


RAD52 influences the effect of BRCA1/2 missense variants on homologous recombination and gene reversion in *Saccharomyces cerevisiae*

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One sentence summary: Yeast is a good model to investigate the complex genetic interaction in cancer.

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Abstract

The breast and ovarian cancer susceptibility genes, *BRCA1* and *BRCA2*, are key players in the homologous recombination (HR) repair pathway and act as tumor suppressors by maintaining genome stability. The yeast *Saccharomyces cerevisiae* has no *BRCA1/2* homolog; however, a number of HR genes are evolutionary conserved between human and yeast. Among them, *RAD52* is involved in DNA double strand break (DSB) repair by HR, and promotes genome stability. We previously reported that the heterologous expression of cancer-associated *BRCA1/2* missense variants in growing yeast cultures affects both spontaneous HR and gene reversion (GR) suggesting that yeast could be a reliable system to assess the functional impact of variants. Because inhibition of Rad52p is lethal in *BRCA1/2* mutated tumors, and Rad52p is conserved between humans and yeast, we asked if the effect of *BRCA1/2* variants on HR and GR could be affected by loss of *RAD52*. We found that the *rad52Δ* mutation predominantly suppressed the stimulation of HR in yeast by pathogenic *BRCA1* variants but also facilitated increased GR by pathogenic variants. Conversely, the *rad52Δ* mutation stimulated HR by a pathogenic *BRCA2* variant in yeast but had no effect on GR. These results demonstrate a functional interplay between the pathogenic *BRCA1/2* variants and Rad52p in budding yeast, supporting the use of budding yeast as a suitable system for evaluating potential chemotherapeutic strategies.

Keywords: *RAD52*, *BRCA1*, *BRCA2*, homologous recombination, gene reversion, the yeast *Saccharomyces cerevisiae*

Introduction

Germline mutations of the DNA repair genes *BRCA1* and *BRCA2* increase the lifetime risk of hereditary breast and ovarian cancer (Kuchenbaecker et al. 2017, Angeli et al. 2020). *BRCA1/2* are mainly involved in homologous recombination (HR) repair that is fundamental to genome stability maintenance; therefore, mutations of HR genes lead to genome instability and tumorigenesis (Wood et al. 2007, Moynahan and Jasin 2010, Negrini et al. 2010, Zamborszky et al. 2017). The yeast *Saccharomyces cerevisiae* has largely been considered an excellent model in cancer research (Guaragnella et al. 2014a, Ferreira et al. 2019), and a reliable genetic system to evaluate the functional impact of variants of DNA repair genes including *BRCA1* and *BRCA2*, although yeast counterparts of these proteins do not exist (Cervelli et al. 2020).

The HR and DNA double strand break (DSB) repair gene *RAD52* is functionally and structurally conserved between yeast and humans. The expression of human *RAD52* gene in yeast *rad52* mutants suppresses the HR repair defect confirming a functional homology between human and *S. cerevisiae* *RAD52* (Manthey et al. 2017, Clear et al. 2020). In yeast, Rad52p plays a fundamental role in DSB repair by HR; in humans, it is reported no to be so essential for HR repair, although new crucial activities were recently discovered (Ngo et al. 2020, Oshidari et al. 2020, Stefanovic et al. 2020, Cano-Linares et al. 2021, Hatchi et al. 2021). During DSB re-

pair, *RAD52* acts downstream of *BRCA1/2*, when single strand DNA is generated. *RAD52* has been shown to interact with *RAD51*, and *BRCA2* interacts with both *RAD51* and *BRCA1* to allow DSB repair by HR to maintain genome stability; moreover, *RAD52* function is needed to tether together the new ssDNA and the homologous DNA strand allowing HR repair. Therefore, *RAD52* is mainly involved in HR and single strand annealing (SSA) pathways (Hanamshet et al. 2016, Jalan et al. 2019, Hendrickson 2020, Malacaria et al. 2020).

Rad52p plays a key role in the survival of cells lacking the *BRCA1/2* function, as Rad52p inactivation is lethal in *BRCA1/2* deficient tumor cells (Lok et al. 2013). When *BRCA1/2* genes are mutated, like in hereditary breast and ovarian cancers, Rad52p inactivation by specific inhibitors selectively kills cancer cells (synthetic lethality). Therefore, Rad52p is a potential target for therapy in *BRCA*-deficient tumors (Toma et al. 2019). The finding of synthetic lethality between *RAD52* and *BRCA1/2* opened a significant area of research with the aim to investigate other players in this pathway and to determine their epistasis and synthetic lethality with Rad52 (Nogueira et al. 2019, Lodovichi et al. 2020a). Since when *BRCA1/2* genes have been identified, thousands of variants of uncertain significance (VUS) have been annotated in the database ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>); therefore, reliable functional assays are needed to help evaluating the

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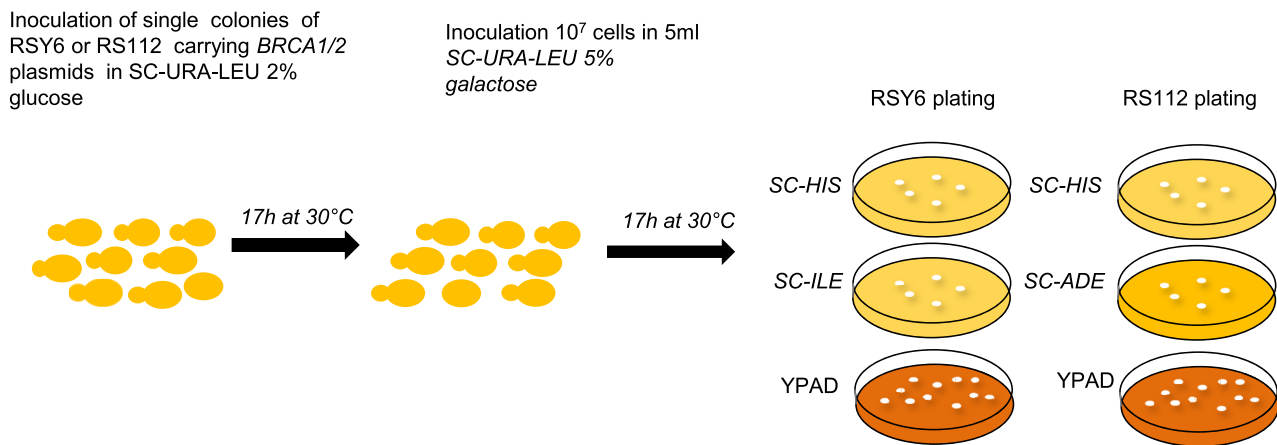


