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Characterisation of gene expression profiles of yeast cells expressing *BRCA1* missense variants

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ABSTRACT

Germline mutations in breast cancer susceptibility gene 1 (*BRCA1*) confer high risk of developing breast and ovarian cancers. Even though most *BRCA1* cancer-predisposing mutations produce a non-functional truncated protein, 5–10% of them cause single amino acid substitutions. This second type of mutations represents a useful tool for examining *BRCA1* molecular functions. Human *BRCA1* inhibits cell proliferation in transformed *Saccharomyces cerevisiae* cells and this effect is abolished by disease-associated mutations in the BRCT domain. Moreover, *BRCA1* mutations located both inside and outside the BRCT domain may induce an increase in the homologous recombination frequency in yeast cells. Here we present a microarray analysis of gene expression induced in yeast cells transformed with five *BRCA1* missense variants, in comparison with gene expression induced by wild-type *BRCA1*. Data analysis was performed by grouping the *BRCA1* variants into three sets: Recombination (R)-set (Y179C and S1164I), Recombination and Proliferation (RP)-set (I1766S and M1775R) and Proliferation (P)-set (A1789T), according to their effects on yeast cell phenotype. We found 470, 740 and 1136 differentially expressed genes in R-, P- and RP-set, respectively. Our results point to some molecular mechanisms critical for the control of cell proliferation and of genome integrity providing support to a possible pathogenic role of the analysed mutations. They also confirm that yeast, despite the absence of a *BRCA1* homologue, represents a valid model system to examine *BRCA1* molecular functions, as the molecular pathways activated by *BRCA1* variants are conserved in humans.
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1. Introduction

Germline mutations in the breast cancer susceptibility gene 1, *BRCA1*, predispose to breast and ovarian cancers.[1]

Human *BRCA1* encodes a full-length protein of 1863 amino acids containing some known functional domains: a highly conserved N-terminal RING finger domain, two nuclear localisation signals, an 'SQ' cluster, a branched DNA-binding domain and C-terminal BRCT domains.[2]

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BRCA1 appears to coordinate multiple activities linked to the maintenance of genomic integrity and to function as a tumour suppressor, through the direct or indirect interaction with a large number of molecules, including tumour suppressors, oncogenes, DNA damage repair proteins, cell cycle regulators, transcriptional activators and repressors.[3] *BRCA1* plays a role in homologous recombination (HR) during both mitosis and meiosis and regulates the homology-directed DNA repair and crossing over.[4,5] It is also involved in non-homologous end-joining[6] and mismatch repair, both in response to DNA damage[7] and during chromosome segregation.[8] *BRCA1* growth suppressor function in mammalian cells depends at least in part on its interaction with retinoblastoma protein (RB) but also on interaction with a series of other cell cycle-related proteins such as p53 and p21.[9,10]

Most pathogenic *BRCA1* mutations originate a truncated protein, but a number of missense mutations, whose role in the disease is often difficult to ascertain, have also been detected in hereditary breast cancer patients. An almost complete list of genetic variants detected till now in *BRCA1* and *BRCA2* genes is reported in BIC (Breast Cancer Information Core) database (http://research.nhgri.nih.gov/bic/).

Saccharomyces cerevisiae has been shown to represent a good system to discriminate between pathogenic and polymorphic missense mutations located in *BRCA1* BRCT domains, as inactivating mutations within BRCT domains abolish *BRCA1* ability to inhibit yeast cell proliferation.[11] To elucidate the *BRCA1* BRCT domain effects on gene expression, Skibbens et al.[12] compared yeast cells transformed with vector or with vector containing the *BRCA1* BRCT domain by microarray analysis. Even though their analysis was limited to the BRCT domain, they showed that the yeast model is also useful to elucidate molecular aspects of *BRCA1* function.

Caligo et al.,[13] as well, have shown that some *BRCA1* missense mutations isolated from patients' breast cancers and located outside the BRCT domains, in scattered positions along the sequence, induce an increase in HR. This suggests that yeast may represent a model for the study of both *BRCA1* molecular functions: growth suppression and maintenance of genome integrity.

Here we present a study in which gene expression induced in *S. cerevisiae* by each of five *BRCA1* missense variants was compared by microarray analysis to that induced by wild-type *BRCA1*. All five variants had been isolated from familial breast cancers and had induced a phenotypic change in yeast cells, either on proliferation, or on HR or both:[13] three carried missense mutations within the BRCT domains, and two outside the BRCT domains.

