RESEARCH ARTICLE

A High-Throughput Functional Complementation Assay for Classification of *BRCA1* Missense Variants

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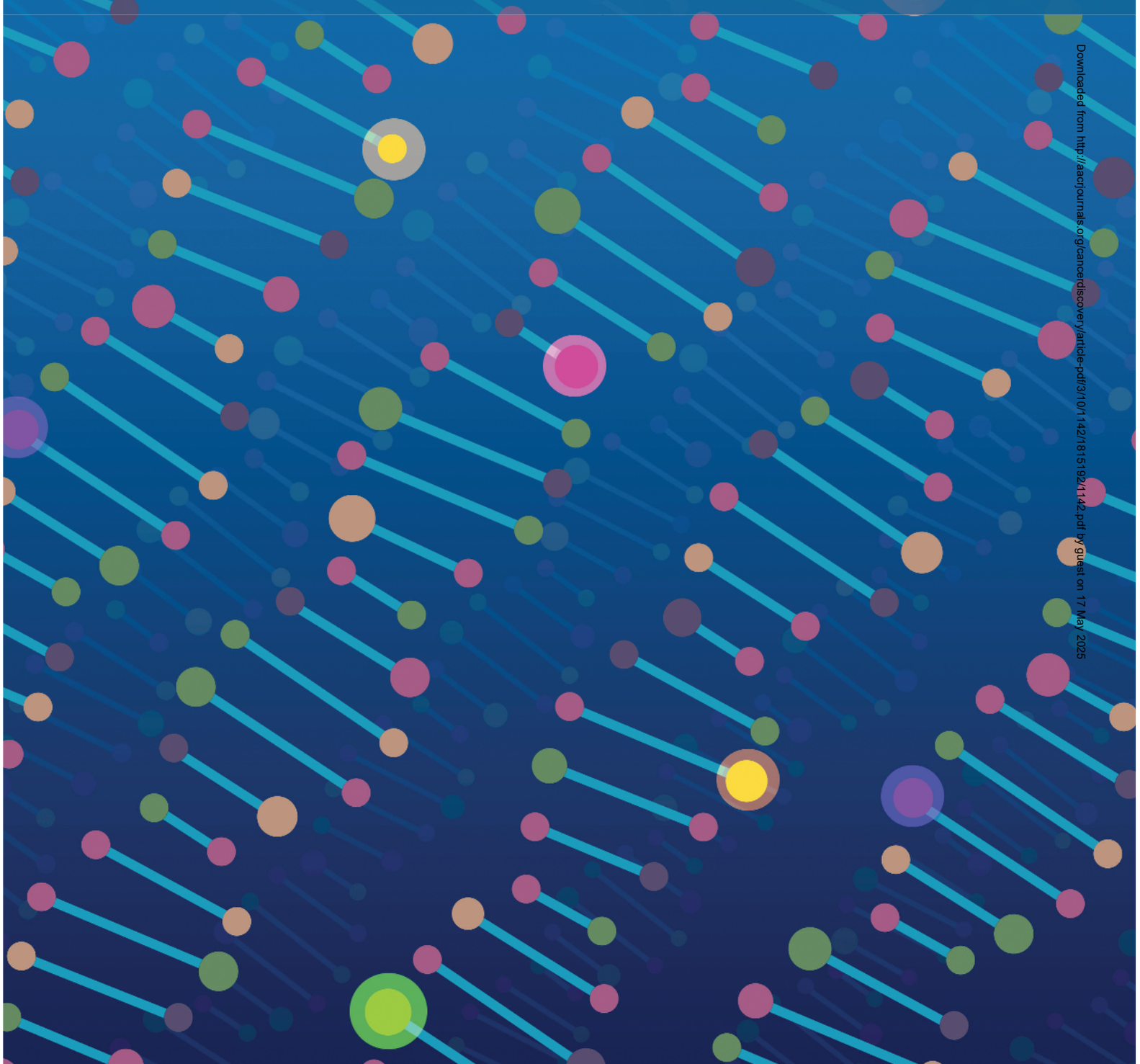

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ABSTRACT Mutations in *BRCA1* and *BRCA2* account for the majority of hereditary breast and ovarian cancers, and therefore sequence analysis of both genes is routinely conducted in patients with early-onset breast cancer. Besides mutations that clearly abolish protein function or are known to increase cancer risk, a large number of sequence variants of uncertain significance (VUS) have been identified. Although several functional assays for *BRCA1* VUSs have been described, thus far it has not been possible to conduct a high-throughput analysis in the context of the full-length protein. We have developed a relatively fast and easy cDNA-based functional assay to classify *BRCA1* VUSs based on their ability to functionally complement BRCA1-deficient mouse embryonic stem cells. Using this assay, we have analyzed 74 unclassified *BRCA1* missense mutants for which all predicted pathogenic variants are confined to the BRCA1 RING and BRCT domains.

SIGNIFICANCE: *BRCA1* VUSs are frequently found in patients with hereditary breast or ovarian cancer and present a serious problem for clinical geneticists. This article describes the generation, validation, and application of a reliable high-throughput assay for the functional classification of *BRCA1* sequence variants of uncertain significance. *Cancer Discov; 3(10); 1142–55. ©2013 AACR.*

INTRODUCTION

Germline loss-of-function mutations in *BRCA1* and *BRCA2* are known to result in an approximately tenfold increased lifetime risk of developing breast or ovarian cancer. Thus far, no other genes have been identified with such a strong link to hereditary breast and ovarian cancer (HBOC), and in the past decades, many women have been screened for germline mutations in *BRCA1* or *BRCA2*. This has resulted in the identification of numerous pathogenic mutations as well as a large number of sequence variants for which the clinical relevance is not clear. In the most recent publication of the ENIGMA (Evidence-based Network for the Interpretation of Germline Mutant Alleles) group, an international consortium for the evaluation of *BRCA1* or *BRCA2* sequence variants, a total of 1,273 unique *BRCA1* variants is mentioned (1), but this number may increase even further because of the implementation of high-throughput sequencing methods. Of the *BRCA1* variants of uncertain significance (VUS), 920 are nontruncating exonic mutations that may affect protein function or mRNA splicing, but for which there is not enough linkage information to indicate whether they are pathogenic. To aid genetic counseling of individuals with *BRCA1/2* VUS, both genetic and functional classification methods have been developed. Genetic analysis of *BRCA1* or *BRCA2* VUSs relies on cosegregation with disease, cooccurrence with known pathogenic mutations, and family history of cancer. These data have been integrated into computational models to calculate the likelihood that a VUS is disease-causing (2). Additional *in silico* analysis of the evolutionary conservation of the amino acids affected by the mutation and the predicted impact of the mutant amino acids on protein folding are also implemented in such models. Functional assays do not rely on preexisting data, but directly test the effect of *BRCA1/2* VUSs on known functions of the encoded proteins (3). Although this may seem relatively straightforward, it can be difficult to extrapolate data from functional assays into cancer risks for patients. For instance, in most assays only part of the BRCA1 protein is analyzed. In addition, some of the more elegant assays are technically demanding and not suitable to analyze large numbers of mutations. We reasoned that a good functional assay should fulfill three basic requirements: (i) it should investigate the biologic effects of a *BRCA1* VUS in the context of the full-length protein; (ii) it should be conducted under normal physiologic conditions in a noncancerous cell type; and (iii) it should be based on a highly standardized and reproducible protocol.

These considerations led us to develop a functional assay based on physiologic expression of full-length human *BRCA1* cDNA in mouse embryonic stem cells that are genetically engineered to allow conditional deletion of endogenous *Brca1*. Mutant *BRCA1* cDNAs are generated using site-directed mutagenesis (SDM) and introduced in a defined genomic locus of mouse embryonic stem cells by recombinase-mediated cassette exchange (RMCE). In this way, we have analyzed 86 *BRCA1* variants for their effects on cell proliferation and drug sensitivity, including 74 clinically relevant VUSs.

