

Award Number: W81XWH-14-1-0441

TITLE: Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer

PRINCIPAL INVESTIGATOR: Neil Johnson, Ph.D.

CONTRACTING ORGANIZATION:

The Research Institute of Fox Chase Cancer Center
Philadelphia, PA 19111

REPORT DATE: Dec 2019

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

REPORT DOCUMENTATION PAGE				Form Approved OMB No. 0704-0188		
Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Department of Defense, Washington Headquarters Services, Directorate for Information Operations and Reports (0704-0188), 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302. Respondents should be aware that notwithstanding any other provision of law, no person shall be subject to any penalty for failing to comply with a collection of information if it does not display a currently valid OMB control number. PLEASE DO NOT RETURN YOUR FORM TO THE ABOVE ADDRESS.						
1. REPORT DATE (DD-MM-YYYY) Dec 2019		2. REPORT TYPE Final		3. DATES COVERED 09/15/2014 - 09/14/2019		
4. TITLE AND SUBTITLE Identifying Determinants of PARP Inhibitor Sensitivity in Ovarian Cancer				5a. CONTRACT NUMBER		
				5b. GRANT NUMBER W81XWH-14-1-0441		
				5c. PROGRAM ELEMENT NUMBER		
6. AUTHOR(S) Neil Johnson, Ph.D. E-Mail: Neil.Johnson@fcc.edu				5d. PROJECT NUMBER		
				5e. TASK NUMBER		
				5f. WORK UNIT NUMBER		
7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES) The Research Institute of Fox Chase Cancer Center 333 Cottman Avenue Philadelphia, Pennsylvania 19111 E-Mail: osr@fcc.edu				8. PERFORMING ORGANIZATION REPORT NUMBER		
9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES) U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012				10. SPONSOR/MONITOR'S ACRONYM(S)		
				11. SPONSOR/MONITOR'S REPORT NUMBER(S)		
12. DISTRIBUTION / AVAILABILITY STATEMENT Approved for public release; distribution unlimited						
13. SUPPLEMENTARY NOTES						
14. ABSTRACT During the course of the grant, we have discovered several novel insights into PARP inhibitor (PARPi) and cisplatin resistance mechanisms. We showed that the BRCA1-Δ11q protein is responsible for promoting PARPi resistance in ovarian cancers with exon 11 located BRCA1 mutations. In another aspect of our studies encompassed within this grant, we discovered that <i>BRCA1</i> ^{185delAG} alleles were capable of initiating translation at a start site downstream of the mutation-induced stop codon. We also made a BRCA1 null mouse model and subsequently discovered that 53BP1 knockout only partially restores HR in the absence of BRCA1 hypomorphic proteins. In another recent study, our laboratory showed that BRCA2 mutant alleles are capable of expressing proteins that contribute to PARP inhibitor resistance. These studies were published and OC130212 acknowledged in Wang et al., <i>Cancer Research</i> , 2016; Wang et al., <i>Journal of Clinical Investigation</i> , 2016; Nacson et al., <i>Cell Reports</i> , 2018; Park et al., <i>Molecular Cancer Therapeutics</i> , 2019; and Wang et al., <i>Nature Communications</i> , 2019. In terms of career development, I was promoted to associate professor with tenure in May 2019.						
15. SUBJECT TERMS Ovarian cancer, BRCA1, RAD51, PARP inhibitors, platinum, biomarkers, drug resistance						
16. SECURITY CLASSIFICATION OF:				17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES 114	19a. NAME OF RESPONSIBLE PERSON USAMRMC
a. REPORT Unclassified	b. ABSTRACT Unlimited	c. THIS PAGE Unclassified	19b. TELEPHONE NUMBER (Include area code)			

Standard Form 298 (Rev. 8-98)
Prescribed by ANSI Std. Z39.18

Table of Contents

1. Introduction 4

2. Keywords 4

3. Accomplishments 4

4. Impact 7

5. Changes/Problems 8

6. Products 8

7. Participants & Other Collaborating Organizations 10

8. Special Reporting Requirements 11

9. Appendices 11

DOD Final Report

Principal Investigator: Neil Johnson, Ph.D.

Institution: Institute for Cancer Research

Grant Number: W81XWH-14-1-0441

INTRODUCTION:

Cells that are deficient in homologous recombination (HR) DNA repair, such as those lacking functional BRCA1 are highly sensitive to poly(ADP-ribose) polymerase (PARP) inhibitors. Ovarian cancer patients that harbored germ-line *BRCA1* mutations treated with PARP inhibitors exhibited meaningful responses in early phase clinical trials. However, emerging clinical trial data indicates that PARP inhibitor therapy may benefit only a subset of *BRCA1* mutation carriers. Our preliminary data suggests that PARP inhibitor selection pressure results in genetic adaptations that enable cells to utilize severely truncated BRCA1 proteins for RAD51 loading and HR repair. Our objectives are to define the BRCA1 peptide region minimally required for PARP inhibitor resistance, and discover genetic alterations that activate DNA end resection as well as mutant *BRCA1* protein stabilization in ovarian carcinomas. The expression of mutant BRCA1 or novel proteins identified to be important for drug resistance will be assessed for their ability to be used as biomarkers of PARP inhibitor or platinum response. *Specific Aims.* 1: Identify the region of mutant BRCA1 protein critical for PARP inhibitor resistance; 2: Identify genetic alterations essential for PARP inhibitor resistance; 3: Determine the ability of identified genetic aberrations to serve as predictive biomarkers.

KEYWORDS:

Ovarian cancer, BRCA1, RAD51, PARP inhibitors, platinum, biomarkers, drug resistance.

ACCOMPLISHMENTS:

What were the major goals of the project?

Major Task 1: Determine the minimum region of BRCA1 protein required for PARP inhibitor resistance.

Milestone(s) Achieved: Drug resistant cell lines are derived that express different types of mutant BRCA1 proteins. Target date for completion: 12 months (from start of award). Current status: 100% completed.

Major Task 2: Determine the minimum region of BRCA1 protein required for RAD51 focus formation.

Milestone(s) Achieved: The minimum region of mutant BRCA1 protein is identified that can contribute to RAD51 loading. Target date for completion: 16 months (from start of award). Current status: 100% completed.

Major Task 3: Generate whole exome sequencing and gene expression data sets for PARP inhibitor sensitive and resistant cell lines.

Milestone(s) Achieved: Identification of genes that are mutated or differentially expressed in PARP inhibitor resistant cells. Target date for completion: 30 months (from start of award). Current status: 100% completed. Completion date: 36 months.

Major Task 4: Validate identified genes as resistance causing through RNAi and cDNA overexpression techniques.

Milestone(s) Achieved: Validation of genes that are mutated or differentially expressed and result in PARP inhibitor resistance. Target date for completion: 36 months (from start of award). Current status: 100% completed.

Major Task 5: Obtain IRB approval for assessing tumor DNA and assembling tumor microarrays.

Milestone(s) Achieved: IRB protocol approved. Target date for completion: 40 months (from start of award). Current status: 100% completed.

Major Task 6: Analyze tumor DNA for mutations.

Milestone(s) Achieved: Identification of gene mutation or protein expression changes in ovarian tumors. Target date for completion: 55 months (from start of award). Current status: 100% completed.

Major Task 7: Determine the ability of potential biomarkers to predict therapeutic outcome.

Milestone(s) Achieved: Biomarkers are ready for application in clinical trials of BRCA1 mutant ovarian cancer and data is published in peer-reviewed journals. Target date for completion: 60 months (from start of award). Current status: 100% completed.

What was accomplished under these goals?

My work investigating the effects of BRCA1 function provoked our interest in the role of the BRCA1 protein in homologous recombination DNA repair and the cellular response to DNA damaging agents. Of particular interest, clinical trial data demonstrated that the *BRCA1* and *BRCA2* mutation patient groups have a mixed response to PARP inhibitor monotherapy. Over the course of the grant period, we have started to address the following question: why do ovarian tumors harboring mutations in the same DNA repair gene respond differently to PARP inhibitors? For *BRCA1* mutation carriers, I hypothesized that the primary determinant of tumor response is the cellular capacity to repair DNA damage. We discovered that PARP inhibitor and cisplatin resistance mechanisms often depend on the location of the BRCA1 mutation. During the course of the grant, we have discovered several novel insights into PARP inhibitor (PARPi) and cisplatin resistance mechanisms.

We showed that germline mutations in BRCA1 exon 11 are subject to splicing and removal, resulting in the restoration of the correct reading frame and generating a BRCA1-Δ11q protein containing the N- and C-terminal, but without the deleted exon 11 coding amino acids. Importantly, the BRCA1-Δ11q protein is responsible for promoting PARPi resistance in ovarian cancers with exon 11 located BRCA1 mutations. This work was published and OC130212 acknowledged in Wang et al., *Cancer Research*. 2016.

In another aspect of our studies encompassed within this grant, we discovered that *BRCA1*^{185delAG} alleles were capable of initiating translation at a start site downstream of the mutation-induced stop codon. These protein products retained hypomorphic activity and promoted therapy resistance. This work was published and OC130212 acknowledged in Wang et al., *Journal of Clinical Investigation*. 2016.

Homologous recombination (HR) DNA repair not only impacts the cellular response to PARPi therapy, but also the ability of embryos to develop and organismal health. Thus, biological mechanisms that promote PARP inhibitor resistance, may also enhance the viability of BRCA1 mutant embryos. 53BP1 is an inhibitor of DNA end resection, the first step of HR, and 53BP1 knockout restores HR and the viability of BRCA1 mutant mice. We hypothesized the BRCA1 mutation location, which determines the protein product generated and functional domains that are absent, could play a role in the ability of 53BP1 to restore HR and development. We made a BRCA1 null mouse model and subsequently discovered that 53BP1 knockout only partially restores HR in the absence of BRCA1 hypomorphic proteins. Loss of 53BP1 activates DNA end resection, but BRCA1 isoforms are required for RAD51 loading and optimal HR. Consequently, 53BP1 and BRCA1 null mice are tumor prone and have short life-spans. Importantly, the biology uncovered using transgenic mice also reflected that of human cancers. When we constructed the same genetic alterations in human cancer cell lines and tumors, we found that 53BP1 only effectively promoted HR and PARPi resistance in cells with specific types of BRCA1 mutations that generated semi-functional protein products. This work was published and OC130212 acknowledged in Nacson et al., *Cell Reports*. 2018.

In another recent study, our laboratory showed that BRCA2 mutant alleles are capable of expressing proteins that contribute to PARP inhibitor resistance. This work was published and OC130212 acknowledged in Park et al., *Molecular Cancer Therapeutics*. 2019.

In our most recent study, we found Alu-mediated BRCA1 rearrangements were responsible for promoting chemotherapy resistance. This work was published and OC130212 acknowledged in Wang et al., *Nature Communications*. 2019.

In terms of career development, I was promoted to Associate Professor with tenure in May 2019.

What opportunities for training and professional development has the project provided?

During the course of this award, I have taken part in the following training activities:

- Discussed progress with Drs. Boyd, Connolly and Golemis.
- Discussed progress with my Chief Scientific Officer, Dr. Chernoff.
- Attended Junior Faculty Mentoring meetings.
- Presented research at the monthly Faculty Seminar.
- Presented research at Molecular Therapeutics Departmental meetings.
- Presented research at FCCC Gynecologic Oncology meetings.

During the course of this award, I have taken part in the following professional development activities:

- I attended FCCC Gynecologic Cancer meetings.
- I attended and presented at the several Biennial Ovarian Cancer Research Symposium, presented by the Rivkin Center for Ovarian Cancer and the American Association for Cancer Research in Seattle, WA.
- I attended all of the Annual DoD Ovarian Cancer Academy meetings.
- I attended the Bassett Center for BRCA Annual Scientific Symposiums, Philadelphia, PA.
- I was invited to give a lecture on my work at the Gordon Conference, Benzon symposium, and the Marsha Rivkin symposium.
- I have had papers from my lab accepted for publication in Cancer Research, JCI, Cell Reports, Molecular Cancer Therapeutics and Nature Communications that address the work proposed in the DoD project.

How were the results disseminated to communities of interest?

I have presented my work to FCCC meeting groups described above. Additionally, I gave oral presentations at the following venues during the course of this DoD project.

- 2013 Invited Seminar Speaker, Penn State, Hershey, PA. Host: Lucian Moldovan, PhD
- 2014 Invited Seminar Speaker, Vall d'Hebron Institute of Oncology, Barcelona, Spain. Host: Judith Balmana, MD and Violeta Serra, PhD
- 2014 Invited Seminar Speaker, Temple University, Philadelphia, PA. Host: Xavier Grana, PhD
- 2014 Invited Seminar Speaker, Universidad Nacional Autónoma de México, Mexico City, Mexico. Host: Luis Aria Romero, PhD
- 2015 Invited Seminar Speaker, University of Pennsylvania, Philadelphia, PA. Host: Penn Genome Integrity Group
- 2015 Invited Seminar Speaker, Bassett Center for BRCA Annual Scientific Symposium, Philadelphia, PA Host: Annual Symposium organizers
- 2015 Invited Seminar Speaker, Cardinal Bernardin Cancer Center of Loyola University, Chicago, IL. Host: Clodia Osipo, PhD
- 2016 Invited Seminar Speaker, Rutgers Cancer Institute, Rutgers University, New Brunswick, NJ. Host: Bing Xia, PhD
- 2016 Invited Speaker, 11th Biennial Rivkin-AACR Cancer Research Symposium, Seattle, WA
- 2016 Expert, 'Meet the expert session', 11th Biennial Rivkin-AACR Cancer Research Symposium, Seattle, WA
- 2017 Invited Seminar Speaker, Gynecologic Oncology seminar series, University of Pennsylvania, Host: Ronny Drapkin, MD, PhD
- 2017 Invited Seminar Speaker, Mayo Clinic, Rochester, MN. Host: Scott Kaufmann, MD PhD
- 2018 Invited Speaker, 'DNA damage, Mutation and Cancer' Gordon Conference
- 2018 Invited Speaker, 12th Biennial Rivkin-AACR Cancer Symposium, Seattle, WA
- 2018 Invited Seminar Speaker, Bassett Center UPENN, Philadelphia, PA. Host: Susan Domchek, MD
- 2018 Invited Seminar Speaker, National Cancer Institute, Bethesda, MD. Host: Andre Nussenzweig, PhD
- 2019 Invited Speaker, Benzon Symposium on DNA damage, Copenhagen

Moreover, we published the following studies:

1. Wang Y, Bernhardt AJ, Cruz C, Kraiss JJ, Nacson J, Nicolas E, Peri S, van der Gulden H, van der Heijden I, O'Brien SW, Zhang Y, Harrell MI, Johnson SF, Candido Dos Reis FJ, Pharoah PD, Karlan B, Gourley C, Lambrechts D, Chenevix-Trench G, Olsson H, Benitez JJ, Greene MH, Gore M, Nussbaum R, Sadetzki S, Gayther SA, Kjaer SK; kConFab Investigators, D'Andrea AD, Shapiro GI, Wiest DL, Connolly DC, Daly MB, Swisher EM, Bouwman P, Jonkers J, Balmaña J, Serra V, **Johnson N**. The BRCA1- $\Delta 11q$ Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin. *Cancer Res*. 2016 May 1;76(9):2778-90. doi: 10.1158/0008-5472.CAN-16-0186. PMID: PMC4874568.
2. Wang Y, Kraiss JJ, Bernhardt AJ, Nicolas E, Cai KQ, Harrell MI, Kim HH, George E, Swisher EM, Simpkins F, **Johnson N**. RING Domain-Deficient BRCA1 Promotes PARP Inhibitor and Platinum Resistance. *The Journal of Clinical Investigation*. 2016; 126(8):3145-57. PMID: PMC4966309.
3. Nacson J, Kraiss JJ, Bernhardt AJ, Clausen E, Feng W, Wang Y, Nicolas E, Cai KQ, Tricarico R, Hua X, DiMarcantonio D, Martinez E, Zong D, Handorf EA, Bellacosa A, Testa JR, Nussenzweig A, Gupta GP, Sykes SM, **Johnson N**. BRCA1 mutation-specific responses to 53BP1 loss-induced homologous recombination and PARP inhibitor resistance. *Cell Reports*. 2018; 24(13):3513-3527. PMID: PMC6219632.
4. Park PH, Yamamoto TM, Li H, Alcivar AL, Xia B, Wang Y, Bernhardt AJ, Turner KM, Kossenkova AV, Watson ZL, Behbakht K, Casadei S, Swisher EM, Mischel PS, **Johnson N**, Bitler BG. Amplification of the mutation-carrying BRCA2 allele promotes RAD51 loading and PARP inhibitor resistance in the absence of reversion mutations. *Mol Cancer Ther*. 2019 Oct 1; pii: molcanther.0256.2019. doi: 10.1158/1535-7163.MCT-17-0256. [Epub ahead of print] PubMed PMID: 31575654.
5. Wang Y, Bernhardt AJ, Nacson J, Kraiss JJ, Tan YF, Nicolas N, Radke MR, Handorf E, Guevara AL, Balmaña J, Swisher EM, Serra V, Peri S and **Johnson N**. BRCA1 intronic Alu elements drive gene rearrangements and PARP inhibitor resistance. *Nature Communications*. In press.

What do you plan to do during the next reporting period to accomplish the goals?

Not applicable.

IMPACT:

What was the impact on the development of the principal discipline(s) of the project?

PARPi therapy is an exciting new treatment option for ovarian cancer patients that has been incorporated into the standard of care. Tumor resistance to therapy is the primary cause ovarian cancer related death. Because we have now revealed new insights into resistance, our findings could help reveal new strategies to prolong the effectiveness of PARPi therapy. Understanding why tumors become drug resistant is paramount for the design of new strategies that effectively target drug resistant tumors and reduce ovarian cancer deaths. We have developed in depth knowledge of how BRCA1 mutant cancers become drug resistant, based on their mutation location, and this knowledge can be used going forward for the application of new therapies. Our work suggests that resistance mechanisms are diverse, and in the case of BRCA1, allele-specific drug resistance mechanisms exist. To improve the current understanding of ovarian cancer drug resistance so that resistant tumors can be effectively treated, mutation-focused studies are crucial and we have provided a foundation for the rational design of approaches that target resistant disease.

The observation that BRCA1 mutant tumors harboring RING, exon 11, and BRCT domain located mutations will likely develop mechanisms of PARPi resistance via translation, splicing, and folding, suggests drug resistance can be reversed using inhibitors of the latter pathways. These results have important, immediate implications for

the clinical management and application of PARPi therapies. When PARPi resistance develops, patients with specific *BRCA1* allelic mutations may benefit from alternative therapies with different mechanisms of action. Furthermore, our work will enable the mechanism of drug resistance to be predicted, based on the location of patients' *BRCA1* mutation. The long-term implications of our work is that the development of small molecules that specifically and potently kill BRCA1 mutant protein expressing drug resistant ovarian tumors, but leave full-length BRCA1 expressing normal cells unharmed, will improve ovarian cancer patient's survival and reduce deaths. In summary, our discoveries during the current 5-year grant term have helped lay the foundation for new clinical approaches that may successfully target drug resistant ovarian cancers.

What was the impact on other disciplines?

Nothing to Report.

What was the impact on technology transfer?

Nothing to Report.

What was the impact on society beyond science and technology?

Nothing to Report.

CHANGES/PROBLEMS:

Changes in approach and reasons for change

Nothing to Report.

Actual or anticipated problems or delays and actions or plans to resolve them

Nothing to Report.

Changes that had a significant impact on expenditures

Nothing to Report.

Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents

Nothing to Report.

Significant changes in use or care of human subjects

Nothing to Report.

Significant changes in use or care of vertebrate animals.

Nothing to Report.

Significant changes in use of biohazards and/or select agents

Nothing to Report.

PRODUCTS:

Publications, conference papers, and presentations

Journal publications.

1. Wang Y, Bernhardt AJ, Cruz C, Krais JJ, Nacson J, Nicolas E, Peri S, van der Gulden H, van der Heijden I, O'Brien SW, Zhang Y, Harrell MI, Johnson SF, Candido Dos Reis FJ, Pharoah PD, Karlan B, Gourley C, Lambrechts D, Chenevix-Trench G, Olsson H, Benitez JJ, Greene MH, Gore M, Nussbaum R, Sadetzki S, Gayther SA, Kjaer SK; kConFab Investigators, D'Andrea AD, Shapiro GI, Wiest DL, Connolly DC, Daly MB, Swisher EM, Bouwman P, Jonkers J, Balmaña J, Serra V, **Johnson N**. The BRCA1-Δ11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin. *Cancer Res*. 2016 May 1;76(9):2778-90. doi: 10.1158/0008-5472.CAN-16-0186. PMCID: PMC4874568.

Principal Investigator: Neil Johnson, Ph.D.

2. Wang Y, Kraiss JJ, Bernhardt AJ, Nicolas E, Cai KQ, Harrell MI, Kim HH, George E, Swisher EM, Simpkins F, **Johnson N**. RING Domain-Deficient BRCA1 Promotes PARP Inhibitor and Platinum Resistance. *The Journal of Clinical Investigation*. 2016; 126(8):3145-57. PMCID: PMC4966309.
3. Nacson J, Kraiss JJ, Bernhardt AJ, Clausen E, Feng W, Wang Y, Nicolas E, Cai KQ, Tricarico R, Hua X, DiMarcantonio D, Martinez E, Zong D, Handorf EA, Bellacosa A, Testa JR, Nussenzweig A, Gupta GP, Sykes SM, **Johnson N**. BRCA1 mutation-specific responses to 53BP1 loss-induced homologous recombination and PARP inhibitor resistance. *Cell Reports*. 2018; 24(13):3513-3527. PMCID: PMC6219632.
4. Park PH, Yamamoto TM, Li H, Alcivar AL, Xia B, Wang Y, Bernhardt AJ, Turner KM, Kossenkova AV, Watson ZL, Behbakht K, Casadei S, Swisher EM, Mischel PS, **Johnson N**, Bitler BG. Amplification of the mutation-carrying BRCA2 allele promotes RAD51 loading and PARP inhibitor resistance in the absence of reversion mutations. *Mol Cancer Ther*. 2019 Oct 1; pii: molcanther.0256.2019. doi: 10.1158/1535-7163.MCT-17-0256. [Epub ahead of print] PubMed PMID: 31575654.
5. Wang Y, Bernhardt AJ, Nacson J, Kraiss JJ, Tan YF, Nicolas N, Radke MR, Handorf E, Guevara AL, Balmaña J, Swisher EM, Serra V, Peri S and **Johnson N**. BRCA1 intronic Alu elements drive gene rearrangements and PARP inhibitor resistance. *Nature Communications*. In press.

Books or other non-periodical, one-time publications. Nothing to report.

Other publications, conference papers, and presentations.

Meeting Abstracts

1. 2016. AACR Advances in ovarian cancer research. Pittsburgh. 53BP1-driven homologous recombination and PARP inhibitor resistance requires intact BRCA1-PALB2 association. Joseph Nacson, Andrea Bernhardt, Xiang Hua, Yifan Wang, John Kraiss and Neil Johnson.
2. 2016. 11th Biennial Rivkin-AACR Cancer Research Symposium. Seattle. BRCA1 RING Domain-Deficient Proteins Promote PARP Inhibitor and Platinum Resistance. Yifan Wang, John J. Kraiss, Andrea J. Bernhardt, Emmanuelle Nicolas, Kathy Q. Cai, Maria I. Harrell, Hyoung H. Kim, Erin George, Elizabeth M. Swisher, Fiona Simpkins and Neil Johnson.
3. 2016. DNA repair: Tumor Development and therapeutic response. Montreal. BRCA1 mutations in the BRCT domain can be removed through alternative splicing and BRCT-less proteins promote PARP inhibitor resistance. Yifan Wang, Andrea J. Bernhardt and Neil Johnson.
4. 2018. 'DNA damage, Mutation and Cancer' Gordon Conference. Ventura. RNF168 promotes continued DNA replication at stalled forks and loss of viability in BRCA1 mutant cancers. John Kraiss, Andrea Bernhardt, Joseph Nacson, Yifan Wang, and Neil Johnson.
5. 2019. AACR Advances in ovarian cancer research. Georgia. Assessment of PARPi and cisplatin resistance in BRCA1 exon 11 mutant patient -derived xenografts. John Kraiss, Emma Clausen, Vladimir Khazak, Igor Astsaturov, Clare L. Scott and Neil Johnson.

Website(s) or other Internet site(s)

Nothing to Report.

Technologies or techniques

Nothing to Report.

Inventions, patent applications, and/or licenses

Nothing to Report.

Other Products

Nothing to Report.

PARTICIPANTS & OTHER COLLABORATING ORGANIZATIONS**What individuals have worked on the project?**

Name:	<i>N. Johnson, Ph.D.</i>
Project Role:	Principal Investigator
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Overall administration and guidance of research; Management and training of personnel
Funding Support:	N/A
Name:	<i>J. Boyd, Ph.D.</i>
Project Role:	Mentor
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Dr. Boyd is the mentor on this project. He provides advice, assistance and support.
Funding Support:	N/A
Name:	<i>A. Johnson</i>
Project Role:	Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Helped generate a range of BRCA1 mutant cancer cell lines.
Funding Support:	N/A
Name:	<i>E. Clausen</i>
Project Role:	Scientific Technician
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	4
Contribution to Project:	Supported postdoc and student in their efforts characterizing BRCA1 mutant cancers.
Funding Support:	N/A
Name:	<i>G. Conway, Ph.D.</i>
Project Role:	Postdoctoral Associate
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	1
Contribution to Project:	Discovered several insights into PARPi resistance mechanisms.
Funding Support:	N/A
Name:	<i>J. Nacson</i>
Project Role:	Graduate Student
Researcher Identifier (e.g. ORCID ID):	
Nearest person month worked:	12
Contribution to Project:	Discovered a role for 53BP1 in PARPi resistance and BRCA1 mutant cancers.
Funding Support:	N/A

Has there been a change in the active other support of the PD/PI(s) or senior/key personnel since the last reporting period?

Not applicable.

What other organizations were involved as partners?

Organization Name: Florida International University

Location of Organization: Miami, Florida

Partner's contribution to the project: Mentor

- o Dr. Boyd devoted effort as a mentor on this project. Funds were also allotted for travel.

Organization Name: University of Pennsylvania

Location of Organization: Philadelphia, PA

Partner's contribution to the project: Consultant

- o Dr. Burger provided advice and guidance on the development of biomarkers that predict tumor PARP inhibitor sensitivity.

Organization Name: University of Washington

Location of Organization: Seattle, WA

Partner's contribution to the project: Consultant

- o Dr. Swisher provided advice and guidance on the development of on the biomarkers that predict tumor PARP inhibitor sensitivity.

SPECIAL REPORTING REQUIREMENTS

COLLABORATIVE AWARDS: Nothing to Report.

QUAD CHARTS: Nothing to Report.

APPENDICES:

Publications:

1. Wang Y, Bernhardt AJ, Cruz C, Krais JJ, Nacson J, Nicolas E, Peri S, van der Gulden H, van der Heijden I, O'Brien SW, Zhang Y, Harrell MI, Johnson SF, Candido Dos Reis FJ, Pharoah PD, Karlan B, Gourley C, Lambrechts D, Chenevix-Trench G, Olsson H, Benitez JJ, Greene MH, Gore M, Nussbaum R, Sadetzki S, Gayther SA, Kjaer SK; kConFab Investigators, D'Andrea AD, Shapiro GI, Wiest DL, Connolly DC, Daly MB, Swisher EM, Bouwman P, Jonkers J, Balmaña J, Serra V, **Johnson N**. The BRCA1- $\Delta 11q$ Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin. *Cancer Res*. 2016 May 1;76(9):2778-90. doi: 10.1158/0008-5472.CAN-16-0186. PMCID: PMC4874568.
2. Wang Y, Krais JJ, Bernhardt AJ, Nicolas E, Cai KQ, Harrell MI, Kim HH, George E, Swisher EM, Simpkins F, **Johnson N**. RING Domain-Deficient BRCA1 Promotes PARP Inhibitor and Platinum Resistance. *The Journal of Clinical Investigation*. 2016; 126(8):3145-57. PMCID: PMC4966309.
3. Nacson J, Krais JJ, Bernhardt AJ, Clausen E, Feng W, Wang Y, Nicolas E, Cai KQ, Tricarico R, Hua X, DiMarcantonio D, Martinez E, Zong D, Handorf EA, Bellacosa A, Testa JR, Nussenzweig A, Gupta GP, Sykes SM, **Johnson N**. BRCA1 mutation-specific responses to 53BP1 loss-induced homologous recombination and PARP inhibitor resistance. *Cell Reports*. 2018; 24(13):3513-3527. PMCID: PMC6219632.
4. Park PH, Yamamoto TM, Li H, Alcivar AL, Xia B, Wang Y, Bernhardt AJ, Turner KM, Kossenkov AV, Watson ZL, Behbakht K, Casadei S, Swisher EM, Mischel PS, **Johnson N**, Bitler BG. Amplification of the mutation-carrying BRCA2 allele promotes RAD51 loading and PARP inhibitor resistance in the absence of

reversion mutations. *Mol Cancer Ther.* 2019 Oct 1; pii: molcanther.0256.2019. doi: 10.1158/1535-7163.MCT-17-0256. [Epub ahead of print] PubMed PMID: 31575654.

5. Wang Y, Bernhardt AJ, Nacson J, Kraus JJ, Tan YF, Nicolas N, Radke MR, Handorf E, Guevara AL, Balmaña J, Swisher EM, Serra V, Peri S and **Johnson N**. BRCA1 intronic Alu elements drive gene rearrangements and PARP inhibitor resistance. *Nature Communications*. In press.

The BRCA1- Δ 11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin

Yifan Wang¹, Andrea J. Bernhardt¹, Cristina Cruz^{2,3}, John J. Krais¹, Joseph Nacson¹, Emmanuelle Nicolas⁴, Suraj Peri¹, Hanneke van der Gulden⁵, Ingrid van der Heijden⁵, Shane W. O'Brien¹, Yong Zhang⁶, Maribel I. Harrell⁷, Shawn F. Johnson⁸, Francisco J. Candido Dos Reis^{9,10}, Paul D. P. Pharoah¹⁰, Beth Karlan¹¹, Charlie Gourley¹², Diether Lambrechts¹³, Georgia Chenevix-Trench¹⁴, Håkan Olsson¹⁵, Javier J. Benitez¹⁶, Mark H. Greene¹⁷, Martin Gore¹⁸, Robert Nussbaum¹⁹, Siegal Sadetzki²⁰, Simon A. Gayther²¹, Susanne K. Kjaer²², kConFab Investigators²³, Alan D. D'Andrea^{24,25}, Geoffrey I. Shapiro^{8,26}, David L. Wiest⁶, Denise C. Connolly¹, Mary B. Daly²⁷, Elizabeth M. Swisher⁷, Peter Bouwman⁵, Jos Jonkers⁵, Judith Balmaña², Violeta Serra³, and Neil Johnson¹

Abstract

Breast and ovarian cancer patients harboring *BRCA1/2* germline mutations have clinically benefitted from therapy with PARP inhibitor (PARPi) or platinum compounds, but acquired resistance limits clinical impact. In this study, we investigated the impact of mutations on *BRCA1* isoform expression and therapeutic response. Cancer cell lines and tumors harboring mutations in exon 11 of *BRCA1* express a *BRCA1- Δ 11q* splice variant lacking the majority of exon 11. The introduction of frameshift mutations to exon 11 resulted in nonsense-mediated mRNA decay of full-length, but not the *BRCA1- Δ 11q* isoform. CRISPR/Cas9 gene editing as well as

overexpression experiments revealed that the *BRCA1- Δ 11q* protein was capable of promoting partial PARPi and cisplatin resistance relative to full-length *BRCA1*, both *in vitro* and *in vivo*. Furthermore, spliceosome inhibitors reduced *BRCA1- Δ 11q* levels and sensitized cells carrying exon 11 mutations to PARPi treatment. Taken together, our results provided evidence that cancer cells employ a strategy to remove deleterious germline *BRCA1* mutations through alternative mRNA splicing, giving rise to isoforms that retain residual activity and contribute to therapeutic resistance. *Cancer Res*; 76(9); 2778–90. ©2016 AACR.

¹Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania. ²High Risk and Cancer Prevention Group, Vall d'Hebron Institute of Oncology, Barcelona, Spain. ³Experimental Therapeutics Group, Vall d'Hebron Institute of Oncology, Barcelona, Spain. ⁴Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania. ⁵Division of Pathology, The Netherlands Cancer Institute, Amsterdam, the Netherlands. ⁶Immune Cell Development and Host Defense Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania. ⁷Department of Obstetrics and Gynecology and Medicine, University of Washington, Seattle, Washington. ⁸Department of Medical Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. ⁹Department of Gynecology and Obstetrics, Ribeirão Preto Medical School, University of São Paulo, São Paulo, Brazil. ¹⁰Centre for Cancer Genetic Epidemiology, University of Cambridge, Cambridge, United Kingdom. ¹¹Women's Cancer Program at the Samuel Oschin Comprehensive Cancer Institute, Cedars-Sinai Medical Center, Los Angeles, California. ¹²University of Edinburgh Cancer Research UK Centre, MRC IGMM, Edinburgh, United Kingdom. ¹³VIB Vesalius Research Center, University of Leuven, Leuven, Belgium. ¹⁴QIMR Berghofer Medical Research Institute, Herston, Australia. ¹⁵Departments of Cancer Epidemiology and Oncology, Lund University, Lund, Sweden. ¹⁶Human Genetics Group and Human Genotyping Unit Spanish National Cancer Research Centre (CNIO), Madrid, Spain. ¹⁷Division of Cancer Epidemiology and Genetics, National Cancer Institute, Rockville, Maryland. ¹⁸Royal Marsden NHS Foundation Trust, London, United Kingdom. ¹⁹University of California

San Francisco, Cancer Risk Program, San Francisco, California. ²⁰Gertner Institute for Epidemiology and Health Policy Research, Sheba Medical Center, Tel Hashomer, Israel. ²¹Department of Preventive Medicine, Keck School of Medicine, University of Southern California Norris Comprehensive Cancer Center, Los Angeles, California. ²²Department of Virus, Lifestyle and Genes, Danish Cancer Society Research Center, Copenhagen, Denmark. ²³University of Melbourne, Melbourne, Australia. ²⁴Department of Radiation Oncology, Dana-Farber Cancer Institute and Harvard Medical School, Boston, Massachusetts. ²⁵Department of Pediatrics, Children's Hospital and Harvard Medical School, Boston, Massachusetts. ²⁶Department of Medicine, Brigham and Women's Hospital and Harvard Medical School, Boston, Massachusetts. ²⁷Risk Assessment Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania.

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

Y. Wang and A.J. Bernhardt are the co-first authors of this article.

Corresponding Author: Neil Johnson, Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA 19111. Phone: 215-728-7016; Fax: 215-728-2741; E-mail: neil.johnson@fccc.edu

doi: 10.1158/0008-5472.CAN-16-0186

©2016 American Association for Cancer Research.

Introduction

Germline mutations in the *BRCA1* gene are associated with an increased risk of developing breast and ovarian cancer (1, 2). Mutations often result in reading frameshifts and nonsense-mediated mRNA decay (NMD; ref. 3). The *BRCA1* protein is essential for efficient homologous recombination (HR)-mediated repair of double-stranded DNA breaks (4, 5). Inhibitors of PARP, as well as platinum agents, induce double-stranded DNA breaks that are repaired by the HR DNA repair pathway (6, 7). Consequently, cells that have defective HR DNA repair, such as those with dysfunctional *BRCA1* or *BRCA2* proteins, are highly sensitive to PARP inhibitor (PARPi) or platinum treatments (8–11).

Although PARP inhibitors have been shown to provide survival improvements, many patients that harbor germline *BRCA1* or *BRCA2* mutations do not gain benefit from PARPi therapy (12–14). In addition, many patients that first benefit from either PARPi or platinum therapy develop disease progression and resistance (15). PARPi or platinum resistance has been demonstrated to arise by a variety of mechanisms, including reversion mutations (16, 17), loss of 53BP1 pathway activity (18, 19), expression of hypomorphic *BRCA1* proteins (20, 21), and drug efflux (22).

BRCA1 mRNA isoforms generated by alternative splicing lack specific exons and have been shown to be expressed in cells and tissues (23–25). In particular, the relative levels of *BRCA1* exon 11 splice isoforms differ between normal and cancer tissues and in discrete phases of the cell cycle (26–29). These isoforms include *BRCA1* full-length (inclusion of all coding exons), Δ 11 (skipping of exon 11), and Δ 11q (partial skipping of exon 11). The *BRCA1- Δ 11q* isoform derives from use of an alternative exon 11 splice donor site, resulting in the exclusion of most exon 11 nucleotides (c.788-4096; Supplementary Fig. S1; refs. 27, 28). In human cells and tissues, the *BRCA1- Δ 11q* isoform expression is more readily detectable than the *BRCA1- Δ 11* isoform (26, 29, 30).

The *BRCA1- Δ 11* isoform has previously been implicated in both cell death and proliferation in mouse studies. Both *Brca1*-null and *Brca1* ^{Δ 11/ Δ 11} mice that exclusively express *Brca1- Δ 11* undergo embryonic lethality, but *Brca1* ^{Δ 11/ Δ 11} embryos die at a later stage, suggesting that *Brca1- Δ 11* isoform can partially compensate for the lack of other *Brca1* isoforms during embryogenesis (31–33). *BRCA1* mutations located in exon 11 represent approximately 30% of the overall number of mutation carriers that develop breast and ovarian cancer in the United States (34–37). Here, we examined the impact of exon 11 mutations on *BRCA1* isoform expression and therapy response.

Materials and Methods

Cell lines and reagents

Cells were purchased from Asterand or ATCC. Cycloheximide (CHX), actinomycin D (ACT), puromycin, blasticidin, and DMSO were purchased from Sigma-Aldrich, cisplatin was from APP/Fresenius Kabai USA LLC, and paclitaxel from Sagent Pharmaceuticals. Pladienolide B (PI-B) was purchased from Calbiochem. Clovis Oncology provided rucaparib (CO-338) and olaparib (AZD2281) was purchased from Selleckchem. *BRCA1*-mutated cell lines were validated through DNA sequencing and the identification of specific *BRCA1* mutations that are uniquely present in individual cell lines as well as DNA fingerprinting. Cell lines tested negative for mycoplasma.

Colony formation assays

Depending on colony-forming potential, cells were seeded at a density ranging from 500 to 4,000 cells per well in 6-well plates in the presence of increasing concentrations of either rucaparib or olaparib. For cisplatin and taxol treatments, exponentially growing cells were cultured in 24-well plates, treated with increasing concentrations of cisplatin and taxol for 24 hours, and replated in 6-well plates for colony formation. For shRNA or cDNA add back colony formation experiments, cells were treated as above, but with the addition of either puromycin or blasticidin in the media. For siRNA treatments, exponentially growing cells were reverse transfected in 24-well plates, and 2 days after transfection, cells were treated with rucaparib for 72 hours and then replated in 6-well plates for colony formation. For PI-B colony assays, cells were treated with PI-B (1.25 nmol/L) and rucaparib (100 nmol/L) for 72 hours and then replated into 6-well plates for colony formation. Colony formation was assessed 2 weeks postplating with crystal violet staining. Mean colony formation from three experiments was expressed as percentage of colonies $\pm$ SE relative to vehicle-treated cells.

Gene sequencing and RT-PCR analysis

Genomic DNA was isolated from cells using the DNeasy tissue kit (Qiagen). To determine whether gene rearrangements had taken place that would have excluded the exon 11q region from genomic DNA of cell lines and patient-derived xenograft (PDX) tumors, we carried out PCR using OneTaq Hot Start 2X Master Mix (NEB) and gDNA as templates. Primers were located in exon 10 (forward, F) and 12 (reverse, R): F:aatcaccct-caaggaacca; R:ctcacccagatgctgcttc. Total RNA was isolated from cell lines using RNeasy Kit (Qiagen). For quantitative RT-PCR, RNA was tested for quality on a Bioanalyzer (Agilent). RNA concentrations were determined with a spectrophotometer (NanoDrop; Thermo Fisher Scientific). RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Ambion) and a mixture of anchored oligo-dT and random decamers. Two reverse transcription reactions were performed for each sample using either 100 or 25 ng of input RNA. Assays were used in combination with TaqMan Universal Master mix or Power SybrGreen Master Mix and run on a 7900 HT sequence detection system (Applied Biosystems). Cycling conditions were 95°C, 15 minutes, followed by 40 (two-step) cycles (95°C, 15 seconds; 60°C, 60 seconds). C_t (cycle threshold) values were converted to quantities (in arbitrary units) using a standard curve (four points, 4-fold dilutions) established with a calibrator sample. For each sample, the values were averaged and SD of data derived from two independent PCRs. For the *BRCA1* splice variants, amplicons quantified by on-chip electrophoresis on an Agilent 2100 Bioanalyzer were used for absolute quantification assays. Primers that specifically recognize the following *BRCA1* mRNA isoforms were used: exon +11 containing: F:tagcaaggagccaacataacagat; R:cttattcattctttctctcacacag; Δ 11q: F:gattctgcaaaaaaggctgct; R:cgatgctgcttcacctga.

For *RBFOX2* F:aagccagtagtgagctgt, R:tgcttagggacacatctgctt; and *POLR2F*: F:tgccatgaaggaaactcaagg R:tcatagtccatctggcag. *BRCA1* expression values were routinely normalized to a *POLR2F* housekeeping gene (HKG) control and expressed as a percentage of the values calculated for MDA-MB-231 cells or to a relevant control sample.

BRCA1-minigene generation and analysis

Genomic DNA derived from blood lymphocytes of healthy individuals was tested for the absence of mutations by BROCA sequencing. The entire genomic region of *BRCA1* from exon 8 to exon 12 was amplified with the following 5 PCR reactions and primer sets:

1F: ccgctcgagAACCTTGGAACCTGTGAGAAGCTCTG
 1R: ccggatatCAATTTGAGAGCCAGTTTGAAT
 2F: ccgctcgagAACCTGGGTGACAGAGCAAGA
 2R: ccggatatcAGGGAAGACAGAGTCCTAATAAGA
 3F: ccgctcgagAGAGCTAAATGTTTGTCTTGGTC
 3R: ccggatatCTTTGATAAAATCCTCAGGATGAAG
 4F: ccgctcgagATTTGGGAAAACCTATCGGAAG
 4R: ccggatatTAATACTGGAGCCACTTCATTAGT
 5F: ccgctcgagCCAGCTCAAGCAATATTAATGAAGT
 5R: ccggatatGTAAATGTCACTCTGAGAGGATAGC

HA-tag was cloned into pENTRA vector using *Sall* and *XhoI* sites. Each *BRCA1* fragment was first cloned into pENTRA-HA vector using *XhoI* (shown in lowercase forward primers above) and *EcoRV* (shown in lowercase reverse primers above) sites. The 5 cloned fragments were assembled into a minigene using the following restriction sites that corresponded with cut sites in each fragment: *EcoRI*, *SpeI*, *StuI*, and *ScaI* sites. An eGFP fragment was cloned into the 3' end of the exon 12 fragment using *EcoRV* site. The minigene was then shuttled into pcDNA6.2 Destination vector using the LR Clonase system (Invitrogen). We confirmed by Sanger sequencing that mutations were not introduced into exons or introns within 500 bp flanking each exon. The indicated mutations (see Supplementary Fig. S3A) were introduced using site-directed mutagenesis (Agilent) using the following primers:

2288delT F: ccagtgaaactaaagaattgtcaacctagcctccaaga
 2288delT R: tcttggaggtaggttgacaaattcttaagttcactgg
 2529C>T F: caaatgtctgcacactaactcacattatttggttctgttttg
 2529C>T R: caaaaacagaaacaaataatgtgtgagttagtgtgcagcatttg
 3960C>T F: ctagggtgattgttctaagatgcctttgccaatattacc
 3960C>T R: ggtaattattggcaaggcatcttaggaacatcaccttag
 1stFOXMut F: tcagggtatgtctgtttcaacttacacgtggagcatgtg
 1stFOXMut R: cacatggctccagctgttaagttgaaacagaactacacctga
 2ndFOXMut F: gagtaataaactgctgttctgtgtgttaagtgagctggcagtagta
 2ndFOXMut R: tactcatgccagctcattacaacagcagacagcagttattactc
 Ex11qsplce F: tgcaagtttgaaacagaactccctgatactttctggatg
 Ex11qsplce R: catccagaaaagtatcaggggagtctgttcaaaactgca

To measure *BRCA1-Δ11q*-reporter expression, total RNA was isolated from transfected cells using RNeasy Kit (Qiagen). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems). PCR was performed using OneTaq Hot Start 2X Master Mix (NEB). Primers that specifically recognize *BRCA1-Δ11q*-reporter mRNA were: F: gattctgcaaaaaggctgct; R: agtctgtctgcttcattgtgtg; and BSD: F: gccttgtctcaagaagaatcca; R: tagcctccacacataacca. In addition, Western blots using HA antibody detected *BRCA1-Δ11q*-reporter protein expression.

Western blotting

Western blotting was carried out as described previously and proteins detected using the following antibodies: BRCA1: N-terminal: (MS110, EMD), C-terminal: (D9, Santa Cruz Biotechnology), BRCA1 (9010, Cell Signaling Technology), tubulin

(2148, Cell Signaling Technology), HA (23675, Cell Signaling Technology), RAD51 (H-92, Santa Cruz Biotechnology), Pol II [C-18, (Santa Cruz Biotechnology)], CtIP (A300-438-A, Bethyl Laboratories), PALB2 (A301-247A1, Bethyl Laboratories), BARD1 (A300-263A, Bethyl Laboratories), and BRCA2 (OP-95, EMD). HA (23675, Cell Signaling Technology) antibody was used for immunoprecipitation of HA-BRCA1 complexes from 2 mg of nuclear extract using Pierce Classic IP Kit (Thermo Scientific) according to the manufacturer's instructions. Nuclear extracts were derived using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Scientific) according to the manufacturer's instructions. Densitometric analyses were carried out using ImageJ software.

Immunofluorescence and microscopy

For immunofluorescence HA (HA.11, Covance), BRCA1 (MS110, EMD), γ -H2AX (pS139; N1-4131, EMD) and RAD51 (H-92, Santa Cruz Biotechnology), geminin (10802-1-AP, ProteinTech Group) and geminin (Abnova, E7071-1A8) antibodies were followed by secondary antibodies conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories). We acquired immunofluorescence images using Nikon NIU Upright Fluorescent Microscope and generated images using Nikon NIS Elements software. For IR experiments, we routinely fixed cells 8 hours after treatment with 10 Gy. For analyses, we counted a minimum of 200 cells per condition or cell line. Foci-positive cells were expressed as a percentage of geminin-positive cells to account for any differences in S-G₂-M populations between cell lines. Each experiment was carried out three times with biologic replicates. γ -H2AX IRIF was present equally in all cell lines and routinely measured as a positive control for IRIF.

Immunofluorescence and immunohistochemical analyses of tumors

For BRCA1 focus formation analysis, tissue sections were deparaffinized with xylene and hydrated with decreasing concentrations of ethanol. For target antigen retrieval, sections were microwaved in Dako Antigen Retrieval Buffer pH 9.0 for 4 minutes at 110°C [a T/T MEGA multifunctional Microwave Histoprocessor (Milestone)]. Sections were cooled down in distilled water for 5 minutes, and then permeabilized with Dako Wash Buffer containing Tween 20 for 5 minutes, followed by incubation in blocking buffer (Dako Wash Buffer with 1% BSA) for 5 minutes. Primary antibodies were diluted in Dako Antibody Diluent and incubated at room temperature for 1 hour [anti-BRCA1 Abcam MS110 diluted 1:200; anti-Geminin (ProteinTech Group, 10802-1-AP, diluted 1:400)]. Sections were washed for 5 minutes in Dako Wash Buffer followed by 5 minutes in blocking buffer. Secondary antibodies (Alexa Fluor 488 or 568) were diluted 1:500 in blocking buffer and incubated for 30 minutes at room temperature. The two-step washing was repeated followed by 5 minutes incubation in distilled water. Dehydration was performed with increasing concentrations of ethanol. Sections were mounted with DAPI ProLong Gold antifade reagent and stored at -20°C. For analyses, tumors from 2 mice per treatment group were analyzed and counted. For each tumor, a minimum of 100 geminin-positive cells were identified and counted for those that had at least 5 BRCA1 foci. BRCA1 foci-positive cells were calculated as a percentage of geminin-positive cells.

For assessment of Ki-67 and γ -H2AX by IHC, slides were deparaffinized and hydrated. Antigen retrieval was performed using pH 9 EDTA buffer (DAKO, S2368). Endogenous peroxidases were quenched by immersion of slides in 3% hydrogen peroxide solution (30% H_2O_2 , Fisher BP2633-500, diluted in methanol). Primary antibody Ki-67 (Clone EP5, Epitomics, 1:1,500), or γ -H2AX (pS139; N1-4131, EMD; 1:20,000) were diluted with DaVinci Green Diluant (Biocare Medical, PD900) and incubated on slides overnight at 4 degrees in a humidified slide chamber. Slides were then washed 3 times in TBST and incubated with Envision+ System HRP Labelled Polymer Anti-Rabbit (Dako, K4003) for 1 hour at room temperature. Specimens were washed 3 times in TBST and then developed with DAB solution (Dako, K3468) and counterstained in Meyer Hematoxylin (Dako, S3309). For analyses of Ki67 and γ -H2AX expression, a minimum of 2 tumors derived from 2 separate mice were used. Mice were treated with rucaparib 150 mg/kg two times per day for 4 continuous days. For cisplatin, mice were treated with a single dose of 6 mg/kg. Tumors were resected and formalin fixed 4 days from the first dose. A minimum of 2 tumors and 3 images per tumor were used to calculate staining intensities. Image staining intensities were measured using ImageJ analysis software.

PDX tumor derivation, xenograft treatments, and analyses

For PDXs, patient consent for tumor use in animals was completed under a protocol approved by the Vall d'Hebron Hospital Clinical Investigation Ethical Committee and Animal Use Committee. Mice were maintained and treated in accordance with institutional guidelines of Vall d'Hebron University Hospital Care and Use Committee. Tumors were subcutaneously implanted in 6-week-old female HsdCpb:NMRI-Foxn1^{nu} mice (Harlan Laboratories). Animals were supplemented with 1 $\mu\text{mol/L}$ estradiol (Sigma) in the drinking water. Upon xenograft growth, tumor tissue was reimplanted into recipient mice, which were randomized upon implant growth. Mice were allocated in control/treated groups to deliver similar mean and SE when tumor volume reached between 150 and 300 mm^3 without any blinding. Vehicle or olaparib was administered at 50 mg/kg per oral gavage 6 days per week. Tumor xenografts were measured with calipers and tumor volumes were determined using the formula: $(\text{length} \times \text{width}^2)$. At the end of the experiment, animals were killed by CO_2 inhalation. Tumor volumes are plotted as mean $\pm$ SEM. Relative tumor volume RTV for vehicle-treated mice and individual RTVs are shown for olaparib-treated mice. For xenograft studies, MDA-MB-436 cells were subcutaneously implanted in 6-week-old female NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ). When tumors reached approximately 300 mm^3 , tumors were harvested and cut up into smaller pieces followed by subcutaneous reimplantation. Treatment was initiated when tumors reached between 150 and 180 mm^3 . Rucaparib was administered at 150 mg/kg twice daily for 10 continuous days with a 2-day break after the first 5 days. Cisplatin was administered at a single dose of 6 mg/kg. Tumors were measured with calipers and tumor volumes calculated as described above. Measurements were carried out every 2 days and mice were euthanized when tumors reached 1,500 mm^3 in accordance with institutional guidelines of Fox Chase Cancer Center (Philadelphia, PA).

Statistical analyses

Mean and SE values were compared using unpaired *t* tests (GraphPad Software). *P* < 0.05 was considered statistically sig-

nificant. Asterisks indicate statistically significant *P* values. There were similar variances between statistical groups compared. All statistical tests were unpaired *t* tests unless otherwise stated next to the *P* value.

Results

Cell lines with exon 11 mutations express BRCA1-Δ11q

To examine the impact of *BRCA1* exon 11 mutations on the expression of *BRCA1* proteins, we employed a panel of human cancer cell lines that were either *BRCA1* wild-type or harbored known deleterious *BRCA1* mutations (Supplementary Fig. S2A). Full-length *BRCA1* protein was detectable in MDA-MB-231, MCF7, and MDA-MB-468 wild-type *BRCA1* cell lines, but was absent in all *BRCA1* mutation-containing cell lines. A band corresponding with the predicted molecular weight (~89 kDa) of the *BRCA1*-Δ11q isoform was present in *BRCA1* wild-type cell lines, as well as L56Br-C1, SUM149PT, and UWB1.289 cell lines that harbored *BRCA1* exon 11 frameshift mutations (Fig. 1A).

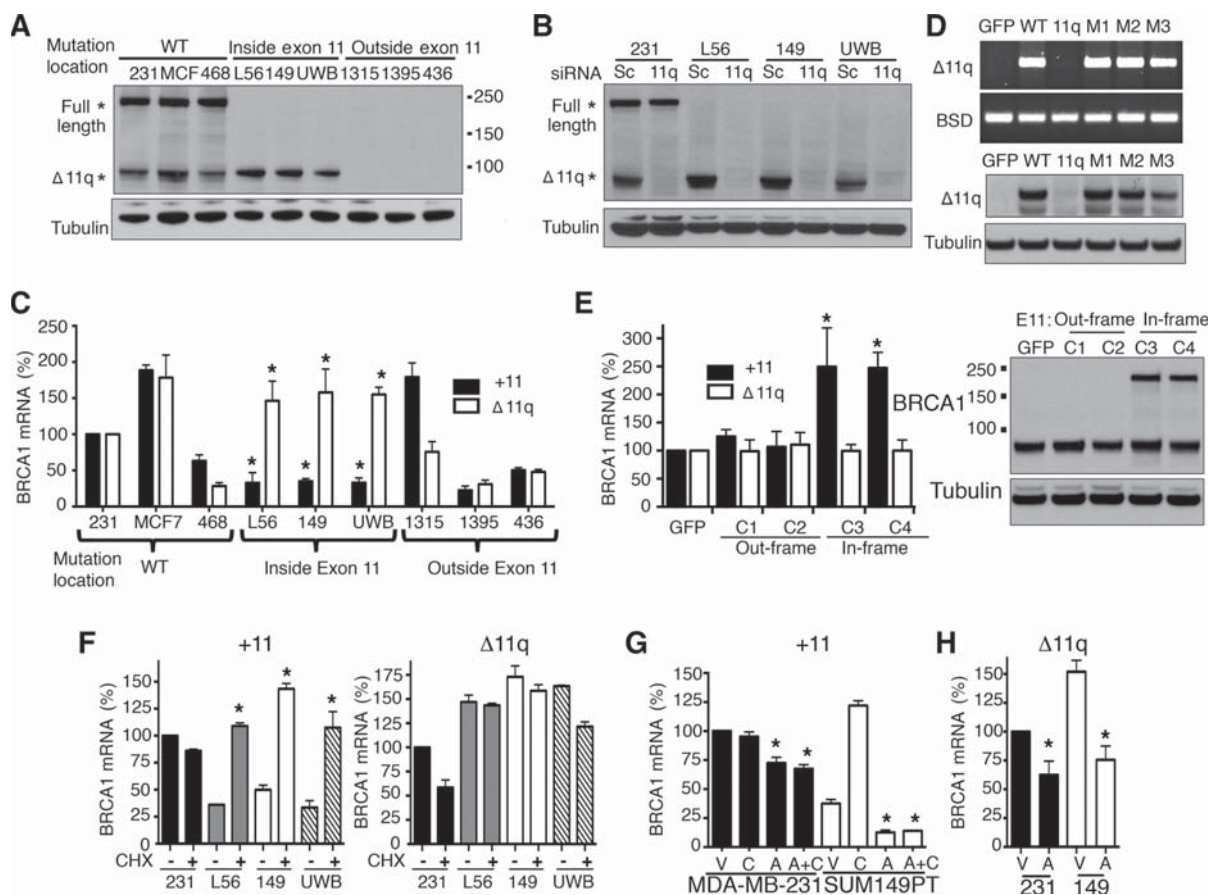
To establish the identity of the isoform detected below the 100-kDa mark, we transfected cells with a siRNA designed to specifically target *BRCA1*-Δ11q and measured protein expression. *BRCA1*-Δ11q siRNA did not impact the levels of the full-length, but dramatically reduced the expression of the predicted *BRCA1*-Δ11q isoform (Fig. 1B). Furthermore, we confirmed that cells did not harbor secondary *BRCA1* reversion mutations or genomic rearrangements (Supplementary Fig. S2).

Next, we investigated the levels of *BRCA1* mRNA containing exon 11 (+11) as well as the *BRCA1*-Δ11q (Δ11q) isoform expression. L56Br-C1, SUM149PT, and UWB1.289 exon 11-mutant cell lines exhibited 3- (*P* < 0.001), 3- (*P* < 0.001), and 2.9-fold (*P* < 0.001) lower expression of +11 transcripts, respectively, relative to MDA-MB-231 cells (Fig. 1C). In contrast, relative Δ11q expression was 1.46- (*P* = 0.0148), 1.55- (*P* < 0.001), and 1.58-fold (*P* = 0.0136) higher in L56Br-C1, SUM149PT, and UWB1.289, respectively, relative to MDA-MB-231 cells expression levels (Fig. 1C).

Robust *BRCA1* gene transcription promotes *BRCA1*-Δ11q expression

To investigate the impact of germline mutations on exon 11 splicing, we introduced a series of exon 11 frameshift mutations into a *BRCA1*-minigene system (Supplementary Fig. S3). Mutation of the exon 11q splice junction disrupted Δ11q-reporter mRNA and protein expression. However, three different frameshift mutations at various locations throughout exon 11 had no effect on Δ11q-reporter expression (Fig. 1D). Moreover, RNA-seq analyses of mRNA splice junctions did not indicate that higher levels of *BRCA1*-Δ11q expression in cell lines with exon 11 mutations were due to increased global alterations in splicing (Supplementary Table S1).

To further investigate the effects of exon 11 mutations on *BRCA1*-Δ11q levels, we used CRISPR/Cas9 technology to generate SUM149PT cells that either had additional out of frame mutations, or reversion mutations that restored the reading frame (Supplementary Fig. S4). In line with minigene data, the introduction of additional out-of-frame or frame-restoring mutations in exon 11 did not affect *BRCA1*-Δ11q expression (Fig. 1E). However, clones 3 and 4 that had restored *BRCA1* reading frames, expressing the *BRCA1*-long-form protein product, had 2.5- (*P* < 0.0001) and 2.47-fold (*P* < 0.0001) increased levels of +11

**Figure 1.**

BRCA1 exon 11-mutant cell lines express *BRCA1*-Δ11q. A, cell lines were analyzed for *BRCA1* and tubulin levels by Western blot analysis. *, predicted *BRCA1* locations, molecular weights are indicated. B, cells were treated with scrambled (Sc) or *BRCA1*-Δ11q (11q) siRNA and analyzed by Western blot analysis. C, exon 11 containing (+11) *BRCA1* transcripts and the *BRCA1*-Δ11q (Δ11q) isoform were detected using qRT-PCR. Values were normalized to a HKG, expressed as a percentage of MDA-MB-231 cells. D, 293T cells were transfected with either GFP-control or *BRCA1*-minigene reporter constructs that were wild-type (WT) or carrying mutations that disrupted the cryptic 11q splice site (11q), or with frameshift mutations (M1:2288delT; M2:2529C>T; M3:3960C>T). *BRCA1*-Δ11q-reporter mRNA and protein expression was measured by RT-PCR (top) and Western blot analysis (bottom); see Supplementary Fig. S3. E, CRISPR/Cas9 targeting the mutation-containing region of exon 11 (sg_exon11)-generated SUM149PT clones (C) 1-4; see Supplementary Fig. S4. +11 and Δ11q mRNA was measured using qRT-PCR (left); values were normalized to a HKG and expressed as a percentage of sg_GFP control cells. *BRCA1* protein was detected by Western blot analysis (right). F, the indicated cell lines were treated with vehicle or 10 μg/mL CHX for 5 hours, followed by assessment of +11 (left) and Δ11q (right) levels by qRT-PCR. G, cells were treated with either vehicle (V), 10 μg/mL CHX (C), 5 μg/mL ACT (A), or A and C simultaneously for 5 hours and assessed for +11 expression by qRT-PCR. H, cells were treated with either vehicle (V) or 5 μg/mL ACT (A) for 5 hours and assessed for Δ11q expression by qRT-PCR. *, $P < 0.05$.

expression, respectively, relative to sg_GFP control cells (Fig. 1E), likely resulting from premature translation termination codon (PTC) removal, enabling +11 containing transcripts to avoid NMD (3).

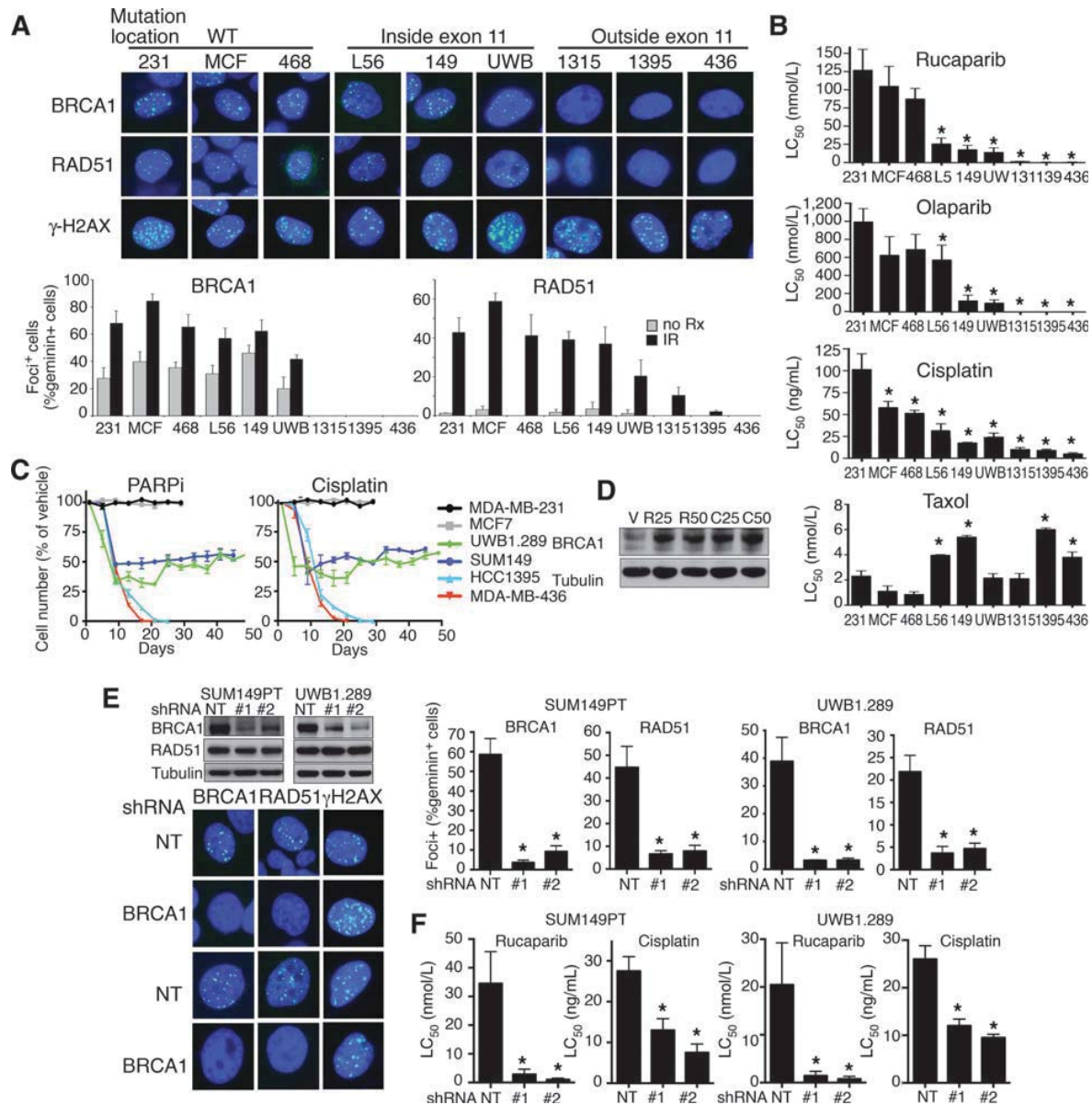
We confirmed that NMD contributed to the low levels of +11 mRNA detected in exon 11 mutation containing cell lines by treating cells with CHX, an inhibitor of NMD (Fig. 1F; ref. 38). CHX treatment for 5 hours resulted in a 2.8- ($P = 0.0007$), 3.2- ($P = 0.023$), and 2.9-fold ($P = 0.0024$) increase in +11 levels in L56Br-C1, SUM149PT, and UWB1.289 exon 11-mutant cell lines, respectively, but did not affect +11 levels in wild-type MDA-MB-231 cells, or Δ11q mRNA levels in any of the cell lines (Fig. 1F).

