Analysis of matched primary and recurrent BRCA1/2 mutation-associated tumors identifies recurrence-specific drivers

Jennifer Shah

University of Pennsylvania <https://orcid.org/0000-0001-9908-9874>

Dana Pueschl

University of Pennsylvania

Bradley Wubbenhorst

University of Pennsylvania

John Pluta

University of Pennsylvania

Kurt D.Andrea

University of Pennsylvania

Anna Hubert

University of Pennsylvania

Jake Shilan

University of Pennsylvania

Wenting Zhou

University of Pennsylvania

Adam Kraya

University of Pennsylvania

Alba Llop-Guevara

Vall d'Hebron Institut d'Oncologia

Catherine Ruan

University of Pennsylvania

Violeta Serra

Vall d'Hebron Institut d'Oncologia <https://orcid.org/0000-0001-6620-1065>

Judith Balmaña

Vall d'Hebron Institut d'Oncologia <https://orcid.org/0000-0002-0762-6415>

Michael Feldman

University of Pennsylvania

Anupma Nayak

University of Pennsylvania

Kara Maxwell

University of Pennsylvania <https://orcid.org/0000-0001-8192-4202>

Susan Domchek

University of Pennsylvania <https://orcid.org/0000-0002-5914-7272>

Katherine Nathanson (✉ knathans@upenn.edu)

University of Pennsylvania <https://orcid.org/0000-0002-6740-0901>

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Authors, affiliations, and contact information

1 Jennifer B. Shah¹, jshah413@gmail.com
2 Dana Pueschl¹, dana.pueschl@pennmedicine.upenn.edu
3 Bradley Wubbenhorst¹, bwubb@pennmedicine.upenn.edu
4 John Pluta¹, jpluta@pennmedicine.upenn.edu
5 Kurt D'Andrea¹, dandreak@upenn.edu
6 Anna P. Hubert¹, anna.hubert@pennmedicine.upenn.edu
7 Jake S. Shilan¹, shilanjake@gmail.com
8 Wenting Zhou¹, wezhou@pennmedicine.upenn.edu
9 Adam A. Kraya¹, krayaa@chop.edu
10 Alba Llop Guevara², allop@vhio.net
11 Catherine Ruan¹, ruan.catherine@gmail.com
12 Violeta Serra³, vserra@vhio.net
13 Judith Balmaña², jbalmama@vhio.net
14 Michael Feldman^{4,6}, michael.feldman2@pennmedicine.upenn.edu
15 Anupma Nayak⁴, anupma.nayak@pennmedicine.upenn.edu
16 Kara N. Maxwell^{5,6,7}, kara.maxwell@pennmedicine.upenn.edu
17 Susan M. Domchek^{5,6,7}, susan.domchek@pennmedicine.upenn.edu
18 Katherine L. Nathanson^{*1,6,7}, knathans@upenn.edu

¹ Division of Translational Medicine and Human Genetics, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

² High Risk and Familial Cancer Group, Vall d'Hebron Institut d'Oncologia, Barcelona; Department of Medical Oncology, Hospital Vall d'Hebron, Universitat Autònoma de Barcelona, Barcelona, Spain

³ Experimental Therapeutics Group, Vall d'Hebron Institut d'Oncologia, Barcelona, Spain.

⁴ Division of Surgical Pathology, Department of Pathology and Laboratory Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

⁵ Division of Hematology-Oncology, Department of Medicine, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

⁶ Abramson Cancer Center, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

⁷ Basser Center for BRCA, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, USA

*Materials and correspondence should be addressed to K.L.N. (email: knathans@upenn.edu)

Abstract

Recurrence is a major cause of death among BRCA1/2 mutation carriers with breast (BrCa) and ovarian cancers (OvCa). We performed multi-omic sequencing on 67 paired primary and recurrent BrCa and OvCa from 27 BRCA1/2 mutation carriers to identify potential recurrence-specific drivers. *PARP1* amplifications were identified in recurrences (FDR $q=0.05$), and *PARP1* was significantly overexpressed across primary BrCa and recurrent BrCa and OvCa, independent of amplification status. RNA-seq analysis found two *BRCA2* isoforms, *BRCA2-201/Long* and *BRCA2-001/Short*, predicted sensitive and insensitive to nonsense-mediated decay, respectively. *BRCA2-001/Short* was expressed more frequently in recurrences and associated with reduced overall survival in breast cancer (87 vs. 121 months; HR=2.535 [1.179-5.45]). Loss of heterozygosity (LOH) status was discordant in a subset of tumors, with both LOH and lack of LOH found in recurrences. Our study revealed multiple potential drivers of recurrent disease in *BRCA1/2* mutation-associated cancer, improving our understanding of tumor evolution and suggesting potential biomarkers.

Introduction

Pathogenic variants in *BRCA1/2* are the most common cause of hereditary breast and ovarian cancer, underlying 5-10% of breast and 20% of ovarian cancers^{1,2}. *BRCA1* and *BRCA2* function independently to maintain genomic integrity, with both playing critical roles in the homologous recombination (HR) pathway of DNA repair³. The loss of *BRCA1* or *BRCA2* allows cells to accrue mutations and genomic rearrangements facilitating a transition to cancer⁴. Carriers of pathogenic germline variants in *BRCA1/2* have an increased risk of breast and ovarian carcinomas, along with prostate and pancreatic cancers^{3,5}.

Women with pathogenic variants in *BRCA1/2* are diagnosed with high-grade serous ovarian carcinomas⁶. *BRCA1* mutation carriers are predisposed to hormone receptor-negative breast cancers, whereas *BRCA2* mutation carriers more commonly develop hormone-positive breast cancers³. Ovarian and triple-negative breast cancers tend to recur within a few years of diagnosis^{6,7}. Recurrences are usually associated with therapeutic resistance, and both recurrent ovarian cancer and metastatic breast cancer are considered incurable. The standard of care in *BRCA1/2* mutation-associated ovarian cancers is platinum-based chemotherapy (cisplatin, carboplatin) as first line treatment, with poly (ADP-ribose) polymerase inhibitors (PARPi) used increasingly in both the maintenance and metastatic settings. For *BRCA1/2* mutation-associated breast cancer, systemic therapy is given as appropriate to the stage and receptor status of a patient's disease. PARPi are employed for late-stage breast cancer, and the OlympiA trial has suggested benefit in the adjuvant setting⁸⁻¹⁰. Although tumors in *BRCA1/2* mutation carriers may initially respond to platinum and PARPi, many develop resistance over time. Preclinical studies have demonstrated that platinum and PARPi resistance are mediated by several of the same mechanisms, such as expression of MDR1 (ABCB1) efflux pumps or loss of DNA damage response regulators (TP53BP1, REV7, CHD4, and PARP1)¹¹⁻¹⁷. However, in tumors from *BRCA1/2* germline mutation carriers, the most common identified

resistance mechanism to platinum and PARPi are reversion mutations affecting the mutant *BRCA1/2* allele^{11,17–24}.

Prior studies of matched primary and recurrent *BRCA1/2* mutation-associated breast and ovarian cancers have been limited by small numbers (3–8 patients) or focused on tumors resistant to PARPi, which is not representative of current frontline therapy and resistance^{17,25–29}. At present, most *BRCA1/2* mutation carriers with breast cancer do not receive PARPi prior to their first recurrence. Therefore, acquired resistance mechanisms to conventional frontline breast and ovarian cancer therapies are largely unknown. In addition, clinical trials have evaluated the efficacy of immune checkpoint blockade on *BRCA1/2* mutation-associated breast and ovarian cancers, with variable objective response rates^{30–37}. Characterizing expression of checkpoint proteins in primary and recurrent *BRCA1/2* mutation-associated tumors may help to identify markers of response to immunomodulatory therapies.

To address outstanding questions about cancer recurrence in *BRCA1/2* mutation carriers, we performed an analysis of paired primary and recurrent *BRCA1/2* mutation-associated tumors. The sample set consisted of 67 primary and recurrent tumors from 13 *BRCA1/2* germline mutation carriers with breast cancer and 14 *BRCA1/2* germline mutation carriers with ovarian cancer. We performed a combination of whole exome, targeted, and RNA sequencing on this cohort of specimens, in addition to multiplex immunofluorescent microscopy^{38,39}. Our goal was to identify genetic, transcriptomic, and immune events that underlie acquired therapeutic resistance. We hypothesized that differences between paired tumors would indicate novel mechanisms of tumor evolution associated with post-treatment tumor recurrence in *BRCA1/2* mutation-associated cancers.

Results

Cohort selection and clinical characteristics

We studied matched primary and recurrent breast cancers from 13 subjects, nine

BRCA1 and four *BRCA2* pathogenic variant carriers (**Table 1**). All but one *BRCA2* mutation carrier with breast cancer were female. Nine of 13 primary breast tumors were estrogen receptor expression negative (ER-), predominantly triple negative breast cancer (TNBC) with concordant ER- recurrences. We evaluated matched primary and recurrent ovarian cancer from 14 women, 10 *BRCA1* and four *BRCA2* pathogenic variant carriers. Most (9/14) primary ovarian tumors were diagnosed at stage III or IV. As treatment for their primary tumor, all patients received chemotherapy (**Table 1**). All 14 (100%) ovarian cancer patients received platinum and 4/14 (29%) received PARPi prior to a collected recurrence. Three of 13 breast cancer patients (23%) received platinum and a single breast cancer patient (1/13; 8%) received PARPi. Breast cancer patients received hormonal therapy and radiation (38% and 54%) more frequently than ovarian cancer patients (21% and 21%). Additional clinical and sequencing details for each tumor are documented in **Supplementary Data 1**.

