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RESEARCH ARTICLE

Long-term survival of an ovarian cancer patient harboring a RAD51C missense mutation

Meghan R. Sullivan,[1,7] Rohit Prakash,[2,7] Yashpal Rawal,[3,7] Weibin Wang,[4] Patrick Sung,[3] Marc R. Radke,[5] Scott H. Kaufmann,[6] Elizabeth M. Swisher,[5] Kara A. Bernstein,[1] and Maria Jasin[2]

[1]Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, Pennsylvania 15261, USA; [2]Developmental Biology Program, Memorial Sloan Kettering Cancer Center, New York, New York 10021, USA; [3]Department of Biochemistry and Structural Biology, UT Health Science Center at San Antonio, San Antonio, Texas 78229, USA; [4]Department of Radiation Medicine, Peking University Health Science Center, Beijing, 100191, China; [5]Division of Gynecologic Oncology, Department of Obstetrics and Gynecology, University of Washington School of Medicine, Seattle, Washington 98195, USA; [6]Departments of Oncology and Molecular Pharmacology and Experimental Therapeutics, Mayo Clinic, Rochester, Minnesota 55905, USA

Abstract Mutations in homologous recombination (HR) genes predispose to cancer but also sensitize to chemotherapeutics. Although therapy can initially be effective, cancers frequently cease responding, leading to recurrence and poor prognosis. Here we identify a germline mutation in *RAD51C*, a critical HR factor and known tumor suppressor, in an ovarian cancer patient with exceptionally long, progression-free survival. The RAD51C–T132P mutation is in a highly conserved residue within the nucleotide-binding site and interferes with single-strand DNA binding of the RAD51 paralog complex RAD51B–RAD51C–RAD51D–XRCC2 and association with another RAD51 paralog XRCC3. These biochemical defects lead to highly defective HR and drug sensitivity in tumor cells, ascribing RAD51C–T132P as a deleterious mutation that was likely causal for tumor formation. Conversely, its position within a critical site suggests that it is refractory to secondary mutations that would restore *RAD51C* gene function and lead to therapy resistance. A need for a greater understanding of the relationship between mutation position and reversion potential of HR genes is underscored, as it may help predict the effectiveness of therapies in patients with HR-deficient cancers.

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Corresponding authors: prakashr@mskcc.org; karab@pitt.edu; m-jasin@ski.mskcc.org

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INTRODUCTION

Homologous recombination (HR) is required to maintain genomic integrity in response to DNA-damaging agents and replication stress (Chen et al. 2018; Zhao et al. 2019). Monoallelic germline mutations in the HR genes *BRCA1* and *BRCA2* predispose individuals to several cancers, especially breast and ovarian, with frequent loss of the wild-type allele observed in the cancers. This loss of heterozygosity (LOH) results in HR deficiency specifically within the tumor, and thus sensitizes it to platinum drugs, which are standard in the treatment of ovarian cancer together with surgery. Poly(ADP-ribose) polymerase (PARP) inhibitors,

[7]These authors contributed equally to this work.

which trap PARP1 on DNA, also target HR-deficient cancers, leading to their clinical approval for the treatment of these cancers (Curtin and Szabo 2020). Although not as frequently as in *BRCA1/2*, mutations in other HR genes are observed in cancer families, including the RAD51 paralog *RAD51C*, leading to its identification as a tumor suppressor (Meindl et al. 2010). A recent meta-analysis of approximately 23,000 ovarian cancers ranked *RAD51C* as a relatively high cancer risk gene (Suszynska et al. 2020). We identified an ovarian cancer patient with a germline *RAD51C* c.394A > C mutation causing a T132P missense mutation within the RAD51C nucleotide binding fold, specifically in the conserved Walker A motif (Fig. 1A). She presented with a Stage IIIC high-grade serous primary peritoneal carcinoma with loss of the wild-type *RAD51C* allele. This patient underwent surgery followed by cisplatin/paclitaxel therapy and showed no recurrence >10 yr after diagnosis. Given the apparently complete and exceptional patient response, we performed cellular and biochemical analyses of RAD51C–T132P. Our findings provide abundant evidence that RAD51C–T132P is highly HR-defective. We discuss the deleterious nature of this missense mutation and its relationship to