The increase in +11 mRNA resulting from inhibition of NMD led us to predict that exon 11-mutant cell lines have robust *BRCA1* gene transcription. To test this possibility, we treated

cells with the transcription inhibitor ACT and measured *BRCA1* mRNA levels. MDA-MB-231 cells treated with ACT had a 1.4-fold ($P = 0.0002$) reduction in +11 mRNA levels compared with vehicle-treated cells. However, ACT treatment reduced +11 levels 3-fold ($P = 0.0133$) in SUM149PT cells compared with vehicle-treated cells, and completely abrogated the CHX-mediated increase in +11 mRNA (Fig. 1G). ACT also reduced Δ11q levels 1.6-fold ($P = 0.002$) and 2-fold ($P = 0.021$) in MDA-MB-231 and SUM149PT cells, respectively, compared with vehicle treatments (Fig. 1H).

Exon 11-mutant cells demonstrate partial PARPi and cisplatin resistance

We next measured the ability of exon 11-mutant cells to form *BRCA1* and RAD51 foci. *BRCA1* wild-type as well as exon 11-

**Figure 2.**

Exon 11-mutant cells are less sensitive to PARPi and cisplatin treatment. A, cells were untreated (no Rx) or treated with IR (10 Gy) and subject to immunofluorescence to detect BRCA1, RAD51, and γ-H2AX foci; representative images of IR-treated cells. Mean ± SEM foci-positive cells are expressed as a percentage of total geminin-positive cells. B, cell lines were treated with rucaparib, olaparib, cisplatin, or taxol and colony formation assessed; graphs represent three independent experiments, mean ± SEM LC₅₀ values; see Supplementary Table S2 for fold changes and *P* values. C, cells were maintained in the presence of vehicle, 100 nmol/L rucaparib (left), or 20 ng/mL cisplatin (right) and counted every 4 days. Cell line growth was expressed as a percentage of vehicle-treated cell numbers counted on the same day. Mean ± SEM from three technical replicates. D, UWB1.289 vehicle-, rucaparib (R)-, and cisplatin (C)-treated cell lysates were collected at days 25 and 50 for Western blot analysis. E, assessment of BRCA1, RAD51, and γ-H2AX foci by immunofluorescence as for A. Western blot analysis (top) shows BRCA1-Δ11q was depleted using two individual BRCA1 targeting shRNAs. Representative images (bottom) of IR-treated cells and mean ± SEM foci-positive cells are expressed as a percentage of total geminin-positive cells (right). F, cells described in E were treated with rucaparib or cisplatin and colony formation assessed; three independent experiments, mean ± SEM; LC₅₀ concentrations are shown; see Supplementary Table S2. *, *P* < 0.05.

mutant cell lines formed robust BRCA1 and RAD51 γ-irradiation-induced foci (IRIF). In contrast, BRCA1 and RAD51 IRIF were low or undetectable in cells that harbored *BRCA1* mutations outside of exon 11 (Fig. 2A).

SUM1315MO2, HCC1395, and MDA-MB-436 cell lines were highly sensitive to treatment with the PARPi rucaparib and olaparib. In contrast, exon 11 mutation containing L56Br-C1, SUM149PT, and UWB1.289 cells displayed intermediate PARPi

sensitivity. SUM1315MO2, HCC1395, and MDA-MB-436 cell lines were also more sensitive to cisplatin treatment compared with L56Br-C1, SUM149PT, and UWB1.289 cells. The presence of *BRCA1* exon 11 mutations did not impact taxol sensitivity (Fig. 2B; Supplementary Table S2).

In cell growth experiments, *BRCA1* wild-type cells continuously cultured in the presence of PARPi or cisplatin proliferated at the same rate as vehicle-treated cells (Fig. 2C). Exon 11-mutant cell lines proliferated at a reduced rate in the presence of PARPi or cisplatin (Fig. 2C). MDA-MB-436 and HCC1395 cell lines harbor BRCT domain mutations and lost viability in the presence of PARPi or cisplatin. PARPi and cisplatin initially had a greater impact on the proliferation rate of UWB1.289 cells, but over time proliferation gradually increased, and corresponded with increased expression of *BRCA1*- Δ 11q (Fig. 2D). Importantly, depletion of the *BRCA1*- Δ 11q using 2 *BRCA1* shRNAs reduced

RAD51 IRIF 6.8- ($P = 0.0021$) and 5.7-fold ($P = 0.0027$) in SUM149PT, as well as 6- ($P = 0.0014$) and 4.7-fold ($P = 0.0016$) in UWB1.289 cells, compared with nontarget (NT) shRNA cells (Fig. 2E). Furthermore, *BRCA1* shRNA sensitized SUM149PT and UWB1.289 cells to both PARPi and cisplatin treatments (Fig. 2F; Supplementary Table S2).

BRCA1- Δ 11q is functional but inferior to *BRCA1* full-length

To more finely assess the ability of *BRCA1*- Δ 11q to provide resistance to therapy, we used CRISPR/Cas9-mediated gene editing to modify *BRCA1* in SUM149PT cells (Supplementary Fig. S4). SUM149PT cells were subject to an sg_GFP control, or sg_exon11 targeting the mutation-containing region of exon 11. Mutations introduced by sg_exon11 were either out of frame (out-frame) or restored the *BRCA1* reading frame (in-frame). Moreover, cell lines that expressed sg_exon22 demonstrated frameshift mutations in

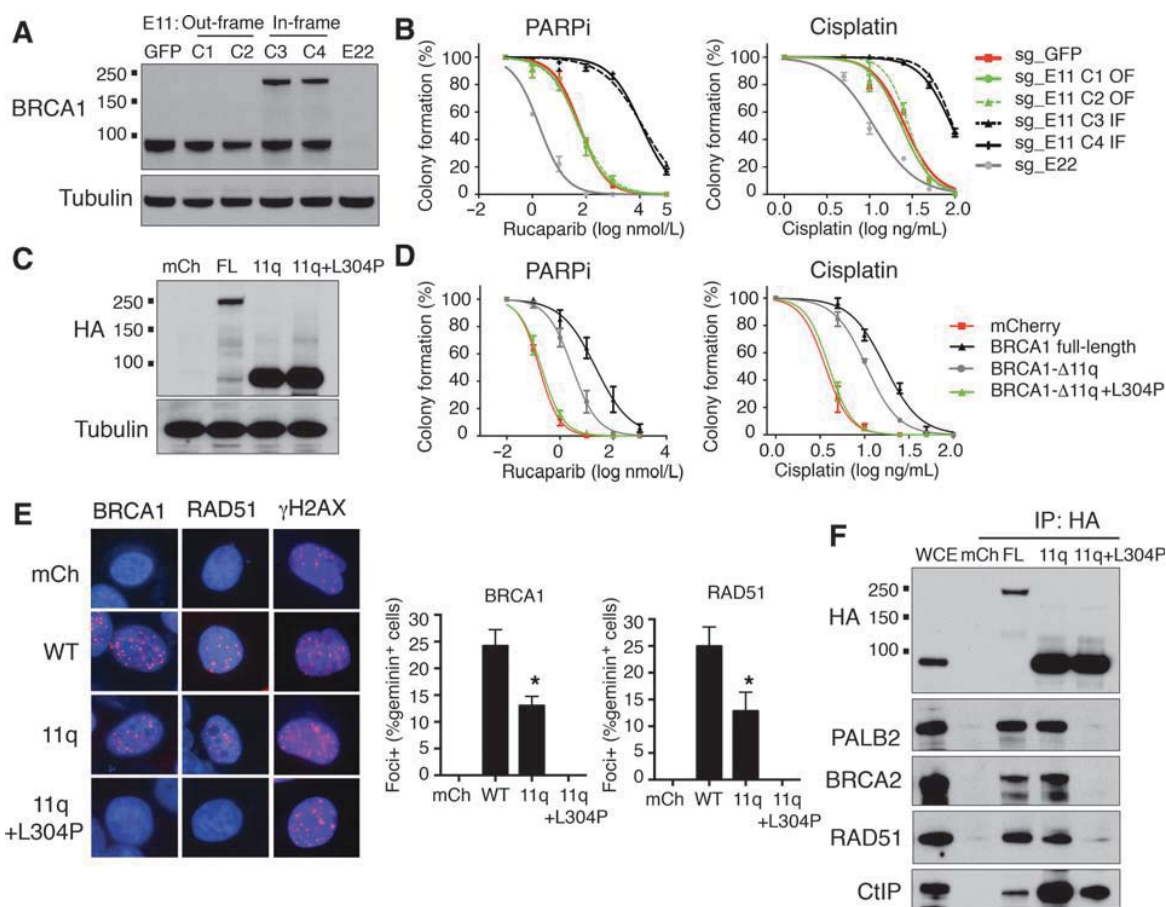
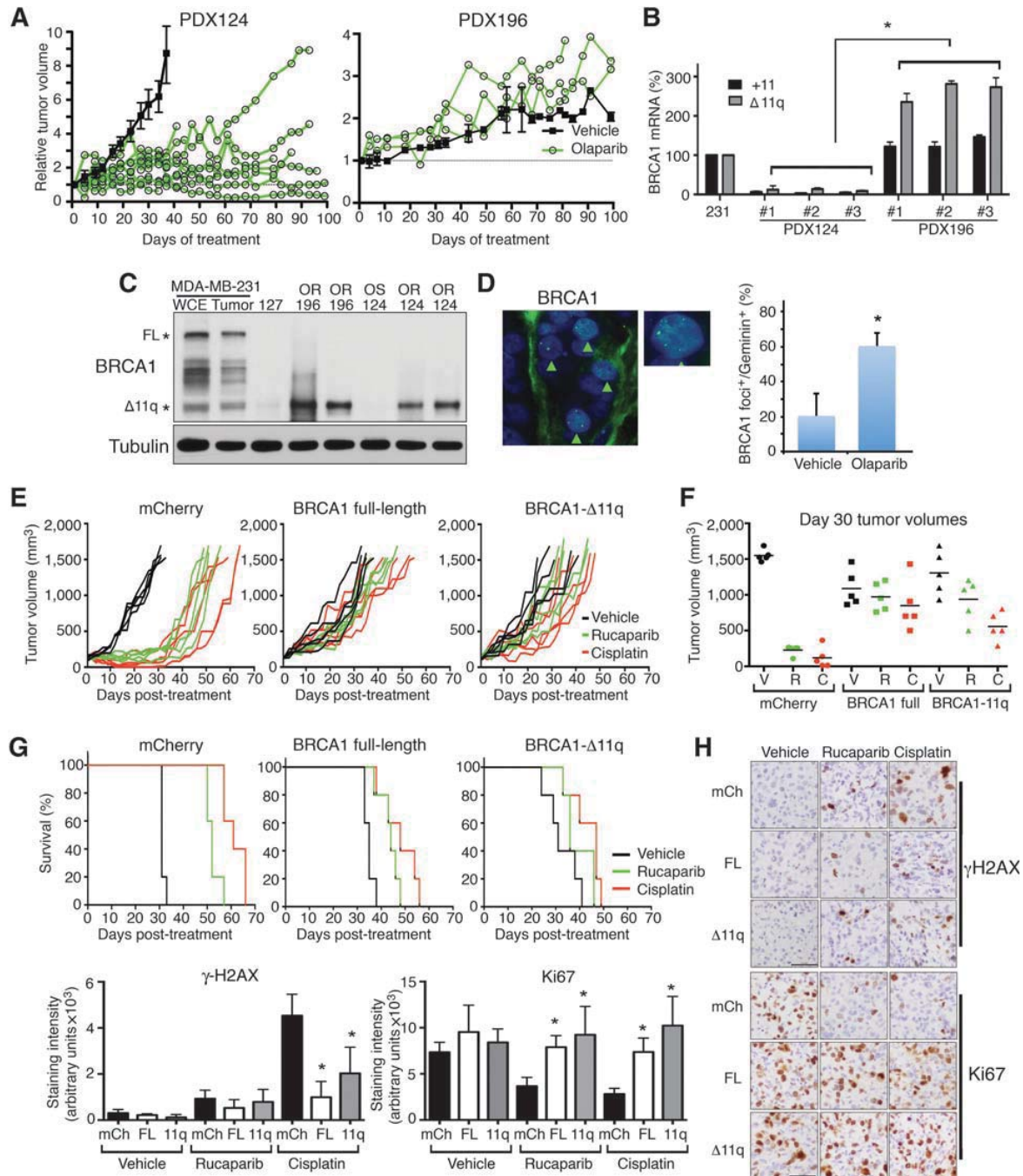


Figure 3.

BRCA1- Δ 11q provides partial resistance to therapy *in vitro*. A, CRISPR/Cas9 gene targeting the SUM149PT *BRCA1* mutation-containing region of exon 11 (sg_exon11) did not affect the reading frame (OF) in clones 1, 2, or restored the reading frame (IF) in clones 3, 4. Targeting of exon 22 (sg_exon22) resulted in frameshift mutations and loss of *BRCA1* expression. Cells treated with sg_GFP were used as a control. *BRCA1* protein was detected by Western blot analysis. See Supplementary Fig. S4 for more details. B, cells described in A were treated with rucaparib or cisplatin and colony formation assessed. C, MDA-MB-436 cells expressing mCherry, *BRCA1* full-length, *BRCA1*- Δ 11q, or *BRCA1*- Δ 11q+L304P were assessed for *BRCA1* protein expression by Western blot analysis. D, cells described in C were treated with rucaparib or cisplatin and colony formation assessed. E, cells described in C were treated with IR (10 Gy) and subject to immunofluorescence to detect *BRCA1*, RAD51, and γ -H2AX foci, as well as geminin and DAPI staining; representative images of IR-treated cells. Double geminin and *BRCA1*- or RAD51-positive cells were counted and expressed as a percentage of total geminin-positive cells; bars show mean $\pm$ SEM of geminin+*BRCA1* or RAD51 foci-positive cells from three independent experiments. F, cells described in C were subject to immunoprecipitation using an anti-HA antibody and Western blotting with the indicated antibodies. *, $P < 0.05$.

**Figure 4.**

BRCA1-Δ11q promotes resistance *in vivo*. **A**, PDX124 ($n \geq 8$ mice; left) and PDX196 ($n \geq 3$ mice; right) tumors were treated with vehicle (black lines) or olaparib (green lines) and tumor volume measured. **B**, PDX tumors were harvested from three individual untreated mice and assessed for +11 and Δ11q expression by qRT-PCR. Values were normalized to the *POL2RF* HKG control and expressed as a percentage of the values calculated for MDA-MB-231 cells. **C**, MDA-MB-231 cells were prepared for whole cell extract (WCE) as well as tumor xenografts and used as positive controls, and compared with PDX127 (*BRCA1*^{B5delAG} mutant control, $n = 1$), PDX196 ($n = 2$), PDX124 OS (growth inhibition with olaparib, $n = 1$), and OR (growth slowed with olaparib, $n = 2$) tumors for Western blotting. **D**, mice harboring PDX196 tumors were treated with vehicle or olaparib and tumors assessed for BRCA1 foci formation as well as geminin staining; representative images (left) and quantification of BRCA1 mean $\pm$ SEM foci geminin-positive cells. **E**, MDA-MB-436 tumor xenografts expressing mCherry, BRCA1 wild-type, and BRCA1-Δ11q were treated with vehicle (black lines), rucaparib (green lines), or cisplatin (red lines) and tumor growth measured; lines represent individual tumors and mice ($n = 5$). **F**, individual tumor volumes at day 30 are shown. **G**, Kaplan-Meier survival analyses for mice described in **E**. **H**, Mice were treated as in **E** for 4 days, tumors were assessed for γ -H2AX and Ki67 staining by IHC. Representative images, scale bars, 50 μ m. Mean $\pm$ SEM quantification of staining intensities. *, $P < 0.05$.

exons 22, resulting in loss of BRCA1- $\Delta 11q$ expression (Fig. 3A and Supplementary Fig. S4). BRCA1- $\Delta 11q$ expression in sg_exon11-treated clones with unrestored reading frames was identical to sg_GFP control cells and there were no differences in PARPi and cisplatin sensitivity in colony formation assays. However, sg_exon11 in-frame clone 3 was 226- ($P = 0.0174$) and 3.7-fold ($P = 0.0009$) more resistant to PARPi and cisplatin, respectively, and clone 4 was 207- ($P < 0.0001$) and 3.6-fold ($P = 0.0058$) more resistant to PARPi and cisplatin, respectively, compared with sg_GFP cells. In contrast, sg_exon22 cells were 31.4- ($P = 0.0183$) and 2.3-fold ($P = 0.0059$) more sensitive to PARPi and cisplatin, respectively, compared with sg_GFP cells (Fig. 3B).

We also compared the ability of ectopic BRCA1 full-length and BRCA1- $\Delta 11q$ to rescue PARPi and cisplatin sensitivity (Fig. 3C). MDA-MB-436 cells harbor a *BRCA1*^{5396+1G>A} mutation that results in protein misfolding, undetectable BRCA1 protein and RAD51 IRIF, as well as exquisite PARPi and cisplatin sensitivity (20, 39, 40). MDA-MB-436 cells expressing BRCA1 full-length demonstrated robust rescue and were 179- ($P = 0.0063$) and 4.6-fold ($P = 0.0002$) more resistant to PARPi and cisplatin, respectively, compared with mCherry control cells. BRCA1- $\Delta 11q$ was less effective at rescue, and cells were 19- ($P = 0.0493$) and 2.8-fold ($P = 0.0004$) more resistant to PARPi and cisplatin, respectively, compared with mCherry control cells (Fig. 3D). Moreover, when we introduced an L304P mutation (equivalent to L1407P in full-length BRCA1) that blocks the BRCA1-PALB2 interaction (41), BRCA1- $\Delta 11q$ -mediated PARPi and cisplatin rescue was abolished (Fig. 3D).

Both BRCA1 full-length and BRCA1- $\Delta 11q$ -expressing cells formed BRCA1 and RAD51 IRIF. However, BRCA1- $\Delta 11q$ -expressing cells exhibited 1.9- ($P = 0.0053$) and 1.9-fold ($P = 0.0141$) lower levels of BRCA1 and RAD51 IRIF, respectively, than full-length BRCA1-expressing cells, while BRCA1- $\Delta 11q$ + L304P-expressing cells had undetectable BRCA1 and RAD51 IRIF (Fig. 3E). Immunoprecipitation of ectopic BRCA1 from MDA-MB-436 cells suggested that BRCA1- $\Delta 11q$ interacted, although less efficiently, with functional protein partners for full-length BRCA1 (Fig. 3F). We also demonstrated ectopic BRCA1- $\Delta 11q$ provided

therapy resistance in HCC1395 cells and in a mouse embryonic stem cell system (Supplementary Fig. S5; ref. 42).

BRCA1- $\Delta 11q$ promotes resistance *in vivo*

To examine the significance of *BRCA1- $\Delta 11q$* expression in tumors, we first measured PARPi responsiveness and *BRCA1* isoform expression in two individual *BRCA1* exon 11-mutant PDX models. Despite both PDX models harboring the same *BRCA1*-exon 11 2080delA mutation (Supplementary Fig. S2), PDX124 tumors were sensitive and PDX196 tumors were resistant to olaparib treatment (Fig. 4A). We confirmed that tumors did not have reversion mutations or gene rearrangements (Supplementary Fig. S2). Similar to exon 11-mutant cell lines, both PDX124 and PDX196 expressed higher levels of $\Delta 11q$ compared with +11 mRNA, relative to MDA-MB-231 control cells. However, *BRCA1* mRNA levels were significantly greater in PDX196 tumors than in PDX124 tumors, with 26- ($P = 0.0001$) and 23-fold ($P < 0.0001$) higher levels of both +11 and $\Delta 11q$, respectively (Fig. 4B). BRCA1- $\Delta 11q$ protein expression was detectable in olaparib-resistant PDX196 tumors, as well as PDX124 tumors that eventually grew through olaparib treatment (Fig. 4C). BRCA1 foci formation was also readily detectable in PDX196 tumors (Fig. 4D).

To further assess the impact of BRCA1- $\Delta 11q$ on therapy resistance *in vivo*, we utilized the MDA-MB-436 isogenic cell line panel for xenograft experiments. At 30 days post tumor implantation, rucaparib or cisplatin treatment delayed mean tumor growth 6.8- ($P < 0.0001$) and 12.9-fold ($P < 0.0001$), respectively, compared with vehicle-treated mice with mCherry-overexpressing MDA-MB-436 tumors. In contrast, rucaparib and cisplatin treatment delayed mean tumor growth 1.1-fold ($P = 0.4455$) and 1.28-fold ($P = 0.2537$) in BRCA1 wild-type and 1.4-fold ($P = 0.0872$) and 2.3-fold ($P = 0.019$) in BRCA1- $\Delta 11q$ -overexpressing tumors, respectively, compared with vehicle-treated mice (Fig. 4E and F).

Kaplan-Meier analyses indicated that the median overall survival (OS) of mice harboring mCherry-expressing tumors that were treated with rucaparib or cisplatin increased 1.7-fold ($P = 0.0016$, log-rank test) and 2-fold ($P = 0.0016$, log-rank test), respectively, compared with vehicle treated mice. In contrast, in

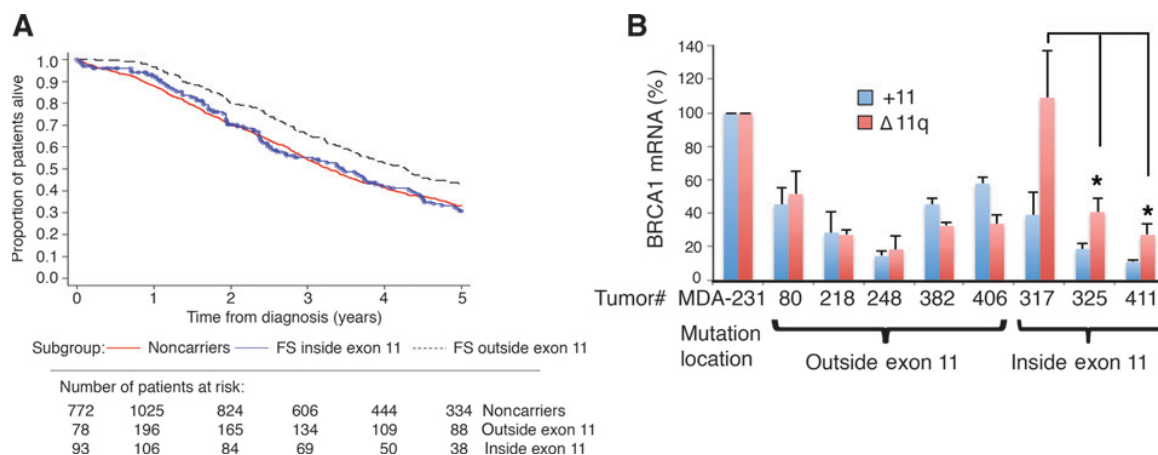
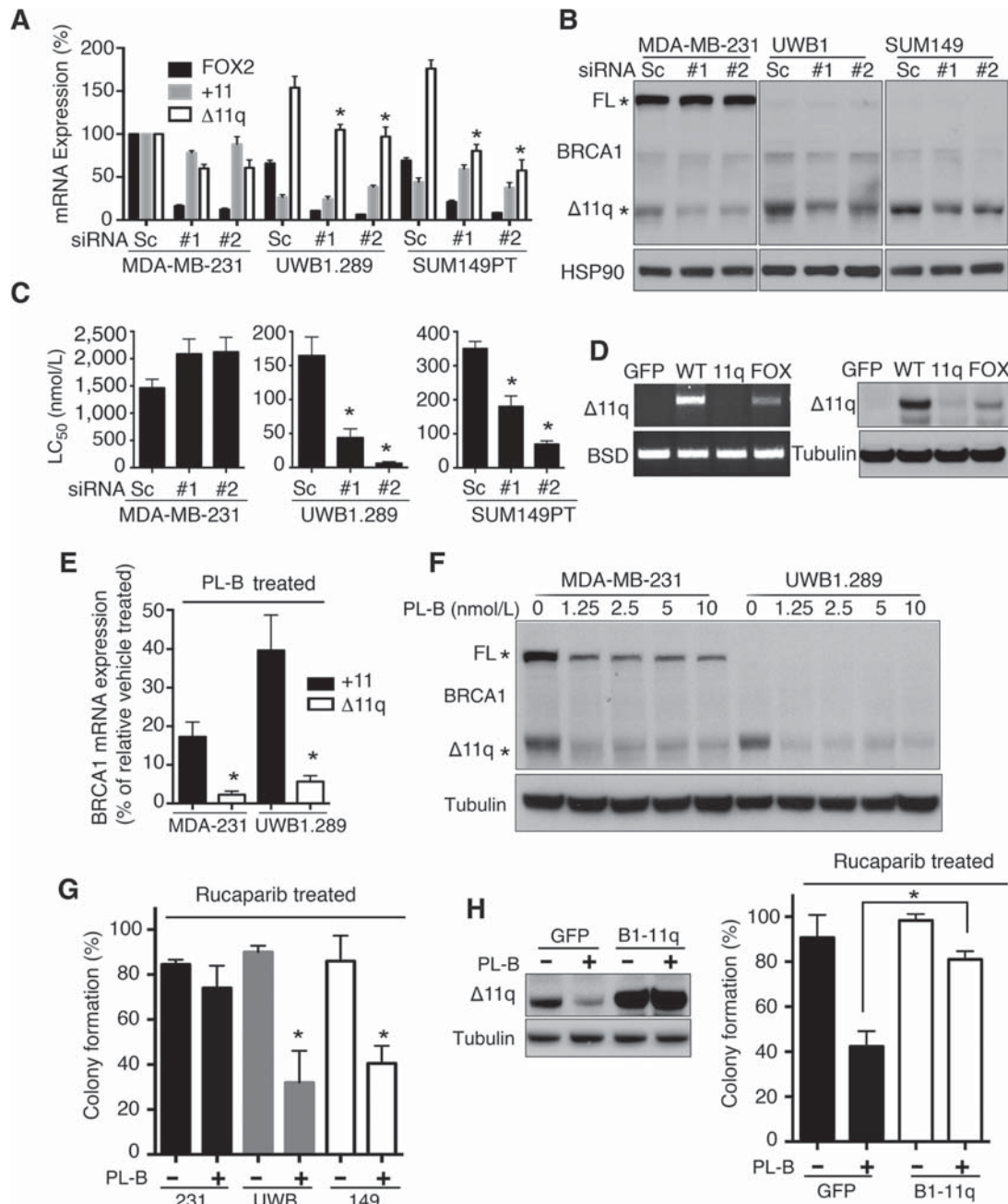


Figure 5.

BRCA1 exon 11 mutations and patient survival. A, Kaplan-Meier estimates of cumulative survival according to *BRCA1* mutation group of serous ovarian cancer patients (see Supplementary Tables S3 and S4). B, primary breast and ovarian cancer patient tumors (unrelated to studies described in A) were subject to qRT-PCR analysis for +11 and $\Delta 11q$ expression. Values were normalized to a HKG control and expressed as a percentage of the values calculated for MDA-MB-231 cells. *, $P < 0.05$.

**Figure 6.**

Splicing inhibition sensitizes exon 11-mutant cells to PARPi. A, MDA-MB-231, UWB1.289, and SUM149PT cells were transfected with scrambled (Sc) or FOX2#1 and FOX2#2 siRNA and FOX2, POLR2F, +11 and Δ11q *BRCA1* isoform mRNA levels measured by qRT-PCR. Values were normalized to *POLR2F* HKG expression and expressed as a percentage of MDA-MB-231 cells. B, cells treated as in A were subject to Western blotting. C, cells treated as in A were subject to 3-day rucaparib and cisplatin exposure and reseeded for colony formation. Mean ± SEM. LC₅₀ values are shown. D, 293T cells were transfected with BRCA1 minigene reporter constructs as in Fig. 1D and Supplementary Fig. S3, with FOX2-binding sites mutated (FOX). BRCA1-Δ11q-reporter construct mRNA and protein expression was measured by RT-PCR (left) and Western blot analysis (right), respectively. E, MDA-MB-231 and UWB1.289 cells incubated with PL-B (10 nmol/L) and mRNA levels measured by qRT-PCR. Values were expressed as a percentage of vehicle-treated MDA-MB-231 cells or UWB1.289 cells. F, cells were treated with increasing concentrations of PL-B and subject to Western blotting. G, cells were treated with vehicle (–) or PL-B (+; 1.25 nmol/L) and either vehicle or rucaparib (100 nmol/L) for 72 hours and reseeded for colony formation assay; mean ± SEM colony formation of rucaparib-treated cells calculated as a percentage of vehicle-treated cells. H, SUM149PT cells engineered to ectopically express GFP or BRCA1-Δ11q were treated as in G and assessed for colony formation. Western blot analysis (left) and mean ± SEM colony formation of rucaparib-treated cells, calculated as a percentage of vehicle-treated cells (right). *, *P* < 0.05.

mice harboring BRCA1 wild-type tumors that were treated with rucaparib or cisplatin, median OS increased 1.3-fold ($P = 0.0071$, log-rank test) and 1.4-fold ($P = 0.0048$, log-rank test), respectively, and in mice harboring BRCA1- $\Delta 11q$ tumors treated with rucaparib or cisplatin median OS increased 1.2-fold ($P = 0.1536$, log-rank test) and 1.5-fold ($P = 0.0333$, log-rank test), respectively, compared with vehicle-treated mice (Fig. 4G).

Short-term assessment of pharmacodynamics markers showed that rucaparib increased γ -H2AX positivity similarly in all tumors, but wild-type and BRCA1- $\Delta 11q$ tumors had 4.5- ($P < 0.0001$) and 2.2-fold ($P = 0.005$) lower γ -H2AX positivity, respectively, after cisplatin treatment compared with mCherry-expressing tumors. In addition, wild-type and BRCA1- $\Delta 11q$ tumors treated with rucaparib had 2.2- ($P = 0.0007$) and 2.5-fold ($P = 0.0059$) higher Ki67 positivity, respectively; and tumors treated with cisplatin had 2.6- ($P < 0.0001$) and 3.7-fold ($P = 0.0003$) higher Ki67 positivity, respectively, compared with mCherry-expressing tumors (Fig. 4G). In contrast to *in vitro* data, the degree of PARPi or cisplatin rescue afforded by BRCA1- $\Delta 11q$ overexpression was more similar to BRCA1 full-length *in vivo*.

Assessment of BRCA1 exon 11 mutations and patient survival

Because exon 11-mutant tumors were capable of expressing BRCA1- $\Delta 11q$, we assessed survival outcomes of patients with frameshift mutations located inside (IE11) versus outside (OE11) of exon 11. We analyzed 5-year OS data from the time of initial diagnosis in patients with serous ovarian carcinoma from a previously reported study (43). Here, participants with BRCA1 frameshift mutations OE11 ($n = 231$, 43%; 95% CI, 36%–49%) presented better OS than noncarriers ($n = 1,333$, 33%; 95% CI, 30%–36%; $P = 0.002$, log-rank test) as well as participants with frameshift mutations IE11 ($n = 148$, 31%; 95% CI, 23%–39%; $P = 0.02$, log-rank test) at 5 years of follow up. Moreover, participants with frameshift mutations IE11 presented with 5-year OS similar to noncarriers ($P = 0.84$, log-rank test; Fig. 5A, Supplementary Tables S3 and S4).

Although patients with exon 11 mutations demonstrated worse 5-year survival, we hypothesized that, similar to our PDX data, BRCA1- $\Delta 11q$ might only be highly expressed in a fraction of exon 11-mutant tumors, and so not all exon 11-mutant patients would necessarily be resistant to therapy. Analyses of primary tumors (unrelated to the above studies) with mutations IE11 showed variable levels of BRCA1- $\Delta 11q$ expression. Tumor #317 expressed 2.7- ($P = 0.0227$) and 4-fold ($P = 0.0117$) greater levels of $\Delta 11q$ compared with tumor #325 and tumor #411, respectively, despite all tumors harboring deleterious exon 11 mutations (Fig. 5B).

Inhibition of splicing increases PARPi sensitivity

Because high levels of BRCA1- $\Delta 11q$ expression were required for residual function, we hypothesized that limiting splicing-dependent excision of exon 11q might provide an opportunity to enhance PARPi sensitivity. We first identified two binding elements for the FOX2 splicing site selection factor at position +18 and +63 downstream from the cryptic splice site location (Supplementary Fig. S6; refs. 44, 45). Depletion of FOX2 using 2 individual siRNAs did not impact +11 expression levels, but reduced $\Delta 11q$ expression 1.7- ($P = 0.0043$) and 1.6-fold ($P = 0.0179$) in MDA-MB-231, 1.5- ($P = 0.048$) and 1.6-fold ($P = 0.0301$) in UWB1.289, 2.2- ($P = 0.0056$) and 3.1-fold ($P = 0.0017$) in SUM149PT cells, compared with scrambled

siRNA-treated cells, respectively (Fig. 6A). At the protein level, densitometric analyses of Western blots indicated FOX2 siRNA did not impact full-length BRCA1 protein expression, but BRCA1- $\Delta 11q$ levels were reduced 2.9- and 2.2-fold in MDA-MB-231, 2- and 2.8-fold in UWB1.289, 1.9- and 1.8-fold in SUM149PT cells, compared with scrambled siRNA-treated cells (Fig. 6B). FOX2 siRNA had no impact on MDA-MB-231 cells PARPi sensitivity, but reduced the LC₅₀ value of rucaparib 3.8- ($P = 0.0063$) and 27.4-fold ($P = 0.0017$) in UWB1.289 cells, as well as 1.9- ($P = 0.0237$) and 5-fold ($P = 0.0002$) in SUM149PT cells, compared with scrambled siRNA-treated cells (Fig. 6C). Furthermore, BRCA1-minigene analyses demonstrated that mutation of FOX2-binding motifs also partially decreased $\Delta 11q$ -reporter mRNA and protein levels (Fig. 6D).

We next investigated the ability of PI-B, a small-molecule inhibitor of the U2 snRNP spliceosome machinery (46), to disrupt BRCA1- $\Delta 11q$ production. PI-B treatment reduced both +11 and $\Delta 11q$ mRNA expression; however, the relative reduction in $\Delta 11q$ levels was 7.7- ($P = 0.002$) and 7-fold ($P = 0.016$) greater compared with the reduction in +11 levels in MDA-MB-231 and UWB1.289 cells, respectively (Fig. 6E). Furthermore, full-length BRCA1 protein levels were reduced but remained readily detectable after treatment with up to 10 nmol/L PI-B in MDA-MB-231 cells. However, BRCA1- $\Delta 11q$ protein levels were barely detectable at 1.25 nmol/L PI-B in both MDA-MB-231 and UWB1.289 cells (Fig. 6F). Simultaneous incubation with both PI-B and rucaparib did not significantly impact MDA-MB-231 cells, but resulted in a 2.8- ($P = 0.0296$) and 2.1-fold ($P = 0.0426$) reduction in colony formation compared with cells treated with rucaparib only in UWB1.289 and SUM149PT cells, respectively (Fig. 6G). Furthermore, ectopic overexpression of a BRCA1- $\Delta 11q$ cDNA that did not depend on exon 11q splicing, resulted in a 1.9-fold ($P = 0.001$) rescue in colony formation in cells treated with the PI-B and rucaparib combination, compared with GFP-expressing control cells (Fig. 6H), suggesting that PI-B-induced PARPi sensitization was mediated, in part, through the reduction in BRCA1- $\Delta 11q$ levels.

Discussion

In the current study, we provide evidence that BRCA1 splice isoforms lacking exon 11 are capable of producing truncated but hypomorphic proteins. It was previously shown that the exon 11-deficient Brca1- $\Delta 11$ isoform partially compensates for the lack of other Brca1 isoforms during embryogenesis (31–33, 47). Here, we show that BRCA1- $\Delta 11q$ can also partially compensate for full-length BRCA1 in response to homologous recombination targeting therapeutics. BRCA1- $\Delta 11q$ retains, although less efficiently, many of the protein–protein interactions carried out by full-length BRCA1. We identified the BRCA1- $\Delta 11q$ –PALB2 interaction as critical for BRCA1- $\Delta 11q$ -mediated RAD51 IRIF and resistance.

BRCA1- $\Delta 11q$ was expressed in BRCA1 wild-type as well as exon 11-mutant cell lines. Although frameshift mutations in exon 11 resulted in the NMD of exon 11 containing transcripts, they did not impact the rate of alternative splicing or directly affect BRCA1- $\Delta 11q$ levels. In support of this, PDX124 and PDX196 tumors both harbored identical 2080delA exon 11 mutations; however, both +11 and $\Delta 11q$ transcript levels were significantly higher in PDX196 relative to PDX124 tumors, suggesting that overall BRCA1 gene transcription, rather than the rate of alternative exon splicing, was elevated. Both patients whose tumors were used to derive PDX124 and PDX196 initially demonstrated clinical

responsiveness to olaparib therapy; while PDX124 was derived prior to the patient starting olaparib therapy, PDX196 was derived at the time of clinical tumor progression on olaparib. We anticipate that high levels of *BRCA1-Δ11q* expression may be selected for in response to chemotherapy. Primary patient tumors harboring exon 11 mutations also demonstrated variable *BRCA1-Δ11q* levels, and this isoform might only be overexpressed in a subset of exon 11 mutation carriers. Additional resistance mechanisms such as secondary reversion mutations have also been shown to occur in tumors with exon 11 mutations (48, 49).

Intriguingly, our analyses indicated that exon 11 mutation carriers with ovarian cancer had a worse 5-year overall survival compared with non-exon 11 mutation carriers (Fig. 5A). Another recent study of mutation-specific cancer risks identified the exon 11 region of *BRCA1* as an ovarian cancer cluster region, where mutation carriers had a relative decrease in breast relative to ovarian cancer risk (50). Further work is required to determine whether *BRCA1-Δ11q* has any impact on long-term survival outcomes or breast versus ovarian tissue-specific phenotypes.

Currently, an understanding of PARPi and platinum resistance is incomplete. Our findings provide evidence for a role of the *BRCA1-Δ11q* alternative splice isoform in promoting resistance. Moreover, we show that inhibition of the spliceosome reduced *BRCA1-Δ11q* levels and sensitized exon 11-mutant cell lines to PARPi, potentially offering a strategy to resensitize tumors that express high levels of *BRCA1-Δ11q*.

Disclosure of Potential Conflicts of Interest

C. Gourley reports receiving a commercial research grant, has received speakers bureau honoraria, and is a consultant/advisory board member for AstraZeneca. G.I. Shapiro reports receiving commercial research support and is a consultant/advisory board member for Clovis. No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: Y. Wang, C. Cruz, D. Lambrechts, A.D. D'Andrea, G.I. Shapiro, E.M. Swisher, N. Johnson

Development of methodology: Y. Wang, A.J. Bernhardt, C. Cruz, E. Nicolas, V. Serra, N. Johnson

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): Y. Wang, A.J. Bernhardt, C. Cruz, J.J. Krais, J. Nacson, E. Nicolas, I. van der Heijden, S.W. O'Brien, M.I. Harrell, S.F. Johnson, F.J. Candido dos Reis, P.D.P. Pharoah, B. Karlan, C. Gourley, D. Lambrechts, G. Chenevix-Trench, H. Olsson, M.H. Greene, M. Gore, R. Nussbaum,

S. Sadetzki, S.K. Kjaer, kConFab Investigators, G.I. Shapiro, D.C. Connolly, M.B. Daly, E.M. Swisher, P. Bouwman, J. Jonkers, J. Balmana, V. Serra, N. Johnson

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): Y. Wang, C. Cruz, E. Nicolas, S. Peri, I. van der Heijden, Y. Zhang, F.J. Candido dos Reis, P.D.P. Pharoah, B. Karlan, J.J. Benitez, S.K. Kjaer, G.I. Shapiro, D.L. Wiest, P. Bouwman, J. Jonkers, J. Balmana, V. Serra, N. Johnson

Writing, review, and/or revision of the manuscript: Y. Wang, C. Cruz, F.J. Candido dos Reis, P.D.P. Pharoah, B. Karlan, C. Gourley, G. Chenevix-Trench, H. Olsson, M.H. Greene, M. Gore, R. Nussbaum, S. Sadetzki, S.A. Gayther, S.K. Kjaer, kConFab Investigators, A.D. D'Andrea, G.I. Shapiro, D.L. Wiest, D.C. Connolly, M.B. Daly, E.M. Swisher, J. Balmana, V. Serra, N. Johnson

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): A.J. Bernhardt, B. Karlan, C. Gourley, D. Lambrechts, R. Nussbaum, P. Bouwman, V. Serra

Study supervision: S.K. Kjaer, P. Bouwman, J. Balmana, V. Serra, N. Johnson

Other (performed experiments): H. van der Gulden

Acknowledgments

The authors thank the FCCC Biostatistics and bioinformatics, Biorepository and Genomics facilities and Yasir H. Ibrahim, Pilar Antón, Ana Vivancos, Orland Diaz, Michael Slifker, Yusheng Li for helpful experimental support and data analysis.

Grant Support

This work was supported by US NIH grants P50 CA083638 (Fox Chase Cancer Center (FCCC) Specialized Program of Research Excellence (SPORE) in Ovarian Cancer and R21CA191690, 5P30 CA006927 (FCCC Developmental New Investigator funds), Susan G. Komen Career Catalyst Award CCR12226280, and OC130212 Department of Defense Ovarian Academy Award, Basser Center for BRCA pilot project award (N. Johnson), P50 CA83636 (Pacific Ovarian Cancer Research Consortium SPORE in Ovarian Cancer), the Wendy Feuer Ovarian Cancer Research Fund (E.M. Swisher), M.H. Greene was supported by the Intramural Research Program of the US National Cancer Institute. J. Balmana is recipient of an Instituto de Salud Carlos III (ISCIII) grant PI12/02606. C. Cruz is supported by a Sociedad Española de Oncología Médica (SEOM)-BUCKLER 0'0 grant; Conquer Cancer Young Investigator Award; and Asociación Española contra el Cáncer (AECC). This work was also supported by a GHD/FERO (V. Serra).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received January 21, 2016; accepted February 15, 2016; published online May 2, 2016.

References

- Szabo CI, King MC. Inherited breast and ovarian cancer. *Hum Mol Genet* 1995;4:1811-7.
- Friedman LS, Ostermeyer EA, Szabo CI, Dowd P, Lynch ED, Rowell SE, et al. Confirmation of *BRCA1* by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nat Genet* 1994;8:399-404.
- Perrin-Vidol L, Sinilnikova OM, Stoppa-Lyonnet D, Lenoir GM, Mazoyer S. The nonsense-mediated mRNA decay pathway triggers degradation of most *BRCA1* mRNAs bearing premature termination codons. *Hum Mol Genet* 2002;11:2805-14.
- Moynahan ME, Cui TY, Jasin M. Homology-directed dna repair, mitomycin-c resistance, and chromosome stability is restored with correction of a *Brc1* mutation. *Cancer Res* 2001;61:4842-50.
- Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M, Feunteun J, et al. Dynamic changes of *BRCA1* subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* 1997;90:425-35.
- Lord CJ, Tutt AN, Ashworth A. Synthetic lethality and cancer therapy: lessons learned from the development of PARP inhibitors. *Annu Rev Med* 2015;66:455-70.
- Lord CJ, Ashworth A. Mechanisms of resistance to therapies targeting *BRCA*-mutant cancers. *Nat Med* 2013;19:1381-8.
- Bryant HE, Schultz N, Thomas HD, Parker KM, Flower D, Lopez E, et al. Specific killing of *BRCA2*-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 2005;434:913-7.
- Farmer H, McCabe N, Lord CJ, Tutt AN, Johnson DA, Richardson TB, et al. Targeting the DNA repair defect in *BRCA* mutant cells as a therapeutic strategy. *Nature* 2005;434:917-21.
- Kennedy RD, Quinn JE, Mullan PB, Johnston PG, Harkin DP. The role of *BRCA1* in the cellular response to chemotherapy. *J Natl Cancer Inst* 2004;96:1659-68.
- Quinn JE, Kennedy RD, Mullan PB, Gilmore PM, Carty M, Johnston PG, et al. *BRCA1* functions as a differential modulator of chemotherapy-induced apoptosis. *Cancer Res* 2003;63:6221-8.
- Gelmon KA, Tischkowitz M, Mackay H, Swenerton K, Robidoux A, Tonkin K, et al. Olaparib in patients with recurrent high-grade serous or poorly differentiated ovarian carcinoma or triple-negative breast cancer: a phase 2, multicentre, open-label, non-randomised study. *Lancet Oncol* 2011;12:852-61.

13. Sandhu SK, Schelman WR, Wilding G, Moreno V, Baird RD, Miranda S, et al. The poly(ADP-ribose) polymerase inhibitor niraparib (MK4827) in BRCA mutation carriers and patients with sporadic cancer: a phase 1 dose-escalation trial. *Lancet Oncol* 2013;14:882–92.
14. Ang JE, Gourley C, Powell B, High H, Shapira-Frommer R, Castonguay V, et al. Efficacy of chemotherapy in BRCA1/2 mutation carrier ovarian cancer in the setting of poly(ADP-ribose) polymerase inhibitor resistance: a multi-institutional study. *Clin Cancer Res* 2013;19:5485–93.
15. Ledermann J, Harter P, Gourley C, Friedlander M, Vergote I, Rustin G, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol* 2014;15:852–61.
16. Edwards SL, Brough R, Lord CJ, Natrajan R, Vatcheva R, Levine DA, et al. Resistance to therapy caused by intragenic deletion in BRCA2. *Nature* 2008;451:1111–5.
17. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, et al. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* 2008;451:1116–20.
18. Bouwman P, Aly A, Escandell JM, Pieterse M, Bartkova J, van der Gulden H, et al. 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol* 2010;17:688–95.
19. Bunting SF, Callen E, Wong N, Chen HT, Polato F, Gunn A, et al. 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* 2010;141:243–54.
20. Johnson N, Johnson SF, Yao W, Li YC, Choi YE, Bernhardt AJ, et al. Stabilization of mutant BRCA1 protein confers PARP inhibitor and platinum resistance. *Proc Natl Acad Sci U S A* 2013;110:17041–6.
21. Drost R, Bouwman P, Rottenberg S, Boon U, Schut E, Klarenbeek S, et al. BRCA1 RING function is essential for tumor suppression but dispensable for therapy resistance. *Cancer Cell* 2011;20:797–809.
22. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, et al. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci U S A* 2008;105:17079–84.
23. Colombo M, Blok MJ, Whiley P, Santamarina M, Gutierrez-Enriquez S, Romero A, et al. Comprehensive annotation of splice junctions supports pervasive alternative splicing at the BRCA1 locus: a report from the ENIGMA consortium. *Hum Mol Genet* 2014;23:3666–80.
24. Thomassen M, Blanco A, Montagna M, Hansen TV, Pedersen IS, Gutierrez-Enriquez S, et al. Characterization of BRCA1 and BRCA2 splicing variants: a collaborative report by ENIGMA consortium members. *Breast Cancer Res Treat* 2012;132:1009–23.
25. Romero A, Garcia-Garcia F, Lopez-Perolio I, Ruiz de Garibay G, Garcia-Saenz JA, Garre P, et al. BRCA1 Alternative splicing landscape in breast tissue samples. *BMC Cancer* 2015;15:219.
26. Orban TI, Olah E. Expression profiles of BRCA1 splice variants in asynchronous and in G1/S synchronized tumor cell lines. *Biochem Biophys Res Commun* 2001;280:32–8.
27. Tammaro C, Raponi M, Wilson DI, Baralle D. BRCA1 exon 11 alternative splicing, multiple functions and the association with cancer. *Biochem Soc Trans* 2012;40:768–72.
28. Raponi M, Smith LD, Silipo M, Stuani C, Buratti E, Baralle D. BRCA1 exon 11 a model of long exon splicing regulation. *RNA Biol* 2014;11:351–9.
29. Orban TI, Olah E. Emerging roles of BRCA1 alternative splicing. *Mol Pathol* 2003;56:191–7.
30. Wilson CA, Payton MN, Elliott GS, Buaas FW, Cajulis EE, Grosshans D, et al. Differential subcellular localization, expression and biological toxicity of BRCA1 and the splice variant BRCA1-delta11b. *Oncogene* 1997;14:1–16.
31. Ludwig T, Chapman DL, Papaioannou VE, Efstratiadis A. Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. *Genes Dev* 1997;11:1226–41.
32. Xu X, Qiao W, Linke SP, Cao L, Li WM, Furth PA, et al. Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat Genet* 2001;28:266–71.
33. Xu X, Wagner KU, Larson D, Weaver Z, Li C, Ried T, et al. Conditional mutation of Brca1 in mammary epithelial cells results in blunted ductal morphogenesis and tumour formation. *Nat Genet* 1999;22:37–43.
34. NIH National Human Genome Research Institute. Breast Cancer Information Core. Available from: <https://research.nhgri.nih.gov/projects/bic/index.shtml>.
35. Thompson D, Easton D, Breast Cancer Linkage C. Variation in BRCA1 cancer risks by mutation position. *Cancer Epidemiol Biomarkers Prev* 2002;11:329–36.
36. Risch HA, McLaughlin JR, Cole DE, Rosen B, Bradley L, Fan I, et al. Population BRCA1 and BRCA2 mutation frequencies and cancer penetrances: a kin-cohort study in Ontario, Canada. *J Natl Cancer Inst* 2006;98:1694–706.
37. Risch HA, McLaughlin JR, Cole DE, Rosen B, Bradley L, Kwan E, et al. Prevalence and penetrance of germline BRCA1 and BRCA2 mutations in a population series of 649 women with ovarian cancer. *Am J Hum Genet* 2001;68:700–10.
38. Barbier J, Dutertre M, Bittencourt D, Sanchez G, Gratadou L, de la Grange P, et al. Regulation of H-ras splice variant expression by cross talk between the p53 and nonsense-mediated mRNA decay pathways. *Mol Cell Biol* 2007;27:7315–33.
39. Williams RS, Chasman DI, Hau DD, Hui B, Lau AY, Glover JN. Detection of protein folding defects caused by BRCA1-BRCT truncation and missense mutations. *J Biol Chem* 2003;278:53007–16.
40. Williams RS, Glover JN. Structural consequences of a cancer-causing BRCA1-BRCT missense mutation. *J Biol Chem* 2003;278:2630–5.
41. Sy SM, Huen MS, Chen J. PALB2 is an integral component of the BRCA complex required for homologous recombination repair. *Proc Natl Acad Sci U S A* 2009;106:7155–60.
42. Bouwman P, van der Gulden H, van der Heijden I, Drost R, Klijn CN, Prasetyanti P, et al. A high-throughput functional complementation assay for classification of BRCA1 missense variants. *Cancer Discov* 2013;3:1142–55.
43. Bolton KL, Chenevix-Trench G, Goh C, Sadetzki S, Ramus SJ, Karlan BY, et al. Association between BRCA1 and BRCA2 mutations and survival in women with invasive epithelial ovarian cancer. *JAMA* 2012;307:382–90.
44. Venables JP, Klinck R, Koh C, Gervais-Bird J, Bramard A, Inkel L, et al. Cancer-associated regulation of alternative splicing. *Nat Struct Mol Biol* 2009;16:670–6.
45. Huang SC, Ou AC, Park J, Yu F, Yu B, Lee A, et al. RBFOX2 promotes protein 4.1R exon 16 selection via U1 snRNP recruitment. *Mol Cell Biol* 2012;32:513–26.
46. Kotake Y, Sagane K, Owa T, Mimori-Kiyosue Y, Shimizu H, Uesugi M, et al. Splicing factor SF3b as a target of the antitumor natural product pladienolide. *Nat Chem Biol* 2007;3:570–5.
47. Huber LJ, Yang TW, Sarkisian CJ, Master SR, Deng CX, Chodosh LA. Impaired DNA damage response in cells expressing an exon 11-deleted murine Brca1 variant that localizes to nuclear foci. *Mol Cell Biol* 2001;21:4005–15.
48. Norquist B, Wurzel KA, Pennil CC, Garcia R, Gross J, Sakai W, et al. Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J Clin Oncol* 2011;29:3008–15.
49. Patch AM, Christie EL, Etemadmoghadam D, Garsed DW, George J, Fereday S, et al. Whole-genome characterization of chemoresistant ovarian cancer. *Nature* 2015;521:489–94.
50. Rebbeck TR, Mitra N, Wan F, Sinilnikova OM, Healey S, McGuffee L, et al. Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. *JAMA* 2015;313:1347–61.

Cancer Research

The Journal of Cancer Research (1916–1930) | The American Journal of Cancer (1931–1940)

The BRCA1- Δ 11q Alternative Splice Isoform Bypasses Germline Mutations and Promotes Therapeutic Resistance to PARP Inhibition and Cisplatin

Yifan Wang, Andrea J. Bernhardt, Cristina Cruz, et al.

Cancer Res 2016;76:2778-2790.

Updated version Access the most recent version of this article at:
<http://cancerres.aacrjournals.org/content/76/9/2778>

Cited articles This article cites 49 articles, 22 of which you can access for free at:
<http://cancerres.aacrjournals.org/content/76/9/2778.full.html#ref-list-1>

E-mail alerts [Sign up to receive free email-alerts](#) related to this article or journal.

Reprints and Subscriptions To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.

RING domain–deficient BRCA1 promotes PARP inhibitor and platinum resistance

Yifan Wang,¹ John J. Krais,¹ Andrea J. Bernhardt,¹ Emmanuelle Nicolas,² Kathy Q. Cai,¹ Maria I. Harrell,³ Hyoung H. Kim,⁴ Erin George,⁴ Elizabeth M. Swisher,³ Fiona Simpkins,⁴ and Neil Johnson¹

¹Molecular Therapeutics Program and ²Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, Pennsylvania, USA. ³Department of Obstetrics and Gynecology, University of Washington, Seattle, Washington, USA. ⁴Department of Obstetrics and Gynecology, University of Pennsylvania, Philadelphia, Pennsylvania, USA.

Patients with cancers that harbor breast cancer 1 (*BRCA1*) mutations initially respond well to platinum and poly(ADP-ribose) polymerase inhibitor (PARPi) therapy; however, resistance invariably arises in these patients and is a major clinical problem. The *BRCA1*^{T85delAG} allele is a common inherited mutation located close to the protein translation start site that is thought to produce a shortened, nonfunctional peptide. In this study, we investigated the mechanisms that lead to PARPi and platinum resistance in the SUM1315M02 breast cancer cell line, which harbors a hemizygous *BRCA1*^{T85delAG} mutation. SUM1315M02 cells were initially sensitive to PARPi and cisplatin but readily acquired resistance. PARPi- and cisplatin-resistant clones did not harbor secondary reversion mutations; rather, PARPi and platinum resistance required increased expression of a really interesting gene (RING) domain–deficient BRCA1 protein (Rdd-BRCA1). Initiation of translation occurred downstream of the frameshift mutation, probably at the BRCA1-Met-297 codon. In contrast to full-length BRCA1, Rdd-BRCA1 did not require BRCA1-associated RING domain 1 (BARD1) interaction for stability. Functionally, Rdd-BRCA1 formed irradiation-induced foci and supported RAD51 foci formation. Ectopic overexpression of Rdd-BRCA1 promoted partial PARPi and cisplatin resistance. Furthermore, Rdd-BRCA1 protein expression was detected in recurrent carcinomas from patients who carried germline *BRCA1*^{T85delAG} mutations. Taken together, these results indicate that RING-deficient BRCA1 proteins are hypomorphic and capable of contributing to PARPi and platinum resistance when expressed at high levels.

Introduction

Germline breast cancer 1 (*BRCA1*) mutations dramatically increase a carrier's lifetime risk of developing breast and ovarian cancer (1–3). Pathogenic *BRCA1*-mutant alleles fail to generate protein products with tumor-suppressor activity, and cancer initiation usually results from loss of heterozygosity (LOH) affecting the *BRCA1* WT allele (3–5). The BRCA1 protein is an 1863-aa peptide containing several highly conserved domains. The N-terminal contains a RING domain that is commonly found in E3 ubiquitin ligases and is required for heterodimerization with BRCA1-associated RING domain 1 (BARD1). BRCA1 interacts with partner and localizer of BRCA2 (PALB2) through a coiled-coil region that facilitates the formation of a BRCA1-PALB2-BRCA2-RAD51 complex. The BRCA1 C-terminus domains (BRCT) bind phosphorylated proteins such as CtIP and abraxas (5–7).

The BRCA1 protein is necessary for efficient homologous recombination (HR) DNA repair (8), and mutations in the *BRCA1* gene that render the protein product dysfunctional result in cellular sensitivity to poly(ADP-ribose) polymerase inhibitors (PARPi) (9, 10), as well as to DNA-damaging platinum agents (8, 11, 12). Ovarian and breast cancer patients who harbor

BRCA1 mutations respond well to initial platinum-based therapy (13–15), and several PARPi are currently under advanced-stage clinical investigation, as they have demonstrated clinical activity in patients with *BRCA* mutations (15–17). Despite survival improvements, resistance to both PARPi and platinum therapy invariably arises and is a major clinical problem (18–21). Secondary *BRCA1* and *BRCA2* gene-reversion mutations that restore the reading frame represent the most well-validated mechanism of platinum resistance (22–25). Several mechanisms of PARPi resistance have been described in *Brca1*-mutated mouse mammary tumors, including activation of P-glycoprotein (26) and loss of 53BP1 expression (27–29).

Cellular mechanisms that result in increased levels of truncated, but hypomorphic, BRCA1 proteins have been shown to result in both PARPi and platinum resistance. We previously demonstrated that cells harboring a BRCT domain-truncating mutation were able to develop resistance to PARPi and cisplatin through overexpression of a BRCT domain–deficient BRCA1 protein (30). Despite lacking the BRCT domain, the retention of all other critical regions, including the PALB2-interacting domain, facilitated RAD51 loading and therapy resistance. Similarly, when the *Brca1*^{C61G} allele was introduced into a conditional mouse model of BRCA1-associated breast cancer, tumors formed at a rate similar to that seen in *Brca1*-deficient mice. However, tumors carrying *Brca1*^{C61G} responded poorly to platinum and PARPi (31). Intriguingly, a similar study that introduced the *Brca1*^{I26A} synthetic missense variant that reduces

► Related Commentary: doi:10.1172/JCI89209

Conflict of interest: The authors have declared that no conflict of interest exists.

Submitted: February 12, 2016; **Accepted:** May 9, 2016.

Reference information: *J Clin Invest.* doi:10.1172/JCI87033.

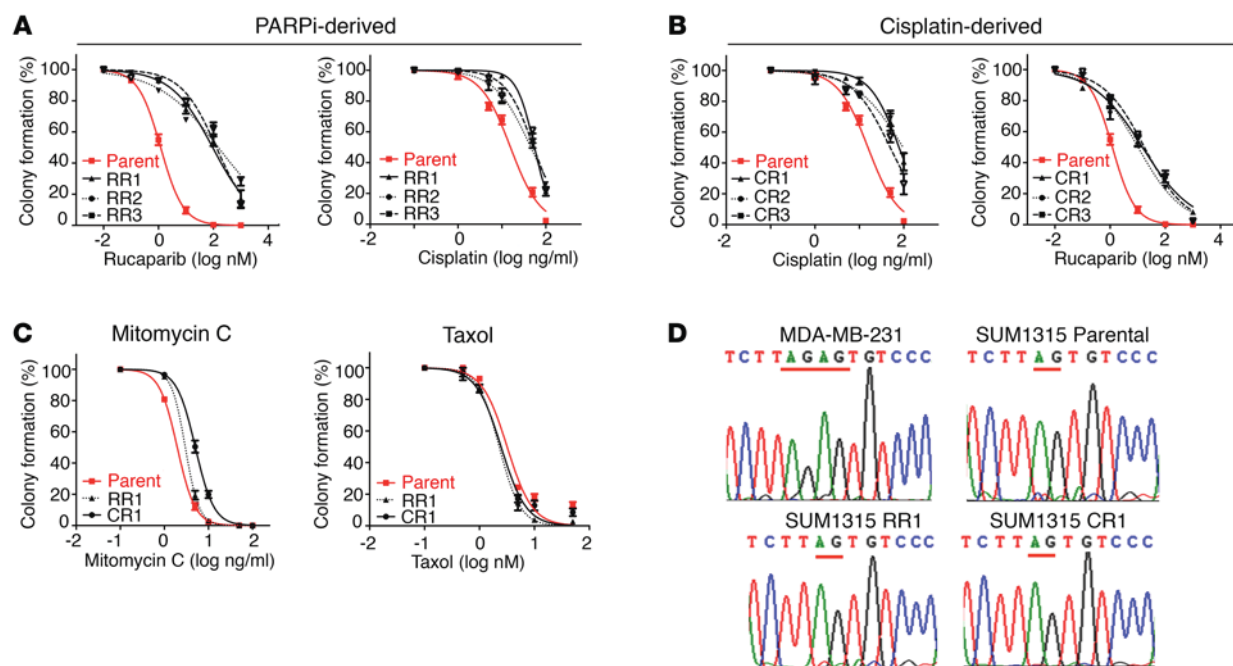


Figure 1. SUM1315MO2 PARPi- and cisplatin-resistant cells do not harbor reversion mutations. (A) SUM1315MO2 parental cells and RR1–3 cells were measured for primary resistance to rucaparib and cross-resistance to cisplatin. (B) SUM1315MO2 parental cells and CR1–3 cells were measured for primary resistance to cisplatin and cross-resistance to rucaparib. (C) SUM1315MO2 parental cells and RR1 and CR1 cells were assessed for mitomycin C and taxol sensitivity. Colony formation assays were employed throughout and three independent experiments were performed. (D) *BRCA1* introns and exons from parental cells and resistant clones were subjected to Sanger sequencing; representative *BRCA1* electropherograms are shown for parental cells and 6 resistant clones that were sequenced.