Analysis of somatic mutations from whole exome and targeted sequencing

We used whole exome sequencing (WES) to assess the landscape of somatic mutations in 67 paired primary/recurrent tumors and matched germline samples from 27 *BRCA1/2* mutation carriers with breast or ovarian cancer. We also re-sequenced 44 tumors and 18 respective germline samples at high depth (>300x) using a custom targeted capture of 209 genes (**Supplementary Table 1**).

We assessed individual gene variants to identify shared oncogenes or tumor suppressors. The majority of the cohort (53/67 tumors; 80%) had loss of function (LoF) mutations in *TP53* (**Fig. 1a, Supplementary Fig. 1a-c**). Breast tumors without *TP53* mutations were mostly *BRCA2* mutation-associated and estrogen receptor positive (ER+) in origin (8/9 tumors from patients 10-13). Five ovarian tumors from three patients (17, 20, 25) also lacked *TP53* mutations. *KMT2C* was mutated in 21/67 tumors, 16 of which were ovarian tumors. *NOTCH1* mutations were identified exclusively in (six) breast tumors, and *NF1* was mutated in

six breast and ovarian tumors. One *BRCA2* mutation carrier (Patient 10) had an ER+ primary breast tumor and two recurrences lacking *TP53* mutations, which appeared to be driven by the *AKT1* E17K mutation instead^{40,41}. Gain of function (GoF) mutations were identified in *PIK3CA* and *TBX3* in one breast and one ovarian tumor, respectively.

We next examined the global landscape of mutations by tumor. Mutational signatures, were dominated by Signature 3 (*BRCA1/2* inactivating mutations and defective HR) and Signatures 1A/B (aging)⁴² (**Fig. 1b**). Sixteen of 27 (60%) tumors had mutations associated with Signature 4 (smoking)⁴². In paired tumor comparisons, tumor mutational burden (TMB) did not change from primary tumor to recurrence (**Supplementary Fig. 1d, Supplementary Data 2**). To investigate functional impact, we determined whether LoF variants accumulated in common pathways across the cohort. We ranked genes by prevalence of LoF variants within primary and recurrent tumor groups, then performed Gene Set Enrichment Analysis (GSEA) Preranked with Hallmark Gene Sets (**Fig. 1c, Supplementary Data 3**)⁴³. LoF variants from recurrences were enriched for sixteen gene sets not found in primary tumor LoF variants (all FDR $q < 0.25$). These gene sets highlighted potential differences in metabolic processes (oxidative phosphorylation, glycolysis), apoptosis, *TP53* signaling, and pathways regulating cell growth and identity (*TGF β* , Notch, *MTORC1*, *KRAS*).

To identify additional driver mutations in *BRCA1/2* mutation-associated tumors, we performed MutSigCV analysis on primary and recurrent tumor groups (**Fig. 1d,e; Supplementary Data 4**)⁴⁴. *TP53* and *BAGE2* were the only significantly mutated genes within both tumor groups (FDR $q < 0.05$). As *BAGE2* encodes a noncoding RNA not directly targeted by the exome captures used, *TP53* is the only gene that can be considered significantly mutated in this sample set⁴⁵.

Analysis of *BRCA1/2* loss of heterozygosity

Using whole exome and targeted sequencing, we assessed *BRCA1/2* allele-specific loss

of heterozygosity (LOH). First, we assessed whether somatic variants mediated LOH in our sample set of tumors. We found pathogenic variants in the respective *BRCA1* or *BRCA2* genes in 20 of 67 tumors, all with low alternative allele fraction (< 4.5%), suggesting that these mutations only affected a minority of cells. Next, we used allele-specific copy number to determine whether tumors underwent LOH via copy number loss of their wild-type *BRCA1/2* allele⁴⁶. Overall, 22/27 (81%) primary tumors and 33/40 (82%) recurrences demonstrated LOH (**Fig. 2a**). Most primary/recurrent tumor pairs had concordant *BRCA1/2* allele-specific LOH status (20/27 patients; 74%). Seven patients showed discordant LOH status when comparing their primary tumor to recurrence(s). Discordant tumor LOH status was more common in *BRCA2* mutation carriers, likely due to the increased prevalence of nonLOH ER+ breast cancers⁴⁷.

We observed discordant LOH status more frequently in breast cancers (five – 38%) than in ovarian cancer patients (two - 14%). Notably, three of four ER+ breast cancer patients in the cohort had discordant LOH in their tumors. We noticed overlap between patients with LOH transitions and those with *TP53* wild-type tumors (6/7 patients – 86%). In three patients (6, 11, 12), the primary tumor did not have LOH, but first exhibited LOH upon recurrence (nonLOH to LOH, **Supplementary Fig. 2a, b**). Conversely, patients 10, 13, 17, and 20 showed the opposite effect: primary tumors exhibited LOH but developed into a nonLOH recurrence (LOH to nonLOH, **Supplementary Fig. 3a, b**). This “LOH reversal” was observed in two *BRCA2* mutation carriers with breast cancer, treated with non-platinum chemotherapy and radiation, and two *BRCA1* mutation carriers with ovarian cancer, both treated with platinum. Mutational signatures reflected the change in HR proficiency (and potential for other drivers) among tumors with LOH reversal; loss of Signature 3 was observed in patients 10, 13, and 20 (**Fig. 1b**). In patients 10, 17, and 20, nonLOH recurrences also had a lower HRD score compared to primary tumors with LOH (**Fig. 2b**). For all three tumors that first developed LOH upon recurrence, nonLOH primary tumors had a lower HRD score than recurrences (**Fig. 2b**). We did not find any

other associations between LOH discordance and treatment history.

Assessment of genomic scarring and copy number variation

We next evaluated whether recurrent tumors showed more chromosomal instability and genomic scarring than primary tumors. Neither aneuploidy nor HRD scores (individual metrics or combined) were significantly different between tumor pairs overall, although some primary and recurrent tumors did have different scores (**Supplementary Fig. 4a-e; Supplementary Data 2**). To identify commonly amplified and deleted genes in primary and recurrent tumors, we performed GISTIC analysis, stratified by tumor type (**Fig. 2c, d; Supplementary Fig. 4f, g; Supplementary Fig. 5a-d; Supplementary Data 5**)⁴⁸. We assessed individual genes that fell within significant amplifications or deletions (90% confidence interval and FDR $q < 0.05$) and were biologically relevant to breast and ovarian cancer. Both breast and ovarian tumors demonstrated significant *CHD2* and *MYC* amplifications, as have been widely observed in *BRCA1/2* mutation-associated cancers (**Supplementary Fig. 5a, b**)^{49–51}. Breast tumors demonstrated amplifications of *SOX4*, which has been shown to promote PI3K/AKT signaling (**Supplementary Fig. 5a**)⁵². We observed deletions of putative breast tumor suppressor *RUNX3*, DNA repair genes *RAD18* and *XPC*, and chromatin remodelers (*HDAC4/10/11* and *BRD1*) (**Supplementary Fig. 5c**)^{53–55}. Specific to ovarian tumors were amplifications in *ABCB11*, an ABC transporter associated with chemoresistance (**Supplementary Fig. 5b**)⁵⁶. Other notable ovarian-specific deletions included DNA damage response genes (*ERCC1/2*, *CHEK1*, *FANCF*), apoptotic regulators (*BAX*, *BAD*), *PTEN*, *WEE1*, and *CDKN1C* (**Supplementary Fig. 5d**).

In a comparison of GISTIC segments between breast and ovarian tumors, we identified intersections (>50% overlap) for sixteen deletions and eight amplifications (**Supplementary Data 5**). As the tumor types exhibited similar copy number profiles, we combined all primary and all recurrent tumors to identify shared and distinct amplifications and deletions. Significant

(90%CI and FDR $q < 0.05$) deletions were more similar between primary and recurrent tumors than significant amplifications (Jaccard similarity index of 0.20 vs. 0.06). Primary tumors had amplifications in *MYC* and *CHD2* (**Fig. 2c**). Recurrent tumors shared these amplifications and were also uniquely enriched for amplifications of *ABCB10*, another ABC transporter associated with chemotherapy resistance (**Fig. 2d**)⁵⁶. Further, we noted recurrence-specific amplifications in *TGFB2*, *TRAF5*, *WNT3A*, *SOX4*, and *E2F3*, suggesting that recurrences may have increased dosage of growth and lineage signal pathway genes. In recurrences, unique deletions included *CASP9*, *CDKN2C*, *JUN*, *MUTYH*, and *RAD54L*, suggesting dysregulation of cell cycle, DNA damage response, and apoptosis (**Supplementary Fig. 4g**).

We found *PARP1* to be significantly (90% confidence interval, FDR $q = 0.05$) amplified in recurrences but not primary tumors (**Fig. 2d**). Given the use of PARPi for treatment in *BRCA1/2* mutation-associated tumors, we investigated further. Over half of tumors (35/67, 52%) in the cohort had somatic gains (copy number ≥ 4) or amplifications (copy number ≥ 6) in *PARP1* (**Supplementary Data 6**). *PARP1* gains and amplifications were present in primary ovarian tumors (43%), recurrent ovarian tumors (32%), primary breast tumors (62%), and recurrent breast tumors (78%) (**Fig. 2e**). Seventy percent of all patients (19/27) in the cohort had a *PARP1* gain or amplification in at least one tumor (85% of breast patients, 57% of ovarian patients). Most of these patients (11/19, 60%) had a *PARP1* gain or amplification in both primary and recurrent tumors. Gains and amplifications tended to be focal and centered around the *PARP1* locus, rather than arm-level, underscoring a potential selection effect (**Fig. 2f**)⁴⁸. We also evaluated *PARP1* copy number status in TCGA PanCancer cohorts of primary breast and ovarian tumors. We grouped TCGA tumors into *BRCA1/2* germline mutation-associated (*gBRCA1/2*) and HR wild-type (HR-WT) groups, as previously described (**Supplementary Fig. 6a, Supplementary Data 7**)^{47,57}. After replicating the Sequenza analysis performed in the primary/recurrent cohort, we found a high prevalence of *PARP1* gains and amplifications in both tumor types, with no differences based on *BRCA1/2* status (**Supplementary Fig. 6b**,

Supplementary Data 6).