2. Materials and methods

2.1. Yeast strain and BRCA1 missense variants

We used the diploid strain RS112 of *S. cerevisiae* (from Robert Schiestl, UCLA, Los Angeles, CA, USA) transformed with the vector YCp GAL::*BRCA1* which contains the human *BRCA1* gene under the control of galactose inducible promoter GAL1p (obtained from Craig Bennett, Duke University, Durham, NC, USA) or with the derivative vectors carrying the following *BRCA1* missense variants: Y179C, S1164I, I1766S, M1775R and A1789T.[13] Except for M1775R that had been described as deleterious by a transcriptional activation assay[14] and, more recently, by a multi-modal approach,[15] all variants were either not reported or described as missense variants of unknown pathological significance in BIC database. However, *in silico* analyses with Sorting Intolerant From Tolerant (SIFT) (http://blocks.fhcrc.org/sift/SIFT.html) and Polymorphism Phenotyping (PolyPhen) (http://tux.embl-heidelberg.de/ramensky/polyphen.cgi) show that all variants probably inactivate protein function.[13] The I1766S variant has been reported as deleterious in one paper,[16] while the Y179C has previously been studied without reaching any conclusive result;[17,18] S1164I and A1789T had never been studied before the work of Caligo and colleagues, 2009.[13]

Concerning the effects on yeast cell phenotype, only the mutations in the BRCT domains (I1766S, M1775R and A1789T) reverted the growth suppression (small colony) phenotype,[13] typically observed in yeast cells transformed with wild-type *BRCA1*.[11] Two of them, I1766S and M1775R, also induced HR. As expected, the two variants, Y179C and S1164I, mapping outside the BRCT domains, did not revert the small colony phenotype, but induced HR.[13] Table 1 resumes all five variants' features.

2.2. Induction of BRCA1 expression

The expression of *BRCA1* was induced as follows: stable transformants obtained as reported by Caligo et al.[13] were pregrown in 10–20 ml of glucose medium for 24 h at 30 °C. Then, cell pellets were washed in water and were split into two aliquots: one was inoculated in 20 ml of glucose and the other

Table 1 – Description of the analysed BRCA1 missense variants.

Variant	Nucleotide	*BRCA1* functional domain	Predicted pathogenicity[a]	Reported pathogenicity	Small colony phenotype assay	Homologous recombination induction
Y179C	c.655A > G	cMyc interaction domain	Damaging	Neutral[16,17]	–	+
S1164I	c.3610G > T	DNA-binding domain	Damaging	Never studied before	–	+
I1766S	c.5416T > G	BRCT domain	Damaging	Deleterious[15]	+	+
M1775R	c.5443T > G	BRCT domain	Damaging	Deleterious[13,14]	+	+
A1789T	c.5484G > A	BRCT domain	Probable	Never studied before	+	–

a Based on SIFT, PolyPhen and BIC.

was inoculated in 20 ml of galactose medium. The cultures were incubated at 30 °C for 24 h under constant shaking. Thereafter, cells were washed twice in sterile water and were immediately used to extract RNA or stored at −20 °C.

The expression of *BRCA1* protein was assessed by Western blot analysis.[13] The observed phenotypes (proliferation or HR) were independent from the level of expression of *BRCA1* variants.[13]

2.3. Isolation of RNA

Total RNA extraction and DNase treatment were performed with the MasterPureTM Yeast RNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA), according to the manufacturer's instructions. The RNA was further cleaned using the chromatographic system RNeasy MinElute Cleanup (Qiagen, Valencia, CA, USA).

The concentration and purity of total RNA were measured by 260 nm UV absorption and by 260/280 ratios, respectively, by using NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Inc. Wilmington, Del, USA): all RNAs displayed a 260/280 OD ratio > 1.9.

The RNA integrity was verified by electrophoresis on a 1.2% agarose-formaldehyde gel.

2.4. Microarray hybridisation

One microgram of each RNA sample was amplified and dye-labelled with Alexa 647 (red) or Alexa 555 (green) (Invitrogen, Carlsbad, CA, USA) with the Amino Allyl MessageAmp™ II aRNA Amplification Kit (Ambion, Austin, TX, USA).