E3-ubiquitin ligase activity, but does not prevent BRCA1-BARD1 assembly, did not impact BRCA1 tumor-suppressor activity, and mice were resistant to tumor formation (32).

The *BRCA1*^{185delAG} allele is a common founder mutation, prevalent in approximately 1% of the Ashkenazi Jewish population and predisposes carriers to early-onset breast and ovarian cancer (33, 34). Here, we report that *BRCA1*^{185delAG} frameshift mutation-carrying cell lines and tumors are capable of producing RING domain-deficient BRCA1 proteins (Rdd-BRCA1) that facilitate RAD51 foci formation and PARPi and cisplatin resistance.

Results

SUM1315MO2 PARPi- and cisplatin-resistant cells do not acquire secondary reversion mutations. The SUM1315MO2 cell line was derived from a skin metastasis of a female patient with invasive ductal carcinoma. SUM1315MO2 cells have LOH at the *BRCA1* locus and harbor the common pathogenic *BRCA1*^{185delAG} allele (35, 36). To understand the impact of the *BRCA1*^{185delAG} allele on drug resistance, we cultured SUM1315MO2 cells in the presence of increasing concentrations of either the PARPi rucaparib or cisplatin, until 3 individual resistant clones were derived for each agent. SUM1315MO2 cells rapidly acquired PARPi and cisplatin resistance, and resistant clones were derived within 1 to 2 months from initial exposure.

We confirmed by colony formation assay that the clones demonstrated drug resistance. Rucaparib-resistant clones 1–3 (RR1–3) were 84- ($P = 0.024$), 128- ($P = 0.008$), and 110-fold ($P = 0.01$) more resistant to rucaparib treatment than were SUM1315MO2 parental cells. Additionally, RR1–3 were 3.6- ($P < 0.001$), 3- ($P = 0.002$), and 3.5-fold

($P < 0.001$) more resistant to cisplatin compared with parental cells (Figure 1A). SUM1315MO2 cells cultured in the presence of cisplatin were also measured for cisplatin and PARPi sensitivity. Cisplatin-resistant clones 1–3 (CR1–3) were 5.1- ($P = 0.006$), 6.3- ($P < 0.001$), and 3.3-fold ($P = 0.008$) more resistant to cisplatin treatment and were 12- ($P = 0.002$), 9- ($P = 0.021$), and 14-fold ($P = 0.005$) more resistant to rucaparib than were SUM1315MO2 parental cells (Figure 1B). Furthermore, RR clone 1 (RR1) and CR clone 1 (CR1) were 1.5- ($P = 0.008$) and 2.6-fold ($P = 0.001$) more resistant than parental cells to the DNA cross-linking agent mitomycin C, respectively. In contrast, parental, RR1 and CR1 cells demonstrated similar levels of sensitivity to the microtubule inhibitor taxol (Figure 1C).

Platinum-resistant *BRCA1*^{185delAG} patient carcinomas have previously been shown to develop reversion mutations that restore the WT sequence (22). We sequenced the *BRCA1* gene introns and exons, as well as mRNA, in parental and resistant clones and found that the original *BRCA1*^{185delAG} mutation remained present; moreover, no additional *BRCA1* mutations were acquired in any of the resistant clones (Figure 1D). Because we could not detect reversion mutations in SUM1315MO2 resistant cell lines, we investigated the possibility that mutations in other DNA repair genes could be providing resistance by sequencing a panel of candidate genes using BROCA analysis (37). BROCA sequencing confirmed the presence of the known *BRCA1* and *TP53* mutations present in both parental and resistant clones, but there were no additional mutations in a series of genes associated with resistance, including *TP53BP1* (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI87033DS1).

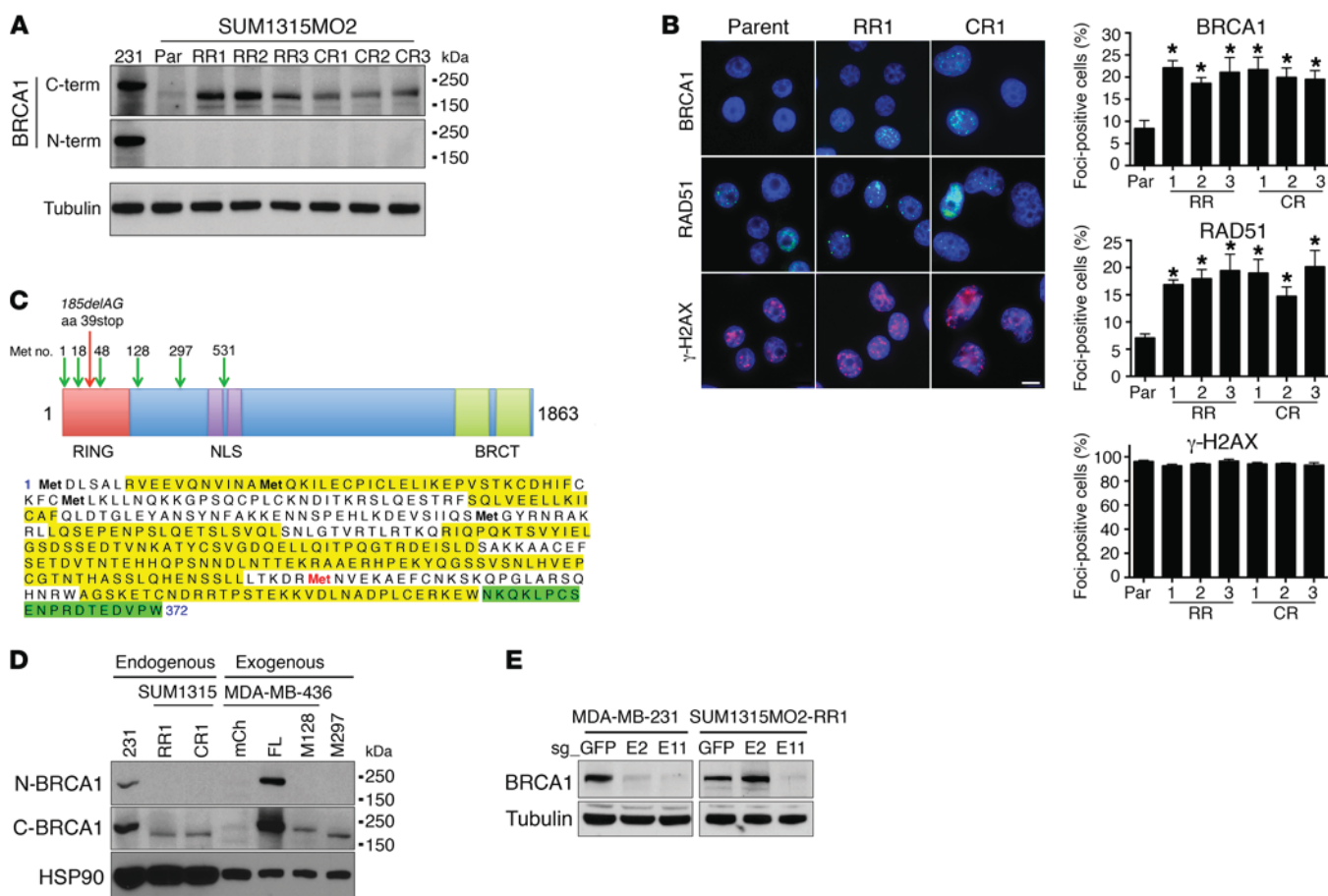


Figure 2. SUM1315MO2 cells express a RING domain-deficient BRCA1 protein. (A) Cell lysates were generated from MDA-MB-231 (abbreviated as 231 in the figure), SUM1315MO2 parental (Par), RR1–3, and CR1–3 cells, and BRCA1 protein was detected by Western blot analysis using C-terminal-specific (C-term) and N-terminal-specific (N-term) BRCA1 Abs. Molecular weight markers are indicated. Three independent experiments were performed. (B) SUM1315MO2 parental, RR1–3, and CR1–3 cells were treated with IR, and BRCA1, RAD51, γ-H2AX, and DAPI were detected by immunofluorescence (representative images are shown). Scale bar: 10 μm. Bar graphs show the mean and SEM percentage of cells containing more than 5 foci ($n = 3$ independent experiments). * $P < 0.05$, 2-tailed Student's t test. (C) In-frame methionine translation start sites are depicted, along with the location of the stop codon generated from the *BRCA1*^{185delAG} mutation (above). BRCA1 was immunoprecipitated from MDA-MB-231 and SUM1315MO2 RR1 cells and subjected to mass spectrometry. BRCA1 peptides detected from MDA-MB-231 cells are highlighted in yellow and SUM1315MO2 RR1 cells in green, Met-297 is highlighted in red (below). See Supplemental Figure 1 for complete information on peptide coverage. (D) Cell lysates were collected from MDA-MB-231 and SUM1315MO2 RR1 and CR1 cells, as well as from MDA-MB-436 cells that expressed exogenous mCherry (mCh), BRCA1 full-length (FL), BRCA1-Met128 (M128), or BRCA1-Met-297 (M297). BRCA1 gel migration was detected by Western blot analysis using N- and C-terminal-specific Abs; HSP90 was measured as a loading control. (E) CRISPR/Cas9 targeting sg_GFP, sg_exon 2 (E2), or sg_exon 11 (E11) in MDA-MB-231 and SUM1315MO2 RR1 cells. Western blot analyses of BRCA1 levels using a C-terminal-specific Ab (see Supplemental Figure 2 for additional information). Three independent experiments were performed.

SUM1315MO2 resistant cells express a RING domain-deficient BRCA1 protein. To investigate potential BRCA1 protein products generated from the *BRCA1*^{185delAG} allele in SUM1315MO2 parental and resistant clones, we measured BRCA1 protein levels by Western blotting with Abs that specifically recognize the N- and C-terminal regions of BRCA1. As a control, WT BRCA1 protein expressed in MDA-MB-231 cells was detectable with both N- and C-terminal Abs. In contrast, BRCA1 protein was undetectable in both SUM1315MO2 parental and resistant clones using the N-terminal-specific Ab. However, the C-terminal Ab identified a band migrating slightly below full-length BRCA1 that was of low abundance in parental cells, but with elevated expression in both RR and CR clones (Figure 2A). Moreover, the BRCA1 protein detected in SUM1315MO2 cells was capable of forming γ irradiation-induced foci (IRIF), and we readily detected both BRCA1 and

RAD51 foci in parental SUM1315MO2 cells. However, both RR and CR clones had a 2.22- to 2.64-fold ($P = 0.004$ – 0.021) increase in BRCA1 IRIF and a 2.1- to 2.85-fold ($P = 0.0001$ – 0.03) increase in RAD51 IRIF compared with parental cells, respectively. The levels of γ-H2AX, a marker for DNA damage, did not significantly differ between cell lines (Figure 2B).

We hypothesized that cells were utilizing an in-frame translation start site downstream of the *BRCA1*^{185delAG} mutation to generate a RING domain-deficient BRCA1 protein. To identify the possible downstream translation start site, we immunoprecipitated endogenous BRCA1 from SUM1315MO2 RR1, as well as WT-expressing MDA-MB-231 cells and analyzed BRCA1 peptides by mass spectrometry. Total peptide coverage was overall greater in MDA-MB-231 cells (58% total peptide coverage) compared with that in SUM1315MO2 RR1 cells (19% total peptide cover-

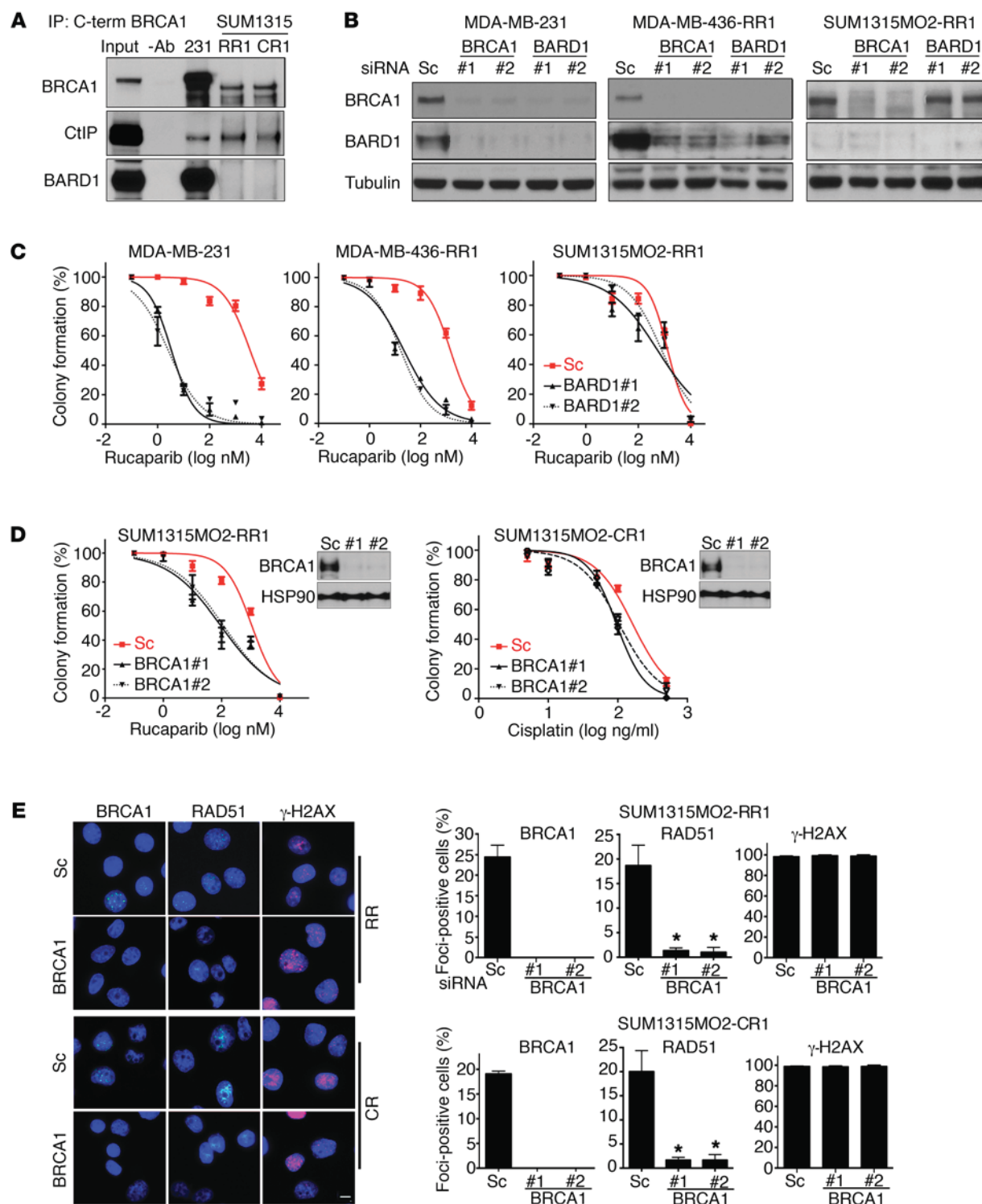


Figure 3. Rdd-BRCA1 does not require BARD1 for stability or function. (A) BRCA1 was immunoprecipitated from MDA-MB-231 and SUM1315MO2 RR1 and CR1 cells using a C-terminal-specific Ab. Immunoprecipitates were measured for BRCA1, BARD1, and CtIP protein levels by Western blot analysis. Three independent experiments were performed. (B) MDA-MB-231, MDA-MB-436-RR1, and SUM1315MO2 RR1 cells were treated with scrambled (Sc) or 2 independent siRNAs targeting BRCA1 or BARD1, and BRCA1 and tubulin measured by Western blot analysis. Samples were run on parallel gels. The tubulin blot was derived from duplicate samples run on a parallel gel. (C) Cells described in B were transfected with scrambled or 2 independent BARD1 siRNAs, treated with increasing concentrations of rucaparib, and colony formation assessed. (D) Cells were transfected with scrambled, BRCA1 no. 1, or BRCA1 no. 2 siRNA, followed by treatment with increasing concentrations of either rucaparib or cisplatin for either SUM1315MO2 RR1 or CR1 cells, respectively, and then reseeded for colony formation assay ($n = 3$). Insets show Western blots of BRCA1 knockdown. Three independent experiments were performed. (E) SUM1315MO2 RR1 and CR1 cells were treated with scrambled or 2 independent BRCA1 siRNAs and subjected to IR. BRCA1, RAD51, and γ -H2AX IRIF were assessed by immunofluorescence. Representative images are shown. Scale bar: 10 μ m. Bar graphs show the mean and SEM percentage of cells containing more than 5 foci ($n = 3$ independent experiments; $*P < 0.05$, 2-tailed Student's t test).

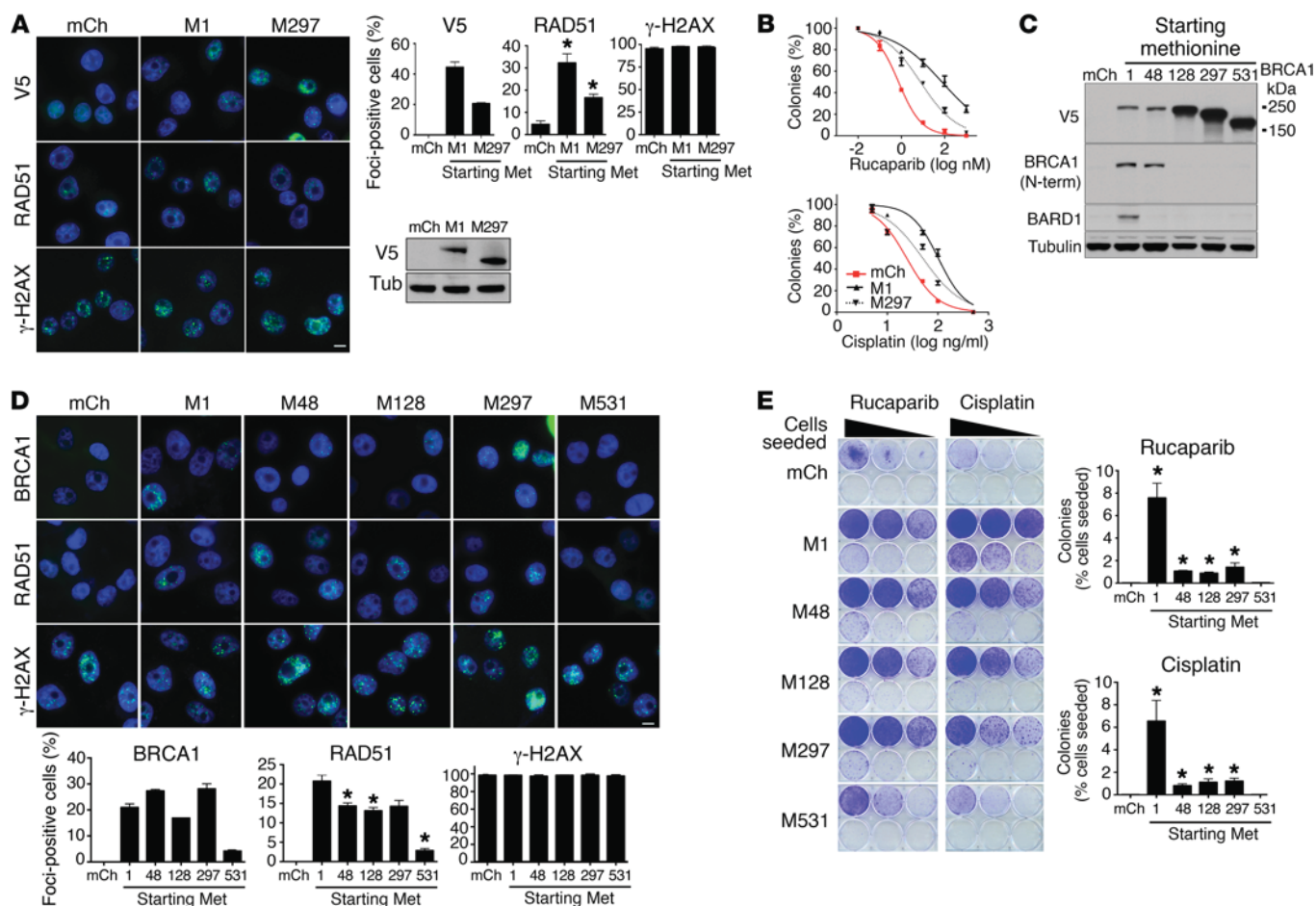


Figure 4. Ectopic overexpression of Rdd-BRCA1 proteins provides partial resistance. (A) SUM1315MO2 parental cells engineered to overexpress mCherry, BRCA1 full-length (M1), and BRCA1-Met-297 (M297) constructs were treated with IR, and V5, RAD51, γ -H2AX, and DAPI were detected by immunofluorescence. Representative images are shown. Scale bar: 10 μ m. Bar graphs show the mean and SEM percentage of cells containing more than 5 foci ($n = 3$ independent experiments; $*P < 0.05$, 2-tailed Student's t test). Western blot shows exogenous V5-tagged BRCA1 protein expression. (B) SUM1315MO2 cell lines described in A were treated with increasing concentrations of rucaparib or cisplatin and colony formation assessed. Three independent experiments were performed. (C) MDA-MB-436 cells stably expressing mCherry and BRCA1 constructs starting at Met-1 (full-length), Met-48, Met-128, Met-297, and Met-531 were assessed for V5, BRCA1 (N-terminal-specific Ab), BARD1, and tubulin expression by Western blot analysis. (D) MDA-MB-436 cells described in C were treated with IR, and V5, RAD51, γ -H2AX, and DAPI were detected by immunofluorescence. Representative images are shown. Scale bar: 10 μ m. Bar graphs show the mean and SEM percentage of cells containing more than 5 foci ($n = 3$ independent experiments; $*P < 0.05$, 2-tailed Student's t test). (E) MDA-MB-436 cells described in C were seeded at 6 different seeding densities and maintained in either 20 nM rucaparib or 20 ng/ml cisplatin for 3 weeks, until resistant colonies emerged or cells died. Representative 6-well plates are shown. Bar graphs show the mean and SEM number of colonies expressed as a percentage of the number of cells originally seeded ($n = 3$; $*P < 0.05$, 2-tailed Student's t test). Three independent experiments were performed.

age). However, MDA-MB-231 cells demonstrated robust coverage before Met-297 (79% coverage), with the first peptide detected at Arg-7. In contrast, we were unable to detect any peptides before Met-297 (0% coverage), with the first peptide detected at Asn-354 in SUM1315MO2 RR1 cells (Figure 2C and Supplemental Figure 1).

Furthermore, we compared the gel migration of endogenous BRCA1 from SUM1315MO2 RR1 and CR1 cells with exogenously expressed, full-length Met-128- and Met-297-initiating BRCA1 proteins. Gel migration patterns suggested that endogenous BRCA1 from SUM1315MO2 resistant cells migrated in a manner most similar to that of exogenous BRCA1-Met-297 cells (Figure 2D). To confirm that Met-297 was the initiating start site, we used CRISPR/Cas9 gene editing to disrupt the *BRCA1* reading frame before (sg_ex2) as well as at the Met-297 coding region (sg_ex11). MDA-MB-231 cells that were *BRCA1* WT demonstrated a similar

reduction in BRCA1 protein levels with both sg_ex2 and sg_ex11. In contrast, Rdd-BRCA1 protein expression in SUM1315MO2 RR1 cells was not affected by sg_ex2, but decreased with sg_ex11 treatment (Figure 2E and Supplemental Figure 2). These data support the notion that the Rdd-BRCA1 reading frame is initiated at Met-297.

There are several in-frame methionine start sites downstream of the *BRCA1*^{185delAG} mutation; we sought to understand why cells favored Met-297. First, we examined the possibility that alternative mRNA splicing was responsible for removing exons encoding Met-1 (exon 2) and translation start sites before Met-297 (located in exon 11). Reverse transcription-PCR (RT-PCR) analyses using a forward primer located in the 5'-UTR and a reverse primer in exon 11 (downstream of Met-297) detected 1 band that was the predicted size of full-length *BRCA1* (Supplemental Figure 3A). Additionally, we carried out a series of quantitative RT-PCR reactions using primers that

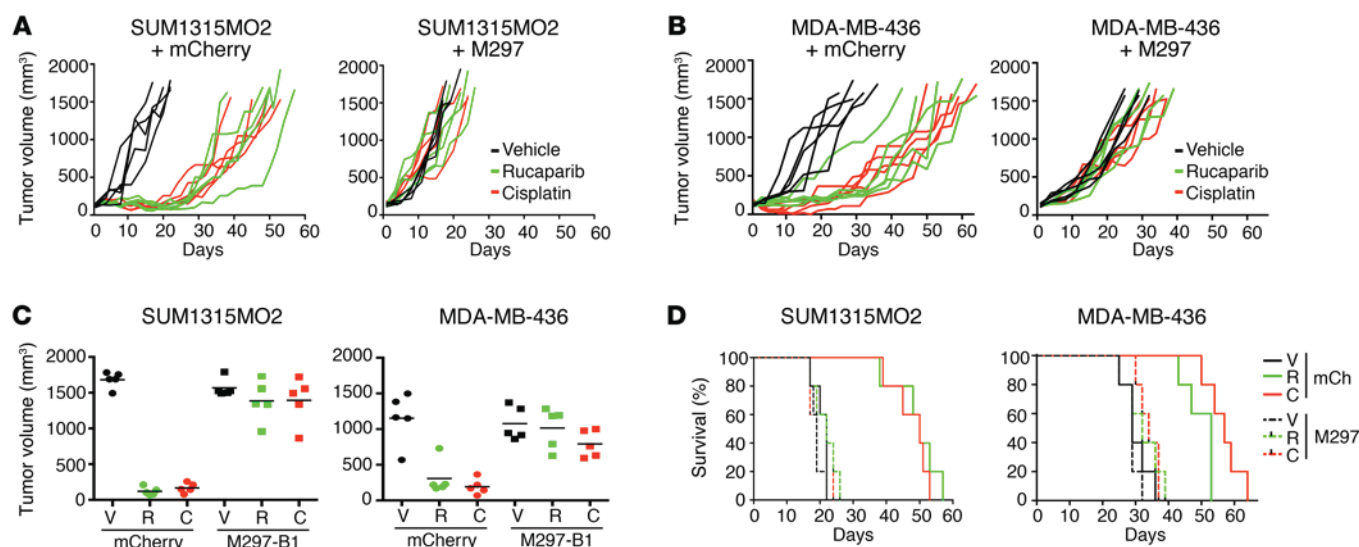


Figure 5. Ectopic overexpression of Rdd-BRCA1 proteins provides resistance in vivo. (A) SUM1315MO2 and (B) MDA-MB-436 tumor xenografts expressing ectopic mCherry or BRCA1-Met-297 were treated with vehicle (black lines), rucaparib (green lines), or cisplatin (red lines), and tumor growth was measured. Each line represents an individual tumor. (C) Individual tumor volumes at day 20 are shown. (D) Kaplan-Meier survival analyses. $n = 5$ mice per treatment group.

specifically detected *BRCA1* mRNA that contained exons 2, 6, 11, or 16. We found similar relative expression levels for each exon in all cell lines, indicating that full-length *BRCA1* mRNA, rather than an exon-deficient alternative splice isoform, was dominant (Supplemental Figure 3B). We next examined mRNA features that could impact the translation start site choice. Previous studies demonstrated that Met-48 had a weak, but Met-128 and Met-297 had similar-strength, Kozak consensus motifs (38). Hairpin structures at the 5' end of mRNA translation start sites commonly block translation initiation by preventing the 40S ribosomal subunit from binding (39–41). Analyses of a *BRCA1* mRNA secondary structure revealed a higher than 80% probability of the presence of a translation initiation inhibitory hairpin structure located immediately before Met-128; however, we found no such structures in proximity to the Met-297 start site (Supplemental Figure 4A). These data reveal the possibility that mRNA sequence and structural features resulted in a preference for Met-297 as the translation initiation site.

DNA damage-induced alternative protein translation is a process in which transcripts utilize downstream translation start sites for protein production, but is accompanied by a global decrease in total cellular translation (42, 43). We measured phosphorylated eIF2 α (p-eIF2 α) and total activating transcription factor 4 (ATF4) protein levels, which were previously shown to be markers of alternative translation (42). SUM1315MO2 RR and CR clones had elevated levels of eIF2 α phosphorylation and ATF4 compared with levels detected in the parental cell line. Additionally, puromycin incorporation into newly generated peptides was lower in resistant cell lines, suggesting that total levels of global translation were decreased (Supplemental Figure 4, B and C). These data support the notion that the alternative translation pathway contributed to the increased levels of Rdd-BRCA1 protein present in SUM1315MO2 resistant derivatives.

Rdd-BRCA1 does not require BARD1 for stability or function. The BRCA1 RING domain mediates interaction with BARD1 and is absent in the BRCA1-Met-297 protein. Both C- and N-termi-

nally interacting proteins CtIP and BARD1, respectively, were detected in immunoprecipitates of full-length BRCA1 from MDA-MB-231 cells. However, BARD1 was not present in immunoprecipitates of BRCA1 from SUM1315MO2 RR1 or CR1 cells, while CtIP was readily detectable (Figure 3A). Because BARD1 interaction with full-length BRCA1 is required for the stability of both proteins (44), we investigated the impact of siRNAs targeting both BRCA1 and BARD1 on relative protein levels. BRCA1 and BARD1 siRNA treatments resulted in reciprocal depletion of both proteins in MDA-MB-231 and MDA-MB-436 RR1 cells that expressed WT BRCA1 or a C-terminal truncated form of BRCA1, respectively (30). In contrast, BRCA1 siRNA decreased BRCA1 protein levels, but basal BARD1 protein levels were barely detectable and did not change with siRNA treatment in SUM1315MO2 RR1 cells; additionally, BARD1 siRNA did not affect BRCA1 protein levels (Figure 3B). BARD1 siRNA-treated MDA-MB-231 cells were 1,121- ($P < 0.001$) and 945-fold ($P < 0.001$) more sensitive to rucaparib with 2 independent BARD1 siRNAs than were scrambled siRNA-treated cells. Similarly, MDA-MB-436 RR1 cells were 67- ($P = 0.0414$) and 91-fold ($P = 0.0411$) more sensitive to rucaparib with 2 independent BARD1 siRNAs than were scrambled siRNA-treated cells. In contrast, BARD1 siRNA did not sensitize SUM1315MO2 RR1 cells to rucaparib treatment (Figure 3C).

To begin to investigate the role of the Rdd-BRCA1 protein in HR DNA repair and drug resistance, we treated RR1 and CR1 cells with either scrambled or 2 independent BRCA1 siRNAs and assessed their impact on rucaparib and cisplatin sensitivity by colony formation assays. BRCA1 siRNA-treated RR1 cells were 11- ($P = 0.0011$) and 12-fold ($P = 0.001$) more sensitive to rucaparib than were scrambled siRNA-treated control cells ($P = 0.0002$). Similarly, CR1 cells treated with BRCA1 siRNAs were 1.9- ($P = 0.048$) and 1.7-fold ($P = 0.049$) more sensitive to cisplatin than were scrambled siRNA-treated cells (Figure 3D). Furthermore, BRCA1 siRNA reduced RAD51 foci by 14- ($P = 0.002$) and 19-fold ($P = 0.002$) in RR cells and by 12- ($P = 0.0019$) and 12-fold ($P = 0.0021$) in CR cells,

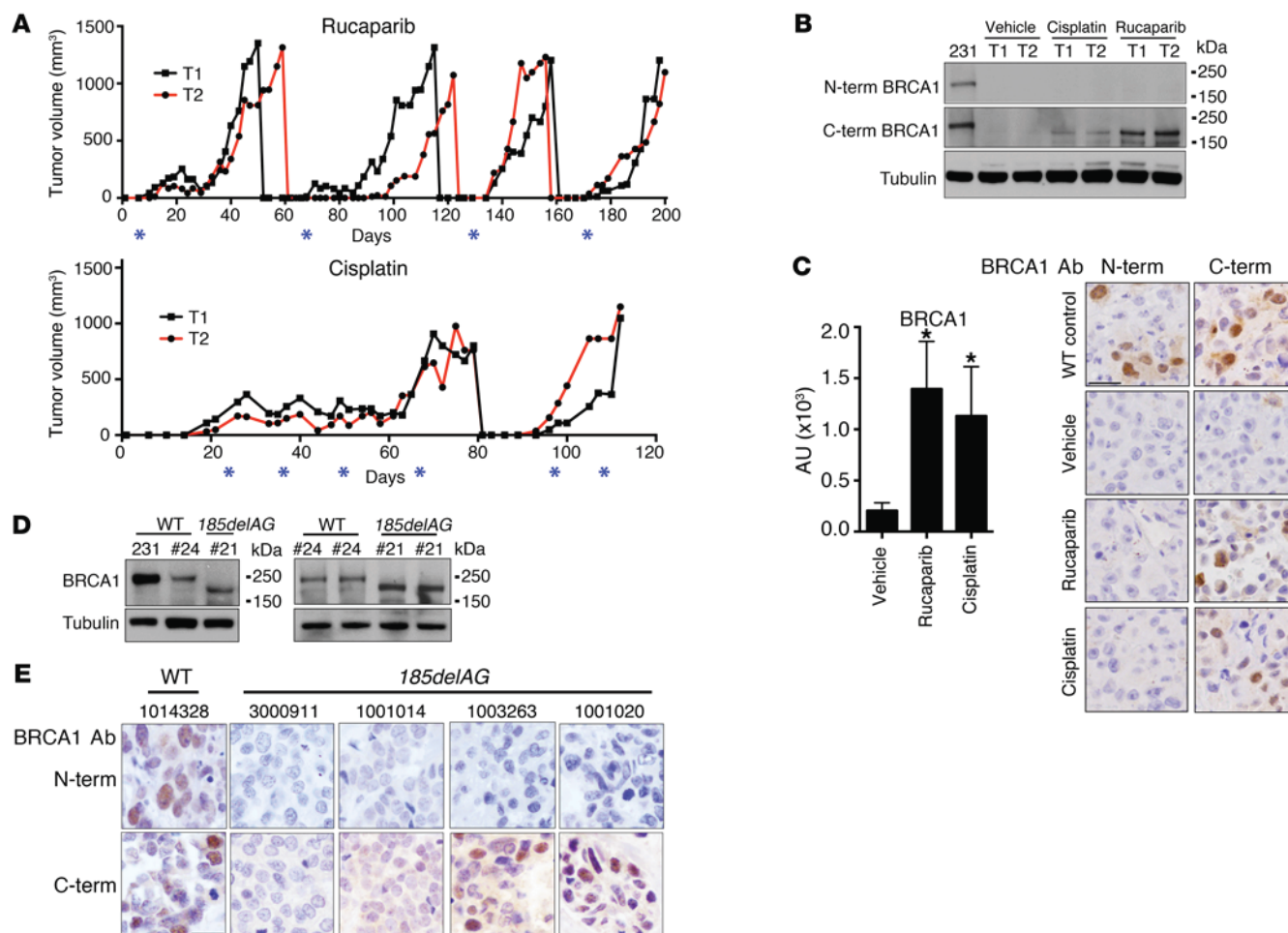


Figure 6. Rdd-BRCA1 proteins are expressed in tumors. (A) Mice carrying SUM1315M02 parental tumors were treated with rucaparib or cisplatin and tumor growth monitored. When tumors reached approximately 1,000 mm³ in size, they were harvested and reimplanted. Red and black lines represent individual tumors from each set of treatments; asterisks represent the days of drug administration. *n* = 2 mice per treatment group. (B) Assessment of BRCA1 protein levels from tumors described in A by Western blot analysis. MDA-MB-231 cells were used as BRCA1 WT controls. (C) Bar graph shows quantification of staining intensities using C-terminal Ab of at least 2 individual tumors for each treatment. MDA-MB-231 xenografts were used as BRCA1 WT controls; SUM1315M02 tumors were treated with vehicle or rucaparib, and cisplatin-resistant tumors from A were assessed by IHC for BRCA1 expression using N- and C-terminal-specific Abs. Scale bar: 20 μm. Two tumors for each treatment group were assessed. **P* < 0.05, 2-tailed Student's *t* test. Bar graph shows quantification of staining intensities using C-terminal Ab of at least 2 individual tumors for each treatment. (D) MDA-MB-231, PDX WO-24 (#24), and PDX WO-21 (#21) were assessed by Western blot analysis for BRCA1 protein expression using a C-terminal-specific Ab. Three individual tumors were assessed for each PDX model. Samples were run on parallel gels, and the tubulin blot was derived from duplicate samples run on a parallel gel. (E) Primary tumors from patients with WT or BRCA1^{185delAG} germline mutations were stained with BRCA1 N- and C-terminal-specific Abs by IHC. Scale bar: 20 μm. See Supplemental Table 2 for more details.

but γ-H2AX foci were unaffected (Figure 3E), with little impact on cell-cycle progression (Supplemental Figure 5).

Rdd-BRCA1 proteins are hypomorphic and provide partial resistance in vitro. We next examined the impact of exogenous BRCA1-Met-297 overexpression in SUM1315M02 parental cells. Exogenous full-length and BRCA1-Met-297 both formed IRIF and increased RAD51 IRIF by 6.9- (*P* = 0.0027) and 3.6-fold (*P* = 0.0158), respectively, compared with mCherry control-expressing cells. The levels of γ-H2AX were not affected by exogenous protein expression (Figure 4A). Furthermore, SUM1315M02 cells overexpressing exogenous BRCA1 full-length or Met-297 were 101.3- (*P* = 0.0255) and 10.3-fold (*P* = 0.0169) more resistant to rucaparib, respectively, as well as 4.3- (*P* = 0.0003) and 2.1-fold (*P* = 0.0004) more resistant to cisplatin than were mCherry-expressing cells, respectively (Figure 4B).

We further assessed the ability of a series of Rdd-BRCA1 proteins to provide PARPi and cisplatin rescue in a cellular background that lacked the endogenous BRCA1^{185delAG} allele. MDA-MB-436 cells were selected to assess exogenous Rdd-BRCA1 activity, as these cells harbor a hemizygous BRCA1^{5396+1G>A} mutation that disrupts BRCT domain protein folding, resulting in BRCA1 protein degradation and loss of detectable expression; additionally, these cells lack RAD51 foci and are exquisitely PARPi and cisplatin sensitive (30, 35). MDA-MB-436 cells expressing mCherry control and BRCA1 proteins initiating at Met-1, -48, -128, -297, and -531 were first confirmed by Western blot analysis for exogenous protein expression (Figure 4C). As expected, expression of Met-1, but not the RING-deficient Met-48-Met-531 proteins, resulted in a reemergence of BARD1. BRCA1-Met-1, -48, -128, and -297, but not Met-531, formed

efficient BRCA1 IRIF. However, BRCA1-Met-48, -128, -297, and -531 had 1.4- ($P = 0.026$), 1.6- ($P = 0.0138$), 1.5- ($P = 0.0671$), and 7.3-fold ($P = 0.0023$) less RAD51 IRIF compared with that detected in full-length BRCA1-Met-1-expressing cells. The levels of γ -H2AX were not affected by exogenous protein expression (Figure 4D).

To assess the ability of Rdd-BRCA1 proteins to provide therapy resistance, cells were seeded at a range of densities and incubated with a single rucaparib and cisplatin concentration that selected for drug-resistant colonies. Met-1, -48, -128, and -297 BRCA1-expressing cells had 253- ($P = 0.0016$), 36- ($P < 0.001$), 30- ($P < 0.001$), and 47-fold ($P = 0.0033$) more rucaparib-resistant colonies than did mCherry control cells, respectively; these cells also had 321-, 41-, 55-, and 60-fold (all $P < 0.001$) more cisplatin-resistant colonies than did mCherry control cells, respectively (Figure 4E). Interestingly, BRCA1-Met-531 had no impact on PARPi or cisplatin rescue. Similar results were obtained using standard colony formation assays exposing cells to increasing concentrations of drug (Supplemental Figure 6, A and B). Additionally, exogenous BRCA1-Met-297 provided rescue in both SUM1315MO2 and MDA-MB-436 cells in the presence of an shRNA targeting only endogenous BRCA1, suggesting that residual endogenous BRCA1 was not contributing to the observed rescue (Supplemental Figure 6, C and D).

BRCA1-Met-297 overexpression promotes resistance in vivo. We assessed the ability of exogenous BRCA1-Met-297 to provide rucaparib and cisplatin resistance in vivo. SUM1315MO2 and MDA-MB-436 cells that overexpressed mCherry control or BRCA1-Met-297 readily formed tumors and were used for xenograft experiments (Supplemental Figure 7). Rucaparib and cisplatin treatments both inhibited tumor growth in mCherry-expressing SUM1315MO2 and MDA-MB-436 tumors. In contrast, the degree of growth inhibition was markedly reduced in BRCA1-Met-297-expressing tumors (Figure 5, A and B). Twenty days after tumor implantation, rucaparib and cisplatin treatment delayed mean tumor growth by 14- ($P < 0.0001$) and 10-fold ($P < 0.0001$) in mCherry-overexpressing SUM1315MO2 tumors, respectively, compared with that observed in vehicle-treated mice. Similarly, rucaparib and cisplatin treatment delayed mean tumor growth by 3.7- ($P = 0.0023$) and 5.9-fold ($P = 0.0004$) in mCherry-overexpressing MDA-MB-436 tumors, respectively, compared with that seen in vehicle-treated mice. In contrast, neither rucaparib nor cisplatin treatment resulted in significant delays in tumor growth in BRCA1-Met-297-overexpressing SUM1315MO2 or MDA-MB-436 tumors compared with what was observed in vehicle-treated mice (Figure 5C).

Kaplan-Meier analyses indicated that the median overall survival (OS) of mice harboring SUM1315MO2 mCherry-expressing tumors that were treated with rucaparib or cisplatin increased by 2.3-fold ($P = 0.0034$, log-rank test) and 2.3-fold ($P = 0.0034$, log-rank test), respectively, compared with the median OS of vehicle-treated mice. Similarly, for mice harboring MDA-MB-436 mCherry-expressing tumors that were treated with rucaparib or cisplatin, OS increased by 1.8-fold ($P = 0.0018$, log-rank test) and 2-fold ($P = 0.0018$, log-rank test), respectively, compared with the OS of vehicle-treated mice. In contrast, mice harboring BRCA1-Met-297-expressing tumors that were treated with either rucaparib or cisplatin did not demonstrate significant increases in OS compared with vehicle-treated mice (Figure 5D).

Short-term assessment of tumor response by immunohistochemical analyses demonstrated that rucaparib and cisplatin treatments significantly increased γ -H2AX staining intensity in all tumors. Furthermore, rucaparib and cisplatin treatments decreased Ki67 staining intensity by 3- ($P < 0.0001$) and 4.3-fold ($P < 0.0001$), respectively, in mCherry-expressing SUM1315MO2 tumors and by 2.3- ($P = 0.0046$) and 2.4-fold ($P = 0.0217$), respectively, in mCherry-expressing MDA-MB-436 tumors. In contrast, rucaparib and cisplatin treatment did not significantly impact Ki67 staining in BRCA1-Met-297-expressing tumors (Supplemental Figure 8).

Rdd-BRCA1 is expressed in resistant tumors. To assess the expression of endogenous Rdd-BRCA1 protein in vivo, SUM1315MO2 xenografts that were initially responsive to rucaparib and cisplatin treatment were subjected to multiple rounds of rucaparib or cisplatin treatment until tumors acquired resistance (Figure 6A). Rucaparib- and cisplatin-resistant tumors demonstrated increased expression of Rdd-BRCA1 as measured by Western blot analysis (Figure 6B). Additionally, Abs were optimized to distinguish between N- and C-terminal-containing BRCA1 proteins by immunohistochemical analyses (Supplemental Figure 7). SUM1315MO2 tumors that were rucaparib and cisplatin resistant had a 6.8- ($P < 0.0001$) and 5.5-fold ($P < 0.0001$) increase in BRCA1 C-terminal Ab immunohistochemical staining intensity compared with that seen in parental tumors. However, there was no change in N-terminal BRCA1-staining intensity (Figure 6C).

We next compared BRCA1 protein expression levels in a *BRCA1*^{185delAG} mutant high-grade serous ovarian cancer (HGSOC) patient-derived xenograft (PDX), WO-21, with levels in a *BRCA1* WT HGSOC PDX, WO-24 (Figure 6D). PDX WO-21 was obtained from a patient whose tumor progressed on olaparib therapy and who subsequently underwent tertiary cytoreduction surgery, at which point tumor was collected for orthotopic ovarian transplantation and PDX generation. PDX WO-24 was obtained from an untreated patient during primary cytoreduction surgery. BRCA1 protein expression levels in WO-24 was similar levels in MDA-MB-231 cells. However, WO-21, harboring the *BRCA1*^{185delAG} mutation, expressed a truncated BRCA1 protein resembling BRCA1-Met-297 (Figure 6D).

Furthermore, we analyzed breast and ovarian carcinomas resected from individual patients with germline *BRCA1*^{185delAG} mutations for the expression of Rdd-BRCA1 proteins using N- and C-terminal-specific Abs (Supplemental Table 2). A patient's carcinoma that was WT for *BRCA1* was used as a positive control (specimen 1014328). Here, BRCA1 protein could be detected with both N- and C-terminal Abs. We did not detect positive BRCA1 staining with either Ab in 2 primary ovarian carcinomas with *BRCA1*^{185delAG} mutations. Interestingly, we were able to detect nuclear BRCA1 staining with the C-terminal, but not the N-terminal, BRCA1 Ab in 2 recurrent breast carcinomas (specimens 1003263 and 1001020) from individuals with *BRCA1*^{185delAG} germline mutations (Figure 6E and Supplemental Table 2).

Discussion

In the current study, we present evidence to support a mechanism of PARPi and platinum resistance, whereby BRCA1 protein products are generated using translation start sites located downstream of the mutation-induced stop codon. BRCA1 proteins pro-

duced from downstream translation start sites were truncated at the N-terminal region and lacked the RING domain. However, Rdd-BRCA1 proteins were hypomorphic, contributing to RAD51 loading and PARPi and cisplatin resistance.

Multiple pathogenic frameshifting mutations are located toward the 5' end of the *BRCA1* gene, including the most commonly reported *BRCA1*^{185delAG} allele. The *BRCA1*^{185delAG} mRNA is predicted to produce a short, approximately 5-kDa peptide (E23fsX17). Although this peptide has not been detected endogenously, exogenous overexpression of a BRCA1 peptide consisting of aa 1–23fsX17 was reported to induce phenotypes not associated with full-length BRCA1, including enhanced IL-1 β expression, and to promote apoptosis (45, 46). However, endogenously generated small peptide products normally located in the structured RING region of the protein may not be folded and are likely to be degraded. In line with this, we did not detect any N-terminal region-containing peptide products in SUM1315MO2 parental or resistant cells.

In contrast to the majority of premature termination codon-inducing (PTC-inducing) *BRCA1* mutations, previous studies demonstrated that *BRCA1*^{185delAG} and *BRCA1*^{188del11} alleles were not subject to nonsense-mediated mRNA decay (NMD), leading to speculation that translation may continue after the PTC and prevent NMD (38, 47). Using C-terminal-specific BRCA1 Abs, we confirmed that translation does occur after the *BRCA1*^{185delAG}-induced PTC, and we readily identified a protein product that was only slightly smaller in size compared with that of full-length BRCA1 in SUM1315MO2 parental and resistant cells, probably starting at Met-297. Previous *in silico* analyses predicted Met-128 and Met-297 to be downstream *BRCA1* translation start sites (38). Our analyses suggest that *BRCA1* mRNA structural features may result in a preference for the Met-297 translation initiation site.

BRCA1-Met-297 was present in parental SUM1315MO2 cells but more abundant in rucaparib and cisplatin resistant clones. Interestingly, expression of BRCA1-Met-297 tended to be higher in cells in which resistance was rucaparib induced as opposed to cisplatin induced. This could be reflective of the type of DNA repair mechanisms that are induced by each agent, whereby PARPi primarily activates BRCA1 mediated-HR DNA repair, whereas platinum is likely to elicit both HR and nucleotide excision repair (NER) DNA repair pathways (48). Alternatively, PARPi may have a greater impact on DNA damage-induced alternative translation pathways (43). DNA damage has previously been associated with translational reprogramming, allowing for selective synthesis of DNA damage response proteins, and relies on upstream ORFs; DNA damage has also been associated with a general reduction in global translation (42, 43, 49). Our data suggested that SUM1315MO2 resistant cells had lower rates of global translation as well as increased levels of p-eIF2 α and total ATF4, potentially indicating that the alternative translation pathway contributed to increased BRCA1-Met-297 expression.

In contrast to full-length BRCA1, Rdd-BRCA1 did not require BARD1 for stability, probably due to an absence of the RING-located nuclear export signal usually masked by BARD1 binding (50). However, BARD1 was unstable and present at low or undetectable levels in the absence of a BRCA1 RING domain-containing peptide. Previous studies using genetic mouse mod-

els of mammary carcinogenesis demonstrated that the *Brca1*^{C61G} allele product, which is unable to interact with BARD1, promoted RAD51 loading as well as platinum and PARPi resistance (31). The mechanism we describe may not be limited to cancers with *BRCA1*^{185delAG} mutations and could be relevant to multiple frameshifting, 5'-located *BRCA1* mutations. However, mutations located after Met-297 (c.891) are unlikely to develop resistance through this mechanism, as the next downstream translation start site at Met-531 (c.1593) produced a functionless protein. Truncated constructs expressing BRCA1 BRCT domains have previously been shown to be capable of forming foci (51–55). However, BRCA1-Met-531 did not form IRIF, despite retaining BRCT domains, suggesting that the peptide length or specific regions retained may impact the ability of BRCT domains to be recruited to sites of DNA damage.

SUM1315MO2 cells and tumors were initially PARPi and platinum sensitive, in line with the patients' clinical response data, in which many patients harboring *BRCA1*^{185delAG} mutations had robust PARPi or platinum responses, but eventually acquired resistance (17, 56). Although RNAi-mediated depletion of endogenous Rdd-BRCA1 from SUM1315MO2 RR and CR cells reduced RAD51 IRIF and restored PARPi and cisplatin sensitivity, ectopic overexpression of BRCA1-Met-297 did not provide the same degree of resistance that was observed in SUM1315MO2 RR and CR cells. Furthermore, RING domain-deficient BRCA1 proteins were less effective at rescuing PARPi and cisplatin sensitivity than were ectopic full-length BRCA1-expressing cells. These data suggest that Rdd-BRCA1 proteins were important for the development of resistance; however, additional epi/genetic events may work in conjunction with BRCA1-Met-297 to generate a more robust resistance phenotype.

BRCA1^{185delAG} tumors from patients who had developed either platinum or PARPi resistance were previously shown to frequently harbor genetic reversion mutations that restored the WT sequence (22). Recent analyses of therapy-resistant ovarian carcinomas confirmed that approximately half of *BRCA1*-mutant carcinomas tested harbored secondary mutations that restored the ORF (25). Interestingly, the allele frequency of reversion mutations often varied within a tumor, potentially suggesting that intratumoral heterogeneity facilitates the existence of more than one mechanism of resistance within individual carcinomas. It is possible that reversion mutation-containing cells coexist with RING-deficient BRCA1 protein-expressing cells within neoplasms.

In conclusion, we show that BRCA1 RING domain-deficient proteins retain hypomorphic activity and, when expressed at high enough levels, can contribute to HR DNA repair and therapy resistance. In light of the increasing number of resistance mechanisms described to date, multiple biological events may contribute to therapy resistance in *BRCA1* mutation carriers, as well as within individual tumors. Further work is required to assess the incidence of these events in patients' malignancies as well as their impact on chemotherapeutic response and survival outcomes.

Methods

Cell lines and reagents. Cell lines were obtained from the ATCC or Asterand. Cell lines tested negative for mycoplasma. Cells were authenticated by DNA fingerprinting (IDEXX BioResearch). Chem-

icals were purchased from Sigma-Aldrich. Clovis Oncology provided rucaparib (CO-338). Cisplatin was from APP/Fresenius Kabai USA and paclitaxel from Sagent Pharmaceuticals.

Derivation of resistant clones. SUM1315MO2 cells were cultured in the presence of either 1 μ M rucaparib or 30 ng/ml cisplatin in 6-well plates until 3 independent resistant clones emerged (approximately 1 month). Clones were labeled RR1 to RR3 and CR1 to CR3. Cells were cultured in the absence of rucaparib or cisplatin for a minimum of 2 weeks before they were used for experiments.

Colony formation assays. Cells were seeded in 6-well plates at 1,000 cells per well in the presence of increasing concentrations of rucaparib. For cisplatin, mitomycin C, or taxol treatments, exponentially growing cells were cultured in 24-well plates, treated with increasing concentrations or doses for 24 hours, and replated at 1,000 cells per well in 6-well plates for colony formation. For siRNA treatments, exponentially growing cells were reverse transfected in 24-well plates, and 2 days after transfection, cells were treated with rucaparib for 72 hours or cisplatin for 24 hours and then replated in 6-well plates for colony formation. For the resistance emergence assays described in Figure 4E, cells were seeded at decreasing densities in 6-well plates and maintained in the presence of either 20 nM rucaparib or 20 ng/ml cisplatin until resistant colonies emerged. Colony formation was assessed 2 weeks after plating with crystal violet staining. The mean colony formation from 3 experiments was expressed as a percentage of colonies $\pm$ SEM relative to vehicle-treated cells. Lethal concentration (LC_{50}) values (concentration required to reduce colony formation by 50% compared with vehicle) were calculated using GraphPad Prism software (Version 6) and used to compare fold changes in drug sensitivity.

Immunoprecipitation, mass spectrometry, Western blotting, immunofluorescence, and focal microscopy. BRCA1 Ab (Santa Cruz Biotechnology Inc.; catalog sc-6954) was used for immunoprecipitation of BRCA1 complexes from 2 mg nuclear extract using a Pierce Classic IP Kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Nuclear extracts were derived using NE-PER Nuclear and Cytoplasmic Extraction Reagents (Thermo Fisher Scientific) according to the manufacturer's instructions. For peptide analyses, after immunoprecipitation, bands corresponding to BRCA1 were cut out and gel purified, digested using chymotrypsin, and analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS). The following Abs were used to detect proteins by Western blotting: tubulin (Cell Signaling Technology; catalog 2148); HSP90 (Enzo Life Sciences; catalog ADI-SPA-830); RAD51 (Santa Cruz Biotechnology Inc., catalog sc-8349); N-terminal BRCA1, MS110 (Calbiochem; catalog OP92); C-terminal BRCA1 (Santa Cruz Biotechnology Inc.; catalog sc-6954); C-terminal BRCA1 (EMD Millipore; catalog 07-434); V5 (Bethyl Laboratories; catalog A190-120A); CtIP (Bethyl Laboratories; catalog A300-488A); BARD1 (Bethyl Laboratories; catalog A300-263A); p-S51 eIF2 α (Cell Signaling Technology; catalog 3398); total eIF2 α (Cell Signaling Technology; catalog 9722); and ATF4 (Cell Signaling Technology; catalog 11815). For assessment of newly translated peptides, cells were incubated with 1 μ M puromycin for 30 minutes. Cells were then collected and subjected to Western blot analysis using anti-puromycin Ab (Kerafast; catalog EQ0001). For immunofluorescence, V5 (Bethyl Laboratories; catalog A190-120A); BRCA1 (Santa Cruz Biotechnology Inc.; catalog sc-6954); γ -H2AX [pS139] (EMD Millipore; catalog 05-636); and RAD51 (Santa Cruz Biotechnology Inc.; catalog sc-8349). Abs were followed by second-

ary Abs conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories; codes 711-585-152, 711-095-152, 715-095-150, and 715-585-150). We acquired immunofluorescence images using a Nikon NIU Upright Fluorescence Microscope and generated images using Nikon NIS Elements software. For IR experiments, we routinely fixed cells 8 hours after treatment with 10 Gy. For analyses, we counted a minimum of 200 cells per condition or cell line. Each experiment was performed at least 3 times with biological replicates.

Cell-cycle analysis. Cells were harvested and fixed with ethanol. The cells were washed with PBS and resuspended in 0.5 ml FxCycle PI/RNase Staining Solution (Life Technologies, Thermo Fisher Scientific) and incubated at room temperature for 30 minutes. The data were acquired using a BD LSR II Flow Cytometer and analyzed using FlowJo software.

CRISPR/Cas9 gene editing. The CRISPR/Cas9 system was a gift of Christoph Seeger (Fox Chase Cancer Center) (57). MDA-MB-231 and SUM1315MO2 cells were first infected with the lentiviral vector pCW-Cas9 and selected with puromycin (2 μ g/ml). Cas9 expression was conditionally induced by the presence of doxycycline (4 μ g/ml). A Cas9 high-expression clone was selected for sequential genome editing. Gene-specific single-guide RNA (sgRNA) sequences were identified using the CRISPR Design Tool (<http://crispr.mit.edu/>). We used the following DNA oligonucleotides to generate the following sgRNAs: sg_GFP, forward: CACCGAGCTGGACGGCGACGTAA; sg_GFP, reverse: AAACCTTTACGTCGCCGTCCAGCTC; sg_exon 2, forward: CACCGTTGTGCTGACTTACCAGAT; sg_exon 2, reverse: AAACATCTGGTAAGTCAGCACAAAC; sg_exon 11, forward: CACCGTGAAGTTAAACAATGCACC; sg_exon 11, reverse: AAACGGTGCATTGTGTTAACTTCAC. sgRNA oligonucleotides were cloned into the sgRNA expression vector pLX-SG1 using BsmBI sites.

MDA-MB-231-Cas9 and SUM1315MO2-Cas9 cells were then infected with the lentiviral vector pLX-SG1 and selected with blasticidin (10 μ g/ml). After a 5-day incubation with doxycycline (4 μ g/ml), cells were collected. For validation of targeted mutations, genomic DNA was isolated from cells, followed by PCR amplification of targeted loci using the forward and reverse primers listed below, and PCR products were directly sequenced using the primers listed below. The expression of BRCA1 was further confirmed by Western blot analysis. Exon 2, forward: GATAGGAAGTGAATATGCCTTGA, exon 2, reverse: CAGCCTCTC-GACAGAGATCCTAT, exon 2, sequenced: ATGGAGAAAGCTAAG-GCTACCA; exon 11, forward: GAGTGGTTTTCAGAAAGTGATGA, exon 11, reverse: TAATACTGGAGCCCACTTCATTAGT, exon 11, sequenced: TGGCTCAGTAACAAATGCTCCTAT.

RNA interference and cDNA add-back treatments. We purchased Hs_BRCA1_FlexiTube siRNA constructs (construct 1, SI00096313 and construct 2, SI02654575) and AllStars Negative Control siRNA (scrambled control) from QIAGEN. BARD1 siRNA 1 (J-003873-09) and siRNA 2 (J-003873-10) were obtained from Dharmacon. Mission shRNA non-target control (SHC002) was purchased from Sigma-Aldrich, and shRNA targeting endogenous BRCA1 was a gift of Jos Jonkers (Netherlands Cancer Institute, Amsterdam, Netherlands). Transfections were carried out according to standard protocols. mCherry and HA-BRCA1 cDNA were cloned into the pENTR1A Gateway Entry vector (Invitrogen) and shuttled into a pDest-IRES-GFP Destination vector (Life Technologies, Thermo Fisher Scientific). To generate BRCA1 proteins where translation initiated at methionines 48–531, a small fragment corresponding to the specific methionine of interest was first amplified using the follow-

ing primers: Met-48, forward: CCCAAGCTTATGCTGAACTTCT-CAACCAGAAG, reverse: ATCTGTTATGTTGGCTCCTTGCTA; Met-128, forward: CCCAAGCTTATGGGCTACAGAAACCGTGC, reverse: ATCTGTTATGTTGGCTCCTTGCTA; Met-297, forward: AGCTTAT-CAATGTAGAAAAGGCTG, reverse: AATTCAGCCTTTTCTACATTCA-TA; Met-531, forward: CCCAAGCTTATGATAATCAGGGAACAAAC, reverse: CGAGATACTTTCCTGAGTGCCA. The amplified fragments were used to replace the region spanning the HA affinity tag to the ATG of interest in a pENTR-HA-BRCA1-V5 (full-length) construct using HindIII and EcoRI (M48, M128, M297) and KpnI (M531) sites. cDNAs were shuttled into pLenti-IRES-GFP Destination vectors using the LR Clonase system (Invitrogen). Lentiviral generation and infections were carried out according to standard protocols. Protein knockdown or reexpression was routinely assessed 72 hours after transfection or 96 hours after infection. Cells infected with cDNAs were sorted for GFP positivity using the BD FACSAria II cell sorter and routinely checked for GFP positivity to maintain stable cell lines.

Gene-sequencing and RNA analyses. Genomic DNA was isolated from cells using the DNeasy Blood and Tissue kit (QIAGEN). *BRCA1* gene sequencing was carried out as previously described (58). PCR amplicons were sequenced bidirectionally using the Applied Biosystems Big Dye Terminator version 3.1 Cycle Sequencing Kit and an ABI 3130xl genetic analyzer. Trace sequences were analyzed using Sequencher version 4.9 software (Gene Codes Corporation) and ABI Sequence Scanner version 1.0 software. *BROCA* sequencing included the following genes: *ATM*, *ATR*, *BABAM1*, *BAP1*, *BARD1*, *BLM*, *BRCA1*, *BRCA2*, *BRCC36*, *BRE*, *BRIP1*, *CDK12*, *CHEK1*, *CHEK2*, *DCLRE1C*, *FAM175A*, *FANCC*, *ID4*, *LIG4*, *MLH1*, *MRE11A*, *MSH2*, *MSH6*, *NBN*, *PALB2*, *PIK3CA*, *PMS2*, *PRKDC*, *PTEN*, *RAD50*, *RAD51*, *RAD51B*, *RAD51C*, *RAD51D*, *RBBP8*, *SLX4*, *TOPBP1*, *TP53*, *TP53BP1*, *UIMC1*, *USP28*, *XRCC2*, *XRCC3*, *XRCC4*, *XRCC5*, and *XRCC6*, and only clear loss-of-function mutations were reported (37).

Total RNA was isolated from cell lines using the RNeasy Plus Mini Kit (QIAGEN). RT-PCR assays were performed with cDNA generated using the SuperScript III First-Strand Synthesis System (Life Technologies, Thermo Fisher Scientific) and Hot Start Taq DNA Polymerase (New England BioLabs). Primers were located in exon 1, forward: GTATTCTGAGAGGCTGCTGCTTAG and exon 11, reverse: TTCATTGGCTTGTTACTCTTCTTG.