To assess copy number variation by pathway, we next performed GSEA Preranked on lists of genes ranked by prevalence of copy number gain or loss within each tumor group (**Fig. 2g; Supplementary Data 3**). Primary tumors were enriched for gains in gene sets for UV response, TNF α signaling via NF- κ B, IFN α and IFN γ response, and angiogenesis. Recurrent tumors were uniquely enriched for gains in MTORC1 signaling genes and deletions in Hedgehog signaling genes (consistent with our finding that recurrent tumors accumulate LoF mutations in Hedgehog signaling genes).

Global transcriptomic analysis of primary and recurrent tumors

We performed RNA sequencing (RNA-seq) on 50 primary and recurrent tumors from the WES cohort, adding in recurrences from four additional patients (subjects 28-31, **Fig. 3a; Supplementary Data 1**). We also conducted RNA-seq on 12 normal breast and fallopian tube samples from prophylactic surgeries in *BRCA1/2* mutation carriers (subjects 32-43, **Fig. 3a, Supplementary Fig. 7a, Supplementary Data 1**). We used hierarchical clustering to assess sample relatedness across the cohort (**Supplementary Fig. 7b**). In the resulting dendrogram, many paired primary and recurrent tumors were not each other's closest relatives (11/19 tumor pairs). In general, ER- breast tumors clustered closer to ovarian tumors than ER+ breast tumors, which in turn clustered closest to normal samples. Sample relatedness appeared to be most informed by ER status and tissue of origin. We further assessed sample clustering using principal components (**Supplementary Fig. 7c**). We did not observe any clustering associated with tumor origin (breast or ovarian), tumor type (primary or recurrent), or germline mutation (*BRCA1* vs. *BRCA2*).

To identify transcriptomic differences by tumor type, we compared gene expression in primary and recurrent tumors to that of tissue-matched normal controls (**Fig. 3b, c; Supplementary Fig. 8a, b**). Gene expression was highly similar between primary and recurrent

tumors of the same origin; the average $\log_2$ (counts per million) for individual genes was highly correlated between breast ($R^2=0.972$) and ovarian ($R^2=0.965$) primary and recurrent tumors. *ASPM*, assembly factor for spindle microtubules, was highly expressed across all ovarian and breast tumors (**Fig. 3b, c; Supplementary Fig. 8a, b**). Genes with the highest log-fold changes in breast cancer included *CENPF*, *CDK1*, *TOP2A*, and *MKI67* (**Fig. 3b, Supplementary Fig. 8a**). Among the genes with the highest log-fold changes in ovarian cancer were *BIRC5*, *MYBL2*, *RERGL*, *CRISP2*, and *ZCCHC12* (**Fig. 3c, Supplementary Fig. 8b**)⁵⁸.

As the average $\log_2$ (counts per million) for individual genes were similar between breast and ovarian tumors overall ($R^2=0.934$), we combined all *BRCA1/2* mutation-associated tumors to identify the major transcriptomic differences compared to normal tissues. Using GSEA, we found enrichment of 25 Hallmark gene sets (all adj. $p<0.01$, **Fig. 3d, Supplementary Data 8**). The identified gene sets included several pathways also enriched for copy number gains (**Fig. 2g**): $IFN\alpha$ and $IFN\gamma$ signaling, MTORC1 signaling, response to UV light, and $TNF\alpha$ signaling via NF- κ B. Increased expression of these gene sets was also identified in GSEA stratified by tumor type (**Supplementary Fig. 8c, d, Supplementary Data 8**).

To identify transcriptomic trends driving tumorigenesis, we next interrogated genes that were significantly different ($|\log_2(\text{fold change})|>1$ and adj. $p<0.05$) in tumor vs. normal tissue. Hierarchical clustering identified two modules: 1) 4715 genes with low expression in tumors; and 2) 2492 genes with high expression in tumors (**Supplementary Fig. 8e, Supplementary Data 9**). Low expression genes (module 1) were enriched for transcription factor binding motifs from lineage-related transcription factors (Forkhead and Homeobox) and myogenic regulators *MEF2A* and *SRF* (all Fisher score ≥ 7 , Z-score ≥ 10) (**Supplementary Data 9**). Using Gene Ontology (GO), we evaluated the genes in module 1. Among the top hits for transcription factor binding sites lost in tumors, we identified TEF-3, a transcription factor for Hippo signaling ($p=1.8\times 10^{-30}$); and PARP, a predicted PARP1 binding motif ($p=6.6\times 10^{-29}$)⁵⁹ (**Fig. 3e, Supplementary Data 9**). We also used GO to establish trends for genes with increased

expression in tumors (module 2) and found enrichment of terms for mitosis and cell cycle (**Fig. 3f, Supplementary Data 9**). In agreement with GSEA results, we also found enrichment of several immune activation terms in tumors (all $p < 1 \times 10^{-7}$) (**Fig. 3f, Supplementary Data 9**).

PARP1 expression was significantly increased in breast and ovarian recurrences compared to normal tissue ($\log_2FC = 1.62$, adj. $p = 2.52 \times 10^{-6}$ and $\log_2FC = 9.44$, adj. $p = 1.58 \times 10^{-6}$) (**Fig. 3b, c**). *PARP1* expression was also increased in breast primary tumors ($\log_2FC = 1.43$, adj. $p = 3.26 \times 10^{-5}$) (**Supplementary Fig. 8a**). Primary and recurrent ovarian tumors both showed increased expression of *MYBL2*, for which *PARP1* is a putative coactivator (**Fig. 3c, Supplementary Fig. 8b**)⁶⁰. Primary and recurrent breast tumors showed increased expression of *MKI67* (Ki67), which has been positively correlated with *PARP1* expression in other breast tumor cohorts (**Fig. 3b, Supplementary Fig. 8a**)⁶¹. Overall, *PARP1* expression in all tumors exceeded the mean *PARP1* expression in normal tissue samples, an effect that did not depend on the presence of a *PARP1* gain or amplification ($p = 3.17 \times 10^{-7}$ and $p = 5.01 \times 10^{-5}$ for tumors with and without *PARP1* gain or amplification, respectively, compared to normal tissue; **Supplementary Fig. 9**).

Analysis of gene fusions in primary and recurrent tumors

We investigated whether gene fusions could be potential drivers in *BRCA1/2* mutation-associated tumors. We limited our analysis to previously described gene fusions with >5 junction spanning reads (range 5-228 reads) found in ≥ 3 patients. Six of 54 (11%) tumors had fusions involving genes for immunoglobulin light and/or heavy chains (IGL@, IGH@) (**Fig. 4a, b; Supplementary Data 10**). Fusions of *MALAT1*, a lncRNA associated with poor survival in metastasis in several tumor types, were recently observed at high frequency (13/18, 72%) in ovarian cancer and TNBC patients after PARPi treatment^{62,63}. We found *MALAT1* fusions in one breast and two ovarian (3/54, 5.5%) tumors. We did not identify any fusions involving efflux pump *ABCB1*, which have been previously associated with multidrug resistance^{17,25}.

Differential BRCA2 isoform usage in primary and recurrent tumors

As mRNA isoform switching events are a well-recognized phenomenon in cancer, we evaluated differential transcript usage among primary tumors, recurrent tumors, and normal tissue samples⁶⁴. We identified an isoform switching event between recurrences and normal tissue, involving two protein-coding *BRCA2* transcripts. Overall, *BRCA2* gene expression was significantly higher in breast and ovarian recurrent tumors compared to normal breast and fallopian tube tissue (adj. $p=7.13 \times 10^{-6}$, **Fig. 4c**). The increase in *BRCA2* expression was also observed in breast and ovarian recurrences when compared separately to matched normal tissue (both $\log FC \geq 1.79$, adj. $p < 1 \times 10^{-3}$). We found that recurrent tumors preferentially expressed a shorter transcript (ENST00000380152.3 or *BRCA2-001*, hereafter referred to as “*BRCA2-001/Short*,” $q=1.7 \times 10^{-4}$). In contrast, normal samples favored a longer transcript (ENST00000544455.1 or *BRCA2-201*, hereafter referred to as “*BRCA2-201/Long*,” $q=6.0 \times 10^{-4}$) (**Fig. 4d**). *BRCA2-201/Long* has a longer 3' UTR, and is predicted to be sensitive to nonsense-mediated decay (NMD); whereas with a shorter 3' UTR, *BRCA2-001/Short* is predicted to be NMD-insensitive (**Fig. 4e**)⁶⁵. The differing region of the *BRCA2* 3' UTR contains binding sites for RNA binding proteins ELAVL1 (HuR) and PABPC1 (both FDR $q < 0.01$, **Supplementary Fig. 10a**), based on RNA immunoprecipitation sequencing (RIP-seq) experiments in human B cells (GEO Accession GSE35585). The presence of HuR binding sites in pre-mRNA introns and 3' UTR has been directly associated with increased mRNA stability⁶⁶. PABPC1 has been to shown to inhibit NMD in mRNA transcripts with long 3' UTRs^{67,68}. Of note, expression of *PABPC1* and *ELAVL1* genes was significantly higher in tumors compared to normal tissue (module 2, **Supplementary Data 9**).

