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Heterozygous germline mutations in breast cancer 1 (*BRCA1*) strongly predispose women to breast cancer. *BRCA1* plays an important role in DNA double-strand break (DSB) repair via homologous recombination (HR), which is important for tumor suppression. Although *BRCA1*-deficient cells are highly sensitive to treatment with DSB-inducing agents through their HR deficiency (HRD), *BRCA1*-associated tumors display heterogeneous responses to platinum drugs and poly(ADP-ribose) polymerase (PARP) inhibitors in clinical trials. It is unclear whether all pathogenic *BRCA1* mutations have similar effects on the response to therapy. Here, we have investigated mammary tumorigenesis and therapy sensitivity in mice carrying the *Brca1^{185stop}* and *Brca1^{5382stop}* alleles, which respectively mimic the 2 most common *BRCA1* founder mutations, *BRCA1^{185delAG}* and *BRCA1^{5382insC}* . Both the *Brca1^{185stop}* and *Brca1^{5382stop}* mutations predisposed animals to mammary tumors, but *Brca1^{185stop}* tumors responded markedly worse to HRD-targeted therapy than did *Brca1^{5382stop}* tumors. Mice expressing *Brca1^{185stop}* mutations also developed therapy resistance more rapidly than did mice expressing *Brca1^{5382stop}* . We determined that both murine *Brca1^{185stop}* tumors and human *BRCA1^{185delAG}* breast cancer cells expressed a really interesting new gene domain–less (RING-less) BRCA1 protein that mediated resistance to HRD-targeted therapies. Together, these results suggest that expression of RING-less BRCA1 may serve as a marker to predict poor response to DSB-inducing therapy in human cancer patients.
BRCA1<sup>185delAG</sup> tumors may acquire therapy resistance through expression of RING-less BRCA1

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Heterozygous germline mutations in breast cancer 1 (BRCA1) strongly predispose women to breast cancer. BRCA1 plays an important role in DNA double-strand break (DSB) repair via homologous recombination (HR), which is important for tumor suppression. Although BRCA1-deficient cells are highly sensitive to treatment with DSB-inducing agents through their HR deficiency (HRD), BRCA1-associated tumors display heterogeneous responses to platinum drugs and poly(ADP-ribose) polymerase (PARP) inhibitors in clinical trials. It is unclear whether all pathogenic BRCA1 mutations have similar effects on the response to therapy. Here, we have investigated mammary tumorigenesis and therapy sensitivity in mice carrying the Brca<sup>185delAG</sup> and Brca<sup>5382stop</sup> alleles, which respectively mimic the 2 most common BRCA1 founder mutations, BRCA1<sup>185delAG</sup> and BRCA1<sup>5382stop</sup>. Both the Brca<sup>185delAG</sup> and Brca<sup>5382stop</sup> mutations predisposed animals to mammary tumors, but Brca<sup>185delAG</sup> tumors responded markedly worse to HRD-targeted therapy than did Brca<sup>5382stop</sup> tumors. Mice expressing Brca<sup>185delAG</sup> mutations also developed therapy resistance more rapidly than did mice expressing Brca<sup>5382stop</sup>. We determined that both murine Brca<sup>185delAG</sup> tumors and human BRCA<sup>185delAG</sup> breast cancer cells expressed a really interesting new gene domain-less (RING-less) BRCA1 protein that mediated resistance to HRD-targeted therapies. Together, these results suggest that expression of RING-less BRCA1 may serve as a marker to predict poor response to DSB-inducing therapy in human cancer patients.

Introduction

Breast cancer is one of the most common malignancies in women, accounting for almost 1 in 3 diagnosed cancers, and it is the second leading cause of cancer death among women in Western countries (1). Five percent to ten percent of all breast cancer cases have a hereditary component, and thirty percent to eighty percent of all hereditary cases are attributable to mutations in the breast cancer 1 or breast cancer 2 (BRCA1 or BRCA2) gene. Germline mutations in the BRCA1 gene predispose women to hereditary breast and ovarian cancer (HBOC), with an 80%–90% lifetime risk of developing breast cancer and a 40%–50% risk of developing ovarian cancer (2).