For quantitative RT-PCR, RNA was tested for quality on a Bioanalyzer (Agilent). RNA concentrations were determined with a spectrophotometer (NanoDrop; Thermo Fisher Scientific). RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Ambion, Thermo Fisher Scientific) and a mixture of anchored oligo-dT and random decamers. Two reverse transcription reactions were performed for each sample using either 100 or 25 ng input RNA. Assays were used in combination with Taqman Universal Master Mix or Power SYBR Green Master Mix and run on a 7900 HT Sequence Detection System (all from Applied Biosystems, Thermo Fisher Scientific). Cycling conditions were 95°C for 15 minutes, followed by 40 (2-step) cycles (95°C, 15 s; 60°C, 60 s). Ct values were converted to quantities (in AU) using a standard curve (4 points, 4-fold dilutions) established with a calibrator sample. *BRCA1* and the housekeeping gene *POLR2F* mRNA levels were measured using the following forward and reverse primers: *BRCA1*, exons 2/3, forward: TTATCTGCTCTTCGCGTTGAAG, *BRCA1*, exons 2/3, reverse: TTGTGGAGACAGGTTCTCTTGAT; *BRCA1*, exons 6/7, forward:

TGACACAGGTTTGGAGTATGCA, *BRCA1*, exons 6/7, reverse: CTGTAGCCCATACTTTGGATGATAGA; *BRCA1*, exon 11, forward: TAG-CAAGGAGCCAACATAACAGAT, *BRCA1*, exon 11, reverse: CTGTAGCCCATACTTTGGATGATAGA; *BRCA1*, exons 15/16, forward: GAATAGAAACTACCCATCTCAAGAGGA, *BRCA1*, exons 15/16, reverse: CAGGTAAGGGGTTCCCTCTAGAT; *POLR2F*, forward: TGCCATGAAGGAACTCAAGG, *POLR2F*, reverse: TCATAGCTC-CCATCTGGCAG.

For each experiment, the *BRCA1* mRNA values were normalized to *POLR2F* values and expressed as a fraction of expression levels detected in MDA-MB-231 cells.

RNA secondary structure predicted was made using the RNA-structure web server (<http://rna.urmc.rochester.edu/RNAstructureWeb/Servers/Predict1/Predict1.html>). Briefly, we entered the 70-bp mRNA sequence upstream of Met-128 and Met-297 for secondary mRNA prediction using the default settings.

PDX tumor derivation, xenograft treatments, and analyses. NSG (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ) mice were purchased from The Jackson Laboratory. Patients' tumors were harvested at the time of surgery, and small tumor chunks (5-mm) were transplanted into the ovary of 5- to 8-week-old female mice. Once tumors were established and reached approximately 700 to 1,000 mm³ by ultrasound (M-Turbo; SonoSite), tumors were harvested for expansion and frozen for DNA, RNA, and protein analyses. Whole-exome sequencing was performed for *BRCA1* and *BRCA2* genes.

For xenograft studies, SUM1315MO2 and MDA-MB-436 cells were s.c. implanted into 6-week-old female NSG mice. When tumors reached approximately 300 mm³, they were harvested and cut into smaller pieces, followed by s.c. reimplantation. Treatment was initiated when tumors reached between 150 and 180 mm³ in size. Rucaparib was administered at 150 mg/kg twice daily for 10 consecutive days, with a 2-day break after the first 5 days. Cisplatin was administered in a single dose of 6 mg/kg. Vehicle treatment consisted of 0.5% methylcellulose in water. Tumors were measured with calipers and tumor volumes calculated using the formula: (length × width²). Measurements were made every 3 days and mice euthanized when tumors reached 1,500 mm³, in accordance with the institutional guidelines of Fox Chase Cancer Center.

Histologic and immunohistochemical staining. Slides were deparaffinized and hydrated. Antigen retrieval was performed using EDTA buffer, pH 9 (Dako; code S2368). Endogenous peroxidases were quenched by the immersion of slides in 3% hydrogen peroxide solution (30% H₂O₂, Fisher BP2633-500, diluted in methanol). The following primary Abs were used: Ki-67 (Epitomics; clone EP5; 1:1,500) or γ-H2AX (pS139) (EMD Millipore; N1-431; 1:20,000); *BRCA1* N-terminal (Calbiochem; MS110; 1:400); and *BRCA1* C-terminal (EMD Millipore; 07-434; 1:7,500). Abs were diluted with DaVinci Green Diluant (Biocare Medical; code PD900) and incubated on slides overnight at 4°C in a humidified slide chamber. Slides were then washed 3 times in Tris-buffered saline with Tween-20 (TBST) and incubated with EnVision+ System HRP Labelled Polymer Anti-Rabbit (Dako; code K4003) for 1 hour at room temperature. Specimens were washed 3 times in TBST and then developed with DAB solution (Dako; code K3468) and counterstained in Meyer's Hematoxylin (Sigma-Aldrich; catalog MHS32-1L). For analyses of Ki67 and γ-H2AX expression, a minimum of 2 tumors derived from 2 separate mice were used. Mice were treated with rucaparib 150 mg/kg 2 times per day for 4 consecutive

days. For cisplatin, mice were treated with a single dose of 6 mg/kg. Tumors were resected and formalin fixed 4 days after the first dose. A minimum of 2 tumors and 3 images per tumor were used to calculate staining intensities. Image-staining intensities were measured using ImageJ software (NIH). For BRCA1 patient tumor analyses, staining was scored either positively or negatively, on the basis of the presence or absence of nuclear staining.

Statistics. All results are presented as the mean $\pm$ SEM and were analyzed by a 2-tailed Student's *t* test. Statistical significance was accepted at a *P* value of less than 0.05.

Study approval. Fox Chase Cancer Center (IRB no. 14-805) and the University of Pennsylvania (IRB no. 702679) approved the work carried out in this study. Animals were maintained according to IACUC guidelines of the Fox Chase Cancer Center (IACUC no. 12-13) and the Wistar Institute (Philadelphia, Pennsylvania, USA; IACUC no. 112635).

Author contributions

YW, AJB, JJK, EN, KQC, HHK, EG, FS, and NJ designed and performed the experiments. MIH and EMS carried out BROCA analyses. YW, AJB, JJK, EN, HHK, EG, FS, and NJ analyzed and interpreted the data. NJ wrote the manuscript and supervised the project.

Acknowledgments

This work was supported by the National Cancer Institute (NCI) (5P30CA006927, to the Fox Chase Cancer Center); the Fox Chase Cancer Center–University of Pennsylvania (FCCC-UPENN) Specialized Program of Research Excellence (SPORE) in Ovarian Cancer; a Susan G. Komen Career Catalyst Award (CCR12226280, to NJ); a Department of Defense Pilot Award and Nested Teal Postdoctoral Scholar Award (OC140040, to NJ and YW) and OC130212 Department of Defense Ovarian Academy Award (NJ); an NIH T32 Postdoctoral Trainee Grant (T32 CA 9035-39, to JJK); the Pacific Ovarian Cancer Research Consortium SPORE in Ovarian Cancer (P50CA83636) and the Wendy Feuer and OCRF Program Project Development Grant (both to EMS). Clovis Oncology supplied rucaparib. We are grateful to Cara Dubyk for help with IHC staining and to the Fox Chase Cancer Center Biorepository, Cell Sorting, Genomics, and Histopathology facilities. We thank Hsin Yao Tang at the Wistar Proteomics facility for help with mass spectrometry.

Address correspondence to: Neil Johnson, Fox Chase Cancer Center, 333 Cottman Avenue, Philadelphia, Pennsylvania 19111, USA. Phone: 215.728.7016; Email: neil.johnson@fccc.edu.

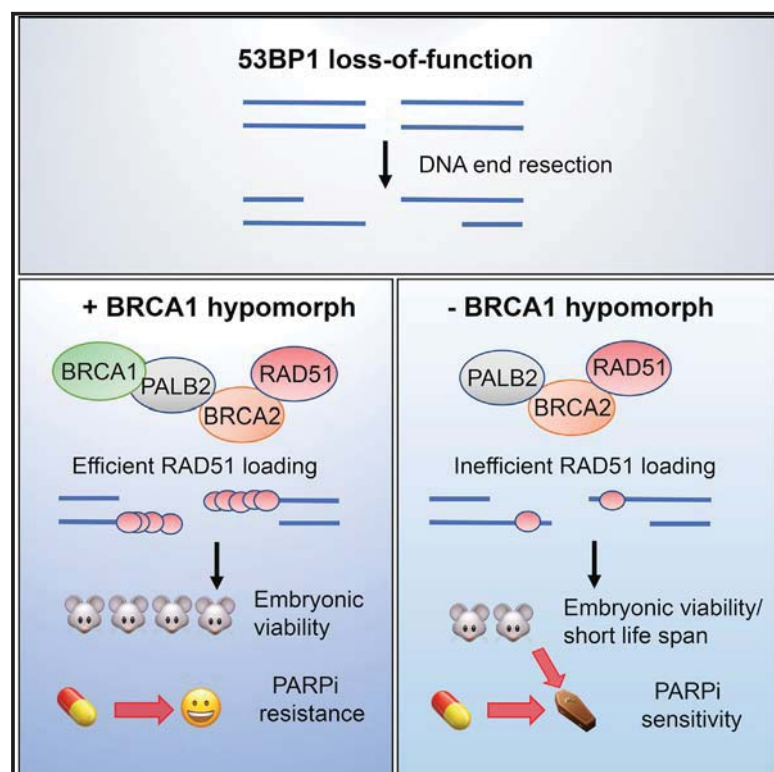
- Szabo CI, King MC. Inherited breast and ovarian cancer. *Hum Mol Genet.* 1995;4(spec no):1811–1817.
- Friedman LS, et al. Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian cancer in ten families. *Nat Genet.* 1994;8(4):399–404.
- Yang X, Lippman ME. BRCA1 and BRCA2 in breast cancer. *Breast Cancer Res Treat.* 1999;54(1):1–10.
- Petrucelli N, Daly MB, Feldman GL. Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. *Genet Med.* 2010;12(5):245–259.
- Roy R, Chun J, Powell SN. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat Rev Cancer.* 2012;12(1):68–78.
- Huen MS, Sy SM, Chen J. BRCA1 and its toolbox for the maintenance of genome integrity. *Nat Rev Mol Cell Biol.* 2010;11(2):138–148.
- Jiang Q, Greenberg RA. Deciphering the BRCA1 Tumor Suppressor Network. *J Biol Chem.* 2015;290(29):17724–17732.
- Moynahan ME, Chiu JW, Koller BH, Jasin M. Brca1 controls homology-directed DNA repair. *Mol Cell.* 1999;4(4):511–518.
- Bryant HE, et al. Specific killing of BRCA2-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature.* 2005;434(7035):913–917.
- Farmer H, et al. Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature.* 2005;434(7035):917–921.
- Moynahan ME, Cui TY, Jasin M. Homology-directed dna repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brca1 mutation. *Cancer Res.* 2001;61(12):4842–4850.
- Quinn JE, et al. BRCA1 functions as a differential modulator of chemotherapy-induced apoptosis. *Cancer Res.* 2003;63(19):6221–6228.
- Silver DP, et al. Efficacy of neoadjuvant Cisplatin in triple-negative breast cancer. *J Clin Oncol.* 2010;28(7):1145–1153.
- Safra T, Rogowski O, Muggia FM. The effect of germ-line BRCA mutations on response to chemotherapy and outcome of recurrent ovarian cancer. *Int J Gynecol Cancer.* 2014;24(3):488–495.
- Jayson GC, Kohn EC, Kitchener HC, Ledermann JA. Ovarian cancer. *Lancet.* 2014;384(9951):1376–1388.
- Scott CL, Swisher EM, Kaufmann SH. Poly (ADP-ribose) polymerase inhibitors: recent advances and future development. *J Clin Oncol.* 2015;33(12):1397–1406.
- Kaufman B, et al. Olaparib monotherapy in patients with advanced cancer and a germline BRCA1/2 mutation. *J Clin Oncol.* 2015;33(3):244–250.
- Ledermann J, et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by BRCA status in a randomised phase 2 trial. *Lancet Oncol.* 2014;15(8):852–861.
- Luvero D, Milani A, Ledermann JA. Treatment options in recurrent ovarian cancer: latest evidence and clinical potential. *Ther Adv Med Oncol.* 2014;6(5):229–239.
- Ledermann JA, Kristeleit RS. Optimal treatment for relapsing ovarian cancer. *Ann Oncol.* 2010;21(suppl 7):vii218–vii222.
- Lord CJ, Ashworth A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat Med.* 2013;19(11):1381–1388.
- Norquist B, et al. Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J Clin Oncol.* 2011;29(22):3008–3015.
- Sakai W, et al. Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature.* 2008;451(7182):1116–1120.
- Edwards SL, et al. Resistance to therapy caused by intragenic deletion in BRCA2. *Nature.* 2008;451(7182):1111–1115.
- Patch AM, et al. Whole-genome characterization of chemoresistant ovarian cancer. *Nature.* 2015;521(7553):489–494.
- Rottenberg S, et al. High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci U S A.* 2008;105(44):17079–17084.
- Bunting SF, et al. 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell.* 2010;141(2):243–254.
- Bouwman P, et al. 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat Struct Mol Biol.* 2010;17(6):688–695.
- Jaspers JE, et al. Loss of 53BP1 causes PARP inhibitor resistance in Brca1-mutated mouse mammary tumors. *Cancer Discov.* 2013;3(1):68–81.
- Johnson N, et al. Stabilization of mutant BRCA1 protein confers PARP inhibitor and platinum resistance. *Proc Natl Acad Sci U S A.* 2013;110(42):17041–17046.
- Drost R, et al. BRCA1 RING function is essential for tumor suppression but dispensable for therapy resistance. *Cancer Cell.* 2011;20(6):797–809.
- Shakya R, et al. BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. *Science.* 2011;334(6055):525–528.
- Friedman LS, et al. Novel inherited mutations and variable expressivity of BRCA1 alleles, including the founder mutation 185delAG in Ashkenazi Jewish families. *Am J Hum Genet.* 1995;57(6):1284–1297.
- Struwing JP, et al. The carrier frequency of the BRCA1 185delAG mutation is approximately 1 percent in Ashkenazi Jewish individuals. *Nat Genet.* 1995;11(2):198–200.
- Elstrodt F, et al. BRCA1 mutation analysis of 41

- human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res.* 2006;66(1):41–45.
36. Ignatoski KM, Ethier SP. Constitutive activation of pp125fak in newly isolated human breast cancer cell lines. *Breast Cancer Res Treat.* 1999;54(2):173–182.
 37. Walsh T, et al. Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. *Proc Natl Acad Sci U S A.* 2011;108(44):18032–18037.
 38. Buisson M, Anczuków O, Zetoune AB, Ware MD, Mazoyer S. The 185delAG mutation (c.68_69delAG) in the BRCA1 gene triggers translation reinitiation at a downstream AUG codon. *Hum Mutat.* 2006;27(10):1024–1029.
 39. Gebauer F, Hentze MW. Molecular mechanisms of translational control. *Nat Rev Mol Cell Biol.* 2004;5(10):827–835.
 40. Kozak M. Circumstances and mechanisms of inhibition of translation by secondary structure in eucaryotic mRNAs. *Mol Cell Biol.* 1989;9(11):5134–5142.
 41. Kozak M. Constraints on reinitiation of translation in mammals. *Nucleic Acids Res.* 2001;29(24):5226–5232.
 42. Hinnebusch AG. The scanning mechanism of eukaryotic translation initiation. *Annu Rev Biochem.* 2014;83:779–812.
 43. Powley IR, et al. Translational reprogramming following UVB irradiation is mediated by DNA-PKcs and allows selective recruitment to the polysomes of mRNAs encoding DNA repair enzymes. *Genes Dev.* 2009;23(10):1207–1220.
 44. Joukov V, Chen J, Fox EA, Green JB, Livingston DM. Functional communication between endogenous BRCA1 and its partner, BARD1, during *Xenopus laevis* development. *Proc Natl Acad Sci U S A.* 2001;98(21):12078–12083.
 45. Woolery KT, Mohamed M, Linger RJ, Dobrinski KP, Roman J, Kruk PA. BRCA1 185delAG mutation enhances interleukin-1 β expression in ovarian surface epithelial cells. *Biomed Res Int.* 2015;2015:652017.
 46. O'Donnell JD, Linger RJ, Kruk PA. BRCA1 185delAG mutant protein, BRAT, up-regulates maspin in ovarian epithelial cells. *Gynecol Oncol.* 2010;116(2):262–268.
 47. Perrin-Vidoz L, Sinilnikova OM, Stoppa-Lyonnet D, Lenoir GM, Mazoyer S. The non-sense-mediated mRNA decay pathway triggers degradation of most BRCA1 mRNAs bearing premature termination codons. *Hum Mol Genet.* 2002;11(23):2805–2814.
 48. Ceccaldi R, et al. A unique subset of epithelial ovarian cancers with platinum sensitivity and PARP inhibitor resistance. *Cancer Res.* 2015;75(4):628–634.
 49. Spriggs KA, Bushell M, Willis AE. Translational regulation of gene expression during conditions of cell stress. *Mol Cell.* 2010;40(2):228–237.
 50. Fabbro M, Rodriguez JA, Baer R, Henderson BR. BARD1 induces BRCA1 intranuclear foci formation by increasing RING-dependent BRCA1 nuclear import and inhibiting BRCA1 nuclear export. *J Biol Chem.* 2002;277(24):21315–21324.
 51. Sobhian B, et al. RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science.* 2007;316(5828):1198–1202.
 52. Wang B, et al. Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science.* 2007;316(5828):1194–1198.
 53. Kim H, Chen J, Yu X. Ubiquitin-binding protein RAP80 mediates BRCA1-dependent DNA damage response. *Science.* 2007;316(5828):1202–1205.
 54. Pathania S, et al. BRCA1 is required for postreplication repair after UV-induced DNA damage. *Mol Cell.* 2011;44(2):235–251.
 55. Au WW, Henderson BR. The BRCA1 RING and BRCT domains cooperate in targeting BRCA1 to ionizing radiation-induced nuclear foci. *J Biol Chem.* 2005;280(8):6993–7001.
 56. Chetrit A, Hirsh-Yechezkel G, Ben-David Y, Lubin F, Friedman E, Sadetzki S. Effect of BRCA1/2 mutations on long-term survival of patients with invasive ovarian cancer: the national Israeli study of ovarian cancer. *J Clin Oncol.* 2008;26(1):20–25.
 57. Seeger C, Sohn JA. Targeting Hepatitis B virus with CRISPR/Cas9. *Mol Ther Nucleic Acids.* 2014;3:e216.
 58. Swisher EM, Sakai W, Karlan BY, Wurz K, Urban N, Taniguchi T. Secondary BRCA1 mutations in BRCA1-mutated ovarian carcinomas with platinum resistance. *Cancer Res.* 2008;68(8):2581–2586.

Cell Reports

BRCA1 Mutation-Specific Responses to 53BP1 Loss-Induced Homologous Recombination and PARP Inhibitor Resistance

Graphical Abstract



Authors

Joseph Nacson, John J. Krais, Andrea J. Bernhardt, ..., Gaorav P. Gupta, Stephen M. Sykes, Neil Johnson

Correspondence

neil.johnson@fccc.edu

In Brief

Using a *Brca1*^{4C} mouse model and a panel of *BRCA1* mutant cancer cell lines, Nacson et al. show that 53BP1 loss of function induced homologous recombination and PARP inhibitor resistance is suboptimal in the absence of hypomorphic *BRCA1* proteins that retain the coiled-coil domain and ability to interact with PALB2.

Highlights

- The embryonic viability of *Brca1*^{4C/4C} mice can be rescued with 53bp1 knockout
- *Brca1*^{4C/4C};53bp1^{-/-} mice are tumor prone and sensitive to PARP inhibitor treatment
- 53BP1 loss provides varying levels of PARPi resistance in *BRCA1* mutant cell lines
- Efficient RAD51 loading and HR requires *BRCA1* hypomorphs that interact with PALB2



Nacson et al., 2018, Cell Reports 24, 3513–3527
September 25, 2018 © 2018 The Author(s).
<https://doi.org/10.1016/j.celrep.2018.08.086>

CellPress

BRCA1 Mutation-Specific Responses to 53BP1 Loss-Induced Homologous Recombination and PARP Inhibitor Resistance

Joseph Nacson,^{1,2} John J. Krais,¹ Andrea J. Bernhardt,¹ Emma Clausen,¹ Wanjun Feng,³ Yifan Wang,¹ Emmanuelle Nicolas,^{1,4} Kathy Q. Cai,⁵ Rossella Tricarico,⁶ Xiang Hua,⁷ Daniela DiMarcantonio,⁷ Esteban Martinez,⁷ Dali Zong,⁸ Elizabeth A. Handorf,⁹ Alfonso Bellacosa,⁶ Joseph R. Testa,¹⁰ Andre Nussenzweig,⁸ Gaorav P. Gupta,³ Stephen M. Sykes,⁷ and Neil Johnson^{1,11,*}

¹Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

²Temple University, Lewis Katz School of Medicine, Philadelphia, PA 19140, USA

³Department of Radiation Oncology, UNC Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599, USA

⁴Genomics Facility, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

⁵Histopathology Facility, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

⁶Cancer Epigenetics Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

⁷Blood Cell Development and Function Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

⁸Laboratory of Genome Integrity, National Cancer Institute, NIH, Bethesda, MD 20892, USA

⁹Bioinformatics and Bioinformatics Facility, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

¹⁰Cancer Biology Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA

¹¹Lead Contact

*Correspondence: neil.johnson@fccc.edu

<https://doi.org/10.1016/j.celrep.2018.08.086>

SUMMARY

BRCA1 functions in homologous recombination (HR) both up- and downstream of DNA end resection. However, in cells with 53BP1 gene knockout (KO), BRCA1 is dispensable for the initiation of resection, but whether BRCA1 activity is entirely redundant after end resection is unclear. Here, we found that 53bp1 KO rescued the embryonic viability of a *Brca1*^{ΔC/ΔC} mouse model that harbors a stop codon in the coiled-coil domain. However, *Brca1*^{ΔC/ΔC}; 53bp1^{-/-} mice were susceptible to tumor formation, lacked Rad51 foci, and were sensitive to PARP inhibitor (PARPi) treatment, indicative of suboptimal HR. Furthermore, BRCA1 mutant cancer cell lines were dependent on truncated BRCA1 proteins that retained the ability to interact with PALB2 for 53BP1 KO induced RAD51 foci and PARPi resistance. Our data suggest that the overall efficiency of 53BP1 loss of function induced HR may be BRCA1 mutation dependent. In the setting of 53BP1 KO, hypomorphic BRCA1 proteins are active downstream of end resection, promoting RAD51 loading and PARPi resistance.

INTRODUCTION

BRCA1 germline mutations predispose carriers to an increased lifetime risk of developing breast and ovarian cancer. Malignancies often demonstrate loss of heterozygosity, where the

wild-type allele is lost but the mutant allele remains present. The ability of BRCA1 mutant alleles to generate protein products that contribute to biological processes normally undertaken by wild-type BRCA1 are beginning to emerge, and although mutant proteins lack tumor-suppressor activity, several truncated BRCA1 protein isoforms have been shown to promote residual DNA repair and chemotherapy resistance (Drost et al., 2011, 2016; Johnson et al., 2013; Shakya et al., 2011; Wang et al., 2016a, 2016b).

BRCA1 is an 1863-amino-acid protein with several conserved domains that function in homologous recombination (HR) DNA repair. The RING domain is located toward the N-terminal region of the protein and is required for BRCA1-BARD1 heterodimer formation. Exon 11 is the largest exon within the BRCA1 gene and encodes the central portion of the protein. The coiled-coil (CC) region directly interacts with PALB2, forming a larger BRCA1-PALB2-BRCA2-RAD51 protein complex (Sy et al., 2009; Xia et al., 2006, 2007; Zhang et al., 2009a, 2009b). BRCA1 harbors two BRCT repeats toward the C-terminal end of the protein that mediate interactions with phosphorylated proteins. The BRCT domain accounts for the BRCA1-CtIP interaction, potentially enhancing CtIP localization to double-strand DNA breaks (DSBs) and activating MRE11-RAD50-NBS1 (MRN) nuclease activity (Huen et al., 2010; Roy et al., 2011).

BRCA1 has been shown to contribute to HR at distinct steps, first promoting DNA end resection (Bouwman et al., 2010; Bunting et al., 2010) and later recruiting PALB2-BRCA2-RAD51 complexes to resected single-stranded DNA (ssDNA) (Sy et al., 2009; Zhang et al., 2009a, 2009b). When a DSB initially arises, 53BP1 is rapidly localized to surrounding chromatin and blocks the initiation of DNA end resection. BRCA1 promotes the displacement of



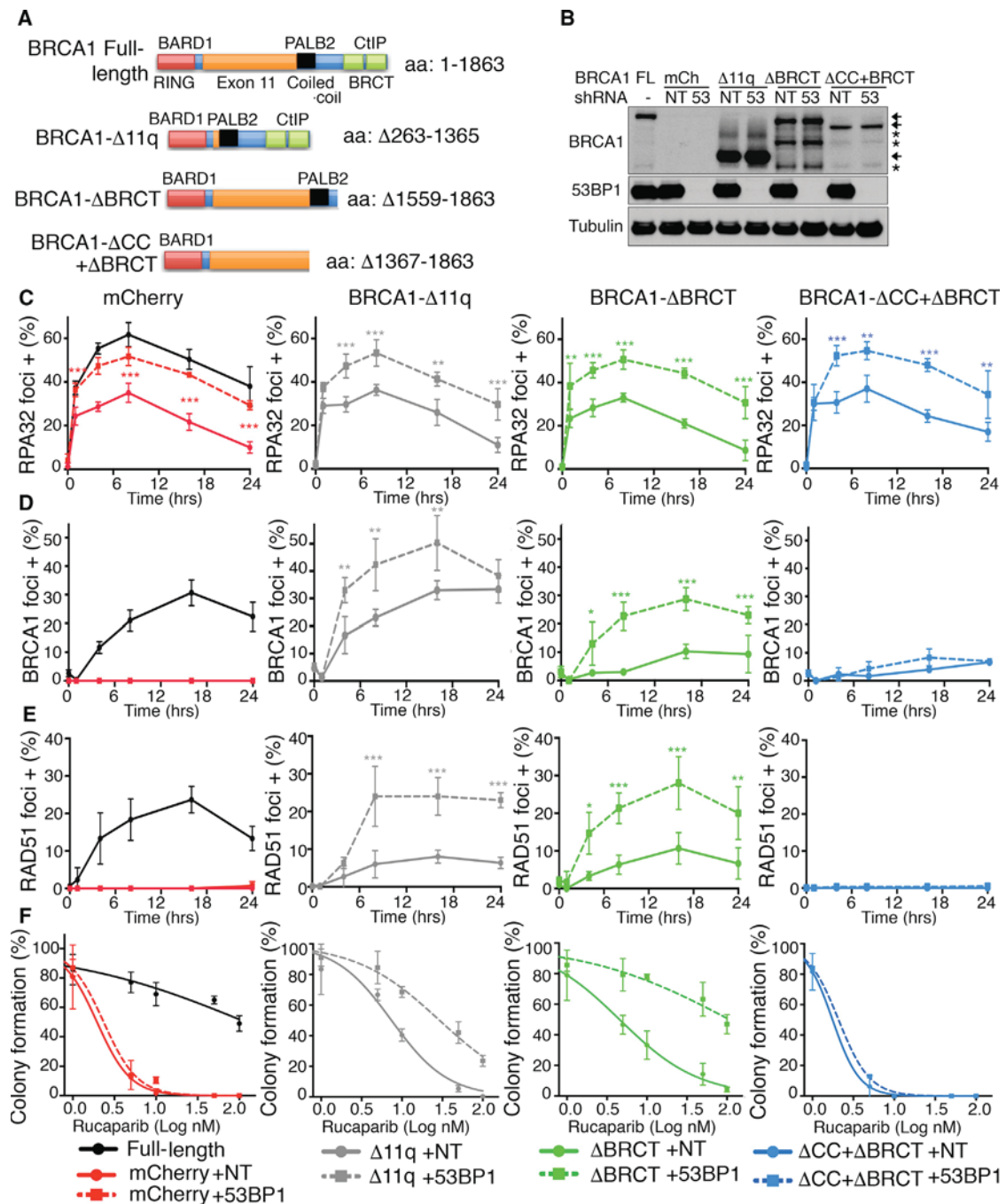


Figure 1. Effects of 53BP1 Depletion on BRCA1 Protein Activity

(A) Cartoon showing BRCA1 constructs expressed in MDA-MB-436 cells: BRCA1 full-length, BRCA1-Δ11q, BRCA1-ΔBRCT, and BRCA1-ΔCC+BRCT. Retained peptide domains and protein interactions are indicated. Red indicates the RING domain location, orange indicates exon-11-encoded amino acids, black indicates coiled-coil domain amino acids, green shows BRCT domain locations, and blue shows non-assigned-domain amino acids.

(B) Western blot analyses of MDA-MB-436 cells engineered to express mCherry, BRCA1 full-length (FL) protein, BRCA1-Δ11q (Δ11q), BRCA1-ΔBRCT (ΔBRCT), and BRCA1-ΔCC+BRCT (ΔCC+BRCT) truncated proteins as well as non-target (NT) or 53BP1 shRNA (53). Arrows indicate truncated BRCA1 proteins, and asterisks indicate nonspecific bands.

(C) Cells from (B) were assessed for RPA32 IRIF formation using immunofluorescence assays. For comparison, FL-BRCA1-expressing cells are included as solid black bars in graphs showing data for mCherry-expressing cell lines. At the indicated time points post-γ-irradiation (IR) (10 Gy), cells were fixed and those with at least 10 foci per nucleus counted positive for IRIF. For each protein, a minimum of 100 nuclei per time point and cell line were counted per experiment. Mean and SEM percentages of cells containing RPA32 foci are shown from three independent experiments. Solid lines represent NT shRNA, and dashed lines represent

(legend continued on next page)

53BP1 from chromatin surrounding DSBs and consequently activates DNA end resection (Chapman et al., 2012; Densham et al., 2016). After DNA end resection, BRCA1 directly interacts with and recruits PALB2 to DSB sites, where BRCA2-RAD51 forms a complex with PALB2, and mutations that block the BRCA1-PALB2 interaction have been shown to reduce the efficiency of RAD51 foci formation and HR (Anantha et al., 2017; Foo et al., 2017; Sy et al., 2009).

Cells that carry deleterious *BRCA1* mutations have a reduced capacity to perform HR; consequently, *BRCA1* mutant cancers are highly sensitive to DNA-damaging chemotherapies and PARP inhibitors (PARPis) (Bryant et al., 2005; Farmer et al., 2005; Moynahan et al., 1999, 2001). Several PARPis have been incorporated into the standard of care for the treatment of ovarian and breast cancer patients. *BRCA1* and *BRCA2* mutation carriers treated with PARPi therapy have demonstrated improved outcomes compared to patients without these mutations. However, PARPi remains ineffective in a portion of *BRCA1*-mutation-carrying patients, and most tumors that are responsive eventually acquire resistance (Ledermann et al., 2016; Robson et al., 2017).

Mutations or downregulation of proteins that regulate DNA repair pathway choice have been shown to promote HR and PARPi resistance in the absence of *BRCA1* activity (Bouwman et al., 2010; Bunting et al., 2010; Tkáč et al., 2016; Xu et al., 2015). Specifically, when 53BP1 is depleted or knocked out, DNA end resection and HR ensue in the absence of *BRCA1* activity and result in PARPi resistance (Bouwman et al., 2010; Bunting et al., 2010). *53bp1* knockout (KO) was shown to be capable of fully rescuing embryonic development in mice harboring homozygous *Brca1* mutant alleles (Bunting et al., 2010, 2012; Cao et al., 2009; Li et al., 2016). Moreover, derived cells demonstrated Rad51 foci formation and PARPi resistance, potentially indicating that *Brca1* activity may be dispensable for Rad51 loading in the setting of 53bp1 deficiency.

We and others have shown that HR and PARPi resistance can occur when *BRCA1*-mutation-carrying alleles produce truncated proteins that are capable of promoting RAD51 foci formation (Drost et al., 2016; Johnson et al., 2013; Wang et al., 2016a, 2016b). Furthermore, previous studies demonstrating 53bp1-KO-induced rescue of embryonic viability utilized *Brca1*^{Δ11} and *Brca1*^{Δ2} alleles that have been shown to produce exon-11-deficient and RING-domain-deficient hypomorphic *Brca1* proteins (Bunting et al., 2010, 2012; Cao et al., 2009; Li et al., 2016). To explore the relationship between 53BP1 KO and *BRCA1* protein expression, we generated a novel *Brca1*^{ΔC} allele that harbors a stop codon in the CC domain and fails to generate a detectable *Brca1* protein. Herein, we assessed the relationship between

BRCA1 mutational status and 53BP1 loss of function in promoting HR and PARPi resistance.

RESULTS

53BP1 Depletion Augments the Activity of *BRCA1* CC-Containing Proteins

BRCA1 mutant alleles are often capable of generating truncated *BRCA1* proteins that lack functional domains usually present in the full-length protein (Drost et al., 2016; Hill et al., 2014; Johnson et al., 2013; Wang et al., 2016a, 2016b; Zhou et al., 2003). To examine the DSB recruitment dynamics of *BRCA1* proteins that are deficient for various functional domains, we examined γ -irradiation-induced foci (IRIF) formation kinetics. In addition, we measured the impact of *BRCA1* protein status on 53BP1 foci formation. MDA-MB-436 cells have a *BRCA1* 5396+1G > A mutation that results in loss of *BRCA1* protein expression (Elstrodt et al., 2006), undetectable RAD51 foci, and exquisite PARPi sensitivity (Johnson et al., 2013; Wang et al., 2016a). We therefore used this cell line to add back *BRCA1* proteins that lacked the following peptide regions: exon 11 coding (*BRCA1*- Δ 11q), BRCT domain (*BRCA1*- Δ BRCT), and both the CC and BRCT domains (*BRCA1*- Δ CC+ Δ BRCT) (Figure 1A). We first confirmed the cytoplasmic and nuclear expression of *BRCA1* proteins (Figure S1A). As expected, full-length *BRCA1* was entirely nuclear, while *BRCA1*- Δ 11q that lacks an exon 11 located nuclear import signal was expressed similarly in both cytoplasmic and nuclear fractions (Chen et al., 1996; Korlimarla et al., 2013). The *BRCA1*- Δ BRCT and *BRCA1*- Δ CC+ Δ BRCT proteins were detected in cytoplasmic fractions but were predominantly nuclear (Figure S1A). Of note, all truncated proteins demonstrated similar or higher nuclear expression than ectopic full-length *BRCA1*, indicating that observations related to loss of activity would not result from reduced nuclear expression. As expected, mCherry-expressing negative control cells lacked *BRCA1* foci. Interestingly, full-length *BRCA1* and *BRCA1*- Δ 11q proteins that retain BRCT and CC domains formed IRIF with similar kinetics (Figure S1B). In contrast, slower kinetics were observed, and fewer cells expressing the *BRCA1*- Δ BRCT protein demonstrated foci positivity. These dynamics were further decreased in cells expressing the *BRCA1*- Δ CC+ Δ BRCT protein (Figure S1B). Although *BRCA1* has been shown to exclude 53BP1 to the periphery of the focus (Chapman et al., 2012; Densham et al., 2016), we did not observe differences between cell lines in the overall 53BP1 IRIF kinetics in our MDA-MB-436 isogenic model (Figure S1B).

We next measured the effects of 53BP1 depletion on truncated *BRCA1* protein activity. MDA-MB-436 cells expressing

53BP1 shRNA. Data were analyzed using two-way ANOVA. Significant differences between NT and 53BP1 shRNA at each time point are denoted (***p < 0.001; **p < 0.01; *p < 0.05). See Figure S1C for representative images.

(D) Cells were assessed as in (C) for mean and SEM percentages of cells containing at least 5 *BRCA1* foci per nucleus from three independent experiments. See Figure S2 for representative images.

(E) Cells were assessed as in (C) for mean and SEM percentages of cells containing at least 5 RAD51 foci per nucleus from three independent experiments. See Figure S2 for representative images.

(F) Cells were incubated with rucaparib and assessed for colony formation. Mean and SEM colony formation was calculated as a percentage of DMSO-vehicle-treated cells from three independent experiments. Full-length *BRCA1* colony formation data (black line) is shown as a comparator in graphs with mCherry-expressing cells. NT and 53BP1 shRNA-treated cells are shown with solid and dashed lines, respectively.

the above proteins were further engineered to express non-target (NT) or 53BP1-targeting short hairpin RNA (shRNA) (Figure 1B), and foci formation dynamics were compared to MDA-MB-436 cells that expressed full-length BRCA1. BRCA1 and 53BP1 both regulate the resection of DSBs, and we measured RPA32 IRIF as an indicator of DNA end-resection proficiency. Here, mCherry-, BRCA1- $\Delta 11q$ -, BRCA1- Δ BRCT-, and BRCA1- Δ CC+ Δ BRCT-expressing cell lines all had reduced RPA32 IRIF compared with full-length BRCA1-expressing cells. However, 53BP1 shRNA significantly elevated RPA32 IRIF, regardless of BRCA1 protein status, to similar kinetics as that observed in full-length-BRCA1-expressing cells (Figures 1C and S1C). 53BP1 shRNA did not affect BRCA1 IRIF in mCherry-negative control cells. However, 53BP1 shRNA markedly increased the number of BRCA1- $\Delta 11q$ and BRCA1- Δ BRCT IRIF-positive cells compared with NT shRNA-expressing cells. The CC domain appeared crucial for 53BP1 shRNA-promoted IRIF, as the number of BRCA1-foci-positive cells expressing the BRCA1- Δ CC+ Δ BRCT protein was similar between NT and 53BP1 shRNA treatments (Figures 1D and S2). Furthermore, 53BP1 shRNA had no impact on RAD51 IRIF in mCherry-expressing cells but dramatically increased RAD51 IRIF in BRCA1- $\Delta 11q$ - and BRCA1- Δ BRCT-expressing cells. Similar to BRCA1 IRIF, the ability of 53BP1 shRNA to promote RAD51 IRIF appeared dependent on the BRCA1 CC domain, as 53BP1 shRNA failed to elevate RAD51 IRIF in BRCA1- Δ CC+ Δ BRCT-expressing cells (Figures 1E and S2).

Next, the effect of 53BP1 depletion in combination with truncated BRCA1 protein expression on PARPi sensitivity was measured and compared to full-length BRCA1-expressing cells in colony-formation assays. Here, 53BP1 shRNA failed to significantly impact PARPi resistance in mCherry-expressing cells. However, BRCA1- $\Delta 11q$ - and BRCA1- Δ BRCT-expressing cells had a 4-fold ($p = 0.013$, unpaired t test) and 21-fold ($p < 0.001$, unpaired t test) increase, respectively, in rucaparib LC50 (lethal concentration reducing colony formation by 50%) values compared with NT shRNA-expressing cells. In contrast, 53BP1 shRNA did not significantly impact rucaparib sensitivity in cells expressing BRCA1- Δ CC+ Δ BRCT (Figure 1F). All in, these data show that BRCA1 protein status does not affect the ability of 53BP1 depletion to stimulate DNA end resection and RPA32 foci formation. However, in the MDA-MB-436 cell line isogenic system, BRCA1 truncated proteins that retain the CC domain were critical for 53BP1-depletion-induced RAD51 foci formation and robust PARPi resistance.

Brca1 ^{$\Delta C/\Delta C$} Embryonic Viability Is Rescued by 53bp1 KO

Previous studies showed that 53bp1 KO rescued HR and the embryonic viability of mice harboring hypomorphic *Brca1* ^{$\Delta 11/\Delta 11$} and *Brca1* ^{$\Delta 2/\Delta 2$} mutant alleles. Furthermore, mice had normal lifespans and did not demonstrate increased tumor susceptibility (Bunting et al., 2010, 2012; Cao et al., 2009). Because 53BP1 depletion failed to induce RAD51 foci and PARPi resistance in BRCA1- Δ CC+ Δ BRCT-expressing MDA-MB-436 cells, we explored the possibility that similar *Brca1* mutations in mice would result in an impotent 53bp1-KO-induced rescue of HR and embryonic development. We aimed to introduce mutations in the *Brca1* CC-encoding region using clustered regularly inter-

spaced short palindromic repeats (CRISPR)/Cas9 genetic editing. Here, we generated mice harboring a *Brca1* c.4080del4 frameshift mutation. This mutation results in a p.1361fs28X stop codon that blocks translation in the CC domain, predicted to cause loss of the C-terminal region of the protein, that includes both the CC and BRCT domains; we therefore refer to this allele as *Brca1* ^{ΔC} (Figure 2A). Moreover, this mutation is downstream of exon 11 and unlikely to be removed by alternative splicing (Ludwig et al., 1997; Xu et al., 1999). As expected, homozygosity for the *Brca1* ^{ΔC} allele resulted in embryonic lethality, and homozygous embryos were unable to be detected at embryonic day 9.5 (E9.5), indicating that *Brca1* ^{$\Delta C/\Delta C$} induced early embryonic lethality (Table 1).

Contrary to our supposition that loss of the CC domain would abrogate 53bp1-KO-induced embryonic development, interbreeding *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} or *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} mice resulted in *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} progeny at close to the expected Mendelian ratios (Table 1). *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} mice develop normally and are similar to *Brca1* ^{$+/+$} ;*53bp1* ^{$-/-$} mice in terms of size (Figure 2B) and weight (Figure 2C). However, in contrast to previous reports describing *Brca1* ^{$\Delta 11/\Delta 11$} ;*53bp1* ^{$-/-$} homozygous mice (Bunting et al., 2010; Cao et al., 2009), the lifespan of *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} mice was significantly shorter (Figure 2D), with 4 out of 4 mice that were subjected to pathological analyses demonstrating thymic lymphomas at 3–6 months of age (Figure 2E), similar to those reported in older *Brca1* ^{$+/+$} ;*53bp1* ^{$-/-$} mice (Ward et al., 2003).

Brca1 ^{$\Delta 11/\Delta 11$} and *Brca1* ^{$\Delta 2/\Delta 2$} mutant mouse embryonic fibroblasts (MEFs) were previously shown to express Brca1 hypomorphic proteins that lack either the exon 11 coding region or the RING domain, respectively, with both proteins retaining the CC domain (Li et al., 2016; Xu et al., 1999). Due to severe early lethality, we were unable to generate *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$+/+$} MEFs; however, *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} MEFs were readily generated. We detected full-length Brca1 in wild-type MEFs as well as the hypomorphic Brca1 ^{$\Delta 11$} protein in *Brca1* ^{$\Delta 11/\Delta 11$} MEFs. However, we were routinely unable to detect Brca1 protein in *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} MEFs (Figure 2F). *Brca1* mRNA was similarly expressed in *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} and wild-type MEFs (Figure S3A), suggesting that nonsense-mediated mRNA decay (NMD) did not account for loss of protein expression. We predict that the *Brca1* ^{ΔC} mutation induces protein folding defects that result in proteasomal degradation. However, relatively few hours of MG132 treatment killed *Brca1* ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} MEFs, with concentrations and time points that enabled live cells to be collected insufficient to detect truncated Brca1 protein by western blotting (Figure S3B). In summation, 53bp1 KO rescued the embryonic development of *Brca1* ^{$\Delta C/\Delta C$} mice, but mice are susceptible to tumor development. Moreover, in contrast to hypomorphic *Brca1* ^{$\Delta 11$} and *Brca1* ^{$\Delta 2$} alleles that generate CC-domain-containing proteins, the *Brca1* ^{ΔC} allele does not generate a detectable protein product.

Brca1 ^{$\Delta C/\Delta C$} ;*53bp1* ^{$-/-$} Mice Have Impaired HR

We next examined HR DNA repair in *Brca1* and *53bp1* mutant mice. MEFs that are *Brca1* mutated or DNA repair defective often senesce at early passages due to oxidative DNA damage and activation of p53-p21 (Cao et al., 2009; Parrinello et al., 2003).

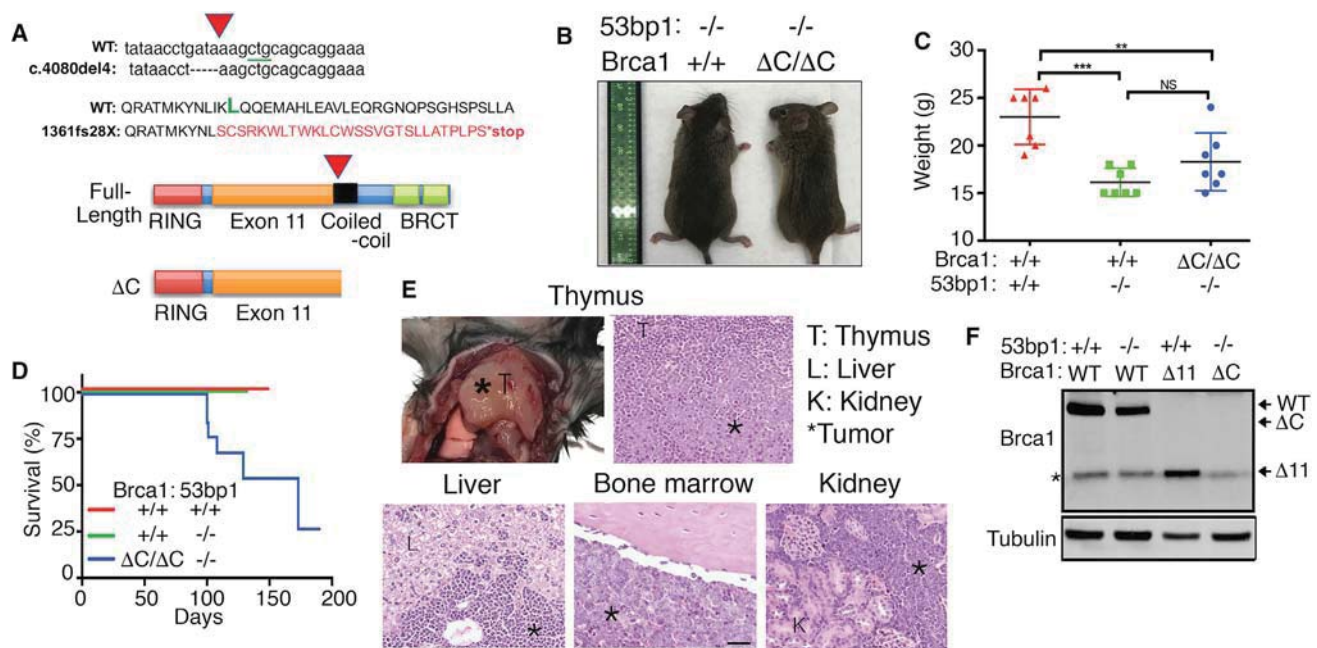


Figure 2. Generation and Assessment of *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} Mice

(A) *Brca1* coiled-coil genomic region targeted using CRISPR/Cas9. Red arrow indicates single guide RNA (sgRNA)-targeting location and the resulting c.4080-4083 4-bp deletion mutation. The *Brca1* coiled-coil wild-type peptide region is shown with the critical L1363 residue (equivalent to human L1407) highlighted green. Amino acid changes resulting from the 1361fs28X mutation are red. Bottom: cartoon of predicted truncated protein product.

(B) Representative photograph of 6-week-old littermates with indicated genotypes. See also Table 1.

(C) Weights of individual mice with the indicated genotypes at 6 weeks of age. **p < 0.01, ***p < 0.001, NS, not significant (one-way ANOVA with multiple comparisons).

(D) Kaplan-Meier survival curves of mice with the indicated genotypes.

(E) Representative photograph of *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} mouse with thymic lymphoma. H&E of thymus, liver, bone marrow, and kidney showing tumor infiltrates (*). Scale bar, 20 μm.

(F) Primary MEFs (before passage 4) with the indicated genotypes were assessed for *Brca1* protein expression by western blotting. Arrows indicate the expected gel migrations of *Brca1* wild-type (WT) (top arrow), a potential *Brca1*^{ΔC} protein (middle arrow), and the *Brca1*^{Δ11} protein (lower arrow). Asterisk indicates a nonspecific band.

A previous study reported that senescence occurred in early passages of *Brca1*^{Δ11/Δ11};*53bp1*^{+/+} MEFs but was rescued in *Brca1*^{Δ11/Δ11};*53bp1*^{-/-} cells (Cao et al., 2009), presumably because *53bp1* KO restored HR DNA repair. In our experiments, over 80% of *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} cells were positive for senescence-associated β-galactosidase (SA-β-gal) staining by passage 6 (Figure S3C). Senescence was delayed when cells were maintained in 2% oxygen (Figure S3C), suggesting that *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} MEFs are sensitive to oxidative DNA damage. Therefore, in contrast to *Brca1*^{Δ11/Δ11} MEFs, *53bp1* KO did not rescue the ability of *Brca1*^{ΔC/ΔC} MEFs to proliferate beyond six passages, pointing toward the possibility that *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} MEFs remain DNA-repair defective. To reduce the onset of senescence, all subsequent experiments were set up when cells were within three passages of their derivation.

In foci formation experiments, *Brca1*^{Δ11/Δ11} MEFs had fewer Rpa32 and Rad51 IRIF-positive cells than wild-type cells (Figure 3A). Similar to previous reports, *53bp1* KO fully rescued both Rpa32 and Rad51 foci formation in cells harboring the *Brca1*^{Δ11/Δ11} allele. In contrast, although *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} MEFs had robust Rpa32 IRIF, there were 5-fold (p < 0.001, un-

paired t test) fewer Rad51-foci-positive cells compared to wild-type MEFs (Figure 3A). Of note, *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} cells had marginally higher Rad51 foci positivity compared to *Brca1*^{Δ11/Δ11} MEFs (Figure 3A). We also directly assessed HR DNA repair using a previously described CRISPR/Cas9-based reporter assay targeting the *Rosa26* locus (Wyatt et al., 2016) (Figures S3D–S3G). Here, *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} MEFs had 2-fold (p < 0.001, unpaired t test) fewer gene conversion events compared with *Brca1*^{Δ11/Δ11};*53bp1*^{-/-} MEFs (Figure 3B). Differences in Rad51 IRIF or HR proficiency were unlikely to be a result of disparate cell-cycle distributions, as MEFs had similar S/G2 portions (Figure S3H).

Inappropriate recombination often results in deleterious chromosomal rearrangement events commonly manifested in the formation of multi-radial chromosome structures. In analyses of metaphase chromosomes from cells treated with 500 nM rucaparib, *Brca1*^{Δ11/Δ11};*53bp1*^{+/+} and *Brca1*^{ΔC/ΔC};*53bp1*^{-/-} MEFs demonstrated 17- and 3.3-fold greater numbers of radial chromosomes and 6.4- and 3.9-fold greater numbers of chromosome breaks, respectively, compared with *Brca1*^{Δ11/Δ11};*53bp1*^{-/-} MEFs (Figure 3C). Furthermore, in cell growth experiments, the mean GI50 (growth inhibition by 50%)

Table 1. Predicted and Observed *Brca1* and *53bp1* Genotype Frequencies

<i>Brca1</i> ^{+/ΔC} $\times$ <i>Brca1</i> ^{+/ΔC}			
	<i>Brca1</i> ^{+/+}	<i>Brca1</i> ^{+/ΔC}	<i>Brca1</i> ^{ΔC/ΔC}
E9.5 embryos expected	6.75	13.5	6.75
(27 screened) observed	13	14	0
Pups expected	27	54	27
(108 screened) observed	36	72	0
<i>Brca1</i> ^{+/ΔC} <i>53bp1</i> ^{+/-} $\times$ <i>Brca1</i> ^{+/ΔC} <i>53bp1</i> ^{+/-}			
	<i>Brca1</i> ^{ΔC/ΔC} <i>53bp1</i> ^{+/+} or <i>Brca1</i> ^{ΔC/ΔC} <i>53bp1</i> ^{+/-}	<i>Brca1</i> ^{+/+} <i>53bp1</i> ^{-/-} or <i>Brca1</i> ^{+/ΔC} <i>53bp1</i> ^{-/-}	<i>Brca1</i> ^{ΔC/ΔC} <i>53bp1</i> ^{-/-}
Pups expected	24.75	24.75	8.25
(132 screened) observed:	0	26	3
<i>Brca1</i> ^{+/ΔC} <i>53bp1</i> ^{-/-} $\times$ <i>Brca1</i> ^{+/ΔC} <i>53bp1</i> ^{-/-}			
	<i>Brca1</i> ^{+/+} <i>53bp1</i> ^{-/-}	<i>Brca1</i> ^{+/ΔC} <i>53bp1</i> ^{-/-}	<i>Brca1</i> ^{ΔC/ΔC} <i>53bp1</i> ^{-/-}
Pups expected	27.25	54.5	27.25
(109 screened) observed	32	52	25

value for rucaparib was 11,131-fold ($p < 0.001$, unpaired t test) lower in *Brca1* ^{Δ 11/ Δ 11};53bp1^{+/+} MEFs than wild-type MEFs (0.4 versus 4,440 nM). In contrast, no significant differences in GI50 values were observed between *Brca1* ^{Δ 11/ Δ 11};53bp1^{-/-} and wild-type MEFs. The mean GI50 value for rucaparib was also 1,776-fold ($p < 0.001$, unpaired t test) lower in *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} MEFs than wild-type MEFs (2.5 versus 4,440 nM). However, *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} MEFs were also 6.3-fold ($p < 0.001$, unpaired t test) less sensitive to rucaparib than *Brca1* ^{Δ 11/ Δ 11};53bp1^{+/+} MEFs (2.5 versus 0.4 nM) (Figure 3D). These data show that in contrast to the *Brca1* ^{Δ 11/ Δ 11};53bp1^{-/-} genotype, *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} cells are PARPi sensitive. Nevertheless, the latter cells were also mildly less PARPi responsive than *Brca1* ^{Δ 11/ Δ 11};53bp1^{+/+} MEFs, indicating that 53bp KO likely provides some residual HR in the setting of *Brca1* ^{Δ C/ Δ C}.

To determine if PARPi sensitivity extended beyond MEFs, mice were treated with 200 mg/kg rucaparib twice daily for 5 days; this dose is routinely well tolerated by NSG mice in xenograft experiments. While rucaparib had no impact on *Brca1*^{+/+};53bp1^{+/+}, *Brca1*^{+/+};53bp1^{-/-}, or *Brca1* ^{Δ 11/ Δ 11};53bp1^{-/-} mice, 5 out of 5 *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} mice treated with rucaparib demonstrated sickness that required euthanasia according to institutional guidelines within 9 days of treatment initiation (Figure 3E). Additionally, rucaparib-treated *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} mice had significantly decreased peripheral blood neutrophil and lymphocyte counts (Figure 3F). Taken together, these data show that the *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} genotype confers defective HR and PARPi sensitivity.

Because *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} mice showed markers of HR deficiency, we explored potential HR-independent mechanisms that might account for 53bp1-KO-associated rescue of embryonic development. p53 KO is an established mechanism that rescues *Brca1* mutant embryo development (Hakem et al., 1997; Ludwig et al., 1997; Xu et al., 2001), and 53bp1 KO was previously shown to abrogate p53 transcriptional activity in human cancer cell lines (Cuella-Martin et al., 2016). However, we did not find evidence of reduced p53 transcriptional activity

in 53bp1 KO MEFs, with γ -irradiation and nutlin treatments providing robust increases in p21 mRNA expression in all MEF genotypes (Figure S4A). Furthermore, in line with previous reports (Ray Chaudhuri et al., 2016), 53bp1 KO did not rescue *Brca1*-mutation-associated replication fork protection defects (Figure S4B). We therefore considered the possibility that 53bp1 KO restored the viability of mice through residual HR. Despite low levels, we were nevertheless routinely able to detect Rad51 foci in a minority of *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} cells (Figure 3A). We hypothesized that 53bp1 KO-induced hyperactivation of DNA end resection might be sufficient to stimulate residual *Brca1*-independent Rad51 loading that in turn provides a level of HR activity capable of supporting organismal development. Indeed, RNAi-mediated depletion of Palb2 or *Brca2* completely abolished Rad51 IRIF in *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} cells (Figures S4C–S4E). These findings indicate that *Brca1* ^{Δ C/ Δ C};53bp1^{-/-} cells have decreased HR proficiency and are more sensitive to PARPi treatment than *Brca1* wild-type or *Brca1* ^{Δ 11/ Δ 11};53bp1^{-/-} cells. However, 53bp1 KO likely promotes some residual HR through *Brca1*-independent but Palb2-*Brca2*-dependent and inefficient Rad51 loading.

53BP1 Depletion Promotes PARPi Resistance in Subsets of Cancer Cell Lines

We aimed to gain broader insight into the ability of 53BP1 loss of function to promote HR and PARPi resistance in human *BRCA1* mutant cancers. In addition to full-length *BRCA1*, human cell lines often express the *BRCA1*- Δ 11q splice isoform that is homologous to the mouse *Brca1*- Δ 11 protein and lacks the majority of exon-11-coding amino acids (Figure 4A) (Hill et al., 2014; Wang et al., 2016a; Xu et al., 1999). We confirmed that *BRCA1* exon-11-mutation-containing SUM149PT, UWB1.289, and L56BRC1 cell lines do not express full-length *BRCA1* but do express the *BRCA1*- Δ 11q splice variant (Figures 4A and 4B). In contrast, MDA-MB-436, HCC1395, and SUM1315MO2 cells have *BRCA1* mutations located outside of exon 11 that result in low or undetectable levels of truncated *BRCA1* proteins due to folding issues and proteasomal degradation (Figures 4A

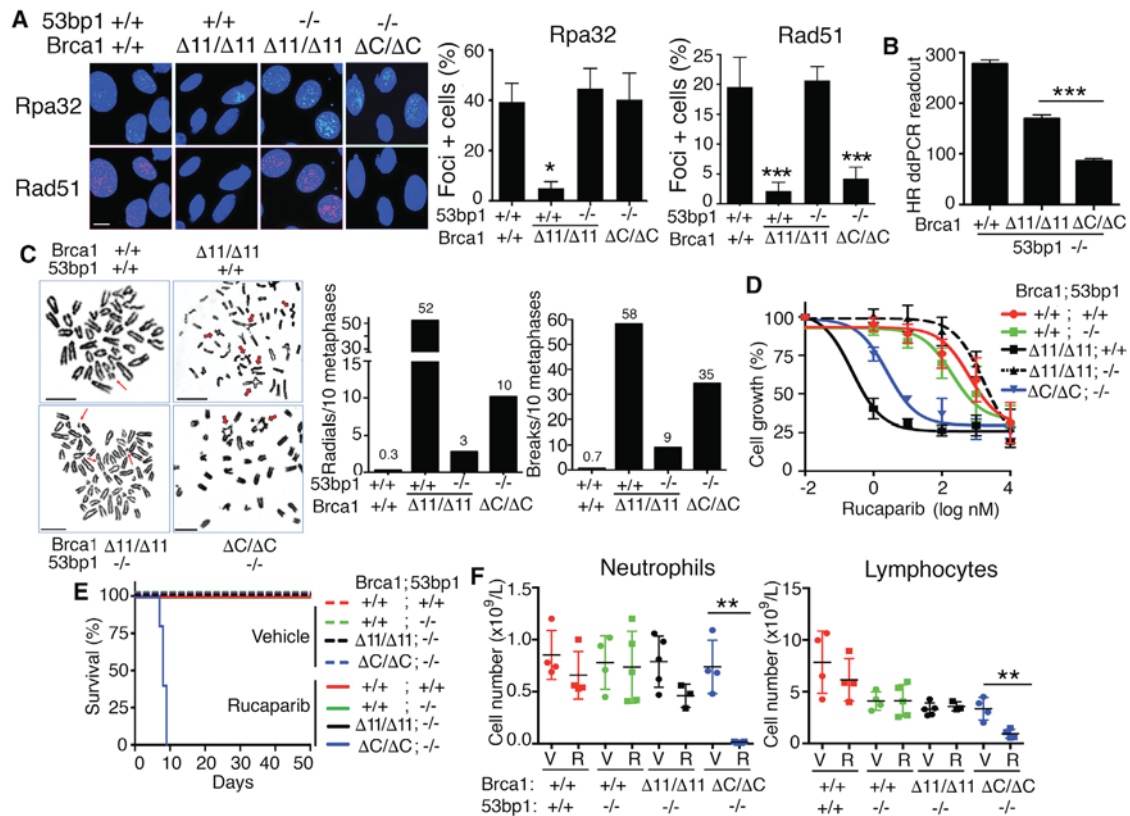


Figure 3. Assessment of HR and PARPi Sensitivity

(A) MEFs were subject to 10-Gy IR and assessed for Rpa32 and Rad51 foci formation as described in Figure 1. The number of foci-positive cells (mean and SEM) from three independent experiments is shown (* $p < 0.05$, *** $p < 0.001$, compared to WT, unpaired t test). Representative images of MEFs with the indicated genotypes are shown. Scale bar, 10 μ m.

(B) Cell lines were electroporated with plasmids expressing *Rosa26*-targeting sgRNA and Cas9 cDNA, plasmid enhanced green fluorescent protein (pEGFP), and a homology donor sequence. The number of gene conversion events from three independent repeats is shown. *** $p < 0.001$ (unpaired t test). See Figures S3D–S3G for additional controls and information.

(C) Metaphase spreads were prepared from the indicated genotypes of MEFs treated with 500 nM rucaparib for 24 hr. The mean number of breaks and radial chromosomes per 10 metaphases are shown. The number of metaphases assessed were 29 for *Brca1* $^{+/+}$;53bp1 $^{+/+}$, 46 for *Brca1* $^{\Delta 11/\Delta 11}$;53bp1 $^{+/+}$, 29 for *Brca1* $^{\Delta 11/\Delta 11}$;53bp1 $^{-/-}$, and 56 for *Brca1* $^{\Delta C/\Delta C}$;53bp1 $^{-/-}$ MEFs. Scale bars, 10 μ m.

(D) MEFs were treated with rucaparib for 7 days and stained with Sulforhodamine B (SRB). Solubilized stains were quantified, and cell growth (mean and SEM) is expressed as percentage of vehicle-treated cells from three independent experiment.

(E) Mice were treated with vehicle or 200 mg/kg rucaparib twice daily for 5 continuous days, and survival was measured by Kaplan-Meier analyses.

(F) Peripheral blood from mice described in (E) was assessed at the end of vehicle (V) or rucaparib (R) treatment for neutrophil and lymphocyte cell numbers. ** $p < 0.01$ compared with vehicle (unpaired t test).

and 4B). Cell lines expressed similar levels of 53BP1, RPA32, PALB2, BRCA2, and RAD51 (Figure 4B).

BRCA1 mutant cell lines were engineered to express either NT or two unique 53BP1 shRNAs (Figure 4C), and PARPi sensitivity was compared to *BRCA1* wild-type control MDA-MB-231 cells that are known to be HR proficient and PARPi resistant. Two individual 53BP1-targeting shRNAs significantly increased the LC50 values of rucaparib-treated SUM149PT, UWB1.289, and L56BRC1 cells compared to NT shRNA, with cells demonstrating similar or higher levels of resistance than *BRCA1* wild-type MDA-MB-231 cells (Figure 4D). In contrast, although MDA-MB-436, HCC1395, and SUM1315MO2 cells gained relatively small increases in LC50 values with 53BP1 shRNA, cells remained between 106- and 311-fold more sen-

sitive to rucaparib treatment than MDA-MB-231 cells (Figures 4D and S5A). Of note, observed phenotypes were not due to differential effects of shRNA on cell-cycle distributions (Figure S5B).

We investigated the impact of 53BP1 depletion on the initial steps of HR by measuring markers of DNA end resection and RAD51 loading. As expected, 53BP1 shRNA significantly increased RPA32 IRIF, a marker of DNA end resection, in all cell lines (Figures 4E and S5C). In contrast, RAD51 IRIF significantly increased in SUM149PT, UWB1.289, and L56BRC1 cells but remained low in MDA-MB-436, HCC1395, and SUM1315MO2 cells treated with 53BP1 shRNA (Figures 4F and S5D).