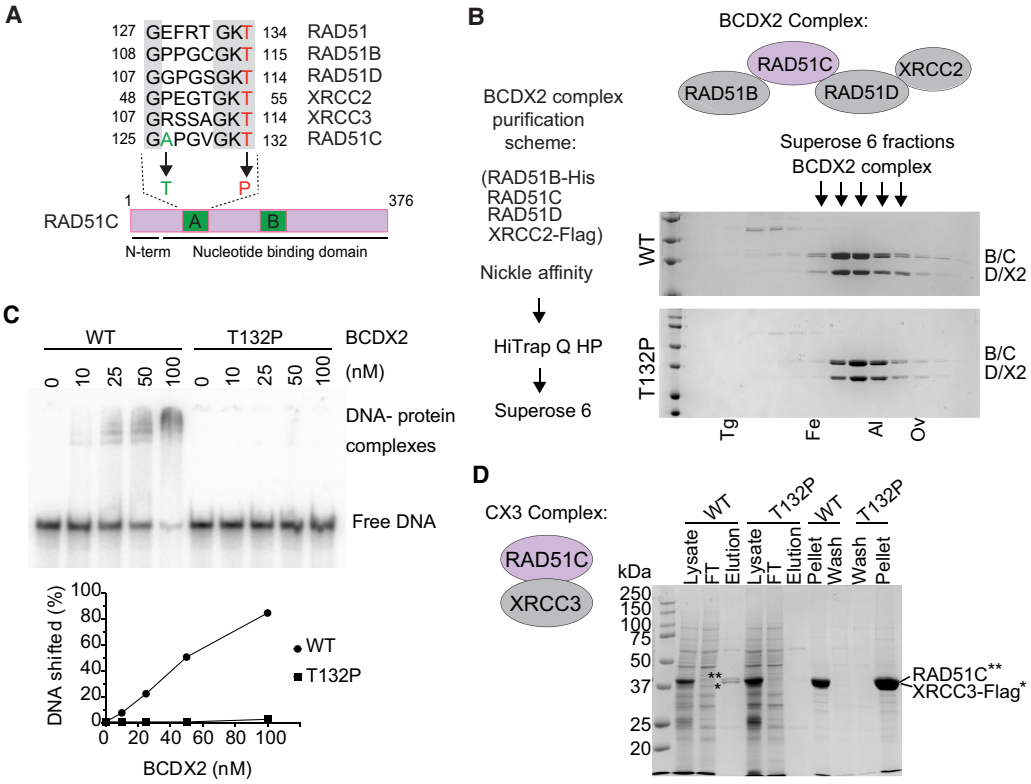

Figure 1. Defective RAD51 paralog complexes with RAD51C–T132P. (*A*) RAD51C contains a conserved Walker A motif found in RAD51 and other RAD51 paralogs. Highly conserved residues in the Walker A motif are shaded (GKT and the preceding G). The patient-derived RAD51C–T132P (red) mutation is in the terminal conserved residue of the motif, whereas the functional population variant RAD51C–A126T (green) is in a non-conserved residue. (*B*,*C*) RAD51C–T132P fractionates in the BCDX2 complex with RAD51B, RAD51D, and XRCC2 (*B*), but renders the complex defective in single-stranded DNA binding (*C*). Protein markers: Tg, thyroglobulin; Fe, ferritin; Al, aldolase; Ov, ovalbumin. (*D*) Unlike RAD51C–T132P, RAD51C–WT coelutes with XRCC3-FLAG in a single step FLAG affinity pulldown from insect cells. Although a substantial amount of both RAD51C proteins is found in the pellet as insoluble protein, a portion of the wild-type RAD51C and XRCC3-FLAG is soluble and able to form the CX3 complex. (FT) Flowthrough.

the long-term, progression-free survival of the patient and the need for a greater understanding of the reversion potential of HR gene mutations.

