Exploring the link between MORF4L1 and risk of breast cancer

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Published in:
Breast Cancer Research

DOI:
10.1186/bcr2862

2011

Link to publication

Citation for published version (APA):

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Download date: 30. Dec. 2018
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Abstract

Introduction: Proteins encoded by Fanconi anemia (FA) and/or breast cancer (BrCa) susceptibility genes cooperate in a common DNA damage repair signaling pathway. To gain deeper insight into this pathway and its influence on cancer risk, we searched for novel components through protein physical interaction screens.

Methods: Protein physical interactions were screened using the yeast two-hybrid system. Co-affinity purifications and endogenous co-immunoprecipitation assays were performed to corroborate interactions. Biochemical and functional assays in human, mouse and Caenorhabditis elegans models were carried out to characterize pathway components. Thirteen FANCD2-monoubiquitinylation-positive FA cell lines excluded for genetic defects in the downstream pathway components and 300 familial BrCa patients negative for BRCA1/2 mutations were analyzed for genetic mutations. Common genetic variants were genotyped in 9,573 BRCA1/2 mutation carriers for associations with BrCa risk.

Results: A previously identified co-purifying protein with PALB2 was identified, MRG15 (MORF4L1 gene). Results in human, mouse and C. elegans models delineate molecular and functional relationships with BRCA2, PALB2, RAD51 and RPA1 that suggest a role for MRG15 in the repair of DNA double-strand breaks. Mrg15-deficient murine embryonic fibroblasts showed moderate sensitivity to γ-irradiation relative to controls and reduced formation of Rad51 nuclear foci. Examination of mutants of MRG15 and BRCA2 C. elegans orthologs revealed phenocopy by accumulation of RPA-1 (human RPA1) nuclear foci and aberrant chromosomal compactions in meiotic cells. However, no alterations or mutations were identified for MRG15/MORF4L1 in unclassified FA patients and BrCa familial cases. Finally, no significant associations between common MORF4L1 variants and BrCa risk for BRCA1 or BRCA2 mutation carriers were identified: rs7164529, \( P_{\text{trend}} = 0.45 \) and 0.05, \( P_{2\text{df}} = 0.51 \) and 0.14, respectively; and rs10519219, \( P_{\text{trend}} = 0.92 \) and 0.72, \( P_{2\text{df}} = 0.76 \) and 0.07, respectively.

Conclusions: While the present study expands on the role of MRG15 in the control of genomic stability, weak associations cannot be ruled out for potential low-penetrance variants at MORF4L1 and BrCa risk among BRCA2 mutation carriers.

Introduction

Genes that when mutated cause Fanconi anemia (FA) and/or influence breast cancer (BrCa) susceptibility functionally converge on a homology-directed DNA damage repair process [1]. That is, 15 FA genes (FANCs) and genes with high-penetrance, moderate-penetrance or low-penetrance mutations for BrCa encode for proteins cooperating in a defined FA/BrCa signaling pathway [2-6]. Remarkably, germline bi-allelic and mono-allelic loss-of-function mutations in four of these genes cause FA and BrCa, respectively: FANCD1/BRCA2 [7,8], FANC/BRIP1 [9-12], FANCN/PALB2 [13-15], and the recently identified FA-like/BrCa mutated gene FANCO/RAD51C [3,4]. These observations partially endorse perturbation of the DNA damage repair response as fundamental in leading to breast carcinogenesis. In addition to the main effects on susceptibility, variation in RAD51 - a gene encoding for a component of this pathway and paralog of RAD51C - modifies BrCa risk among BRCA2 but not BRCA1 mutation carriers [16]. Notably, RAD51 interacts with BRCA1 and BRCA2 [17,18] to regulate double-strand breaks repair by homologous recombination [19].

While genes with low-penetrance and/or modifier alleles can be linked to diverse biological processes, the FA/BrCa pathway is still incomplete [2,20]. To gain deeper insight into the molecular and functional FA/BrCa wiring diagram and the fundamental biological process(es) influencing cancer risk, we screened for novel protein physical interactions of known pathway components. Consistent with previous results on protein complex memberships [21,22], we identified a physical interaction between PALB2 and MRG15. Results from the analysis of MRG15/MORF4L1 in unclassified FA patients and familial BrCa cases did not reveal pathological alterations; nonetheless, a weak modifier effect among carriers of BRCA2 mutations cannot be ruled out.