We observed that expression of each *BRCA2* isoform was mutually exclusive in 64 of 66 (97%) RNA-seq samples, with significant association between sample group (normal, primary tumor, or recurrence) and isoform usage ($p=0.001$, **Fig. 4f**, **Supplementary Data 11**). Across all

tumor samples, expression of *BRCA2-001/Short* was slightly more common in breast tumors (10/20; 50%) than in ovarian tumors (14/34; 42%). *BRCA2-001/Short* was expressed in 8/11 (73%) breast recurrences compared to 12/23 (52%) ovarian recurrences. In general, *BRCA2-001/Short* was expressed more frequently in *BRCA1* mutation-associated tumors (21/42; 50%) than in *BRCA2* mutation-associated tumors (3/12; 25%). This difference was largest within the recurrence group; *BRCA2-001/Short* was expressed in 18/27 (67%) *BRCA1* mutation-associated recurrences compared to 2/7 (29%) *BRCA2* mutation-associated recurrences, but non-significant. There were no apparent associations with treatment history or LOH status.

To validate these results, we assessed *BRCA2* isoform expression in an independent cohort of 42 *BRCA1/2* mutation-associated primary breast and ovarian tumors (**Supplementary Data 1, Supplementary Fig. 10b**). As in our discovery set, primary tumors in the validation cohort more commonly expressed *BRCA2-201/Long* (33/42; 79%, **Supplementary Data 11**). Expression of the shorter isoform *BRCA2-001/Short* was similarly uncommon in breast vs. ovarian primary tumors (28%, 29%) and in *BRCA1* vs. *BRCA2* mutation-associated primary tumors (28%, 31%).

Next, we tested whether expression of *BRCA2-001/Short* affected overall survival across 67 *BRCA1/2* mutation carriers (from discovery and validation cohorts) for whom we had survival data and RNA sequenced from at least one tumor (**Supplementary Data 11**). Survival time for breast cancer patients did not have a significant association with ER status ($p=0.16$), age at diagnosis ($p=0.47$), tumor stage at diagnosis ($p>0.74$), or patient recurrence status ($p=0.58$) (**Supplementary Data 11**). Out of 34 breast cancer patients assessed, those with *BRCA2-001/Short* expression in any tumor had significantly worse overall survival compared to patients whose tumors did not express this alternative isoform ($p=0.017$, HR=2.535, 95% CI 1.179-5.45) (**Fig. 4g**). Median overall survival was nearly three years shorter for patients whose tumor(s) expressed *BRCA2-001/Short* (87 months, 95%CI 71.2 - 121) compared to those whose tumors did not (121 months, 95%CI 100.0 – 159). Expression of this shorter *BRCA2* isoform was not

correlated with overall survival in 33 ovarian cancer patients after adjustment for patient recurrence status (**Supplementary Fig. 10c**).

Analysis of DNA Damage Response and Immune Checkpoint Proteins

We next assessed whether *PARP1* copy number gains and mRNA expression translated to high PARP1 protein expression. We performed immunohistochemistry (IHC) for PARP1 on three tissue microarrays (TMAs) containing 23 primary and recurrent *BRCA1/2* mutation-associated tumors from our sequencing cohort (**Supplementary Fig. 11a-d, Supplementary Data 1, Supplementary Methods**). PARP1 protein was highly expressed across the cohort of tumors (mean H-score 252.5, median H-score 276.4) with no significant differences by tumor type (**Fig. 5a**). As in the RNA-seq results, high PARP1 expression did not depend on the presence of a *PARP1* gain or amplification (**Fig. 5b**). However, the only tumor with a *PARP1* copy number loss had a relatively low H-score of 112. Overall, our results from RNA-seq and IHC suggest that PARP1 is highly expressed in *BRCA1/2* mutation-associated breast and ovarian cancers.

Next, we assessed DNA damage markers and immune checkpoint proteins using the CODEX platform (**Supplementary Fig. 12a-d, Supplementary Table 2, Supplementary Methods**). We found no significant differences in percent of RAD51+ tumor cells between primary and recurrent tumors (**Supplementary Fig. 13a, b**). However, we found that the percent of (tumor and stromal) RAD51+ cells trended higher in tumors expressing *BRCA2-001/Short* compared to those expressing *BRCA2-201/Long* (**Fig. 5c, d**). We found no differences in percent of (tumor and stromal) CTLA-4+ or PD-1+ cells between primary and recurrent tumors (**Supplementary Fig. 13c-f**).

Tumor Evolution

We present the multi-omic results from three patients for an in-depth analysis of the

relationship between primary and recurrent tumors. Patient 20 was a *BRCA1* mutation carrier with an ovarian primary tumor and four recurrences (**Fig. 6a**). Over nine years during which the patient received multiple lines of therapy, the tumor maintained *BRCA1* LOH, copy number gains in *ABCB10/11*, high aneuploidy scores (≥ 13), *MYC* amplifications ($CN \geq 10$) and *PARP1* gains ($CN=4$) (**Supplementary Fig. 3a**). However, *BRCA2* isoform usage evolved, with her primary tumor expressing *BRCA2-201/Long* and recurrences expressing *BRCA2-001/Short*. Recurrence #2 had different genomic features (including LoF mutations in *TP53BP1*, *NF1*, *TSC1*, and *ATM*) suggesting evolution from a distinct clone (**Supplementary Fig 3b**). The patient eventually progressed on PARPi, and a *BRCA1* reversion was identified (**Supplementary Fig. 14a, b**).

Patient 13 was a *BRCA2* mutation carrier with TNBC. Her LOH primary yielded a nonLOH recurrence, suggesting outgrowth of nonLOH sub-clones (**Fig. 6b**). This recurrent tumor was the only one that underwent LOH reversal without a concomitant decrease in HRD score (**Fig. 2b**, left panel). The tumor also showed *BRCA2* isoform switching and a *MYC* amplification. The recurrence had copy number losses and mutations in HR and other DNA damage response genes, including *MUTYH*, *RAD54L*, *RAD52*, and *RAD50*. *RAD52* specifically is required for survival of *BRCA2*-deficient cells⁶⁹. TMB, aneuploidy, and HRD scores all increased upon recurrence, indicating continued accumulation of genomic instability and mutations.

For Patient 6, a *BRCA1* mutation carrier, we evaluated one primary tumor and three recurrences of TNBC (**Fig. 6c**). The recurrent tumor had allele-specific LOH and a *BRCA2* isoform switch (**Supplementary Fig. 2b**). Two recurrences had copy number gains in *ABCB10/11*. Over the course of tumor progression, recurrences underwent loss of DNA damage response and repair genes (by copy number loss or LoF mutation). These deleterious alterations varied across recurrences but clustered into the same pathways.

Discussion

In this study, we evaluated multi-omic features of paired primary and recurrent tumors from 27 *BRCA1/2* mutation carriers, representing 13 breast cancers (primarily TNBC) and 14 ovarian cancers (mostly high-grade serous). Patients received a mix of chemotherapy and radiation, in addition to platinum and PARPi, prior to collected recurrences. We assessed HRD, aneuploidy, somatic mutations, copy number variation, and global transcription in paired *BRCA1/2* mutation-associated breast and ovarian tumors, focusing on features associated with recurrence.

HRD scores and aneuploidy scores did not change significantly with recurrence, consistent with prior findings that HRD scores do not increase upon post-platinum recurrence in *BRCA1/2* mutation-associated ovarian cancer²⁷. We also did not observe a significant increase in TMB. Although a prior observation suggested TMB increases upon recurrence post-platinum therapy in *BRCA1/2* mutation-associated ovarian cancers, the study included only five primary/recurrent ovarian tumor pairs¹⁷. Consistent with prior studies of ovarian cancers, our results suggest that *BRCA1/2* mutation-associated malignancies accrue most of their genomic scarring and mutations early in tumorigenesis, and increasing genomic instability is not a major factor driving recurrence. *TP53* was the only established cancer gene consistently mutated in primary and recurrent tumors, necessary to allow cell cycle progress in genomically unstable tumors^{29,47,70–73}. We found that ER+ *BRCA2* mutation-associated breast tumors, as well as tumors with discordant LOH (primary vs. recurrence), were less likely to have *TP53* mutations.

We found that *PARP1* was gained or amplified across all tumor groups. However, *PARP1* amplifications only reached statistical significance (per GISTIC) in recurrent tumors and were observed most frequently (78%) in breast cancer recurrences. We identified up to 8-fold amplifications centering on a minimal common region of chr1:226,474,131-227,148,258, which fully encompasses only three other genes (*ITPKB*, *PSEN2*, *STUM*) in addition to *PARP1*. In our

and the TCGA cohorts of primary tumors, we found a similar frequency of *PARP1* gains and amplifications across *gBRCA1/2* and sporadic HR-WT breast and ovarian tumors. Variation in methods likely accounts for the slightly different prevalence of *PARP1* amplification reported in cBioPortal for TCGA PanCancer ovarian (53%) and breast (74%) cohorts⁷⁴. We also observed over two-fold increases for *PARP1* expression in primary and recurrent breast tumors, as well as ovarian recurrences, independent of *PARP1* copy number. Our results agree with prior reports of high *PARP1* expression in unselected ovarian and hormone receptor-negative breast cancers^{75–77}. Increased nuclear PARP1 protein has also been correlated with decreased relapse-free and overall survival in breast cancer and AML^{78,79}. In this study, we present the first evidence of *PARP1* overexpression as a near-ubiquitous feature of primary and recurrent *BRCA1/2* mutation-associated tumors, corroborated at the protein level. We propose that *PARP1* copy number gains represent a common (but not exclusive) route to *PARP1* overexpression, and that such gains are common in breast and ovarian tumors regardless of *BRCA1/2* status. Increased levels of PARP1 protein may stoichiometrically dilute PARPi, which trap PARP1/2 complexes on DNA for synthetic lethality^{80–82}. Further, *PARP1* overexpression may promote PARP1-dependent alternative DNA repair pathways and transcriptional programs to enhance tumor cell survival^{83,84}. *PARP1* amplifications and overexpression should be explored as biomarkers of therapeutic response or clinical outcomes in recurrences in patients treated with PARPi^{85–88}.