RESULTS

Generation of Mouse Embryonic Stem Cells Expressing *BRCA1* Sequence Variants

Although BRCA1-deficient tumor cells proliferate rapidly *in situ*, loss of BRCA1 in normal cells leads to a severe proliferation defect (4). We decided to make use of this phenotype for the functional analysis of *BRCA1* variants in

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Note: Supplementary data for this article are available at Cancer Discovery Online (http://cancerdiscovery.aacrjournals.org/).

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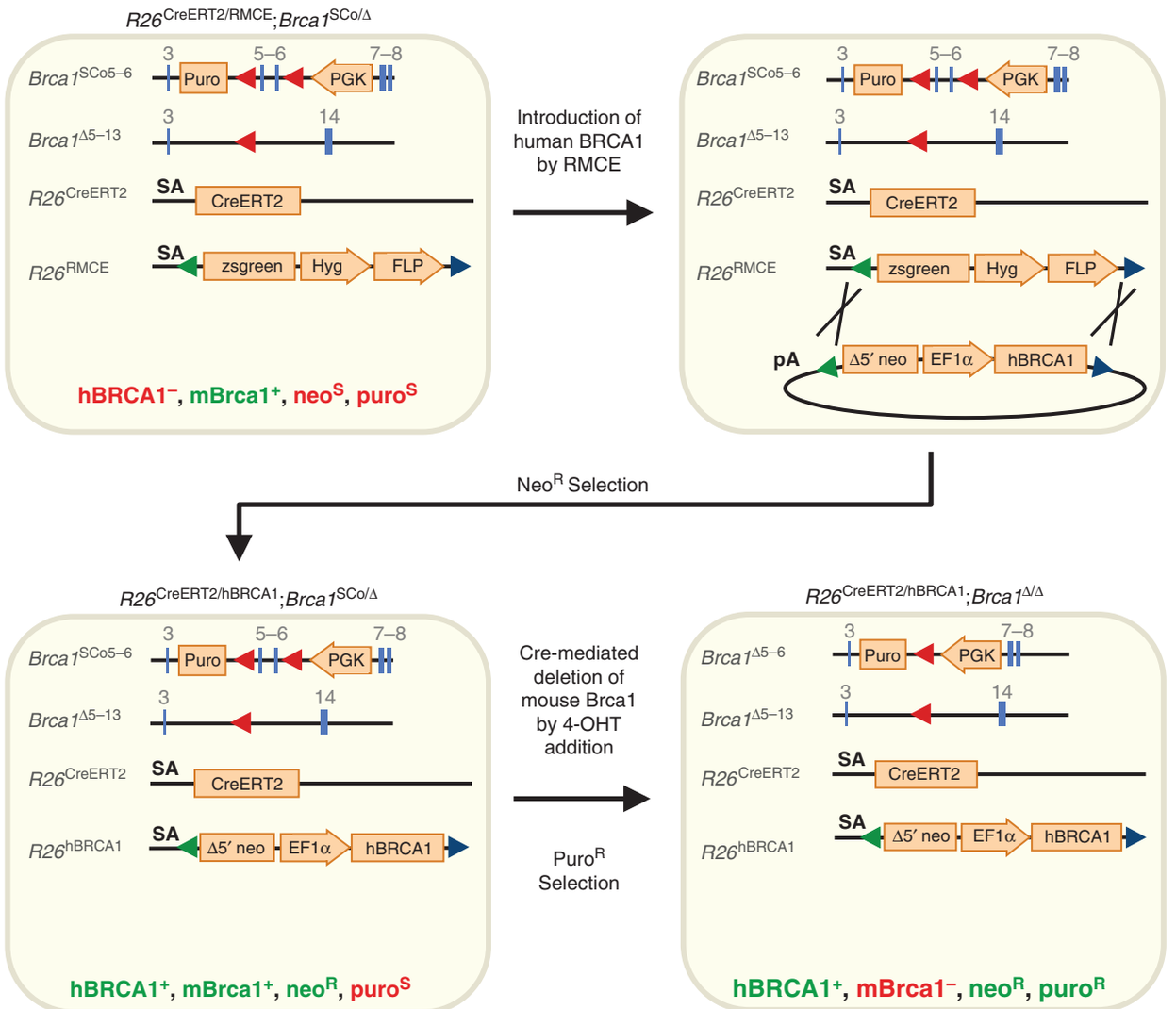

Figure 1. Schematic overview of the RMCE procedure in *R26*$^{CreERT2/RMCE}$;*Brca1*$^{SCo/\Delta}$ embryonic stem cells. Before the introduction of a human *BRCA1* cDNA, *R26*$^{CreERT2/RMCE}$;*Brca1*$^{SCo/\Delta}$ embryonic stem cells are mouse BRCA1-proficient and sensitive to both neomycin and puromycin. Targeting of single-copy human *BRCA1* cDNA variants to the *Rosa26* locus by Flp RMCE results in expression of human BRCA1 and neomycin resistance. Addition of 4-OHT leads to CreERT2-mediated deletion of mouse *Brca1* exons 5 and 6, resulting in loss of mouse BRCA1 protein and concomitant expression of puromycin from the *phosphoglycerate kinase* (PGK) promoter. This enables selection of mouse BRCA1-deficient, human BRCA1-expressing *R26*$^{CreERT2/hBRCA1}$;*Brca1*$^{\Delta/\Delta}$ embryonic stem cells that can be used in functional complementation assays.

Brca1-selectable conditional knockout (SCo) mouse embryonic stem cells (5). These cells carry one *Brca1*-null allele and a selectable conditional *Brca1*^{SCo} allele, which contains, in addition to *loxP* sites around exons 5–6, a split puromycin resistance marker that is activated upon Cre-mediated deletion of exons 5–6. They also contain a *CreERT2* allele in the *Rosa26* locus, which allows for controlled activation of Cre via administration of 4-hydroxytamoxifen (4-OHT). Thus, BRCA1-deficient embryonic stem cells can be easily obtained via 4-OHT–induced inactivation of the *Brca1*^{SCo} allele and subsequent selection for puromycin resistance (Fig. 1; ref. 5). To allow efficient integration of human *BRCA1* variants in one specific genomic locus, we supplied the other *Rosa26* allele with *F3* and *Frt* recombination sites for RMCE by the site-specific recombinase Flp (Fig. 1 and Supplementary Fig. S1; ref. 6). Cells that have undergone successful RMCE can be selected because they express a truncated neomycin selection marker under control of the endogenous *Rosa26* promoter, which further increases targeting efficiency. Flp-mediated recombination ensures single-copy integration of *BRCA1* expression constructs at the same *Rosa26* locus, thus avoiding position-effect variegation and copy number–dependent differences in expression.

