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Germline mutations in *RAD51D* confer susceptibility to ovarian cancer

Chey Loveday[1,30], Clare Turnbull[1,30], Emma Ramsay[1], Deborah Hughes[1], Elise Ruark[1], Jessica R Frankum[2], Georgina Bowden[1], Bolot Kalmyrzaev[1], Margaret Warren-Perry[1], Katie Snape[1], Julian W Adlard[3], Julian Barwell[4], Jonathan Berg[5], Angela F Brady[6], Carole Brewer[7], Glen Brice[8], Cyril Chapman[9], Jackie Cook[10], Rosemarie Davidson[11], Alan Donaldson[12], Fiona Douglas[13], Lynn Greenhalgh[14], Alex Henderson[15], Louise Izatt[16], Ajith Kumar[17], Fiona Lalloo[18], Zosia Miedzybrodzka[19], Patrick J Morrison[20], Joan Paterson[21], Mary Porteous[22], Mark T Rogers[23], Susan Shanley[24], Lisa Walker[25], Breast Cancer Susceptibility Collaboration (UK)[26], Diana Eccles[27], D Gareth Evans[28], Anthony Renwick[1], Sheila Seal[1], Christopher J Lord[2], Alan Ashworth[2], Jorge S Reis-Filho[2], Antonis C Antoniou[29] & Nazneen Rahman[1]

Recently, *RAD51C* mutations were identified in families with breast and ovarian cancer[1]. This observation prompted us to investigate the role of *RAD51D* in cancer susceptibility. We identified eight inactivating *RAD51D* mutations in unrelated individuals from 911 breast-ovarian cancer families compared with one inactivating mutation identified in 1,060 controls ($P = 0.01$). The association found here was principally with ovarian cancer, with three mutations identified in the 59 pedigrees with three or more individuals with ovarian cancer ($P = 0.0005$). The relative risk of ovarian cancer for *RAD51D* mutation carriers was estimated to be 6.30 (95% CI 2.86–13.85, $P = 4.8 \times 10^{-6}$). By contrast, we estimated the relative risk of breast cancer to be 1.32 (95% CI 0.59–2.96, $P = 0.50$). These data indicate that *RAD51D* mutation testing may have clinical utility in individuals with ovarian cancer and their families. Moreover, we show that cells deficient in RAD51D are sensitive to treatment with a PARP inhibitor, suggesting a possible therapeutic approach for cancers arising in *RAD51D* mutation carriers.

Homologous recombination is a mechanism for repairing stalled replication forks, DNA interstrand crosslinks and double-strand breaks[2]. Constitutional inactivating mutations in several genes that encode proteins crucial for DNA repair by homologous recombination have been shown to predispose to cancer[3]. In particular, these mutations have a strong association with female cancers, and mutations in genes such as *BRCA1*, *BRCA2*, *ATM*, *BRIP1*, *CHEK2*, *PALB2*, *RAD50* and *RAD51C* have been shown to confer susceptibility to breast and/or ovarian cancer[1,4]. Indeed, the analysis of families with breast and ovarian cancer was crucial to mapping *BRCA1* (ref. 5). For many years, it was widely believed that the genetic contribution to families with breast and ovarian cancer was largely attributable to mutations in *BRCA1* and *BRCA2* (refs. 6–8). Last year, however, researchers[1] identified mutations in *RAD51C* in breast-ovarian cancer families. This suggested that analysis of such families may still have utility in the discovery of cancer predisposition genes.

In eukaryotic cells, DNA repair by homologous recombination involves several proteins, of which a central player is the DNA