Materials and methods

Yeast two-hybrid design and screens

Following indications of increased sensitivity in the yeast two-hybrid (Y2H) system [23,24], we designed multiple baits of each FA/BrCa pathway protein according to family domains defined by Pfam [25] and intrinsically disordered regions predicted by PONDR [26], as well as full-length ORFs. Proteome-scale Y2H screens were carried out using the mating strategy [27] and two different cDNA libraries as sources of prey, of human fetal brain or spleen (ProQuest; Invitrogen, Carlsbad, CA, USA). Bait fragments were obtained by RT-PCR using cDNAs derived from healthy lymphocytes, with the primers

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indicated in Additional file 1 and were subsequently cloned into the Gateway pDONR201 (Invitrogen) vector. Baits were 5'-sequenced so that they were confirmed, they did not show changes relative to publicly available sequence information and they were in-frame. Fragments were then transferred to the pPC97 yeast expression vector (Invitrogen) to be fused with the DNA-binding domain of Gal4. Constructs were transformed into the AH109 (Clontech, Palo Alto, CA, USA) yeast strain for screens (Y187 mate strain) using selective medium lacking histidine and supplemented with 10 mM 3-amino-triazole (Sigma-Aldrich, Taufkirchen, Germany) to test the interaction-dependent transactivation of the HIS3 reporter. Baits had previously been examined for self-activation at 3-amino-triazole concentrations in the range 10 to 80 mM. Under these conditions, >10^7 transformants were screened for each bait. Positive colonies were grown in selective medium for three cycles (10 to 15 days) to avoid unspecific cDNA contaminants, prior to PCR amplification and sequence identification of prey [28].

**Microarray data analysis**

The similarity of expression profiles was evaluated by calculating Pearson correlation coefficients using normalized (gcRMA) expression levels from the Human GeneAtlas U133A dataset [29] [Gene Expression Omnibus:GSE1133]. Comparisons were made for all possible microarray probe pairs.

**Co-immunoprecipitation and co-affinity purification assays**

For co-affinity purification (co-AP) assays, plasmids (1.5 μg) were transfected into HEK293/HeLa cells in six-well format using Lipofectamine 2000 (Invitrogen). Cells were then cultured for 48 hours and lysates prepared in buffer containing 50 mM Tris-HCl (pH 7.5), 100 to 150 mM NaCl, 0.5% Nonidet P-40, 1 mM ethylenediamine tetraacetic acid, and protease inhibitor cocktail (Roche Molecular Biochemicals). Lysates were clarified with centrifugation and washed four times with 0.5 × 10^7 to 1 × 10^7 cells/ml in NETN buffers (20 mM Tris pH 7.5, 1 mM ethylenediamine tetraacetic acid and 0.5% NP-40) containing 100 to 350 mM NaCl plus protease inhibitor cocktail (Roche Molecular Biochemicals). In some assays, supplementary phosphatase (10 to 50 mM NaF) or proteasome (MG132; Sigma-Aldrich) inhibitors were added to the solutions. Lysates were pre-cleared with protein-A sepharose beads (GE Healthcare), incubated with antibodies (2.5 to 5 μg) for 2 hours to overnight at 4°C with rotation, and then with protein-A beads for 1 hour at 4°C with rotation. Beads were collected by centrifugation and washed four times with lysis buffer prior to gel analysis.

**Survival and siRNA-based assays**

For evaluation of survival, 3 × 10^5 cells were seeded in duplicate in 60-mm dishes and left to recover for 24 hours. Cultures were then exposed to mitomycin-C or γ-radiation at the indicated doses. Next, 72 hours after the treatment, cells were rinsed with PBS, harvested by trypsinization and counted. Survival is reported as the percentage relative to untreated controls. Each siRNA (Additional file 2) was transfected for two successive rounds (24 hours apart) at a final concentration of 20 nM using Lipofectamine RNAiMAX reagent (Invitrogen) according to the manufacturer's instructions. After 4 days, cultures were treated with mitomycin-C or γ-radiation. Stealth siRNA Lo GC (12935-200; Invitrogen) was used as a negative control.

**Immunofluorescence microscopy and antibodies**

Cells were grown on glass cover slips and fixed using standard paraformaldehyde solution. Pre-extraction with PBS containing 0.5% Triton X-100 for 5 minutes at room temperature was used in some experiments. Staining was performed overnight at 4°C using appropriate primary antibody dilutions. Samples were then washed three times with 0.02% Tween 20 in PBS, incubated for 30 minutes at room temperature with Alexa fluor-conjugated secondary antibodies (Molecular Probes, Invitrogen), washed three times with 0.02% Tween 20 in PBS, and mounted on 4,6-diamidino-2-phenylindole-containing VECTASHIELD solution (Vector Laboratories, Peterborough, UK). Images were obtained using a Leica CTR-6000 microscope (Leica, Buffalo Grove, IL, USA).