RMCE vectors were equipped with a human *BRCA1* cDNA expression construct, which was modified using SDM to introduce defined mutations in *BRCA1*. The focus of our analysis was on Dutch and Belgian VUSs that were found in families with HBOC. We also included a number of variants

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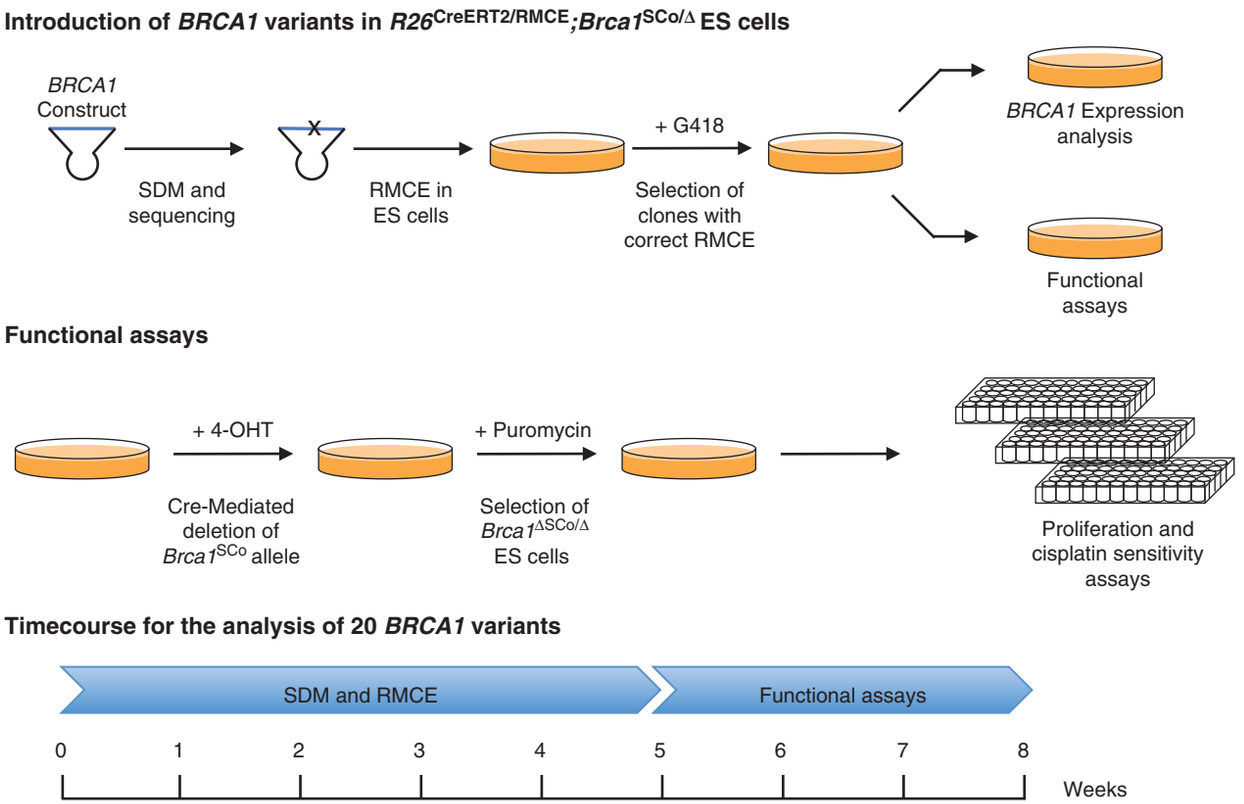

Figure 2. Workflow for the functional classification of *BRCA1* sequence variants in *Brca1*-null embryonic stem (ES) cells. Outline of the generation of mouse *Brca1*-deficient embryonic stem cells expressing human *BRCA1* variants and functional complementation assays. Indicated are the experimental steps and the time it takes one person to analyze 20 mutants.

that were previously classified using functional assays (7, 8) or a multifactorial likelihood model (9), as well as M1400V, L1407P, and M1411T, which have been reported to attenuate the interaction between BRCA1 and PALB2 (10). To allow validation of our functional complementation assay, we included a series of eight *BRCA1* variants that are known to be deleterious or neutral according to the Breast Cancer Information Core database (BIC; http://research.nhgri.nih.gov/bic/; Supplementary Table S1). These controls include the well-known pathogenic *BRCA1* founder mutations 185delAG and 5382insC and the neutral polymorphisms Y105C, R866C, and E1250K. The BIC designation is supported by the classification according to the International Agency for Research on Cancer (http://iarc.fr/), which includes the Align-GVGD score that indicates biophysical and evolutionary alterations (http://agvgd.iarc.fr/; Supplementary Table S1; refs. 11, 12). Align-GVGD scores vary between C0 (likely neutral) and C65 (likely deleterious). Sequence-verified constructs that contained the intended VUS, but no other *BRCA1* mutations, were transfected into $R26^{CreERT2/RMCE};Brca1^{SCo/\Delta}$ embryonic stem cells to undergo RMCE (Fig. 2). Neomycin-resistant clones were pooled and RMCE was confirmed by PCR analysis. Subsequently, protein expression of the human BRCA1 variants was analyzed by Western blot analysis with a human BRCA1-specific antibody (Fig. 2 and Supplementary Fig. S2). Comparison with BRCA1 protein levels in embryonic stem cells expressing human *BRCA1* from a bacterial artificial chromosome (BAC), which is known to rescue embryonic lethality of *Brca1*-null mice (7, 13), showed that transcription of the human *BRCA1* cDNA from the *EF1A* gene promoter results in physiologic levels of BRCA1 protein (Supplementary Fig. S1). Most *BRCA1* variants were expressed at equal levels, allowing comparison of their functional activities. A number of C-terminal BRCA1 mutants showed low levels of expression (Supplementary Table S2; Supplementary Fig. S2). Real-time RT-PCR analysis showed that decreased protein expression was not caused by decreased mRNA expression (Supplementary Fig. S3), suggesting that the low abundance of BRCA1 protein results from posttranslational events. In fact, for most of these variants, mutation-associated protein instability has already been documented in previous publications (refs. 8, 14, 15; Supplementary Table S2).

Functional Complementation Assay of *BRCA1* Sequence Variants in Mouse *Brca1*-Null Embryonic Stem Cells

As a first functional test, we assayed the ability of *BRCA1* variants to restore the proliferation defect of switched $R26^{CreERT2/RMCE};Brca1^{SCo/\Delta}$ embryonic stem cells (Fig. 2). Expression of endogenous mouse *Brca1* was shut off through overnight induction of Cre activity by 4-OHT, and 7 days

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after switching, cells were plated in 96-well plates for proliferation assays, and were analyzed using Sulphorhodamine B staining. For each group of mutants tested, we included positive and negative controls consisting of embryonic stem cells containing, respectively, wild-type human *BRCA1* cDNA and an empty RMCE vector. *BRCA1* variants were evaluated on their ability to support growth compared with these controls (Supplementary Tables S3 and S4). Although we tested 28 mutations in the central domain encoded by exon 11 (aa 224–1366), *BRCA1* variants that were unable to rescue the proliferation defect of *Brca1*-null mouse embryonic stem cells to BRCA1 wild-type levels were confined to the conserved N- and C-terminal domains of BRCA1.

Cisplatin Sensitivity Assay for Classification of *BRCA1* Variants

Although the ability of *BRCA1* variants to support proliferation appears to be indicative of VUS function, results were not always clear-cut (Supplementary Tables S3 and S4). BRCA1 is known to be important for DNA interstrand crosslink (ICL) repair through mechanisms that are both dependent (5) and independent (16) of its function in homologous recombination (HR). The role of BRCA1 in ICL repair is stressed by the occurrence of genetic reversion mutations restoring BRCA1 protein expression in platinum-resistant ovarian tumors in *BRCA1*-mutation carriers (17, 18). We therefore decided to conduct a 96-well–based cisplatin sensitivity assay to allow a more stringent evaluation of *BRCA1* VUS functionality.