Germline BRCA1 mutations are scattered throughout the 81-kb-long gene that encompasses 22 coding exons (3). Most of the known pathogenic BRCA1 mutations are predicted to result in premature termination of protein translation and nonsense-mediated mRNA decay (NMD) (4, 5). These mutations include small deletions and insertions that generate frameshifts, single-base substitutions that produce termination codons, and splice site errors (2). Although there are many different BRCA1 mutations, in certain ethnic populations, only a few founder mutations account for almost all BRCA1-associated breast and/or ovarian cancer families. Two founder mutations, BRCA1<sup>185delAG</sup> and BRCA1<sup>5382stop</sup>, account for the vast majority of BRCA1 mutations in the Ashkenazi Jewish population (2, 6). The BRCA1<sup>185delAG</sup> and BRCA1<sup>5382stop</sup> mutations are carried, respectively, by 1% and 0.15% of Ashkenazi Jews (7, 8). The prevalence of these mutations in unselected patients of Ashkenazi Jewish ethnicity who have ovarian cancer was found to be close to 30% and may even exceed 50% in patients with a family history of breast and/or ovarian cancer (9–11).

BRCA1 has been implicated in various cellular processes, including DNA repair, cell-cycle control, and transcriptional regulation (12). In particular, the role of BRCA1 in homologous recombination-mediated (HR-mediated) repair of DNA double-strand breaks (DSBs) appears to be important in the maintenance of genomic stability and tumor suppression (13). Impaired HR also renders BRCA1-deficient cells extremely sensitive to DSB-inducing agents, like platinum drugs (14). In line with this, patients

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with BRCA1-mutated ovarian cancer had a better prognosis after platinum-based chemotherapy than did nonmutation carriers (15–18). More recently, it was also shown that triple-negative breast cancers in BRCA1 mutation carriers were highly sensitive to neoadjuvant cisplatin chemotherapy (19). Moreover, patients harboring breast tumors with a BRCA1-like genomic profile had a significantly greater benefit from high-dose, platinum-based chemotherapy versus conventional chemotherapy than did patients with non-BRCA1-like tumors (20). Also, chemical inhibitors of poly(ADP-ribose) polymerase (PARP), an enzyme involved in DNA single-strand break (SSB) repair, are effective against BRCA-deficient tumors in preclinical models (21–23) and in patients carrying BRCA mutations (24–29). Recently, the clinical PARP inhibitor olaparib (Lynparza) has been approved as maintenance therapy for BRCA mutation carriers with platinum-sensitive ovarian cancer (30). PARP inhibition results in an increased number of DSBs for olaparib (Lynparza) has been approved as maintenance therapy than did patients with non-BRCA1-like tumors. In addition, tumors carrying BRCA1 185delAG mutation carriers with platinum-sensitive ovarian can-
tion, no obvious differences in TFS could be detected between
or knockout of Msh3 (44–47). In order to mimic the BRCA
resistant tumors in preclinical models (21–23) and in patients carrying BRCA mutations (24–29). Recently, the clinical PARP inhibitor olaparib (Lynparza) has been approved as maintenance therapy for BRCA mutation carriers with platinum-sensitive ovarian cancer (30). PARP inhibition results in an increased number of DSBs due to replication fork collapse at SSBs. PARP inhibition is therefore selectively toxic in cells that lack HR-mediated DSB repair, such as BRCA1–2-deficient tumor cells.