Figure 1. Scheme of the yeast assays. Single colonies of the RS112 and RSY6 strain containing the plasmid expressing BRCA1/2 protein or the empty vector were first pre-grown in SC-URA-LEU glucose. Thereafter, 10⁷ cells were inoculated in 5 ml of 5% galactose medium. Then, cells were counted and plated to score for cell surviving fraction. RS112 cells were plated onto SC-HIS and SC-ADE solid medium to determine intra- and inter-chromosomal HR, respectively. RSY6 cells were plated onto SC-ILE and SC-HIS medium to score GR and intra-chromosomal HR respectively. Total cell number was determined by plating cells from both strains in YPAD medium. Plates were incubated for 4–5 days at 30°C, colonies counted and GR/HR frequency calculated as reported in the Materials and Methods.

pathogenicity of novel VUSs (Toland and Andreassen 2017). Functional assays in model organisms have been very helpful to classify VUSs, although they need validation that is crucial to determine cancer risk. The biological validity of a functional assay should be assessed by determining to what extent the assay relies on a natural function of the BRCA1/2 protein, and how this particular function contributes to tumor suppression (Milot et al. 2012, Guidugli et al. 2014)(Cervelli et al. 2020). We previously reported that the expression of pathogenic BRCA1 variants in yeast increased HR and gene reversion (GR); although many variants should be analyzed to validate these assays, these results indicate that yeast could be a reliable system to develop functional assays (Caligo et al. 2009). Moreover, as HR and GR are considered indicators of genome instability, yeast is a valuable model system to study genetic factors involved in carcinogenesis (Schar 2001) (Pikor et al. 2013). To investigate the mechanisms by which BRCA1 variants affect yeast HR and GR, we performed a genetic analysis in yeast mismatch and DSB repair mutants. We demonstrated that yeast DNA-repair pathways differentially affect BRCA1-induced HR and GR suggesting that pathogenic BRCA1 variants could have specific genetic requirements that might have a role in cancer occurrence (Maresca et al. 2015, Maresca et al. 2018). More recently, we studied the effect of BRCA1 variants in cell cycle arrested cells and showed that BRCA1 pathogenic variants induced higher GR in S-phase arrested yeast cells suggesting that BRCA1 interfere with yeast DNA repair functions that are active in S-phase (Lodovichi et al. 2020b). As BRCA1 has a role in replication fork stability, we could hypothesize that expression of BRCA1 pathogenic variants in yeast destabilizes DNA replication fork leading to the formation of a higher level of endogenous DNA damage that can stimulate both HR and GR (Schlachter et al. 2012).

Expression of wild type BRCA2 increases HR events in a diploid yeast strain, suggesting that BRCA2 might interfere with yeast DNA repair pathways. In the same strain, BRCA2 neutral variants also induce HR whereas the pathogenic variant does not (Spugnese et al. 2013). Notably, BRCA1 and BRCA2 have opposite effect on yeast HR; this could reflect the distinct roles that BRCA1 and BRCA2 have in HR. BRCA1 mainly works in DNA damage signaling and repair; BRCA2 regulates the activity of RAD51 recombinase (Tutt and Ashworth 2002, Venkitaraman 2002).

Although many pathogenic BRCA1/2 variants have been identified, the role of DNA repair in the BRCA1/2 pathogenicity or tumorigenesis is not yet completely clear. Here, we expressed several BRCA1 and BRCA2 missense variants in yeast *rad52* deletion strains in order to determine whether RAD52 affects genome instability (HR and GR) induced by the pathogenic, neutral variants or VUS. We believe that this system can be helpful to develop more tailored synthetic lethality-based therapies.

Materials and methods

Yeast strains

The diploid RS112 (*MATa/MATα ura3-52/ura3-52 leu2-3112/leu2-Δ98 trp5-27/TRP5 ade2-40/ade2-101 ilv1-92/ilv1-92 arg4-3/ARG4 his3Δ5'-LEU2-his3Δ3'/his3-Δ200 LYS2/lys2-801*) and the haploid RSY6 (*MATa ura3-52 leu2-3,-112 trp5-27 ade2-40 ilv1-92 arg4-3 his3Δ5'-LEU2-his3Δ3'*) strain of *Saccharomyces cerevisiae* were originally obtained by Robert Schiestl (UCLA, Los Angeles CA), and have been used in our laboratory for many years. The deletion strains RSY6 *rad52Δ* and RS112 *rad52Δ/rad52Δ*, isogenic to RSY2 and RS112 respectively, were constructed by standard gene replacement procedure and already described (Rothstein 1991, Galli et al. 2003). Complete medium (YPAD), and synthetic complete media lacking uracil (SC-URA), leucine (SC-LEU), adenine (SC-ADE), histidine (SC-HIS), isoleucine (SC-ILE), or uracil and leucine (SC-URA-LEU) were prepared according to the standard techniques (Sherman 2002). Chemicals were purchased from Merck (Darmstadt, Germany), powder for media from Thermo-Fisher Scientific (Milan, Italy).

Plasmids

The plasmid YCpGAL::BRCA1 which contains the human BRCA1 gene under the galactose-inducible promoter GAL1p was obtained from Craig Bennett (Duke University, Durham, NC).