Half-maximal inhibitory concentrations (IC_{50}) of cisplatin were determined using a resazurin cell viability assay, and *BRCA1* variants were again classified in comparison to wild-type *BRCA1* and an empty RMCE vector. To obtain corrected cisplatin IC_{50} values, we fitted a log-logistic curve constrained at 1 and 0. We excluded a fit if the residual squared error (RSE) exceeded 0.1. We then applied a Bayesian predictor to classify *BRCA1* VUS as pathogenic or benign. Most variants that showed less than wild-type activity in the proliferation assay also scored as functionally impaired in the cisplatin sensitivity assay and were classified as deleterious (Table 1,

Table 1. Functional classification of *BRCA1* VUS based on cisplatin response

Variant	DNA change	Type of mutation[a]	Classification
S4F	c.11C>T	VUS	Not Clear
M18T	c.53T>C	VUS	Deleterious
185delAG	c.68_69delAG	Deleterious control	Deleterious[b]
K45Q	c.133A>C	VUS	Neutral
C61G	c.181T>G	Deleterious control	Deleterious
C64G	c.190T>G	VUS	Deleterious[b]
D67Y	c.199G>T	VUS	Neutral
Y105C	c.314A>G	Neutral control	Neutral
N132K	c.396C>A	VUS	Neutral
P142H	c.425C>A	VUS	Neutral
L147F	c.441G>C	VUS	Neutral
L165P	c.494T>C	VUS	Neutral
R170W	c.508C>T	VUS	Neutral
S186Y	c.557C>A	VUS	Neutral
V191I	c.571G>A	VUS	Neutral
T231M	c.692C>T	VUS	Neutral
D245V	c.734A>T	VUS	Neutral
L246V	c.736T>G	VUS	Neutral
V271L	c.811G>C	VUS	Neutral
S308A	c.922A>G; c.923G>C	Artificial[h]	Not Clear
L358R; C360R; E362H	c.1073T>G; c.1078T>C; c.1084G>C; c.1086G>T	Artificial	Neutral
L668F	c.2002C>T	VUS	Neutral
D695N	c.2083G>A	VUS	Neutral
P798L	c.2393C>T	VUS	Neutral
N810Y	c.2428A>T	VUS	Neutral
T826K	c.2477C>A	VUS	Neutral

(*continued*)

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Table 1. Functional classification of *BRCA1* VUS based on cisplatin response (*Continued*)

Variant	DNA change	Type of mutation[a]	Classification
R841Q	c.2522G>A	VUS	Neutral
Y856H	c.2566T>C	VUS	Neutral
R866C	c.2596C>T	Neutral control	Neutral
S988A	c.2962T>G	Artificial	Neutral
M1008I	c.3024G>A	VUS	Neutral
E1060A	c.3179A>C	VUS	Neutral
S1101N	c.3302G>A	VUS	Neutral
K1110del	c.3328_3330delAAG	VUS	Neutral
S1140G	c.3418A>G	VUS	Neutral
E1214K	c.3640G>A	VUS	Neutral
N1236K	c.3708T>G	VUS	Neutral
E1250K	c.3748G>A	Neutral control	Neutral
L1267S	c.3800T>C	VUS	Neutral
E1282V	c.3845A>T	VUS	Neutral
S1297del	c.3891_3893delTTC	VUS	Neutral
S1301R	c.3903T>A	VUS	Neutral
E1346K	c.4036G>A	VUS	Neutral
V1378I	c.4132G>A	VUS	Neutral
M1400V	c.4198A>G	VUS	Neutral
L1407P	c.4220T>C	VUS	Neutral[b]
M1411T	c.4232T>C	VUS	Neutral
R1443G	c.4327C>G	VUS	Neutral
S1448G	c.4342A>G	VUS	Neutral
S1486C	c.4456A>T	VUS	Neutral
S1497A	c.4489T>G	Artificial	Neutral
V1534M	c.4600G>A	VUS	Neutral
R1589P	c.4766G>C	VUS	Neutral
M1628T	c.4883T>C	VUS	Neutral
S1651P	c.4951T>C	VUS	Not Clear
S1651F	c.4952C>T	VUS	Not Clear
M1652I	c.4956G>A	VUS	Neutral
S1655F	c.4964C>T	VUS	Deleterious[b]
H1686R	c.5057A>G	VUS	Deleterious
H1686Q	c.5058T>A	VUS	Deleterious
V1688del	c.5062_5064delGTT	VUS	Deleterious
T1691I	c.5072C>T	VUS	Not clear
R1699W	c.5095C>T	Deleterious control	Deleterious[b]
R1699Q	c.5096G>A	VUS	Deleterious[b]
G1706E	c.5117G>A	VUS	Deleterious[b]
G1706A	c.5117G>C	VUS	Neutral
A1708E	c.5123C>A	Deleterious control	Deleterious
W1718C	c.5154G>T	VUS	Deleterious
T1720A	c.5158A>G	VUS	Neutral
E1735K	c.5203G>A	VUS	Not clear

(*continued*)

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Table 1. Functional classification of *BRCA1* VUS based on cisplatin response (*Continued*)

Variant	DNA change	Type of mutation[a]	Classification
V1736A	c.5207T>C	VUS	Not clear[b]
D1739G	c.5216A>G	VUS	Deleterious
D1739V	c.5216A>T	VUS	Deleterious
H1746Q	c.5238C>G	VUS	Not clear
R1753T	c.5258G>C	VUS	Not clear[b]
5382insC	c.5266dupC	Deleterious control	Not clear[b]
L1764P	c.5291T>C	VUS	Deleterious[b]
C1767S	c.5300G>C	VUS	Neutral
G1770V	c.5309G>T	VUS	Deleterious[b]
W1782C	c.5346G>T	VUS	Neutral
A1789T	c.5365G>A	VUS	Deleterious
E1794D	c.5382G>C	VUS	Neutral
V1804D	c.5411T>A	VUS	Neutral
P1812R	c.5435C>G	VUS	Neutral
W1837R	c.5509T>C	VUS	Deleterious
H1862L	c.5585A>T	VUS	Neutral

[a]Type of mutation indicates if a variant is a VUS according to the BIC database (http://research.nhgri.nih.gov/bic/).
[b]Functionally impaired in the DR-GFP and/or combined PARP inhibitor/cisplatin sensitivity assay.

Fig. 3). Variants showing increased cisplatin sensitivity were tested at least twice before they were classified. The positive and negative controls classified as expected, although the known pathogenic truncation mutation 5382insC scored as neutral in one of three assays, stressing the need for repeat experiments. Also *BRCA1* variants that were previously tested in other assays conducted as expected. It should be noted that the artificial variants S308A and S1497A rescued proliferation and cisplatin responses of mouse *Brca1*-deficient embryonic stem cells in BAC complementation assays, but are predicted to be deleterious based on their effects on embryonic stem cell differentiation and their response to γ-irradiation, respectively (7). The V1804D mutation scored as a neutral variant in our assay, which is in line with most published data (8, 9), except for the results of an embryonic stem cell–based BAC complementation assay (7). Again, all *BRCA1* mutations that were classified as deleterious were confined to regions encoding the conserved N- and C-terminal domains (Fig. 4), despite the observation that deletion of the central region encoded by exon 11 leads to genetic instability in mice (12). Remarkably, the three mutations that diminish the interaction between BRCA1 and PALB2 (10), just C-terminal of the region encoded by exon 11, had no effect on cisplatin sensitivity in this assay. Of note, there was in general good correlation between our classification and the Align-GVGD score (Supplementary Table S4). Notable exceptions were the neutral control variant R866C, which validated our assay but scored as likely deleterious (C65) using Align-GVGD, and G1770V, which scored as likely neutral (C0) by Align-GVGD but was classified as deleterious in our assay. Our assay also classified some variants for which the Align-GVGD scores were less clear, emphasizing the usefulness of functional assays to complement *in silico* analysis.

We also analyzed the possible effects of all *BRCA1* variants on mRNA splicing, which may have deleterious consequences but cannot be assessed in our cDNA-based assay. The predictive value for exonic variants outside the consensus splice sites is questionable (19, 20), but four missense mutations were present in existing splice sites (Supplementary Table S4). Although c.5154G>T (encoding W1718C) was deleterious, three of these variants were classified as neutral in our assay. One of them, c.441G>C (encoding L147F), had an increased probability to damage the splice donor site of exon 7. Another variant, c.5072C>T (encoding T1691I), might also affect splicing, but no defect was measured in blood samples of mutation carriers (19). The third exonic splice site mutation that was classified as neutral in our cisplatin sensitivity assay, c.133A>C (K45Q), is not predicted to lead to aberrant splicing.