[1]Section of Cancer Genetics, The Institute of Cancer Research, Sutton, UK. [2]The Breakthrough Breast Cancer Research Centre, The Institute of Cancer Research, London, UK. [3]Yorkshire Regional Centre for Cancer Treatment, Cookridge Hospital, Leeds, UK. [4]Leicestershire Genetics Centre, University Hospitals of Leicester National Health Service (NHS) Trust, Leicester, UK. [5]Human Genetics, Division of Medical Sciences, University of Dundee, Dundee, UK. [6]North West Thames Regional Genetics Service, Kennedy Galton Centre, London, UK. [7]Peninsula Regional Genetics Service, Royal Devon & Exeter Hospital, Exeter, UK. [8]South West Thames Regional Genetics Service, St. George's Hospital, London, UK. [9]West Midlands Regional Genetics Service, Birmingham Women's Hospital, Birmingham, UK. [10]Sheffield Regional Genetics Service, Sheffield Children's NHS Foundation Trust, Sheffield, UK. [11]West of Scotland Regional Genetics Service, FergusonSmith Centre for Clinical Genetics, Glasgow, UK. [12]South Western Regional Genetics Service, University Hospitals of Bristol NHS Foundation Trust, Bristol, UK. [13]Northern Genetics Service, Newcastle upon Tyne Hospitals NHS Foundation Trust, Newcastle upon Tyne, UK. [14]Cheshire and Merseyside Clinical Genetics Service, Alder Hey Children's NHS Foundation Trust, Liverpool, UK. [15]Northern Genetics Service (Cumbria), Newcastle upon Tyne Hospitals NHS Trust, Newcastle upon Tyne, UK. [16]South East Thames Regional Genetics Service, Guy's and St. Thomas NHS Foundation Trust, London, UK. [17]North East Thames Regional Genetics Service, Great Ormond St. Hospital, London, UK. [18]University Department of Medical Genetics & Regional Genetics Service, St. Mary's Hospital, Manchester, UK. [19]University of Aberdeen and North of Scotland Clinical Genetics Service, Aberdeen Royal Infirmary, Aberdeen, UK. [20]Northern Ireland Regional Genetics Service, Belfast Health and Social Care (HSC) Trust & Department of Medical Genetics, Queen's University Belfast, Belfast, UK. [21]East Anglian Regional Genetics Service, Cambridge University Hospitals NHS Foundation Trust, Cambridge, UK. [22]South East of Scotland Clinical Genetics Service, Western General Hospital, Edinburgh, UK. [23]All Wales Medical Genetics Service, University Hospital of Wales, Cardiff, UK. [24]Royal Marsden NHS Foundation Trust, London, UK. [25]Oxford Regional Genetics Service, Oxford Radcliffe Hospitals NHS Trust, Oxford, UK. [26]A full list of members appears in the **Supplementary Note**. [27]Faculty of Medicine, University of Southampton, Southampton University Hospitals NHS Trust, Southampton, UK. [28]University Department of Medical Genetics & Regional Genetics Service, St. Mary's Hospital, Manchester, UK. [29]Center for Cancer Genetic Epidemiology, Department of Public Health and Primary Care, University of Cambridge, Cambridge, UK. [30]These authors contributed equally to this work. Correspondence should be addressed to N.R. (nazneen.rahman@icr.ac.uk).

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Table 1 Cancer history and pathology in *RAD51D* mutation carriers

Family ID	Mutation; protein alteration[a]	Person ID	Cancer history (age)[b]	Pathology	Tumor analysis
Fam1	c.363delA	1	Breast cancer, left (34)	Invasive ductal carcinoma of no special type, grade 3	NA
			Breast cancer, right (52)	Invasive ductal carcinoma of no special type, grade 3	Loss of wild-type allele
Fam2	c.803G>A; p.Trp268X	1	Ovarian cancer (58)	Bilateral serous adenocarcinoma	Loss of wild-type allele
Fam3	c.556C>T; p.Arg186X	1	Ovarian cancer (38)	NA	NA
		2	Breast cancer (39)	High grade ductal comedo carcinoma *in situ*	NA
		3	Breast cancer (58)	Invasive carcinoma with medullary features	NA
		4	Breast cancer (53)	Invasive ductal carcinoma of no special type	NA
Fam4	c.480+1G>A	1	Breast cancer (51)	Invasive ductal carcinoma of no special type, grade 3	NA
Fam5	c.345G>C; p.Gln115His[c]	1	Ovarian cancer (45)	Bilateral serous adenocarcinoma	NA
		2	Ovarian cancer (74)	NA	NA
Fam6	c.556C>T; p.Arg186X	1	Breast cancer (35)	Invasive ductal carcinoma of no special type, grade 3	NA
Fam7	c.757C>T; p.Arg253X	1	Ovarian cancer (51)	Differentiated endometrioid adenocarcinoma	NA
		2	Breast cancer (47)	NA	NA
Fam8	c.270_271dupTA	1	Ovarian cancer (58)	Differentiated adenocarcinoma	Loss of mutant allele
			Breast cancer (65)	Invasive ductal carcinoma of no special type, grade 3	Reduction of wild-type allele
Control	c.748delC		NA	NA	NA