We identified several significant differences between primary and recurrent tumors with respect to somatic mutations, copy number variation, and gene expression. Several cancer-associated gene sets were exclusively enriched for LoF mutations within breast and ovarian recurrences, including those for apoptosis; glycolysis; P53, TGF β , Notch, and MTORC1 signaling. Breast and ovarian recurrences had distinct copy number profiles, despite having similar HRD and aneuploidy scores. Notably, recurrences of both type had *ABCB10* amplifications, suggesting a shared mechanism of chemoresistance only observed *in vitro*

previously⁵⁶. Hedgehog signaling genes accumulated LoF variants and copy number deletions exclusively within recurrences, suggesting that Hedgehog signaling is required for primary tumorigenesis (as in sporadic breast and ovarian cancers) but dispensable or dysregulated in recurrence^{89,90}. Ultimately, the genetic and transcriptomic features identified as unique to recurrences could represent drivers and therapeutic targets for late-stage *BRCA1/2* mutation-associated tumors.

We also assessed LOH status in matched tumors to determine whether *BRCA1/2* deficiency varied over the course of tumor evolution. Seven pairs of primary and recurrent tumors demonstrated changes in allele-specific LOH status, more commonly in breast cancer. We observed the highest frequency of nonLOH tumors (50% of tumors, 100% of patients) in *BRCA2* mutation-associated breast cancers, as has been previously reported⁴⁷. We also found that LOH discordance was more common in patients with ER+ breast cancer and those without somatic *TP53* LoF mutations. Three breast cancers (one *BRCA1*, two *BRCA2*) developed allele-specific LOH upon recurrence, associated with a significant increase in HRD score. Interestingly, two breast (both *BRCA2*) and two ovarian (both *BRCA1*) tumors underwent an apparent reversal of allele-specific LOH, reflected by lower HRD scores in nonLOH recurrences. Prior studies have suggested LOH reversal occurs in a subset of *BRCA1* mutation-associated ovarian cancers after neoadjuvant platinum treatment^{29,72,91}. We observed the same, and we also identified LOH reversal in *BRCA2* mutation-associated breast cancers treated with non-platinum chemotherapy and radiation. NonLOH recurrences likely arose from clones resistant to DNA damaging agents, although no specific pattern was observed with treatment. Our results suggest that a few tumors evolve towards HR proficiency (as evidenced by nonLOH recurrences) but most evolve towards HR deficiency (as evidenced by recurrences with LOH).

We also assessed transcriptomic differences in primary and recurrent tumors and found two different isoforms of *BRCA2*. *In silico* predictions suggest that *BRCA2-001/Short* and *BRCA2-201/Long* differ in regulation of mRNA stability and/or localization, as dictated by NMD

sensitivity and RNA binding proteins^{64,65}. Expression of *BRCA2-001/Short* was significantly more common in recurrent tumors and further enriched among breast tumors and *BRCA1* mutation carriers. We hypothesize that the isoform switch may represent a mechanism by which *BRCA1* mutation-associated tumors can modulate *BRCA2* transcript stability to retain HR function and enhance tumor survival. In nonLOH *BRCA2* mutation-associated tumors, expression of the shorter isoform could also protect mRNA expression from the wild-type *BRCA2* allele. Our survival analysis offers further support for a correlation between *BRCA2* isoform expression and clinical outcomes. We found that breast cancer patients lacking *BRCA2-001/Short* expression in any tumor (primary or recurrences) had significantly improved overall survival (median 87 vs 121 months), suggesting it could be a prognostic biomarker. We did not observe differences in survival based on isoform usage in ovarian cancer patients. In existing literature, multiple mechanisms have been shown to restore *BRCA2* function in platinum and PARPi resistance, and isoform switching could represent an additional mechanism^{23,24,92–94}. This hypothesis is also supported by increased RAD51 positivity (a readout of *BRCA2* activity) among tumors expressing *BRCA2-001/Short*. Taken together, our results suggest that *BRCA1* mutation-associated tumors, as well as nonLOH *BRCA2* mutation-associated tumors, could benefit from enhanced stability of *BRCA2* mRNA. We therefore propose that *BRCA2* isoform switching represents a novel tumorigenic driver in *BRCA1/2* mutation-associated cancers.

Although this cohort is the largest of paired primary and recurrent *BRCA1/2* mutation-associated tumors published to date, the number of matched primary and recurrent tumors was still relatively small. As a result, we were limited in our ability to identify correlations between genomic or transcriptomic features and treatment, which was quite heterogeneous. The cohort includes few post-PARPi recurrences, as PARPi use was relatively rare at the time of sample collection. We focused our efforts on identifying similarities and differences between paired tumors across the cohort. Since we employed bulk sequencing techniques, we may not fully appreciate tumor heterogeneity associated with recurrent disease. We attempted to use the

544 same FFPE block and samples for multi-omic sequencing, but it was not always possible due to
545 sample availability. For the same reason, we could not assess *BRCA1* promoter methylation,
546 another possible route to biallelic *BRCA1* loss in *BRCA1* mutation-associated tumors. However,
547 we did confirm *BRCA1* expression in all nonLOH *BRCA1* mutation-associated tumors with RNA-
548 seq. Additionally, *BRCA1* promoter methylation does not always abrogate *BRCA1* expression^{95–}
549 ⁹⁷. Further validation of key results (LOH transitions, *PARP1* amplifications, *BRCA2* isoform
550 switching) in an independent cohort of paired primary and recurrent *BRCA1/2* mutation-
551 associated breast and ovarian tumors will be important. Although we were able to determine
552 that the *BRCA2* isoform switching event was associated with decreased survival in two
553 independent sample sets, additional cohorts are needed to confirm, and mechanistic studies
554 remain to be done to confirm functionality.

555 Ultimately, these results suggest key biological features of therapy-resistant recurrences,
556 thereby highlighting novel therapeutic possibilities for *BRCA1/2* mutation carriers with cancer.
557 We identified allele-specific LOH transitions in 25% of patients, underlining the plasticity of
558 *BRCA1/2* status after treatment with DNA damaging agents. These results suggest that that
559 sequencing the most recent tumor or circulating DNA will be most informative in planning
560 personalized treatment. Lastly, we found that *BRCA2* isoform switching may be a novel pro-
561 tumorigenic event that appears in both breast and ovarian recurrences and is associated with
562 reduced breast cancer patient survival. Further studies are needed to assess whether these
563 mechanisms are specific to *BRCA1/2* mutation-associated breast and ovarian cancers, or also
564 observed in the context of other tumor types or other DNA repair defects.

565

Methods

Acquisition of tumor, germline, and normal tissue specimens from *BRCA1/2* mutation carriers

Patients gave informed consent for research use of germline DNA, tumor specimens, and clinical data, under a University of Pennsylvania IRB approved protocol. Eligible patients met the following criteria: (1) diagnosis of breast or ovarian cancer; (2) positive genetic test result for pathogenic germline mutation in *BRCA1* or *BRCA2* from a Clinical Laboratory Improvement Amendments (CLIA)-approved laboratory; (3) available archived germline DNA from blood or saliva; (4) available formalin-fixed paraffin-embedded (FFPE) blocks from the primary tumor; and (5) available FFPE blocks from at least one recurrence matching the primary tumor. Matched recurrent tumor samples were identified using manual review of patient charts and pathology reports, supervised by KNM and SMD. All clinical phenotypic data were collected through manual chart review. Patients 1-27 met all study criteria and comprised the primary/recurrent tumor cohort. An additional four patients (Patients 28-31) lacked an available primary tumor sample (meeting all but criterion 4) and were included only in groupwise RNA sequencing analyses.

We also identified 12 *BRCA1/2* mutation carriers as a control group for RNA sequencing analyses. Eligible patients met the following criteria: (1) positive genetic test result for pathogenic germline mutation in *BRCA1* or *BRCA2* from a CLIA-approved laboratory; (2) no prior history of cancer or cancer treatment; and (3) available FFPE blocks from prophylactic mastectomy or salpingo-oophorectomy surgeries. We extracted and sequenced RNA from normal breast and fallopian tube samples from these patients. Of note, fallopian tube samples were used as controls for ovarian cancer, for which the tissue of origin is the fallopian tube⁹⁸.

Sample acquisition of primary tumor validation cohorts from TCGA and Penn

For validation of *PARP1* copy number gains, we used WES from primary breast and ovarian tumors in TCGA cohorts (**Supplementary Data 7**). Level 1 WES binary alignment map (bam)

files from TCGA Breast and Ovarian cohorts were obtained from Genomic Data Commons (<https://gdc.cancer.gov/>) as previously described^{47,57}. Tumors were excluded from analysis for any of the following reasons: (1) failed QC; (2) somatic *BRCA1/2* loss as indicated by mutation, low expression, or *BRCA1* promoter methylation; or (3) germline or somatic mutations in genes related to homologous recombination (see **Supplementary Data 7**). TCGA tumors were assessed for these mutations, and grouped in g*BRCA1/2* or HR-WT groups, as previously described and visualized in **Supplementary Fig. 6a**^{47,57}.