Results from Proliferation and Cisplatin Sensitivity Assays Correlate with HR Activity of BRCA1 Variants

BRCA1 and BRCA2 are involved in DNA repair via HR (12, 21). Together with non-homologous end joining (NHEJ), HR forms the cellular defense against DNA double-strand breaks (DSB), a severe type of DNA damage that is lethal if unrepaired. Although HR is essentially error-free, NHEJ is error-prone, and therefore defects in HR are known to lead to genomic instability. Although it is not clear whether other

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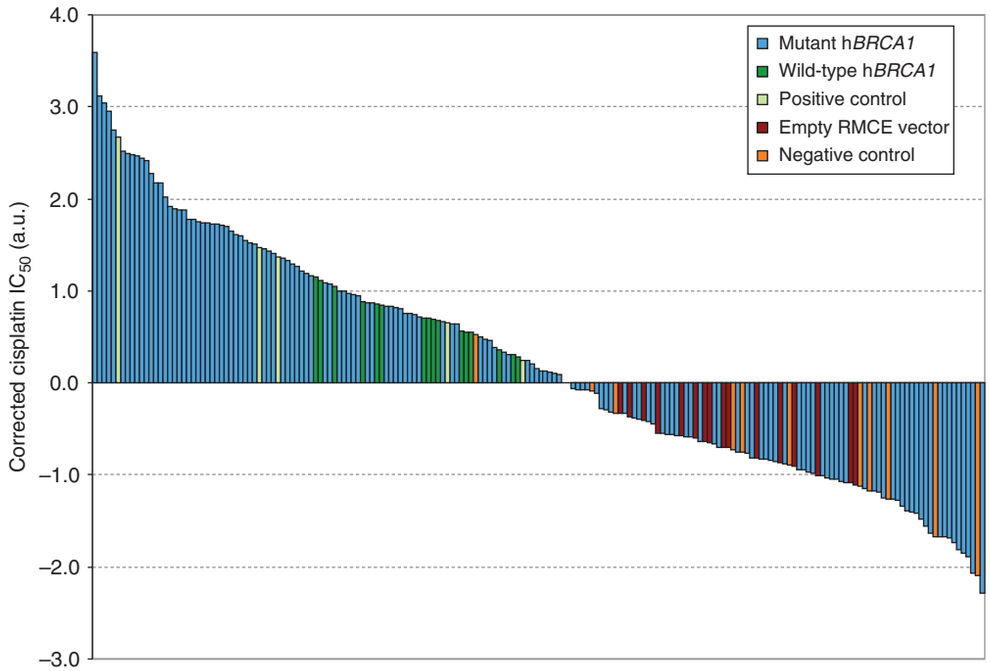

Figure 3. Waterfall chart of cisplatin IC_{50} values normalized for wild-type human *BRCA1* and empty RMCE vector controls. Corrected cisplatin IC_{50} values for all tested human *BRCA1* variants and controls, including repeat experiments. *BRCA1* variants are classified as functionally impaired or neutral when the corrected IC_{50} values are similar to either the empty RMCE vector or the wild-type human *BRCA1* controls ($P < 0.05$).

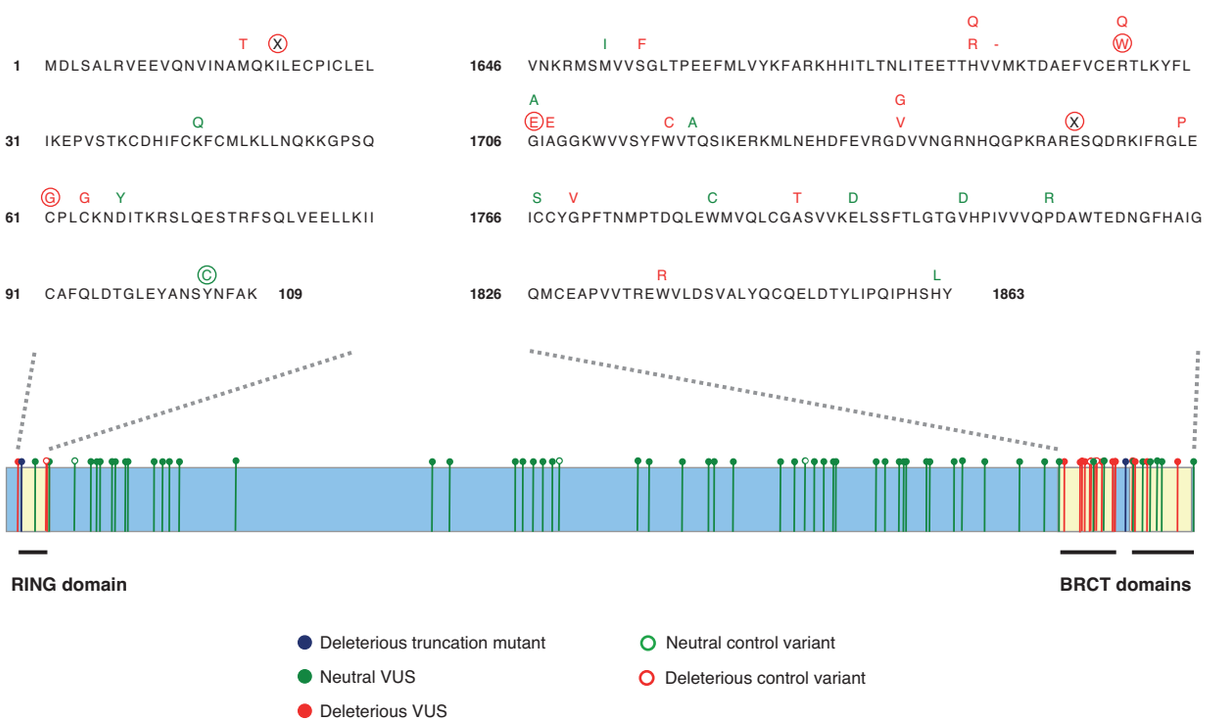

Figure 4. Predicted pathogenic BRCA1 amino acid substitutions are confined to the evolutionarily conserved N- and C-terminal domains. Schematic representation of the BRCA1 protein with the positions of variants classified as neutral (green) or deleterious (red) indicated. Positive and negative controls are depicted by open pinheads and the known deleterious truncation mutations 185delAG (N-terminal) and 5382insC (C-terminal) are marked in blue. The amino acid sequences of the evolutionarily conserved RING (N-terminal) and BRCT (C-terminal) domains are specified to show the exact positions of deleterious (red) and neutral (green) variants. The amino acid substitutions or nonsense (X) mutations of positive and negative controls are encircled.