NA, not available.
[a]Mutation nomenclature corresponds to Ensembl Transcript ID ENST00000345365. [b]Age at which cancer occurred in years. [c]This mutation is at the final base of exon 4, disrupts the splice site and results in skipping of exons 3 and 4. Person IDs correspond to those shown in **Figure 1**.

recombinase RAD51, the ortholog of bacterial recA[9]. RAD51 forms helical filaments on DNA and catalyzes DNA strand invasion and exchange. Many other proteins are involved in these processes, including five RAD51 paralogs (RAD51B, RAD51C, RAD51D, XRCC2 and XRCC3)[10]. Here, through a case-control mutation study, we show that mutations in *RAD51D* (also known as *RAD51L3*) predispose to cancer in humans.

We sequenced the full coding sequence and intron-exon boundaries of *RAD51D* in DNA from unrelated probands from 911 breast-ovarian cancer families and 1,060 population controls (**Supplementary Table 1**). The breast-ovarian cancer families included at least one case of breast cancer and at least one case of ovarian cancer and all families were negative for mutations in *BRCA1* and *BRCA2* (**Supplementary Table 2**).

We identified inactivating mutations in *RAD51D* in 8 of the 911 cases and in 1 of the 1,060 controls ($P = 0.01$) (**Table 1**). The mutations were not equally distributed within the series, with a higher prevalence of mutations present in families with more than one case of ovarian cancer: we detected four mutations in 235 families with two or more cases of ovarian cancer ($P = 0.005$) and three mutations in the 59 families with three or more cases of ovarian cancer ($P = 0.0005$) (**Fig. 1**).