The plasmid pYES2-BRCA2, which contains the human BRCA2 gene under the galactose-inducible promoter GAL1p, was constructed from pcDNA3-BRCA2 (a gift from Chris Lord). As previously reported, BRCA2 was cut out from the vector by restriction with *KpnI* and *ApaI* and inserted in the yeast expression vector

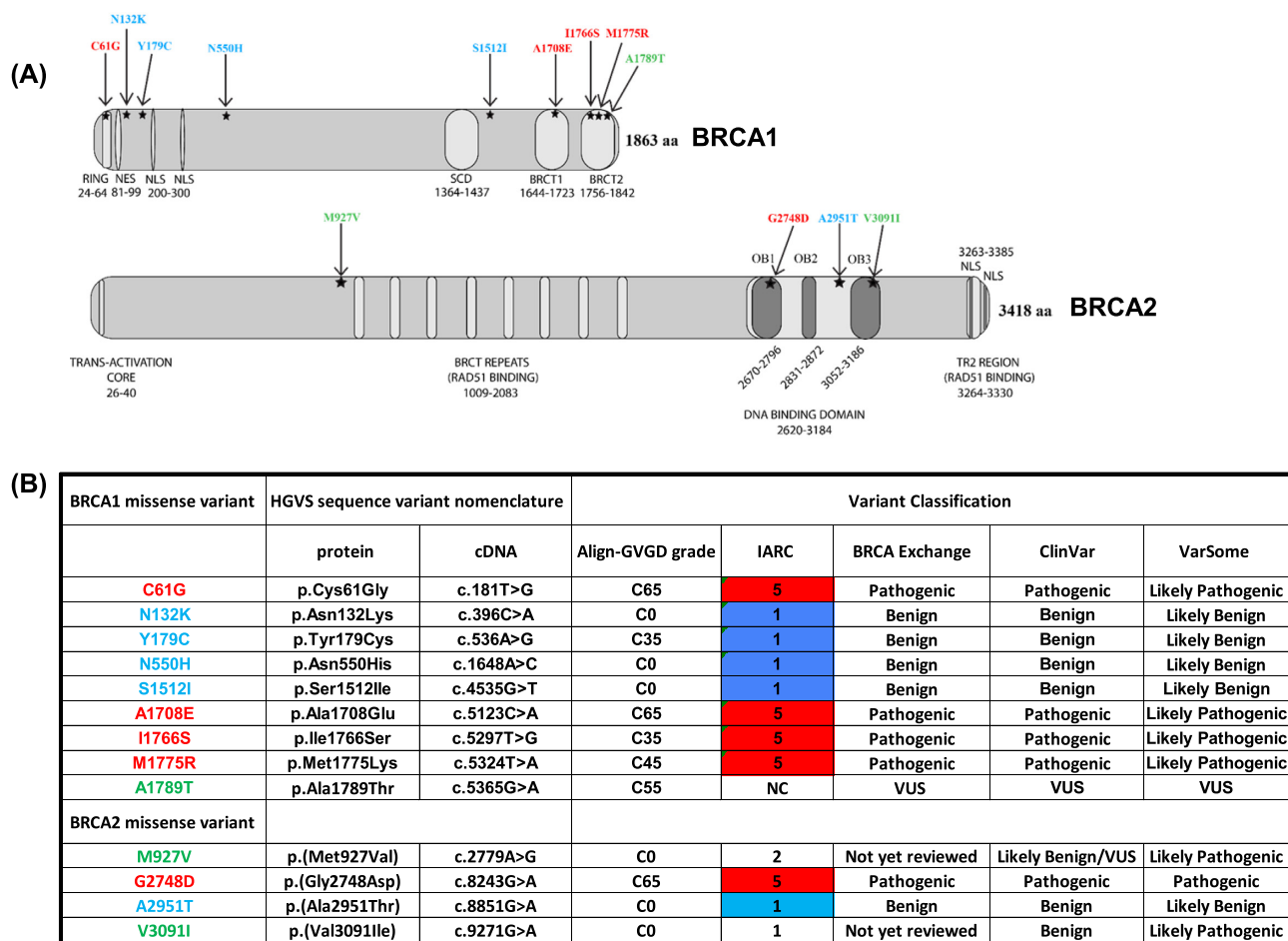


Figure 2. Selection and classification of BRCA1/2 variants. **(A)** BRCA1 and BRCA2 proteins are composed of 1863 and 3418 amino-acids (aa) respectively. The distribution of pathogenic (red) and neutral variants (light blue), and VUS (green) tested in *S. cerevisiae* is shown. Functional domains are depicted on the protein sequence; in BRCA1: the RING (Really Interesting New Gene) finger, NES and NLS (Nuclear export and localization signal), the SCD (Serine/Threonine-Q cluster) and the BRCT domain (BRCA1 C terminus); in BRCA2: the trans-activation core, the BRC repeats (Rad51-binding domain) that consists of 8 repeated peptide sequences of about 35 aa the DNA binding domain composed of 3 OB-fold (oligonucleotide/oligosaccharide binding) motifs, the NLS and TR2 regions which bind Rad51. **(B)** For each BRCA1/2 variant, the HGVS nomenclature and the classification annotated in the following databases is reported: Align-GVGD (<http://agvgd.hci.utah.edu/>), IARC (<http://hci-exlovd.hci.utah.edu/home.php>), BRCA Exchange (<https://brcaexchange.org/>), ClinVar (<https://www.ncbi.nlm.nih.gov/clinvar/>) and VarSome (<https://varsome.com/>). Variants with Align-GVGD grade higher than 25 are considered to be pathogenic or likely pathogenic; IARC has grouped the variants in the following classes: IARC class 5, pathogenic; class 4, likely pathogenic; class 3, VUS; class 3, likely benign; class 1, benign.

pYES2 (Invitrogen) digested with KpnI and EcoRI (Spugnesi et al. 2013).

The plasmids expressing wild type BRCA1/2 and the missense variants were constructed by site-directed mutagenesis with specific oligonucleotides using QuikChange II XL site-directed mutagenesis kit (Stratagene, La Jolla, CA USA) (Caligo et al. 2009, Spugnesi et al. 2013). Yeast cells were transformed with plasmid DNA by using lithium acetate and single strand DNA as carrier, and selected in SC-URA as reported previously (Gietz and Schiestl 2007). Colonies were grown for 4/5 days in selective medium at 30°C and further analyzed.