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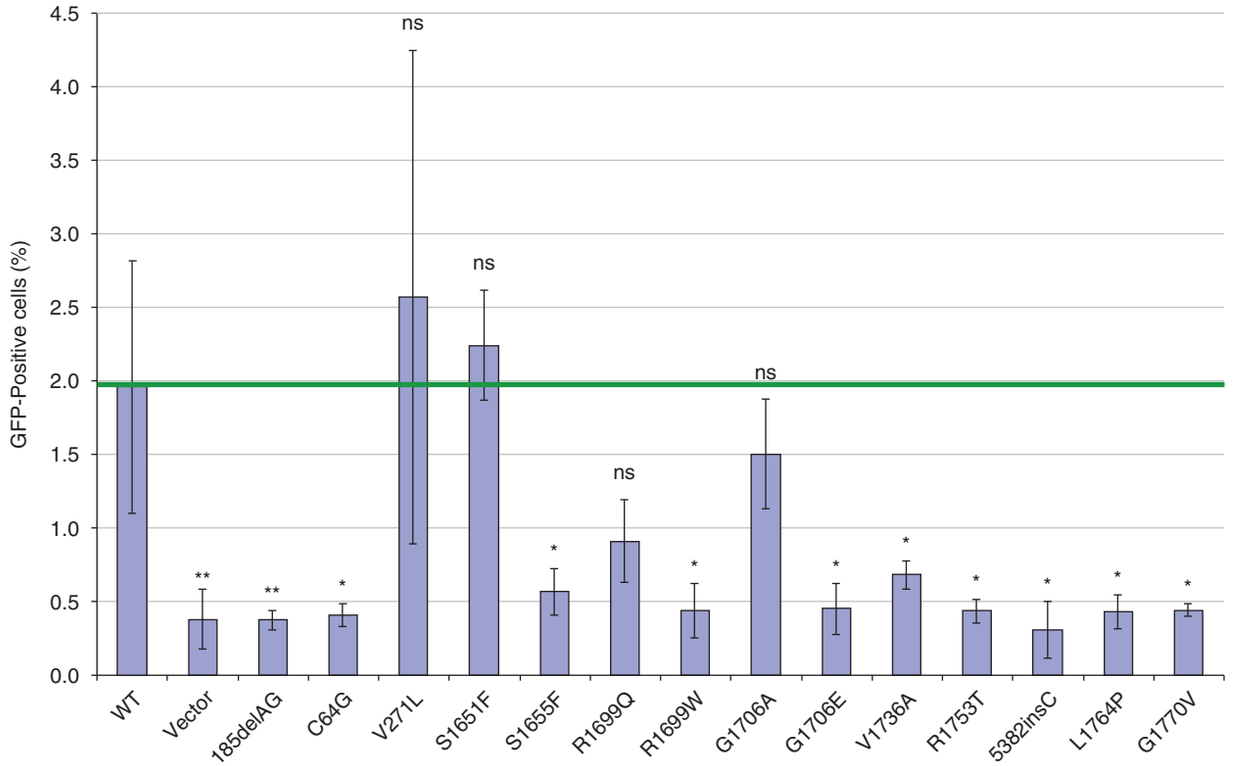

Figure 5. *BRCA1* sequence variants classified as pathogenic do not restore HR. $R26^{CreERT2/hBRCA1}$;$Brca1^{SCo/\Delta}$;$Pim1^{DR\text{-}GFP/wt}$ embryonic stem cells carrying the DR-GFP reporter gene in the *Pim1* locus and mutant *BRCA1* or controls in the *Rosa26* locus were switched using 4-OHT and transfected with a vector expressing I-SceI and mCherry. Transfected cells were analyzed for GFP expression as a measure of HR activity. Expression of *BRCA1* wild-type (WT) cDNA resulted in increased HR compared to the empty RMCE vector (Vector) control. Error bars indicate the SD between the results of three independent I-SceI transfections. Significantly decreased HR activity compared with the wild-type control (green line) is indicated.

functions of BRCA1 are also important for tumor suppression (16, 22, 23–26), its role in HR is likely to be relevant. Therefore, we used the direct repeat (DR)-GFP assay (27) to measure the effects on HR for a subset of our *BRCA1* VUSs. A number of predicted pathogenic variants and controls were shuttled into $R26^{CreERT2/RMCE}$;$Brca1^{SCo/\Delta}$;$Pim1^{DR\text{-}GFP/wt}$ embryonic stem cells carrying the DR-GFP reporter in the *Pim1* locus. Expression of endogenous mouse *Brca1* was switched off, and cells were transfected with a plasmid encoding the I-SceI meganuclease as well as an mCherry fluorescent marker to control for transfection efficiency. Repair of I-SceI–induced DNA DSBs in DR-GFP via HR leads to expression of GFP, which can be monitored by flow cytometry. All but one of the predicted pathogenic variants tested resulted in defective HR, thereby confirming our functional classification (Fig. 5). The only predicted deleterious variant that did not significantly differ from wild-type *BRCA1*, R1699Q, seemed to support intermediate levels of HR activity.

PARP Inhibitor Sensitivity Assay for Classification of BRCA1 Variants with Intermediate Activity

Deleterious effects of variants with intermediate or partial activity may escape detection in certain functional assays. Although cisplatin sensitivity assays allow robust and reproducible classification of several functionally impaired *BRCA1* variants, assays using other compounds may have additional value. It is known that BRCA1- and BRCA2-deficient cells are extremely sensitive to PARP1 inhibition (28, 29), leading to a larger dynamic range between BRCA2-deficient cells and isogenic BRCA2-proficient controls than for cisplatin (30). We therefore tested complementation of PARP inhibitor sensitivity for a number of *BRCA1* mutants and the *BRCA1* wild-type control. Given the unexpected neutral effects of the M1400V, L1407P, and M1411T mutations in the PALB2 interaction domain, we decided to include these variants in this series, as well as the R1699Q and V1736A variants that have recently been shown to confer (intermediate) breast and ovarian cancer risk (31, 32). To allow direct comparison of results from different assays, we repeated the cisplatin sensitivity and proliferation assays in parallel to the olaparib sensitivity assay. Our results indicate that *BRCA1* wild-type and empty vector controls indeed show a larger difference in sensitivity for olaparib than for cisplatin (Fig. 6). However, this increase in dynamic range is accompanied by an increased variation between repeat experiments for BRCA1-proficient samples. Nevertheless, the functional defect of the R1699Q and V1736A mutations becomes more evident, and there also

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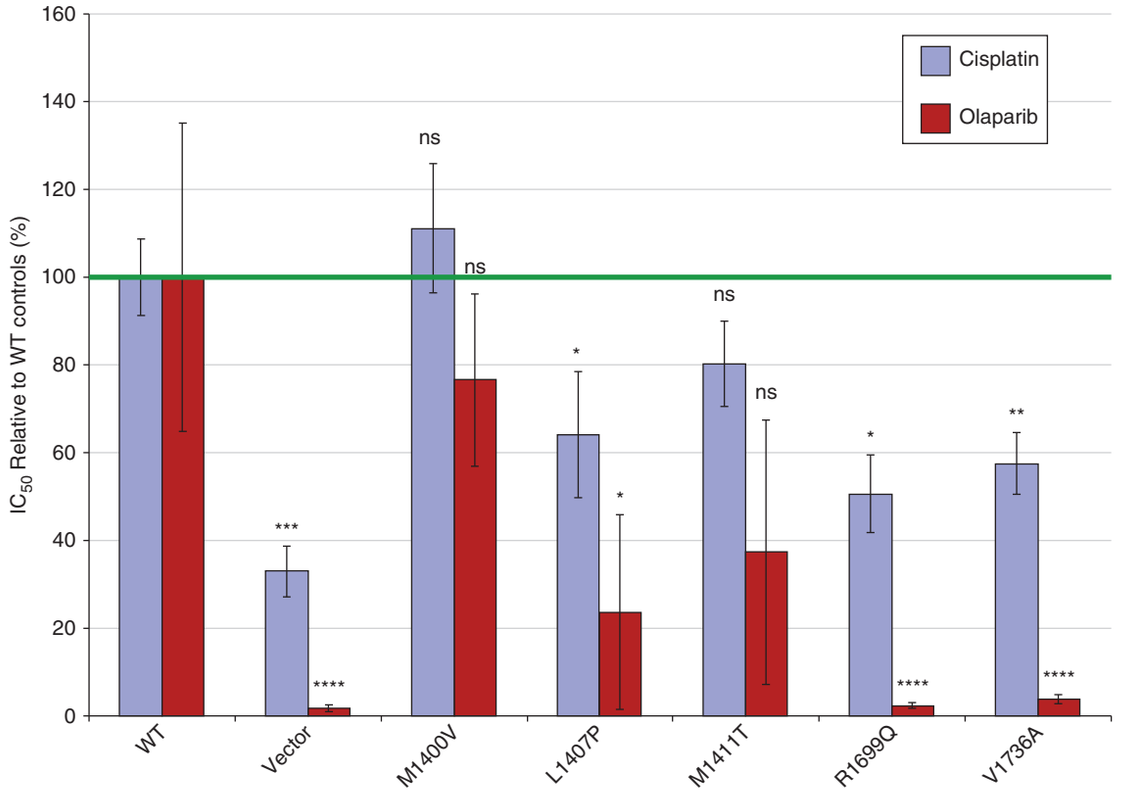