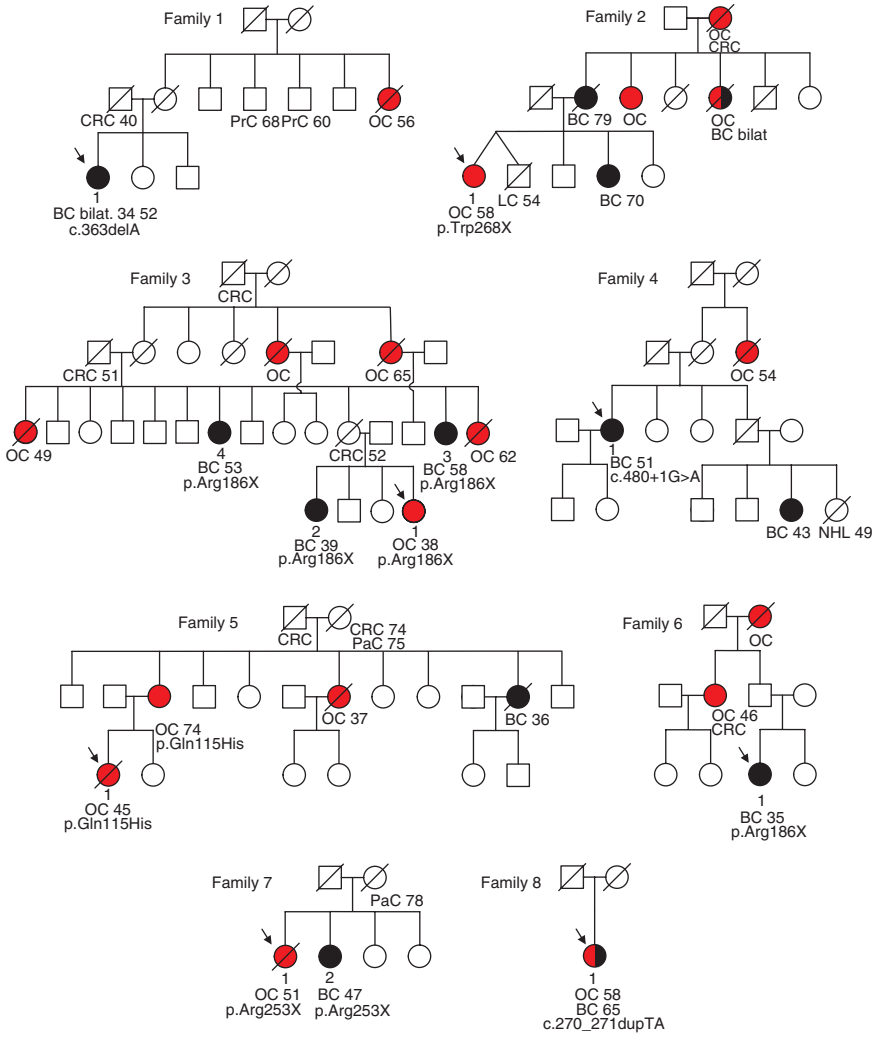

Figure 1 Abridged pedigrees of eight families with *RAD51D* mutations. Individuals with ovarian cancer are shown as red circles; individuals with breast cancer are shown as black circles. Other cancers are shown as unfilled circles or squares. Where known, the age of cancer diagnosis is listed under the individual, with two ages given for metachronous bilateral breast cancers. The relevant *RAD51D* mutation is listed under the affected individuals analyzed, but not the unaffected individuals, to preserve confidentiality. BC, breast cancer; BC bilat., bilateral breast cancer; OC, ovarian cancer; CRC, colorectal cancer; LC, lung cancer; NHL, non-Hodgkin lymphoma; PaC, pancreatic cancer; PrC, prostate cancer.

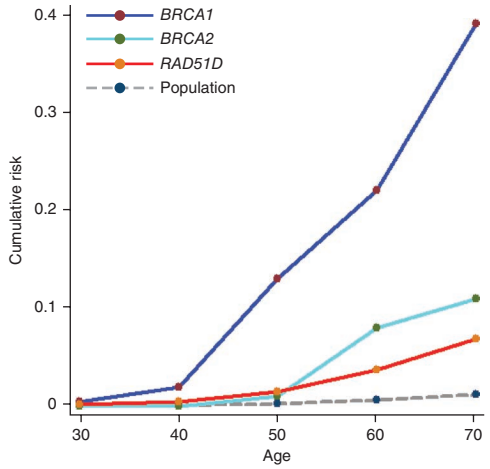

Figure 2 Average age-related cumulative risk of ovarian cancer in *RAD51D* mutation carriers, *BRCA1* and *BRCA2* mutation carriers[22] and the general population[23].

All the mutations are predicted to result in protein truncation through frameshifting insertions or deletions (n = 3), the generation of nonsense codons (n = 4) or splice defects (n = 2) (**Table 1**). We also identified 5 intronic, 3 synonymous and 15 nonsynonymous variants. Three coding variants, rs9901455 (p.Ser78Ser), rs4796033 (p.Arg165Gln) and rs28363284 (p.Glu233Gly), have minor allele frequencies >1%; none of these variants was associated with risk of breast or ovarian cancer in our dataset (**Supplementary Table 3**). Of the remaining rare variants, three were present in both cases and controls, nine were detected in a single case and eight were detected in a single control (**Supplementary Table 4**). Thus, there was no overall difference in the frequency of nontruncating *RAD51D* variants between cases and controls. Moreover, there was no difference in the position or predicted functional effects of these variants, and it is noteworthy that an equal number (n = 5) of nonsynonymous variants detected in cases and controls are predicted to affect function (**Supplementary Fig. 1** and **Supplementary Table 4**). These data indicate that mutations that result in inactivation of RAD51D function predispose to cancer but that other variants are likely to be predominantly nonpathogenic.

We tested for the family mutations in samples from 13 relatives. This showed that five of five individuals affected with ovarian or breast cancer carried the family mutation, whereas six of eight unaffected relatives did not carry the family mutation. Several other cancers were present in relatives, such as pancreatic, prostate and colorectal cancer (**Fig. 1**). However, the mutation status of these individuals is not known, and additional studies will be required to evaluate whether *RAD51D* mutations predispose to other cancers. Pathology information was available for four ovarian cancers from *RAD51D* mutation carriers; three of the cancers were serous adenocarcinoma and one was an endometrioid cancer. Pathology information was available for eight breast cancers, of which seven were ductal in origin and one was a carcinoma with medullary features. Receptor status was available from five breast cancers, of which three were estrogen-receptor positive and two were estrogen-receptor negative. Tumor material was available from two ovarian cancers and two breast cancers. We detected loss of the wild-type allele in one ovarian and one breast cancer and reduction of the proportion of the wild-type allele in a further breast cancer. In the last ovarian cancer, the mutant allele was lost and the wild-type allele was retained (**Table 1** and **Supplementary Fig. 2**).