RESULTS

RAD51C–T132P Patient Information

The patient presented in January, 2009 at age 57 with Grade 3 Stage IIIC serous primary peritoneal carcinoma. She underwent extensive resection to no visible disease and received six cycles of chemotherapy that consisted of intravenous paclitaxel on Day 1, intraperitoneal cisplatin on Day 2, and intraperitoneal paclitaxel on Day 8, ending in June 2009. She received all chemotherapy except one dose of intraperitoneal paclitaxel (i.e., >90% of planned paclitaxel and 100% of planned cisplatin). There was no further therapy and no evidence of recurrence as of November 2019.

Through BROCA sequencing (Norquist et al. 2018), a *RAD51C* variant c.394A > C (NM_058216, Chr 17:56772540) was identified in germline DNA, leading to a T132P missense mutation in *RAD51C*. In the tumor, the variant allele frequency was 0.80, indicating loss of the wild-type *RAD51C* allele (Table 1). A somatic *TP53* mutation was also observed (c.1024C > T, p.R342X; allele frequency 0.69) (Table 1). No other mutations were identified in the panel of BROCA genes.

Defective RAD51 Paralog Complexes with RAD51C–T132P

RAD51C shares a high degree of homology with other RAD51 paralogs and the RAD51 recombinase itself within the Walker A nucleotide binding motif (Fig. 1A; Prakash et al. 2015). RAD51C–T132P falls at the highly conserved terminal residue within the motif, GXXXXGKT, predicting that it would impair RAD51C nucleotide binding and/or hydrolysis and hence HR function (French et al. 2003).

RAD51C is a member of two RAD51 paralog complexes (Zhao et al. 2019), the "BCDX2" complex (RAD51B, RAD51C, RAD51D, XRCC2) and the "CX3" complex (RAD51C, XRCC3). To examine the impact of this mutation on the BCDX2 complex, we expressed all four proteins in insect cells. Using a multistep purification, the BCDX2 complex with either RAD51C–WT or RAD51C–T132P eluted in similar fractions (Fig. 1B). However, although the BCDX2 complex containing RAD51C–T132P was largely intact, single-stranded DNA binding, which was assayed in the presence of ATP, was highly defective (Fig. 1C).

Table 1. BROCA sequencing results

Sample	Gene	Chromosome (hg19)	HGVS DNA reference	HGVS protein reference	Variant type	Predicted effect[a]	dbSNP/ dbVar ID	Genotype	Allele frequency	Target coverage
Germline	*RAD51C*	Chr 17:56772540	NM_002876: c.394A > C	p.T132P	Substitution	Pathogenic	None	Heterozygous	44%	235
	TP53	Chr 17:7574003	NM_000546: c.1024C > T	p.R342X	Substitution	Stop-gain	None	NA	0%	123
Tumor	*RAD51C*	Chr 17:56772540	NM_002876: c.394A > C	p.T132P	Substitution	Pathogenic	None	Homozygous	75%	20
	TP53	Chr 17:7574003	NM_000546: c.1024C > T	p.R342X	Substitution	Stop-gain	None	Homozygous	67%	15

[a]Predicted pathogenic effect for RAD51C–T132P is based on the predictive algorithms PolyPhen, SIFT, and GERP.
BROCA sequencing has been described (Norquist et al. 2018).

We also attempted to purify the CX3 complex by coexpressing RAD51C–WT or RAD51C–T132P with XRCC3-FLAG in insect cells. Cells were lysed, pelleted, and the supernatant incubated with FLAG-affinity beads. Although a portion of RAD51C–WT and XRCC3 were observed to be soluble and coeluted from the beads, indicating CX3 complex formation, little or no complex was observed with RAD51C–T132P (Fig. 1D). Thus, both RAD51 paralog complexes are defective with RAD51C–T132P in this biochemical analysis.