Yeast protein extracts and Western blot

BRCA1 and BRCA2 protein level was determined in total protein extracts from yeast cells, transformed with the expression vector, after 17-hour induction in 5% galactose medium. Single clones were pre-grown in 10–20 ml of SC-URA glucose medium for 24 hours at 30°C. Then, cell pellet was washed in sterile distilled wa-

ter, and split in two aliquots: one was inoculated in 20 ml of SC-URA-LEU glucose and the other one in 20 ml of SC-URA-LEU 5% galactose. The cultures were incubated at 30°C for 17 hours, under constant shaking at 200 rpm. Thereafter, pellets were washed twice in ice cold water and re-suspended in 0.5 ml of suspension buffer [50 mM KCl, 5 mM MgCl₂, 0.1 M EDTA, 25 mM HEPES, 5 mM DTT, 0.3 M (NH₄)₂SO₄, 10% glycerol, pH 7.4] plus 10 µl of protease inhibitor solution [PMSF: 4.4 mg phenylmethylsulphonyl fluoride, 62 mg pepstatin, 50 mg chemostatin and 725 µl DMSO in 1 ml H₂O]. Total protein extracts were prepared according to the method previously reported (Maresca et al. 2015) (Guaragnella et al. 2014a). Brca1p and Brca2p are analyzed using Anti-BRCA1 MoAb Ab4 (clone SD118-Calbiochem, Darmstadt Germany) diluted 1:300 and rabbit anti-H-300 anti-BRCA2 antibody from Santa Cruz Biotechnology, Inc. (sc-8326, Santa Cruz, CA USA). As loading control, we determined the level of the 3-Phosphoglycerate kinase (PGK) with the PGK Monoclonal Antibody (22C5D8, Invitrogen) diluted 1:5000. Signal of proteins analyzed has been evaluated by chemoluminescence using the ChemiDoc™ MP Imager

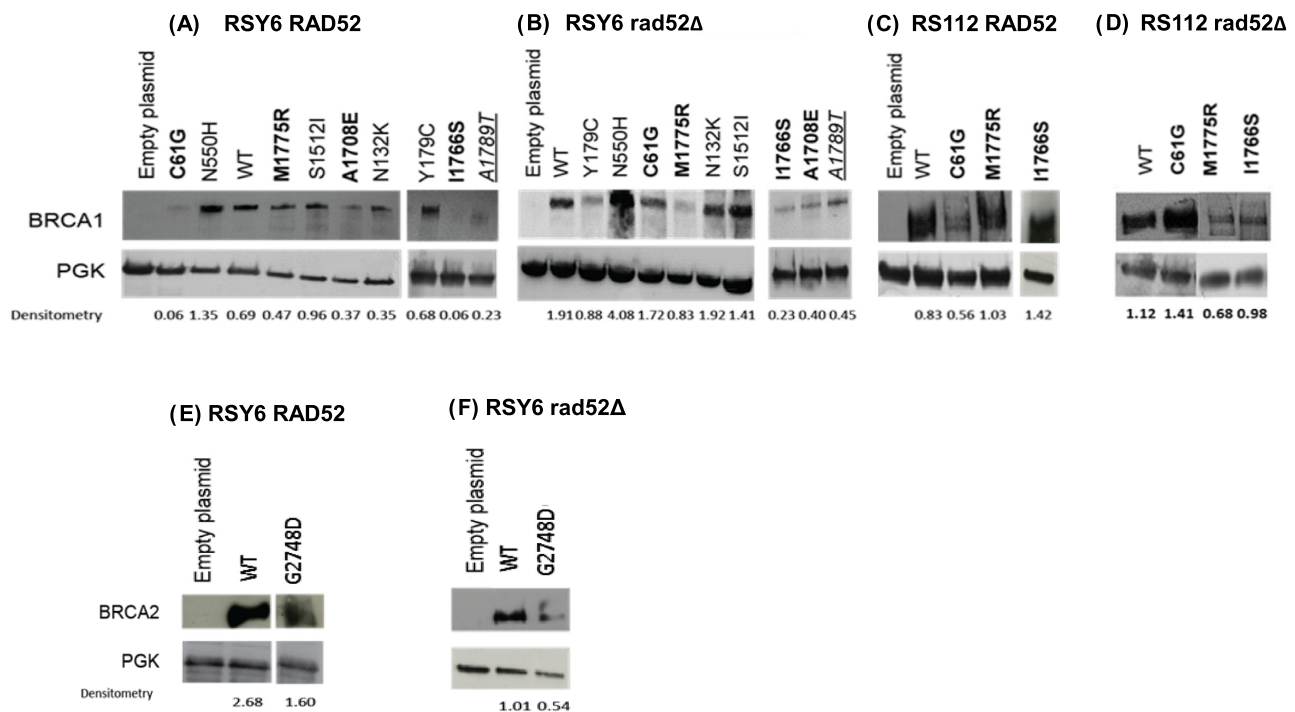


Figure 3. Expression of BRCA1/2 in RAD52 and *rad52Δ* strains of *Saccharomyces cerevisiae*. Total extracts from yeast cells grown in galactose medium and expressing BRCA1 or BRCA2 were analyzed by Western blot using the mouse anti-BRCA1 antibody and rabbit anti-BRCA2 antibody as described in Materials and Methods. PGK level was evaluated as loading control. Densitometry is reported below each lane. BRCA1 was detected in protein extracts from RSY6 RAD52 (A), RSY6 *rad52Δ* (B), RS112 RAD52 (C) and RS112 *rad52Δ* (D). BRCA2 protein level was detected in extracts from RSY6 RAD52 (E) and RSY6 *rad52Δ* (F).

System (Bio-Rad, CA, USA). Densitometry was performed by ImageJ (<https://imagej.nih.gov>).