We also examined the importance of *BRCA1* on the ability of 53BP1 depletion to rescue RAD51 IRIF in a panel of

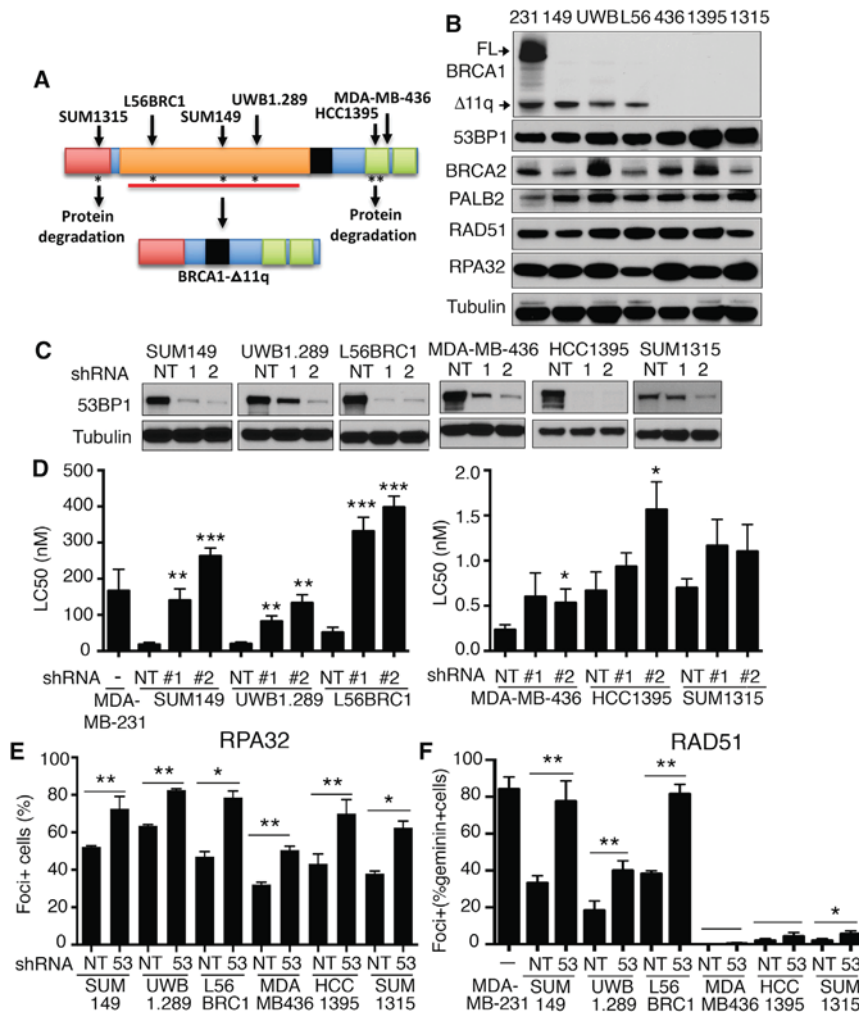


Figure 4. 53BP1 Depletion in *BRCA1* Mutant Cancer Cell Lines

(A) Cartoon showing the FL BRCA1 protein and the relative location of mutations (*) in *BRCA1* mutant cancer cell lines. The predicted effects of mutations are indicated. L56BRC1, SUM149PT, and UWB1.289 harbor exon 11 mutations and are capable of expressing the BRCA1- Δ 11q splice isoform (shown below).

(B) Western blot analyses of the indicated proteins in MDA-MB-231 (231), SUM149PT (149), UWB1.289 (UWB), L56BRC1 (L56), MDA-MB-436 (436), HCC1395 (1395), and SUM1315MO2 (1315) cells. Full-length and BRCA1- Δ 11q proteins are indicated by arrows.

(C) Western blot analyses of 53BP1 protein expression after the indicated cell lines were subjected to NT or 53BP1 shRNA sequence 1 or 2.

(D) Cell lines were treated with increasing concentrations of rucaparib and colony formation assessed. BRCA1 WT MDA-MB-231 cells were also assessed for rucaparib sensitivity as a comparator. Values represent mean and SEM. LC50 values for 3 independent experiments are shown. *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$ compared with NT shRNA for each cell line (unpaired t test). See Figure S5A for fold change values.

(E) Cells expressing NT or 53BP1 shRNA#2 were assessed for RPA32 IRIF by immunofluorescence as described in Figure 1. The number of foci-positive cells (mean and SEM) for 3 independent experiments is shown. ** $p < 0.01$; * $p < 0.05$ compared to NT (unpaired t test). See Figure S5C for representative images.

(F) Cells were assessed for RAD51 IRIF as described in Figure 1. MDA-MB-231 BRCA1 WT cells were included as a positive control for RAD51 foci. To account for potential cell cycle or growth differences between cell lines, RAD51-foci-positive cells were normalized to geminin-positive cells. The number of foci-positive cells (mean and SEM) that were also geminin staining positive for 3 independent experiments is shown. ** $p < 0.01$; * $p < 0.05$ compared to NT (unpaired t test). See Figure S5D for representative images.

non-transformed and cancer cell lines. In non-transformed cell lines, BRCA1 siRNA reduced RAD51 IRIF, but combined BRCA1 and 53BP1 siRNA did not significantly increase RAD51 foci (Figure S6A). In *BRCA1* wild-type cancer cell lines, although mild RAD51 IRIF increases could be detected with combined 53BP1 and BRCA1 siRNA relative to BRCA1 depletion alone in OVCAR5 and MCF7 cell lines, RAD51 IRIF remained undetectable when only BRCA1-foci-negative cells were counted (Figure S6B), indicating that 53BP1 siRNA-induced RAD51 IRIF was associated with residual BRCA1 foci. In *BRCA1* mutant cancer cell lines, 53BP1 siRNA did not rescue RAD51 IRIF in BRCA1 siRNA-treated UWB1.289 cells. In contrast, we were able to detect a portion of SUM149PT cells that lacked BRCA1 foci but did contain RAD51 foci in combined BRCA1 and 53BP1 siRNA-treated cells. Furthermore, HCC1397 cells completely lacked BRCA1 foci regardless of siRNA treatment, and cells demonstrated robust RAD51 IRIF (Figure S6C). Altogether, these

data point toward cell line to cell line variation in the absolute dependence on BRCA1 for RAD51 loading, with the majority of cell lines tested requiring BRCA1 for efficient RAD51 IRIF, including cells with 53BP1 depletion.

BRCA1 Hypomorphs Promote 53BP1-Gene-KO-Induced PARPi Resistance

It is possible that incomplete RNAi-mediated depletion of 53BP1 accounted for milder rescue phenotypes observed in a subset of cell lines. To compare the effects of RNAi to a more comprehensive loss of 53BP1 activity in human cancer cell lines, we generated CRISPR/Cas9-mediated 53BP1 gene KOs in *BRCA1* mutant SUM149PT and MDA-MB-436 cells. For each cell line, three clones with loss of detectable 53BP1 protein expression were selected for further characterization (Figure 5A). Here, SUM149PT 53BP1 KO clones 3, 12, and 18 displayed 55-, 54-, and 67-fold ($p < 0.05$, unpaired t test) increased rucaparib

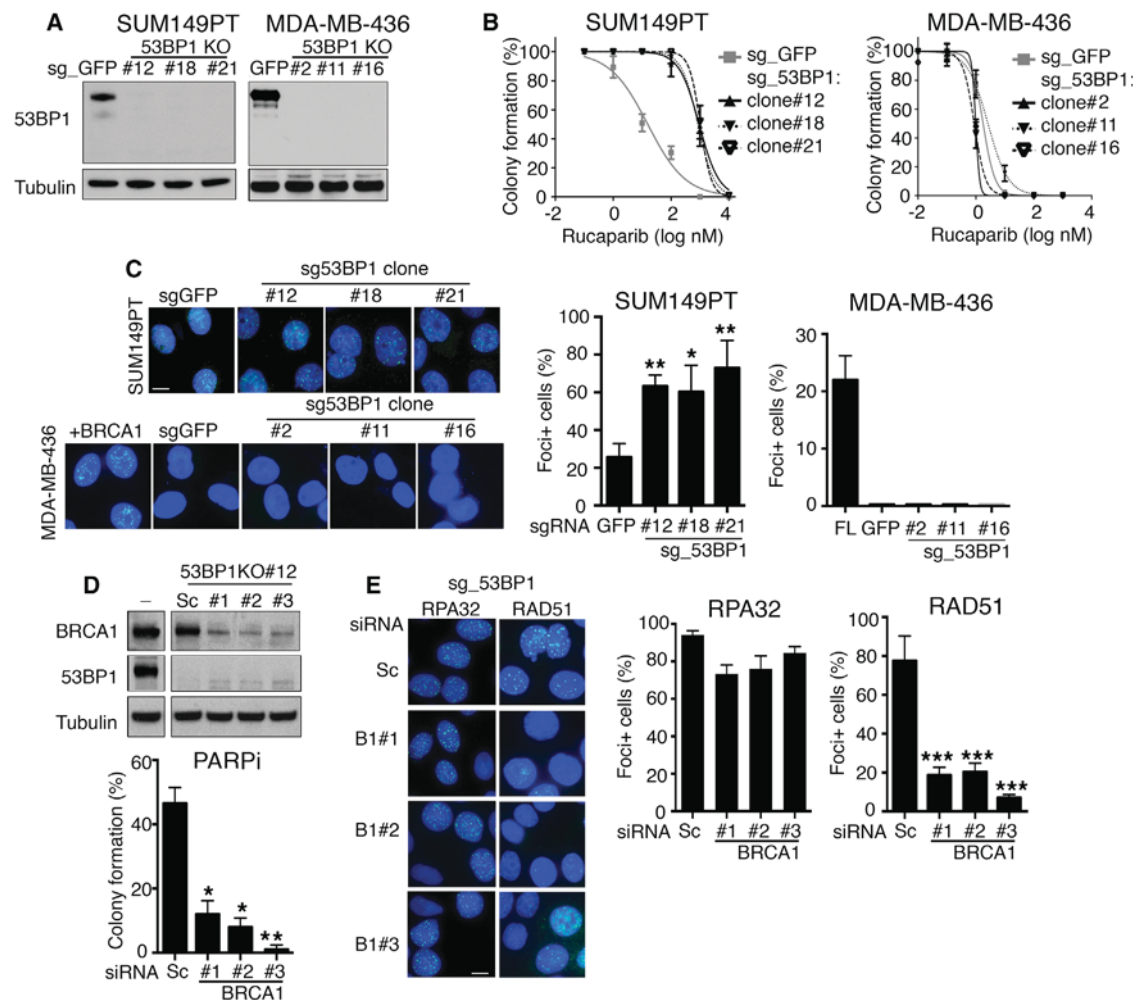


Figure 5. 53BP1 Gene KO Using CRISPR/Cas9

(A) Cells were treated with sg_GFP or sg_53BP1. For sg_53BP1-treated cells, we established multiple clones per cell line and assessed 53BP1 protein expression by western blotting. Selected clones with undetectable 53BP1 protein expression are shown for protein expression relative to the sg_GFP control cells.

(B) Cells from (A) were treated with increasing concentrations of rucaparib and colony formation assessed. Mean and SEM colony formation is shown from 3 independent experiments.

(C) Cells from (A) were assessed for RAD51 IRIF as described in Figure 1. The number of foci-positive cells (mean and SEM) from 3 independent experiments and representative images are shown. * $p < 0.05$, ** $p < 0.01$ compared to GFP (unpaired t test). MDA-MB-436+BRCA1 FL proteins were also assessed as a positive control. Scale bar, 10 μ m.

(D) SUM149PT sg_53BP1 KO clone#12 cells were subjected to scrambled (Sc) or each of 3 different BRCA1-targeting siRNAs. Top: western blot showing BRCA1- Δ 11q depletion. Bottom: mean and SEM colony formation of cells treated with 1 μ M rucaparib normalized to vehicle-treated cells from 3 independent experiments. * $p < 0.05$, ** $p < 0.01$ compared to Sc (unpaired t test).

(E) Cells from (D) were assessed for RPA32 and RAD51 IRIF as described in Figure 1. The number of foci-positive cells (mean and SEM) from 3 independent experiments and representative images are shown. *** $p < 0.001$ compared to Sc (unpaired t test). Scale bar, 10 μ m.

LC50 values compared to sg_GFP control cells. In contrast, MDA-MB-436 53BP1 KO clones did not demonstrate any significant level of PARPi resistance (Figure 5B). Furthermore, SUM149PT 53BP1 KO clones exhibited increased RAD51 IRIF, whereas RAD51 IRIF remained undetectable in MDA-MB-436 53BP1 KO clones (Figure 5C).

SUM149PT cells harbor a *BRCA1* exon 11 mutation that blocks the synthesis of full-length BRCA1, but cells are capable of expressing the BRCA1- Δ 11q splice variant. To determine if 53BP1 KO-induced PARPi resistance required BRCA1 hypo-

morphic protein activity, SUM149PT+53BP1KO#12 cells were treated with scrambled or 3 individual BRCA1 siRNAs capable of depleting the BRCA1- Δ 11q protein, as well as 1 μ M rucaparib. BRCA1 siRNA resulted in 4-, 6-, and 46-fold ($p < 0.05$, unpaired t test) decrease in the number of colonies forming in the presence of rucaparib compared to scrambled-siRNA-treated cells (Figure 5D). Furthermore, BRCA1 siRNA had little impact on RPA32 IRIF, but RAD51 IRIF was reduced 4-, 4-, and 11-fold ($p < 0.001$, unpaired t test) compared to scrambled-siRNA-treated cells (Figure 5E). Therefore, 53BP1 gene KO produced

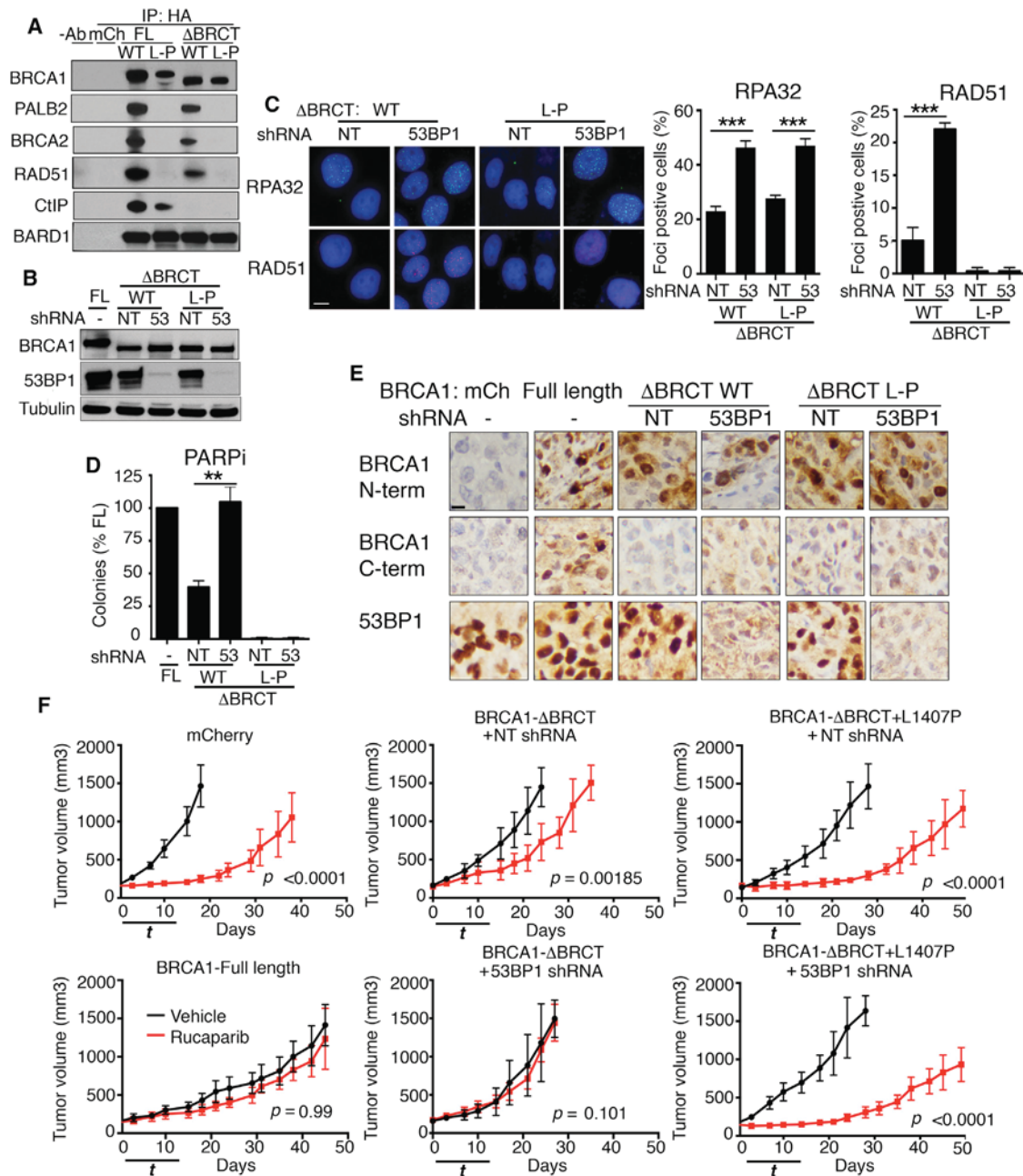


Figure 6. Effect of BRCA1 and 53BP1 Mutational Status on Tumor PARPi Sensitivity

(A) Western blot analyses of MDA-MB-436 cells engineered to express mCherry (mCh), FL BRCA1 (FL), and BRCA1- Δ BRCT (Δ BRCT) that were WT for the coiled-coil region (WT) or harbored the L1407P (L-P) missense mutation and subjected to immunoprecipitation using hemagglutinin (HA) antibody and immunoblotted for the indicated proteins.

(B) Western blot analyses of MDA-MB-436 cells expressing either BRCA1 FL or BRCA1- Δ BRCT (Δ BRCT) proteins, which are either coiled-coil WT or harbor the L1407P (L-P) mutation. Additionally, cells expressed NT or 53BP1 (53) shRNA.

(C) Cells from (B) were assessed for RPA32 and RAD51 IRIF as described in Figure 1. Left: representative images and number of foci-positive cells (mean and SEM) are shown from 3 independent experiments. ***p < 0.001 compared with relative NT shRNA cells (unpaired t test). Scale bar, 10 μ m.

(D) Cells from (B) were assessed for colony formation in the presence of 100 nM rucaparib. Mean and SEM from 3 independent experiments normalized to colony formation of BRCA1 FL-expressing cells are shown. **p < 0.01 compared with relative NT shRNA cells (unpaired t test).

(E) MDA-MB-436 tumor xenografts expressing or BRCA1- Δ BRCT proteins that are either coiled-coil WT or harbor the L1407P (L-P) mutation and treated with NT or 53BP1 shRNA targeting. BRCA1 and 53BP1 expression status in tumor xenografts was confirmed by IHC staining. MDA-MB-436 cells with mCherry and BRCA1 FL add-back tumors were included as controls for staining. Scale bar, 10 μ m.

(legend continued on next page)

phenotypes similar to RNAi-mediated depletion, whereby BRCA1 hypomorphic protein expression was required for robust RAD51 foci and PARPi resistance.

BRCA1 and 53BP1 Mutational Status Impacts Tumor PARPi Sensitivity

The BRCA1 CC domain facilitates the direct interaction between BRCA1 and PALB2 and enables the formation of a larger BRCA1-PALB2-BRCA2-RAD51 complex. We investigated the importance of the BRCA1-PALB2 interaction for HR and PARPi resistance in the setting of BRCT-less BRCA1 protein expression and 53BP1 depletion. The *BRCA1* L1407P missense mutation, previously shown to abrogate the BRCA1-PALB2 interaction (Anantha et al., 2017; Sy et al., 2009), was introduced into the BRCA1-ΔBRCT constructs and expressed in MDA-MB-436 cells. We confirmed that full-length BRCA1+L1407P was unable to interact with PALB2 and consequently BRCA2 and RAD51 in co-immunoprecipitation experiments. However, RING- and BRCT-domain-mediated interactions with BARD1 and CtIP, respectively, remained intact (Figure 6A). Additionally, we confirmed protein interactions retained by the BRCA1-ΔBRCT protein, where loss of the BRCT domain results in failure to interact with associated proteins such as CtIP but BARD1, PALB2, BRCA2, and RAD51 interactions were intact. In contrast, BRCA1-ΔBRCT+L1407P was unable to complex with PALB2, BRCA2, RAD51, and CtIP and only retained the RING domain interaction with BARD1 (Figure 6A).

Next, we examined the ability of 53BP1 shRNA to restore RPA32 and RAD51 loading in cells that expressed either BRCA1-ΔBRCT wild-type or BRCA1-ΔBRCT+L1407P (Figure 6B). Here, while 53BP1 shRNA increased RPA32 IRIF regardless of BRCA1 status, RAD51 IRIF only increased in BRCA1-ΔBRCT-expressing cells, with BRCA1-ΔBRCT+L1407P demonstrating a complete absence of RAD51 IRIF (Figure 6C). Furthermore, 53BP1 shRNA increased the number of PARPi-resistant BRCA1-ΔBRCT wild-type-expressing cells to a similar level as that observed in cells expressing full-length-BRCA1. However, 53BP1 shRNA had no impact on PARPi sensitivity in cells expressing BRCA1-ΔBRCT+L1407P (Figure 6D). Similar results were obtained with BRCA1-Δ11q+L304P-expressing cells (Figures S7A and S7B).

The latter observations prompted us to explore the role of *BRCA1* and *53BP1* mutational and expression status on PARPi responsiveness in *BRCA1* mutant tumors. We subcutaneously injected MDA-MB-436 cells that expressed mCherry or BRCA1-full-length controls as well as BRCA1-ΔBRCT or BRCA1-ΔBRCT+L1407P with NT or 53BP1 shRNA into the flanks of NSG mice. BRCA1 and 53BP1 expression status was confirmed in tumor xenografts by immunohistochemical (IHC) staining (Figure 6E). Mice were treated with 200 mg/kg rucaparib twice daily for 10 days. Rucaparib strongly inhibited the growth of mCherry-expressing tumors but had little impact

on full-length BRCA1-expressing control tumors (Figure 6F). Rucaparib treatment mildly but significantly delayed tumor growth in BRCA1-ΔBRCT+NT shRNA-expressing tumors but had no impact on the growth of BRCA1-ΔBRCT+53BP1 shRNA-expressing tumors. In contrast, rucaparib resulted in a marked delay of tumor growth in BRCA1-ΔBRCT+L1407P-expressing tumors, regardless of 53BP1 status (Figure 6F). Altogether, these data indicate that 53BP1 knockdown requires an intact BRCA1-PALB2 complex for maximal tumor PARPi resistance.

DISCUSSION

BRCA1 plays a key role in preserving genome integrity through its involvement in HR DNA repair, with several well-characterized functions, including the initiation of DNA end resection, RAD51 loading, and homologous DNA pairing (Bouwman et al., 2010; Bunting et al., 2010; Sy et al., 2009; Zhang et al., 2009a, 2009b; Zhao et al., 2017). However, potential ambiguity regarding whether BRCA1 activity is obligatory for the completion of each of these steps arises from variation in cell lines, organisms, and genetic backgrounds used to study BRCA1. Previous reports demonstrated that *53bp1* gene KO was capable of rescuing embryonic viability, Rad51 loading, HR repair, and PARPi resistance in *Brca1*-deficient mice (Bunting et al., 2010, 2012; Cao et al., 2009). These studies point toward the initiation of DNA end resection as a key function of BRCA1 and suggest that BRCA1 may be dispensable after end resection when RAD51 loading occurs. In the current study, we report that in spite of 53BP1-KO-induced DNA end resection, RAD51 loading and HR remain suboptimal in the absence of BRCA1 activity and that protein products generated from mutant *BRCA1* alleles influence the ability of 53BP1 KO to induce maximal HR and PARPi resistance.

In line with previous reports demonstrating rescue of HR in *Brca1*^{Δ11} mice that express the *Brca1*-Δ11 protein (Cao et al., 2009), we discovered that 53BP1 KO efficiently rescued HR in *BRCA1* exon 11 mutant cancer cell lines that express the BRCA1-Δ11q splice isoform. *53bp1* KO was also previously shown to rescue HR and embryonic viability in *Brca1*^{Δ2/Δ2} mice, initially thought to be *Brca1* null due to a stop codon early in the reading frame (Bunting et al., 2012). However, a subsequent report showed that cells harboring the *Brca1*^{Δ2} allele expressed a RING-less *Brca1* protein that retains the CC domain and formed robust Rad51 IRIF (Li et al., 2016). In another study, 53BP1-KO-induced PARPi resistance was shown in cells harboring the *Brca1*^{SCO} allele, which deletes exons 5 and 6 (Bouwman et al., 2010). We speculate it may be possible for these cells to also produce a *Brca1* RING-less protein from the Met294 in-frame translation start site in exon 11, equivalent to human Met297 (Drost et al., 2016; Wang et al., 2016b). Additional studies have assessed PARPi sensitivity in mammary tumor cells

(F) Mice harboring MDA-MB-436 tumor xenografts described in (E) were treated with vehicle (black line) or 200 mg/kg rucaparib twice daily (red line) for 2 × 5 days with a 2-day interval; treatment period is indicated with a line and (t). Mean and SEM tumor volumes are shown for 5 tumors per cell line per treatment. We used a linear mixed-effects model to test the difference in slopes of log-tumor volume, and p values shown are from tests of differences in growth rates between vehicle- and rucaparib-treated tumors.

derived from mice harboring a more deleterious *Brca1*^{Δ5-13} allele where any protein product would lack the Brca1 CC and be unable to interact with Palb2. Here, similar to our results, 53bp1 shRNA-treated *Brca1*^{Δ5-13} cells had markedly lower levels of Rad51 IRIF compared to *Brca1* wild-type cells (Jaspers et al., 2013).

We were unable to detect any truncated Brca1 protein product in *Brca1*^{ΔC/ΔC};53bp1^{-/-} MEFs, indicating that the *Brca1*^{ΔC} mutation is (or is close to) a null allele. Furthermore, *Brca1*^{ΔC/ΔC};53bp1^{-/-} MEFs demonstrated low levels of Rad51 foci, increased numbers of chromosomal rearrangements, and fewer gene conversion events and were sensitive to PARPi treatment, all indicative of inefficient HR. Despite the latter observations, intriguingly, 53bp1 KO rescued the viability of homozygous *Brca1*^{ΔC} mice, raising the possibility that HR-independent 53bp1 activities contribute to embryonic lethality. However, we were unable to detect a reduction in p53 transcriptional activity in *Brca1*^{+/+};53bp1^{-/-} or *Brca1*^{ΔC/ΔC};53bp1^{-/-} cells, and *Brca1*^{ΔC/ΔC};53bp1^{-/-} MEFs senesced in normoxic conditions, suggesting p53 activity was intact. In line with previous reports, 53bp1 KO also did not rescue replication fork degradation associated with Brca1-deficient cells (Ray Chaudhuri et al., 2016). 53bp1 KO might also direct repair toward the resection-dependent and RAD51-independent single-strand annealing (SSA) or alternative end-joining (Alt-EJ) pathways (Bhargava et al., 2016), although these are highly mutagenic and unlikely to support organismal viability. In contrast, some level of residual HR is likely essential for organismal and cellular viability. Recently, BRCA2-induced HR was shown to be critical for the viability of human mammary epithelial cells (Feng and Jasin, 2017). We hypothesize that *Brca1*^{ΔC/ΔC};53bp1^{-/-} mice are viable, because 53bp1 KO directs DSBs toward HR, and that low levels of endogenous DNA breaks, which arise during embryo development, are tolerated due to residual Brca1-independent Rad51 loading and HR. Nevertheless, cells with inefficient Rad51 loading are unable to withstand greater numbers of DNA breaks encountered in the presence of oxidative damage or PARPi. Indeed, although Rad51 IRIF was impaired, we were able to detect some Rad51-positive *Brca1*^{ΔC/ΔC};53bp1^{-/-} MEFs, and residual foci formation was dependent on Palb2 and Brca2 activity (Figure S7C).

Despite surviving partial or complete embryonic development, both *Brca1*^{Δ11/Δ11};53bp1^{+/+} and *Brca1*^{ΔC/ΔC};53bp1^{-/-} cells are sensitive to PARPi. Residual HR is afforded by either the Brca1^{Δ11} protein in the case of *Brca1*^{Δ11/Δ11};53bp1^{+/+} cells or loss of 53bp1 protein activity in the case of *Brca1*^{ΔC/ΔC};53bp1^{-/-} cells. However, HR is further enhanced to a level that supports embryonic development and PARPi resistance in *Brca1*^{Δ11/Δ11};53bp1^{-/-} mice when HR-promoting alleles are combined resulting in both Brca1^{Δ11} expression and 53bp1 deficiency. Of note, despite exhibiting PARPi resistance, meiotic defects were previously reported in *Brca1*^{Δ11/Δ11};53bp1^{-/-} and *Brca1*^{Δ2/Δ2};53bp1^{-/-} mice (Cao et al., 2009; Li et al., 2016), suggesting that HR remains suboptimal or is not regulated correctly compared with *Brca1* and 53bp1 wild-type animals. Parallel to observations in mice, low levels of HR are sufficient to support human viability, as evidenced by Fanconi anemia patients, who are also highly sensitive to DNA-damaging chemotherapy treatments.

53BP1 depletion had a mild effect on PARPi resistance in human *BRCA1* mutant HCC1395, SUM135MO2, and MDA-MB-436 breast cancer cell lines that have low levels of truncated BRCA1 protein expression. Furthermore, while CRISPR/Cas9-mediated 53BP1 KO resulted in substantial PARPi resistance in SUM149PT cells, 53BP1 KO did not increase PARPi resistance in MDA-MB-436 cells. In contrast, a previous report using transcription activator-like effector nucleases (TALENs) to generate 53BP1 KO MDA-MB-436 cells found that a 53BP1 KO clone showed increased PARPi resistance (Yang et al., 2017). These differences may be a result of variations in the expression levels of truncated BRCA1 between MDA-MB-436 cells or clones. However, similar to our findings, a recent report showed that CRISPR/Cas9-mediated PARP1 KO provided PARPi resistance in SUM149PT, but not MDA-MB-436, cells. The authors also demonstrated that PARP1 KO-induced PARPi resistance in SUM149PT cells was dependent on residual activity provided by the BRCA1-Δ11q protein (Pettitt et al., 2018).

We found that loss of 53BP1 activity alone did not support tumoral PARPi resistance in *in vivo* experiments using the MDA-MB-436 breast cancer cell line xenograft system. Here, an intact BRCA1-PALB2 interaction and loss of 53BP1 expression were both required for robust tumor growth in the presence of rucaparib. Supporting this observation, we previously discovered the co-occurrence of increased BRCA1 hypomorphic protein expression and 53BP1 loss of function in therapy-resistant *BRCA1* mutant cancer cells and patient tumors (Johnson et al., 2013). However, it is important to note that although MDA-MB-436 cells strictly required BRCA1 for RAD51 foci, we demonstrate cell line to cell line variation in the absolute dependency for BRCA1 at the RAD51 loading step of HR. For example, in contrast to MDA-MB-436 cells, *Brca1*^{ΔC/ΔC};53bp1^{-/-} MEFs had low but consistently detectable levels of Rad51 IRIF, despite being null for Brca1 protein expression. Whereas HCC1397 cells demonstrated robust RAD51 IRIF in the absence of a detectable BRCA1 protein. Such cell line variations may stem from the activity of additional factors that contribute to PALB2 recruitment to DNA breaks such as RNF168, ATR or RPA (Luijsterburg et al., 2017; Murphy et al., 2014; Yazinski et al., 2017). Moreover, KO of DNA end resection inhibitory proteins involved in the shieldin complex were recently shown to promote PARPi resistance in a range of human and mouse *BRCA1* mutant cell lines (Barazas et al., 2018; Dev et al., 2018; Mirman et al., 2018; Noordermeer et al., 2018; Tomida et al., 2018). We anticipate that similar to 53BP1 KO, the presence of BRCA1 hypomorphs in combination with shieldin KO may facilitate a greater level of PARPi resistance compared with shieldin KO and the absence of mutant BRCA1 protein expression.

In conclusion, we report that suboptimal HR is capable of supporting organismal viability but is not sufficient for tumor suppression or robust PARPi resistance. Furthermore, our results indicate that specific *BRCA1* mutant alleles and their protein products may impact the route of PARPi resistance acquired by cancer cells. 53BP1 KO has previously been shown to have no effect on the PARPi sensitivity of cells containing *BRCA2* or *PALB2* mutations (Bowman-Colin et al., 2013). *BRCA1* CC domain disruptions may represent another mutational subgroup that is less likely to develop 53BP1 loss-of-function-associated

PARPi resistance. Future work will be necessary to assess potential relationships between specific *BRCA1* mutations and particular PARPi resistance pathways in the clinical setting.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

- **KEY RESOURCES TABLE**
- **EXPERIMENTAL MODEL AND SUBJECT DETAILS**
 - Contact for Reagent and Resource Sharing
 - Cell Lines
 - Mice
- **METHOD DETAILS**
 - *BRCA1* mutant cDNA generation
 - Lentivirus Production
 - Lentivirus cell infection
 - siRNA transfection
 - RNA extraction and qRT-PCR
 - PARPi resistance assays
 - Colony formation assays
 - Sulforhodamine B (SRB) assays
 - DNA replication fork protection
 - SA-β-Gal Staining
 - Cell cycle analyses
 - Immunofluorescence and microscopy
 - Western Blotting and Immunoprecipitation
 - Metaphase spreads
 - Homologous recombination reporter assays
 - Generation of *Brca1*^{ΔC} mutant mice
 - Mouse Genotyping
 - PARPi treatment and peripheral blood analysis
 - MEF generation
 - Embryo dissection
 - Xenograft studies
 - Histologic and immunohistochemical staining
- **QUANTIFICATION AND STATISTICAL ANALYSIS**

SUPPLEMENTAL INFORMATION

Supplemental Information includes seven figures and one table and can be found with this article online at <https://doi.org/10.1016/j.celrep.2018.08.086>.

ACKNOWLEDGMENTS

This work was supported by NIH grants P50 CA083638 and 5P30 CA006927 to Fox Chase Cancer Center (FCCC), as well as grant R01CA214799 and Department of Defense Ovarian Academy Award OC130212 to N.J. Clovis Oncology supplied rucaparib. We are grateful for help from FCCC Genomics, Cell Culture, Histopathology, and Cell Sorting facilities. We thank Dr. Zemin Liu at the FCCC Cytogenetics facility for help analyzing metaphase spreads.

AUTHOR CONTRIBUTIONS

J.N., J.J.K., Y.W., E.C., A.J.B., W.F., E.N., K.Q.C., D.D., E.M., and R.T., designed and performed experiments. X.H., J.N., and N.J. developed *Brca1* mouse models. D.Z., E.A.H., A.N., G.P.G., J.N., A.B., J.R.T., S.M.S., and N.J. analyzed and interpreted data. J.N. and N.J. wrote the manuscript. N.J. supervised the project.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 20, 2018

Revised: August 1, 2018

Accepted: August 29, 2018

Published: September 25, 2018

REFERENCES

- Anantha, R.W., Simhadri, S., Foo, T.K., Miao, S., Liu, J., Shen, Z., Ganesan, S., and Xia, B. (2017). Functional and mutational landscapes of *BRCA1* for homology-directed repair and therapy resistance. *eLife* 6, e21350.
- Barazas, M., Annunziato, S., Pettitt, S.J., de Krijger, I., Ghezraoui, H., Roobol, S.J., Lutz, C., Frankum, J., Song, F.F., Brough, R., et al. (2018). The CST complex mediates end protection at double-strand breaks and promotes PARP inhibitor sensitivity in *BRCA1*-deficient cells. *Cell Rep.* 23, 2107–2118.
- Bhargava, R., Onyango, D.O., and Stark, J.M. (2016). Regulation of single-strand annealing and its role in genome maintenance. *Trends Genet.* 32, 566–575.
- Bouwman, P., Aly, A., Escandell, J.M., Pieterse, M., Bartkova, J., van der Gulden, H., Hiddingh, S., Thanasoula, M., Kulkarni, A., Yang, Q., et al. (2010). 53BP1 loss rescues *BRCA1* deficiency and is associated with triple-negative and *BRCA*-mutated breast cancers. *Nat. Struct. Mol. Biol.* 17, 688–695.
- Bowman-Colin, C., Xia, B., Bunting, S., Klijn, C., Drost, R., Bouwman, P., Fine-man, L., Chen, X., Culhane, A.C., Cai, H., et al. (2013). *Palb2* synergizes with *Trp53* to suppress mammary tumor formation in a model of inherited breast cancer. *Proc. Natl. Acad. Sci. USA* 110, 8632–8637.
- Bryant, H.E., Schultz, N., Thomas, H.D., Parker, K.M., Flower, D., Lopez, E., Kyle, S., Meuth, M., Curtin, N.J., and Helleday, T. (2005). Specific killing of *BRCA2*-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* 434, 913–917.
- Bunting, S.F., Callén, E., Wong, N., Chen, H.T., Polato, F., Gunn, A., Bothmer, A., Feldhahn, N., Fernandez-Capetillo, O., Cao, L., et al. (2010). 53BP1 inhibits homologous recombination in *Brca1*-deficient cells by blocking resection of DNA breaks. *Cell* 141, 243–254.
- Bunting, S.F., Callén, E., Kozak, M.L., Kim, J.M., Wong, N., López-Contreras, A.J., Ludwig, T., Baer, R., Faryabi, R.B., Malhowski, A., et al. (2012). *BRCA1* functions independently of homologous recombination in DNA interstrand crosslink repair. *Mol. Cell* 46, 125–135.
- Cao, L., Xu, X., Bunting, S.F., Liu, J., Wang, R.H., Cao, L.L., Wu, J.J., Peng, T.N., Chen, J., Nussenzweig, A., et al. (2009). A selective requirement for 53BP1 in the biological response to genomic instability induced by *Brca1* deficiency. *Mol. Cell* 35, 534–541.
- Chapman, J.R., Sossick, A.J., Boulton, S.J., and Jackson, S.P. (2012). *BRCA1*-associated exclusion of 53BP1 from DNA damage sites underlies temporal control of DNA repair. *J. Cell Sci.* 125, 3529–3534.
- Chen, C.F., Li, S., Chen, Y., Chen, P.L., Sharp, Z.D., and Lee, W.H. (1996). The nuclear localization sequences of the *BRCA1* protein interact with the importin-α subunit of the nuclear transport signal receptor. *J. Biol. Chem.* 271, 32863–32868.
- Cuella-Martin, R., Oliveira, C., Lockstone, H.E., Snellenberg, S., Grolmusova, N., and Chapman, J.R. (2016). 53BP1 integrates DNA repair and p53-dependent cell fate decisions via distinct mechanisms. *Mol. Cell* 64, 51–64.
- Densham, R.M., Garvin, A.J., Stone, H.R., Strachan, J., Baldock, R.A., Daza-Martin, M., Fletcher, A., Blair-Reid, S., Beesley, J., Johal, B., et al. (2016). Human *BRCA1*-BARD1 ubiquitin ligase activity counteracts chromatin barriers to DNA resection. *Nat. Struct. Mol. Biol.* 23, 647–655.
- Dev, H., Chiang, T.W., Lescale, C., de Krijger, I., Martin, A.G., Pilger, D., Coates, J., Sczaniecka-Clift, M., Wei, W., Ostermaier, M., et al. (2018). Shieldin complex promotes DNA end-joining and counters homologous recombination in *BRCA1*-null cells. *Nat. Cell Biol.* 20, 954–965.

- Drost, R., Bouwman, P., Rottenberg, S., Boon, U., Schut, E., Klarenbeek, S., Klijn, C., van der Heijden, I., van der Gulden, H., Wientjens, E., et al. (2011). BRCA1 RING function is essential for tumor suppression but dispensable for therapy resistance. *Cancer Cell* 20, 797–809.
- Drost, R., Dhillon, K.K., van der Gulden, H., van der Heijden, I., Brandsma, I., Cruz, C., Chondronasiou, D., Castroviejo-Bermejo, M., Boon, U., Schut, E., et al. (2016). BRCA1185delAG tumors may acquire therapy resistance through expression of RING-less BRCA1. *J. Clin. Invest.* 126, 2903–2918.
- Elstrodt, F., Hollestelle, A., Nagel, J.H., Gorin, M., Wasielewski, M., van den Ouweland, A., Merajver, S.D., Ethier, S.P., and Schutte, M. (2006). BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res.* 66, 41–45.
- Farmer, H., McCabe, N., Lord, C.J., Tutt, A.N., Johnson, D.A., Richardson, T.B., Santarosa, M., Dillon, K.J., Hickson, I., Knights, C., et al. (2005). Targeting the DNA repair defect in BRCA mutant cells as a therapeutic strategy. *Nature* 434, 917–921.
- Feng, W., and Jasin, M. (2017). BRCA2 suppresses replication stress-induced mitotic and G1 abnormalities through homologous recombination. *Nat. Commun.* 8, 525.
- Foo, T.K., Tischkowitz, M., Simhadri, S., Boshari, T., Zayed, N., Burke, K.A., Berman, S.H., Blecua, P., Riaz, N., Huo, Y., et al. (2017). Compromised BRCA1-PALB2 interaction is associated with breast cancer risk. *Oncogene* 36, 4161–4170.
- Hakem, R., de la Pompa, J.L., Elia, A., Potter, J., and Mak, T.W. (1997). Partial rescue of Brca1 (5-6) early embryonic lethality by p53 or p21 null mutation. *Nat. Genet.* 16, 298–302.
- Hill, S.J., Clark, A.P., Silver, D.P., and Livingston, D.M. (2014). BRCA1 pathway function in basal-like breast cancer cells. *Mol. Cell. Biol.* 34, 3828–3842.
- Huen, M.S., Sy, S.M., and Chen, J. (2010). BRCA1 and its toolbox for the maintenance of genome integrity. *Nat. Rev. Mol. Cell Biol.* 11, 138–148.
- Jaspers, J.E., Kersbergen, A., Boon, U., Sol, W., van Deemter, L., Zander, S.A., Drost, R., Wientjens, E., Ji, J., Aly, A., et al. (2013). Loss of 53BP1 causes PARP inhibitor resistance in Brca1-mutated mouse mammary tumors. *Cancer Discov.* 3, 68–81.
- Johnson, N., Johnson, S.F., Yao, W., Li, Y.C., Choi, Y.E., Bernhardt, A.J., Wang, Y., Capelletti, M., Sarosiek, K.A., Moreau, L.A., et al. (2013). Stabilization of mutant BRCA1 protein confers PARP inhibitor and platinum resistance. *Proc. Natl. Acad. Sci. USA* 110, 17041–17046.
- Korlimarla, A., Bhandary, L., Prabhu, J.S., Shankar, H., Sankaranarayanan, H., Kumar, P., Remacle, J., Natarajan, D., and Sridhar, T.S. (2013). Identification of a non-canonical nuclear localization signal (NLS) in BRCA1 that could mediate nuclear localization of splice variants lacking the classical NLS. *Cell. Mol. Biol. Lett.* 18, 284–296.
- Ledermann, J.A., Harter, P., Gourley, C., Friedlander, M., Vergote, I., Rustin, G., Scott, C., Meier, W., Shapira-Frommer, R., Safra, T., et al. (2016). Overall survival in patients with platinum-sensitive recurrent serous ovarian cancer receiving olaparib maintenance monotherapy: an updated analysis from a randomised, placebo-controlled, double-blind, phase 2 trial. *Lancet Oncol.* 17, 1579–1589.
- Li, M., Cole, F., Patel, D.S., Misenko, S.M., Her, J., Malhowski, A., Alhamza, A., Zheng, H., Baer, R., Ludwig, T., et al. (2016). 53BP1 ablation rescues genomic instability in mice expressing 'RING-less' BRCA1. *EMBO Rep.* 17, 1532–1541.
- Ludwig, T., Chapman, D.L., Papaioannou, V.E., and Efstratiadis, A. (1997). Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of Brca1, Brca2, Brca1/Brca2, Brca1/p53, and Brca2/p53 nullizygous embryos. *Genes Dev.* 11, 1226–1241.
- Luijsterburg, M.S., Typas, D., Caron, M.C., Wiegant, W.W., van den Heuvel, D., Boonen, R.A., Couturier, A.M., Mullenders, L.H., Masson, J.Y., and van Attikum, H. (2017). A PALB2-interacting domain in RNF168 couples homologous recombination to DNA break-induced chromatin ubiquitylation. *eLife* 6, e20922.
- Mirman, Z., Lottersberger, F., Takai, H., Kibe, T., Gong, Y., Takai, K., Bianchi, A., Zimmermann, M., Durocher, D., and de Lange, T. (2018). 53BP1-RIF1-shieldin counteracts DSB resection through CST- and Pol α -dependent fill-in. *Nature* 560, 112–116.
- Moynahan, M.E., Chiu, J.W., Koller, B.H., and Jasin, M. (1999). Brca1 controls homology-directed DNA repair. *Mol. Cell* 4, 511–518.
- Moynahan, M.E., Cui, T.Y., and Jasin, M. (2001). Homology-directed DNA repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brca1 mutation. *Cancer Res.* 61, 4842–4850.
- Murphy, A.K., Fitzgerald, M., Ro, T., Kim, J.H., Rabinowitsch, A.I., Chowdhury, D., Schildkraut, C.L., and Borowiec, J.A. (2014). Phosphorylated RPA recruits PALB2 to stalled DNA replication forks to facilitate fork recovery. *J. Cell Biol.* 206, 493–507.
- Noordermeer, S.M., Adam, S., Setiawati, D., Barazas, M., Pettitt, S.J., Ling, A.K., Olivieri, M., Álvarez-Quilón, A., Moatti, N., Zimmermann, M., et al. (2018). The shieldin complex mediates 53BP1-dependent DNA repair. *Nature* 560, 117–121.
- Parrinello, S., Samper, E., Krtolica, A., Goldstein, J., Melov, S., and Campisi, J. (2003). Oxygen sensitivity severely limits the replicative lifespan of murine fibroblasts. *Nat. Cell Biol.* 5, 741–747.
- Pettitt, S.J., Krastev, D.B., Brandsma, I., Dréan, A., Song, F., Aleksandrov, R., Harrell, M.I., Menon, M., Brough, R., Campbell, J., et al. (2018). Genome-wide and high-density CRISPR-Cas9 screens identify point mutations in PARP1 causing PARP inhibitor resistance. *Nat. Commun.* 9, 1849.
- Ray Chaudhuri, A., Callen, E., Ding, X., Gogola, E., Duarte, A.A., Lee, J.E., Wong, N., Lafarga, V., Calvo, J.A., Panzarino, N.J., et al. (2016). Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* 535, 382–387.
- Robson, M., Im, S.A., Senkus, E., Xu, B., Domchek, S.M., Masuda, N., Delaloge, S., Li, W., Tung, N., Armstrong, A., et al. (2017). Olaparib for metastatic breast cancer in patients with a germline BRCA mutation. *N. Engl. J. Med.* 377, 523–533.
- Roy, R., Chun, J., and Powell, S.N. (2011). BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat. Rev. Cancer* 12, 68–78.
- Sementino, E., Menges, C.W., Kadariya, Y., Peri, S., Xu, J., Liu, Z., Wilkes, R.G., Cai, K.Q., Rauscher, F.J., 3rd, Klein-Szanto, A.J., and Testa, J.R. (2018). Inactivation of Tp53 and Pten drives rapid development of pleural and peritoneal malignant mesotheliomas. *J. Cell. Physiol.* Published online June 15, 2018. <https://doi.org/10.1002/jcp.26830>.
- Shakya, R., Reid, L.J., Reczek, C.R., Cole, F., Egli, D., Lin, C.S., deRooy, D.G., Hirsch, S., Ravi, K., Hicks, J.B., et al. (2011). BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. *Science* 334, 525–528.
- Sy, S.M., Huen, M.S., and Chen, J. (2009). PALB2 is an integral component of the BRCA complex required for homologous recombination repair. *Proc. Natl. Acad. Sci. USA* 106, 7155–7160.
- Tkác, J., Xu, G., Adhikary, H., Young, J.T.F., Gallo, D., Escobedo-Díaz, C., Krietsch, J., Orthwein, A., Munro, M., Sol, W., et al. (2016). HELB is a feedback inhibitor of DNA end resection. *Mol. Cell* 61, 405–418.
- Tomida, J., Takata, K.I., Bhetawal, S., Person, M.D., Chao, H.P., Tang, D.G., and Wood, R.D. (2018). FAM35A associates with REV7 and modulates DNA damage responses of normal and BRCA1-defective cells. *EMBO J.* 37, e99543.
- Wang, Y., Bernhardt, A.J., Cruz, C., Krais, J.J., Nacson, J., Nicolas, E., Peri, S., van der Gulden, H., van der Heijden, I., O'Brien, S.W., et al.; kConFab Investigators (2016a). The BRCA1- Δ 11q alternative splice isoform bypasses germline mutations and promotes therapeutic resistance to PARP inhibition and cisplatin. *Cancer Res.* 76, 2778–2790.
- Wang, Y., Krais, J.J., Bernhardt, A.J., Nicolas, E., Cai, K.Q., Harrell, M.I., Kim, H.H., George, E., Swisher, E.M., Simpkins, F., and Johnson, N. (2016b). RING domain-deficient BRCA1 promotes PARP inhibitor and platinum resistance. *J. Clin. Invest.* 126, 3145–3157.
- Ward, I.M., Minn, K., van Deursen, J., and Chen, J. (2003). p53 Binding protein 53BP1 is required for DNA damage responses and tumor suppression in mice. *Mol. Cell. Biol.* 23, 2556–2563.

- Wyatt, D.W., Feng, W., Conlin, M.P., Yousefzadeh, M.J., Roberts, S.A., Mieczkowski, P., Wood, R.D., Gupta, G.P., and Ramsden, D.A. (2016). Essential roles for polymerase θ -mediated end joining in the repair of chromosome breaks. *Mol. Cell* 63, 662–673.
- Xia, B., Sheng, Q., Nakanishi, K., Ohashi, A., Wu, J., Christ, N., Liu, X., Jasin, M., Couch, F.J., and Livingston, D.M. (2006). Control of BRCA2 cellular and clinical functions by a nuclear partner, PALB2. *Mol. Cell* 22, 719–729.
- Xia, B., Dorsman, J.C., Ameziame, N., de Vries, Y., Rooimans, M.A., Sheng, Q., Pals, G., Errami, A., Gluckman, E., Llera, J., et al. (2007). Fanconi anemia is associated with a defect in the BRCA2 partner PALB2. *Nat. Genet.* 39, 159–161.
- Xu, X., Weaver, Z., Linke, S.P., Li, C., Gotay, J., Wang, X.W., Harris, C.C., Ried, T., and Deng, C.X. (1999). Centrosome amplification and a defective G2-M cell cycle checkpoint induce genetic instability in BRCA1 exon 11 isoform-deficient cells. *Mol. Cell* 3, 389–395.
- Xu, X., Qiao, W., Linke, S.P., Cao, L., Li, W.M., Furth, P.A., Harris, C.C., and Deng, C.X. (2001). Genetic interactions between tumor suppressors Brca1 and p53 in apoptosis, cell cycle and tumorigenesis. *Nat. Genet.* 28, 266–271.
- Xu, G., Chapman, J.R., Brandsma, I., Yuan, J., Mistrik, M., Bouwman, P., Bartkova, J., Gogola, E., Warmerdam, D., Barazas, M., et al. (2015). REV7 counteracts DNA double-strand break resection and affects PARP inhibition. *Nature* 521, 541–544.
- Yang, Z.M., Liao, X.M., Chen, Y., Shen, Y.Y., Yang, X.Y., Su, Y., Sun, Y.M., Gao, Y.L., Ding, J., Zhang, A., et al. (2017). Combining 53BP1 with BRCA1 as a biomarker to predict the sensitivity of poly(ADP-ribose) polymerase (PARP) inhibitors. *Acta Pharmacol. Sin.* 38, 1038–1047.
- Yazinski, S.A., Comaills, V., Buisson, R., Genois, M.M., Nguyen, H.D., Ho, C.K., Todorova Kwan, T., Morris, R., Lauffer, S., Nussenzweig, A., et al. (2017). ATR inhibition disrupts rewired homologous recombination and fork protection pathways in PARP inhibitor-resistant BRCA-deficient cancer cells. *Genes Dev.* 31, 318–332.
- Zhang, F., Fan, Q., Ren, K., and Andreassen, P.R. (2009a). PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. *Mol. Cancer Res.* 7, 1110–1118.
- Zhang, F., Ma, J., Wu, J., Ye, L., Cai, H., Xia, B., and Yu, X. (2009b). PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr. Biol.* 19, 524–529.
- Zhao, W., Steinfeld, J.B., Liang, F., Chen, X., Maranon, D.G., Jian Ma, C., Kwon, Y., Rao, T., Wang, W., Sheng, C., et al. (2017). BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. *Nature* 550, 360–365.
- Zhou, C., Smith, J.L., and Liu, J. (2003). Role of BRCA1 in cellular resistance to paclitaxel and ionizing radiation in an ovarian cancer cell line carrying a defective BRCA1. *Oncogene* 22, 2396–2404.

STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Mouse Anti-53BP1 Monoclonal antibody	Millipore	Cat# MAB3802; RRID: AB_2206767
Rabbit Anti-BARD1 Polyclonal antibody	Bethyl	Cat# A300-263A; RRID: AB_2061250
Rabbit Anti-BRCA1 Polyclonal antibody (C-terminal)	Millipore	Cat# 07-434; RRID: AB_2275035
Mouse Anti-BRCA1 Monoclonal antibody (N-terminal)	Millipore	Cat# OP92T-10UG; RRID: AB_564282
Anti-Brcal antibody (recognizing mouse Brca1)	Gift from A. Nussenzweig	
Rabbit Anti-BRCA2 Polyclonal antibody	Bethyl	Cat# A303-434A; RRID: AB_10952240
Rabbit Anti-CtIP Polyclonal antibody	Bethyl	Cat# A300-488A; RRID: AB_2175262
Mouse Anti-Geminin Monoclonal antibody	Abnova	H00051053-M01; RRID: AB_606308
Rabbit Anti-Geminin Polyclonal antibody	Protein Tech	Cat# 10802-1-AP; RRID: AB_2110945
Mouse Anti-HA Monoclonal antibody	Cell Signaling	Cat# 2367S; RRID: AB_10691311
Rabbit Anti-PALB2 Polyclonal antibody	Bethyl	Cat# A301-247A; RRID: AB_890608
Rabbit Anti-Rad51 Polyclonal antibody	GeneTex	Cat# GTX100469; RRID: AB_1951602
Rabbit Anti-RPA32 Polyclonal antibody	Santa Cruz	Cat# sc-28709; RRID: AB_2238546
Rat Anti-RPA32/RPA2 Monoclonal antibody	Cell Signaling	Cat# 2208; RRID: AB_2238543
Mouse Anti-RPA2 (Ab-2) Monoclonal antibody	CalBiochem	Cat# NA18-100UG; RRID: AB_213121
Rabbit Anti-Tubulin Polyclonal antibody	Cell Signaling	Cat# 2148; RRID: AB_2288042
Rabbit Anti-Histone3 Polyclonal antibody	Cell Signaling	Cat# 2148; RRID: AB_331563
Alexa Fluor 594-AffiniPure Donkey Anti-Mouse IgG	Jackson ImmunoResearch Labs	Cat# 715-585-150; RRID: AB_2340854
Alexa Fluor 594- AffiniPure Donkey anti-rabbit antibody	Jackson ImmunoResearch Labs	Cat# 711-585-152; RRID: AB_2340621
Fluorescein (FITC)-AffiniPure Donkey Anti Mouse	Jackson ImmunoResearch Labs	Cat# 715-095-150; RRID: AB_2340792
Fluorescein (FITC)-AffiniPure Donkey Anti-Rabbit	Jackson ImmunoResearch Labs	Cat# 711-095-152; RRID: AB_2315776
Donkey Anti-Rabbit IgG ECL Antibody, HRP Conjugated	GE Healthcare	Cat# NA9340-1ml; RRID: AB_772191
Sheep Anti-Mouse IgG ECL Antibody, HRP Conjugated	GE Healthcare	Cat# NA9310-1ml; RRID: AB_772193
Rabbit anti-Mouse IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 594	Thermo Fisher Scientific	Cat# A-11062; RRID: AB_2534109
Chicken anti-Rat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	Cat# A-21470; RRID: AB_2535873
Mouse anti-BrdU antibody	BD Biosciences	Cat# 347580; RRID: AB_400326
Rat anti-BrdU antibody	Bio-Rad	Cat# OBT0030; RRID: AB_609568
Dako EnVision+ System- HRP Labeled Polymer Anti-Rabbit	DAKO	Cat# K4002
Dako EnVision+ System- HRP Labeled Polymer Anti-Mouse	DAKO	Cat#K4000
Chemicals, Peptides, and Recombinant Proteins		
Rucaparib	Clovis Oncology	N/A
Hydroxyurea	Sigma	Cat#H8627
Nutlin-3a	Selleckchem	Cat#S8059
Bpu10I	New England Biolabs	Cat#R0649S
BssSI	New England Biolabs	Cat#R0587
Ascl	New England Biolabs	Cat#R0558

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Vectashield mounting medium with DAPI	Vector	Cat#H-1200
5-Iodo-2'-deoxyuridine	Sigma	Cat#I7125
5-Chloro-2'-deoxyuridine	Sigma	Cat#C6891
Tween-20	Sigma	Cat#1379
Triton X-100	Sigma	Cat#X100
Paraformaldehyde	Sigma	Cat#158127
OneTaq® Hot Start 2X Master Mix	New England Biolabs	Cat#M048S
Lipofectamine RNAimax	Invitrogen	Cat#13778075
Transit-LT1	Mirus	Cat#MIR2304
Sulforhodamine B	Sigma	Cat#230162
Sodium hydroxide	Sigma	Cat#221465
Sodium deoxycholate	Sigma	Cat#D6750
DAKO Target Retrieval Solution	DAKO	Cat#S1699
Dako Liquid DAB+ Substrate Chromogen System	DAKO	Cat#K3467
DA VINCI green diluent	BIOCARE	Cat#PD900L
Background Sniper	BIOCARE	Cat#BS966H
Meyer's Hematoxylin	Sigma	Cat#MHS32
Polybrene	Boston Bioproducts	Cat#BM-862W
Alt-R Cas9	IDT	Cat# 1074181
MG-132	MedChem Express	Cat#HY-13259
Critical Commercial Assays		
FxCycle PI/RNase Staining Solution	Thermo Fisher	Cat#F10797
QuikChange Lightning Site-Directed Mutagenesis Kit	Agilent	Cat#210518
Gateway LR Clonase II Enzyme Mix	Invitrogen	Cat#11791
GuideIT sgRNA <i>in Vitro</i> transcription Kit	Takara	Cat#632636
Pierce Classic IP Kit	Thermo Fisher	Cat#26146
NE-PER Nuclear and Cytoplasmic Extraction Kit	Thermo Fisher	Cat#78833
MEGAclear Transcription Clean-Up	Invitrogen	Cat# AM1908
SA-β-Gal Staining	Sigma	Cat#CS0030
RNeasy Plus Mini Kit	QIAGEN	Cat#74134
Hprt expression Taqman assay	Life Technologies	Mm00446968_m1
Experimental Models: Cell Lines		
Human: MDA-MB-231	ATCC	Cat#CRM-HTB-26; RRID: CVCL_0062
Human: SUM149PT	ASTERAND	Cat#CS-07; RRID: CVCL_3422
Human: UWB1.289	ATCC	Cat#CRL-2945; RRID: CVCL_B079
Human: L56Br-C1	Fox Chase Cancer Center	RRID: CVCL_DF84
Human: MDA-MB-436	ATCC	Cat#HTB-130; RRID: CVCL_0623
Human: HCC1395	ATCC	Cat#CRL-2324; RRID: CVCL_1249
Human: SUM1315MO2	ASTERAND	Cat#CS-014; RRID: CVCL_5589
Human: MCF7	ATCC	Cat#HTB-22; RRID: CVCL_0031
Human: OVCAR3	ATCC	Cat#HTB-161; RRID: CVCL_0465
Human: OVCAR5	Fox Chase Cancer Center	RRID: CVCL_1628
Human: HCC1937	ATCC	Cat#CRL-2336; RRID: CVCL_0290
Human: h-TERT RPE1	ATCC	Cat#CRL-4000; RRID: CVCL_4388
Human: IMR-90	ATCC	Cat#CCL-186; RRID: CVCL_0347
Human: MCF10F	Fox Chase Cancer Center	RRID: CVCL_0598
Human: HEK293T	Fox Chase Cancer Center	RRID: CVCL_0063

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
B6;129-Trp53bp1tm1Jc/J (Trp53BP1 ^{-/-})	The Jackson Laboratory	Cat#006495
B6C3F1/J	The Jackson Laboratory	Cat#100010
B6C3F1/J <i>Brca1</i> ^{ΔC}	This paper	N/A
STOCK <i>Brca1</i> ^{tm2.1Cxd} /Nci (Δ11)	NCI	Cat#01XC9
NOD.Cg- <i>Prkdc</i> ^{scid} 2rg ^{tm1Wjl} /SzJ	FCCC Laboratory Animal Facility	Cat#005557
Oligonucleotides		
Flexitube BRCA1 siRNA 9	QIAGEN	Cat#SI00299495
Flexitube BRCA1 siRNA 13	QIAGEN	Cat#SI02654575
Flexitube BRCA1 siRNA 15	QIAGEN	Cat#SI02664368
SMARTpool: ON-TARGETplus TP53BP1 siRNA	Dharmacon	Cat#L003548
SMARTpool: ON-TARGETplus Palb2 siRNA	Dharmacon	Cat#L042993
SMARTpool: ON-TARGETplus Brca2 siRNA	Dharmacon	Cat#L167423
Primers for site directed mutagenesis, mouse genotyping, qRT-PCR and oligos for 53BP1 shRNA and HR assay, see Table S1.	This study	
Recombinant DNA		
Gateway pENTR 1A vector	Invitrogen	
pDest-IRES-GFP Destination vector	Life Technologies	
pCW-Cas9	Fox Chase Cancer Center	Gift Christoph Seeger
pLX-SG1	Fox Chase Cancer Center	Gift Christoph Seeger
VSV-G/pMD2.G	Addgene	Cat#12259
psPAX2	Addgene	Cat#12260
Software and Algorithms		
Prism Software v6.0	GraphPad Software	
Adobe Photoshop CS v8.0	Adobe	
NIS Elements	Nikon	
FlowJo v10.1	FlowJo	

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Contact for Reagent and Resource Sharing

Further information and requests for reagents should be directed to and will be fulfilled by the Lead Contact, Neil Johnson (neil.johnson@fccc.edu)

Cell Lines

Cell lines were obtained from the ATCC or Asterand and tested negative for mycoplasma. Cell line identities were confirmed by IDEXX analysis. All cell lines were grown in media with 10% FBS and penicillin/streptomycin except otherwise mentioned. MDA-MB-231, MCF7, MDA-MB-436, OVCAR5 cells were grown in RPMI1640, for the OVCAR3 cell line glutamax and insulin were added. HCC1395 and L56BRC1 were grown in RPMI media supplemented with HEPES and Sodium Pyruvate. SUM1315MO2 and SUM149PT were grown in Hams F-12 supplemented with insulin, EGF, HEPES and hydrocortisone. HEK293T, IMR-90 and MEFs were grown in DMEM. HEK293T were grown without penicillin/streptomycin and for the IMR-90 and the MEFs, media was supplemented with 15% FBS, non-essential amino acids, sodium pyruvate and L-glutamine. MCF10F was grown in high calcium media. H-TERT RPE1 was grown in DMEM:F-12 supplemented with L-Glutamine, HEPES, sodium pyruvate and sodium bicarbonate. With the exception of the MEFs which were grown in a 2% oxygen incubator all cell lines were incubated at 37°C with 5% CO₂ in a humidified incubator.