RAD51C–T132P Exhibits Severe HR Defects

To determine the effect of RAD51C–T132P on HR, complementation experiments were performed in *RAD51C* knockout U2OS cells (Garcin et al. 2019). These cells contain an integrated reporter in which HR is measured by GFP fluorescence (Fig. 2A). We expressed RAD51C–T132P in these cells, and in parallel as a control, RAD51C–A126T, which is mutated at a nearby nonconserved residue and is a known functional population variant (Meindl et al. 2010). Although both RAD51C–WT and RAD51C–A126T complemented the HR defect of the *RAD51C* knockout U2OS cells, RAD51C–T132P showed little or no HR activity (Fig. 2A).

HR-deficient cells, including *RAD51C* knockout cells, are defective in RAD51 focus formation upon exposure to DNA-damaging agents (Garcin et al. 2019). We analyzed RAD51 foci formation in *RAD51C* knockout U2OS cells stably expressing RAD51C–T132P and found that it did not restore foci formation above the level seen in uncomplemented cells (0.02 ± 0.01 and 0.04 ± 0.02, respectively) (Fig. 2B). In contrast, expression of either RAD51C–WT or RAD51C–A126T led to a substantial number of RAD51 foci (mean 14.4 ± 0.7 and 16.9 ± 0.8, respectively).

In general, HR-deficient cells are characterized by a high degree of sensitivity to cross-linking agents like cisplatin and to PARP inhibitors like olaparib (Garcin et al. 2019). RAD51C–T132P expressing cells were found to be as sensitive to both agents as the uncomplemented *RAD51C* knockout cell line (Fig. 2C,D). Thus, in these cellular assays that directly measure or reflect HR proficiency, RAD51C–T132P was nonfunctional and, therefore, can be considered to be a pathogenic allele.

Unlike U2OS cells, RAD51C function is essential for the survival of the nontransformed human mammary epithelial cell line MCF10A, presumably because of its role in HR (Garcin et al. 2019). Using a *RAD51C* conditional MCF10A cell line we previously developed (Supplemental Fig. 1A; Garcin et al. 2019), we determined whether RAD51C–T132P allows survival of these cells. Whereas transduction of RAD51C–WT into conditional cells led to colony formation upon Cre-mediated recombination, transduction of RAD51C–T132P abrogated colony survival; all surviving colonies had not undergone Cre-mediated recombination (Fig. 2E; Supplemental Fig. 1B). Thus, this mutant is not able to complement the lethality seen for the RAD51C-deficient MCF10A cells. Likely, tumor cells that have lost the wild-type *RAD51C* allele have compensating mutations to allow their survival and proliferation, such as in *TP53*, which is found mutated in the patient's ovarian cancer.

DISCUSSION

Deleterious *RAD51C* germline mutations are associated with predisposition to ovarian cancer, and so it seems likely that the HR-defective RAD51C–T132P mutation was causative for cancer formation in this patient. However, her long-term tumor-free survival for >10 yr seems remarkable given the usual progression of the disease. The durable patient response to the initial treatment in which the cytoreductive surgery led to no visible disease and the presence of a deleterious mutation, which is chemosensitizing, raises the question as to whether the exceptional outcome was due to the surgery or the nature of the mutation. Surgery with resection to no visible disease in ovarian cancer patients results in significantly better survival,