For validation of *BRCA2* isoform switching, we evaluated an independent cohort of 42 primary breast and ovarian tumors from patients with *BRCA1/2* mutations, also consented under the University of Pennsylvania approved IRB protocol. Eligible patients had a diagnosis of breast or ovarian cancer with available FFPE tumor tissue and positive genetic test result for pathogenic germline mutation in *BRCA1* or *BRCA2* from a CLIA-approved laboratory. RNA extraction, sequencing, and analyses were identical to those used for primary/recurrent cohort samples.

Pathological review of FFPE specimens

FFPE tumors were collated and sectioned by the Tumor Tissue and Biospecimen Bank at the University of Pennsylvania, then stained using hematoxylin and eosin. For tumor samples, staining was reviewed and marked by AN to identify sections with $\geq 70\%$ invasive tumor. Normal tissue (RNA sequencing controls) was reviewed for no evidence of tumor. DNA and RNA were extracted from FFPE sections or rolls of 5-10 μ m thickness. RNA extractions were performed within one week of sectioning to minimize degradation.

Whole exome and targeted sequencing of tumor and germline DNA

DNA was extracted from FFPE using standard laboratory deparaffinization, proteinase K digestion, and ethanol precipitation. Germline DNA was extracted from whole blood or saliva.

Germline DNA was extracted from whole blood using sucrose-based lysis of erythrocytes, followed by proteinase K digestion and ethanol precipitation from leukocytes. Germline DNA was extracted from saliva samples using Oragene kits (DNA Genotek).

All DNA was sheared for library preparation using a Covaris sonicator. Tumor DNA libraries were prepared using the NEBNext FFPE Repair mix and NEBNext Ultra II DNA library prep kit (New England Biolabs), per manufacturer's instructions. Germline DNA libraries were prepared using the NEBNext Ultra DNA library prep kit (New England Biolabs), per manufacturer's instructions. DNA libraries were pooled and hybridized using SureSelect Target Enrichment System for Illumina Multiplex Sequencing (Agilent) and associated protocols. For WES, tumor and germline libraries were hybridized to SureSelect All Exon v5, SureSelect All Exon v6+COSMIC, and SureSelect All Exon v7 captures (Agilent). For targeted sequencing, tumor and germline libraries were hybridized to a custom capture, which largely utilized baits from the SureSelect All Exon platform (Agilent; see **Supplementary Table 1** for gene list). DNA samples, libraries and hybridization pools were quantified using a Qubit (ThermoFisher) and fragment size was determined using a BioAnalyzer 2100 (Agilent). WES was performed using an Illumina HiSeq 4000 and targeted sequencing was performed using an Illumina NovaSeq 6000. All sequencing was performed with 150 paired-end reads by the University of Pennsylvania Next Generation Sequencing Core.

RNA sequencing of tumor and normal RNA

Tumor RNA was extracted from the same FFPE tumor blocks from which DNA was extracted. Tumor and normal RNA samples were extracted from FFPE with the RNeasy FFPE kit (Qiagen). To preserve RNA integrity, RNA samples were extracted within one week of sectioning and stored at -80°C. We used the TruSeq RNA Exome platform (Illumina) for library preparation and hybridization capture, per manufacturer protocols. Sequencing was performed

with 150 paired-end reads using an Illumina HiSeq 4000 at the University of Pennsylvania Next Generation Sequencing Core.

Bioinformatic analysis of DNA and RNA sequencing

Fastq files from whole exome and targeted sequencing were aligned to the hg19 build of the human genome using the Burrows-Wheeler Aligner (BWA v.0.7.17-r1188)⁹⁹. The resulting bam files were processed according to Genome Analysis Toolkit (GATK v3.7) best practices (picardtools v2.20.7)¹⁰⁰. WES achieved a mean depth of 98x in tumor samples (median depth 87x) and 93x in germline samples (median depth 94x). Targeted sequencing achieved a mean depth of 332x in tumor samples (median depth 304x) and 317x in germline samples (median depth 316x).

Fastq files from RNA sequencing were aligned using STAR aligner (v2.7.2a) and gene annotations from the GENCODE Human Release 19 reference assembly (https://www.gencodegenes.org/human/release_19.html)¹⁰¹. RNA sequencing achieved a mean depth of 67.6 million reads (median 55.6 million reads) for tumor and normal samples.

Alignment and quantification of transcripts was performed using StringTie (v2.1.3b) to generate abundance files in Ballgown readable format¹⁰². We used Tximport (v1.16.1) to quantify transcript abundance from t_data.ctab files, generating "lengthScaledTPM" counts by gene¹⁰³. Next, we used EdgeR (v3.30.3) to generate, filter, and normalize counts per million (cpm)¹⁰⁴.

Briefly, gene-level transcripts per million were used to create three separate DGELists: one for all samples, one for ovarian tumor and normal fallopian tube samples, and one for breast tumor and normal breast samples. All DGELists were filtered to include only genes for which transcripts were detected in ≥ 2 or ≥ 3 samples, depending on the size of the smallest biological group. Filtered DGELists were then normalized using the trimmed mean of M values (TMM) method¹⁰⁵. Filtered, normalized DGELists were used to generate filtered, normalized cpm for each of the three comparisons. We assessed the distribution of filtered, normalized cpm across

the cohort to identify any outlying samples (**Supplementary Fig. 7a**). Filtered, normalized cpm were used for all cohort-level and tumor-specific RNA-seq analyses, except for detection of isoform switching.

Identification, filtering, and analysis of somatic variants

We used a union of MuTect2 (v4.1.8.1), Strelka2 (v2.9.2), VarDictJava (v1.5.1), and VarScan2 (2.4.4) to call somatic variants from whole exome and targeted sequencing of matched tumor and germline DNA^{106–109}. VarDictJava and VarScan2 were used to call and filter germline variants^{106,109}. Each patient's germline variant calls were checked to ensure detection of the same pathogenic *BRCA1/2* variant reported in their genetic test results. All variants were annotated with ANNOVAR (October 2019 release)¹¹⁰. We limited our analysis of mutational signatures to variants called by MuTect2 and with alternative allele depth of ≥ 10 reads. Mutational signatures were assessed separately for breast and ovarian tumor cohorts using deconstructSigs (v1.8)^{42,111}.

With the exception of mutational signatures, all downstream analyses were restricted to exonic variants that were called by and passed filtering for at least one caller and had alternative allele read depth of $\geq$ five reads. Tumor mutational burden (TMB) was calculated for each tumor using the following equation: $TMB = \frac{(n \text{ somatic, exonic nonsynonymous SNVs} + \text{indels})}{\text{size of WES capture (Mbp)}}$ ¹¹². In order to exclude common SNPs from further analyses, we next removed somatic variants with frequency in any population (PopFreqMax) ≥ 0.01 ¹¹⁰. Using the filtered variant set, we identified significantly-mutated genes within primary and recurrent tumor groups using MutSigCV (v1.3)⁴⁴.

Next, we identified gain of function (GoF) and loss of function (LoF) mutations in individual genes. LoF mutations were identified as those that met one of the following criteria: (1) frameshift mutation, (2) nonsense mutation, or (3) nonsynonymous SNV predicted to be pathogenic by REVEL score >0.5 ¹¹³. GoF mutations were defined as those that met all of the

following criteria: (1) not a LOF mutation; (2) documentation in Catalog of Somatic Mutations in Cancer (COSMIC v84, <https://cancer.sanger.ac.uk/cosmic>); (3) occurring in a Tier 1 oncogene as defined by the Cancer Gene Census (CGC, <https://cancer.sanger.ac.uk/census>); and (4) matching the mutation type determined to be oncogenic for that gene by CGC.

Pathway analysis was performed on LoF variants using Gene Set Enrichment Analysis (GSEA) Preranked (v4.0.2 for Windows)⁴³. Briefly, genes were ranked based on frequency of LoF mutations in primary and recurrent tumor groups separately. A gene could be counted as mutated only once per patient within each tumor group. This approach was used to prevent skewing of results by patients with multiple recurrences or by individual tumors accruing distinct LOF mutations in the same gene. Ranked gene lists were used as input for GSEA Preranked with Hallmark gene sets (1000 permutations). Gene set enrichment was considered significant using a cutoff of FDR<0.25.

Genome-wide and gene-level analysis of copy number variation

We performed allele specific copy number analysis using Sequenza (v3.0), to identify segments of copy number variation (CNVs) as well as ploidy and cellularity estimates⁴⁶. Segments were used to calculate homologous recombination deficiency (HRD) and aneuploidy scores, using custom scripts and as previously described (<https://github.com/maxwell-lab/HRDex>)^{47,57}. We used GISTIC2.0 (v2.0.23, Gene GISTIC mode, with default cutoffs) to identify significant CNVs at 90% confidence in tumor cohorts⁴⁸. Genes were highlighted from segments with residual $q \leq 0.05$. BEDtools (v2.28.0) was used to compute Jaccard statistics for similarity between CNV sets, limited to segments of $\geq 50\%$ reciprocal overlap¹¹⁴.

CNV segments were binned based on total (integer) copy number using the following convention: CN=0, Deletion; CN=1, Loss; CN=2-3, Neutral; CN=4-5, Gain; CN ≥ 6 , Amplification. Binned segments were annotated with genes using AnnotSV (v2.3)¹¹⁵. Genes were considered to be lost or deleted if >50% of the locus was encompassed by a copy number loss or deletion.