Yeast recombination and reversion assay

The RS112 RAD52/RAD52 and RS112 *rad52Δ/rad52Δ* strain of *Saccharomyces cerevisiae* were used to determine the effect of BRCA1 wild type and missense variants on intra- and inter-chromosomal HR as previously reported (Caligo et al. 2009). Briefly, this strain carries two *his3* alleles deleted at 3' and 5' respectively sharing 400bp of homology; an intra-chromosomal HR event leads to the restoration of *HIS3* gene allowing cells to grow in medium lacking histidine (SC-HIS). To assess inter-chromosomal HR, this strain carries the two alleles, *ade2-40* and *ade2-101* on homologous chromosomes; in this case, an inter-chromosomal HR event leads to the restoration of *ADE2* gene allowing cells to grow in medium lacking adenine (SC-ADE). The parental haploid RSY6 RAD52, and RSY6 *rad52Δ* strains were used to evaluate the effect of wild type BRCA1/2 and several missense variants on intra-chromosomal HR and GR as already reported (Maresca et al. 2018, Lodovichi et al. 2020a). In addition to the same intra-chromosomal HR substrate as in the diploid RS112 strain, RSY6 contains a mutation on *ilv1* gene making it unable to grow in medium lacking isoleucine (SC-ILE); a GR event abrogates the effect of this mutation restoring strain ability to grow in SC-ILE. Single colonies from RSY6 or RS112 strains were inoculated into 5 ml of SC-URA-LEU medium containing glucose and incubated at 30°C for 24 hours. Thereafter, cultures were washed twice in sterile distilled water and counted (Fig. 1). For each BRCA1/2 variant as well as the BRCA1/2 wild type and the controls (strains carrying the pYES2 empty vector), aliquots containing 10^7 cells were inoculated in 5ml of SC-URA-LEU medium containing 5% galactose and incubated at 30°C for 17

hours, under constant shaking (200 rpm) (Fig. 1). The frequencies of Intra- and inter-chromosomal HR were determined in the RS112 strain as total number of His⁺ colonies per 10^{-4} vital cells and, as total number of Ade⁺ colonies per 10^{-5} vital cell. We have also determined the effect of BRCA1/2 variants on GR at *ilv1-92* allele in RSY6 RAD52 and in *rad52Δ* strain by plating yeast-expressing BRCA1/2 wild type or BRCA1/2 missense variants, grown at 30°C for 17 hours in 5ml SC-URA-LEU plus 5% galactose, in SC-ILE medium; the frequency of GR was calculated as total number of Ilv⁺ revertants per 10^{-6} vital cells (Maresca et al. 2018, Lodovichi et al. 2020b). Intra-chromosomal HR frequency was also measured in the RSY6 by plating cells in SC-HIS medium as described before. For each BRCA1/2 variant, as well as for the BRCA1/2 wild type, 4 to 6 independent experiments were set up. Each culture was plated in triplicate.

All the data were evaluated for normal distribution by using the Shapiro-Wilk and D'Agostino & Pearson omnibus normality test. Results, reported as mean \pm standard deviation of 4–5 independent experiments, were statistically analyzed by the two-tailed Student 't' test using 'GraphPad Prism 6' program. Data from BRCA1/2 wild type and from each variant were compared to the empty vector pYES2 (negative control). Statistical analysis was performed for each strain; no data comparison between strains has been made.

Results

BRCA1 and BRCA2 missense variants

Due to the functional conservation of HR machinery between yeast and humans, we determined the effect of several BRCA1/2 missense variants on HR and GR in yeast *rad52* deletion mu-