Purified negative control IgGs of different species were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Anti-tag antibodies used were anti-HA (12CA5 and Y11; Santa Cruz Biotechnology), anti-HIS...
(H15; Santa Cruz Biotechnology) and anti-MYC (9E10; Sigma-Aldrich). Other antibodies used were anti-ACTN (ACTN05 C4; Abcam, Cambridge, UK), anti-Actb (8226; Abcam), anti-ATR (09-070; Millipore), anti-BRCA2 (Ab-1; Calbiochem-EMD Biosciences, San Diego, CA, USA), anti-CHEK2 (H300; Santa Cruz Biotechnology), anti-CHUK (ab54626; Abcam), anti-FANCD2 (ab2187; Abcam), anti-phospho-Ser139-H2AX (JBW301; Millipore), anti-KPNA1 (ab6035 and ab55387; Abcam), anti-MRG15 (N2-14; Novus Biologicals, Littleton, CO, USA; 1-235 ab37602; Abcam; and 15C [31-34]), anti-NFkB1 (H119; Santa Cruz Biotechnology), anti-p84 (ab487; Abcam), anti-PALB2 (675-725; Novus Biologicals), anti-PPHLN1 (ab69569; Abcam), anti-RAD51 (H92; Santa Cruz Biotechnology), anti-RPA1 (C88375; LifeSpan BioSciences, Seattle, WA, USA), anti-TOP3A (N20; Santa Cruz Biotechnology), anti-TRF2 (36; BD Transduction Laboratories, Mississauga, ON, USA), anti-TSNAX (3179C2a; Santa Cruz Biotechnology), and anti-USP1 (AP130a; Abgent, San Diego, CA, USA). Secondary horseradish peroxidase-linked antibodies were purchased from GE Healthcare and Abcam.

**Caenorhabditis elegans studies**

Worms were cultured according to standard protocols, maintained on NGM agar seeded with *Escherichia Coli* OP50 [35]. The Bristol N2 strain was used as the wild-type strain. Strains carrying mutations studied here were provided by the Caenorhabditis Genetics Center (University of Minnesota, Minneapolis, MN, USA): DW104 brc-2(tm1086) III/hT2[bli-4(e937) let-7(q782) qIs48][I; III]; VC1873: rad-51(ok2218) IV/nT1[qIs51][IV;V]; and XA6226 mrg-1(qa62000) qC1 dpy-19(e1259) glp-1(q339) [qIs26]. Gonads from gravid adults were dissected out with fine-gauge needles to perform a standard immunofluorescence. Primary antibodies were rat anti-RPA-1 (1:500) and rabbit anti-RAD-51 (1:100). Secondary antibodies were anti-rat Alexa 488 and anti-rabbit Alexa 568 (Invitrogen). Gonads were mounted with ProLong® Gold antifade reagent with 4,6-diamidino-2-phenylindole (Invitrogen). The cell-permeable SYTO 12 Green-Fluorescent Nucleic Acid Stain (Invitrogen) was used to label apoptotic cell death.

**Study samples, genotyping and statistical analysis**

All participants were enrolled under Institutional Review Boards or ethics committee approval at each participating center, and gave written informed consent. Research was conducted in accordance with the Declaration of Helsinki.

The MORF4L1 genomic sequence was obtained from the University of California at Santa Cruz Genome Browser version hg18 and intronic primers were designed using the web-based program Primer3 [36]. Extracts from 13 unclassified FANCD2 monoubiquitylation-proficient FA cell lines, without mutations in FANCI, FANCD1, FANCN, FANCO, or FANCP, and including six cases with deficient RAD51 nuclear foci formation, were examined by immunoblotting using the anti-MRG15 15C antibody [31-34]. These samples were also sequenced on all annotated MORF4L1 exons and exon-intron boundaries using primers shown in Additional file 3.

**BRCA1 and BRCA2 mutation carriers**

were enrolled through 18 centers participating in the CIMBA and following previously detailed criteria [37,38]. The following individual and clinical data were collected: year of birth, mutation description, ethnicity, country of residence, age at last follow-up, age at diagnosis of BrCa or at ovarian cancer diagnosis, age at bilateral prophylactic mastectomy, and age at bilateral prophylactic oophorectomy.