The Alexa 647 and Alexa 555 dye incorporation rates were measured by UV absorption at 647 nm and 555 nm, respectively. Both fluorophores showed a comparable efficiency of incorporation ranging between 5 and 6 dye molecules per 100 nucleotides. Afterwards, 250 μl of hybridisation mix containing 0.25 μg of Alexa 555-labelled amplified RNA (corresponding to 35–40 picomoles of Alexa 555 dye), 0.25 μg of Alexa 647-labelled amplified RNA (corresponding to 35–40 picomoles of Alexa 647 dye), 25 μl of 10X control targets, 5 μl of 25X fragmentation buffer and 125 μl of 2X hybridisation buffer (the three latter ones from the In situ hybridisation kit plus, Agilent Technologies, Palo Alto, CA, USA) was hybridised to each array on Yeast Oligo 2×11k Microarrays (Agilent Technologies, Palo Alto, CA, USA). Each slide contains 2 arrays with 11,000 60-mer oligonucleotide probes representing 6256 Open Reading Frame (ORF) of *S. cerevisiae* S288C strain. The array hybridisation was performed at 60 °C in an oven (Agilent Technologies, Palo Alto, CA, USA) for 17 h under constant rotation.

After hybridisation, the arrays were washed consecutively in 6X SSC, 0.005% TritonX-102 (In situ Hybridisation kit Plus, Agilent Technologies, Palo Alto, CA, USA) solution for 10 min at room temperature and in 0.1X SSC, 0.005% Triton X-102 solution for 5 min on ice and were air dried.

2.5. Experimental design

Microarray experiments were performed by using a dye swap 'reference design'.

The RNA from yeast cells transformed with each *BRCA1* variant (*BRCA1 mut*$^+$ cells) was labelled twice, with Alexa 555 and with Alexa 647, respectively. Each pair of targets was hybridised on two distinct arrays on the same slide and was compared to RNA from *BRCA1 wt*$^+$ cells as indicated in Table 2. Two experimental replicas in dye swap per sample were thus produced in order to avoid dye effect and mutation effect confounding.

2.6. Microarray data acquisition and analysis

Microarray images were acquired by Gene Pix 4000B dual-laser scanner (Axon Instruments, USA) at 5 μm resolution, 100% gain and variable PMT, depending on the needed colour balancing.

Intensity raw data were extracted from TIF images by using the GenePix PRO 6.0 software (Molecular Devices, Sunnyvale, CA, USA) and were analysed by using LIMMA package,[19] an add-in library of Bioconductor (http://www.bioconductor.org).

Before performing the statistical analysis, background was subtracted from the raw data by using the LIMMA package '*minimum*' method, which sets any null or negative intensity value generated by the classical background subtraction, equal to half the minimum of the positive corrected intensity values for that array.

Data were normalised within arrays by '*LOWESS*' method and between arrays by '*Aquantile*' method, both from the LIMMA package. B-statistic[20] and adjusted *p*-value (adj-*p*)[21] were utilised to assign statistical significance to each differentially

Table 2 – Microarray experimental design.

Slide #	Array #	Alexa 555	Alexa 647
1	1_1	M1775R	*Wild type*
1	1_2	*Wild type*	M1775R
2	2_1	I1766S	*Wild type*
2	2_2	*Wild type*	I1766S
3	3_1	A1789T	*Wild type*
3	3_2	*Wild type*	A1789T
4	4_1	S1164I	*Wild type*
4	4_2	*Wild type*	S1164I
5	5_1	Y179C	*Wild type*
5	5_2	*Wild type*	Y179C

expressed gene. Only genes with $B > 0$ and adj-$p < 0.01$ were considered.

Pathway analyses were performed by Pathway Explorer (http://pathwayexplorer.genome.tugraz.at/). The list of differentially expressed genes was also analysed by an accurate investigation of the literature to search for genes and molecular pathways potentially involved in the observed phenotypes. To this aim we used Saccharomyces Genome Database (SGD) (http://www.yeastgenome.org), Ensembl (http://www.ensembl.org), information Hyperlinked Over Proteins (iHOP) (http://www.ihop-net.org/UniPub/iHOP/), Munich Information centre for Protein Sequences (MIPS) (http://MIPS.gsf.de) and PubMed Central (http://www.ncbi.nlm.nih.gov/sites/entrez?db=pubmed).