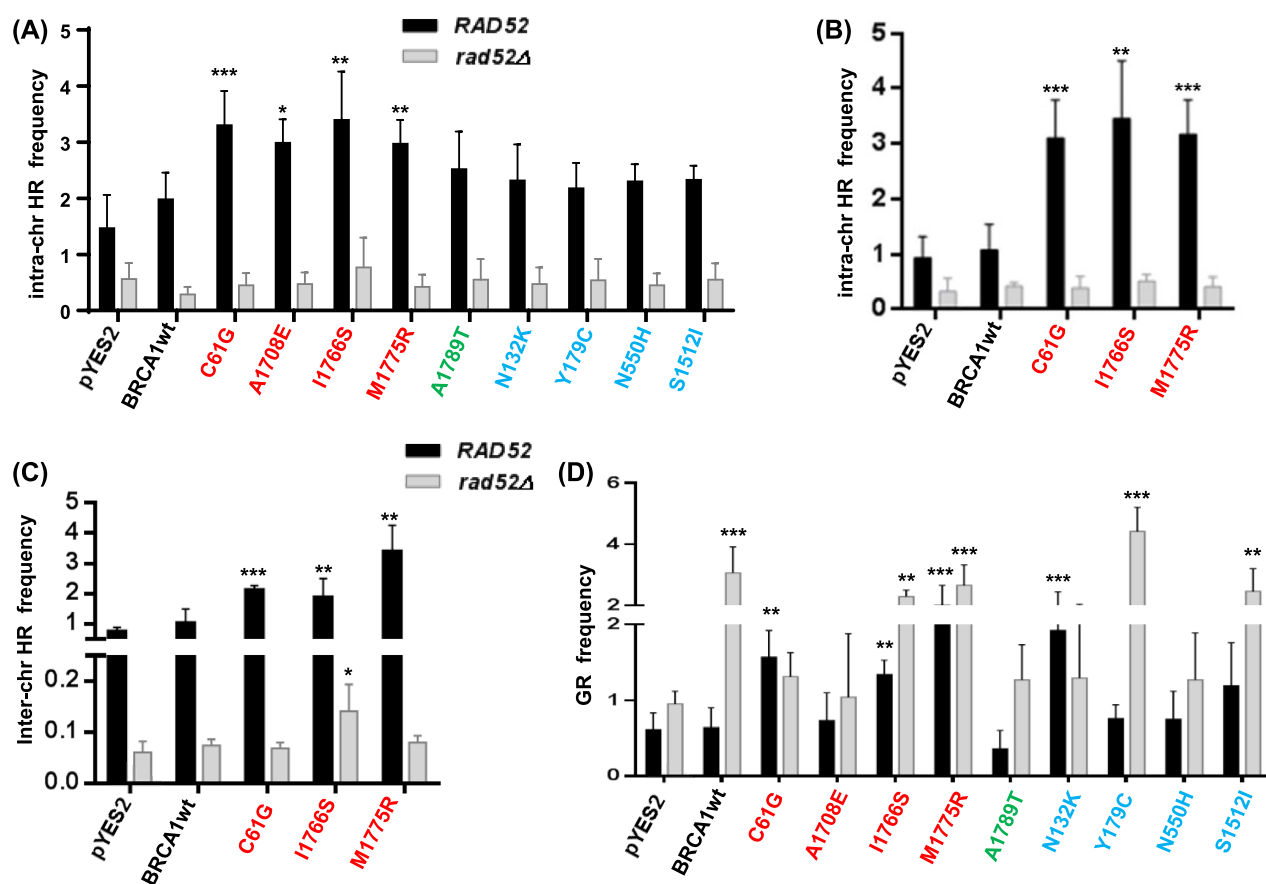


Figure 4. Effect of BRCA1 expression on HR and GR in the RAD52 and *rad52Δ* strains of *Saccharomyces cerevisiae*. The haploid RSY6 strain was used to determine the effect of BRCA1 on intra-chromosomal HR and GR, as described in the Materials and Methods. The RS112 strain is diploid and allows to determine both intra- and inter-chromosomal HR (named in the graphs intra-chr and inter-chr HR). HR and GR events were evaluated after growth in galactose medium and plating in selective media as described in Materials and Methods. Pathogenic missense variants are reported in red, neutral ones in light blue and VUS in green. As negative control, HR and GR were measured in the strains transformed with the empty vector (pYES2). Yeast strains carrying the empty plasmid and expressing the BRCA1 wild type are in black. **(A)** Effect of BRCA1 wild type and missense variants on the intra-chromosomal HR in haploid strains; results are reported as intra-chromosomal HR frequency that was calculated as number of HIS3 recombinants on 10^4 vital cells. Three pathogenic variants were also tested in the diploid strains to evaluate the effect on intra- **(B)** and inter-chromosomal HR **(C)**: intra-chromosomal and inter-chromosomal HR frequencies were expressed as number of His⁺ and Ade⁺ total colonies on 10^4 or 10^5 vital cells, respectively. **(D)** RSY6 derivative strains carry also the *ilv1-92* mutant allele that allows the evaluation of GR events. Results are reported as mean \pm standard deviation from 4–6 independent experiments, each of them plated in triplicate. Normality test and statistical analysis was carried out as described in Materials and Methods. BRCA1 wild type was compared to pYES2 (empty vector) and any variant to BRCA1 wild type. * $P < 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.

tant aiming to get helpful information for a more precise and tailored therapy in patients carrying specific missense variants. As depicted in the Fig. 2A and B, we selected four neutral (pN132K, pY179C, pN550H and pS1512I), four pathogenic (pC61G, pA1708E, pI1766S and pM1775R) BRCA1 variants, and one VUS (pA1789T). We also selected the pathogenic pG2748D and the neutral pA2951T BRCA2 variant, and two BRCA2 variants with contrasting classification reports (pM927V, pV3091I, hereafter referred as VUS) (Fig. 2B). The position of BRCA1/2 amino-acid substitution is shown in Fig. 2A. The classification of the selected variants, as reported in different databases, is shown in Fig. 2B. We have previously demonstrated that our yeast strains are able to sustain the expression of BRCA1 and BRCA2 missense variants. In Fig. 3A, C and G, we reported already published data, just for comparison (Caligo et al. 2009, Di Cecco et al. 2009, Spugnese et al. 2013, Guaragnella et al. 2014a, Maresca et al. 2015, Maresca et al. 2018, Lodovichi et al. 2020b). Western blot experiments showed that *rad52Δ* yeast strains sustain the expression of wild type BRCA1/2 and several missense variants (Fig. 3B, D and F). Moreover, hap-

loid RSY6 *rad52Δ* is also able to express wild type BRCA2 and the pathogenic variant (Fig. 3F). Densitometry showed that some BRCA1 variants such as the neutral pN550H and pS1512I reached an equal or higher protein level than wild type BRCA1 in both RSY6 RAD52 and *rad52Δ* strains (Fig. 3A and B); on the contrary, the pathogenic BRCA1 variants pC61G, pI1766S and pA1708E showed a lower level than the wild type (Fig. 3A and B). In the diploid RS112 strain, pC61G showed less accumulation than pM1775R and pI1766S (Fig. 3C). In all strains, wild type BRCA1 level ranges from 0.69 to 1.91 (Fig. 3A–D). Wild type BRCA2 reached a higher level in both strains RSY6 RAD52 and *rad52Δ* as compared to the pathogenic pG2748D variant (Fig. 3E and F).

BRCA1-induced HR requires RAD52

The effect of BRCA1 missense variants on HR was evaluated first in the RSY6 RAD52 and RSY6 *rad52Δ* strains which allow to measure intra-chromosomal HR events between two deleted *his3* alleles which share 400 base pairs of homology, by directly counting the His⁺ colonies on solid media (Schiestl et al. 1988).