These characteristics are typical of the intermediate-penetrance cancer predisposition genes that we and others have described in breast cancer[1,4,11–14]. To estimate directly the risks associated with *RAD51D* mutations, we performed modified segregation analysis by modeling the risks of ovarian and breast cancer simultaneously and incorporating the information from the controls and full pedigrees of both mutation-positive and mutation-negative breast-ovarian cancer families. The ovarian cancer relative risk for *RAD51D* mutation carriers was estimated to be 6.30 (95% CI 2.86–13.85, $P = 4.8 \times 10^{-6}$) (**Fig. 2**). By contrast, the association with breast cancer risk was not statistically significant (relative risk = 1.32, 95% CI 0.59–2.96, P = 0.50).

To further explore the role of *RAD51D* mutations in breast cancer risk, we sequenced the gene in an additional series of 737 unrelated individuals from pedigrees in which there was familial breast cancer but no ovarian cancer. We did not identify any inactivating mutations (0 out of 737 cases compared to 1 out of 1,060 controls had inactivating mutations; P = 1.0). Although at first glance these data may seem surprising, they are consistent with the results of the segregation analysis. In particular, if *RAD51D* mutations confer a sizeable relative risk of ovarian cancer but only a small or no increase in breast cancer risk, the frequency of *RAD51D* mutations in a series of breast cancer families selected on the basis of not containing ovarian cancer would be anticipated to be very low. The data are also consistent with the detection of *RAD51D* mutations in seven individuals with breast cancer in the breast-ovarian cancer families, as we specifically ascertained the ovarian cancer cases because of their close family history of breast cancer. This selection will inevitably result in an enrichment of breast cancer in relatives of *RAD51D*-mutation–positive ovarian cancer cases irrespective of whether or not such mutations confer a risk of breast cancer. To formally refine the risk of breast cancer associated with *RAD51D* mutations will likely be very challenging because the population frequency of *RAD51D* mutations is so low. Assuming a population mutation frequency of 0.1% and a relative risk of breast cancer of 1.3, full mutational analysis of *RAD51D* in 275,000 cases and 275,000 controls would be required to have 90% power to show the association.

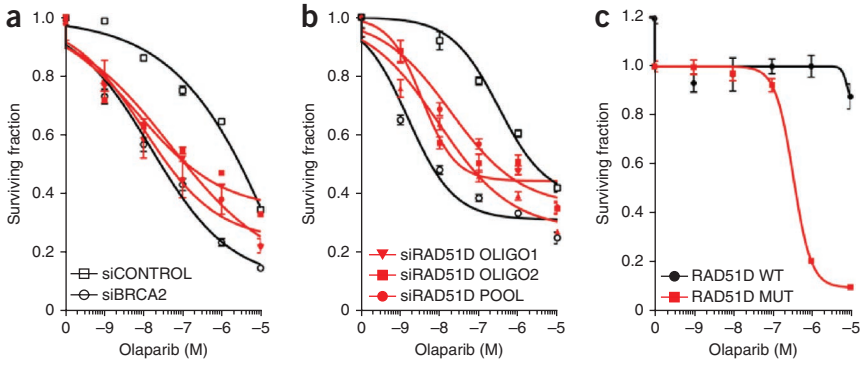

Figure 3 Effect of *RAD51D* silencing on olaparib sensitivity. (**a**,**b**) We transfected CAL51 (**a**) or MCF7 (**b**) cells with siCONTROL, siRNA directed against *RAD51D* or siRNA directed against *BRCA2*. For siRNA targeting *RAD51D*, cells were transfected with one of two individual siRNAs or a pool of both siRNAs combined. We then treated transfected cells with olaparib for 7 days before assaying for cell viability. (**c**) We treated wild-type (WT) CHO cells or CHO cells mutated (MUT) in *RAD51D* with olaparib for 7 days before assaying for cell viability. M, molar concentration.