Mice

All experiments involving animals were approved by the Fox Chase Cancer Center (FCCC) Institutional Animal Care and Use Committee. The mouse *Brca1*^{ΔC} allele was generated by the FCCC transgenic mouse facility (detailed below). Before initiating experiments, germline transmission of the allele was confirmed. Embryos harboring the *Brca1*^{Δ11} allele (Xu et al., 2001) were obtained

from the NCI and mice with the *Trp53bp1*^{-/-} (Ward et al., 2003) allele were obtained from the Jackson Laboratory. For xenograft experiments, 6–8 weeks old NOD-*scid* IL2Rg^{null} females were acquired from the FCCC Laboratory Animal Facility.

METHOD DETAILS

BRCA1 mutant cDNA generation

HA-BRCA1 cDNA was cloned into the pENTR1A Gateway Entry vector (Invitrogen) and shuttled into a pDest-IRES-GFP Destination vector (Life Technologies) using Gateway LR Clonase II Enzyme Mix (Invitrogen). To generate cDNA that would translate to C-terminal truncated proteins an Ascl site was introduced at the end of BRCA1 exon 24 by site-directed mutagenesis (Agilent). Restriction digestion was made using the Ascl site (*BRCA1* 5773 nt) and a BssSI site present at *BRCA1* 4771 nt. This was followed by site-directed mutagenesis with the insertion of a stop codon at the end of the exon 11 coding region (c.4099G > T) generating BRCA1-ΔCC+ΔBRCT or at the end of the exon 15 coding region (c.4675G > T) generating BRCA1-ΔBRCT. The BRCA1-Δ11q construct was previously described (Wang et al., 2016a). The L1407P missense mutation was generated by site directed mutagenesis (Agilent). See Table S1 for primer sequences.

Lentivirus Production

HEK293T were transfected with either pDest-IRES-GFP, pLKO.1, pCW-Cas9 or pLX-SG1 with psPAX2 packaging and VSV-G/pMD2.G envelope plasmids using Transit-LT1 (Mirus). Culture media was collected 60 hr later and filtered through a 0.45 μm filter.

Lentivirus cell infection

Cell lines were infected with lentivirus using polybrene (Boston Bioproducts) and infected cells were selected based on either GFP expression or antibiotic resistance. Clones were generated from the pCW-Cas9 infected cells to select for cells with high Cas9 expression. Cas9 expression was conditionally induced by the addition of doxycycline (4 μg/ml) to culture media. sgRNA targeting *53BP1* or *GFP* was cloned into the pLX-SG1 vector using BsmBI sites. After blasticidin selection clones were generated and *53BP1* protein expression was evaluated by immunoblot.

siRNA transfection

For siRNA experiments cells were transfected using 10 to 40 nM of siRNA with Lipofectamine RNAiMAX (Invitrogen). All the experiments were carried out at least 48 hr after siRNA transfection. See Key Resources Table for details of siRNA sequences.

RNA extraction and qRT-PCR

RNA was extracted using the RNeasy Plus Mini Kit (QIAGEN) following manufacture instructions. For quantitative RT-PCR, RNA was tested for quality on a Bio-analyzer (Agilent). RNA concentrations were determined with a spectrophotometer (NanoDrop; Thermo Fisher Scientific). RNA was reverse transcribed using Moloney murine leukemia virus reverse transcriptase (Ambion, Thermo Fisher Scientific) and a mixture of anchored oligo-dT and random decamers. Two reverse transcription reactions were performed for each sample using either 100 or 25 ng input RNA. Assays were used in combination with Taqman Universal Master Mix or Power SYBR Green Master Mix and run on a 7900 HT Sequence Detection System (all from Applied Biosystems, Thermo Fisher Scientific). Cycling conditions were 95°C for 15 min, followed by 40 (2-step) cycles (95°C, 15 s; 60°C, 60 s). Ct values were converted to quantities (in AU) using a standard curve (4 points, 4-fold dilutions) established with a calibrator sample. For *Hprt* expression the Taqman assay (Life Technologies: Mm00446968_m1) was used. Primers used for *p53*, *p21*, *Palb2*, *Brca2* and *Brca1* are listed in Table S1.

PARPi resistance assays

MDA-MB-436 cells expressing different BRCA1 proteins were seeded in 6 well plates in the following seeding densities: 2x10⁴, 1x10⁴, 0.5x10⁴, 0.25x10⁴, 1.25x10³ and 0.625x10³ cells/ well. The cells were treated with 100 nM of rucaparib for 3 weeks. Media containing rucaparib was changed every 48–72 hr. After 3 weeks resistant colonies were fixed with 3:1 methanol: acid acetic solution and stained with crystal violet. The mean colony formation from 3 different experiments were expressed as the mean percentage of resistant colonies ± SEM relative to BRCA1 full-length expressing PARPi treated cells.

Colony formation assays

Cells were seeded in 6-well plates at 500 and 1000 cells per well in the presence of increasing concentrations of PARPi. Colony formation was assessed after 2 weeks with crystal violet staining. Lethal concentration (LC₅₀) values (concentration required to reduce colony formation by 50%) were calculated using GraphPad Prism software. Experiments were repeated at least 3 times.

Sulforhodamine B (SRB) assays

Depending on growing rate MEFs were seeded at densities ranging from 2000 to 5000 cells per well in 24 well plates. The next day increasing concentrations of PARPi was added to culture media. Media was changed every 3 days. After 10 days cells were fixed with 50% Trichloroacetic acid followed by 30 min incubation at room temperature with 0.4% SRB in a 1% acetic acid solution. Plates were

washed and SRB solubilized with 10 mM Tris base pH 10.5. Staining intensity was read on a plate reader at 510 nm wavelength. Cell growth was calculated as a percentage of the DMSO vehicle treated control wells for each group.

DNA replication fork protection

To measure replication fork stability MEFs were labeled with 50 μ M CldU for 30 min, washed and exposed to 250 μ M IdU for 30 min. This was followed by exposure of 2 μ M of hydroxyurea for 3 hr (Sigma). Cells were collected, and resuspended in PBS. Cells were lysed (200 mM Tris-HCl, 50 mM EDTA, 0.5% SDS, pH 7.4), and DNA fibers stretched onto glass slides (Superfrost Microscope Slides precleaned, Fisher Scientific) by tilting slides 30 degrees, followed by fixing in a methanol: acetic acid solution (3:1) for 10 min. The fibers were denatured with 2.5 M HCl for 2.5 hr, washed with PBS and blocked with 2% BSA in PBST for 40 min. Labeled DNA with CldU and IdU were stained with anti-BrdU antibodies recognizing CldU and IdU (Novus 1:500 and BD Bioscience 1:100, respectively). Anti-mouse Alexa 594 (1:300) and anti-rat Alexa 488 (1:300) were used for secondary antibodies. Images were captured with Nikon NIU Upright Fluorescence Microscope. Fiber length was quantified using ImageJ software.

SA- β -Gal Staining

MEFs were seeded in a 12 well plate and SA- β -Gal Staining was performed using a commercial staining kit (Sigma). Cells were incubated overnight at 37°C in a low CO₂ incubator.

Cell cycle analyses

Cells were harvested and fixed with 50% ethanol. Cells were then washed with PBS and re-suspended in 0.5 mL FxCycle PI/RNase Staining Solution (Life Technologies, Thermo Fisher Scientific) and incubated at room temperature for 30 min. Data were acquired using a BD LSR II Flow Cytometer and analyzed using FlowJo software.

Immunofluorescence and microscopy

Cells were fixed with 4% paraformaldehyde (PFA) and permeabilized with 1% Triton-X in PBS for 10 min. Primary antibodies (BRCA1 MS110 Millipore, 1:1000, RAD51 GeneTex 1:1000, RPA32 CalBiochem 1:1000) were incubated overnight at 4 degrees in 5% goat serum. Alexa Fluor or FITC conjugated antibodies (Jackson ImmunoResearch Labs 1:1000) were incubated for 1 hr at room temperature. For RPA32 foci experiments, we pre-extracted cells prior to fixation as previously described (Di Micco R, d'Adda di Fagagna F. Indirect immunofluorescence with preextraction (*in situ* cell fractionation). *Protocol Exchange*. 2006). Briefly, we treated cells with ice-cold cytoskeleton buffer (10 mM Pipes pH 6.8, 100 mM NaCl, 300 mM sucrose, 3mM MgCl₂, 1mM EGTA, 0.5% Triton X-100) for 5 min on ice followed by 5 min incubation with ice-cold cytoskeleton stripping buffer (10 mM TrisHCl pH 7.4, 10 mM NaCl, 3mM MgCl₂, 1% Tween 40(v/v), 0.5% sodium deoxycholate) then fixed in 4% PFA and continued to be processed as described above. To detect mouse RPA32 we incubated with Rat Anti-RPA32 (4E4, Cell Signaling) and Chicken anti-Rat IgG (Thermo Fisher Scientific) as secondary antibody. Slides were mounted with mounting media containing DAPI. Images were captured using a Nikon NIU Upright Fluorescence Microscope and generated images using Nikon NIS Elements software. For IR experiments, we routinely fixed cells 8 hr after treatment with 10 Gy. For analyses, we counted a minimum of 100 cells per condition per cell line. Foci positive cells were nuclei that contained 5 foci for BRCA1 and RAD51 or 10 foci for RPA32 and 53BP1. Each experiment was performed at least 3 times with biological replicates.

Western Blotting and Immunoprecipitation

Nuclear extracts were derived using the NE-PER Nuclear and Cytoplasmic Extraction Kit following the manufacturer's instructions (Thermo Scientific). Protease inhibitor and phosphatase inhibitors were added (Millipore, Cat#524625 and #539131). Lysates were separated by SDS-PAGE and transferred to a PDVF membrane (ImmobilonP, Millipore). Membranes were blocked for 1 hr with 5% nonfat milk in PBS. Primary Antibodies were incubated overnight at 4 degrees directed against 53BP1 (Millipore 1:1000), BARD1 (Bethyl 1:2000), BRCA1 (Millipore MS110, 1:500), BRCA2 (Bethyl 1:2000), CtIP (Bethyl 1:2000), PALB2 (Bethyl 1:2000), RAD51 (Genetex 1:1000), RPA32 (Santa Cruz 1:1000), Tubulin (Cell signaling 1:2000) and Histone 3 (Cell signaling 1:1000). Brca1 (1:1000 recognizing mouse species Brca1, gift from A. Nussenzweig), HRP conjugated secondary antibodies were used. Immunoprecipitation of BRCA1 complexes were carried out using the Pierce Classic IP Kit (Thermo Fischer). Briefly, 1 mg of nuclear extract was pre-cleared with Control Agarose Resin Slurry for 1 hr at 4 degrees. Each lysate was incubated with 10 μ g of anti-HA antibody (Cell Signaling) and incubated at 4 degrees overnight. To retrieve the complex 20 μ l of Protein A/G agarose beads was incubated with the antibody/lysate sample for 3 hr. The beads were washed three times and eluted with 50 μ l of acidic Elution Buffer provided by the manufacturer. To neutralize the low pH of the buffer, 5 μ l of 1 M Tris pH 9.5 was added.

Metaphase spreads

Assay was performed by the Fox Chase Cancer Center Cytogenetics Core Facility. Briefly, cells were exposed to colcemid (0.01 μ g/ml) for 12 h, harvested with 0.075 M KCl and fixed with methanol-acetic acid (3:1) (Sementino et al., 2018).

Homologous recombination reporter assays

The HR reporter assay was previously described (Wyatt et al., 2016). Briefly, 2×10^6 cells were transfected with 5 μ g pGL3-U6-sgRNA-PGK-puromycin (gift of Xingxu Huang, Addgene # 51133), 5 μ g Flag-Cas9 (gift of Xingxu Huang, Addgene # 44758), 10 μ g HR long donor (Wyatt et al., 2016) and 1 μ g pEGFP-N2 (Clontech) by Neon transfection kit (Invitrogen) using a 1350V, 30 ms pulse in a 100 μ L chamber. The Rosa26-specific sgRNA sequence used here was: 5'-ACTCCAGTCTTTCTAGAAGA. See Table S1 for additional primer sequences. 48 hr post transfection, a portion of the cells were analyzed by flow cytometry to quantify the transfection efficiency, and the remaining cells were harvested for genomic DNA extraction (QIAGEN). Digital PCR was performed to quantify the frequency of gene conversion events using the primers and Taqman probe listed below. The HR signal was normalized to 5000 copies of genomic DNA, measured using a Chromosome 6 control dPCR assay, using primers/probes sequences listed below. Analysis of dPCR data were performed using QuantaSoft (Bio-Rad).

Generation of Brca1^{ΔC} mutant mice

Candidate guide RNAs targeting the CC region of *Brca1* were selected using crispr.mit.edu and tested *in vitro* using Guide-it Complete sgRNA Screening System (Takara), see Table S1 for sequences. The selected guide for the mouse generation was the one that targeted closer to the Leucine 1363 residue, had the highest cut efficiency and low off-target score. The sgRNA was transcribed *in vitro* using the Guide-it Complete sgRNA Screening System (Takara) following manufacture instructions. RNA was purified using the MEGAclear Transcription Clean-Up (Invitrogen). The transcribed RNA together with Alt-R Cas9 (IDT) were resuspended (20 ng/ μ L) in injection buffer (1 mM Tris HCl, pH 7.5; 0.1 mM EDTA) and 1-2 μ L injected in B6C3/F1 single cell 0.5 day embryos (pronuclei). The embryos were transferred to Swiss Webster recipient females (Taconic Tac:SW).

Mouse Genotyping

DNA was extracted using 240 μ L of 50 mM NaOH. Samples were incubated at 97°C degrees for 30 min, followed by addition of 60 μ L of Tris-HCl 1 M pH 8. After centrifugation 1 μ L was used for each PCR reaction. To evaluate *Brca1* status a 2kb fragment was generated using Brca1 Forward and Brca1 Reverse primers (Table S1). Following amplification fragments were incubated at 37°C degrees for 2 hr with the Bpu10I restriction enzyme. The mutant copy sequence displays the restriction site whereas the WT does not. After digestion samples were run on a 1% agarose gel. WT animals showed 1 band, heterozygous 3 bands and homozygous mutant 2 bands. The *Trp53bp1* KO and *Brca1-Δ11* deletion mice were genotyped as previously described (Ward et al., 2003; Xu et al., 2001).

PARPi treatment and peripheral blood analysis

To evaluate tolerability to PARPi, 6 to 8 weeks old, sex matched males and females littermates were randomly selected to receive either 200 mg/kg rucaparib or vehicle orally bi-daily for 5 days. Mice were euthanized in accordance with institutional guidelines. Peripheral blood was also collected from rucaparib and vehicle treated animals and analyzed using an automated analyzer using mouse specific parameters (Abaxis VetScan5).

MEF generation

Mouse embryos (12.5-13.5 days postcoitum) were dissected and internal organs removed. Dissociation was performed by aspirating embryos through a 16-G needle followed by 5-10 min incubation with 0.25% Trypsin at 37°C. The solution was mixed again by trituration and incubated for an additional 5-10 min. After centrifugation cell pellet was resuspended in 75 cm² flasks. Experiments with MEFs were carried out within 3 passages of their derivation to avoid senescence. All MEFs were also cultured in hypoxic incubator prior to being set up for experiments to increase proliferative capacity.

Embryo dissection

Pregnant mice were euthanized 9.5 days postcoitum, uterus was removed and separated from the mesometrium. Individual embryos were dissected under microscope vision (Nikon SMZ1500).

Xenograft studies

MDA-MB-436 cell lines were subcutaneously implanted in 6-8 weeks-old female NSG mice. When tumor size reached between 150 and 180 mm³ animals were randomly selected for rucaparib (Clovis Oncology) or vehicle (PBS) treatment. Rucaparib was administered by oral gavage 200 mg/kg, twice a day for 10 days with a 2-day break after the first 5 days. Tumors were measured twice a week with digital calipers and volume was calculate using the formula (length x width²)/2. When tumors reached 1500 mm³ mice were euthanized in accordance with institutional guidelines.

Histologic and immunohistochemical staining

Slides were deparaffinized with xylene and hydrated with ethanol. Antigen retrieval was performed using Tris/EDTA buffer (DAKO Target Retrieval Solution) and endogenous peroxidase was quenched by immersing slides in 3% hydrogen peroxide solution (30% H₂O₂, Fisher BP2633-500) diluted in methanol. The following primary antibodies were used: BRCA1 N-terminal (MS110 Millipore 1:400), BRCA1 C-terminal (EMD Millipore #07-434, 1:7,500) and 53BP1 (Millipore 1:4000). Antibodies were diluted with D-Vinci Green Diluent (Biocare) and incubated on slides overnight at 4°C in a humidified slide chamber. Slides were then washed and

incubated with EnVision+ System HRP Labeled Polymer Anti-Rabbit or Anti-Mouse for 1 hr at RT. Specimens were washed, then developed with DAB solution (Dako) and counterstained in Meyer's Hematoxylin (Sigma-Aldrich).

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical analysis was performed using GraphPad Prism software. Unpaired t test was used in [Figures 3, 4, 5, and 6](#), one-way ANOVA was used in [Figure 2](#) and two-way ANOVA in [Figure 1](#). Mean and SEM values are shown, $p < 0.05$ was considered statistically significant. Statistically significant p values and number of replicates are indicated in the Figure legends. There were similar variances between statistical groups compared. For the PARPi sensitivity experiments ([Figure 3](#)), 3-5 animals were used for each condition. In xenograft experiments ([Figure 6](#)), 5 animals were used for each condition. Mouse tumor growth curve data were analyzed using linear mixed effects models. For each experiment, the differences in growth rates of log-transformed tumor volumes between vehicle and rucaparib treatments were calculated. We fit linear mixed-effects models with random intercepts and slopes to account for within-mouse correlation, and tested the interaction effects between treatment and time. We assessed whether there was a non-linear effect of time by examining residual plots, and tested models which include a quadratic effect of time for improved fit using likelihood ratio tests. Reported p values are from tests of the interaction terms, interpreted as differences in growth rates between vehicle and rucaparib treatments.

**Amplification of the mutation-carrying BRCA2 allele promotes RAD51 loading and
PARP inhibitor resistance in the absence of reversion mutations.**

Pyoung Hwa Park ^{1#}, Tomomi M. Yamamoto^{2#}, Hua Li¹, Allen L. Alcivar ³, Bing Xia ³,
Yifan Wang⁴, Andrea J. Bernhardt⁴, Kristen M. Turner⁵, Andrew V. Kossenkov¹, Zachary
L. Watson², Kian Behbakht⁶, Silvia Casadei⁷, Elizabeth M. Swisher⁷, Paul S. Mischel⁵,
Neil Johnson⁴, Benjamin G. Bitler^{2*}

¹ Gene Expression and Regulation Program, The Wistar Institute, Philadelphia, PA 19104, USA, ² Division of Reproductive Sciences, The University of Colorado, Aurora, CO 80045, USA, ³ Department of Radiation Oncology, The Cancer Institute of New Jersey, Rutgers University, New Brunswick, NJ 08901, USA, ⁴ Molecular Therapeutics Program, Fox Chase Cancer Center, PA 19111, USA, ⁵ Moores Cancer Center, University of California at San Diego, La Jolla, California 92093, USA, ⁶ Division of Gynecologic Oncology, The University of Colorado, Aurora, CO 80045, USA, ⁷ Department of Ob/Gyn, University of Washington, Seattle, WA 98195, USA

#Authors contributed equally to this work

*Corresponding author:

Benjamin G. Bitler, Ph.D.

12700 East 19th Avenue, MS 8613

Aurora, CO 80045, USA

E-mail: Benjamin.bitler@cuanschutz.edu

Phone: (303) 724-0574

The authors declare no potential conflicts of interest.

Word count – 4,995 and Figures – 6

Running Title: Truncated BRCA2 contributes to PARPi resistance

Abstract

Patients harboring germline Breast Cancer susceptibility genes 1 and 2 (BRCA1/2) mutations are predisposed to developing breast, pancreatic, and ovarian cancers. BRCA2 plays a critical role in homologous recombination DNA repair and deleterious mutations in *BRCA2* confer sensitivity to poly(ADP-ribose) polymerase (PARP) inhibition. Recently, the PARP inhibitors olaparib and rucaparib were FDA approved for the treatment of metastatic breast cancer and recurrent ovarian cancer patients with mutations in BRCA1/2. Despite their initial anti-tumor activity, the development of resistance limits the clinical utility of PARP inhibitor therapy. Multiple resistance mechanisms have been described, including reversion mutations that restore the reading frame of the *BRCA2* gene. In this study, we generated olaparib and rucaparib resistant *BRCA2* mutant Capan1 cell lines. We did not detect secondary reversion mutations in the olaparib or rucaparib resistant clones. Several of the resistant clones had gene duplication and amplification of the mutant *BRCA2* allele, with a corresponding increase in expression of a truncated BRCA2 protein. In addition, homologous recombination (HR)-mediated DNA repair was rescued, as evidenced by the restoration of RAD51 foci formation. Using mass spectrometry, we identified Disruptor Of Telomeric silencing 1-Like (DOT1L), as an interacting partner of truncated BRCA2. RNA-interference-mediated knockdown of *BRCA2* or *DOT1L* was sufficient to re-sensitize cells to olaparib. The results demonstrate that independent of a *BRCA2* reversion mutation amplification of a mutant-carrying *BRCA2* contributes to PARP inhibitor resistance.

INTRODUCTION

Germline mutations in the *BRCA1* or *BRCA2* genes significantly increase an individual's life-time risk of developing breast, prostate, and ovarian cancer [Reviewed in (1)]. The *BRCA1* and *BRCA2* proteins play essential roles in homologous recombination (HR)-mediated repair of DNA double-strand breaks. *BRCA1* has been implicated in DNA end resection as well as *RAD51* loading, whereas *BRCA2* is considered essential for *RAD51* loading onto resected single stranded DNA [reviewed in (2)]. Additionally, both proteins prevent excessive *MRE11*-mediated degradation of DNA replication forks (3). Thus, *BRCA1/2* play critical roles in maintaining genome stability.

Cancers with mutations that disrupt *BRCA1/2* protein activity are highly sensitive to treatment with inhibitors of poly(ADP)-ribose polymerase (PARP). The PARP inhibitors (PARPi) olaparib and rucaparib are now approved for the treatment of *BRCA1/2* wildtype and mutated ovarian cancers and *BRCA1/2*-mutated breast cancers [reviewed in (4)] (5). While not yet approved for pancreatic cancer, a clinical trial reported that olaparib maintenance significantly extended progression free survival (6). However, PARPi resistance poses a significant clinical challenge and is understudied in the context of pancreatic cancer (7). Previously described mechanisms of PARPi resistance include: secondary mutations in the *BRCA2* gene that restore the open-reading frame, increased p-glycoprotein expression, elevated expression of mutated *BRCA1* proteins, stabilization of the replication fork, and loss of DNA end resection inhibitory proteins such as 53BP1 (8-12). Notably, most of these mechanisms have only been demonstrated *in vitro*. Notwithstanding these observations, there are other routes to PARPi resistance.

The Capan1 cell line was derived from a pancreatic adenocarcinoma and harbors a single-base pair deletion in exon 11 (r.6174del) of the *BRCA2* gene, which is

found at an elevated frequency in the Ashkenazi Jewish population and is associated with an increased risk of breast, ovarian, and pancreatic cancers (13-15). Mutations within the region of exon 11 of the *BRCA2* gene result in a premature stop codon. If mRNA is successfully translated, the *BRCA2* protein generated would be predicted to lack the C-terminal DNA binding domain, but retain 7 of the 8 BRC motifs that are needed for RAD51 loading onto DNA (16). Capan1 cells have been characterized as being HR-deficient with low basal levels of RAD51 foci (17). Previous studies have generated cisplatin- and olaparib-resistant Capan1 derivatives and showed that cells acquire secondary reversion mutations that restored the *BRCA2* reading frame and were responsible for generating a functional *BRCA2* protein, capable of promoting HR and therapy resistance (18,19).

In this report, we utilized two independent PARPi to generate multiple Capan1 resistant derivatives. Secondary mutations were not detected in the *BRCA2* gene. Rather, there was a gain in gene copy number of the mutation-carrying *BRCA2* allele that correlated with an increase of a truncated *BRCA2* protein. Knockdown of *BRCA2* re-sensitized resistant Capan1 cells to PARPi. Resistant cells with *BRCA2* amplification had an increase in histone H3 lysine 79 methylation (H3K79Me) and subsequent *BRCA2* knockdown reduced Disruptor of Telomeric silencing 1-like (DOT1L). The findings of this report offer a novel mechanism of PARPi resistance that is mediated through the amplification mutated *BRCA2*.

MATERIALS AND METHODS

Cell culture, and PARP inhibitor resistance establishment

Capan-1 and MDA-MD-231 (positive control for wildtype BRCA2) were purchased from the American Type Culture Collection. TOV-21G (positive control for wildtype BRCA2) from Japanese Cancer Research Resources Bank, and DLD-1 from Horizon (#HD 105-007). Cells were cultured in RPMI1640 with 10% fetal bovine serum and 1% penicillin/streptomycin. Capan-1 olaparib and rucaparib resistant cell line was established by a stepwise exposure to increasing concentrations of olaparib from 15 nM to 128 μ M. The established resistant cells were maintained in 2 μ M olaparib. Cells lines were cultured for a maximum of 8 weeks and monthly tested for mycoplasma using LookOut Mycoplasma PCR based kit (Sigma, cat. # MP0035). Capan-1 and DLD-1 cells were most recently tested on July 2nd 2019 and July 22nd 2019, respectively. Cell lines were authenticated at University of Arizona Genetics Core via small tandem repeat.

Reagents and Antibodies

Olaparib (AZD2281), rucaparib, and pinometostat (EPZ5676) were obtained from Selleckchem. The following antibodies and reagents were obtained from the indicated suppliers: anti-BRCA2 (Bethyl Laboratories, cat. No. A311-267A, 1:5000), anti-BRCA2 ab-1 (Millipore, cat. No. OP95, 1:1000), anti-BRCA2 ab-2 (Millipore, cat. No. CA1033), anti- β -actin (Sigma, cat. No. A1978), anti-GAPDH (Millipore, cat. No. MAB374, 1:20000), anti-DOT1L (Cell Signaling, cat. No.77087), anti-RAD51 (Millipore, cat. No. ABE257 1:500), and anti-BRCA1 (Calbiochem, cat. No. OP92, 1:500), anti-phospho- γ H2AX (Ser139) (Millipore, cat. No. 05-636, 1:400), anti-Vinculin (Cell signaling, cat. No. 13901, 1:1000), anti-H3K79Me (Abcam, cat. No. Ab177185 1:1000) and anti-BrdU (BD Biosciences, cat. No. 347583).

Lentivirus

Lentiviral constructs were packaged using the Virapower Kit and as described previously (20,21). pLKO.1, pLKO.1-shBRCA2 #1 (TRCN0000040194) and pLKO.1-shBRCA2 #2 (TRCN0000040197) were obtained from at the Wistar Institute. shDOT1L #1 (TRCN0000236343) and shDOT1L #2 (TRCN0000236345) were obtained from Functional Genomics Facility at the University of Colorado Denver. Dr. Neil Johnson's laboratory developed pLenti-IRES-GFP-mCherry, pLenti-IRES-GFP-Full Length BRCA2, and pLenti-IRES-GFP-Truncated BRCA2 plasmids. Following pLenti-IRES-GFP transduction GFP positive cells were sorted twice to establish stable cell lines.

Reverse-transcriptase quantitative PCR and RNA Sequencing

RNA was isolated from cells with RNeasy Mini Kit (Qiagen) followed by on-column RNase-free DNase I treatment (Qiagen). *BRCA2* expression (FWD, 5'-GGGAAGCTTCATAAGTCAGTC-3', and REV, 5'-TTTGTAAATGAAGCATCTGATACC-3') was determined using SYBR green 1-step iScript kit (Bio-Rad) on a Bio-Rad Chromo4 machine. β -2-microglobulin (*B2M*) was used as an internal control (FWD, 5'-GGCATTCTGAAGCTGACA-3', and REV, 5'-CTTCAATGTCGGATGGATGAAAC-3'). *DOT1L* expression (FWD, 5'-CACCAGACTGACCAACTCGC-3' and REV, 5'-TCCTAGTTACCTCCAACCTGTGC-3') was determined using Luna universal One-Step RT-qPCR kit (New England Biolabs). Isolated RNA was utilized for global next-generation sequencing (RNA-seq) at The Wistar Genomics Facility. RNA-seq data (GSE86394) was aligned with bowtie2 (22) algorithm and RSEM v1.2.12 software (23) was used to estimate read counts and FPKM values on transcript level using Ensemble transcript information. DESeq algorithm (24) was used to compare two conditions and differences of at least 2 fold that passed False Discovery Rate (FDR)<15% threshold were considered significant. Single nucleotide polymorphisms (SNP) were called using

VarScan2 software and annotated using SnpEff tool (25,26). Results that had $p < 0.001$ by Fisher Exact Test differences and $FDR < 15\%$ between resistant and parental cells were considered significant.

DNA sequencing

Genomic DNA from cell line rucaparib resistant subclones were sequenced with the BROCA-HRV7 targeted sequencing assay as previously described (27). BROCA-HRV includes 81 DNA repair genes. The known *BRCA2* mutation site was sequenced with Sanger sequencing.

Colony formation assay

Cell lines were transduced with pLKO.1-shBRCA2 (#1 or #2), pLKO.1-shDOT1L (#1 or #2) or pLKO.1-control followed by puromycin selection ($1 \mu\text{g/mL}$). Cells were seeded in 24-well plates. Indicated doses of compounds were added and cell medium with compounds was refreshed every three days. Cells were cultured for 12 days. Colonies were fixed with 10% methanol/10% acetic acid. Colonies were stained with 0.5% crystal violet. Colonies were counted using ImageJ Software or dissolved into fixation buffer (10% methanol, 10% acetic acid, PBS) and optical density (570 nm) measured.

Copy number variation assay

Total genomic DNA was isolated from cells with Quick-gDNA Universal Kit (Zymo Research). Copy number variation was determined using pre-designed *BRCA2* primers (Life Technologies, cat. No. 440291) and TaqMan Genotyping Mastermix (Life Technologies). TaqMan Copy Number Reference Assay, Human RNase P (Life Technologies, cat. No. 4403326) was used as an internal control.

Immunoblotting

Cells were collected with sample buffer (2% SDS, 10% glycerol, 62.5mM Tris, pH 8.0, 0.25% Bromophenol Blue, and 100 mM DTT) or RIPA buffer (150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, and 50 mM Tris-HCl pH 8.0) supplemented with Complete protease inhibitor cocktail (Roche). Protein concentrations were measured via BCA kit (Pierce, Cat#23225). Protein samples were separated on SDS-PAGE and transferred on PVDF membrane. Membranes were incubated in primary antibody, followed by either incubating with HRP linked anti-IgG antibodies and detected with chemiluminescent substrate (Thermo Fisher Scientific), or IRDye conjugated anti-IgG antibodies (Li-Cor) and detected with Odyssey digital fluorescence system (Li-Cor).

Histone Extraction

Histones were extracted from cells using the Abcam extraction kit (ab113476) per the manufacturer's protocol.

Immunoprecipitation

Cell pellets were suspended into 500 μ l of Buffer A (10 mM HEPES, 1.5 mM MgCl₂, 10 mM KCl, 0.5 mM DTT, 0.05% NP40, Complete protease inhibitor [Roche] and phosphatase inhibitors [10 mM NaF and 0.2 mM Na₃VO₄], pH 7.9) at 4°C. Using 1 mL syringe passed cell suspension through a 26G needle 10X. Incubate lysate at 4°C for 15 min and centrifuge at 720 x g for 5 min at 4°C. The cell pellet was suspended into 374 μ l of Buffer B (5 mM HEPES, 1.5 mM MgCl₂, 0.2 mM EDTA, 0.5 mM DTT, 26% glycerol (v/v), Complete protease inhibitor and phosphatase inhibitors, pH 7.9) and 26 μ l of 4.6 M NaCl. The pellet was homogenized with disposable pestle and incubated at 4°C for 30 min, and centrifuged at maximum speed (13,000 x g) for 20 min at 4°C. Supernatant was collected, sonicated for 10 sec (Branson Sonifier 250, 20% Duty Cycle, 2 output control), and used for the immunoprecipitation. Protein concentration was measured using a

BCA kit (Pierce, Cat#23225), and 50 µg-75 µg of protein was used for immunoprecipitation.

N-terminus targeting anti-BRCA2 (Bethyl Laboratories, cat. No. A303-434A) or rabbit IgG (Santa Cruz, cat. No. SC-2027) was added to protein samples with Dynabeads protein A (Thermo Fisher Scientific). Samples were incubated on a rotator and the beads were washed with NETN buffer (100 mM NaCl, 20 mM Tris-Cl, pH 8.0, 0.5 mM EDTA, 0.5% NP-40). Proteins were eluted with 1X sample buffer. Eluted samples were separated on SDS-PAGE and transferred on PVDF membrane. Each immunoprecipitation set of samples was incubated with isotype control (IgG), N-terminus targeting anti-BRCA2 antibody (Ab-1, Bethyl Laboratories, cat. No. A303-434A, 1:5000), C-terminus targeting anti-BRCA2 antibody (Ab-2, Bethyl Laboratories, cat. No. A303-435A, 1:5000) or anti-DOT1L antibody (Cell Signaling, 1:1000).

Mass Spectrometry

Capan-1 and Capan-1-OR cells were irradiated at 5 Gy, collected 4 hrs after irradiation, and cells were lysed in ice-cold IP lysed buffer (50 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5% TritonX-100, 150 mM NaCl, Complete protease inhibitor (Roche), 10 mM NaF, 0.2 mM Na₃VO₄). Cells were sheared using a 26G needle. Lysates were incubated on ice for 30 min and were centrifuged at 17,000 x g for 20 min at 4°C. The concentration of the supernatant was determined with a BCA protein and 3.2 mg from Capan-1 or 16 mg from Capan-1-OR cells of total protein were used for immunoprecipitation. After being pre-cleared with 75 µl of protein A beads (NEB, Cat# 91425) that had been pre-equilibrated with IP lysis buffer for 30 min at 4°C, lysates incubated with either 15 µl of 1 mg/ml anti-BRCA2 antibody (Bethyl laboratory Inc., Cat# A303-434A) or control normal Rabbit IgG (1mg/ml, R&D, Cat# AB-105-C) overnight at 4°C. Protein A magnetic beads

were added and incubated for 1 hour at 4°C. Beads were washed with IP lysis buffer 3X for 10 min each at 4°C, and proteins were eluted with 2x Laemmli sample buffer (Bio-rad) and boiled for 10 min. 3 µl of the elution were used for immunoblot. 30 µl were used for 4-20% SDS-PAGE (Bio-Rad) and stained with Imperial Protein Stain (Thermo Fisher). The stained gel was processed by the University of Colorado Biological Mass Spectrometry Core Facility.

Immunofluorescence

Cells were treated with 5 Gy irradiation and incubated for 4 hrs or 3 µM olaparib for 48 hrs prior to immunofluorescence staining. Cells were fixed in 4% paraformaldehyde and permeabilized in 0.5% NP-40. Samples were incubated with antibodies against BRCA1, phospho-γH2AX (Ser139) and RAD51 followed by secondary antibodies (Invitrogen). Slides were mounted with prolong anti-fade reagent (Invitrogen), images were captured on a Nikon Fluorescence microscope and analyzed using NIS-Elements software (Nikon).

Fluorescence in situ hybridization

Capan1 and Capan1-RR cells were arrested in metaphase by colcemid treatment (0.1 µg/ml) for 2 hrs. Cells in metaphase were subsequently collected and incubated in 0.075 M KCl for 10 min, followed by 3 washes in Carnoy's fixative (3:1 methanol: glacial acetic acid). Interphase nuclei and cells in metaphase were dropped onto humidified glass slides, aged overnight, and FISH was performed by immersing slides in ascending ethanol series (70%, 80%, and 100%) for 2 min. *BRCA2* FISH probe (Empire Genomics) was applied to slides, and the cellular DNA and FISH probes were co-denatured at 75°C for 3 min. Hybridization was then carried out overnight at 37°C, followed by washing in 0.4X saline-sodium citrate buffer (SSC) and 2X SSC / 0.1% Tween-20. SSC diluted from

a 20X stock solution (3 M NaCl, 0.3 M sodium citrate, pH 7). Anti-fade mounting medium with DAPI was applied to the slide, and cells in metaphase were imaged with an Olympus BX43 fluorescent microscope equipped with a QIClick camera.

Statistical Analysis

Statistical analysis was performed using GraphPad Prism (Prism 8). Two-tailed student *t*-test or ANOVA were utilized to calculate p-value. All quantitative data are graphed as mean with standard error mean (S.E.M). A calculated p-value of less than 0.05 was considered significant.

RESULTS

Derivation of PARP inhibitor resistant cell lines. To identify mechanisms of PARPi resistance in a pancreatic cell model, we examined Capan1 cells. Capan1 cells have a single-base pair deletion in exon 11 (r.del6174 or 6174delT) of the *BRCA2* gene, which is predicted to produce a protein containing the RAD51 binding sites (BRC-repeats) and lacking the DNA binding domain (Fig. 1A). Examination of The Cancer Genome Atlas detected 35 tumor types and 269 individual tumors with a *BRCA2* mutation beyond the BRC repeats (Table 1). Furthermore, 20 tumor types and 11.8% of tumors with *BRCA2* mutations are predicted to result in a truncated protein lacking the DNA binding domain, which recapitulates the 6174delT mutation (Table 1). To establish resistance, Capan1 cells were incubated with increasing concentrations of either olaparib or rucaparib. Initially, we examined a heterogeneous population of olaparib resistant cells (Capan1-OR-Het). To confirm resistance, colony formation assays on Capan1 and Capan1-OR-Het cells were performed using increasing concentrations of olaparib. We observed the Capan1-OR-Het cells were an average of 743-fold ($p < 0.0001$) more resistant compared to the Capan1 parental population (Fig. 1B-C). Capan1-OR-Het cells were also an average of 1000-fold resistant to rucaparib (Fig. S1A). Colony formation assays revealed changes in proliferation rates between Capan1 and Capan1-OR-Het cells, which was confirmed via cell counting and BrdU incorporation assays (Fig. S1B-D).

PARPi resistance can be associated with cross-resistance to additional DNA damaging agents, including cisplatin (18). We observed that Capan1-OR-Het cells were 110-fold ($p = 0.0036$) more resistant to cisplatin compared to Capan1 (Fig. 1D-E). In contrast, we examined the response of Capan1-OR-Het cells to a microtubule stabilization agent, docetaxel, and found Capan1 and Capan1-OR-Het cells displayed similar sensitivities (Fig. 1F-G). To limit genetic heterogeneity, we established clonal

populations from Capan1-OR-Het cells, referred to as Capan1-OR-1 and -2. Utilizing colony formation assays, we observed Capan1-OR-1 and 2 cells were an average of 387 and 279-fold ($p < 0.0001$ and $p < 0.0001$) resistant to olaparib compared to Capan1 cells (Fig. 1H). Similar results were obtained with Capan1 cells cultured in the presence of an independent PARPi, rucaparib, and seven individual resistant (Capan1-RR) subclones were developed. All of the subclones were observed to be highly resistant to rucaparib, olaparib, and cisplatin (Fig. S1E-G). These data demonstrate that the Capan1 rucaparib and olaparib resistant cells are cross-resistant to other PARPi and platinum-based agents.

Examination of resistant clones for BRCA2 secondary mutations. Previous studies showed that olaparib and cisplatin resistance results from the upregulation of p-glycoprotein or secondary mutations that restore the open-reading frame (ORF) of the *BRCA2* gene (11,18,19). We examined changes in functional efflux activity in Capan1 versus resistant clones. We did not observe a significant difference between Capan1 and resistant clones for the rate of drug efflux based on doxorubicin release assays (Fig. S2A). To further investigate the mechanism of PARPi resistance, we performed next generation sequencing of total RNA (RNA-seq) isolated from parental and Capan1-OR-Het cells (GEO: GSE86394). p-glycoproteins were not differentially upregulated in the Capan1-OR-Het cells compared to the parental cells (Table S1). Examination of *BRCA2* single nucleotide polymorphisms (SNPs) allele frequencies between Capan1-OR-Het and Capan1 cells did not detect any changes suggesting there was not a reversion event (Table S2). Furthermore, Sanger sequencing of DNA from Capan1-OR-1 and -2 for *BRCA2* reversion mutations did not detect reversions in the region of the 6174delT (Fig. S2B). In Capan1-RR cells, we assessed *BRCA2* mutational status by examination of genomic DNA via BROCA-HR testing (27). We detected the original *BRCA2*

(r.del6174) mutation but no other *BRCA2* mutations. Furthermore, there were no new mutations in DNA repair genes in the resistant clones compared to parental cells including *TP53BP1*, *CHD4*, or other genes that might confer resistance (Fig. S2C). The lack of *BRCA2* reversion mutations and efflux activity indicate these mechanisms are not playing a role in PARPi resistance.

Immunoprecipitation analysis of BRCA2. To further ensure a *BRCA2* reversion event had not occurred, we evaluated total *BRCA2* protein levels. We immunoprecipitated (IP) *BRCA2* using antibodies that recognize the epitopes before (Ab-1) and after (Ab-2) the predicted truncation (Fig. S3A). As a control for antibody specificity, we transduced Capan1-OR-2 with two short-hairpins specific for *BRCA2* (sh*BRCA2*). In the Capan1-OR-1 IP, we did not detect full-length *BRCA2*, but observed an increase in a truncated form (Fig. 2A-B and S3B). In the rucaparib resistant sub-clones 8 and 13, Ab-1 was utilized for *BRCA2* IP. We observed that the truncated form of *BRCA2* was IPed from the two sub-clones compared to Capan1, and was only detected by Ab-1, not by Ab-2 (Fig. 2C). In addition, we detected an interaction of the truncated form of *BRCA2* with two mediators of HR, PALB2 and RAD51 (Fig. 2C). These data demonstrate that *BRCA2* reversion was not observed in Capan1 PARPi resistant clones, but there was an increase in truncated *BRCA2* expression, and *BRCA2* interacted with HR proteins.

Amplification of mutated BRCA2. RNA-seq analysis revealed a significant increase in the number of aligned reads to the *BRCA2* gene in the resistant cells compared to Capan1 cells (7585 vs. 1381, Fig. 3A and Table S1). mRNA overexpression was confirmed by quantitative PCR of *BRCA2* in Capan1 parental and resistant (Capan1-OR-Het, -1 and -2) populations (Fig. 3B). In both Capan1-OR and -RR cells, we observed that the clones overexpress the truncated *BRCA2* protein (Fig. 3C and S3C). Not all of the Capan1-RR cells had an increase in *BRCA2* expression, but clones 8 and 13 had the highest

truncated *BRCA2* expression. Genomic localization of differentially expressed genes showed an enrichment of upregulated genes on chromosome 13q (Fig. 3D) suggesting a chromosomal aberration. Given this observation and increase in both mRNA and protein expression, using a PCR based approach we examined changes in the *BRCA2* gene copy number. In the Capan1-OR cells, *BRCA2* gene copy number was significantly increased in Capan1-OR-1 and -2 compared to parental Capan1 cells (9.9 [p=0.0004] and 13.32 [p<0.0001]; Fig. 3E). *BRCA2* copy number gain was also confirmed in Capan1-RR-8 and RR-13 (4.21 [p=0.0024] and 6.05 [p=0.0002]; Fig. 3F). Furthermore, fluorescence in situ hybridization (FISH) on Capan1 and Capan1-RR clones (# 8 and 13) demonstrated multiple *BRCA2*-containing chromosomal rearrangements including, amplification and gene duplication (white arrows, Fig. 3G). These data indicate the truncated *BRCA2* gene is amplified and its resulting protein are overexpressed in PARPi resistant populations.

HR repair is functional in PARPi resistant cells. PARPi resistance has been attributed to a restoration in the HR DNA repair pathway (18). We therefore evaluated the HR repair pathway response by inducing double-strand DNA breaks (DSB) through irradiating (IR; 5 Gy) Capan1 cells and Capan1-OR-1 and -2. To assess the HR repair pathway activity on a single cell level, we performed immunofluorescence analysis following IR to examine the presence of BRCA1 and RAD51 foci. We observed that IR induced a significant increase in BRCA1 foci positive cells in Capan1-OR-1, -2 clonal and heterogeneous populations compared to Capan1 cells (44.8% vs 81.5%, p=0.0317 and 44.8% vs. 79.3%, p=0.0307) (Fig. 4A-B and S4A-B). RAD51 is recruited to DSBs through *BRCA2*'s BRC motifs to facilitate strand invasion during HR repair (28). The formation of RAD51 foci after DNA damage is an output of functional HR repair (29). IR-induced DNA damage led to a significant increase in RAD51 foci-positive cells in

Capan1-OR-1, -2 clonal populations versus Capan1 cells (0% vs 22.0%, $p=0.0099$ and 0% vs. 23.6%, $p=0.0067$) (Fig. 4A-B). Similar results were observed in rucaparib resistant Capan-1 cells (Fig. S4C-D). These data suggest that the HR DNA repair pathway is functional in PARPi resistant Capan1 cells.

Modulation of truncated BRCA2. To directly address the role truncated BRCA2 in mediating HR repair we performed loss-of and gain-of-function studies. In PARPi resistant Capan1 cells, we observed increased expression of truncated BRCA2. Therefore, we assessed the sensitivity of DNA damaging agents after knocking down truncated *BRCA2*. We transduced shControl and two independent shBRCA2 (#1 and #2) into Capan1-OR-1 and observed a varying degree of *BRCA2* knockdown (Fig. 5A-B). shControl and shBRCA2 expressing cells were utilized for colony formation assays with increasing doses of olaparib or cisplatin. We observed that knockdown of *BRCA2* in Capan1-OR-1 cells significantly restored sensitivity to olaparib by 1.7 and 20-fold ($p=0.0027$ and $p<0.0001$) (Fig. 5C). We also confirmed these findings in the Capan1-OR-Het cells (Fig. S5A-B). Next, transduction of two shBRCA2s into Capan1-RR-8 significantly restored rucaparib sensitivity by 7.5 and 44-fold ($p=0.0023$ and $p=0.0015$) (Fig. S5C). Knocking down truncated *BRCA2* significantly sensitized Capan1-OR-1 to cisplatin by 3.9 and 11.7-fold ($p=0.0004$ and $p<0.0001$) (Fig. 5D). There was a concordance of the level of *BRCA2* knockdown and degree of re-sensitization.

For the gain-of-function studies, we were unable to stably transduce full length or truncated *BRCA2* in Capan1 cells. Therefore, we stably overexpressed GFP/HA-tagged full length or truncated *BRCA2* in the DLD-1 *BRCA2*^{-/-} cell line (30). DLD-1 is a colorectal adenocarcinoma cell line that is *BRCA2* wildtype; however, cells were genetically engineered with exon 11 deleted, which results in a loss *BRCA2*^{-/-} cell line (30). Following transduction into DLD-1 *BRCA2*^{-/-} cells, we confirmed expression of both

full length and truncated *BRCA2* (Fig. 5E). As expected, the DLD-1 *BRCA2*^{-/-} expressing the full length *BRCA2* lead to olaparib resistance compared to the DLD-1 *BRCA2*^{-/-} mCherry control cells (765 nM vs. 0.462 nM, respectively) (Fig. 5F). The DLD-1 *BRCA2*^{-/-} expressing the truncated form, however failed to promote olaparib resistance compared to the DLD-1 *BRCA2*^{-/-} mCherry control cells (2.04nM vs. 0.462 nM, respectively) (Fig. 5F). In contrast, knockdown of truncated *BRCA2* in PARPi resistant Capan1 cells led to resensitization, which suggests truncated *BRCA2* in the Capan1 cells is promoting PARPi resistance through a secondary adaptation.

Truncated BRCA2 interactome. To investigate the possibility that an additional adaptation is required to support the ability of truncated *BRCA2* to promote resistance in Capan1 and Capan1-OR-2 cells, we irradiated cells and immunoprecipitated (IP) *BRCA2*. Subsequently, *BRCA2* interacting proteins were identified via mass spectrometry. As a loading control for the IP-mass spectrometry, non-specific tubulin and heat shock proteins were observed at similar levels between Capan1 and Capan1-OR (Table S3). Notably, *BRCA2* was effectively IPed and peptide mapping of *BRCA2*-associated peptides failed to identify any C-terminal peptides confirming the absence of a reversion mutation (Fig. S6A). IP-mass spectrometry data was filtered based on peptide counts from the IgG non-specific pulldown and by the CRAPome prevalence (<10% of all proteomic experiments, (31)). 37 proteins differentially interact with truncated *BRCA2* in Capan1-OR-2 compared to Capan1 cells (Table S3). Similar to Capan1-RR (Fig. 2C), the *BRCA2* pulled down in Capan1-OR cells showed an enrichment of HR proteins, PALB2 and RAD51 (Fig. 6A). Notably, the histone methyltransferase (HMT), DOT1L, and its cofactor, MLLT10, were significantly enriched in the *BRCA2* IP in Capan1-OR-2 cells compared to Capan1 cells (Fig. 6A).

DOT1L functions in a complex with its cofactor, MLLT10, and is the only enzyme known to methylate histone H3 lysine 79 (H3K79Me_{1/2/3}) and is linked to DNA damage repair [reviewed in (32)]. In Mixed Lineage Leukemia (MLL), aberrant DOT1L and MLLT10 activity are responsible for chromosomal rearrangements (33,34). We validated the BRCA2 and DOT1L IP in Capan1-OR-Het cells (Fig. 6B). In Capan1-OR-Het cells, *DOT1L* mRNA expression was only modestly upregulated by 1.3-fold (Fig. 6C). In contrast, DOT1L protein was increased in Capan1-OR-Het cells compared to Capan1 cells, and knockdown of *BRCA2* promoted the loss of DOT1L protein (Fig. 6D). In the DLD-1 model, we evaluated DOT1L and were unable to detect protein expression in either the BRCA2-null cells or in truncated BRCA2 overexpressing cells. However, we subsequently overexpressed *DOT1L* in these cells and DOT1L protein overexpression was only observed in cells expressing truncated BRCA2 (Fig. S6B). These data provide a possible explanation as to why overexpressing truncated BRCA2 in the DLD-1 cell line failed to induce olaparib resistance.

We next wanted to assess the role of DOT1L in Capan1 PARPi resistant cells. A control and two independent shRNAs specific for *DOT1L* were transduced into Capan1-OR-Het cells. Varying levels of DOT1L knockdown were achieved (Fig. 6E) and corresponded with the loss of H3K79Me (Fig. 6F). Notably, DOT1L's enzymatic product was also decreased following *BRCA2* knockdown (Fig. 6F). In the Capan1-OR-Het cells, DOT1L #1 and #2 shRNA-mediated knockdown resensitized cells to olaparib by 2.7 and 6.1-fold, respectively (Fig. 6G). Taken together these data suggests that DOT1L and truncated BRCA2 are potentially cooperating to promote PARPi resistance.

We next determined whether the loss of DOT1L expression (shRNA) or its enzymatic activity (DOT1L inhibitor – pinometostat [EPZ5676] (35)) attenuated HR repair via RAD51 loading. *DOT1L* knockdown or inhibited cells were irradiated with 5 Gy,

incubated for 4 hrs, and RAD51 foci positive cells were quantified. Similarly, *DOT1L* knockdown or inhibited cells were treated with olaparib and RAD51 foci positive cells were quantified. As previously observed in Capan1-OR cells, about 20% of cells were positive for IR-induced RAD51 foci, however in either IR or olaparib treated cells *DOT1L* knockdown significantly reduced RAD51 foci (Fig. 6H-K). Treatment with a DOT1L inhibitor reduced H3K79Me in a dose dependent fashion (Fig. S6C) and H3K79Me was significantly downregulated by 2 μ M pinometostat (Fig. 6L). Doses of pinometostat used are consistent with previous literature (36-38). Treatment with 2 μ M pinometostat in combination with olaparib slightly reduced olaparib's IC50 in Capan1-OR-Het cells (Fig. 6M) and attenuated RAD51 foci formation by an average of 38%, which is a lesser extent than the shRNA knockdowns, 75% reduction in RAD51 foci formation (Fig. 6N-P). These data suggests DOT1L potentially contributes to olaparib resistance by increasing DNA repair and that it could be partially through a methyltransferase independent function.

While PARPi are FDA approved for ovarian cancer they are not currently approved for BRCA-mutated pancreatic cancer. We examined DOT1L expression using the ovarian cancer TCGA data. Increased expression *DOT1L* conveyed a worse progression-free survival and was associated with resistance to the DNA damaging platinum-based chemotherapy (Fig. S6D-E). Moreover, in a previously published BRCA2-mutated PARPi resistant model of ovarian cancer (39), *DOT1L* knockdown significantly inhibited colony formation (Fig. S6F-H). These data highlight that targeting DOT1L in PARPi resistant tumors could be more broadly applicable.

DISCUSSION

PARPi (olaparib and rucaparib) have entered the clinic for the treatment of *BRCA1/2*-mutated cancers; however, the development of resistance remains a significant clinical challenge and elucidating resistance mechanisms is critical. Therefore, we established olaparib and rucaparib resistant cells in the context of a mutated form of *BRCA2*. PARPi resistant cells had an amplification of the truncated form of *BRCA2*, which led to an increase in mRNA and protein expression. Truncated *BRCA2* was observed to interact with DNA damage repair effectors, RAD51 and DOT1L. Subsequently, knockdown of truncated *BRCA2* restored sensitivity to PARPi.

In contrast to previous reports, we did not detect *BRCA2* reversion mutations, but similarly found that HR DNA repair had been restored in resistant cells (18,19). In the original report describing olaparib-resistant Capan1 cells the authors utilized two methods for generation of resistant cells: a step-wise increase in olaparib (1 nM to 50 μ M) or a constant concentration (100 nM). While reversion mutations were observed under both conditions authors noted that only with the step-wise method did they observe amplified *BRCA2* (18). Comparatively, in our study we used the step-wise method for olaparib with a higher maximum dose, suggesting that the degree of selective pressure likely mediates an alternative resistance mechanism.

In the clinical setting, several studies have detected secondary reversion mutations of *BRCA1/2*. A small study examining sixteen germline *BRCA1/2* matched primary and recurrent high grade serous ovarian cancer patients detected five recurrent tumors with reversion mutations. All five patients were treated with platinum-based chemotherapy and three of the five patients had been treated with PARP inhibitors (40). The therapeutic agent (platinum-based chemotherapy or PARP inhibitors) driving the observed reversion mutations is not clear. These data suggest that although reversion

mutations occur, other mechanisms are also likely playing a role in the development of systemic resistance.

In Capan1 PARPi resistant cells *BRCA2* knockdown led to olaparib and rucaparib re-sensitization. Given the significant increase in *BRCA2* copy number and significant upregulation of truncated *BRCA2*, we further evaluated expression of truncated *BRCA2* in an independent *BRCA2*^{-/-} cell line. Overexpression of truncated *BRCA2* was not sufficient to promote resistance suggesting that in the context of amplified truncated *BRCA2*, Capan1 cells have potentially acquired a second adaptation to facilitate the restoration of HR repair.

We demonstrated that the HR DNA repair pathway was restored in the PARP inhibitor resistant cells measured *via* *BRCA1* and *RAD51* foci formation. These findings are consistent with previous reports showing the *BRCA2* protein-containing BRC repeats and lacking the C-terminal domain is sufficient to interact with *RAD51* (41). Through *BRCA2* pull-down and mass spectrometry, we identified *DOT1L* as a *BRCA2* associated protein. The histone methyltransferase, *DOT1L*, directly interacts with DNA and promotes methylation on H3K79 and is the only known methyltransferase to catalyze H3K79. *DOT1L* is an established mediator of cell cycle regulation and DNA double strand break repair [reviewed in (32)]. In mixed-lineage leukemia, *DOT1L* contributes to chromosomal rearrangements and fusions, which suggest that *DOT1L* could be playing an active role in the chromosomal instability in the Capan-1 PARPi resistant cells (Fig. 2H). To-date the only known “reader” of the *DOT1L*-dependent H3K79 methylation is the tumor suppressor p53-binding protein 1, which has established roles in dictating specific DNA repair pathways (42). The *DOT1L* co-factor, *MLLT10*, was also observed in the *BRCA2* pull-down. Interestingly, knockdown of *DOT1L* promoted PARPi resensitization to a greater degree than inhibiting *DOT1L* methyltransferase activity. We plan to

determine if DOT1L has methyltransferase independent functions that mediated PARPi resistance. In our study, we predict that truncated BRCA2 and DOT1L are interacting to increase RAD51 loading, HR-mediated DNA damage repair, and PARPi resistance. Future work will investigate the relationship between truncated BRCA2 and DOT1L interaction in DNA damage response and potentially extend our findings into BRCA-wildtype tumors.

ACKNOWLEDGEMENTS

We acknowledge Dr. Rugang Zhang for his invaluable support. We acknowledge Lindsay J. Wheeler and Scott H. Kaufman for their critical discussion. This work was supported by grants from the NIH/NCI to N. Johnson (R01CA214799) and B.G. Bitler (R00CA194318), and Department of Defense Awards to N. Johnson (OC140040/OC130212) and B.G. Bitler (OC170228). Research supported by a Stand Up To Cancer-Ovarian Cancer Research Fund Alliance-National Ovarian Cancer Coalition Dream Team Translational Cancer Research Grant to E.M. Swisher (SU2C-AACR-DT16-15). Stand Up To Cancer is a division of the Entertainment Industry Foundation. Research grants are administered by the American Association for Cancer Research, the Scientific Partner of SU2C. Support of Core Facilities was provided by The Wistar Institute Cancer Center Support Grant CA010815 to and the University of Colorado Cancer Center Support Grant (P30CA046934).

REFERENCES

1. Narod SA. Modifiers of risk of hereditary breast and ovarian cancer. *Nat Rev Cancer* **2002**;2:113-23
2. Gudmundsdottir K, Ashworth A. The roles of BRCA1 and BRCA2 and associated proteins in the maintenance of genomic stability. *Oncogene* **2006**;25:5864-74
3. Ying S, Hamdy FC, Helleday T. Mre11-dependent degradation of stalled DNA replication forks is prevented by BRCA2 and PARP1. *Cancer Res* **2012**;72:2814-21
4. Bitler BG, Watson ZL, Wheeler LJ, Behbakht K. PARP inhibitors: Clinical utility and possibilities of overcoming resistance. *Gynecol Oncol* **2017**;147:695-704
5. Robson M, Im SA, Senkus E, Xu B, Domchek SM, Masuda N, *et al.* Olaparib for Metastatic Breast Cancer in Patients with a Germline BRCA Mutation. *N Engl J Med* **2017**;377:523-33
6. Golan T, Hammel P, Reni M, Van Cutsem E, Macarulla T, Hall MJ, *et al.* Maintenance Olaparib for Germline BRCA-Mutated Metastatic Pancreatic Cancer. *N Engl J Med* **2019**;381:317-27
7. Lord CJ, Ashworth A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat Med* **2013**;19:1381-8
8. Barber LJ, Sandhu S, Chen L, Campbell J, Kozarewa I, Fenwick K, *et al.* Secondary mutations in BRCA2 associated with clinical resistance to a PARP inhibitor. *J Pathol* **2013**;229:422-9
9. Jaspers JE, Kersbergen A, Boon U, Sol W, van Deemter L, Zander SA, *et al.* Loss of 53BP1 causes PARP inhibitor resistance in Brca1-mutated mouse mammary tumors. *Cancer Discov* **2013**;3:68-81
10. Ray Chaudhuri A, Callen E, Ding X, Gogola E, Duarte AA, Lee JE, *et al.* Replication fork stability confers chemoresistance in BRCA-deficient cells. *Nature* **2016**;535:382-7
11. Rottenberg S, Jaspers JE, Kersbergen A, van der Burg E, Nygren AO, Zander SA, *et al.* High sensitivity of BRCA1-deficient mammary tumors to the PARP inhibitor AZD2281 alone and in combination with platinum drugs. *Proc Natl Acad Sci U S A* **2008**;105:17079-84
12. Wang Y, Kraus JJ, Bernhardt AJ, Nicolas E, Cai KQ, Harrell MI, *et al.* RING domain-deficient BRCA1 promotes PARP inhibitor and platinum resistance. *J Clin Invest* **2016**;126:3145-57
13. Ferrone CR, Levine DA, Tang LH, Allen PJ, Jarnagin W, Brennan MF, *et al.* BRCA germline mutations in Jewish patients with pancreatic adenocarcinoma. *J Clin Oncol* **2009**;27:433-8
14. Moslehi R, Chu W, Karlan B, Fishman D, Risch H, Fields A, *et al.* BRCA1 and BRCA2 mutation analysis of 208 Ashkenazi Jewish women with ovarian cancer. *Am J Hum Genet* **2000**;66:1259-72
15. Struwing JP, Hartge P, Wacholder S, Baker SM, Berlin M, McAdams M, *et al.* The risk of cancer associated with specific mutations of BRCA1 and BRCA2 among Ashkenazi Jews. *N Engl J Med* **1997**;336:1401-8
16. Wong AK, Pero R, Ormonde PA, Tavtigian SV, Bartel PL. RAD51 interacts with the evolutionarily conserved BRC motifs in the human breast cancer susceptibility gene brca2. *J Biol Chem* **1997**;272:31941-4
17. Yuan SS, Lee SY, Chen G, Song M, Tomlinson GE, Lee EY. BRCA2 is required for ionizing radiation-induced assembly of Rad51 complex in vivo. *Cancer Res* **1999**;59:3547-51

18. Edwards SL, Brough R, Lord CJ, Natrajan R, Vatcheva R, Levine DA, *et al.* Resistance to therapy caused by intragenic deletion in BRCA2. *Nature* **2008**;451:1111-5
19. Sakai W, Swisher EM, Karlan BY, Agarwal MK, Higgins J, Friedman C, *et al.* Secondary mutations as a mechanism of cisplatin resistance in BRCA2-mutated cancers. *Nature* **2008**;451:1116-20
20. Bitler BG, Nicodemus JP, Li H, Cai Q, Wu H, Hua X, *et al.* Wnt5a Suppresses Epithelial Ovarian Cancer by Promoting Cellular Senescence. *Cancer Res* **2011**
21. Ye X, Zerlanko B, Kennedy A, Banumathy G, Zhang R, Adams PD. Downregulation of Wnt signaling is a trigger for formation of facultative heterochromatin and onset of cell senescence in primary human cells. *Mol Cell* **2007**;27:183-96
22. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **2012**;9:357-9
23. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **2011**;12:323
24. Anders S, Huber W. Differential expression analysis for sequence count data. *Genome Biol* **2010**;11:R106
25. Cingolani P, Platts A, Wang le L, Coon M, Nguyen T, Wang L, *et al.* A program for annotating and predicting the effects of single nucleotide polymorphisms, SnpEff: SNPs in the genome of *Drosophila melanogaster* strain w1118; iso-2; iso-3. *Fly (Austin)* **2012**;6:80-92
26. Koboldt DC, Zhang Q, Larson DE, Shen D, McLellan MD, Lin L, *et al.* VarScan 2: somatic mutation and copy number alteration discovery in cancer by exome sequencing. *Genome Res* **2012**;22:568-76
27. Norquist BM, Brady MF, Harrell MI, Walsh T, Lee MK, Gulsuner S, *et al.* Mutations in Homologous Recombination Genes and Outcomes in Ovarian Carcinoma Patients in GOG 218: An NRG Oncology/Gynecologic Oncology Group Study. *Clin Cancer Res* **2018**;24:777-83
28. Milne GT, Weaver DT. Dominant negative alleles of RAD52 reveal a DNA repair/recombination complex including Rad51 and Rad52. *Genes Dev* **1993**;7:1755-65
29. Graeser M, McCarthy A, Lord CJ, Savage K, Hills M, Salter J, *et al.* A marker of homologous recombination predicts pathologic complete response to neoadjuvant chemotherapy in primary breast cancer. *Clin Cancer Res* **2010**;16:6159-68
30. Hucl T, Rago C, Gallmeier E, Brody JR, Gorospe M, Kern SE. A syngeneic variance library for functional annotation of human variation: application to BRCA2. *Cancer Res* **2008**;68:5023-30
31. Mellacheruvu D, Wright Z, Couzens AL, Lambert JP, St-Denis NA, Li T, *et al.* The CRAPome: a contaminant repository for affinity purification-mass spectrometry data. *Nat Methods* **2013**;10:730-6
32. Wood K, Tellier M, Murphy S. DOT1L and H3K79 Methylation in Transcription and Genomic Stability. *Biomolecules* **2018**;8
33. Bernt KM, Zhu N, Sinha AU, Vempati S, Faber J, Krivtsov AV, *et al.* MLL-rearranged leukemia is dependent on aberrant H3K79 methylation by DOT1L. *Cancer Cell* **2011**;20:66-78
34. Monroe SC, Jo SY, Sanders DS, Basrur V, Elenitoba-Johnson KS, Slany RK, *et al.* MLL-AF9 and MLL-ENL alter the dynamic association of transcriptional regulators with genes critical for leukemia. *Exp Hematol* **2011**;39:77-86 e1-5

35. Daigle SR, Olhava EJ, Therkelsen CA, Basavapathruni A, Jin L, Boriack-Sjodin PA, *et al.* Potent inhibition of DOT1L as treatment of MLL-fusion leukemia. *Blood* **2013**;122:1017-25
36. Godfrey L, Crump NT, Thorne R, Lau IJ, Repapi E, Dimou D, *et al.* DOT1L inhibition reveals a distinct subset of enhancers dependent on H3K79 methylation. *Nat Commun* **2019**;10:2803
37. Kari V, Raul SK, Henck JM, Kitz J, Kramer F, Kosinsky RL, *et al.* The histone methyltransferase DOT1L is required for proper DNA damage response, DNA repair, and modulates chemotherapy responsiveness. *Clin Epigenetics* **2019**;11:4
38. Zhang W, Zhao C, Zhao J, Zhu Y, Weng X, Chen Q, *et al.* Inactivation of PBX3 and HOXA9 by down-regulating H3K79 methylation represses NPM1-mutated leukemic cell survival. *Theranostics* **2018**;8:4359-71
39. Yamamoto TM, McMellen A, Watson ZL, Aguilera J, Ferguson R, Nurmemmedov E, *et al.* Activation of Wnt signaling promotes olaparib resistant ovarian cancer. *Mol Carcinog* **2019**
40. Christie ELF, S.; Doig, K.; Pattnaik, S.; Dawson, S.J.; Bowtell, D.D. Reversion of BRCA1/2 Germline Mutations Detected in Circulating Tumor DNA From Patients With High-Grade Serous Ovarian Cancer. *J Clin Oncol* **2017**
41. Shivji MK, Davies OR, Savill JM, Bates DL, Pellegrini L, Venkitaraman AR. A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange. *Nucleic Acids Res* **2006**;34:4000-11
42. Huyen Y, Zgheib O, Ditullio RA, Jr., Gorgoulis VG, Zacharatos P, Petty TJ, *et al.* Methylated lysine 79 of histone H3 targets 53BP1 to DNA double-strand breaks. *Nature* **2004**;432:406-11

TABLES

Total Patients with <i>BRCA2</i> Mutation	# of Cancer Types Examined	Mutations beyond BRC Repeats		Mutations Predicted to Result in Truncated <i>BRCA2</i> with intact BRC repeats	
		# of patients (%)	# of Cancer Types	# of patients (%)	# of Cancer Types
458	45	269/458 (58.7)	35	54/458 (11.8)	20

Table 1. Examination of *BRCA2* mutations in The Cancer Genome Atlas. BRC repeats = RAD51 binding domains.