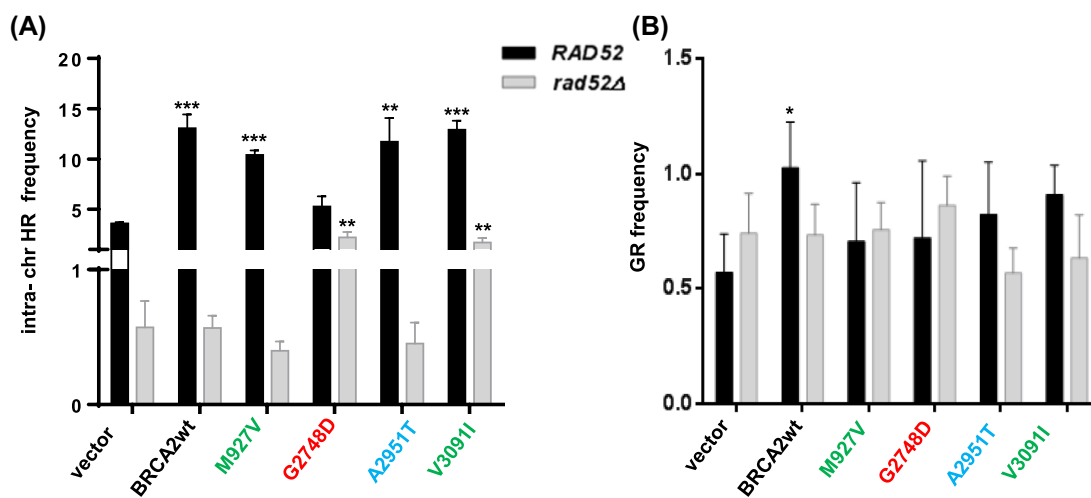


Figure 5. Effect of BRCA2 expression on HR and GR in RSY6RAD52 and RSY2rad52Δ strains of *Saccharomyces cerevisiae*. Yeast strains carrying the BRCA2 expression vectors were grown first in glucose, then shifted to galactose and plated as described in the Materials and Methods. As negative control, HR and GR were measured in the RSY6 strains transformed with the vector. Pathogenic missense variants are reported in red, neutral ones in light blue and VUS in green. Yeast strains carrying the empty pYES2 plasmid and expressing the BRCA2 wild type are in black. (A) Frequency of intra-chromosomal HR events was calculated as total number of His⁺ colonies per 10⁻⁴ vital cells. (B) Frequency of GR events was calculated as total number of Ilv⁺ colonies per 10⁻⁶ vital cells; Results represent the mean ± standard deviation from 4–5 independent experiments. Data were evaluated for normal distribution as described in Materials and Methods. Results were statistically analyzed as described in materials and Methods. BRCA2 wild type was compared to the (empty) vector and each variant to BRCA2 wild type. **P* < 0.05, ***P* ≤ 0.01, ****P* ≤ 0.001.

Notably, in RAD52 and *rad52Δ* strains, we did not observe any substantial effect on cell growth while cultures are incubated for 17 hours in galactose media and expressing BRCA1/2. During this incubation time, cells undergo 3–4 generations, indicating that BRCA1/2 did not affect growth in liquid medium. We have already demonstrated that expression of BRCA1 pathogenic variants used in this study, increased HR and GR as compared to negative control in the RAD52 strains, and results are shown here for comparison (Fig. 4A–D, black bars) (Caligo et al. 2009, Maresca et al. 2015, Maresca et al. 2018, Lodovichi et al. 2020b). Precisely, the expression of the pathogenic variants pC61G, pI1766S, pA1708E and M1775R, (in red) significantly increased intra-chromosomal HR in the haploid RSY6 (Fig. 4A, black bars). In the *rad52Δ* mutant, the expression of pathogenic BRCA1 variants did not affect intra-chromosomal HR as compared to BRCA1 wild type (Fig. 4A, grey bars). Then, we evaluated the effect of the BRCA1 pathogenic variants pC61G, pI1766S and M1775R in the diploid RS112 *rad52Δ/rad52Δ* and in its parental wild type strain to measure intra-chromosomal HR, and inter-chromosomal HR between two *ade2* mutated alleles by directly counting the Ade⁺ colonies. The BRCA1 pathogenic variants induced a statistically significant increase of intra-chromosomal HR in the RAD52/RAD52 strain (Fig. 4B, black bars); this effect is not seen in the *rad52Δ/rad52Δ* mutant confirming the data obtained in the haploid strain (Fig. 4B, grey bars). Moreover, the expression of the pathogenic variant pC61G, pI1766S and pM1775R induced inter-chromosomal HR in the RS112 RAD52/RAD52 strains (Fig. 4C, black bars). In the RS112 *rad52Δ/rad52Δ* mutant, only the pathogenic variant pI1766S induced a weak but significant increase of inter-chromosomal HR as compared to control (vector) (Fig. 4C, grey bars); this suggests that RAD52 is required for BRCA1-induced HR both in diploid and haploid genetic backgrounds.

BRCA1-induced GR is differentially affected by RAD52

The RSY6 strains also carry the revertible *ilv1-92* allele that enables to determine GR by directly counting the Ilv⁺ colonies on

SC-ILE solid medium. In the haploid RSY6 RAD52 strain, the BRCA1 pathogenic pC61G, pI1766S and pM1775R variants, and the neutral pN132K variant induced a statistically significant increase of GR as compared to the negative control (pYES2 plasmid) (Fig. 4D, black bars) (Maresca et al. 2018, Lodovichi et al. 2020a). On the contrary, the pathogenic variant pA1708E did not increase GR in RAD52 strain. This suggests that the results of the GR assay are not 100% reliable for reporting the pathogenicity of BRCA1 variants because it generates false positives (neutral variants that increase GR) and false negatives (pathogenic variants that do not affect GR). In the *rad52* deletion strain, BRCA1 wild type, the pathogenic pI1766S and pM1775R and the neutral pY179C and pS1512I variant induced a statistically significant increase of GR as compared to the negative control (Fig. 4D, grey bars). On the other hand, the pathogenic pC61G and pA1708E variants, the neutral pN132K and pN550H, and the VUS pA1789T showed no effect on GR, in *rad52* deletion strain (Fig. 4D, grey bars). These results indicate that GR induced by BRCA1 variants is differentially dependent on RAD52.

BRCA2-induced HR and GR are affected by RAD52

The expression of wild type BRCA2 and several variants in the diploid yeast RS112 strain has been reported to affect both intra- and inter-chromosomal HR (Spugnese et al. 2013). Here, we have evaluated the effect of wild type BRCA2 and four missense variants on intra-chromosomal HR and GR in the haploid RSY6 RAD52 and *rad52Δ* strains. In the RAD52 strain, BRCA2 wild type, the VUS pM927V and pV3091I, and the neutral variant pA2951T significantly increased HR as compared to the control (Fig. 5A, black bars). In the same strain, the pathogenic BRCA2 variant pG2748D had no effect on intra-chromosomal HR, (Fig. 5A, black bar). Interestingly, wild type BRCA2 did not induce intra-chromosomal HR in *rad52Δ* mutant indicating that RAD52 is required for BRCA2-induced HR (Fig. 5A, grey bar). In the RSY6 *rad52Δ* mutant strain, the variant pV3091I (VUS) and pG2748D showed significant increase of HR as compared to control (Fig. 5A, grey bars). These results suggest that the DNA repair function encoded by RAD52 functionally interacts with BRCA2, in yeast as well as in humans.