Our data clearly show that *RAD51D* is an ovarian cancer predisposition gene, but further studies in familial and sporadic ovarian cancer series would be of value to further clarify the risks of ovarian cancer. *RAD51D* mutation analysis in individuals with Fanconi anemia and Fanconi-like disorders would also be of interest, given that biallelic mutations in *BRCA2*, *PALB2*, *BRIP1* and *RAD51C* have been shown to cause these phenotypes[15–18].

Our discovery has potential clinical utility both for individuals with cancer and their relatives. For example, cancer patients with *RAD51D* mutations may benefit from specific therapies such as poly (ADP-ribose) polymerase (PARP) inhibitors, which have shown efficacy in patients with impaired homologous recombination caused by mutations in *BRCA1* or *BRCA2* (ref. 19). To investigate this, we used RNA interference (RNAi) and assessed the relationship between *RAD51D* loss of function and the sensitivity of tumor cells to a clinical PARP inhibitor, olaparib (AstraZeneca). Short interfering RNAi (siRNAi) reagents targeting *RAD51D* caused olaparib sensitivity of a magnitude similar to that achieved following silencing of *BRCA2* (**Fig. 3a**,**b**), an observation in keeping with the homologous recombination defect observed in *Rad51d*-null rodent cell lines[20]. To extend this analysis, we also observed the *RAD51D*-selective effect of olaparib in *RAD51D*-deficient Chinese hamster ovary (CHO) cells in which both alleles of *RAD51D* had been rendered dysfunctional by gene targeting (**Fig. 3c**)[20]. These data suggest that PARP inhibitors may have clinical utility in individuals with *RAD51D* mutations. We estimate that only ~0.6% of unselected individuals with ovarian cancer will harbor *RAD51D* mutations, but as we enter an era in which genetic testing will become routine, such individuals will be readily identifiable. Their identification will also be of potential value to female relatives, as those relatives with mutations will be, on average, at an approximately sixfold increased risk of ovarian cancer, which equates to an ~10% cumulative risk by age 80. An appreciable proportion of women at this level of risk may consider strategies such as laparoscopic oophorectomy, which is well tolerated and is undertaken in many women with *BRCA* mutations[21].

URLs. Centre for Longitudinal Studies, National Child Development Study, http://www.cls.ioe.ac.uk/studies.asp?section=000100020003; Mutation Surveyor software, http://www.softgenetics.com/.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturegenetics/.

Accession codes. *RAD51D* mutation nomenclature corresponds to Ensembl Transcript ID ENST00000345365.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

N.R., C.L. and C.T. designed the experiment. M.W.-P., C.T. and N.R. coordinated recruitment to the FBCS. J.W.A., J. Barwell, J. Berg, A.F.B., C.B., G. Brice, C.C., J.C., R.D., A.D., F.D., D.G.E., D.E., L.G., A.H., L.I., A.K., F.L., Z.M., P.J.M., J.P., M.P., M.T.R., S. Shanley and L.W. coordinated the FBCS sample recruitment from their respective Genetics centers. C.L., E. Ramsay, D.H., G. Bowden, B.K., K.S., A.R. and S. Seal performed sequencing of *RAD51D*. J.R.F., C.J.L. and A.A. designed and conducted drug sensitivity experiments. J.S.R.-F. undertook examination and dissection of pathological specimens. C.T., E. Ruark and A.C.A. performed statistical analyses. C.L., C.T. and N.R. drafted the manuscript with substantial input from D.G.E., D.E., A.C.A., A.A. and J.S.R.-F. C.T. and N.R. oversaw and managed all aspects of the study.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Cases. We used lymphocyte DNA from 1,648 families with breast-ovarian cancer or breast cancer only. These families were ascertained from 24 genetics centers in the UK through the Genetics of Familial Breast Cancer Study (FBCS), which recruits women ≥18 years of age who have had breast and/or ovarian cancer and have a family history of breast and/or ovarian cancer. At least 97% of the families studied are of European ancestry. Index cases from each family were screened and were negative for germline mutations, including large rearrangements, in *BRCA1* and *BRCA2*. Informed consent was obtained from all participants and the research was approved by the London Multicentre Research Ethics Committee (MREC/01/2/18).