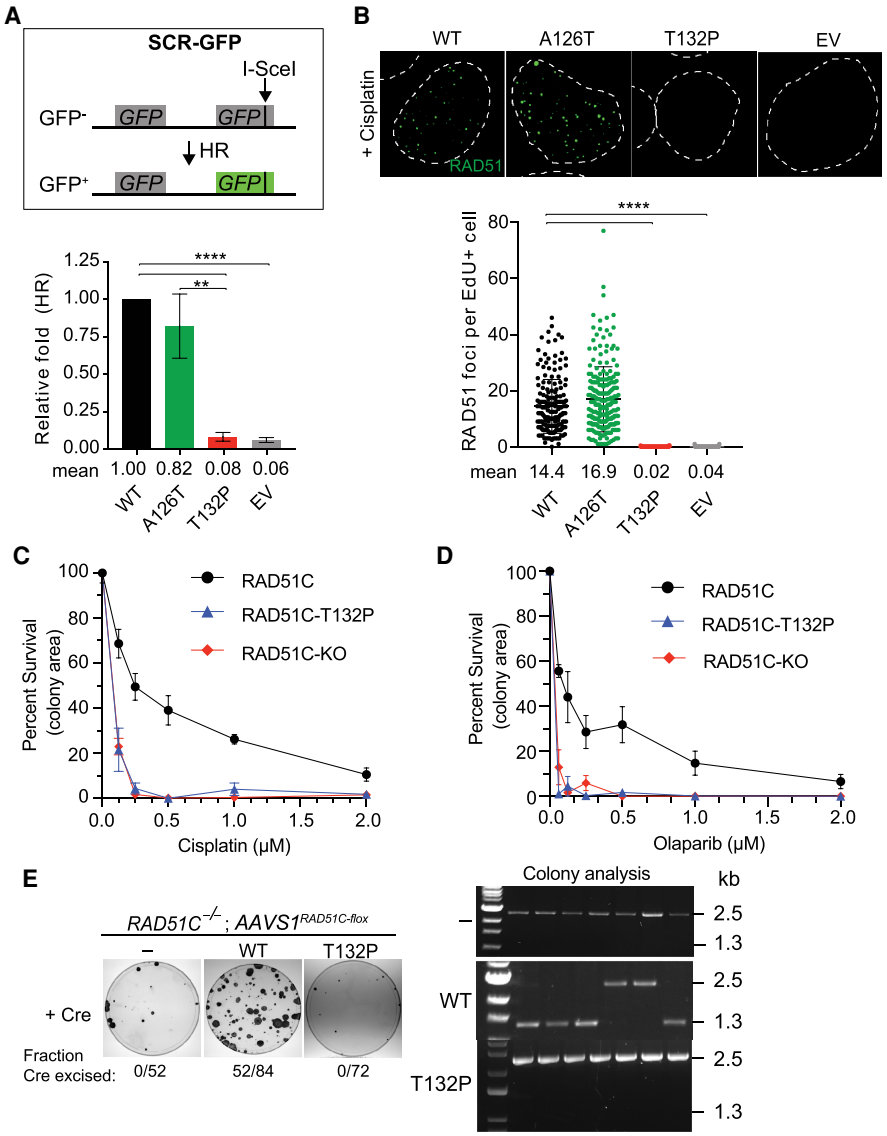

Figure 2. RAD51C–T132P exhibits severe HR defects. (*A*) RAD51C–T132P does not complement the severe HR defects of *RAD51C* knockout U2OS cells, unlike RAD51C–WT or the population variant RAD51C–A126T. HR is measured using the sister chromatid reporter (SCR) that contains two nonfunctional GFP genes and also contains a site for I-SceI endonuclease cleavage. After induction of a double-strand break by I-SceI in the downstream GFP gene, repair from upstream repeat will result in GFP+ cells that are quantified by flow cytometry. (*B*) RAD51 focus formation is defective in cisplatin-treated *RAD51C* knockout cells expressing RAD51C–T132P, but is proficient in cells expressing RAD51C–WT or the population variant RAD51C–A126T. (*C*,*D*) RAD51C–T132P-expressing cells are highly sensitive to cisplatin (*C*) and olaparib (*D*) for clonogenic survival. (*E*) RAD51C–T132P is not compatible with the survival of nontransformed MCF10A cells. Conditional MCF10A cells transduced with either RAD51C–T132P or an empty vector show reduced colony formation following transduction of Cre recombinase. Those colonies that survive have not undergone Cre-mediated excision of RAD51C, as seen by PCR analysis of genomic DNA.

yet most tumors still recur (Tewari et al. 2015). On the contrary, the position of the RAD51C–T132P mutation at a residue that is critical for RAD51C function may present few options for reversion.