We have also evaluated the effect of BRCA2 on GR in the haploid RSY6 strains by plating and counting the *Ilv*⁺ colonies grown in SC-ILE medium after galactose induction. In the RAD52 strain, the expression of wild type BRCA2 significantly increased GR as compared to negative control; no BRCA2 variant significantly affected GR in the same strain (Fig. 5B, black bar). In the RSY6 *rad52Δ* strain, expression of wild type BRCA2 or all the variants had no significant effect on GR (Fig. 5B, grey bars). Again, these results show a functional interaction between BRCA2 and yeast RAD52.

Discussion

Loss-of function mutations in the tumor suppressor gene BRCA1 and BRCA2 are associated with breast and ovarian cancer. In addition, BRCA1/2 defective tumor cells are deficient in DSB repair by HR (Kuchenbaecker et al. 2017, Angeli et al. 2020). The finding that RAD52 is essential for survival of BRCA-deficient cells treated with DNA damaging agents (such as ionizing radiation or other DSB inducers) makes it an attractive target for synthetic lethality-based anticancer therapy. Targeting RAD52 in BRCA1/2-mutated cancer cells sensitizes them to DNA damaging agents while cells or tissues with wild type BRCA1/2 are not influenced (Nogueira et al. 2019, Lodovichi et al. 2020a). In *Saccharomyces cerevisiae*, Rad52 is crucial for most HR events; in human cells, some RAD52 functions are carried out by BRCA2 that physically interacts with BRCA1 to exert its DNA repair activity (Malacaria et al. 2020). Recently, it has been shown that human RAD52 affects HR in the yeast *Saccharomyces cerevisiae* indicating that RAD52 is evolutionary conserved between human and yeast (Manthey et al. 2017, Clear et al. 2020). Moreover, yeast has been considered a highly reliable model in cancer research and can give fundamental contribution to understand and identify new genetic factors involved in tumorigenesis (Guaragnella et al. 2014b) (Ferreira et al. 2019) (Cervelli and Galli 2021).

Previously, we have reported that yeast can give important genetic information on functional interaction between DNA repair pathways and BRCA1-induced genome instability; particularly, we have demonstrated that the mismatch repair genes MSH2 and MSH6 and the HR gene RAD50 differentially affect HR and GR induced by BRCA1 variants (Maresca et al. 2015, Maresca et al. 2018, Lodovichi et al. 2020b). Although, we have not completely understood which mechanisms are involved in BRCA1/2-induced genome instability (determined as HR and GR events), results obtained in the present study strongly suggest that DSB repair pathway modulates BRCA1-induced HR and GR. Here, we found that spontaneous intra- and inter-chromosomal HR induced by pathogenic BRCA1 variants is RAD52-dependent both in haploid and diploid genetic background indicating that RAD52 might affect BRCA1-induced genome instability. We hypothesize that expression of pathogenic BRCA1 variants could increase the production of endogenous DNA damage leading to higher HR. Spontaneous intra-chromosomal recombination can occur by single strand annealing that has been shown to require RAD52 (Rossi et al. 2021); inter-chromosomal recombination may take place by gene conversion that is mainly RAD52-dependent (Coic et al. 2008). Notably, inter-chromosomal HR induced by the BRCA1 pathogenic variant p.I1766S is partially RAD52-dependent because, in the RS112 *rad52Δ* strain, this variant significantly increased HR; this can be due to crossover events that are found to account for more than 30% of recombinants in *rad52Δ* background (Coic et al. 2008).

Spontaneous gene mutations are caused by different mechanisms related to errors in DNA replication and to endogenous

DNA lesions (Maki 2002). RAD52 has been previously reported to be involved in spontaneous mutagenesis although the exact mechanism is not completely known (Kunz et al. 1998) (Mercado-Saenz et al. 2017). The increased GR induced by the expression of wild type BRCA1, and the neutral variant pY179C and pS1512I in *rad52Δ* strain suggests that BRCA1 affects the level of endogenous DNA damage and that RAD52 is genetically required to repair it. As we did not observe any remarkable effect on the growth of *rad52Δ* strains expressing these variants or wild type BRCA1 as well, an effect of BRCA1 on DNA replication fidelity could be ruled out. Interestingly, GR increase induced by the pathogenic variant pC61G and neutral pN132K is RAD52-dependent, whereas the enhancement of GR obtained by the expression of the two pathogenic variants pM1775R and pI1766S is not RAD52-dependent. This may reflect different mechanisms involved in BRCA1-induced GR. In the present study, we have also demonstrated that BRCA1 wild type, pathogenic and neutral variants differentially affect GR in *rad52Δ* yeast mutant. These results strongly suggest that different mechanisms are involved in BRCA1-induced GR and other genetic studies are necessary to understand them.

Intra-chromosomal HR induced by wild type BRCA2, the neutral variant pA2951T and the two VUS is RAD52-dependent; in addition, the expression of the BRCA2 pathogenic variant pG2748D increased HR only in the *rad52Δ* strain suggesting that in a RAD52 wild type background, BRCA2 increased HR by different mechanisms than in *rad52Δ* mutant background. The finding that BRCA2 pathogenic variant decreased HR in RAD52, but increased HR in *rad52Δ* background suggests for the first time that BRCA2 may functionally interact with yeast HR repair. This also indicates that RAD52 depletion could increase genome instability in human cancer cells carrying that BRCA2 variant.

BRCA2 did not induce a strong effect on GR in our yeast strains presumably because BRCA2 is primarily involved in HR. Our study implies that it is more reliable to evaluate the effect of RAD52 on HR than on GR. If a newly identified BRCA1 variant gives significant HR induction in RAD52, but not in *rad52Δ* genetic background, it could be a further indication of its pathogenicity and also give useful hints to design more personalized therapies. Moreover, if a BRCA2 new variant increases HR only in *rad52Δ* strain, this could suggest that RAD52 might be a crucial target for therapy. Therefore, yeast could represent a valuable genetic system to assess genetic interactions between DNA repair and BRCA1 variants and also to design new therapies based on RAD52 depletion.

In conclusions, this study confirms that yeast could be a helpful genetic system that can give information about the pathogenicity of BRCA1/2 missense variants and how to design novel and possibly more efficient therapeutic strategies.

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