Breast-ovarian cancer pedigrees. We included 911 unrelated index cases from breast-ovarian cancer pedigrees. The index cases were diagnosed with breast and/or ovarian cancer. Each family contained an individual with both breast and ovarian cancer or contained at least one case of breast cancer and at least one case of ovarian cancer with ≤1 intervening unaffected female relative. Cases of ovarian cancer below the age of 20 were excluded from the analysis, as an appreciable proportion of these cases are likely to represent non-epithelial ovarian tumors, for example germ cell cancers. Of the 911 probands, 271 had ovarian cancer (with or without breast cancer) and 617 had breast cancer only. The number of family members (including the probands) diagnosed with breast cancer and/or ovarian cancer in the 911 breast-ovarian cancer pedigrees included in the analysis is shown in **Supplementary Table 2.**

Breast-cancer–only pedigrees. We included 737 unrelated index cases from breast cancer–only pedigrees. The index case from each family was diagnosed with breast cancer and had bilateral disease and/or a family history of breast cancer. There was no known case of ovarian cancer in any of these pedigrees. The number of family members (including the probands) diagnosed with breast cancer in the 737 breast cancer–only pedigrees included in the analysis is shown in **Supplementary Table 2**. The six cases of isolated breast cancer all had bilateral disease.

Samples and pathology information from mutation-positive families. For families in which a mutation in *RAD51D* was detected, we sought DNA samples from relatives, and we genotyped all obtainable samples for the family mutation. We also requested tumor material, pathology information and receptor status in probands and affected relatives from the hospitals where they had been treated.

Controls. We used lymphocyte DNA from 1,060 population-based controls obtained from the 1958 Birth Cohort Collection, an ongoing follow-up of persons born in Great Britain during one week in 1958. Biomedical assessment was undertaken during 2002–2004, at which blood samples and informed consent were obtained for the creation of a genetic resource; phenotype data for these individuals were not available (see URLs). At least 97% of the controls were of European ancestry.

Mutation analysis of *RAD51D*. We analyzed genomic DNA extracted from lymphocytes for mutations by direct sequencing of the full coding sequence and the intron-exon boundaries of *RAD51D*. Primer sequences and PCR conditions are given in **Supplementary Table 1**. The PCR reactions were performed in multiplex using the QIAGEN Multiplex PCR Kit (QIAGEN) according to the manufacturer's instructions. Amplicons were unidirectionally sequenced using the BigDye Terminator Cycle sequencing kit and an ABI3730 automated sequencer (ABI PerkinElmer). Sequencing traces were analyzed using Mutation Surveyor software (see URLs) and by visual inspection. All mutations were confirmed by bidirectional sequencing from a fresh aliquot of the stock DNA. Samples from members of *RAD51D*-mutation–positive families were tested for the family mutation by direct sequencing of the appropriate exon.

***In silico* analyses of identified variants.** We computed the predicted effects of *RAD51D* missense variants on protein function using PolyPhen[24] and SIFT[25]. All variants (intronic and coding) were analyzed for their potential effect on splicing. Variants were analyzed using two splice prediction algorithms, NNsplice[26] and MaxEntScan[27], using the Alamut software interface (Interactive Biosoftware). If both the NNsplice and MaxEntScan scores were altered by >20% (that is, a wild-type splice-site score decreased and/or a cryptic splice-site score increased), three further prediction algorithms were used; NetGene2 (ref. 28), HumanSplicingFinder[29] and GENSCAN[30]. A consensus decrease in a wild-type splice-site score and/or a consensus increase in a cryptic splice-site score across all algorithms was considered indicative of disruption of normal splicing.

Tumor analysis. Representative tumor sections were stained with Nuclear Fast Red and microdissected using a sterile needle and a stereomicroscope (Olympus SZ61) to ensure that the proportion of tumor cells was >90%, as previously described[31]. DNA was extracted using the DNeasy kit (QIAGEN) according to the manufacturer's instructions. DNA concentration was measured using the PicoGreen assay (Invitrogen) according to the manufacturer's instructions. *RAD51D*-specific fragments encompassing the relevant mutations were PCR amplified using the primers listed in **Supplementary Table 1** and bidirectionally sequenced using the BigDye Terminator Cycle sequencing kit and an ABI3730 automated sequencer (ABI PerkinElmer). Sequence traces from tumor DNA were compared to sequence traces from lymphocyte DNA from the same individual.