The BRCA1 gene encodes for a protein of 1863 aa that con-
tains a highly conserved amino-terminal really interesting new gene (RING) domain and tandem BRCA1 C-terminus domain (BRCT) repeats at its carboxyl terminus (31). The RING domain of BRCA1 is required for stable interaction with BRCA1-associated RING domain 1 (BARD1), and the BRCA1/BARD1 heterod-
imer has E3 ubiquitin ligase activity with the class of UBC5 E2 ubiquitin–conjugating enzymes (32, 33). The observation that BRCA1/BARD1-dependent ubiquitin conjugates occur at DSBs suggests that the BRCA1/BARD1 heterodimer is important for DNA repair and thereby for the tumor-suppressive function of BRCA1 (34). BRCA1 has been reported to interact with numerous other proteins involved in DNA repair, cell-cycle checkpoint control, transcription, and chromatin remodeling, mainly through its BRCT domains (35–40).

Recent studies have shown that not all biochemical activities of BRCA1 are equally important for its role in tumor suppression and therapy response (41). Using genetically engineered mouse models, Shyaka and coworkers showed that loss of BRCA1 E3 ligase activity does not lead to tumor formation, while loss of BRCT phosphoprotein binding does (42). We showed that BRCA1 RING function is essential for tumor suppression, but does not lead to hypersensitivity to homologous recombination deficien-
ty–targeting (HRD-targeting therapy (43). Mouse mammary tumors that express a mutant BRCA1-C61G protein, which lacks a functional RING domain, respond much worse to DSBinducing therapy than do Brca1-null tumors. In addition, tumors carrying the Brca1C61G mutation rapidly develop therapy resistance, while retaining the Brca1 mutation. These data suggest that the mutant BRCA1-C61G protein has some residual activity in the DNA damage response. This may not only hold true for the BRCA1C61G missense mutation, but also for other BRCA1 mutations, and could indicate the existence of differences in therapeutic response and resistance between different BRCA1 mutation carriers.

In the present study, we investigated the effects of the 2 most common BRCA1 frameshift mutations, BRCA1185delAG and BRCA1182delC, on tumor development and therapy response and resistance in genetically engineered mouse models.

**Results**

**Generation of Brca1185stop and Brca15382stop alleles.** In order to mimic the human BRCA1185delAG and BRCA15382delC mutations in mice, we used short, synthetic, single-stranded oligodeoxyribonucleotides to introduce mutations into the genome of mouse embryonic stem cells (mESCs). It has previously been shown that this technique requires (transient) suppression of DNA mismatch repair (MMR) by knockdown of Mlh1 or knockout of Msh2 or Msh3 (44–47). In order to mimic the BRCA1185delAG mutation, we introduced the Brca1185stop mutation into Mlh1-knockdown mESCs by substitution of 3 nucleotides (TCC to AAG), thereby creating an early STOP codon at aa 24 (Figure 1A). We used Msh3-knockout mESCs to insert 4 nucleotides (AGGA) to generate the Brca15382stop mutation, which resulted in premature protein truncation at aa 1713 and closely resembled the human BRCA15382delC mutation (Figure 1B). Brca1185stop and Brca15382stop mutant mESCs were injected into 3.5-day-old C57BL/6J blasto-
cysts to generate chimeric mice. Chimeric mice were mated with FVB female mice, and germline transmission of the mutant alleles was verified by melting-curve genotyping, PCR, and sequencing (Figure 1, C and D, and data not shown).

**Embryonic lethality of homozygous Brca1185stop and Brca15382stop mice.** To determine the effect of the Brca1185stop and Brca15382stop mutations on normal mouse development, we intercrossed heterozygous Brca1185stop or Brca15382stop mice to produce homozy-
ous offspring. No homozygous pups were born (Supplemental Table 1; supplemental material available online with this article; doi:10.1172/JCI7096DS1), indicating that homozygous Brca1185stop or Brca15382stop mutations lead to embryonic lethality. To study at which stage of embryonic development homozygous Brca1185stop and Brca15382stop mice die, embryos were harvested and genotyped at several time points after gestation. Although (resorbed) homozy-
gous Brca1185stop and Brca15382stop embryos could still be recovered from E12.5 to E13.5 (Supplemental Table 1), they were already severely delayed in development at E19.5 compared with WT and heterozygous embryos (Figure 1, E and F).