2.7. *Microarray data validation by real-time PCR*

The total RNA samples that were used for microarray experiments were also used for real-time PCR analysis.

Total RNAs were reverse transcribed with random and oligo-dT primers using the QuantiTect Reverse Transcription kit (Qiagen, Valencia, CA, USA).

PCR primers were designed using the Beacon Designer 4.0 software (Premier Biosoft. International, Palo Alto, CA, USA) and were synthesised by Invitrogen (Carlsbad, CA, USA). Primer sequences are listed out in Table 3.

Real-time PCRs were performed in the iCycler iQ instrument (Biorad, Hercules, CA, USA) with the Brilliant®SYBR® GreenQPCR Master Mix (Stratagene, La Jolla, CA, USA) mixed with the uracil–DNA-glycosylase (Fermentas, M-Medical, Milan, Italy) and fluorescein (Biorad, Hercules, CA, USA).

For each primer pair, we tested the amplification efficiency by using five serial dilutions of cDNA carried out in duplicate: all primer pairs displayed an efficiency between 85% and 100%.

The stability of three housekeeping genes (*PGK1*, *PDA1* and *ORC5*) was evaluated by using *geNorm* software.[22] Only two of them, *PGK1* and *PDA1*, were used to normalise the expression values of the target genes because the third one, *ORC5*, did not show a M stability parameter value higher than 1.5 (threshold established by geNorm).

Each sample was run in triplicate to calculate the standard deviation (SD) for the three experimental replicates. We considered only the experiments with SD < 0.4 for each group of replicates.

The relative expression levels for the target genes in *BRCA1 mut*$^+$ cells with respect to *BRCA1 wt*$^+$ cells were calculated by the Pfaffl method[23] with the Gene Expression Macro™ 1.1 application (Bio-Rad, Hercules, CA, USA). They were reported as fold increase or decrease.

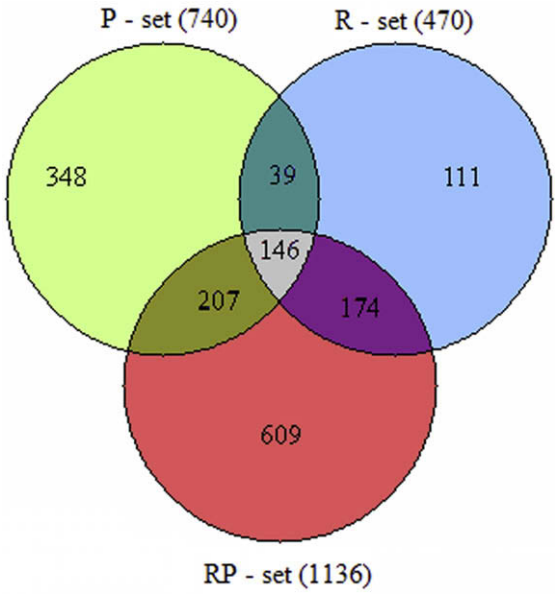

Fig. 1 – Venn diagram of differentially expressed genes by P-, R- and RP-set variants.

3. Results

3.1. *Microarray results*

Data analysis was performed by grouping the *BRCA1* variants (Y179C, S1164I, I1766S, M1775R and A1789T) into three sets: Recombination (R)-set (Y179C and S1164I), Recombination and Proliferation (RP)-set (M1775R and I1766S) and Proliferation (P)-set (A1789T), according to their effects on yeast cell phenotype observed by functional assays,[13] which are summarised in Table 1.

The analysis revealed 470, 740 and 1136 differentially expressed genes in R-, P- and RP-set, respectively (Fig. 1; Supplementary Tables S1–S3); 353 (207+146) genes were modulated by both P- and RP-mutations (P ∩ RP), 320 (174+146) by R- and RP-mutations (R ∩ RP), 185 (39+146) by R- and P-mutations (R ∩ P) and 146 by R-, RP- and P-mutations (R ∩ RP ∩ P) (Fig. 1). Complete information about the microarray experiments and results can be retrieved from the ArrayExpress

Table 3 – Real-time PCR primer pairs and corresponding genes.