Although initially responsive, a significant fraction of cancers develop therapy resistance, leading to progression. In a long-term follow-up study of a large number of *BRCA1/2* ovarian cancer patients, the 5-yr disease-free interval treated with platinum drugs is only ~15% (Jorge et al. 2019). The major mode of verified therapy resistance is reversion of the mutated gene (Chen et al. 2018), with approximately 300 reversion alleles reported thus far from 91 patients (Pettitt et al. 2020). Although reversion to wild type can occur, most often a secondary mutation restores the reading frame from a primary truncation mutation. Secondary mutations typically leave a "scar" around the mutation site (i.e., a deletion and/or de novo insertion, usually <20 amino acids), although larger scars can occur while apparently still restoring function.

BRCA1 and BRCA2 are large proteins, 1863 and 3418 residues, respectively, and several segments are nonessential for HR or even redundant (Chen et al. 2018; Zhao et al. 2019). Thus, these genes may be especially tolerant to secondary mutations that leave scars within the reading frame yet restore protein function. Perhaps not surprisingly then, most *BRCA1/2* pathogenic mutations cause protein truncations, with missense mutations comprising only ~5% of mutations (Pettitt et al. 2020). Notably, a recent meta-analysis of therapy resistance suggested that pathogenic missense mutations in *BRCA1/2* are refractory to reversion and thereby less likely associated with therapy resistance (Pettitt et al. 2020). Reversions of only two missense mutations were identified (Pettitt et al. 2020), which in both cases restored the wild-type sequence (Norquist et al. 2011).

Compared with BRCA1/2, RAD51C is a comparatively compact protein (376 residues), comprised mostly of a conserved nucleotide binding fold and a small amino-terminal domain. Both domains have been shown to be important for function, including for interaction with other RAD51 paralogs (Somyajit et al. 2012; Prakash et al. 2015). Thus, RAD51C may lack or have few nonessential regions and thus be less able to cope with structural perturbations than BRCA1/2.

Despite its discovery as a tumor suppressor more than a decade ago, reports of long-term patient follow-up are scarce. In this regard, it is notable that the one report of RAD51C reversion, in this case a truncation (R193X), revealed frame-restoring secondary mutations that led to either one or two amino acid substitutions at the mutation site (Kondrashova et al. 2017), which is considerably smaller than most scars found at reversion mutations for BRCA1/2 (Pettitt et al. 2020). Similarly, a RAD51D truncation in a therapy-resistant ovarian cancer has been reported to revert through frame restoration with only a three amino acid scar (one residue insertion/two residue deletion) (Kondrashova et al. 2017). However, even with BRCA1/2 truncations, scarring is less pronounced in more conserved domains (Pettitt et al. 2020; Tobalina et al. 2021), indicating more constraint in protein sequence changes.

Since its discovery as a tumor suppressor in 2010 (Meindl et al. 2010), approximately 300 *RAD51C* missense and truncation mutations, both germline and somatic, have been reported throughout the length of the protein in cancer patients (Suszynska et al. 2020) (cbioportal.org). Several have been shown to disrupt HR function in cellular assays (Meindl et al. 2010), although to our knowledge, the analysis of RAD51C–T132P is the first to show defective DNA binding. However, except for the case of R193X, reports of patient outcomes are typically lacking. A systematic analysis of mutations in *RAD51C* (and other RAD51 paralogs) to determine which residues are essential for function together with long-term patient follow-up is warranted to determine whether mutations at critical residues are associated with better patient outcome, and whether this is due to reduced capability of these mutations to successfully revert to cause therapy resistance.

METHODS

BROCA Sequencing

Sequencing coverage details can be found in Supplemental Table 1.