FIGURE LEGENDS

Figure 1. PARP inhibitor resistant cells are cross resistant to DNA damaging agent. **A)** The Capan1 pancreatic adenocarcinoma cell line has a deletion at position 6174. **B)** Capan1 and heterogeneous population of olaparib resistant Capan1 (Capan1-OR-Het) cells were plated on 24-well plates, treated with indicated doses of olaparib, and subjected to colony formation after 12 days. Representative images of colony formation are shown. **C)** Dose response curve was calculated with Capan1 (gray) and Capan1-OR-Het (black). Calculated IC50 values with 95% Confidence Intervals (CI) are indicated. **D)** Same as B, but examined the sensitivity to a DNA damaging agent, cisplatin, in Capan1 and Capan1-OR-Het cells. Cells were treated with indicated doses. Representative images of foci formation assay are shown. **E)** Same as C, but a cisplatin dose response curve for Capan1 (gray) and Capan1-OR-Het (black) was calculated. **F)** Same as B, but sensitivity to a microtubule stabilization agent, docetaxel, was assessed in Capan1 and Capan1-OR-Het cells. Cells were treated with indicated doses. Representative images of foci formation assay are shown. **G)** Same as C, but a

docetaxel dose response curve for Capan1 (gray) and Capan1-OR-Het (black) was calculated. **H)** Same as C, but clonal olaparib resistant populations (Capan1-OR-1 [squares] and -2 [triangles]) were isolated and examined for olaparib sensitivity compared to Capan1 cells (circles). Colonies were counted with ImageJ software. Data is representative of 3 independent experiments. Error bars = S.E.M.

Figure 2. PARP resistant cells express truncated BRCA2. **A)** Capan1-OR-1 cells were transduced with shControl and shBRCA2 (#1 and #2). Protein was extracted from Capan1, Capan1-OR-1 (shControl, shBRCA2 #1 and #2) and BRCA2-wildtype control (TOV-21G) cells. Protein was used for immunoprecipitation (IP) against isotype control (IgG) and BRCA2 (N-terminus, Ab-1). IPed protein was used for immunoblot against BRCA2. **B)** Same as A, but IP against BRCA2 (C-terminus, Ab-2). **C)** Nuclear protein was extracted from Capan1, Capan1-RR clones 8 and 13, and a BRCA2-wildtype control. Protein was used for IP against BRCA2 (N-terminus, Ab-1). IP protein was used for immunoblot against BRCA2 (Ab-1 and Ab-2), RAD51, and PALB2.

Figure 3. Truncated BRCA2 is overexpressed in CAPAN1 PARP inhibitor resistant cells. **A)** Total RNA was isolated from Capan1 and Capan1-OR-Het cells and subsequently used for next generation sequencing (RNA-seq). Aligned-reads for the *BRCA2* gene are shown for Capan1 (parental, blue) and Capan1-OR-Het (resistant, red) cells. **B)** RNA was isolated from Capan1, Capan1-OR-Het, Capan1-OR-1 and 2 and used for qPCR against *BRCA2* (*B2M* = internal control). ANOVA, **p*=0.0068, ***p*=0.007 and ****p*=0.0027. **C)** Protein was extracted from Capan1, Capan1-OR-Het, Capan1-OR-1 and 2, and BRCA2-wildtype control (TOV-21G) cells. Protein was immunoblotted

against BRCA2 (N-terminus). Arrows indicate full-length and truncated BRCA2. β -actin = loading control. **D)** RNA-seq analysis of Capan1 versus Capan1-OR-Het detected 411 differentially expressed genes ($|\text{Fold Change}| > 2$ and False Discovery Rate [FDR] < 0.15). Differentially regulated genes were mapped based on genomic location. **E)** Genomic DNA was isolated from Capan1, CAPAN1-OR-1 and 2 and used for qPCR on *BRCA2* intron-exon junction to determine changes in gene copy number. RNase P = internal control. ANOVA, $*p=0.0004$ and $**p<0.0001$. **F)** Genomic DNA was isolated from Capan1, Capan1-RR-8 and 13 and utilized for qPCR *BRCA2* to determine changes in gene copy number. RNase P = internal control. ANOVA, $**p=0.0024$ and $***p=0.0002$. **G)** Fluorescence in situ hybridization against *BRCA2* (green) in Capan1 and Capan1-RR-8 and -13 cells. Chromosomes = DAPI/blue. White arrows = positive *BRCA2* regions. ? = possible extrachromosomal DNA. Data is representative of 3 independent experiments. Error bars = S.E.M.

Figure 4. Irradiated PARP resistant cells demonstrate restored homologous recombination DNA repair. **A)** Capan1 (parental) and Capan1-OR-1 cells were irradiated (5 Gy) and used for immunofluorescence against BRCA1 (red) and RAD51 (green). White arrowheads indicate BRCA1 and RAD51 foci. **B)** Quantified BRCA1 and RAD51 foci in 200 Capan1, Capan1-OR 1 or 2 cells treated without (-IR) or with (+IR) 5 Gy and graphed as a percentage. Statistical test = ANOVA, $*p < 0.05$. Data is representative of at least 3 independent experiments. Error bars = S.E.M.

Figure 5. BRCA2 knockdown in CAPAN1-PR cells restores sensitivity to DNA damaging agents. **A)** Capan1-OR-1 cells were transduced with shControl or shBRCA2

(#1 or #2). After drug selection RNA was collected and followed by quantitative PCR for *BRCA2*. ANOVA, * $p < 0.001$. **B)** Same as A, but protein was extracted and immunoblotted for *BRCA2*. GAPDH = loading control. **C)** Capan1, Capan1-OR-1 shCtrl, or Capan1-OR-1 sh*BRCA2* (#1 and #2) cells were plated in 24-well plates, treated with increasing doses of olaparib, and cultured for 12 days. Cells were then fixed and used for colony formation assays. Dose response curve shown with indicated IC50 values. **D)** Same as C, but cells were treated with cisplatin. Dose response curve shown with indicated IC50 values. **E)** DLD-1 *BRCA2*^{-/-} cells were transduced with mCherry or GFP/HA-tagged full length (*BRCA2*-HA) or truncated *BRCA2* (*BRCA2*(del6174T)-HA). RNA was isolated and *BRCA2* expression was measured via qPCR. ANOVA, *** $p < 0.001$. **F)** Same as E, but cells were used for a colony formation assay with increasing doses of olaparib. FL = full length. Colonies were counted with ImageJ software. Data is representative of 3 independent experiments. Error bars = S.E.M.

Figure 6. *BRCA2* interacts with *DOT1L* and *DOT1L* contributes to olaparib

resistance. A) Protein from irradiated (5 Gy, 4 hrs) Capan1-OR 1 cells was used for an immunoprecipitation (IP) against *BRCA2* (Ab-1) and isotype control (Rb IgG). IPed protein was separated on a SDS-PAGE and used for mass spectrometry. Peptide count table of Capan1-OR-1 and Capan1 cells. **B)** Capan1 and Capan1-OR-Het cells used for IP against *BRCA2* (Ab-1) and isotype control (Rb IgG). IPed protein was separated on a SDS-PAGE and immunoblotted for *DOT1L* and *BRCA2*. **C)** RNA extracted from Capan1 and Capan1-OR-Het was used for qPCR against *DOT1L*. Internal control = *B2M*. Two-sided t-test, *** $p < 0.001$. **D)** Protein from Capan1, Capan1-OR-Het shControl or sh*BRCA2* (#1 and #2) was separated on a SDS-PAGE and immunoblotted for *DOT1L*. Loading control = β -actin. **E)** Capan1-OR-Het cells were transduced with a control

shRNA (shCtrl) and two *DOT1L* specific-shRNAs (#1 and #2). RNA was extracted from cells and used for qPCR against *DOT1L*. Internal control = *B2M*. ANOVA, *** $p < 0.001$.

F) Total histones were extracted and immunoblotted for H3K79Me. Loading control = Histone H3 (H3). **G)** Capan1-OR-Het shCtrl and shDOTL1 (#1 and #2) cells were used for an olaparib dose response colony formation. IC50 were calculated and graphed. ANOVA, * $p < 0.05$. **H)** Immunofluorescence (IF) on non-irradiated (-IR) and irradiated (5 Gy, +IR) Capan1-OR-Het cells against γ H2Ax (green) and RAD51 (red). White arrowheads = RAD51-foci positive cell. **I)** Same as I, but examined Capan1-OR-Het shDOT1L #1 and #2 cells. **J)** Quantification of RAD51-foci positive cells of H and I. At least 200 cells were counted in triplicate. ANOVA, *** $p < 0.001$. **K)** Capan1-OR-Het shCtrl and shDOTL1 (#1 and #2) cells incubated with olaparib [3 μ M for 48 hrs] used for IF against RAD51. At least 200 cells were counted in triplicate. ANOVA, *** $p < 0.001$. **L)** Capan1-OR-Het cells treated with vehicle control (Ctrl) or 2 μ M pinometostat (Pino). Histones were extracted and immunoblotted for H3K79Me. Loading control = Histone H3. **M)** Capan1-OR-Het shCtrl cells were treated with vehicle control or 2 μ M pinometostat and cells were used for an olaparib dose response colony formation. IC50 were calculated and graphed. P-value calculated with a two-sided t-test. **N)** Non-irradiated (-IR) and irradiated (5 Gy, +IR) Capan1-OR-Het cells treated with pinometostat [2 μ M]. IF against γ H2Ax (green) and RAD51 (red). White arrowheads = RAD51-foci positive cell. **O)** Quantification of RAD51-foci positive cells. At least 200 cells were counted in triplicate. ANOVA, ** $p < 0.01$. **P)** Capan1-OR-Het shCtrl cells were treated with vehicle control or 2 μ M pinometostat followed by olaparib [3 μ M for 48 hrs] used for IF against RAD51. Quantification of RAD51-foci positive cells. At least 200 cells were counted in triplicate. ANOVA, *** $p < 0.001$. Data is representative of 3 independent

experiments. Colony formation was quantified by dissolving crystal violet. Error bars = S.E.M.

Figure 1, Park et al 2019

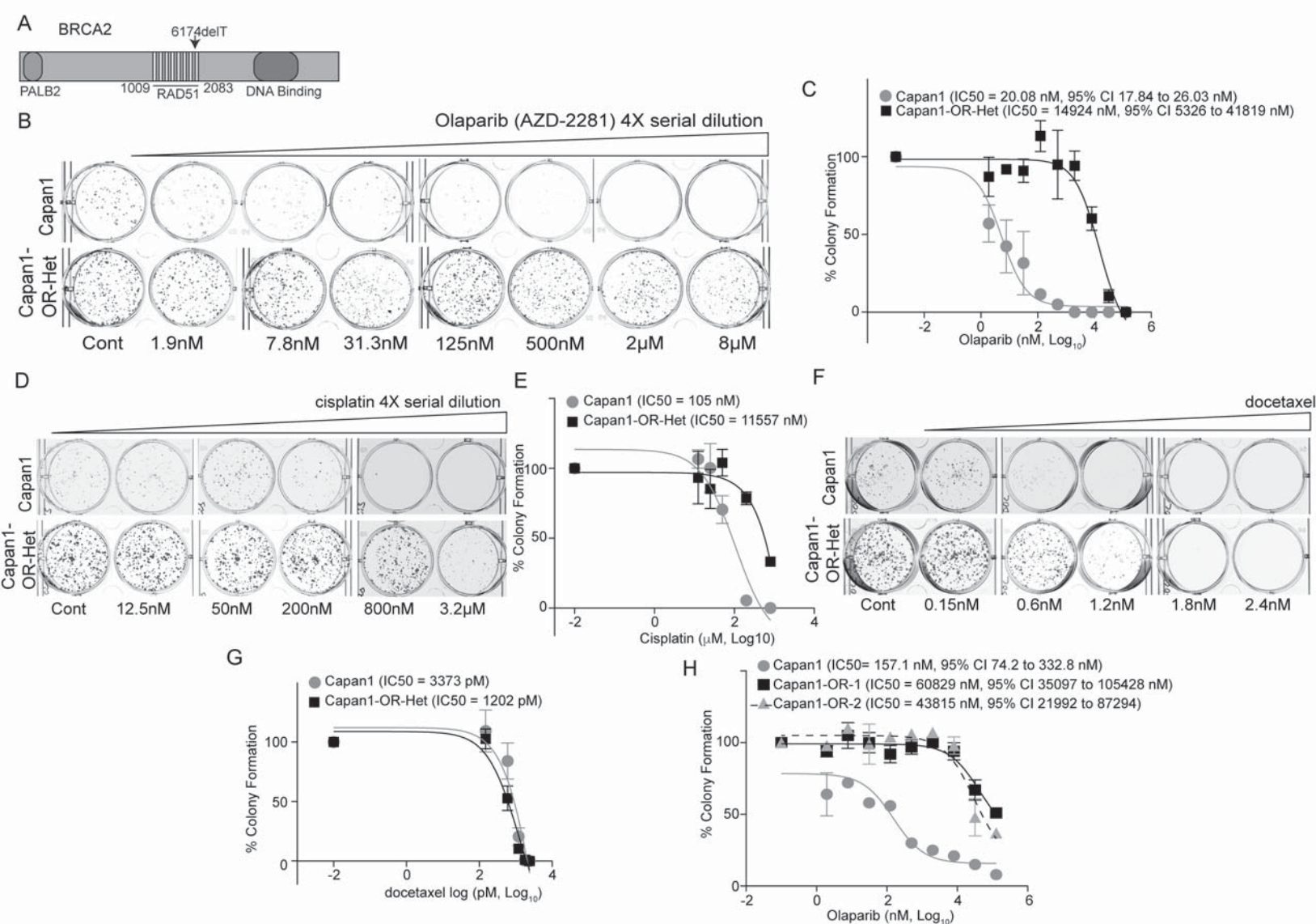
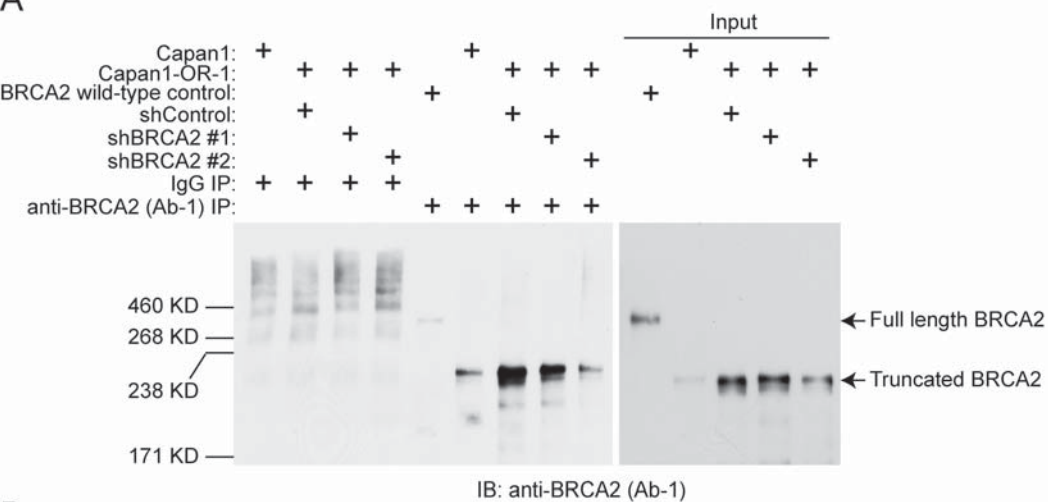
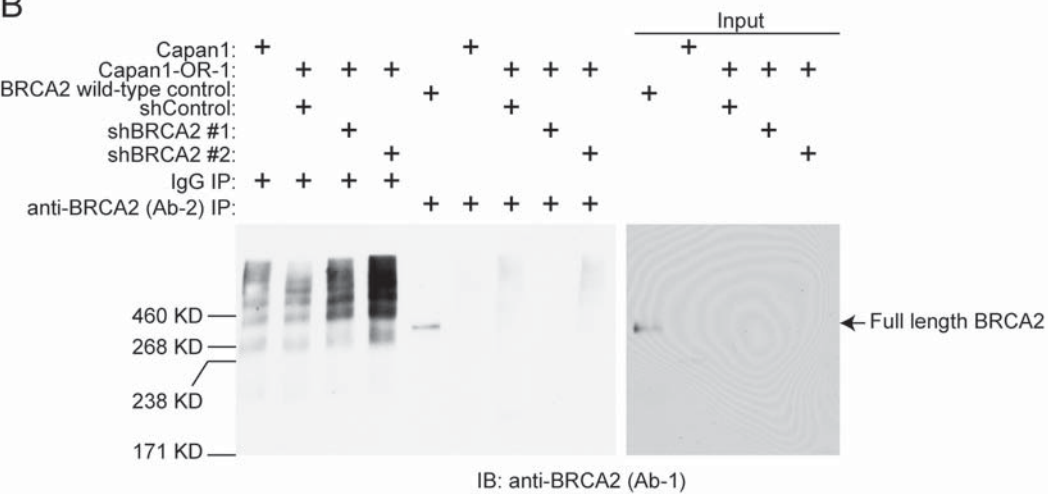


Figure 2, Park and Yamamoto et al 2019

A



B



C

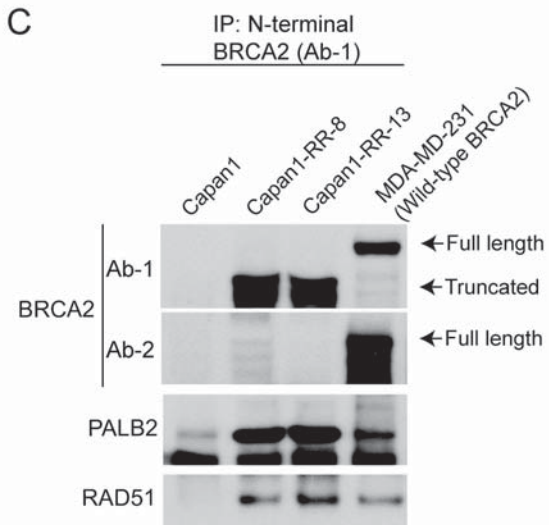


Figure 3, Park and Yamamoto et al 2019

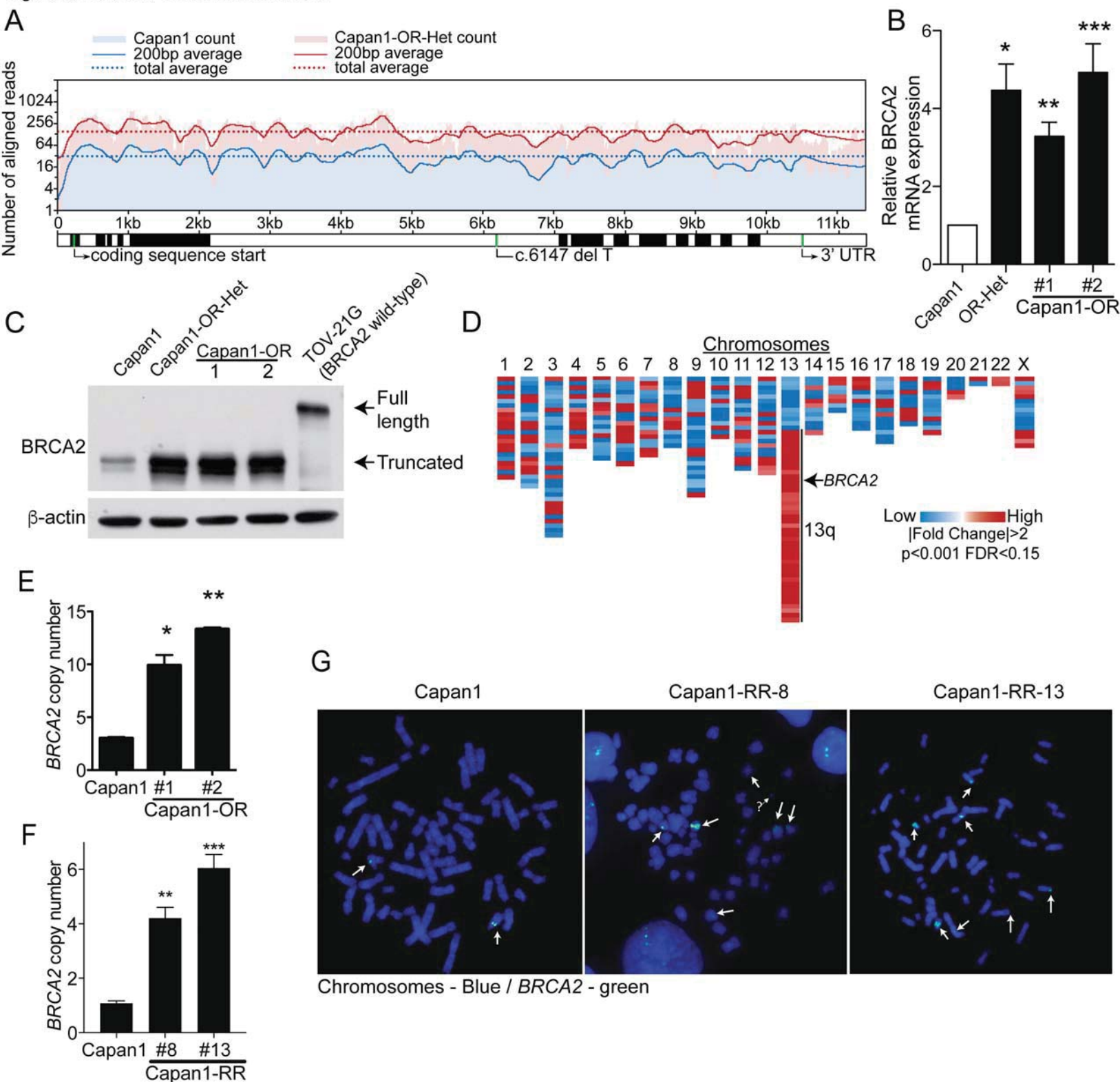


Figure 4, Park and Yamamoto et al, 2019

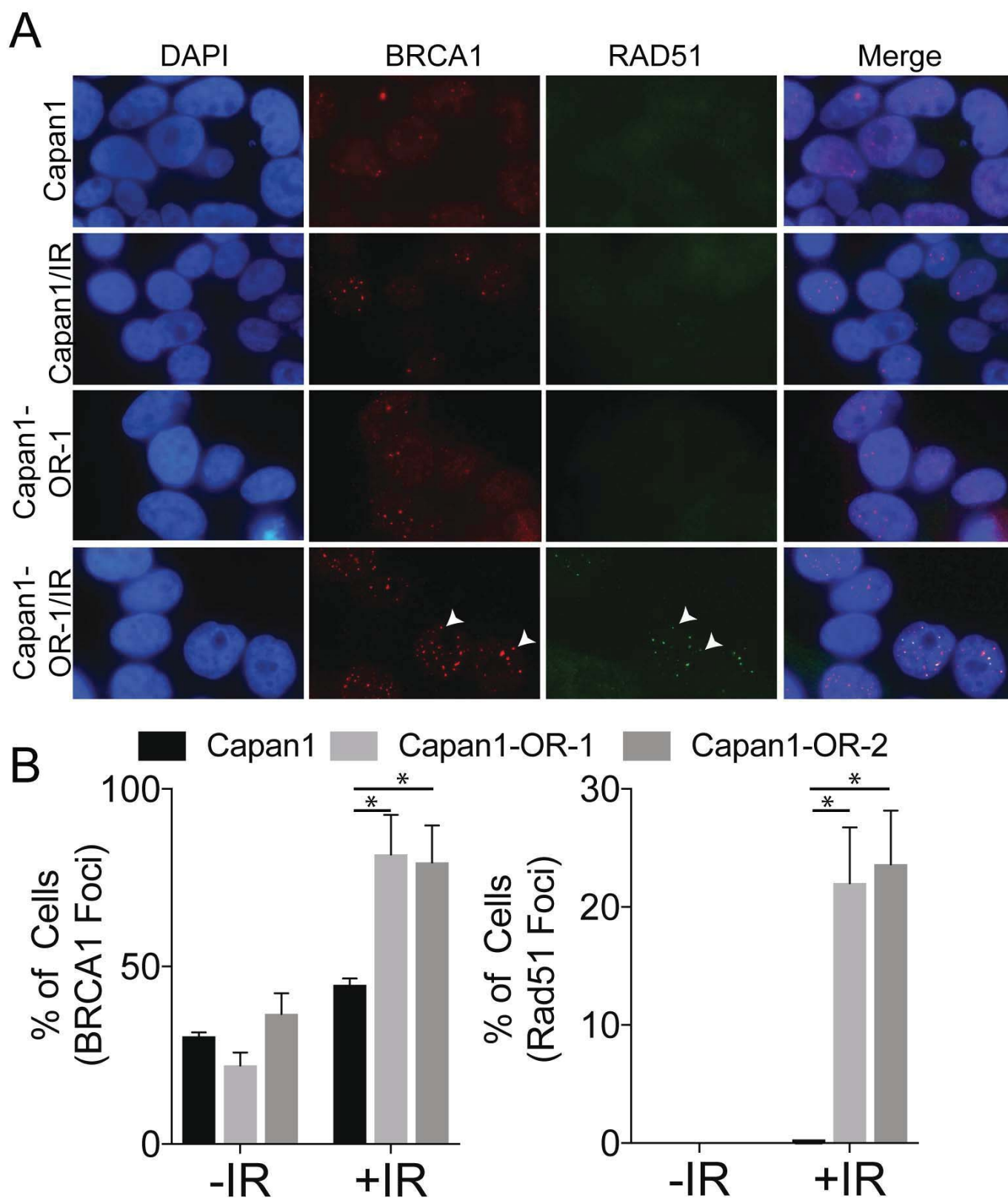


Figure 5, Park and Yamamoto et al 2019

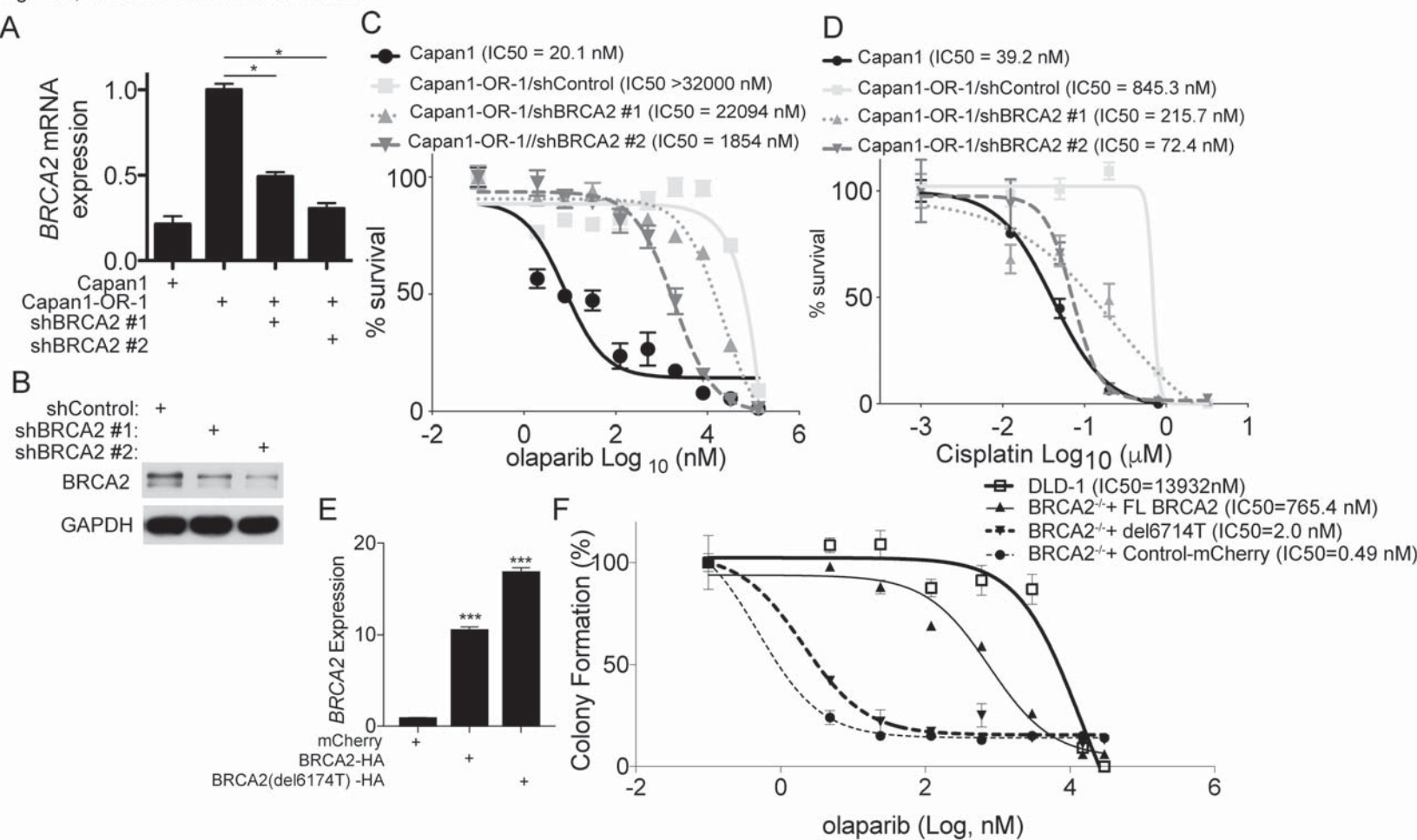
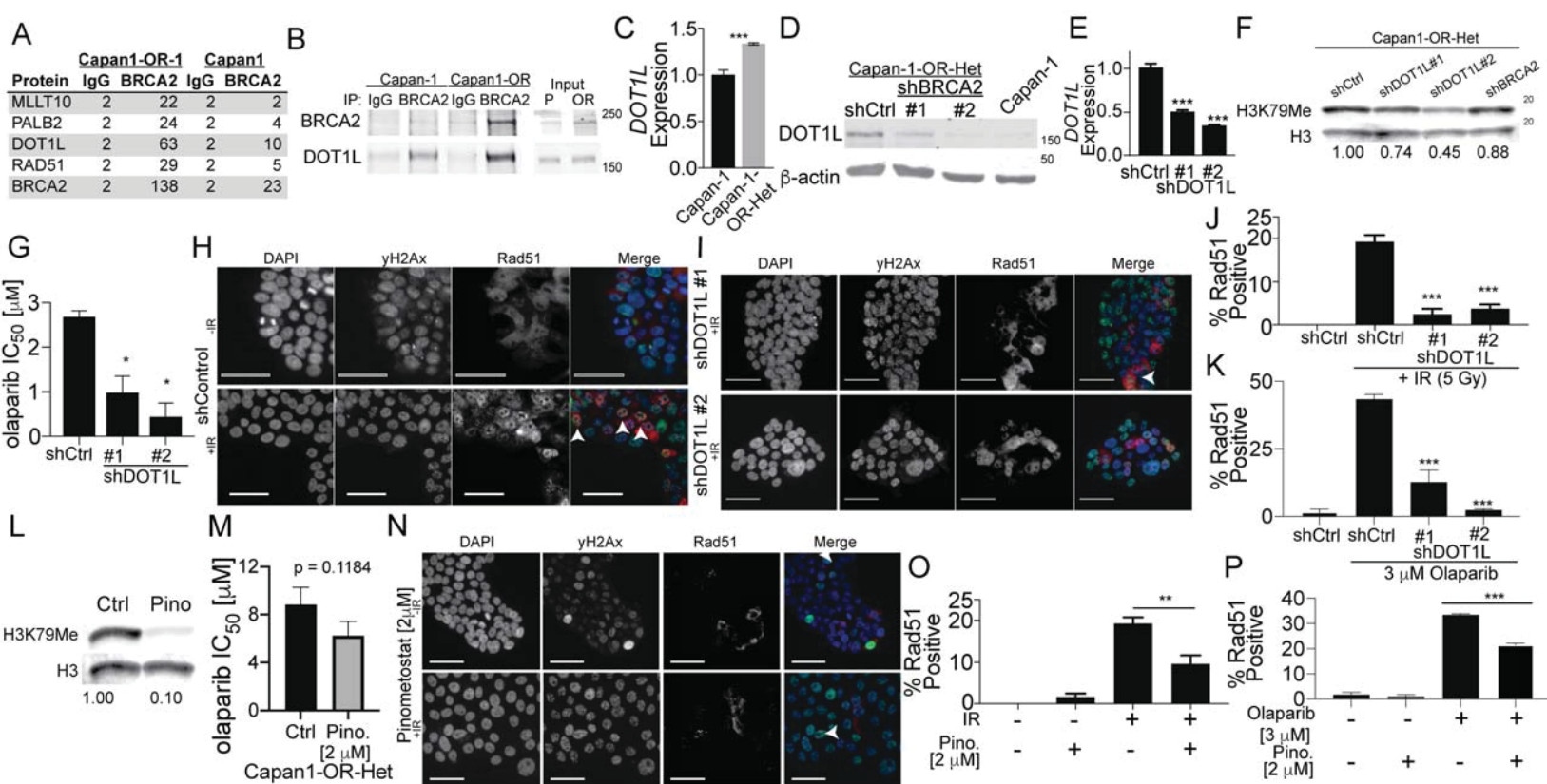


Figure 6, Park and Yamamoto et al 2019



Molecular Cancer Therapeutics

Amplification of the mutation-carrying BRCA2 allele promotes RAD51 loading and PARP inhibitor resistance in the absence of reversion mutations.

Pyoung Hwa Park, Tomomi M Yamamoto, Hua Li, et al.

Mol Cancer Ther Published OnlineFirst October 1, 2019.

Updated version	Access the most recent version of this article at: doi: 10.1158/1535-7163.MCT-17-0256
Supplementary Material	Access the most recent supplemental material at: http://mct.aacrjournals.org/content/suppl/2019/10/01/1535-7163.MCT-17-0256.DC1
Author Manuscript	Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts	Sign up to receive free email-alerts related to this article or journal.
Reprints and Subscriptions	To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org .
Permissions	To request permission to re-use all or part of this article, use this link http://mct.aacrjournals.org/content/early/2019/10/01/1535-7163.MCT-17-0256 . Click on "Request Permissions" which will take you to the Copyright Clearance Center's (CCC) Rightslink site.

ARTICLE

<https://doi.org/10.1038/s41467-019-13530-6>

OPEN

BRCA1 intronic *Alu* elements drive gene rearrangements and PARP inhibitor resistance

Yifan Wang¹, Andrea J. Bernhardt¹, Joseph Nacson^{1,2}, John J. Krais¹, Yin-Fei Tan³, Emmanuelle Nicolas^{1,3}, Marc R. Radke⁴, Elizabeth Handorf⁵, Alba Llop-Guevara⁶, Judith Balmaña⁷, Elizabeth M. Swisher⁴, Violeta Serra⁶, Suraj Peri⁵ & Neil Johnson^{1*}

BRCA1 mutant carcinomas are sensitive to PARP inhibitor (PARPi) therapy; however, resistance arises. *BRCA1* BRCT domain mutant proteins do not fold correctly and are subject to proteasomal degradation, resulting in PARPi sensitivity. In this study, we show that cell lines and patient-derived tumors, with highly disruptive BRCT domain mutations, have readily detectable *BRCA1* protein expression, and are able to proliferate in the presence of PARPi. Peptide analyses reveal that chemo-resistant cancers contain residues encoded by *BRCA1* intron 15. Mechanistically, cancers with BRCT domain mutations harbor *BRCA1* gene break-points within or adjacent to *Alu* elements in intron 15; producing partial gene duplications, inversions and translocations, and terminating transcription prior to the mutation-containing BRCT domain. *BRCA1* BRCT domain-deficient protein isoforms avoid mutation-induced proteasomal degradation, support homology-dependent DNA repair, and promote PARPi resistance. Taken together, *Alu*-mediated *BRCA1* gene rearrangements are responsible for generating hypomorphic proteins, and may represent a biomarker of PARPi resistance.

¹ Molecular Therapeutics Program, Fox Chase Cancer Center, Philadelphia, PA 19111, USA. ² Lewis Katz School of Medicine, Temple University, Philadelphia, PA 19111, USA. ³ Genomics Facility, Fox Chase Cancer Center, Philadelphia, PA 19111, USA. ⁴ Department of Obstetrics and Gynecology, University of Washington, Seattle, WA, USA. ⁵ Bioinformatics and Statistics, Fox Chase Cancer Center, Philadelphia, PA 19111, USA. ⁶ Experimental Therapeutics Group, Vall d'Hebron Institute of Oncology, Barcelona, Spain. ⁷ Hereditary Cancer Genetics Group, Vall d'Hebron Institute of Oncology, Barcelona, Spain. *email: neil.johnson@fccc.edu

B *BRCA1* mutations predispose carriers to an increased lifetime risk of developing cancer¹. A recent study reported that patients harboring *BRCA1* germline mutations had a 72% and 44% cumulative risk of developing breast and ovarian cancer before 80 years of age, respectively². *BRCA1* mutations are also associated with improved therapy response and survival outcomes^{3,4}. Breast and ovarian cancer patients with tumors harboring somatic or germline *BRCA1* mutations have demonstrated robust and lasting responses to PARP inhibitor (PARPi) treatments^{5–9}. PARPi's selectively induce cell death in homologous recombination (HR)-deficient *BRCA1/2* mutant cells, while leaving wild-type cells that are HR-proficient intact^{10,11}. Despite the overall efficacy of PARPi therapy, subsets of *BRCA1* mutant tumors have innate resistance, and others acquire PARPi resistance during the course of treatment¹².

The *BRCA1* gene is located on chromosome 17 and consists of 24 exons, 22 of which are protein coding. Mutations associated with cancer-predisposition are found throughout the gene in all coding exons as well as exon–intron splice sites^{13,14}. The longest *BRCA1* isoform generates an 1863 amino acid (aa) length protein that consists of several highly conserved domains. The N-terminal RING domain facilitates heterodimerization with BARD1. Toward the C-terminal end, the coiled-coil (CC) domain interacts with PALB2 and spans approximately aa's 1393–1424. Further downstream, the BRCT domain consists of two repeats, the first BRCT repeat includes aa's 1642–1736 and the second repeat aa's 1756–1855^{15,16}. The BRCT domain binds to proteins containing a phosphorylated serine-proline-x-phenylalanine (pSPxF) motif, including CtIP, Abraxas, and BRIP1^{17,18}.

BRCA1 plays a critical role in HR DNA repair, and mutations that disrupt protein activity result in defective HR^{19–22}. Moreover, cells that are HR-deficient are highly sensitive to PARPi and platinum treatments^{10,11}. *BRCA1* contributes to HR at distinct steps through the formation of various protein complexes. The *BRCA1*–CtIP interaction has been associated with efficient DNA end resection^{23–25}, and *BRCA1*–PALB2 interaction is required for the formation of a larger *BRCA1*–PALB2–*BRCA2*–RAD51 complex that promotes RAD51 loading^{26–28}. *BRCA1*–BARD1 has been shown to displace 53BP1 and activate end resection^{29,30}, as well as stimulate RAD51-mediated DNA joint formation³¹.

The *BRCA1* BRCT domain is critical for tumor suppression, and a significant portion of germline mutations can be found in this region^{32,33}. The two BRCT repeats pack together through a conserved triple-helical interface that mediate BRCT–BRCT contacts^{34–36}. Previous studies using proteolysis-based assays showed that the majority of commonly arising truncating frameshift and missense mutations that occur within the BRCT domain coding sequence alter the protein folding state. In turn, unfolded and destabilized proteins were subject to proteasomal degradation^{32,34,35}. However, when the entire BRCT domain is absent due to stop codons arising prior to the BRCT domain coding sequence, protein products avoid proteasomal degradation and can be abundantly expressed^{37,38}. *BRCA1* BRCT domain mutant cancers have previously been characterized and demonstrated low or undetectable protein expression^{21,39,40}. In the current study, we identified a mechanism for generating *BRCA1* isoforms that lack the entire BRCT domain (BRCTless) involving *Alu*-mediated genomic rearrangements. We show that *BRCA1* BRCTless isoforms are hypomorphic and promote PARPi resistance.

Results

***BRCA1* intron 15 is translated in SNU-251 cells.** To search for mechanisms of PARPi resistance in cancers specifically with BRCT domain-disrupting mutations, we initially characterized

the effects of the PARPi rucaparib on SNU-251 cells, an endometrioid ovarian cancer cell line that carries a *BRCA1* 5564 G > A (c.5445 G > A) non-sense mutation in exon 23⁴¹. This mutation stops translation toward the middle of the second BRCT repeat after aa 1814. *BRCA1* wild-type MDA-MB-231 cells and *BRCA1* BRCT domain mutant MDA-MB-436 cells were used as comparators (Supplementary Table 1). Rucaparib treatment resulted in a small increase in annexin-positive SNU-251 cells, but markedly increased annexin-positive MDA-MB-436 cells, despite both cell lines harboring *BRCA1* BRCT domain-disrupting mutations (Fig. 1a)^{39–41}. In long-term culture, while rucaparib killed MDA-MB-436 cells; SNU-251 cells demonstrated slower but maintained growth compared to vehicle-treated cells (Fig. 1b and Supplementary Fig. 1a). SNU-251 cells that were subject to long-term rucaparib culture were used in subsequent experiments, referred to as SNU-251-rucaparib resistant (RR) cells. In colony assays, MDA-MB-436 cells formed significantly fewer colonies compared with *BRCA1* wild-type MDA-MB-231 cells in the presence of the PARPi's rucaparib, olaparib, niraparib, as well as cisplatin and γ -irradiation (IR) treatments. However, SNU-251 cells had mild reductions in colony formation, and SNU-251-RR cells formed similar numbers of colonies as MDA-MB-231 cells (Fig. 1c and Supplementary Fig. 1b).

Previously established PARPi resistance mechanisms, including reversion mutations, 53BP1 and PARP1 loss of expression, as well as *BRCA1* protein folding^{12,38,40,42–45}, were not detected (Supplementary Fig. 1c–g and Supplementary Data 2). Protein analyses showed *BRCA1* expression was undetectable in MDA-MB-436 cells; however, SNU-251 parental cells expressed a truncated *BRCA1* protein that had elevated expression in SNU-251-RR cells, and was detected with an N-terminal-specific, but not a C-terminal-targeting *BRCA1* antibody (Fig. 1d). Despite lacking only 49 aa's (6 kDa) from full-length *BRCA1*, the protein expressed in SNU-251 cells showed a more substantial difference in gel migration than predicted by the mutation location (Fig. 1d), and migrated in line with an ectopic *BRCA1* cDNA lacking regions corresponding to exons 16–24 (Fig. 1e). To reveal the protein identity, *BRCA1* was immunoprecipitated from MDA-MB-231 and SNU-251-RR cells and analyzed by mass spectrometry. Peptide coverage was 70% and 50% in exons 2–15 coding regions, but 63% and 0% in exons 16–24 coding regions, in MDA-MB-231 control and SNU-251-RR cells, respectively (Fig. 1f). Surprisingly, we identified 12 aa's followed by a stop codon that are predicted from translation of intron 15 mRNA (Fig. 1f). We confirmed that intron 15 was being retained in *BRCA1* transcripts in SNU-251 cells (Fig. 1g), and expression was increased in SNU-251-RR cells (Fig. 1h). The *BRCA1*-intron 15 containing protein (*BRCA1*-I15) was also the single detectable protein isoform in SNU-251 cells (Supplementary Fig. 1e), and has similar post-translational features as full-length *BRCA1* (Supplementary Fig. 1f–g).

***BRCA1* gene rearrangements generate *BRCA1*-I15.** We next aimed to determine the biological mechanism of *BRCA1* intron 15 translation. DNA sequence alterations were not detected at the *BRCA1* exon-intron 15 junction (Supplementary Fig. 1h). Interestingly, in RNA-seq analyses, SNU-251-RR cells showed reads mapping to the first half of intron 15, but there was an absence of reads at the 3' end of the intron (Fig. 2a). We also readily detected reads mapping to all exons downstream of intron 15, potentially indicating the presence of two transcripts that were terminating at the bonafide 3'UTR, and a 3'UTR located in intron 15. Indeed, two *BRCA1* transcripts were cloned and identified as full-length and intron 15-containing isoforms (Fig. 2b). Sanger sequencing of the 3' ends revealed that the intron 15 ending transcript contained

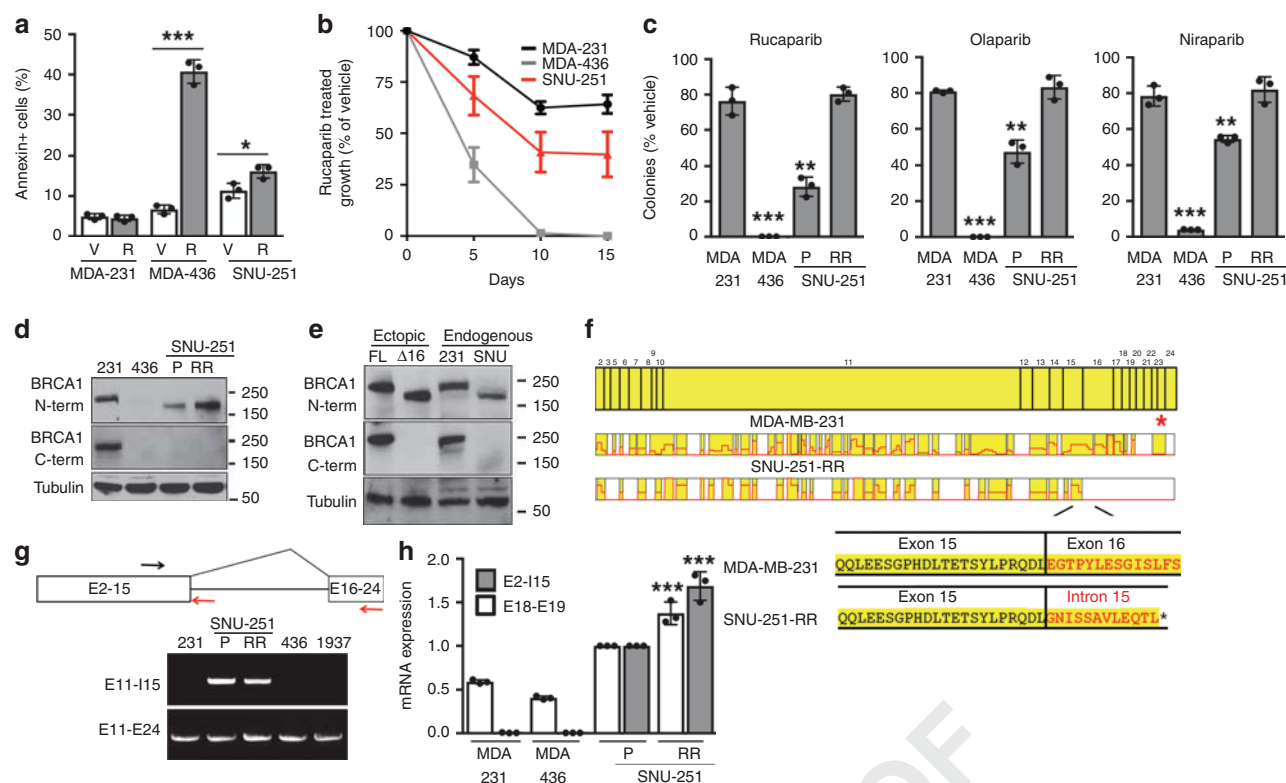


Fig. 1 Characterization of BRCA1 in SNU-251 cells. **a** MDA-MB-231, MDA-MB-436 and SNU-251 cells were cultured in the presence of vehicle (V) or 1 μ M rucaparib (R) for 5 days, followed by Annexin staining and flow cytometry. *** p < 0.001, * p < 0.05 compared to vehicle. **b** Cells were maintained in the presence of vehicle or 1 μ M rucaparib and counted every 5 days. Cell growth is expressed as a percentage of vehicle-treated cells. **c** MDA-MB-231, MDA-MB-436, SNU-251 parental (P), SNU-251-RR cells were seeded at decreasing densities in the presence of either vehicle, 100 nM rucaparib, 100 nM olaparib, or 100 nM niraparib and colonies counted 2-week post-seeding. Cell survival is expressed as a percentage of vehicle-treated cells. *** p < 0.001, ** p < 0.01, compared to MDA-MB-231. See Supplementary Fig. 1b for representative plates as well as γ -irradiation (IR) and cisplatin treatments. **d** MDA-MB-231 (231), MDA-MB-436 (436), SNU-251 parental (P), SNU-251-RR (RR) cells were examined for BRCA1 protein expression using N- or C-terminal-specific antibodies by Western blot. **e** MDA-MB-231 (231), SNU-251 (SNU) cells were assessed for BRCA1 expression using N- or C-terminal-specific antibodies by Western blot. Ectopic full-length BRCA1 (FL) and BRCA1- Δ exons-16-24 (Δ 16) cDNA expressing MDA-MB-436 cells were used as comparators for BRCA1 isoform molecular weights. **f** BRCA1 was immunoprecipitated from MDA-MB-231 and SNU-251-RR cells and subject to mass spectrometry. BRCA1 exons are shown and aligned with peptides that were detected (yellow). Peptides encoded by exon 15 and intron 15 were detected in SNU-251-RR cells and are shown below. **g** MDA-MB-231 (231), SNU-251 parental (P), SNU-251-RR (RR), MDA-MB-436 (436), HCC1937 (1937) cells were assessed for BRCA1 mRNA expression by RT-PCR with the indicated forward and reverse primer locations. **h** BRCA1 intron 15 and exons 18-19 specific mRNA expression was examined by quantitative (q)RT-PCR. *** p < 0.001 compared to SNU-251 parental cells. Data are the mean $\pm$ standard deviation (SD) of n = 3 biological replicates. Statistical significance was assessed by unpaired, two-tailed t tests.

a polyA tail that was approximately 18 base pair (bp) downstream of an AAUAAA polyadenylation signal sequence (chr17:43073282) and 6 bp from a CA cleavage site (Fig. 2c)⁴⁶. Furthermore, the cloned sequence corresponded with the region of intron 15 where reads declined in RNA-seq analyses, confirming transcription termination due to intronic polyadenylation (IPA) (Fig. 2c). In whole-genome sequencing (WGS), SNU-251-RR cells showed evidence of a partial BRCA1 copy number gain, initiating in NBR2 intron 1, and ending midway through BRCA1 intron 15 (Fig. 2d). A PCR-based gene copy number assay showed the partial BRCA1 gene duplication was present in both SNU-251 and SNU-251-RR cells, with the latter gaining an additional copy of the entire locus (Fig. 2e).

Chromosome 17 is rich in short interspersed nuclear elements (SINE), specifically *Alu* elements^{47–51}. A closer inspection of reads revealed that the copy number increase occurred between *AluY* and *AluSP* elements located in NBR2 intron 1 and BRCA1 intron 15, respectively (Fig. 3a and Supplementary Fig. 2)^{52–54}. Further deconvolution showed that the amplified region is flipped and inserted within an adjacent *AluSP* site in BRCA1 intron 15, chromosome 17 (Fig. 3b). Junction 1 generated a chimeric *Alu* via

the joining of two homologous *AluSp* sequences, both from BRCA1 intron 15. Junction 2 was formed by joining *AluY* and *AluJ* sequences from NBR2 intron 1 and BRCA1 intron 15, respectively. The latter sequences were interspaced by a 22 bp insertion that has 55% homology with the NBR2 *AluY* sequence (Fig. 3c). Of note, there was also a 167 bp duplicated sequence present at both junctions (Supplementary Note 1). Non-allelic homologous recombination (NAHR) occurs between two DNA sequences that are not alleles but share sequence similarity^{48,50,55}. Given the sequence homology at junctions, we propose that NAHR may have contributed to generating this rearrangement. The final locus can produce two BRCA1-I15 mRNA transcripts, both of which result in identical IPA events; and importantly, abrogate transcription of the mutation-containing BRCT coding region (Fig. 3d).

BRCA1-15 promotes PARPi resistance. Because the BRCT domain is required for protein interactions and several biological activities¹⁵, we examined the functionality of BRCA1-I15 in SNU-251 cells. Similar to BRCA1 full-length, BRCA1-I15 was located in the nuclear cellular fraction (Supplementary Fig. 3a)³⁷.

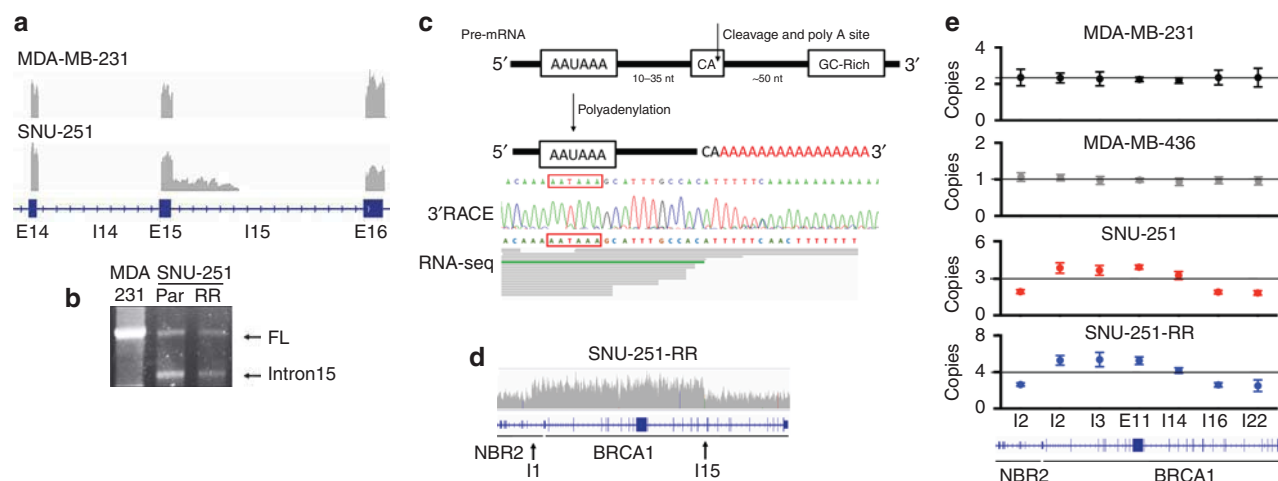


Fig. 2 Intronic polyadenylation occurs in SNU-251 cells. **a** Integrative Genomics Viewer (IGV) showing *BRCA1* exons 14–16 reads detected using RNA-seq in MDA-MB-231 and SNU-251-RR cells. **b** Identification of *BRCA1* transcripts by 3' RACE. Representative gel image indicating *BRCA1* full-length (FL) and intron 15 transcripts. **c** Cartoon showing a consensus AAUAAA polyadenylation signal that is followed by a CA cleavage and polyadenylation site 10–35 nucleotides downstream. Below, Sanger sequencing of *BRCA1* intron 15 mRNA identified by 3' RACE, polyadenylation sequence is highlighted. The corresponding 3' reads detected by RNA-seq are shown for comparison. **d** IGV of SNU-251-RR WGS reads detected mapping to chromosome 17 *NBR2* and *BRCA1* gene locations. **e** Cells were assessed for genomic copy number using q-PCR at *NBR2* intron 2, *BRCA1* intron 2, intron 3, exon 11, intron 14, intron 16 and intron 22. Copy number is normalized to the signal detected in RPE nontransformed cells ($n = 2$ copy number). Line shows the mean copy number for all reactions in each cell line. Data are the mean $\pm$ SD of $n = 3$ biological replicates.

Furthermore, while *BRCA1* BRCT mutant MDA-MB-436 cells lacked both *BRCA1* and RAD51 γ -irradiation-induced foci (IRIF); SNU-251 parental cells demonstrated foci positivity, and the number of *BRCA1* and RAD51 foci positive cells was elevated in SNU-251-RR cells (Fig. 4a and Supplementary Fig. 3b). SNU-251 parental and RR cells also exhibited HR activity, detected using the direct-repeat green fluorescent protein (DR-GFP) reporter system¹⁹ (Fig. 4b and Supplementary Fig. 3c–d). Additionally, *BRCA1* intron 15-targeting siRNA depleted *BRCA1* protein (Fig. 4c), reduced *BRCA1* and RAD51 IRIF (Fig. 4d and Supplementary Fig. 3e), and sensitized SNU-251-RR cells to PARPi treatment, but had no effect on MDA-MB-231 cells (Fig. 4e and Supplementary Fig. 3f). These data indicate that the *BRCA1*-I15 protein isoform is functional and integral to RAD51 loading and PARPi resistance in SNU-251-RR cells.

BRCTless isoforms avoid proteasomal degradation. To determine why a shorter 1570 aa *BRCA1*-I15 protein might be selectively expressed in place of a closer to full-length *BRCA1* c.5445 G > A generated 1814 aa protein, ectopic HA-tagged BRCT domain mutant *BRCA1* proteins were expressed and subject to mRNA and protein expression analyses. While BRCT mutation-containing HA-*BRCA1* mRNA expression was readily detected, protein expression was low or undetectable (Fig. 5a). MG132 treatment increased the expression of *BRCA1* BRCT mutant proteins (Supplementary Fig. 4a), indicating that mutations induced proteasomal degradation.

We hypothesized that due to the location of intron-coded stop codons, specifically intron 14- and intron 15-retaining mRNAs could generate *BRCA1* isoforms that would retain the CC domain, but lack the entire BRCT domain (Supplementary Fig. 4b)¹⁵. Therefore, proteins should be capable of promoting PALB2-BRCA2-RAD51 loading^{26,37}, as well as avoiding BRCT mutation-associated protein degradation³⁷. To assess this possibility, we generated HA-*BRCA1* constructs that contained aa's and translation stop sites encoded by introns 14 and 15 (Supplementary Fig. 4b). Here, HA-*BRCA1*-I14 and -I15-expressing cells had similar mRNA and protein expression as

full-length HA-*BRCA1* (Fig. 5b). Thus, in contrast to BRCT domain mutant proteins, *BRCA1*-I14 and -I15-containing proteins are stable and abundant. Furthermore, while *BRCA1*-I14 and -I15 did not associate with BRCT domain interacting proteins, the CC domain enabled PALB2-BRCA2-RAD51 interaction (Fig. 5c). *BRCA1*-I14 and -I15 were also capable of forming foci and supporting RAD51 IRIF, although to a lesser degree than full-length *BRCA1* (Supplementary Fig. 4c).