Genotyping was performed at the corresponding centers using 5’ to 3’ nuclease-based assays (TaqMan; Applied Biosystems, Foster City, CA, USA), except for an iPLEX assay carried out at the Queensland Institute of Medical Research (Brisbane, Australia) and containing EMBRACE, FCCC, GEORGETOWN, HEBCS, HEBON, ILUH, kConFab, Mayo Clinic, PBCS, SWE-BRCA and UPENN carriers. Results of these assays were centralized and analyzed for quality control as previously described [37]. Based on these criteria, one study was excluded from the analysis.

Hazard ratio (HR) estimates were obtained using Cox regression models under both standard regression analysis and under a weighted cohort approach to allow for the retrospective study design and the nonrandom sampling of affected and unaffected mutation carriers [39]. Analyses were stratified by birth cohort (<1940, 1940 to 1949, 1950 to 1959 and ≥1960), ethnicity and study center. A robust variance estimate was used to account for familial correlations. Time to diagnosis of BrCa from birth was modeled by censoring at the first of the following events: bilateral prophylactic mastectomy, BrCa diagnosis, ovarian cancer diagnosis, death and last date known to be alive. Subjects were considered affected if they were censored at BrCa diagnosis and unaffected otherwise. The weighted cohort approach involves assigning weights separately to affected and unaffected individuals such that the weighted observed incidences in the sample agree with established estimates for mutation carriers [39]. This approach has been shown to adjust for the bias in the HR estimates resulting from the ascertainment criteria used, which leads to an oversampling of affected women. Weights were assigned separately for carriers of mutations in BRCA1 and BRCA2 and by age interval (<25, 25 to 29, 30 to 34, 35 to 39, 40 to 44, 45 to 49, 50 to 54, 55 to 59, 60 to 64,
65 to 69 and ≥70). P values were derived from the robust score test.

Results

Protein physical interactions

The Y2H system was used to identify physical interactions for components of the FA/BrCa signaling pathway. In an initial phase, we screened for interactors of 12 proteins, which included the products of the FANCI and FANCN genes (BRIP1 and PALB2, respectively) [9-11,15], CHEK2 as linked to BrCa risk [40], and known molecular and/or functional interactors of FA/BrCa proteins (ATR, BLM, ERCC1, ERCC4, H2AFX, RAD51, TOP3A, TOPBP1 and USP1; see Additional file 1). To increase interactome coverage, we used specific protein domains or defined regions as baits, in addition to full-length ORFs, and screened >10^7 transformants belonging to two different cDNA sources (see Materials and methods). Multiple baits were thus screened for each protein based on Pfam-based family domain similarities [25] and on predicted intrinsically disordered regions using the PONDR algorithm [26]. Intrinsically disordered regions are defined as lacking a fixed tertiary structure and appear to be more common in nuclear proteins and involved in the cell cycle, transcription and signaling regulation processes [41,42]. A total of 33 baits were screened for the 12 target proteins (Additional file 1).

Two previously demonstrated and six novel, potential physical interactions were identified through the Y2H screens (Additional file 4). Consistent with the physical interaction between their products, analysis of transcriptional data identified significant expression correlations across normal human samples for most gene pairs (Additional file 5). The known interactions were BLM-MLH1 [43] and ERCC4-ERCC1 [44], through a predicted disordered region and a family domain, respectively (Additional file 6). The potential physical interactions included a previously described protein complex membership between PALB2 and MRG15 (also known as the MORF4-like 1 gene product) [21,22]. To corroborate the Y2H results, we performed co-IP and co-IP assays, which suggested reliability for four of the interactions: CHEK2-NFKB1, PALB2-MRG15, TOP3A-TSNAX and USP1-KPNA1 (Additional file 7). TOP3A was originally co-purified with, among others, BLM, FANCA and replication proteins [45]. TSNAX (also known as translin (TSN)-associated factor X) was previously found to interact physically with MORF4 family associated protein 1-like 1 [46], and USP1 and KPNA1 were co-purified [47]. With the exception of MRG15 (see below), however, protein depletion assays did not show cellular sensitivity to γ-irradiation or mitomycin-C for any of the potential pathway components (siRNAs detailed in Additional file 2).