Gene	SGD code	Forward primer	Reverse primer
RNR1	YER070W	5′ CGAACCAGTCACTTCCAATATG 3′	5′ TCATCCCAAATACCTAAATCAACC 3′
POL30	YBR088C	5′ ACCCTGTCATTGCCATCTTC 3′	5′ TTAGCTCCGAACGTCAAGTC 3′
SKM1	YOL113W	5′ CTGGTCAAGGAGCAAGTGGTG 3′	5′ CTACGACTCGGTGGCAATGTG 3′
HHF2	YNL030W	5′ GCTAGAAGAGGTGGTGTCAAG 3′	5′ GTTCAGTGTAAGTAACAGAGTCC 3′
ADE1	YAR015W	5′ CCAAGGCTGAACAAGGTGAAC 3′	5′ TTAGTGTCTGCGATGATGATGC 3′
PGK1	YCR012W	5′ TCACTCTTCTATGGTCGGTTTCG 3′	5′ AATGGTCTGGTTGGGTTCTCC 3′
PDA1	YER178W	5′ TTGCTAAGGACTGGTGTCTATC 3′	5′ AATCTCGTCTCTAGTTCTGTAGG 3′
ORC5	YNL261W	5′ AAGGTAAGGCGGAGAGTGG 3′	5′ CGTGAATATCGCTGAAGTAATCG 3′

database at the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/arrayexpress/) by using the following accession number: E-MEXP-1867.

Pathway Explorer mapped about 20% of each set of differentially expressed genes (Supplementary Tables S4–S6).

3.2. *Microarray data validation by real-time PCR*

Five transcripts among those identified as differentially expressed by microarray analysis were selected for validation by real-time PCR: Ribonucleotide-Diphosphate Reductase, Large Subunit (*RNR1*), Proliferating Cell Nuclear Antigen (*POL30*), STE20/PAK Homologous Kinase Related to Morphogenesis (*SKM1*), Histone H4 protein (*HHF2*) and *N*-Succinyl-5-Aminoimidazole-4-Carboxamide Ribonucleotide Synthetase (*ADE1*), (Table 3). By microarray experiments, *RNR1* and *POL30* resulted down-regulated in all three sets of variants, *SKM1* up-regulated in all three sets and *HHF2* and *ADE1* down-regulated in R- and RP-set (Fig. 2).

Real-time PCR analysis was performed by considering the variants one by one and the differential expression was consistently confirmed for all the five examined genes (Fig. 2).

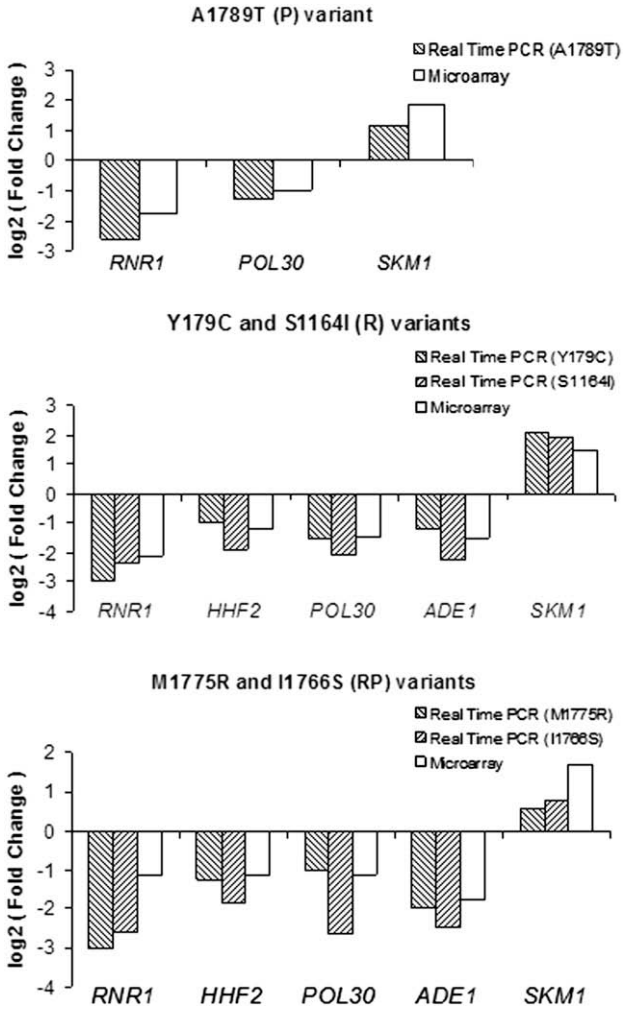

Fig. 2 – Microarray (open bars) and real-time PCR (dashed bars) log-fold changes of genes validated by real-time PCR.

