D	Yes	No	Neutral
4486 G>T	D1420Y	191	+	22/508	N/D	Yes	No	Neutral
8216 A> T	E2663V	9	++++	0/8	0/2	No	N/A	Deleterious
8395 G>C	D2723H	37	++++	0/30	0/13	No	N/A	Deleterious
8410 G>A	V2728I	256 ^c	+	9/256	N/D	Yes	No	Neutral
8795 A>C	E2856A	185	+++	0/148	16/26	Yes	No	Neutral
8930 A>A	G2901D	2	++++	2/2	0/0	Yes	No	Neutral
9190 A>G	S2988G	1	+++	N/D	N/D	Yes	No	Neutral
9382 C> T	R3052W	8	++++	N/D	N/D	No	N/A	Deleterious

N/A, not applicable; N/D, not determined. Number in parenthesis indicates the total number of mutations analyzed for each mutation category.