MRG15 is a chromo domain-containing protein present in histone acetyltransferase and deacetylase complexes [34], and the MRG15 ortholog in Drosophila melanogaster has been co-purified in histone chaperone complexes with a known BRCA2 interactor in humans, EMSY [48]. Consistent with a potential role in DNA damage repair, EAF3, the MORF family ortholog in Saccharomyces cerevisiae, was shown to interact genetically with radiation-sensitive (RAD) genes [49]. As previously shown [21,22], MRGX, a close homolog of MRG15, also co-purified with PALB2 (Additional file 8). Consistent with the interaction domains delineated by the Y2H results, a MRG15 mutant lacking the C-terminal leucine zipper domain but not the N-terminal chromo domain was unable to interact with PALB2 (Additional file 8). Similarly, the helix-loop-helix region in MRGX was necessary for co-purification with PALB2 (Additional file 8). Together, these results support the identification of a physical interaction between PALB2 and MRG15, and probably MRGX.

MRG15 and DNA damage repair

According to the putative role of MRG15 in the repair of DNA double-strand breaks, murine embryonic fibroblasts (MEFs) derived from littermate embryos with the Morf4L1^-/- genotype showed greater sensitivity (measured as cellular survival) to γ-irradiation than wild-type controls (Figure 1). The level of radiation sensitivity was moderate when compared with Atm-deficient MEFs (Figure 1). Milder sensitivity to mitomycin-C of cell cultures depleted of MRG15, relative to BRCA2 and PALB2, was also previously described [21]. In our study, however, deficiency of Mrg15 and depletion of MRG15 in MEFs and in HeLa and MCF10A cells, respectively, did not lead to a statistically significant increase in mitomycin-C-induced cell death or to G2/M phase cell cycle arrest and FANCD2 monoubiquitinylation (Additional file 9 shows results for HeLa cells). The observed milder effect and the use of different cell types may explain the discrepancy regarding mitomycin-C sensitivity when MRG15/Mrg15 is fully or partially depleted.

Contrary to the results for MRG15/Mrg15, radiation sensitivity phenotypes were not observed with assays for MRGX - also consistent with the previous study [21] - and for the potential novel interactor of TOP3A, TSNAX (data not shown). In agreement with the known role of TOP3A in telomere maintenance [50], however, an EmGFP-tagged TSNAX protein co-localized in specific nuclear structures with the telomere-binding protein TRF2 (Additional file 10). The major partner of TSNAX, TSN, was initially identified as a protein that binds to breakpoint junctions [51] and with high affinity to repeat sequences [52]. Although there is no evidence linking TSN to processes where recombination is
necessary, there is some suggestion of a role in the DNA damage response [53]. Intriguingly, telomere shortening has been linked to FA pathology [54-56], and some FANC products were demonstrated to participate in telomere maintenance [57-59]. These observations lead to speculation that interactions between TSN- TSNAX- TOP3A may play a role in DNA damage repair and telomere maintenance by signaling through the FA/BrCa pathway.

In previous work, MRG15 appeared necessary for the association of BRCA2/PALB2/RAD51 with chromatin and the formation of nuclear foci following γ-irradiation [21]. In keeping with these observations, Morf4l1^-/- MEFs showed lower numbers of Rad51 nuclear foci after γ-irradiation - discovered across time points and using clones or unselected cell cultures (Figure 2a shows results for clones). On the other hand, Morf4l1^-/- MEFs showed lower expression levels of Brca1 and Brca2, but results were variable for Rad51 (Figure 2b) - Palb2 levels could not be assessed because the antibodies tested did not cross-react in mouse cell extracts. The result for Brca2 appeared to disagree with a previous study using human cell models [22]; however, another study showed reduction of BRCA2 through transient depletion of MRG15 but not MRGX [21]. This relationship for MRG15 could therefore be reminiscent of the role of PALB2 in stabilizing BRCA2 [60]. Together, these data suggest the involvement of MRG15 in the repair of DNA double-strand breaks through relationships with BRCA2, PALB2 and RAD51.

**Caenorhabditis elegans** mutants of MRG15 and BRCA2 orthologs

The BRCA2 and RAD51 C. elegans orthologs (named BRC-2 and RAD-51, respectively) interact physically and regulate homologous recombination, so that brc-2 mutants fail to locate RAD-51 to sites of double-strand breaks present in meiosis or induced by DNA damage agents [61]. The hallmarks of brc-2 mutants in the germline are therefore lack of RAD-51 foci formation in parallel with an accumulation of RPA-1 at presumptive double-strand breaks, chromosomal abnormalities at diakinesis and, consequently, an increase in apoptotic corpses [61,62]. *C. elegans* has an ortholog for the MORF human protein family (named MRG-1), which, like its mammalian counterparts, associates with chromatin and is required for embryo survival and cell proliferation [63,64]. On the strength of this evidence, the functional link between BRC-2/BRCA2 and MRG-1/ MRG15 was further investigated by assessing the pheno- copy between brc-2 and mrg-1 mutants (tm1086 and qa6200, respectively).