4. Discussion

To investigate the molecular mechanisms that are activated in yeast by potentially deleterious *BRCA1* variants, in this study we hybridised the RNA obtained from yeast cells transformed with five variants exhibiting a phenotypic effect either on proliferation and/or on HR on microarrays,[13] in comparison with the RNA from yeast cells transformed with wild-type *BRCA1* (Tables 1 and 2).

Microarray data relative to the five *BRCA1* variants were analysed by grouping them into three sets, based on the phenotypes described by Caligo et al.[13] We assumed that the genes that are consistently induced or repressed by different variants producing the same phenotype would be functionally correlated with the phenotype itself.

That only 20% of the differentially expressed genes were mapped by *Pathway Explorer* is due to the fact that pathway analysis of yeast genes is still little informative, as a very small number of yeast genes have been placed on KEGG (http://www.genome.jp/kegg/) pathways by means of their homology to human genes, and most of them are involved in metabolism. This is why most differentially expressed genes placed by *Pathway Explorer* were assigned to metabolic pathways, including nucleotide metabolism. Other interesting pathways such as cell cycle, and DNA replication and repair emerged from the analysis, but the small numbers of homologous genes till now assigned to these pathways did not allow for the detection of meaningful differences among the three lists of differentially expressed genes. Thus, the gene lists were analysed by screening the literature and some groups of functionally related genes, which might be more directly involved in the induction of the observed phenotypes, were identified.

4.1. *Genes potentially involved in the induction of homologous recombination*

Chromatin assembly – *HHF2*, *HTA2* and *HTB2*, encoding histone proteins H4, H2A and H2B, respectively, were down-regulated by R- and RP-set variants. *HTB2* was also down-regulated by the A1789T (P) variant. Interestingly, partial depletion of histone H4 has been shown to induce an increase in HR in yeast.[24] H2Ax, the mammalian homologue of H2A, facilitates the assembly of specific DNA repair-complexes on damaged DNA.[25]

The genes encoding for two components of histone acetyltransferase B, *HIF1* and *HAT1*, turned out to be down-regulated by RP-set variants only and by all three sets, respectively. Acetylation of newly synthesised histone H4 by histone acetyltranferase B plays a role in telomeric silencing and double-strand break (DSB) repair.[26]

Nucleotide metabolism – An alteration of nucleotide metabolism can contribute to HR as suggested by Yuen et al.,[27] who, in a systematic screen for genes associated to chromosome instability, showed that several genes coding for enzymes of the adenosine biosynthetic pathway, when deleted, lead to an increased frequency of chromosomal rearrangements. In the present work, three *ADE* genes (*ADE1*, *ADE13* and *ADE17*) turned out to be down-regulated

by R- and RP-set *BRCA1* variants. *ADE6* was down-regulated by RP-set variants. *ADE4* was down-regulated by all three sets of variants.

URA2, which was down-regulated by RP-set variants, and *URA3*, which was down-regulated by both RP- and P-set variants, are structural genes involved in the *de novo* biosynthesis of uridine monophosphate (UMP).[28]

DCD1, which was down-regulated by RP-set variants, is a dCMP deaminase required for dCTP and dTTP synthesis.[29]

PRS4, which was down-regulated by RP-set variants, is a phosphoribosylpyrophosphate synthetase required for nucleotide, histidine and tryptophan biosynthesis.[30]

DUT1, which was down-regulated by all three sets of variants, encodes a dUTPase: by hydrolysing dUTP to dUMP and PPi, DUT1 prevents incorporation of uracil into DNA during replication, thus participating in the maintenance of genome integrity.[31]

Transcription – *SUB2* and *RPB8* are down-regulated by R- and RP-set variants. SUB2 protein is a putative RNA helicase which promotes transcriptional elongation and suppresses transcription-associated recombination.[32] SUB2 inactivation has been shown to increase the recombination rate 800-fold.[33] RPB8 is a highly conserved subunit present in all three eukaryotic RNA polymerases. Although its function is still unclear, both yeast and human RPB8 proteins can bind single-stranded oligonucleotides,[34,35] and human RPB8 is polyubiquitinated by *BRCA1* in response to DNA damage.[36]