**Mammary tumor development in K14-Cre Brca1185stop p53fl/fl and K14-Cre Brca15382stop p53fl/fl mice.** To investigate the influence of Brca1185stop and Brca15382stop mutations on tumor develop-
ment, we independently introduced both alleles into the K14-Cre Brca15382stop p53fl/fl mouse model (referred to hereafter as KBIP mice), in which epithelium-specific deletion of Brca1 and p53 alleles predisposes mice to mammary and skin tumor formation (48). The resulting mice carry 1 Brca1185stop and 1 Brca15382stop allele throughout their body and sporadically lose the remaining Brca1 WT allele in specific tissues, including mammary gland. We crossed heterozy-
gous Brca1185stop and Brca15382stop mice with KBIP animals to generate cohorts of K14-Cre Brca1185stop p53fl/fl mice (referred to hereafter as KB1(185stop)P mice), K14-Cre Brca15382stop p53fl/fl [referred to hereafter as KB1(5382stop)P mice], and KBIP littermate controls. The 3 cohorts were monitored for spontaneous tumor formation and showed equal rates of tumor-free survival (TFS) (Figure 2A). For all cohorts, the median TFS was approximately 200 days, which is similar to what has been described previously for KBIP mice (48) and for mice carrying the Brca1C61G mutation (43). In addi-
tion, no obvious differences in TFS could be detected between cohorts when only mammary tumors (Figure 2B) or skin tumors (Supplemental Figure 1A) were taken into account. Mammary and
ma, characterized by the presence of spindle-shaped cells (Figure 2C and Supplemental Figure 1, E and F). Other mammary tumors that developed in KB1(185stop)P, KB1(5382stop)P, and KB1P mice were grouped as lumen-forming carcinomas with varying degrees of glandular differentiation (Figure 2C and Supplemental Figure 1, E and F). Like the majority of human BRCA1–mutated breast cancers (49), most KB1(185stop)P and KB1(5382stop)P mammary tumors stained negative for the estrogen receptor (Supplemental Figure 1, G and H, and Supplemental Table 2).

A high level of genomic instability is one of the hallmarks of human BRCA1–associated breast cancer (50), and BRCA1-deficient mouse mammary tumors display a considerably larger number of genetic aberrations than do BRCA1-proficient tumors (43, 48, 51).

To investigate the level of genomic instability in KB1(185stop)P and KB1(5382stop)P tumors, we measured DNA copy number aberrations (CNAs) in mammary tumors from KB1(185stop)P (n = 20), skin TFS rates were comparable between KB1P mice derived from the 185delAG cohort and from the 5382insC cohort (Supplemental Figure 1, B and C). Furthermore, the spectrum and incidence of tumors that developed were similar among KB1(185stop)P, KB1(5382stop)P, and KB1P mice (Supplemental Figure 1D).

Characterization of KB1(185stop)P and KB1(5382stop)P mammary tumors. On the basis of their histomorphological characteristics, the majority of mammary tumors that developed in KB1(185stop)P (84%), KB1(5382stop)P (79%), and KB1P mice (85%) were classified as poorly differentiated, solid carcinomas (Figure 2C and Supplemental Figure 1, E and F). In line with this observation, most KB1(185stop)P and KB1(5382stop)P mammary tumors stained (partially) positive for the epithelial marker cytokeratin 8 and negative for the mesenchymal marker vimentin (Supplemental Figure 1, G and H, and Supplemental Table 2). In all cohorts, only a small fraction of mammary tumors (8%) was classified as carcinosarcoma, characterized by the presence of spindle-shaped cells (Figure 2C and Supplemental Figure 1, E and F). Other mammary tumors that developed in KB1(185stop)P, KB1(5382stop)P, and KB1P mice were grouped as lumen-forming carcinomas with varying degrees of glandular differentiation (Figure 2C and Supplemental Figure 1, E and F). Like the majority of human BRCA1–mutated breast cancers (49), most KB1(185stop)P and KB1(5382stop)P mammary tumors stained negative for the estrogen receptor (Supplemental Figure 1, G and H, and Supplemental Table 2).