Conversely, genes were only considered to be gained or amplified if 100% of the locus was encompassed by a gain or amplification. In the case of a gene spanning several different segments of CNV, the copy number of the gene was reported as the minimal copy number of the entire locus.

For pathway analysis of genes subject to copy number gains, genes were ranked based on frequency of their presence in a segment of gain or amplification, within primary and recurrent tumor groups. Similar to our GSEA Preranked analysis of variants (see above), a gene could only be counted as gained once per patient per tumor group. We then repeated this process to rank genes again based on frequency of copy number loss or deletion by tumor group. Ranked gene lists of “gained” genes and “lost” genes were used as input for GSEA Preranked with Hallmark gene sets (1000 permutations)⁴³. Gene set enrichment was considered significant using a cutoff of FDR<0.25.

Detection of *BRCA1/2* Biallelic Loss

We first checked whether *BRCA1/2* biallelic loss occurred via secondary somatic mutations as detected by whole exome or targeted sequencing. Specifically, we determined whether LoF mutations in *BRCA1/2* were present at an alternative allele fraction of ≥ 0.25 . This cutoff reflected whether pathogenic secondary mutations were present in enough of the tumor to facilitate loss of the wild-type *BRCA1/2* allele. Next, we assessed loss of the wild-type *BRCA1* or *BRCA2* allele via allele-specific copy number variation from Sequenza. Briefly, we used tumor and germline bam files from WES for Sequenza input. Zygosity was estimated for all segments of somatic copy number variation based on the number of A (major) and B (minor) alleles detected. Segments with zero B alleles (only A alleles present) were deemed to have undergone LOH. Conversely, if at least one B allele was detected, a segment was considered nonLOH (heterozygous). Tumors were deemed to have undergone loss of heterozygosity (LOH) if the segment containing their pathogenic *BRCA1/2* germline mutation had LOH. When

possible, we confirmed proficient *BRCA1/2* expression in nonLOH tumors with RNA sequencing. For patients with LOH transitions, we assessed the allele fraction of all *BRCA1/2* germline variants (snps and the pathogenic variant) across tumors to further interrogate LOH calls.

Differential gene expression and gene fusion analysis

Filtered, normalized log₂cpm were used to assess sample relatedness. We performed hierarchical clustering (maximum distance, average agglomeration) to generate dendrograms. Dendrograms and principal components analysis (PCA) plots were generated using the stats package in base R (v4.0.2). Between-group fold changes were computed as the difference in average log₂cpm for a given gene between groups. Using log₂cpm fold changes as input, we ran GSEA with Hallmark gene sets using clusterProfiler (v3.16.1) and msigdb (v7.2.1).

We next used the limma R package (v3.44.3) to model mean-variance separately for all-tumor, ovarian-only, and breast-only DGELists. Bayesian statistics were extracted from the resulting linear models to generate adjusted p-values (Benjamini-Hochberg method) and log₂(fold change) by gene for each comparison. Significantly altered genes were extracted using decideTests in limma (global method, adj.p<0.05, |log₂(fold change)|>1).

Significantly altered genes for all-tumor comparisons were clustered into two gene modules (see **Supplementary Data 9**). Hierarchical clustering of genes was based on Pearson coefficients from pairwise correlations (complete method). Hierarchical clustering of samples was based on Spearman coefficients from pairwise correlations (complete method). A heatmap was generated from the resulting dendrograms using the gplots package (v3.1.0). Gene Ontology was performed and plotted for each gene module separately, using gProfiler2 (v0.2.0). Enrichment of transcription factor binding motifs was performed using oPOSSUM (v3.0) Single Site Analysis (<http://opossum.cisreg.ca/oPOSSUM3/>)¹¹⁶. OPOSSUM analysis was conducted

against JASPAR vertebrate core profiles with cutoffs for conservation score >0.40 , oPOSSUM Fisher score ≥ 7 , and oPOSSUM Z-score ≥ 10 .

Gene fusions were identified using STAR-Fusion (v1.7.0) and plots were generated using FusionInspector (v2.1.0)¹¹⁷. We limited our analysis of gene fusions to those with ≥ 5 junction-spanning reads.

Assessment of differential transcript usage

Abundance was re-quantified from bam files per recommendations in the Stringtie manual for novel isoform detection (<http://ccb.jhu.edu/software/stringtie/index.shtml?t=manual>). Briefly, starting from initial bam files, we ran Stringtie in merged mode (using GENCODE Human Release 19 as reference) to generate a merged gene transfer format (gtf) file for the cohort. We quantified abundance from initial bams using Stringtie in Ballgown mode and the merged gtf file as reference. Differential transcript usage was assessed, and switch plots generated, using IsoformSwitchAnalyzer (v1.10.0). The validation cohort of primary tumors was analyzed for isoform switching in an independent use of this workflow. RIP-seq plots were generated using UCSC Genome Browser (hg19 assembly) with the ENCODE RNA Binding Proteins track corresponding to GEO Accession GSE35585. The track pictured for ELAVL1/HuR in **Supplementary Fig. 10a** shows a peak with coordinates chr13:32,973,328-32,973,692, (average enrichment 20.30, FDR $q=4.2 \times 10^{-3}$). The track pictured for PABPC1 in **Supplementary Fig. 10a** shows a peak with coordinates chr13:32,973,450-32,973,693 (average enrichment 31.59, FDR $q=3.7 \times 10^{-3}$).

Statistical analysis

All paired comparisons of continuous variables (HRD scores, aneuploidy scores, TMB) were tested for significance with two-sided Wilcoxon signed rank tests ($\alpha=0.05$). For patients with multiple recurrences, one recurrence was chosen at random for pairwise comparisons

(such that $n=27$ for each group). In the primary/recurrent cohort, groupwise differences in average *PARP1* copy number, *PARP1* mRNA expression, and PARP1 H-score were assessed by Kruskal-Wallis test, with significant results followed by Dunn's test with Bonferroni correction ($\alpha=0.05$). Groupwise differences in CODEX datasets were assessed by two-sided Wilcoxon rank sum test ($\alpha=0.05$). In TCGA cohorts, groupwise differences in average *PARP1* copy number were assessed by two-sided t-tests ($\alpha=0.05$). *PARP1* copy number is reported for primary/recurrent and TCGA tumors in **Supplementary Data 6**.

The adjusted p-value for total *BRCA2* expression was generated by linear modeling with limma (see above). The adjusted p-values for *BRCA2* isoform usage were computed using DEXSeq within isoformSwitchAnalyzer. Association between sample type and *BRCA2* isoform usage was determined by Mantel-Haenszel chi-squared test with continuity correction ($\alpha=0.05$, 1df).

Overall survival was defined as time from diagnosis to death or last follow-up. Survival metrics were calculated based on manual chart review of the electronic health record for each patient. The Penn Medicine electronic health record is indexed routinely against the National Death Index, with a time delay of approximately one year. The survival analysis was limited to 67 patients for whom we collected survival data and RNA-seq from at least one tumor (see **Supplementary Data 11**). For the 33 ovarian cancer patients, we first used a Cox proportional hazards model to control for patient age at diagnosis, tumor stage at diagnosis, and disease recurrent status as confounding variables. We used the same confounding variables, in addition to ER status, in multivariate Cox proportional hazards analysis of the 34 breast cancer patients. In ovarian cancer patients, the only confounding variable associated with survival was disease recurrent status (a logical variable indicating whether the patient's disease eventually recurred). Therefore, we simplified our ovarian Cox proportional hazards model to include recurrent status as the only confounding variable. In breast cancer patients, 0/4 confounding variables were significantly associated with survival, so these were discarded in favor of a univariate analysis.

Cox proportional hazards analyses were performed and plotted using the Survival (v3.2.7) and
Survminer (v0.4.8) R packages. P-values reported on the plots are those corresponding to the
isoform expression term itself. P-values for the models were tested for significance by Wald test
($\alpha=0.05$, 1 df for breast, 2 df for ovarian).

Data availability

Tumor WES and RNA-seq data, as well as RNA-seq from normal tissue samples, have been
deposited in NCBI Sequence Read Archive SUB9821849 (SRA,
<https://www.ncbi.nlm.nih.gov/sra>). Germline WES was not made publicly available to protect
participant privacy but can be made available upon request from the authors. TCGA data is
accessible via the Genomic Data Commons from the National Center Institute:
<https://gdc.cancer.gov/>. All other data are included in Supplementary Information files or will be
made available by the authors upon request.

Code availability

All code used for the study is available at <https://github.com/nathanson-lab/BRCA-PrimaryRecurrent>. Additional code for calculating HRD and aneuploidy scores is available at
<https://github.com/maxwell-lab/HRDex>.

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Author contributions

J.B.S., K.N.M., and K.L.N. conceived the study. J.B.S. performed the majority of DNA/RNA sequencing, bioinformatic analyses, and figure preparation (with exceptions below). J.B.S. and K.L.N. primarily wrote the manuscript. D.P. performed all CODEX staining and imaging, and also prepared associated figures. B.W. performed sequence alignment, variant calling, and Sequenza analysis. J.P. assisted with calculation of HRD and aneuploidy scores and advised on numerous statistical analyses. K.D'A. performed DNA extraction and whole exome sequencing of germline specimens. A.H. and C.R. assisted with clinical data curation. A.A.K. contributed to sample collection and protocols for RNA sequencing. K.N.M. optimized sequencing protocols and initiated tumor collection. J.S.S. performed DNA and RNA extractions and library preparations. W.Z., A.L.G., V.S., J.B., K.N.M., S.M.D., and K.L.N. all assisted in

identifying patients and specimens for the cohort. M.F. supervised collection and sectioning of FFPE specimens. A.N. performed pathological review of FFPE specimens, including IHC grading. S.M.D. offered clinical expertise. All authors contributing to editing the manuscript.