DNA replication and repair – *POL30*, whose expression is down-regulated by all three sets of variants, encodes the Proliferative Cell Nuclear Antigen (PCNA), a homotrimeric complex that functions as a sliding clamp and processivity factor for DNA polymerases;[37] it is also required for sister chromatid cohesion[38] and multiple forms of DNA repair.[39] Interestingly, ectopic expression of wild-type *BRCA1* in human cell lines up-regulates *PCNA*, the human homologue of *POL30*.[40]

The genes coding for three subunits of replication factor C, *RFC3, RFC4* and *RFC5*, were down-regulated by both RP- and P-set variants: replication factor C is a DNA-binding protein and ATPase that acts as a clamp loader of the proliferating cell nuclear antigen (PCNA).[41]

PCNA interacts with RAD27 (whose mRNA is down-regulated by all three sets of variants)[42] and POL32 (whose mRNA is down-regulated by RP-set variants),[43] among other proteins. RAD27 is a flap endonuclease which, in cooperation with the product of *RNH201* (down-regulated by RP-set variants) removes RNA primers from Okazaki fragments during DNA lagging strand synthesis, a critical process for the maintenance of genome integrity.[44] POL32 is the third subunit of DNA polymerase delta; pol32delta mutants show severe defects in DNA repair, replication and mutagenesis.[45]

R-set variants up-regulated *SOH1*, which encodes a protein interacting with factors involved in DNA repair and transcription,[46] and down-regulated *NHP6B*, a member of the high-mobility group box (HMGB) superfamily whose loss leads to genomic instability and hypersensitivity to DNA-damaging agents in yeast; mouse fibroblasts lacking *NHP6B* homologue, *HMGB1*, display higher rates of DNA damage after UV irradiation and chromosomal instability.[47]

The product of *SEM1* (down-regulated by RP-set variants) is one of the components of the regulatory cap of 26S proteasome, a complex involved in protein degradation and DNA DSB repair.[48] The human *SEM1* homologue, DSS1, interacts with BRCA2[49] which, in turn, has been shown to interact with *BRCA1* at sites of DNA damage.[50] Given the conserved role of *SEM1/DSS1* in HR, its down-regulation might represent a possible mechanism through which some mutant forms of *BRCA1* lead to cancer development.

4.2. *Genes potentially involved in proliferation recovery*

Cell cycle – The expression of several cell cycle genes was altered by the P-set variant. These include *CDC6*, encoding a component of the pre-replicative complex,[51] some cyclin genes (*CLN1* and *CLB6*)[52,53] and some genes coding for proteins involved in cell cycle checkpoints (*RFC5*, *DRC1*, *DDC1* and *IPL1*),[54–57] all down-regulated, and the G1 cyclin gene *CLN*,[58]which is up-regulated. The transcription of *CDC6*, *DRC1*, *CLN1*, *CLB6* and *IPL1* is regulated by MBF and SBF transcription complexes,[57,59] suggesting that *BRCA1* might interact, directly or indirectly, with these complexes. Interestingly, in mammalian cells, *BRCA1* interacts with hypophosphorylated RB which, in turn, interacts with the E2F transcription factor, a functional homologue of MBF and SBF which coordinates the transcription of cell cycle genes.[7,9,60]

Nucleotide metabolism – The ribonucleotide reductase small subunit genes *RNR2* and *RNR4*,[61] are up-regulated by RP-set variants. The human homologue of *RNR2* promotes malignant progression in mammalian cells and is up-regulated in pre-malignant breast disease.[62,63] The gene coding for the large subunit of ribonucleotide reductase, *RNR1*, was down-regulated by all three sets of *BRCA1* variants.[64] Interestingly, overexpression of mouse *RNR1* suppresses the tumourigenicity of *ras*-transformed cells.[65]

Invasive and pseudohyphal growth – A group of genes related to invasive (haploid) and pseudohyphal (diploid) growth was up-regulated by the P-set variant: *FLO11, MEP2*, *GPA2*, *HMS1* and *ASH1*. *FLO11* encodes a surface mucin, and represents a key gene of both invasive (haploid) and pseudohyphal (diploid) growth pathways;[66] *MEP2*, *GPA2*, *HMS1* and *ASH1* are all implicated in a number of signal transduction cascades leading to the activation of *FLO11* transcription.[67–69] Consistently, *DIG2*, a negative regulator of *FLO11* transcription,[70,71] is down-regulated in cells transformed with the P-set variant.