Similar to brc-2 mutants, disruption of mrg-1 was linked to a remarkable increase in the number of RPA-1 foci in meiotic cells relative to wild-type animals (Figure 3a). While a wild-type animal presented, on average, three or four RPA-1 foci per nucleus, mrg-1 mutants commonly exhibited nuclei with more than 10 foci (Figure 3b). Two different patterns for RPA-1 staining were observed among mrg-1 mutant germ cell nuclei: one consisted of discrete foci similar to those observed in brc-2 mutants (Figure 3a, arrow), while the other showed more intense and diffuse staining (Figure 3a, arrowhead). Although RAD-51 staining was mainly nuclear in mrg-1 mutants - contrary to brc-2 mutants [61] - it was rather diffuse and often intense when compared with the usual pattern of discrete foci only observed in wild-type animals (Figure 3a and Additional file 11). Finally, mrg-1 mutants frequently showed aberrant chromosomal compaction (Figure 3a, asterisk) and, as expected, an increase in cell death revealed by SYTO-12 staining (Figure 3c). Together, these data further endorse the involvement of MRG-1/MRG15 in the control of genomic stability and suggest that perturbation of its function may activate the nonhomologous end-joining DNA damage repair process, as proposed for alteration of BRC-2 [61].

**MORF4L1, Fanconi anemia and breast cancer risk**

Having identified molecular and functional relationships for MRG15 in the repair of DNA double-strand breaks, we next evaluated the existence of alterations or mutations of MRG15/MORF4L1 in FA and BrCa patients. Immunoblotting of MRG15 using extracts of 13 FANCD2-mono ubiquitinylation-positive FA cell lines - excluded for genetic defects in the downstream genes FANCD1/BRCA2, FANCJ/BRIP1, FANCN/PALB2, FANCO/RAD51C and FANCN/SLX4, and thus unclassifi- able in terms of subtype - failed to show gross reduction...
of protein expression. This negative result included the analysis of six patient-derived FA cell lines defective for RAD51 foci (Additional file 12). Sequencing of MORF4L1 in these lines detected a few base substitutions and single base deletions deeper in the introns, and only annotated common variants in the exons (data not shown). Parallel to FA, we hypothesized that germ-line mutations or common variants in MORF4L1 may confer moderate/low risk of BrCa and/or modify cancer risk among BRCA1 and/or BRCA2 mutation carriers.

Direct sequencing of MORF4L1 exons and flanking sequences in 300 patients with strong familial aggregation of BrCa but without detected mutations in BRCA1 or BRCA2, and belonging to two populations (United Kingdom, Institute of Cancer Research; Spain, Catalan Institute of Oncology), did not reveal pathogenic changes either. This negative result is consistent with a recent report in a similar setting by another group [65]. Nevertheless, given the extremely low frequency of high/moderate-penetrance mutations of other components of the FA/BrCa pathway [3,12,14] and the possible involvement in other cancer types [66], further investigation of MORF4L1 may be warranted.

The public results of the genome-wide association study conducted by the CGEMS initiative [67] suggest that common variation at the linkage disequilibrium block containing MORF4L1 is associated with BrCa risk ($P_{2df} < 0.01$) (Figure 4a). Based on this observation, we genotyped two SNPs in a series of 9,573 BRCA1/2 mutation carriers collected through 18 centers participating in CIMBA: rs7164529 and rs10519219, with $D' = 1$ and $r^2 = 0.08$. After quality control and Hardy-Weinberg equilibrium checks, Cox regression analysis revealed no significant associations between the SNPs and BrCa risk for BRCA1 or BRCA2 mutation carriers (rs7164529, $P_{trend} = 0.45$ and 0.05, $P_{2df} = 0.51$ and 0.14, respectively; rs10519219, $P_{trend} = 0.92$ and 0.72, $P_{2df} = 0.76$ and 0.07, respectively; Table 1). There was some suggestion of association with increased BrCa risk for BRCA2 mutation carriers under the recessive model for rs10519219 ($P = 0.033$) (Figure 4b and Additional file 13). Under the multiplicative model, there was no evidence of heterogeneity in the HRs of rs7164529 between studies ($P = 0.66$ and 0.21 for BRCA1 and BRCA2 mutation carriers, respectively) but some suggestion for rs10519219 among BRCA2 mutation carriers ($P = 0.041$). If an effect exists, the HR estimates for BRCA2 mutation carriers due to minor genotypes of rs7164529 or rs10519219 are in the opposite direction to those obtained in the general population (Table 1). Studying cancer susceptibility in mouse models has revealed opposite allele effects across different genetic backgrounds [68]. In this context, having a potential serial model of function between BRCA2 and MRG15, the
effect of MORF4L1 alleles on BrCa risk might differ depending on the genetic/functional status of BRCA2/BRCA2: that is, wild-type in the general population versus altered or absent in BRCA2 mutation carriers. On the other hand, common predisposition alleles differentially associate with BrCa risk among BRCA1 and BRCA2 mutation carriers [16,37,69], which suggests differences in the influence of a given biological process on carcinogenesis between the two types of carriers.