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reached a volume of 200 mm$^3$, tumor-bearing mice were treated with 50 mg/kg of the PARP inhibitor olaparib (AZD2281) for 28 consecutive days or left untreated (Figure 3A).

We did not observe any differences in overall survival (OS) between mice that did not receive treatment; all mice had to be sacrificed within 25 days because of a large tumor (Figure 3B and Supplemental Figure 2). The $\text{KP}$ and $\text{KB1P}$ data have been published before (43). While mice carrying $\text{KP}$ tumors did not respond at all to olaparib treatment (Figure 3, C and D; black curves; median OS $[t_{50}] = 10$ days), the median OS of mice carrying $\text{KB1P}$ tumors increased from 12 to 60 days following olaparib treatment, and their tumors disappeared completely during the course of treatment (Figure 3, C and D; red curves). However, $\text{KB1P}$ tumors could not be fully eradicated with this 28-day olaparib dosing schedule, and tumors reappeared after the end of the treatment period.

The median OS of mice transplanted with $\text{KB1(5382stop)P}$ tumors increased from 8 to 52 days after olaparib treatment (Figure 3, B and C; blue curves), which was significantly better than the median OS of mice transplanted with $\text{KB1(5382stop)P}$ tumors [KB1(5382stop)P vs. $\text{KP}$, $P < 0.0001$, log-rank test]. No significant difference in OS was observed between mice with $\text{KB1(5382stop)P}$ tumors and those $\text{KB1(5382stop)P}$ (n = 20), and littermate control $\text{KB1P}$ mice (n = 22) using array comparative genomic hybridization (aCGH). When applying the comparative module of the R package KCsmart (52, 53), we did not detect any differences between recurrent CNAs in $\text{KB1(185stop)P}$ or $\text{KB1P}$ tumors (Figure 2D). We also could not find any differences in recurrent CNAs between $\text{KB1(5382stop)P}$ and $\text{KB1P}$ tumors (Figure 2E). On the basis of these results, we conclude that the histological and genetic features of $\text{KB1(185stop)P}$ and $\text{KB1(5382stop)P}$ mammary tumors are indistinguishable from each other and from those of $\text{KB1P}$ control tumors.

Response of $\text{KB1(185stop)P}$ mammary tumors to the PARP inhibitor olaparib. PARP inhibitors have been shown to be effective in breast and ovarian cancer patients carrying $\text{BRCA1/2}$ mutations (24–28). To study the response of $\text{KB1(185stop)P}$ and $\text{KB1(5382stop)P}$ tumors to PARP inhibition, we transplanted several independent $\text{KB1(185stop)P}$, $\text{KB1(5382stop)P}$, $\text{BRCA1-deficient KB1P}$, and $\text{BRCA1-proficient K14-Cre p53fl/fl}$ (referred to hereafter as $\text{KP}$) tumors into the fourth mammary gland of syngeneic female recipient mice. This orthotopic transplantation model ensures that transplanted mouse mammary tumors retain the histomorphological features, molecular characteristics, and drug-sensitivity profiles of their spontaneous counterparts (54, 55). When tumors