SKM1, coding for a Ste20/PAK (p21-activated-kinase)-like serine/threonine protein kinase presumably involved in the activation of polarised growth,[72] is up-regulated by all *BRCA1* variant sets. Invasive growth and pseudohyphal growth are characterised by polarised growth, and alterations of motility, cell–cell and cell–substrate adhesiveness and substrate invasiveness.[73] These morphological changes recall those of cancer cells; indeed, several homologues of yeast genes involved in cytoskeleton remodelling and cell motility are overexpressed in human cancer cells.[74] The mucin family is conserved among eukaryotes and overexpression of one mucin or more mucins has been observed in many types of cancers,[75,76] and the expression level of several human mucins correlates with tumour invasiveness.[75]

Human proteins belonging to the PAK family act downstream of RHO-GTPases, like Cdc42 and Rac proteins, and play a role in modulating the actin cytoskeleton.[77] Overexpression

of RAC proteins is directly correlated with tumour invasiveness and cellular motility in breast cancer.[78] In addition, in breast cancer cell lines the human protein PAK1 interacts with histone H3[79] and its overexpression leads to abnormal assembly of the mitotic spindle.[80]

4.3. *Genes potentially involved in both induction of homologous recombination and proliferation recovery*

Chromatin remodelling – *ARP7*, *ARP9* and *SFH1* were down-regulated by RP-set variants. Arp7, Arp9 and Sfh1 are components of the chromatin remodelling complexes SWI/SNF and RSC,[81,82] which promote transcription elongation,[83] take part in DNA DSB repair[84] and control progression through the cell cycle.[85] Interestingly, in human cells, there is evidence that *BRCA1* controls transcription via a direct interaction with a SWI/SNF-related complex.[86] Yeast *SFH1* is the homologue of the human tumour suppressor gene *SNF5*.[87]

Cell cycle checkpoints – *TOP2* (down-regulated by RP-set variants) encodes a type II topoisomerase which prevents chromosome aberrations by facilitating the separation between replicated sister chromatids and by preventing recombination between rDNA repeats.[88,89] Both yeast *TOP2* and its human homologue are involved in the G2/M decatenation checkpoint, which, in human cells, requires *BRCA1*.[8,90]

The protein encoded by *MSH2*, also conserved in mammals, forms two protein complexes, with either MSH6 or MSH3, which are able to recognise base-base mispairs and single-base insertions/deletions, or larger insertions/deletions, respectively, to initiate the repair process.[91] In human cells, MSH2 takes part in the *BRCA1*-associated surveillance complex,[92] and cooperates with *BRCA1* in activating the G2-M checkpoint following DNA damage.[93] *MSH2* was down-regulated by all three sets of variants.

5. Concluding remarks

The present work highlights a number of genes and molecular pathways which are affected in yeast by five *BRCA1* missense mutations in comparison with wild-type *BRCA1*. These data point to some molecular mechanisms which might be responsible for the effects of *BRCA1* variants on yeast phenotype: transcriptional elongation, and DNA replication and repair, whose alterations might contribute to the rise in HR rate; cell cycle checkpoint control and growth regulation, whose alterations probably contribute to proliferation recovery; chromatin assembly and remodelling and nucleotide metabolism, as pathways which might be implicated in both phenomena. The alteration of molecular mechanisms critical for the control of cell proliferation and of genome integrity provides further support to the hypothesis of a pathogenic role of the analysed mutations, already suggested by the results of Caligo et al.[13] It also supports a dual role of *BRCA1* in cancer protection, both as a caretaker and a gatekeeper gene.[94]

Finally, our results confirm that yeast, despite the absence of a *BRCA1* homologue, represents a valid model system to examine *BRCA1* molecular functions, as the molecular pathways activated by *BRCA1* variants are conserved in humans. Thus, information acquired in the yeast model may contribute to understand the molecular events that occur in human cells as a consequence of *BRCA1*.

Conflict of interest statement

None declared.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ejca.2009.04.025.

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