Competing interests

S.M.D. has received honoraria from AstraZeneca.

Main Text Figures and Figure legends

Figure 1. Integrated analysis of somatic mutations by whole exome and targeted sequencing. A. Phenotype, *BRCA1/2* allele-specific loss of heterozygosity (LOH), *RB1* and *PTEN* status, and somatic mutations found in 67 paired primary and recurrent tumors (from 27 patients) sequenced by whole exome sequencing (WES, n=67) and high-depth targeted sequencing (n=44). Display is limited to genes with ≥ 1 mutation with alternative allele fraction ≥ 0.05 from targeted sequencing. Tumors are displayed in chronological order by patient, with the primary tumor at the top and latest recurrence at the bottom. B. Mutational signatures derived from WES mutations. C. Hallmark Gene Sets enriched in loss of function (LoF) mutations for primary and recurrent tumor groups (all FDR $q < 0.25$). D. MutSigCV results (*FDR $q < 0.05$). E. Variants contributing to MutSigCV results in D, by tumor type.

Figure 2. Genome-wide and gene-specific copy number variation. A. Allele specific loss of heterozygosity (LOH) of *BRCA1/2* in 67 paired primary and recurrent tumors, with treatment exposure. Tumors are displayed in chronological order by patient, with the primary tumor at the top and latest recurrence at the bottom. B. Comparison of HRD score for tumors with a change in LOH status from primary to recurrence. For one patient (Patient 6), who had multiple recurrences with LOH, the first recurrence is displayed. C. GISTIC qplot for 90% confidence interval amplifications in primary tumors. D. GISTIC qplot for 90% confidence interval amplifications in recurrences. For C and D, all highlighted genes have residual $q \leq 0.05$. E. *PARP1* copy number by tumor in primary/recurrent cohort. Groupwise differences in average copy number were determined by Kruskal-Wallis test, followed by Dunn's test with Bonferroni correction ($\alpha=0.05$, $**p<0.01$). F. Segments of copy number gains and amplifications encompassing *PARP1*, by patient. For E and F, total copy number (Sequenza) was binned as follows: Deletion, CN=0; Loss, CN=1; Neutral, CN=2-3; Gain, CN=4-5; Amplification, CN $\geq$ 6. G. Hallmark Gene Sets enriched in primary and recurrent tumor gains and amplifications (CN $\geq$ 4, top) vs. losses and deletions (CN $\leq$ 1, bottom) (all FDR $q<0.25$).

Terms Down in Tumor

id	source	term_id	term_name	term_size	p_value
1	GO:MF	GO:004930	G protein-coupled receptor activity	840	6.3e-40
2	GO:MF	GO:004888	transmembrane signaling receptor activity	1262	7.4e-46
3	REAC	REAC:R-HSA-418555	G alpha (s) signalling events	535	7.5e-50
4	REAC	REAC:R-HSA-388396	GPCR downstream signalling	1100	6.4e-41
5	TF	TF:M07270_1	Factor: TEF-3; motif: GNTATTTTT; match class: 1	4545	1.8e-30
6	TF	TF:M02027_1	Factor: PARP; motif: TTTTCYN; match class: 1	12305	6.6e-29
7	MIRNA	MIRNA:hsa-miR-335-5p	hsa-miR-335-5p	2570	1.7e-43

Terms Up in Tumor

id	source	term_id	term_name	term_size	p_value
1	GO:BP	GO:0007049	cell cycle	1882	2.0e-24
2	GO:BP	GO:0098813	nuclear chromosome segregation	268	9.3e-18
3	GO:BP	GO:0042119	neutrophil activation	500	4.5e-09
4	GO:BP	GO:0036230	granulocyte activation	506	8.2e-09
5	GO:BP	GO:0043312	neutrophil degranulation	484	7.4e-09
6	GO:BP	GO:0034097	response to cytokine	1238	1.7e-08
7	GO:BP	GO:0002274	myeloid leukocyte activation	668	4.1e-08
8	GO:BP	GO:0006260	DNA replication	283	9.0e-17
9	MIRNA	MIRNA:hsa-miR-193b-3p	hsa-miR-193b-3p	846	3.2e-23

Figure 3. Global transcriptomic programs detected by RNA sequencing. A. 66 samples used for RNA sequencing. Tumors are displayed in chronological order by patient, with the primary tumor at the top and latest recurrence at the bottom. Patients 28-31 had recurrent tumors only. Patients 32-43 were *BRCA1/2* mutation carriers with no prior history of cancer or cancer treatment; their samples are normal tissue from breast and fallopian tube. B. Differential gene expression in breast tumor recurrences vs. normal breast tissue. C. Differential gene expression in ovarian tumor recurrences vs. normal fallopian tube tissue. For B and C, a positive $\text{Log}_2(\text{fold change})$ indicates genes with increased expression in recurrent tumors. Adjusted p values were computed based on linear modeling of mean-variance trends (limma). D. Hallmark Gene Sets enriched in genes with increased expression in primary and recurrent breast and ovarian tumors compared to normal breast and fallopian tube tissue (all adj.p<0.05). E. Gene ontology terms enriched in genes with decreased expression in tumor vs. normal comparisons (module 1 [yellow] in **Supplementary Fig. 8e**). F. Gene ontology terms enriched genes with increased expression in tumor vs. normal comparisons (module 2 [blue] in **Supplementary Fig. 8e**).

Figure 4. Gene fusions and isoform switching detected by RNA sequencing. A. Clinical characteristics of patients in which *IGH-@*, *IGL-@*, and *MALAT1* fusions were identified. Tumors are displayed in chronological order by patient, with the primary tumor at the top and latest recurrence at the bottom. “1 fusion” refers to a translocation with one other gene. B. Example of *MALAT1-IGH-@* gene fusion IGV tracks from patient 9. Junction reads (red, middle track) represent split RNA-seq reads used to map the fusion breakpoint. Spanning reads (black, bottom track) represent paired-end reads of fragments that span, but do not directly overlap, the fusion breakpoint. C. Expression of total *BRCA2* (all isoforms) with 95% CI in recurrences vs. normal samples. D. *BRCA2* isoform usage (*BRCA2* isoform expression normalized to total *BRCA2* expression) in recurrences vs. normal samples; q values computed using DEXSeq within isoformSwitchAnalyzer. E. *BRCA2* isoforms involved in isoform switching event. F. *BRCA2* isoform expression by sample and group for entire RNA sequencing cohort. G. Survival curve for patients that expressed *BRCA2-001/Short* in any (primary or recurrent) breast tumor compared to those that did not. Survival proportions and p-value were calculated using a Cox proportional hazards model tested for significant associations with ER status, age at diagnosis, tumor stage at diagnosis, and patient recurrent status ($\alpha=0.05$, see **Methods**).

Figure 5. Validation of prior results by PARP1 and RAD51 protein expression. A. PARP1 nuclear positivity (average tumor H-score) by tumor type, for 23 tumors in tissue microarrays. B. PARP1 nuclear positivity (average tumor H-score) by *PARP1* copy number status across all tumors. For A and B, groupwise differences were determined by Kruskal-Wallis test, followed by Dunn's test with Bonferroni correction ($\alpha=0.05$). C. Percent of RAD51+ cells in primary (n=3) and recurrent (n=4) breast tumors based on *BRCA2* transcript usage. D. Percent of RAD51+ cells in primary (n=6) and recurrent (n=7) ovarian tumors based on *BRCA2* transcript usage. For C and D, groupwise differences were assessed by two-sided Wilcoxon rank sum test ($\alpha=0.05$). Tumors without RNA-seq data (n=2) or expressing both *BRCA2* isoforms (n=1) were excluded from this analysis.

a

b

c

Figure 6. Evolution of *BRCA1/2* mutation-associated breast and ovarian tumors in three patients. A. Genetic and clinical features in Patient 20, a *BRCA1* mutation carrier with ovarian cancer. B. Genetic and clinical features in Patient 13, a *BRCA2* mutation carrier with breast cancer. C. Genetic and clinical features in Patient 6, a *BRCA1* mutation carrier with breast cancer. For A-C, display is limited to tumors sequenced for this study. Copy number losses refer to genes with total copy number of 0 or 1; copy number gains refer to genes with total copy number ≥ 4 . Loss of function (LoF) mutations reported here are from high-depth targeted sequencing.

1483 **Main Text Tables**

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1485 **Table 1:** Characteristics of primary/recurrent patient cohort and tumors

		Breast	Ovarian	Total
Patients		13	14	27
Tumors				
Primary		13	14	27
Recurrent		18	22	40
Total		31	36	67
Number of Recurrences Collected per Patient				
1		9	10	19
>1		4	4	8
Germline Mutation				
BRCA1		9	10	19
BRCA2		4	4	8
ER Status of Primary Tumor				
ER+		4	N/A	4
ER-		9	N/A	9
Stage at Diagnosis				
I		2	1	3
II		8	3	11
III		2	8	10
IV		0	1	1
Unreported		1	1	2
Treatment Received^{a,b}				
Platinums		3	14	17
PARPi ^c		1	4	5
Chemotherapy		13	14	27
Hormonal Therapy		5	3	8
Radiation		7	3	10
^a Treatments are limited to reflect the number of patients from whom a post-treatment recurrence was collected.				
^b Treatment groups are not mutually exclusive; most patients received multiple treatments.				
^c PARPi = Poly (ADP-ribose) polymerase inhibitor				

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Supplementary Files

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