Protein Purification and DNA Binding

Hi5 insect cells were infected for 48 h with baculoviruses expressing CX3 (XRCC3-FLAG) or BCDX2 (RAD51B-His and XRCC2-FLAG) complexes with WT or T132P mutant of RAD51C, respectively. Cell extracts were prepared by sonication in T300 buffer (25 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5 mM EDTA, 300 mM KCl, 1 mM DTT, 0.05% IGEPAL [Millipore], 1 mM PMSF and protease inhibitors) with 5 mM ATP and 2 mM $MgCl_2$, followed by centrifugation at 100,000*g* for 60 min. CX3 and BCDX2 complexes were affinity purified by incubating clarified lysates with anti-FLAG M2 resin (Sigma-Aldrich) and, for the BCDX2 complexes, Ni-NTA resin (QIAGEN). Protein complex elution was achieved with 200 nM FLAG peptide (from the anti-FLAG matrix) and 200 mM imidazole (from the Ni-NTA matrix) in T300 buffer with 2 mM each of ATP and $MgCl_2$. BCDX2 complexes were further purified over 1 ml HiTrap Q HP column fractionated with a 150 to 450 mM KCl gradient in T buffer with each of 2 mM ATP and $MgCl_2$, followed by size exclusion in a Superdex 200 10/300 column (T300 buffer with 2 mM each of ATP and $MgCl_2$).

For DNA binding, 1 nM of 5′ ^{32}P-labeled 80-nt ssDNA (Gaines et al. 2015) was incubated with the indicated concentration of purified BCDX2 complexes in 10 µL reaction buffer (50 mM Tris-HCl, pH 7.5, 155 mM KCl, 1 mM DTT, 1 mM ATP, 1 mM $MgCl_2$, and 100 µg BSA) for 10 min at 37°C. Nucleoprotein complexes were resolved on 5% polyacrylamide gels in Tris-borate buffer. Gels were dried and subjected to phosphorimaging analysis.

Cell Experiments

HR and RAD51 focus assays were previously described (Garcin et al. 2019). RAD51 antibody used for RAD51 focus formation was used 1:1000 (#PC130, EMD Millipore). For clonogenic survival, U2OS cells were seeded at colony forming density (600 cells) on 60-mm dishes (Garcin et al. 2019). Cells were seeded in triplicate and treated 24 h after plating with either cisplatin or olaparib. Cells were exposed to 0–2 µM cisplatin for one cell cycle, as determined from the doubling time. Cells were continuously exposed to 0–2 µM olaparib (Selleck Chem AZD2281), replacing the media with fresh olaparib every 3 d. Cells were grown for 12–14 d (beginning with treatment day 1) and fixed in 100% methanol. Plates were stained with crystal violet, scanned on a FluorChem M (proteinsimple), and quantified for area density using the Colony Count Analysis Tool (AlphaView SA software). Area density was normalized relative to untreated plates. U2OS and MCF10A *RAD51C* derivatives were authenticated and deposited in 2019 at the Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Culture (Garcin et al. 2019).

ADDITIONAL INFORMATION

Data Deposition and Access

The *RAD51C* variant c.394A > C pT132P has been submitted to ClinVar (https://www.ncbi.nlm.nih.gov/clinvar/) under accession number VCV000996829.1.

Ethics Statement

In accord with protocols approved by the Mayo Clinic Institutional Review Board, the patient provided written informed consent for biological study of her ovarian cancer, including DNA

sequencing. BROCA sequencing (Norquist et al. 2018) was performed under Institutional Review Board number 12-010101 (OCRFHG6141G/T, OV2143; Supplemental Table 1). Given that the original diagnosis of this patient was prior to the 2014 adoption of sequencing guidelines by the Society for Gynecological Oncology, no clinical sequencing was performed.

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Author Contributions

M.R.S., R.P., Y.R., P.S., K.A.B., and M.J. designed experiments. M.R.S., R.P., and Y.R. conducted experiments. W.W. provided reagents. M.R.S., R.P., Y.R., P.S., K.A.B., and M.J. wrote the manuscript. M.R.R., S.H.K., and E.M.S. conducted the patient study.

Competing Interest Statement

The authors have declared no competing interest.

Referees

Lorna Rodriguez-Rodriguez
Anonymous

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