Figure 6. PARP inhibitor sensitivity assay of *BRCA1* sequence variants. *R26*$^{CreERT2/hBRCA1}$;*Brca1*$^{SCo/\Delta}$ or *R26*$^{CreERT2/RMCE}$;*Brca1*$^{SCo/\Delta}$ embryonic stem cells carrying mutant *BRCA1*, *BRCA1* wild-type (WT), or empty RMCE vector (Vector) controls in the *Rosa26* locus were switched using 4-OHT and assayed for sensitivity to cisplatin or the PARP inhibitor olaparib. The cytotoxicity assays were conducted in parallel and data were normalized to the average of the wild-type controls. Error bars indicate the SD between the results of biologic triplicates for which the cells were independently switched. Significant deviation from the average IC_{50} values of the wild-type control (green line) is indicated.

seems to be a less than wild-type response for the PALB2 interaction mutant L1407P. In the concurrent proliferation analysis, R1699Q and V1736A both show an intermediate functional defect (Supplementary Fig. S4). Also the M1411T mutation seems to affect the response to PARP inhibition, but the difference with *BRCA1* wild-type is not significant. Interestingly, the L1407P and M1411T variants have previously been shown to be more defective than M1400V in a gene conversion assay (10). It should also be noted that, in contrast with the large-scale classification experiments, the cisplatin sensitivity assay conducted in parallel to the PARP inhibitor assay identified significant functional defects for L1407P and V1736A.

DISCUSSION

Over the past few years several functional assays for classification of *BRCA1* VUSs have been developed. Several of these assays are restricted to functions of the BRCA1 protein that reside in the evolutionarily conserved RING or BRCT domains. Examples include *in vitro* transactivation assays for BRCT peptides (8) and measurement of ubiquitin ligase activity for protein fragments encompassing the N-terminal RING domain (33). Other assays were designed to evaluate the functions of full-length mutant *BRCA1* protein, either by monitoring general effects on proliferation or response to DNA damage (7), or by directly focusing on the role of BRCA1 in DNA repair via HR (34).

Because *BRCA1* VUSs are not restricted to regions encoding the N- or C-terminal domains, and given the observation that interaction between these domains is required for recruitment of BRCA1 to damaged DNA (35), functional assays for the full-length protein would be ideal. In principle, such assays can be conducted in cell lines derived from *BRCA1*-mutated tumors, but there are indications that the outcome of assays for BRCA1 function depends on the cellular context. As absence of BRCA1 leads to loss of cellular viability, it is thought that additional mutations are required for BRCA1-associated tumorigenesis. For example, loss of p53 alleviates the consequences of BRCA1 deficiency both *in vitro* and *in vivo* (4) and is common in BRCA1-deficient tumors (36, 37). Also, depletion of 53BP1 is known to suppress the defects caused by BRCA1 deficiency (5, 38, 39). Therefore, aberrations in BRCA1-deficient tumor cells may mask functional defects of *BRCA1* VUSs. We reasoned that assays in normal cells that can be depleted from endogenous BRCA1 expression are most likely to reveal functional defects of *BRCA1* VUSs. The usefulness of this approach has been previously shown by

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BAC complementation assays in mouse embryonic stem cells (7, 40). However, mutagenesis of large BAC clones by recombineering and functional complementation of cells with these mutant constructs is time-consuming and technically demanding, and therefore cannot be conducted in a high-throughput setting. We therefore set out to develop a cDNA-based functional complementation assay in mouse embryonic stem cells that is easier to control and scale up and more suitable for routine functional classification of *BRCA1* sequence variants.

One advantage of BAC transgenics in complementation assays is that genes are expressed at physiologically relevant levels. This is the result of low copy-number integrations and the presence of natural regulatory elements required for proper gene expression. We decided to use RMCE to allow single-copy integration of *BRCA1* cDNAs at one specific genomic locus. The use of RMCE effectively prevents multiple or partial integrations, concatemers, and position-effect variegation. As a result, all variants are expressed at equal levels. Moreover, transcription of *BRCA1* cDNAs from the *EF1A* gene promoter results in physiologic levels of BRCA1 protein, comparable with those observed in embryonic stem cells stably transfected with a BAC containing the human *BRCA1* locus. Indeed, the wild-type human *BRCA1* cDNA was able to complement *Brca1*-null embryonic stem cells in cellular proliferation, drug sensitivity, and HR assays. Our RMCE strategy uses *BRCA1* cDNA constructs in which mutations can be swiftly introduced using SDM, enabling a higher throughput than introduction of mutations via BAC recombineering. In addition, the introduction of *BRCA1* cDNAs via RMCE obviates the need to analyze multiple embryonic stem cell clones for correct integration and expression.

As a proof of principle, we used our functional complementation assay to analyze exonic *BRCA1* VUSs that were identified in families with HBOC in the Netherlands and Belgium, as well as a set of previously analyzed *BRCA1* variants. A number of mutations resulted in reduced BRCA1 protein levels, most likely because of structural destabilization. In all cases, this led to diminished capacity for functional complementation. Also, several variants that gave rise to normal BRCA1 protein levels were unable to rescue the proliferation defect and cisplatin sensitivity of *Brca1*-null embryonic stem cells. As *BRCA1* loss of function mutations are associated with increased cancer risk, variants that score as functionally impaired in our embryonic stem cell assay system may be causally involved in tumor formation. This notion is supported by the fact that seven of eight known pathogenic or neutral control variants in our validation series were correctly classified by the cisplatin sensitivity assay. The pathogenic 5382insC truncation mutation could not be classified because it scored as neutral in one of three transfection series. This was probably due to technical reasons, as the 5382insC mutation did not restore HR activity in *Brca1*-deficient embryonic stem cells, in contrast with R1699Q, which was recently shown to confer intermediate risk of HBOC (32).

Our assay system yielded ambivalent results for nine other variants: S4F, S308A, S1651P, S1651F, T1691I, V1736A, E1735K, H1746Q, and R1753T. T1691I and E1735K were classified as functionally impaired in only one cisplatin sensitivity test, whereas values from repeat experiments could not be taken into account because of RSE values above 0.1. S4F, S1651P, S1651F, V1736A, H1746Q, and R1753T were differently classified in repeat experiments, which may reflect technical flaws or intermediate activity of these variants. Of note, the V1736A mutation was recently identified as a pathogenic variant with hypomorphic activity in DNA repair (31). Although we did not measure significant HR activity of V1736A in a DR-GFP gene conversion assay, intermediate activity of this variant is supported by the results of the proliferation assays and additional cisplatin sensitivity assays. S1651F showed HR activity similar to wild-type *BRCA1*, whereas R1753T was HR-deficient. S308A is an artificial mutation of a BRCA1 phosphorylation site that was able to support proliferation and resistance to DNA damage in an embryonic stem cell-based BAC complementation assay. However, S308A-complemented embryonic stem cells did show increased apoptosis when cultured in embryoid bodies (7), indicating a partial defect that might explain the ambivalent results for this mutation in our assays.

Our results show that *BRCA1* variants should ideally be assayed in triplicate to avoid misclassification. This also applies to *BRCA1* VUS that we classified as neutral, most of which were tested only once because in our current study we focused on variants that showed functional impairment. The striking restriction of unambiguously predicted pathogenic mutants to the terminal RING and BRCT domains suggests that some plasticity is allowed for the central domain of BRCA1. However, our dataset is still limited and more experiments are required to gain insight into the function of this domain.