In colony assays, *BRCA1*-I14- and -I15 expressing cells formed significantly more colonies in the presence of rucaparib, olaparib and cisplatin compared to mCherry and *BRCA1* c.5445 G > A mutant expressing cells (Fig. 5d). Similar observations were made in xenograft experiments, where differences between vehicle and PARPi treatments were more pronounced in mCherry and *BRCA1* c.5445 G > A, compared with *BRCA1*-full-length and *BRCA1*-I15 expressing tumors (Fig. 5e, f). Of note, PARPi's are known to be moderately effective in the treatment of subsets of *BRCA1* wild-type cancers⁵⁶, and we found that PARPi-treated *BRCA1*-full-length expressing tumors had a statistically significant growth delay compared to vehicle-treated tumors. Additionally, the vehicle-treated *BRCA1*-full-length expressing tumors' growth rate was slower than other groups. Overall, *BRCA1* BRCTless isoforms did not demonstrate the same level of activity as full-length *BRCA1*; nevertheless, they avoid proteasomal degradation and are hypomorphic, promoting RAD51 loading and PARPi resistance.

***BRCA1* rearrangements occur in patient-derived tumors.** We set out to examine *BRCA1*-I15 expression in a panel of five unique patient-derived xenograft (PDX) models that harbored different *BRCA1* BRCT domain mutations (Fig. 6a and Supplementary Table 2)^{57,58}. Here, PDX221 and PDX418 both generated *BRCA1* proteins that had a similar gel migration pattern as SNU-251 cells, and were detectable with N-terminal but not C-terminal-specific *BRCA1* antibodies (Fig. 6b). *BRCA1*-I15 mRNA and protein expression was confirmed using qRT-PCR and mass spectrometry (Fig. 6c and Supplementary Fig. 5a). Interestingly, PDX418, but not PDX221, showed partial *BRCA1* gene

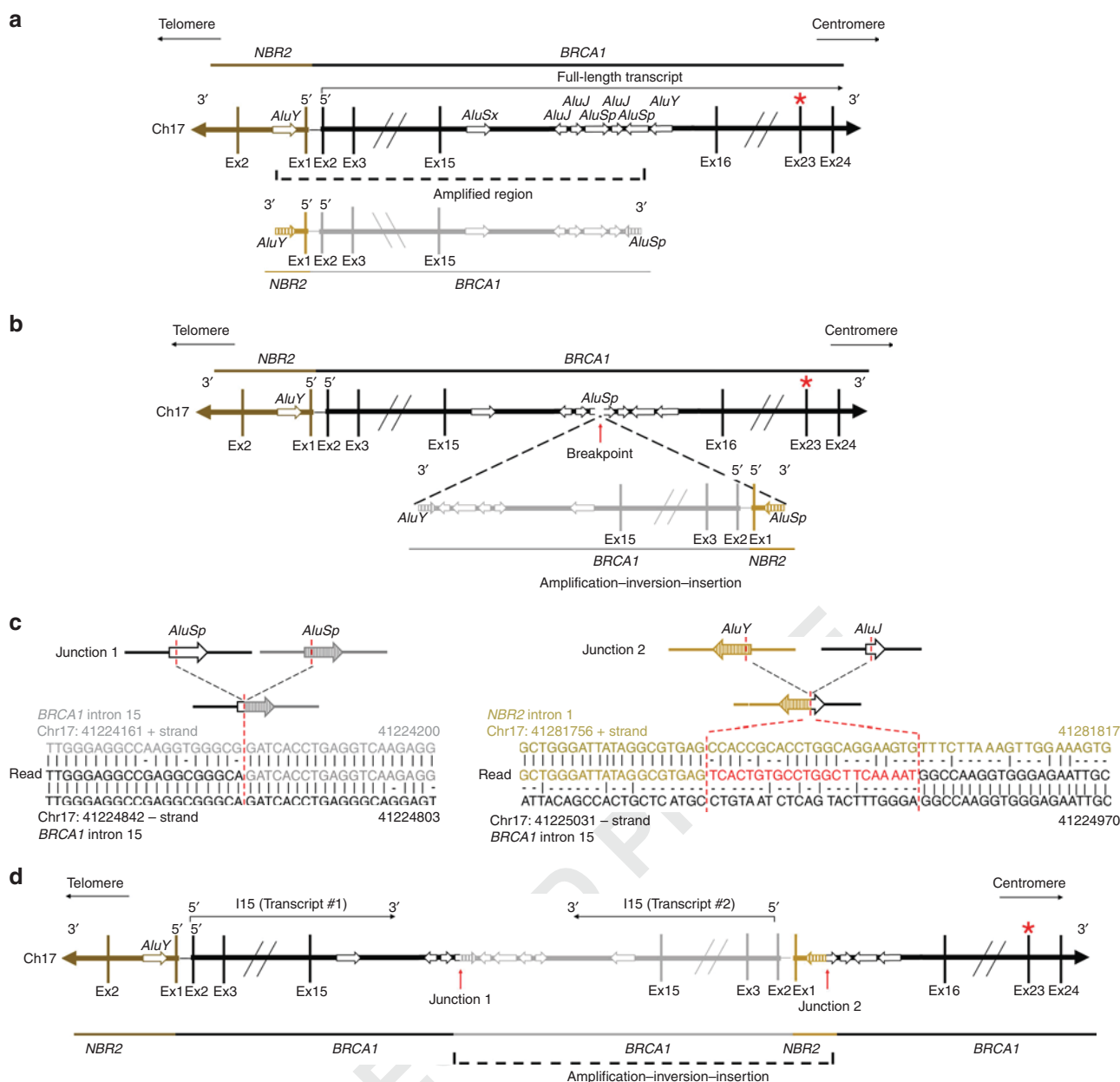


Fig. 3 *Alu*-mediated rearrangements generate *BRCA1*-I15. **a** Schematic showing the *NBR2* (brown) and *BRCA1* (black) gene region of chromosome 17. *Alu* sequences are arrows and their orientation relative to the centromere and telomere of chromosome 17 are indicated. Below, the amplified region detected in SNU-251-RR cells is shown corresponding to *NBR2* (gold) and *BRCA1* (gray) sequences. The *AluY* and *AluSp* sequences at each end of the amplified region are shaded arrows. SNU-251-RR cells also harbor a non-sense mutation c.5445 G > A located in exon 23 that is indicated with a red asterisk. See Supplementary Fig. 2 and Supplementary Note 1 for more details. **b** The breakpoint and insertion site are within an *AluSp* in *BRCA1* intron 15, highlighted by a red arrow. The amplified region shown below is inverted and inserted at the indicated breakpoint. **c** *Alu* sequences that combine to form junctions 1 and 2 are shown as arrows. Reads detected by WGS that map to the indicated regions of *NBR2* intron 1 and *BRCA1* intron 15 are shown. Sequence numbers are the genomic coordinates according to the UCSC genome browser (GRCh37/hg19 assembly). Red dashed lines indicate the exact break location and the corresponding DNA sequences. The read sequence colors correspond to the *Alu* arrows above from which they derive. There is a 22 bp insertion in junction 2, indicated by red colored sequence. **d** Schematic showing the rearranged locus detected in SNU-251-RR cells. Because a polyadenylation signal exists in *BRCA1* intron 15, transcription ends at intron 15. Therefore, the amplified copy is capable of producing a second identical *BRCA1*-I15 transcript. Moreover, the mutation located in exon 23 (red asterisk) is not transcribed in the rearranged locus.

duplication, ending in *BRCA1* intron 15 (Fig. 6d). RNA-seq analyses showed that PDX221 reads ended at an identical location as those detected in SNU-251-RR cells, suggesting the same IPA site (Fig. 6e). WGS analyses of PDX221 showed that a translocation t(17;21)(q21.31;q22.3) had occurred, where a sequence immediately adjacent to the *BRCA1* intron 15 *AluY* sequence was fused to an *ERV3* superfamily *MLT1B* element sequence located

in an intergenic region of chromosome 21q22.3 (Fig. 7a and Supplementary Fig. 5b-d)⁵⁹. Interestingly, the junction site had 3 bp of microhomology (Fig. 7b and Supplementary Note 1). This rearrangement is predicted to result in transcription termination within intron 15, producing *BRCA1*-I15 (Fig. 7c). Additionally, PDX418 showed a chromosome 17:22 translocation t(17;22)(q21.31;q23.1), with break sites immediately 3' of the *AluSx*

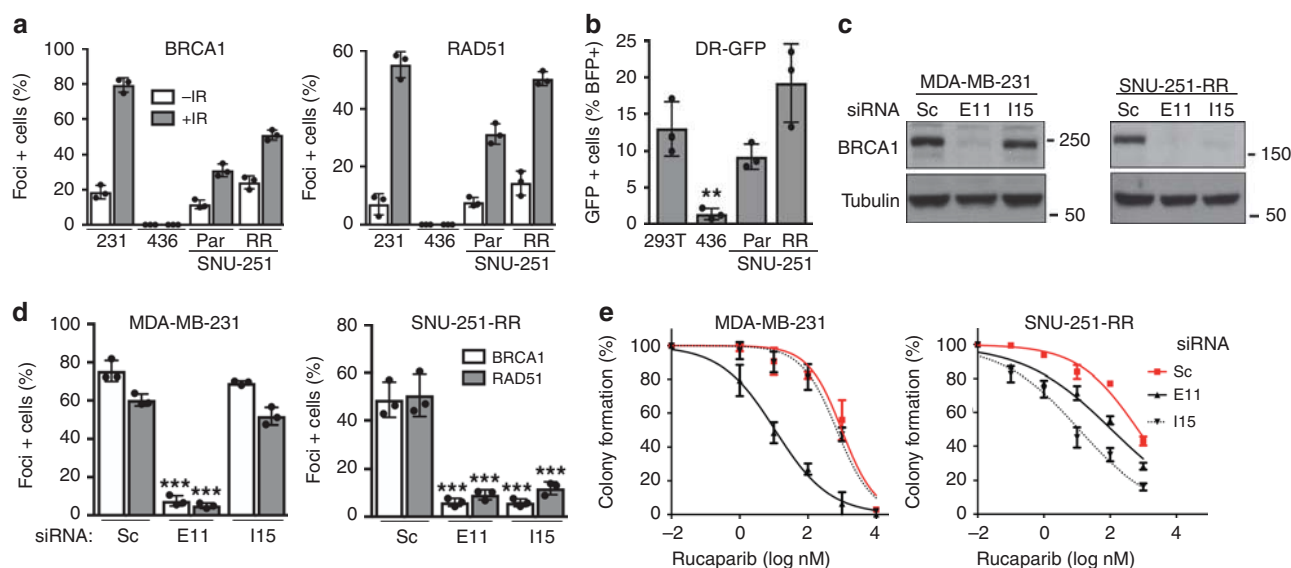


Fig. 4 BRCA1-I15 promotes PARPi resistance. **a** BRCA1 and RAD51 foci formation was assessed by immunofluorescence (IF) with or without 10 Gy IR. See Supplementary Fig. 3b for representative images as well as γ -H2AX and 53BP1 controls. **b** HR repair capacity assessed by DR-GFP assay. ** $p < 0.01$ compared to 293T cells. See Supplementary Fig. 3c–d for representative histograms and summary. **c** MDA-MB-231 and SNU-251-RR cells were treated with scrambled (Sc), BRCA1 exon 11 (E11) or intron 15 (I15)-targeting siRNA and BRCA1 protein expression assessed by Western blotting. **d** Cells were treated as in panel (c) and examined by IF for BRCA1 and RAD51 foci post 10 Gy IR. *** $p < 0.001$ compared to Sc-treated cells. See Supplementary Fig. 3e for representative images. **e** Cells were treated as in panel (c) and subject to increasing concentrations of rucaparib and colony formation assessed. Data are the mean $\pm$ SD of $n = 3$ biological replicates. Statistical significance was assessed by unpaired, two-tailed t tests.

sequence in *BRCA1* intron 15, as well as a region that lacked designated SINE elements within intron 4 of the *CBX6* gene (Fig. 7d and Supplementary Fig. 5b–d). The junction site also demonstrated 2 bp of microhomology (Fig. 7e and Supplementary Note 1), potentially indicating a role of microhomology-mediated end joining (MMEJ) in generating the translocations detected in PDX221 and PDX418^{60,61}. The rearrangement detected in PDX418 is also predicted to result in transcription termination within intron 15 and to produce *BRCA1*-I15 (Fig. 7f). Thus, *BRCA1* intron 15 rearrangements that generate translocations can be detected in chemo-refractory patient-derived tumors.

Discussion

BRCA1 BRCT domain mutations result in loss of tumor suppressor activity and are commonly found in patients with hereditary forms of breast and ovarian cancer. *BRCA1* BRCT domain missense and truncating mutations disrupt BRCT-BRCT repeat contacts and are structurally destabilizing^{32–35}. BRCT mutant proteins that are incapable of folding correctly are subsequently susceptible to proteasomal degradation, and *BRCA1* BRCT mutant protein expression is frequently low or undetectable in tumors and cell lines^{21,39,40}. We previously found MDA-MB-436 cells, which harbor a *BRCA1* BRCT domain mutation, have undetectable *BRCA1* protein expression, lack RAD51 foci, and demonstrated exquisite PARPi sensitivity⁴⁰. In contrast, we report here that SNU-251 cells, which also harbor a disruptive BRCT domain mutation, have detectable *BRCA1* protein expression, RAD51 foci, and are relatively insensitive to PARPi, requiring only a 2–3-week period for unperturbed cell growth in the presence of 1 μ M rucaparib.

The *BRCA1* BRCT domain is required for multiple protein interactions and activities^{62–64}. In agreement, we found that BRCTless *BRCA1* was less efficient compared with full-length *BRCA1* at promoting RAD51 loading and PARPi resistance in an isogenic cell line system. However, BRCTless *BRCA1* was partially proficient for RAD51 loading, and was capable of inducing PARPi resistance at a significantly greater level than the mCherry

control. Therefore, we conclude BRCTless *BRCA1* isoforms are hypomorphs, and promote PARPi resistance when abundantly expressed⁶². Importantly, loss of the entire BRCT domain is required to generate proteins that avoid proteasomal degradation³⁷. Furthermore, BRCTless proteins are dependent on the CC domain for activity, and more severe truncations that are deficient for the CC domain lack any hypomorphic activity³⁸.

Intron retention is a mechanism that regulates gene expression and proteomic diversity, having been extensively studied in organisms such as plants and budding yeast. In mammalian systems, the role of intron retention is more elusive, but several studies have shown that intron retention is a central component of gene expression programs during normal development, as well as in cancer^{65,66}. Analyses of *BRCA1* mRNA indicated that SNU-251 cells likely express two dominant isoforms; the first being full-length, and the second intron 15-retaining. However, rather than epigenetic regulation, intron 15 was translated because of *BRCA1* gene rearrangements.

BRCA1 gene *Alu*-mediated rearrangements are associated with pathogenic loss-of-function mutations^{49,67–69}. In this study, we found *Alu*-mediated gain-of-function events that were coupled with restored DNA repair and chemotherapy resistance. *BRCA1* intron 15 has seven *Alu* sequences, accounting for ~50% of intronic DNA⁵⁰, making this region particularly susceptible to rearrangements. The DNA repair mechanisms that contribute to generating *Alu*-mediated rearrangements may be inferred from mutational signatures at breakpoints^{60,61}. We propose that NAHR contributed to the *Alu*-*Alu*-mediated rearrangement found in SNU-251 cells, while MMEJ may have participated in generating the translocations observed in PDX tumors^{48,50,51,55}.

Overall, the *BRCA1*-I15 hypomorphic isoform was detected in SNU-251 cells as well as 2/5 of BRCT mutant PDX samples assessed, and all were derived from patients who had previously received DNA damaging chemotherapy^{41,57,58}. Thus, *BRCA1* *Alu*-mediated rearrangements represent an additional mechanism of PARPi/chemo-resistance, and could serve as a predictive

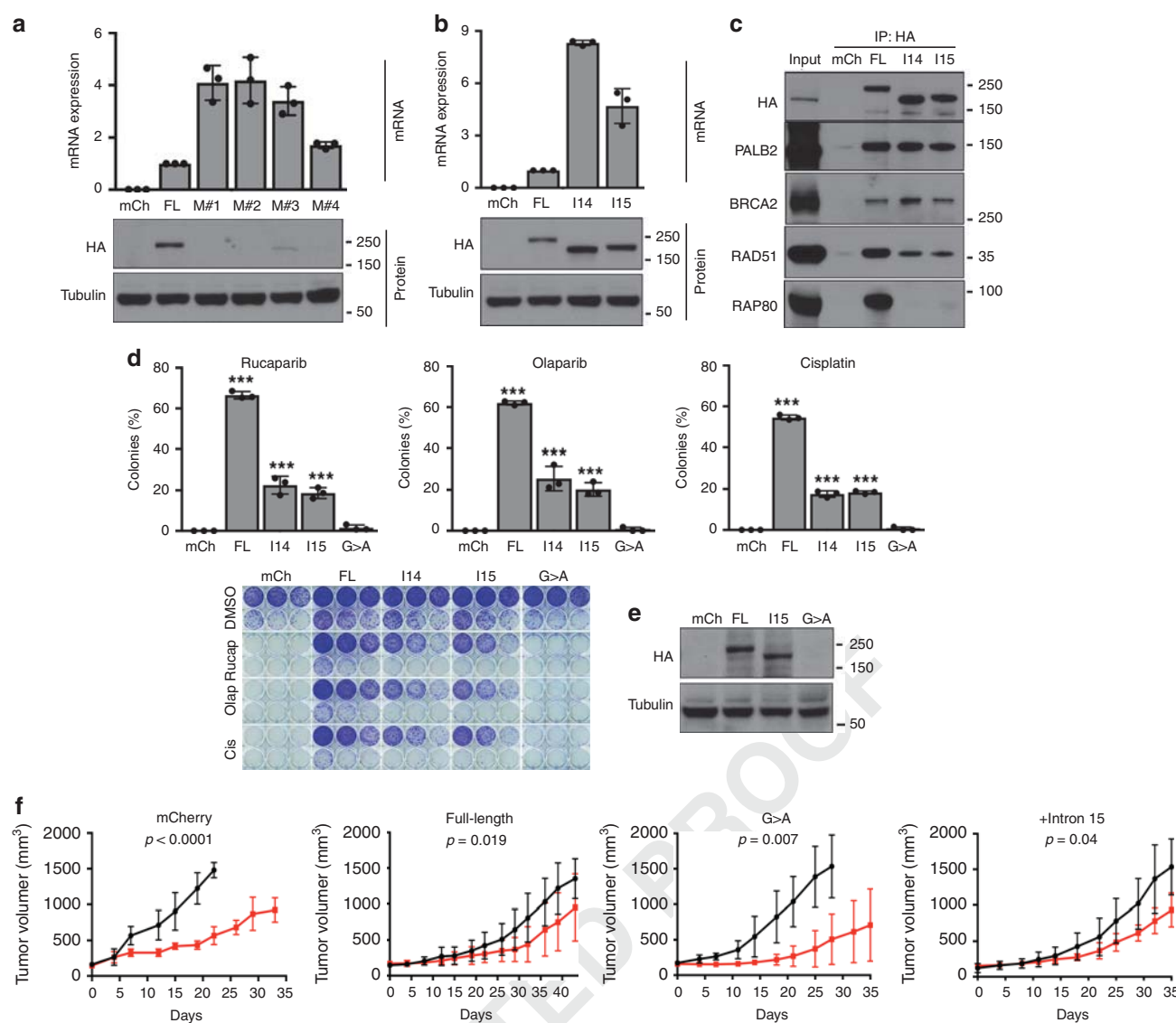


Fig. 5 BRCA1-I15 is stable and hypomorphic. **a** MDA-MB-436 cells engineered to express mCherry (mCh), HA-BRCA1 full-length/wild-type (FL), or the HA-BRCA1 BRCT mutations (M) c.5251 C > T (M#1), c.5263insC (M#2), c.5277insTA (M#3), c.5445 G > A (M#4), that are endogenously present in HCC1395, HCC1937, MDA-MB-436 and SNU-251 BRCA1 BRCT mutant cell lines, respectively; and were assessed for HA mRNA by qRT-PCR. HA expression normalized to FL expressing cells is shown (above). HA protein expression was assessed from the same cells by Western blotting (below). **b** MDA-MB-436 cells engineered to express mCh, BRCA1-FL, BRCA1-I14 (I14), and BRCA1-I15 (I15) were assessed as described in (a). See Supplementary Fig. 4b. **c** Cells from panel (b) were subject to HA-immunoprecipitation with the indicated antibodies. **d** mCh, BRCA1-FL, BRCA1-I14, BRCA1-I15, and BRCA1 c.5445 G > A (SNU-251 mutation) expressing MDA-MB-436 cells were seeded at decreasing densities in the presence of 20 nM rucaparib, 20 nM olaparib or 20 ng/ml cisplatin and assessed for colony formation. *** $p < 0.001$ compared to mCh cells. Below, representative plates. For panels (a–d), data are the mean $\pm$ SD of $n = 3$ biological replicates. Statistical significance was assessed by unpaired, two-tailed t tests. **e** mCh, BRCA1-FL, BRCA1-I15, and BRCA1 c.5445 G > A expressing MDA-MB-436 tumor xenografts were harvested and assessed for HA expression by Western blotting. **f** Mice harboring mCh, BRCA1-FL, BRCA1-I15, and BRCA1 c.5445 G > A expressing MDA-MB-436 tumor xenografts were treated with vehicle (black line) or 200 mg/kg rucaparib bi-daily (red line) for 2×5 days with a 2-day interval. Mean $\pm$ SD. Tumor volumes are shown for five tumors implanted in five separate mice per cell line per treatment from the same experiment. A linear mixed effects model tested the difference in slopes of log- tumor volume, and p values shown are tests of differences in growth rates between vehicle and rucaparib-treated tumors. Moreover, differences-in-slopes (i.e. vehicle to PARPi differences) were compared using Tukey's correction for multiple comparisons: BRCA1 5445 G > A vs. BRCA1-I15 ($p = 0.02$), BRCA1 5445 G > A vs. BRCA1-FL ($p < 0.001$), BRCA1-I15 vs. mCh ($p = 0.04$), and mCh vs. BRCA1-FL ($p = 0.001$), 5445 G > A vs. mCh ($p = 0.99$) or BRCA1-I15 vs. BRCA1-FL ($p = 0.57$). See Supplementary Data 1 file for more details.

biomarker of clinical response in patients with BRCA1 BRCT mutant cancers.

Methods

Cell lines and reagents. SNU-251 cells were obtained from the Korean Cell line bank. All other cell lines were from ATCC. SNU-251 cells were cultured in the presence of $1 \mu\text{M}$ rucaparib until a resistant population emerged (SNU-251-RR).

Cells were cultured in the absence of rucaparib for a minimum of 2 weeks before they were used for experiments. Cell lines tested negative for mycoplasma and identities were confirmed using short-tandem repeat (STR) profiling by IDXX Bioresearch. Chemicals were purchased from Sigma-Aldrich unless stated otherwise. Clovis Oncology provided rucaparib. Olaparib and niraparib were purchased from Selleckchem. Cisplatin was from APP/Fresenius Kabai USA LLC.

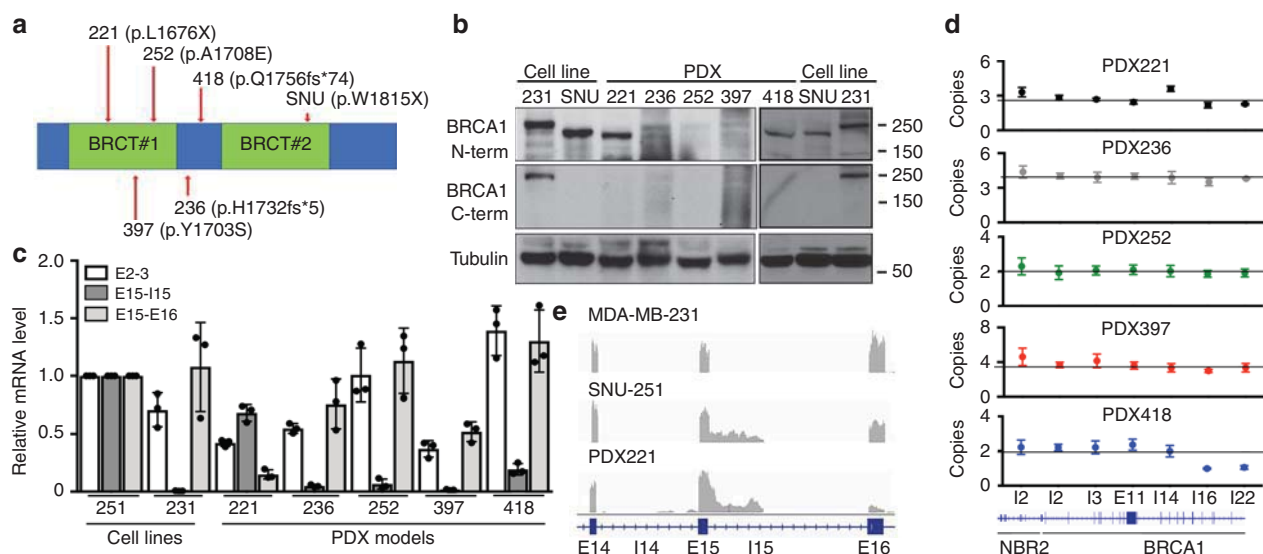


Fig. 6 BRCA1-I15 is expressed in patient-derived tumors. **a** Cartoon showing the location of *BRCA1* mutations present in PDX models relative to *BRCA1* BRCT repeats. See Supplementary Table 2 for more information. **b** PDX tumors were examined for *BRCA1* protein expression using N- or C-terminal-specific antibodies by Western blotting. MDA-MB-231 (231) and SNU-251 (SNU) cells are comparators. **c** *BRCA1* exons 2–3, intron 15, and exons 15–16 mRNA expression in the PDX samples measured by qRT-PCR. MDA-MB-231 (231) and SNU-251 (SNU) cells are included as comparators. **d** Copy number analyses as described in Fig. 2e. **e** *BRCA1* exons 15–16 reads detected using RNA-seq in PDX221 compared to MDA-MB-231, SNU-251-RR cells. Data are the mean $\pm$ SD of $n = 3$ biological replicates.

Colony formation assays. Cells were seeded at decreasing densities in six-well plates and maintained in vehicle or in the presence of 100 nM rucaparib, 100 nM olaparib, 100 nM niraparib or 50 ng/ml cisplatin until resistant colonies emerged. For siRNA treatments, exponentially growing cells were reverse transfected in 24-well plates, 24 h post transfection cells were treated with increasing concentrations of rucaparib for 72 h and re-plated in six-well plates for colony formation. Colony formation was assessed with crystal violet staining. Mean and standard deviation (SD) colony formation from three experiments was expressed as percentage of colonies relative to vehicle group. LC50 values (concentration required to reduce colony formation by 50% compared to vehicle) were calculated using GraphPad Prism software and used to compare fold changes in drug sensitivity.

Immunoprecipitation, mass spectrometry and Western blotting. *BRCA1* (EMD Millipore, catalog# OP92) antibody was used for immunoprecipitation of *BRCA1* complexes from 2 mg of nuclear extract using Pierce Classic IP Kit (ThermoFisher Scientific, catalog# 26146) according to the manufacturer's instructions. Nuclear extracts were derived using NE-PER Nuclear and Cytoplasmic Extraction Reagents (ThermoFisher Scientific, catalog# 78833) according to the manufacturer's instructions. For peptide analyses, after immunoprecipitation, bands corresponding to *BRCA1* were cut out and gel purified, digested using chemotrypsin and analyzed by LC-MS/MS. Proteins detected by Western blotting used the following antibodies: Tubulin (1:2000, Cell Signaling, catalog# 2148), N-terminal *BRCA1*, MS110 (1:500, EMD Millipore, catalog# OP92), C-terminal *BRCA1*, D9 (1:500, Santa Cruz Biotechnology, catalog# sc-6954), HA, 6E2 (1:500, Cell Signaling, catalog# 2367), 53BP1 (1:1000, EMD Millipore, catalog# MAB3802), PARP1 (1:1000, Cell Signaling, catalog# 9542), *BRCA2* (1:1000, Bethyl, catalog# A303-434A), PALB2 (1:1000, Bethyl, catalog# A301-247A), RAD51 (1:500, Santa Cruz Biotechnology, catalog# sc-8349), RAP80 (1:1000, Bethyl, catalog# A300-763A). For assessment of protein stabilization, cells were incubated with either 10 μ M MG132 or 10 μ M cycloheximide for 6 or 12 h or 500 nM HSP90 inhibitor (AT13387, Selleckchem) for 48 h. Cells were then collected and subjected to Western blotting. See Source Data file for uncropped Western blot images.

Immunofluorescence and microscopy. Cells were pre-extracted in cytoskeleton buffer (10 mM PIPES pH 6.8, 100 mM NaCl, 300 mM sucrose, 3 mM $MgCl_2$, 1 mM EGTA, 0.5% Triton X-100) for 5 min and followed by cytoskeleton stripping buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 3 mM $MgCl_2$, 1% Tween 20, 0.5% Sodium deoxycholate) for 5 min on ice. Cells were then fixed with 4% formaldehyde and permeabilized by 1% Triton X-100 in phosphate-buffered saline (PBS). *BRCA1* (1:2000, EMD Millipore, catalog# OP92), HA (1:1000, Covance, catalog# MMS-101R), RAD51 (1:1000, Genetex, catalog# GTX100469), γ -H2Ax (1:1000, R&D Systems, catalog# AF2288) and 53BP1 (1:1000, EMD Millipore, catalog# MAB3802) antibodies were followed by secondary antibodies conjugated to FITC or Texas Red (Jackson ImmunoResearch Laboratories). Cells were mounted in Vectashield containing DAPI (Vector Laboratories Inc.). We acquired immunofluorescence images using Nikon NIU Upright Fluorescent Microscope and

generated images using Nikon NIS Elements software. For IR experiments, we routinely fixed cells 7 h after treatment with 10 Gy. For analyses, we counted a minimum of 200 cells per condition per cell line. Each experiment was carried out at least three times.

Flow cytometric analysis. For cell cycle analysis, cells were harvested and fixed with 70% ethanol. Cells were washed with PBS and resuspended in 0.5 ml of FxCycle™ PI/RNase Staining Solution (ThermoFisher Scientific, catalog# F10797) and incubated at room temperature for 15 min. Data were acquired using BD FACScan Flow Cytometer and analyzed using FlowJo software. For cell death analysis, cells were collected 5 days after 1 μ M rucaparib treatment and analyzed using PE Annexin V Apoptosis Detection Kit I (BD Biosciences, catalog# 55976) according to the manufacturer's instructions. Data were acquired using BD LSR II Flow Cytometer and analyzed using FlowJo software.

DR-GFP assay. In order to account for differences in transfection efficiency between cell lines, we replaced the puromycin cassette in the DR-GFP reporter with BFP⁷⁰. The plasmid was pre-cut by I-SceI (NEB, catalog# R0694). Cells were seeded in 24-well plate and transfected with 0.5 μ g pre-cut plasmid using 1.5 μ l TransIT-2020 (Mirus, catalog# MIR 5400). Forty-eight hours later, cells were collected and analyzed by using BD LSR II Flow Cytometer. The HR efficiency was defined as the percentage of GFP-positive cells in BFP-positive population.

Gene sequencing analyses and qRT-PCR. Genomic DNA was isolated from cells or tumors using the DNeasy Blood and Tissue kit (Qiagen, catalog# 69504). Total RNA was isolated from cell lines using RNeasy Plus Mini Kit (Qiagen, catalog# 74134). RT-PCR with a forward primer located in *BRCA1* exon 11 and reversed primers in exon 24 and intron 15 were used to detect WT and I15 *BRCA1* mRNA. BROCA sequencing was carried out to detect mutations in DNA repair genes^{22,71}. Sanger sequencing was used to detect the *BRCA1* c.5445 G > A mutation in exon 23 and the potential mutations around exon 15/intron 15 splicing site. Primer sequences used in this study are listed in Supplementary Table 3.

RNAseq. RNAseq was performed on HiSeq 2500 system using Truseq stranded mRNA library kit, HiSeq rapid PE cluster kit and HiSeq rapid SBS kit (Illumina, CA) according to the manufacturer's manual. Briefly, 1 μ g total RNAs from each sample were used to make Stranded mRNA-seq library. mRNAs were enriched twice via poly-T-based RNA purification beads, and subjected to fragmentation via a divalent cation method. The first strand cDNA was synthesized by SuperscriptII, followed by second strand synthesis. During second strand synthesis, the dUTP was used to replace dTTP, thereby the second strand was quenched during amplification. A single "A" nucleotide is added to the 3' ends of the blunt fragments. Adapters with illumina P5, P7 sequences as well as indices were ligated to the cDNA fragment. Libraries were again purified using AmPure beads, and subject to a quality check using a bioanalyzer (Agilent) and quantified with Qubit

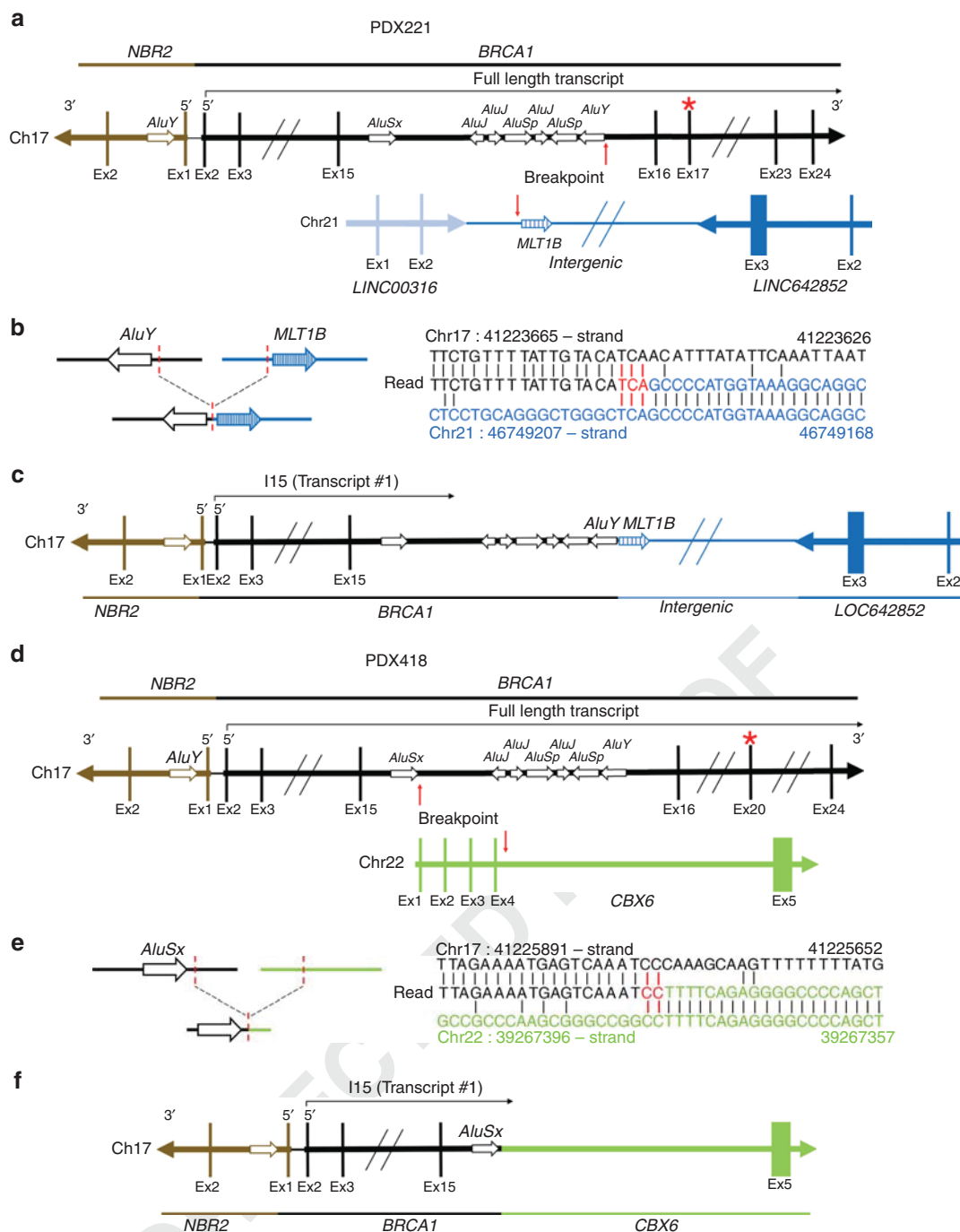


Fig. 7 BRCA1 intron 15 translocations detected in PDX tumors. **a** Schematic depicting the non-rearranged *BRCA1* locus and chr:21q22.3 region. Chr:21q22.3 contains the long intervening/intergenic noncoding RNAs (lincRNAs) *LINC00316* and *LOC642852*. PDX221 has a c.5027delT mutation located in *BRCA1* exon 17 that is noted with a red asterisk. Chromosome breakpoints are indicated (red arrows). **b** The breakpoints and junction are depicted and are within 93 bp of an *AluY* sequence in *BRCA1* intron 15 and within 36 bp of a *MLT1B* sequence in chr21q22.3, dashed red line (see Supplementary Note 1). Right, reads detected by WGS that map to the indicated regions of *BRCA1* intron 15 and chr21 are shown. Sequence numbers are the genomic coordinates according to the UCSC genome browser (GRCh37/hg19 assembly). Red sequence represents a region of microhomology. The read sequence colors correspond to the relative chromosome regions shown (left). **c** Schematic showing the chr17:21 translocation detected PDX221 that results in transcription termination in intron 15 and generates the *BRCA1* intron 15 mRNA isoform. See Supplementary Fig. 5b–d and Supplementary Note 1 for more details. **d** Schematic depicting the non-rearranged *BRCA1* locus and chr:22q23.1 region. PDX418 has a c.5266dupC mutation located in *BRCA1* exon 20 that is noted with a red asterisk, and the breakpoints (red arrows) are indicated. **e** The breakpoints and junction are depicted and are within 117 bp of an *AluSx* sequence in *BRCA1* intron 15; however, a SINE element was not detected in the *CBX6* intron 4 sequence (see Supplementary Note 1). Right, reads detected by WGS that map to the indicated regions of *BRCA1* intron 15 and *CBX6* intron 4 are shown. Sequence numbers are the genomic coordinates according to the UCSC genome browser (GRCh37/hg19 assembly). Red sequence represents a region of microhomology. The read sequence colors correspond to the relative chromosome regions shown (left). **f** Schematic showing the chr17:22 translocation detected PDX418 that results in transcription termination in intron 15 and generates the *BRCA1* intron 15 mRNA isoform. See Supplementary Fig. 5b–d and Supplementary Note 1 for more details.

(Invitrogen). Sample libraries were subsequently pooled and paired end reads at 105 bp generated for downstream bioinformatic analysis. Quality of the RNA-Seq reads were analyzed using FastQC: (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>). Reads were aligned to human genome (Hg38) using Tophat2 (Mapped reads to Hg38 - SNU-251-RR: 96 Million reads; MDA-MB-231: 75 Million reads; PDX-221: 78 Million reads). IGV was used to visually depict the genomic regions and sequencing reads.

3' RACE. Rapid Amplification of cDNA Ends (RACE) was performed to identify the 3' UTR of the I15 *BRCA1* isoform. cDNA was generated using SuperScript III First-Strand Synthesis System (ThermoFisher Scientific, catalog# 18080051) and oligo dT with the adapter sequence. The 3' UTR sequence was determined by Sanger sequencing following two-round nested PCR. Primer sequences are listed in Supplementary Table 3.

Whole-genome sequencing. WGS was performed by Novogene Corporation. A total amount of 1.0 µg DNA per sample was used as input material for the DNA sample preparations. Sequencing libraries were generated using NEBNext® DNA Library Prep Kit following the manufacturer's recommendations and indices were added to each sample. The genomic DNA is randomly fragmented to a size of 350 bp by shearing, then DNA fragments were end polished, A-tailed, and ligated with the NEBNext adapter for Illumina sequencing, and further PCR enriched by P5 and indexed P7 oligos. The PCR products were purified (AMPure XP system) and resulted libraries were analyzed for size distribution by Agilent 2100 Bioanalyzer and quantified using real-time PCR. Libraries were sequenced on an Illumina platform according to an effective concentration and data volume. Burrows–Wheeler Aligner (BWA) was used to map the paired-end clean reads to the human reference genome (hg19). Primary and secondary mapping sites of discordant split-reads that map the breakpoint regions in *BRCA1* loci were identified by parsing CIGAR flags of mapped reads. Integrative Genomics Viewer (IGV) was used to visually depict the genomic regions and sequencing reads. SINE elements were screened using CENSOR (<https://www.girinst.org/censor/index.php>).

Copy number variation assay. Genomic DNA was isolated from cells or tumors using the DNeasy Blood and Tissue kit (Qiagen, catalog# 69504). TaqMan Copy Number Reference Assay, HumanRNase P (LifeTechnologies, catalog# 4403326) was used as an internal control and run with Taqman Universal master mix (ThermoFisher Scientific, catalog# 4304437). *BRCA1* copy number was analyzed using Power SYBR Green master mix (Applied Biosystems, catalog# 4368702). The *BRCA1* copy number of each sample was determined by qPCR targeting different region spanning the *BRCA1* locus using RPE cells as a normalizer. The sequence of primers are listed in Supplementary Table 3.

RNA interference and cDNA add back treatments. We purchased Hs_*BRCA1*_FlexiTube siRNA construct (constructs #9 - SI00299495, targeting exon 11), and AllStars Negative Control siRNA (scrambled control) from Qiagen. Custom-made *BRCA1* intron 15 targeting siRNA sequence: UCUGCUGUAUUG GAACAAUUU (Dharmacon), Δ11q targeting siRNA sequence: GUAUCAGGGU GAAGCAGCAUU (Dharmacon). Transfections were carried out according to standard protocols. mCherry and HA-*BRCA1* cDNA were cloned into pENTR1A Gateway Entry vector and shuttled into a pDest-IRES-GFP Destination vector. To generate *BRCA1* proteins which contained amino acids and translation stop sites encoded by introns 14 and 15 (I14, I15), a small fragment corresponding to the specific intron retaining *BRCA1* was first amplified and then used to replace the C-terminal region of *BRCA1* in a pENTR-HA-*BRCA1*-V5 (full-length) construct using StuI (I14), AclI (I15) and BsaAI sites. cDNAs were shuttled into pLenti-IRES-GFP Destination vectors using the LR Clonase system (ThermoFisher Scientific, catalog# 11791020). Lentiviral generation and infections were carried out according to standard protocols. Protein knockdown was routinely assessed 72 h post transfection. Cells infected with cDNAs were sorted for GFP positivity using the FACS Aria II cell sorter and routinely checked for GFP positivity to maintain stable cell lines.

Xenograft treatments and analyses. MDA-MB-436 cells were subcutaneously implanted in 6-week-old female NSG mice. Treatment was initiated when tumors reached between 150 and 180 mm³. Rucaparib was administered at 150 mg/kg twice daily for 10 continuous days with a 2-day break after the first 5 days. Vehicle treatment consisted of 0.5% methylcellulose in water. Tumors were measured with calipers and tumor volumes calculated using the formula: (length × width²). Measurements were carried out every 3 days and mice euthanized when tumors reached 1500 mm³ in accordance with Institutional Animal Use and Care Committee (IACUC) guidelines of Fox Chase Cancer Center (FCCC). All work involving mice received ethical approval by the IACUC at FCCC.

Patient-derived xenografts. Patient consent for tumor use in animals was completed under a protocol approved by the Vall d'Hebron Hospital Clinical Investigation Ethical Committee and Animal Use Committee. Mice were maintained and treated in accordance with institutional guidelines of Vall d'Hebron University

Hospital Care and Use Committee. Tumors were subcutaneously implanted in 6-week-old female HsdCpb:NMRI-Foxn1nu mice (Harlan Laboratories). Animals were supplemented with 1 mmol/l estradiol (Sigma) in the drinking water. Upon xenograft growth, tumor tissue was reimplanted into recipient mice, which were randomized upon implant growth.

Statistical analyses. Mouse tumor growth curve data were analyzed using linear mixed effects models. For each experiment, the differences in growth rates of log-transformed tumor volumes between vehicle and rucaparib treatments were calculated. We fit linear mixed-effects models with random intercepts and slopes to account for within-mouse correlation, and tested the interaction effects between treatment and time. We assessed whether there was a nonlinear effect of time by examining residual plots, and tested models which include a quadratic effect of time for improved fit using likelihood ratio tests. Reported *p* values are from tests of the interaction terms, interpreted as differences in growth rates between vehicle and rucaparib treatments. Moreover, for xenografts, we fit a single regression model containing all experimental conditions to test the three-way interaction of time × treatment × xenograft model. Similar to the other regression models for log-volume, we fit a linear mixed-effects model with random intercepts and slopes to account for within-xenograft correlation and included a quadratic effect of time. The additional three-way interaction allowed the rates of growth (slopes) to vary by treatment and mouse model. After fitting the model, we tested all pairwise comparisons (contrasts) of the differences-in-slopes between treatment conditions, adjusting for multiple comparisons via Tukey's method (see Supplementary Data 1 for more details). For all other experiments, mean and SD values are shown (GraphPad Software). *p* < 0.05 was considered statistically significant and statistical tests are indicated in the figure legends. Asterisks indicate statistically significant *p* values. There were similar variances between statistical groups compared.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability

All data supporting the findings in this study are available within the paper, Supplementary Information and Source Data files. RNA-Seq and WGS data are available at the Sequence Read Archive (SRA) database under Bioproject ID PRJNA588424. The mRNA sequence of the *BRCA1* intron 15-containing isoform described in this study has been deposited in the NCBI GenBank nucleotide database with GenBank accession number MN577637.

Received: 16 March 2018; Accepted: 14 November 2019;

Published online: xx xxx 2019

References

- Petrucelli, N., Daly, M. B. & Feldman, G. L. Hereditary breast and ovarian cancer due to mutations in *BRCA1* and *BRCA2*. *Genet. Med.* **12**, 245–259 (2010).
- Kuchenbaecker, K. B. et al. Risks of breast, ovarian, and contralateral breast cancer for *BRCA1* and *BRCA2* mutation carriers. *JAMA: J. Am. Med. Assoc.* **317**, 2402–2416 (2017).
- Bolton, K. L. et al. Association between *BRCA1* and *BRCA2* mutations and survival in women with invasive epithelial ovarian cancer. *JAMA: J. Am. Med. Assoc.* **307**, 382–390 (2012).
- Rubin, S. C. et al. Clinical and pathological features of ovarian cancer in women with germ-line mutations of *BRCA1*. *N. Engl. J. Med.* **335**, 1413–1416 (1996).
- Ledermann, J. et al. Olaparib maintenance therapy in patients with platinum-sensitive relapsed serous ovarian cancer: a preplanned retrospective analysis of outcomes by *BRCA* status in a randomised phase 2 trial. *Lancet Oncol.* **15**, 852–861 (2014).
- Robson, M. et al. Olaparib for metastatic breast cancer in patients with a germline *BRCA* mutation. *N. Engl. J. Med.* **377**, 523–533 (2017).
- Pujade-Lauraine, E. et al. Olaparib tablets as maintenance therapy in patients with platinum-sensitive, relapsed ovarian cancer and a *BRCA1/2* mutation (SOLO2/ENGOT-Ov21): a double-blind, randomised, placebo-controlled, phase 3 trial. *Lancet Oncol.* **18**, 1274–1284 (2017).
- Fong, P. C. et al. Inhibition of poly(ADP-ribose) polymerase in tumors from *BRCA* mutation carriers. *N. Engl. J. Med.* **361**, 123–134 (2009).
- Tutt, A. et al. Oral poly(ADP-ribose) polymerase inhibitor olaparib in patients with *BRCA1* or *BRCA2* mutations and advanced breast cancer: a proof-of-concept trial. *Lancet* **376**, 235–244 (2010).
- Farmer, H. et al. Targeting the DNA repair defect in *BRCA* mutant cells as a therapeutic strategy. *Nature* **434**, 917–921 (2005).
- Bryant, H. E. et al. Specific killing of *BRCA2*-deficient tumours with inhibitors of poly(ADP-ribose) polymerase. *Nature* **434**, 913–917 (2005).

Q6

12. Lord, C. J. & Ashworth, A. Mechanisms of resistance to therapies targeting BRCA-mutant cancers. *Nat. Med.* **19**, 1381–1388 (2013).
13. Breast Cancer Information Core. <http://research.nhgri.nih.gov/bic/>.
14. Rebbeck, T. R. et al. Association of type and location of BRCA1 and BRCA2 mutations with risk of breast and ovarian cancer. *JAMA: J. Am. Med. Assoc.* **313**, 1347–1361 (2015).
15. Roy, R., Chun, J. & Powell, S. N. BRCA1 and BRCA2: different roles in a common pathway of genome protection. *Nat. Rev. Cancer* **12**, 68–78 (2012).
16. Deng, C. X. & Brodie, S. G. Roles of BRCA1 and its interacting proteins. *Bioessays* **22**, 728–737 (2000).
17. Yu, X., Chini, C. C., He, M., Mer, G. & Chen, J. The BRCT domain is a phospho-protein binding domain. *Science* **302**, 639–642 (2003).
18. Wu, Q. et al. Structure of BRCA1-BRCT/Abraxas complex reveals phosphorylation-dependent BRCT dimerization at DNA damage sites. *Mol. Cell* **61**, 434–448 (2016).
19. Moynahan, M. E., Chiu, J. W., Koller, B. H. & Jasin, M. Brca1 controls homology-directed DNA repair. *Mol. Cell* **4**, 511–518 (1999).
20. Moynahan, M. E., Cui, T. Y. & Jasin, M. Homology-directed dna repair, mitomycin-c resistance, and chromosome stability is restored with correction of a Brca1 mutation. *Cancer Res.* **61**, 4842–4850 (2001).
21. Wang, Y. et al. The BRCA1-Delta11q alternative splice isoform bypasses germline mutations and promotes therapeutic resistance to PARP inhibition and cisplatin. *Cancer Res.* **76**, 2778–2790 (2016).
22. Wang, Y. et al. RING domain-deficient BRCA1 promotes PARP inhibitor and platinum resistance. *J. Clin. Invest.* **126**, 3145–3157 (2016).
23. Escribano-Diaz, C. et al. A cell cycle-dependent regulatory circuit composed of 53BP1-RIF1 and BRCA1-CtIP controls DNA repair pathway choice. *Mol. Cell* **49**, 872–883 (2013).
24. Cruz-Garcia, A., Lopez-Saavedra, A. & Huertas, P. BRCA1 accelerates CtIP-mediated DNA-end resection. *Cell Rep.* **9**, 451–459 (2014).
25. Yun, M. H. & Hiom, K. CtIP-BRCA1 modulates the choice of DNA double-strand-break repair pathway throughout the cell cycle. *Nature* **459**, 460–463 (2009).
26. Sy, S. M., Huen, M. S. & Chen, J. PALB2 is an integral component of the BRCA complex required for homologous recombination repair. *Proc. Natl Acad. Sci. USA* **106**, 7155–7160 (2009).
27. Zhang, F., Fan, Q., Ren, K. & Andreassen, P. R. PALB2 functionally connects the breast cancer susceptibility proteins BRCA1 and BRCA2. *Mol. Cancer Res.* **7**, 1110–1118 (2009).
28. Zhang, F. et al. PALB2 links BRCA1 and BRCA2 in the DNA-damage response. *Curr. Biol.* **19**, 524–529 (2009).
29. Densham, R. M. et al. Human BRCA1-BARD1 ubiquitin ligase activity counteracts chromatin barriers to DNA resection. *Nat. Struct. Mol. Biol.* **23**, 647–655 (2016).
30. Chapman, J. R., Sossick, A. J., Boulton, S. J. & Jackson, S. P. BRCA1-associated exclusion of 53BP1 from DNA damage sites underlies temporal control of DNA repair. *J. Cell Sci.* **125**, 3529–3534 (2012).
31. Zhao, W. et al. BRCA1-BARD1 promotes RAD51-mediated homologous DNA pairing. *Nature* **550**, 360–365 (2017).
32. Lee, M. S. et al. Comprehensive analysis of missense variations in the BRCT domain of BRCA1 by structural and functional assays. *Cancer Res.* **70**, 4880–4890 (2010).
33. Shakya, R. et al. BRCA1 tumor suppression depends on BRCT phosphoprotein binding, but not its E3 ligase activity. *Science* **334**, 525–528 (2011).
34. Williams, R. S. et al. Detection of protein folding defects caused by BRCA1-BRCT truncation and missense mutations. *J. Biol. Chem.* **278**, 53007–53016 (2003).
35. Williams, R. S. & Glover, J. N. Structural consequences of a cancer-causing BRCA1-BRCT missense mutation. *J. Biol. Chem.* **278**, 2630–2635 (2003).
36. Leung, C. C. & Glover, J. N. BRCT domains: easy as one, two, three. *Cell Cycle* **10**, 2461–2470 (2011).
37. Rodriguez, J. A., Au, W. W. & Henderson, B. R. Cytoplasmic mislocalization of BRCA1 caused by cancer-associated mutations in the BRCT domain. *Exp. Cell Res.* **293**, 14–21 (2004).
38. Nacson, J. et al. BRCA1 mutation-specific responses to 53BP1 loss-induced homologous recombination and PARP inhibitor resistance. *Cell Rep.* **24**, 3513–3527 e3517 (2018).
39. Elstrodt, F. et al. BRCA1 mutation analysis of 41 human breast cancer cell lines reveals three new deleterious mutants. *Cancer Res.* **66**, 41–45 (2006).
40. Johnson, N. et al. Stabilization of mutant BRCA1 protein confers PARP inhibitor and platinum resistance. *Proc. Natl Acad. Sci. USA* **110**, 17041–17046 (2013).
41. Yuan, Y. et al. Establishment and characterization of human ovarian carcinoma cell lines. *Gynecologic Oncol.* **66**, 378–387 (1997).
42. Norquist, B. et al. Secondary somatic mutations restoring BRCA1/2 predict chemotherapy resistance in hereditary ovarian carcinomas. *J. Clin. Oncol.* **29**, 3008–3015 (2011).
43. Bouwman, P. et al. 53BP1 loss rescues BRCA1 deficiency and is associated with triple-negative and BRCA-mutated breast cancers. *Nat. Struct. Mol. Biol.* **17**, 688–695 (2010).
44. Bunting, S. F. et al. 53BP1 inhibits homologous recombination in Brca1-deficient cells by blocking resection of DNA breaks. *Cell* **141**, 243–254 (2010).
45. Pettitt, S. J. et al. Genome-wide and high-density CRISPR-Cas9 screens identify point mutations in PARP1 causing PARP inhibitor resistance. *Nat. Commun.* **9**, 1849 (2018).
46. Tian, B. & Manley, J. L. Alternative polyadenylation of mRNA precursors. *Nat. Rev. Mol. Cell Biol.* **18**, 18–30 (2017).
47. Coppa, A. et al. Optimizing the identification of risk-relevant mutations by multigene panel testing in selected hereditary breast/ovarian cancer families. *Cancer Med.* **7**, 46–55 (2018).
48. Kim, S., Cho, C. S., Han, K. & Lee, J. Structural variation of Alu element and human disease. *Genomics Inf.* **14**, 70–77 (2016).
49. Peixoto, A. et al. Genomic characterization of two large Alu-mediated rearrangements of the BRCA1 gene. *J. Hum. Genet.* **58**, 78–83 (2013).
50. Mazoyer, S. Genomic rearrangements in the BRCA1 and BRCA2 genes. *Hum. Mutat.* **25**, 415–422 (2005).
51. Gu, S. et al. Alu-mediated diverse and complex pathogenic copy-number variants within human chromosome 17 at p13.3. *Hum. Mol. Genet.* **24**, 4061–4077 (2015).
52. De Brakeleer, S., De Greve, J., Lissens, W. & Teugels, E. Systematic detection of pathogenic alu element insertions in NGS-based diagnostic screens: the BRCA1/BRCA2 example. *Hum. Mutat.* **34**, 785–791 (2013).
53. Elliott, B., Richardson, C. & Jasin, M. Chromosomal translocation mechanisms at intronic alu elements in mammalian cells. *Mol. Cell* **17**, 885–894 (2005).
54. Weinstock, D. M., Richardson, C. A., Elliott, B. & Jasin, M. Modeling oncogenic translocations: distinct roles for double-strand break repair pathways in translocation formation in mammalian cells. *DNA Repair (Amst.)* **5**, 1065–1074 (2006).
55. Kolomietz, E., Meyn, M. S., Pandita, A. & Squire, J. A. The role of Alu repeat clusters as mediators of recurrent chromosomal aberrations in tumors. *Genes, Chromosomes Cancer* **35**, 97–112 (2002).
56. Ledermann, J. A. & Pujade-Lauraine, E. Olaparib as maintenance treatment for patients with platinum-sensitive relapsed ovarian cancer. *Therapeutic Adv. Med. Oncol.* **11**, 1758835919849753 (2019).
57. Castroviejo-Bermejo, M. et al. A RAD51 assay feasible in routine tumor samples calls PARP inhibitor response beyond BRCA mutation. *EMBO Mol. Med.* **10**, e9172 (2018).
58. Cruz, C. et al. RAD51 foci as a functional biomarker of homologous recombination repair and PARP inhibitor resistance in germline BRCA-mutated breast cancer. *Ann. Oncol.* **29**, 1203–1210 (2018).
59. Kojima, K. K. Human transposable elements in Repbase: genomic footprints from fish to humans. *Mob. DNA* **9**, 2 (2018).
60. Hastings, P. J., Ira, G. & Lupski, J. R. A microhomology-mediated break-induced replication model for the origin of human copy number variation. *PLoS Genet.* **5**, e1000327 (2009).
61. Hastings, P. J., Lupski, J. R., Rosenberg, S. M. & Ira, G. Mechanisms of change in gene copy number. *Nat. Rev. Genet.* **10**, 551–564 (2009).
62. Chen, L., Nievera, C. J., Lee, A. Y. & Wu, X. Cell cycle-dependent complex formation of BRCA1.CtIP.MRN is important for DNA double-strand break repair. *J. Biol. Chem.* **283**, 7713–7720 (2008).
63. Li, S. et al. Functional link of BRCA1 and ataxia telangiectasia gene product in DNA damage response. *Nature* **406**, 210–215 (2000).
64. Dohrn, L., Salles, D., Siehler, S. Y., Kaufmann, J. & Wiesmuller, L. BRCA1-mediated repression of mutagenic end-joining of DNA double-strand breaks requires complex formation with BACH1. *Biochem. J.* **441**, 919–926 (2012).
65. Dubbury, S. J., Boutz, P. L. & Sharp, P. A. CDK12 regulates DNA repair genes by suppressing intronic polyadenylation. *Nature* **564**, 141–145 (2018).
66. Lee, S. H. et al. Widespread intronic polyadenylation inactivates tumour suppressor genes in leukaemia. *Nature* **561**, 127–131 (2018).
67. Chisholm, K. M., Goff, B. A., Garcia, R., King, M. C. & Swisher, E. M. Genomic structure of chromosome 17 deletions in BRCA1-associated ovarian cancers. *Cancer Genet. Cytogenet.* **183**, 41–48 (2008).
68. Woodward, A. M. et al. Large genomic rearrangements of both BRCA2 and BRCA1 are a feature of the inherited breast/ovarian cancer phenotype in selected families. *J. Med. Genet.* **42**, e31 (2005).
69. Hogervorst, F. B. et al. Large genomic deletions and duplications in the BRCA1 gene identified by a novel quantitative method. *Cancer Res.* **63**, 1449–1453 (2003).
70. Pierce, A. J., Johnson, R. D., Thompson, L. H. & Jasin, M. XRCC3 promotes homology-directed repair of DNA damage in mammalian cells. *Genes Dev.* **13**, 2633–2638 (1999).
71. Walsh, T. et al. Mutations in 12 genes for inherited ovarian, fallopian tube, and peritoneal carcinoma identified by massively parallel sequencing. *Proc. Natl Acad. Sci. USA* **108**, 18032–18037 (2011).

Acknowledgements

This work was supported by the US National Institutes of Health (NIH) Grants P50 CA083638 and 5P30 CA006927, as well as R01CA214799, Susan Komen CCRCR17499048, and OC130212 Department of Defense to N.J., and a Pilot Award and Nested Teal Postdoctoral Scholar supported Y.W. (OC140040). We thank Drs. Judith Balmaña, Cristina Saura and Joaquin Arribas for providing materials. PDXs were established thanks to the GHD-Pink program, the FERO Foundation and the Catalan Agency AGAUR [2017 SGR 540]. V.S. is supported by the Instituto de Salud Carlos III (CP14/00228) and ALG by a PERIS fellowship from the Departament de Salut, Generalitat de Catalunya (SLT002/16/00477). Clovis Oncology supplied rucaparib. We are grateful to FCCC Genomics, Cell Culture and Cell Sorting facilities. We thank Hsin Yao Tang at the Wistar Proteomics facility for help with mass spectrometry.

Author contributions

Y.W., A.J.B., J.N., J.J.K., Y.-F.T., E.N., M.R.R., E.H., A.L.-G., designed and performed experiments. Y.W., Y.-F.T., S.P., and N.J. carried out and analyzed RNA-seq and WGS data. Y.W., J.B., E.M.S., V.S., S.P., and N.J. analyzed and interpreted data. Y.W., and N.J. wrote the manuscript. N.J. supervised the project.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41467-019-13530-6>.

Correspondence and requests for materials should be addressed to N.J.

Peer review information *Nature Communications* thanks the anonymous reviewers for their contribution to the peer review of this work.

Reprints and permission information is available at <http://www.nature.com/reprints>

Publisher's note Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Open Access This article is licensed under a Creative Commons Attribution 4.0 International License, which permits use, sharing, adaptation, distribution and reproduction in any medium or format, as long as you give appropriate credit to the original author(s) and the source, provide a link to the Creative Commons license, and indicate if changes were made. The images or other third party material in this article are included in the article's Creative Commons license, unless indicated otherwise in a credit line to the material. If material is not included in the article's Creative Commons license and your intended use is not permitted by statutory regulation or exceeds the permitted use, you will need to obtain permission directly from the copyright holder. To view a copy of this license, visit <http://creativecommons.org/licenses/by/4.0/>.

© The Author(s) 2019

QUERY FORM

NATURECOMMUNICATIONS	
Manuscript ID	[Art. Id: 13530]
Author	
Editor	
Publisher	

Journal: NATURECOMMUNICATIONS

Author :- The following queries have arisen during the editing of your manuscript. Please answer by making the requisite corrections directly in the e-proofing tool rather than marking them up on the PDF. This will ensure that your corrections are incorporated accurately and that your paper is published as quickly as possible.

Query No.	Description	Author's Response
AQ1	Please check your article carefully, coordinate with any co-authors and enter all final edits clearly in the eproof, remembering to save frequently. Once corrections are submitted, we cannot routinely make further changes to the article.	
AQ2	Note that the eproof should be amended in only one browser window at any one time; otherwise changes will be overwritten.	
AQ3	Author surnames have been highlighted. Please check these carefully and adjust if the first name or surname is marked up incorrectly. Note that changes here will affect indexing of your article in public repositories such as PubMed. Also, carefully check the spelling and numbering of all author names and affiliations, and the corresponding email address(es).	
AQ4	You cannot alter accepted Supplementary Information files except for critical changes to scientific content. If you do resupply any files, please also provide a brief (but complete) list of changes. If these are not considered scientific changes, any altered Supplementary files will not be used, only the originally accepted version will be published.	
AQ5	Please provide document title and year of publication for ref.13.	