Figure 2. Development of genomically unstable mammary tumors in mice carrying $\text{Brca1185stop}$ or $\text{Brca15382stop}$ mutations. (A) TFS curves of $\text{KB1(185stop)P}$ (n = 53, $t_{50} = 186$ days), $\text{KB1(5382stop)P}$ (n = 60, $t_{50} = 200$ days) and $\text{KB1P}$ (n = 128, $t_{50} = 196$ days) mice. $P = 0.4510$ (NS), by log-rank test for $\text{KB1(185stop)P}$ versus $\text{KB1(5382stop)P}$. (B) Mammary TFS curves of $\text{KB1(185stop)P}$ (n = 29, $t_{50} = 184$ days), $\text{KB1(5382stop)P}$ (n = 31, $t_{50} = 206$ days), and $\text{KB1P}$ (n = 68, $t_{50} = 197$ days), $P = 0.0415$ (NS), by log-rank test for $\text{KB1(185stop)P}$ versus $\text{KB1(5382stop)P}$. (C) Distribution of mammary tumor types in $\text{KB1(185stop)P}$, $\text{KB1(5382stop)P}$, and $\text{KB1P}$ mice. Numbers in the pie chart represent percentages. (D) Comparative KCsmart (KC) profiles of $\text{KB1(185stop)P}$ and $\text{KB1P}$ mouse mammary tumors. (E) Comparative KCsmart profiles of $\text{KB1(5382stop)P}$ and $\text{KB1P}$ tumors. $t_{50}$, median survival; n, number of mice; Chr., chromosome.
with KBIP tumors after treatment with olaparib [KB1(5382stop)P vs. KBIP, \( P = 0.0905 \) (NS), log-rank test]. However, in contrast to KBIP tumors, KB1(5382stop)P tumors never completely disappeared during olaparib treatment, but rather entered a phase of tumor stasis (Figure 3D; blue curve).

Interestingly, mice transplanted with KB1(185stop)P tumors had a median OS of 26 days after the start of olaparib treatment (Figure 3C; green curve), which was significantly better than for mice with BRCA1-proficient KP tumors [KB1(185stop)P vs. KP, \( P = 0.0017 \), log-rank test], but significantly worse than for mice with BRCA1-deficient KBIP tumors [KB1(185stop)P vs. KBIP, \( P < 0.0001 \), log-rank test]. While KB1(185stop)P tumors kept growing during the course of olaparib treatment, their growth rate was reduced compared with that of KP tumors (Figure 3D; green curve). Moreover, the olaparib response of KB1(185stop)P tumors was markedly worse than the response of KB1(5382stop)P tumors [Figure 3, C and D and Supplemental Figure 2; KB1(185stop)P vs. KB1(5382stop)P, \( P = 0.0012 \), log-rank test]. The response of KB1(185stop)P tumors to olaparib treatment closely resembled the response of K14-Cre Brca1<sup>−/−C61G</sup> p53<sup>−/−</sup> (referred to hereafter as KB1C61GP) mouse mammary tumors to olaparib (43).

Response of KB1(185stop)P mammary tumors to cisplatin. Since BRCA1-associated tumors are also known to be sensitive to platinum drugs (56), we transplanted several KB1(185stop)P, KB1(5382stop)P, KBIP, and BRCA1-proficient KP mammary tumors to study potential differences in their response to cisplatin (Supplemental Table 3). Tumor-bearing mice were injected with the maximal tolerable dose of cisplatin and re-treated every 2 weeks when the tumor volume was more than 50% of the starting volume. If the tumor size after 2 weeks was smaller than 50%, treatment was postponed until the tumor reached 100% of the starting volume (Figure 4A). As some animals had to be sacrificed because of the toxic side effects of multiple cisplatin doses, we measured OS as well as TFS rates. Again, the data on the KP and KBIP controls have been previously published (43).

The median OS of mice transplanted with KB1(5382stop)P tumors was prolonged from 8 to 159 days after cisplatin treatment (Figure 4, B and C; blue curves), which is identical to the response of mice transplanted with BRCA1-deficient KBIP tumors (43) [Figure 4, B and C; red curves, KB1(5382stop)P vs. KBIP; \( P = 0.9696 \) (NS), log-rank test]. Almost all mice transplanted with KB1(5382stop)P tumors had to be sacrificed because of cisplatin toxicity and not because of therapy resistance (Figure 4D). In contrast, the median OS of mice transplanted with KB1(185stop)P mammary tumors was only prolonged from 15 to 55 days after cisplatin treatment (Figure 4, B and C; green curves) and was indistinguishable from the response of BRCA1-proficient tumors [Figure 4, B and C; black curves, KB1(185stop)P vs. KP, \( P = 0.2550 \) (NS), log-rank test]. After an initial response to cisplatin, KB1(185stop)P tumors rapidly acquired resistance, and 62% of the mice needed to be sacrificed because of therapy-resistant tumors (Figure 4D). Remarkably, the response of KB1(185stop)P tumors
to cisplatin was significantly worse than that of KB1(5382stop)P tumors [Figure 4C; KB1(185stop)P vs. KB1(5382stop)P, P < 0.0001, log-rank test; Supplemental Figure 3, A–E]. The difference in response to cisplatin was even more pronounced when we compared the TFS of mice transplanted with KB1(185stop)P or KB1(5382stop)P mammary tumors (Supplemental Figure 3F).