We performed a number of sensitivity analyses to investigate the robustness of our results. Inclusion of prophylactic oophorectomy as a time-dependent covariate did not influence risk estimations ($P_{\text{regression coefficients}} > 0.10$). Some suggestion of association was revealed when prevalent cases, defined as those diagnosed >5 years before recruitment, were excluded from the analyses: rs7164529 per-allele model, BRCA2 $n = 2,803$, $HR = 1.09$, 95% confidence interval = 1.00 to 1.18, $P = 0.048$; and rs10519219 recessive model, BRCA2 $n = 2,633$, $HR = 1.78$, 95% confidence interval = 1.12 to 2.87, $P = 0.027$. Finally, data were also analyzed using a weighted cohort approach [39] to allow for the retrospective study design and, in particular, the nonrandom sampling of affected and unaffected mutation carriers. This yielded similar results to those shown in Table 1 for the per-allele and two-degrees-of-freedom models (rs7164529, BRCA1 weighted $HR = 1.04$ to 1.08, $BRCA2 \uparrow HR = 1.03$ to 1.12; and rs10519219, BRCA1 $\uparrow HR = 0.98$ to 1.08, BRCA2 $\downarrow HR = 0.95$ to 1.59), but the rs10519219 association under the recessive model

Figure 3 Phenotypic study of Caenorhabditis elegans brc-2 and mrg-1 mutants (a) Representative images of meiotic cells at the distal part, near the gonad bend. RAD-51 foci are bright and nuclear in wild-type (WT) animals whereas RAD-51 foci appear less intense and weakly diffuse in the cytoplasm, reduced but often dispersed and intense in the nuclei, or absent in brc-2, mrg-1 and rad-51 mutants, respectively. There are more RPA-1 nuclear foci in each of the three mutants than in WT animals. 4,6-Diamidino-2-phenylindole (DAPI) panels are merged with the red channel (for WT and brc-2 mutant) and with the green channel (for rad-51 mutant). *Abnormal chromosomal compaction. (b) Quantitation of RAD-51 and RPA-1 foci per nuclei in several germ cell lines of WT animals and brc-2 and mrg-1 mutant animals. Number of cells scored (n) and standard deviation of the mean indicated. **Significant differences relative to WT (Mann-Whitney U test, $P < 0.001$). (c) SYTO-12 staining in synchronized adult worms. Left top panel: an animal heterozygous for the brc-2 mutation (according to green fluorescent protein expression at the pharynx) shows WT SYTO-12 staining (that is, one to two labeled cells at the gonad bend). Right top and left bottom panels: an increase in SYTO-12-positive cells in the germline of brc-2 and mrg-1 mutants, respectively. Right bottom panel: magnification of the highlighted area in the left panel.
Figure 4 Variation at the MORF4L1 locus and breast cancer risk. (a) SNPs with previous suggestive evidence of association with breast cancer (BrCa) risk in the general population ($p_{2df} < 0.01$) [67], genes and the linkage disequilibrium structure around MORF4L1 in HapMap Caucasians (data release 27). (b) Hazard ratio (HR) estimates of association of rs7164529 (top panels) and rs10519219 (bottom panels) with BrCa risk among BRCA1 (left panels) and BRCA2 (right panels) mutation carriers. Graphs show HRs and 95% confidence intervals of heterozygotes and minor allele homozygotes for all participating centers except for rs10519219 and relatively small groups (less than five individuals with the minor genotype). Size of the rectangle is proportional to the corresponding study precision.
Table 1 Association between variants at the MORF4L1 locus and breast cancer risk

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Association study between variants at the MORF4L1 locus and breast cancer risk among BRCA1 and BRCA2 mutation carriers, and in the general population (CGEMS results). n, number of individuals; HR, hazard ratio; CI, confidence interval; OR, odds ratio.