Our cDNA-based system allows for several additional functional assays that have been described previously for BAC transgenic embryonic stem cells (7). These include assays for defects during *in vitro* and *in vivo* embryonic stem cell differentiation but also treatments with other cytotoxic agents. As a proof of principle, we investigated the activity of a number of mutants in the response to the PARP inhibitor olaparib. These included M1400V, L1407, and M1411T, which were previously shown to impair PALB2 binding and have a negative effect on BRCA1 function (10). Although the differences in sensitivity of BRCA1-deficient versus BRCA1-proficient embryonic stem cells are larger for olaparib than for cisplatin, increased variation between repeat experiments allowed us to identify functional defects only for L1407 and not for the other two variants in the PALB2 interaction domain, M1400V and M1411T. However, the R1699Q and V1736A variants, which are known to have hypomorphic activity, clearly showed a defect in the response to olaparib. Interestingly, both mutations are in the BRCT domain, and it has recently been shown that mutation of this domain especially confers sensitivity to PARP inhibition (41). Together, our results show that PARP inhibitor sensitivity assays may have added value, especially for the classification of *BRCA1* VUSs with intermediate phenotypes.

Platinum drugs and PARP inhibitors are selectively toxic to BRCA1-deficient cells because they target HR deficiency. Although the role of BRCA1 in HR is thought to be essential for maintaining genomic integrity and preventing accumulation of (oncogenic) mutations, other activities may also contribute to its tumor suppression function. These activities

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may include the HR-independent role for BRCA1 in ICL repair, which has been attributed to the facilitation of FANCD2 accumulation at cross-linked DNA (16). Nevertheless, we observed a good correlation between the results of the cisplatin sensitivity assay and the results of the DR-GFP HR assay. All *BRCA1* variants that failed to restore the cisplatin response in *Brca1*-null embryonic stem cells were also defective in catalyzing gene conversion, thereby confirming our functional classification. It will be interesting to see if this holds true for all *BRCA1* variants or whether there are also pathogenic mutations that have no effect on HR.

As with any other *in vitro* approach, our functional complementation assay system might still fail to identify all pathogenic variants because it does not necessarily recapitulate all aspects of BRCA1 function *in vivo*. A limitation of our cDNA-based assay is also that it cannot be used to investigate effects on mRNA splicing. Although algorithms have been designed to predict possible splice defects, the consequences of mutations outside of the consensus splice sites especially require functional validation experiments. For this purpose, BAC complementation assays (7), minigene-based splicing assays, or *BRCA1* transcript analysis of patient blood samples (19) may be instrumental. However, transacting factors also affect splicing, and these may be tissue specific (42). A possible solution would be to determine the presence of *BRCA1* splice variants in tumor tissue from *BRCA1* VUS carriers and use this information to generate a cDNA construct for analysis of the functional consequences. To evaluate *BRCA1* VUSs, there remains a need for multifactorial models that combine results from functional assays and *in silico* analyses with genetic evidence and other information from mutation carriers. This also includes DNA copy number data from tumors from *BRCA1* VUS carriers, as it is known that BRCA1-associated breast tumors show distinct genomic aberrations (43, 44). Our functional assay system does however provide a robust and easily implementable tool for the functional characterization of large numbers of *BRCA1* VUSs within the context of the full-length protein. It is our hope that our assay system will find its way to clinical genetics laboratories where it can be used to aid genetic counseling. Ideally, these tests should be coordinated on an international level and in close collaboration with the ENIGMA consortium.

METHODS

Generation of RMCE Vectors Containing Human BRCA1 Sequence Variants

Human *BRCA1* cDNA from a pcDNA3-BRCA1 expression construct (45) was subcloned into the pRNA 251-MCS RMCE exchange vector under control of the *EF1A* gene promoter. *BRCA1* mutations were introduced by site-directed mutagenesis using the QuickChange Lightning protocol (Stratagene), and constructs were verified by sequencing the entire human *BRCA1* cDNA (see Supplementary Methods).

Generation of Human BRCA1 Transgenic Embryonic Stem Cells

$R26^{CreERT2/wt}$;$Brca1^{SCo/\Delta}$ embryonic stem cells were generated by gene targeting in 129/Ola E14 IB10 embryonic stem cells (46). The presence of correctly targeted alleles was verified using Southern blotting, Western blotting, and PCR analysis (5, 47). The wild-type *Rosa26* allele of $R26^{CreERT2/wt}$;$Brca1^{SCo/\Delta}$ embryonic stem cells was equipped with Frt and F3 sites for Flp RMCE as described (ref. 6; see Supplementary Fig. S1). Introduction of human *BRCA1* cDNAs via RMCE was conducted by cotransfection of $R26^{CreERT2/RMCE}$;$Brca1^{SCo/\Delta}$ embryonic stem cells with RMCE vectors and pFlpe (48) using Lipofectamine 2000 (Invitrogen). Cells that had successfully undergone RMCE were selected using 200–400 μg/mL G418. Correct RMCE was confirmed by PCR and expression of human BRCA1 was analyzed by Western blotting using a polyclonal antibody against human BRCA1 (9010; Cell Signaling Technology). The generation of $R26^{CreERT2}$;$Brca1^{SCo/\Delta}$ embryonic stem cells expressing human *BRCA1* from the BAC clone RP11-812O5 was conducted essentially as described (49).

Cytotoxicity and Proliferation Assays

Cre-mediated inactivation of the endogenous mouse $Brca1^{SCo}$ allele was achieved by overnight incubation of cells with 0.5 μmol/L 4-OHT (Sigma). One week after switching, cells were seeded in triplicate at 1,000 cells per well in 96-well plates for cisplatin or olaparib (AZD2281) sensitivity assays essentially as described (5). In addition, cells were seeded in triplicate at 500 cells per well on 96-well plates to monitor proliferation.

HR Reporter Assays

For DR-GFP assays we used a modified version of the p59X DR-GFP construct (ref. 12; kindly provided by T. Ludwig), in which the puromycin resistance marker was inactivated by inversion of an internal SalI fragment. To allow selection of targeted integration of this construct to the *Pim1* locus, we equipped the wild-type *Rosa26* allele of $R26^{CreERT2/wt}$;$Brca1^{SCo/\Delta}$ embryonic stem cells with Frt and F3 sites for RMCE using a targeting vector in which *zsgreen*, the hygromycin resistance marker, and *Flpe* were replaced by a puromycin resistance marker. Subsequently, the DR-GFP construct was targeted to the *Pim1* locus as described (26), *BRCA1* variants were introduced using RMCE, and cells were subcloned to allow HR reporter assays. HR reporter assays were conducted by Lipofectamine 2000 transfections of an I-SceI-mCherry plasmid, which was generated by cloning CMV-mCherry (Clontech) into the cBas I-SceI expression plasmid. Three days after transfection, mCherry/GFP double-positive cells were monitored by flow cytometry on a fluorescence-activated cell sorting CyAn (Beckman Coulter) using Summit software (Beckman Coulter).

Statistical Analysis

We calculated the cisplatin IC_{50} values from the 96-well plate-based cisplatin sensitivity assays for VUS classification by fitting a log-logistic curve, normalized to the no-drug control, constrained between 1 and 0 using the *drc* package in the R programming language. We discarded fits that exceeded a 0.1 RSE. To allow comparison between plates, we normalized using a linear model based on the positive and negative controls, resulting in corrected IC_{50} scores. We estimated normal distributions of the corrected IC_{50} score for both the pooled positive and negative controls across plates. We used these estimated normals to calculate the probability of pathogenicity for each VUS.

Statistical significance for the HR and cytotoxicity assays on selected groups of variants was calculated by two-tailed Student *t* test using Prism 6 Software. Significant differences are indicated by *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; and ****, $P < 0.0001$. NS stands for nonsignificant ($P > 0.05$).

Computational Analysis

Alamut software was used to obtain genomic annotations and Align-GVGD scores (human to sea urchin) for all variants.

See Supplementary Methods for a detailed protocol for the classification of *BRCA1* sequence variants.

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Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): P. Bouwman, H. van der Gulden, I. van der Heijden, E. Wientjens, J. Seibler
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