Drug sensitivity. We used nonsilencing *BRCA2* and *RAD51D* siGENOME siRNAs (Dharmacon). CAL51 and MCF7 cells were grown in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Invitrogen) supplemented with 10% (v/v) FCS (Gibco, Invitrogen). Chinese hamster ovary (CHO) *RAD51D* wild-type (51D1.3 clone) and *RAD51D* dysfunctional (51D1 clone) cells were grown in αMEM (Gibco, Invitrogen) supplemented with 10% FCS (Gibco, Invitrogen). Cells were siRNA transfected using RNAiMAX (Invitrogen), plated in 96-well microtiter plates and then exposed to a titration of olaparib for 7 days. The media and drug were replenished every 3 days. After 7 days of continuous culture, cell viability was estimated using CellTiter-Glo reagent (Promega), and surviving fractions were calculated as previously described[32].

Statistical methods. Statistical analyses were performed using Stata v11 software (StataCorp). The frequency of mutations in cases and controls was compared using a two-sided Fisher's exact test. We estimated the *RAD51D* combined mutation frequency, the breast cancer risk ratio and the ovarian cancer risk ratio relative to non-*RAD51D* mutation carriers simultaneously using modified segregation analysis implemented in the pedigree analysis software MENDEL[33]. The analysis was based on breast and ovarian cancer occurrence in the combined dataset of families and controls. All individuals were censored at age 80 years, the age of their first cancer or their age of death or last observation, whichever occurred first. Females who had bilateral prophylactic mastectomy were censored for breast cancer, and those who had had bilateral prophylactic oophorectomy were censored for ovarian cancer. Thus, only information on the first cancer was included in the primary analysis. We assumed that the breast cancer incidence depends on the underlying genotype through a model of the form: $\lambda(t)=\lambda_0(t)\exp(\beta x)$, where $\lambda_0(t)$ is the baseline incidence at age t in nonmutation carriers, β is the log risk ratio associated with the mutation and x takes value 0 for nonmutation carriers and 1 for mutation carriers. A similar model was assumed for the ovarian cancer incidences. Breast and ovarian cancers were assumed to occur independently, conditional on the genotype[22]. The overall breast and ovarian cancer incidences were constrained to agree with the population incidences for England and Wales in the period of 1993–1997 (ref. 23), as described previously[34,35]. The models were parameterized in terms of the mutation frequencies and log-risk ratios for breast and ovarian cancer. Parameters were estimated using a maximum likelihood estimation. Because *RAD51D* mutation screening was carried out in all index cases and controls, we were able to incorporate information from all controls and the full pedigrees from all cases (including those without a *RAD51D* mutation) together with the segregation information from the families in which a *RAD51D* mutation was detected and genotyping was possible in relatives of the index case. To adjust for ascertainment, we modeled the conditional likelihood of all family phenotypes and mutation status of the index family member and other tested

family members given the disease phenotypes of all family members. For the controls, we modeled the likelihood of the mutation status given that they were unaffected. The variances of the parameters were obtained by inverting the observed information matrix. Log risk ratios were assumed to be normally distributed. Because this model does not explicitly incorporate the effects of other susceptibility genes, it assumes implicitly that the effects of *RAD51D* and other potential susceptibility genes can be regarded as independent, as in a multiplicative model.

Power calculations were based on two-sided association testing with a significance level of $\alpha = 0.05$. We assumed that the observed frequency of truncating mutations in cases from breast-ovarian cancer families (0.88%) and controls (0.094%) reflects the true underlying mutation frequencies in the population, and that the effect calculated from the segregation analysis (odds ratio = 6.30) represents the true risk of ovarian cancer in the population. We assumed that the same ratio of truncating mutations to missense variants (predicted deleterious) would be detected in isolated cases of ovarian cancer as in cases from breast-ovarian cancer families. We assumed that in association testing of mutation frequencies across 25,000 genes, the χ^2 statistics would be normally distributed, and we applied a Bonferroni correction for multiple testing.

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