Discussion

Given the evidence across biological levels and species models, we hypothesized that perturbation of MRG15 function through genetic mutations or common alleles might be at the root of some cases of FA and/or BrCa. The results of our study, in addition to a recent publication on BrCa [65], indicate that in all probability the germline mutations in MORF4L1, if any, are not at the root of FA or BrCa. Next, analysis of common genetic variation at the MORF4L1 locus in BRCA1 and BRCA2 mutation carriers has not identified significant associations under the principal models. However, weak associations for risk among the latter group under the additive (rs7164529) and recessive (rs10519219) models might exist. Notably, in addition to the molecular and functional data presented, while MRG15 was demonstrated to co-purify with both BRCA1 and BRCA2, it only appeared to be necessary for the recruitment of BRCA2 (and PALB2/RAD51), but not of BRCA1, at sites of DNA damage [21]. Taken together, these observations suggest that the potential link between MORF4L1 and risk of BrCa warrants further assessment in larger sets of BRCA2 mutations and in additional case-control studies.

Conclusions

Studies in human, mouse and C. elegans models link MRG15 to the repair of DNA double-strand breaks, possibly through molecular and/or functional interactions with BRCA2, PALB2, RAD51 and RPA1. No pathogenic alterations of MRG15 or MORF4L1 have been observed in FA patients unclassified in terms of subtype or in familial BrCa cases negative for mutations in BRCA1 or BRCA2. Finally, no significant association with BrCa risk among BRCA1 and BRCA2 mutation carriers has been revealed for two common genetic variants at the MORF4L1 locus. Given a potentially weak and specific effect among BRCA2 mutation carriers, however, analyses in a larger series may be warranted.

Additional material

- Additional file 1: Y2H baits for 12 proteins in the FA/BrCa signaling pathway. Supplementary Table 1 containing details of the design of Y2H baits for 12 proteins in the FA/BrCa signaling pathway.
- Additional file 2: siRNAs used in the present study. Supplementary Table 2 containing details of the siRNAs used in the present study.
- Additional file 3: Primers for sequencing of MORF4L1. Supplementary Table 3 containing details of primers used for sequencing of MORF4L1.
- Additional file 4: FA/BrCa signaling pathway components. Supplementary Table 4 containing details of known and potential FA/BrCa signaling pathway components identified through Y2H screens.
- Additional file 5: Gene co-expression. Supplementary Figure 1 containing results of the gene co-expression analysis.
- Additional file 6: Four bait designs and Y2H results. Supplementary Figure 2 containing details of four bait designs and the Y2H results.
- Additional file 7: Co-AP and co-IP assays. Supplementary Figure 3 containing results of the co-AP and co-IP assays.
- Additional file 8: Co-AP assays involving MRG15 and MRGX. Supplementary Figure 4 containing results of co-AP assays involving MRG15 and MRGX.
- Additional file 9: siRNA-mediated depletion of MRG15 and FANCD2 monoubiquitinylation. Supplementary Figure 5 containing results of siRNA-mediated depletion of MRG15 and FANCD2 monoubiquitinylation.
- Additional file 10: TRF2 and TSNAX co-localization. Supplementary Figure 6 containing results of TRF2 and TSNAX co-localization.
Additional file 11: Immunodetection of RAD-S1 and RPA-1
Supplementary Figure 7 containing results for immunodetection of RAD-S1 and RPA-1 in wild-type animals and in brc-2 and mir-1 C. elegans mutant animals.

Additional file 12: MRG15 in extracts of unclassified FA cell lines
Supplementary Figure 8 containing results for the analysis of MRG15 in extracts of unclassified FA cell lines.

Additional file 13: BrCa risk estimates for rs7164529 and rs10519219
Supplementary Table 5 containing BrCa risk estimates (HR and wHR) for rs7164529 (additive model) and rs10519219 (recessive model) among BRCA2 mutation carriers across participating centers.

Additional file 14: Funding support
Supplementary document containing details of funding support.

Abbreviations

Acknowledgements
The authors thank Dr Andre Nussenzweig, Dr Laura Tusell and Dr Anton Gartner for providing Atm-deficient MEFs, anti-TRF2 and anti-RAD-51/RPA-1 antibodies, respectively. They also wish to thank all study participants, clinicians and centers for their valuable contribution, and the CGEMS initiative for making their genome-wide association study results available. The CIMBA data management is supported by Cancer Research - UK. Funding support is further detailed in Additional file 14.

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