To investigate whether these findings might also be relevant for human patients, we evaluated the cisplatin sensitivity of several human BRCA1-mutant breast cancer cell lines. These included the SUM1315MO2 and HCC1937 cell lines, which carry BRCA1<sup>185delAG</sup> and BRCA1<sup>5382insC</sup> mutations, respectively. In line with our observations in the mouse models, BRCA1<sup>185delAG</sup>-mutant SUM1315MO2 human breast cancer cells were less sensitive to cisplatin than were other BRCA1-mutated human breast cancer cell lines, including the BRCA1<sup>5382insC</sup>-mutant HCC1937 cell line (Figure 4E). However, BRCA1<sup>185delAG</sup>-mutant SUM1315MO2 cells were more sensitive to cisplatin than were BRCA1-proficient U2OS cells or WT BRCA1–complemented HCC1937 cells (Figure 4E).

Figure 4. Poor cisplatin response of KB1(185Stop)P mouse mammary tumors. (A) Schematic representation of cisplatin treatment schedule. t<sub>0</sub>, start of treatment with 6 mg/kg cisplatin i.v. at a tumor volume of 200 mm<sup>3</sup>, corresponding to a relative tumor volume (RTV) of 100%. t<sub>13</sub>, if the RTV on day 13 was ≥50%, the mice received an additional treatment that was repeated every 2 weeks until an RTV of ≤50% was observed. If the RTV at t<sub>13</sub> was ≤50%, re-treatment was postponed until an RTV of >100% was observed. (B) OS curves of mice transplanted with KB1(185Stop)P, KB1(5382stop)P, KBIP, or KP tumors without treatment. t<sub>15</sub>, median OS. KB1(185Stop)P: t<sub>15</sub> = 15 days, n = 4 mice; KB1(5382stop)P: t<sub>15</sub> = 8 days, n = 6 mice; KBIP: t<sub>15</sub> = 12 days, n = 4 mice; KP: t<sub>15</sub> = 11 days, n = 4 mice. (C) OS curves of mice transplanted as indicated in B after cisplatin treatment. KB1(185Stop)P: t<sub>50</sub> = 55 days, n = 35 mice; KB1(5382stop)P: t<sub>50</sub> = 159 days, n = 47 mice; KBIP: t<sub>50</sub> = 48 days, n = 21 mice. P < 0.0001, by log-rank test for KB1(185Stop)P vs. KB1(5382stop)P; P < 0.0001, by log-rank test for KB1(185Stop)P vs. KBIP; P = 0.9696 (NS), by log-rank test for KB1(5382stop)P vs. KBIP; P < 0.0001, by log-rank test for KB1(5382stop)P vs. KP; and P < 0.0001, by log-rank test for KBIP vs. KP. (D) Causes of death of tumor-bearing mice after treatment with cisplatin. The stacked bars indicate the percentage of mice that were sacrificed because of cisplatin-resistant mammary tumors, cisplatin-associated toxicity, or another (mesenteric) tumor. (E) Cisplatin sensitivity of various human BRCA1-mutated breast cancer cell lines, including SUM149PT (BRCA1<sup>2288delT</sup>), SUM1315MO2 (BRCA1<sup>185delAG</sup>), and HCC1937 (BRCA1<sup>5382insC</sup>). The U2OS osteosarcoma and WT BRCA1–complemented HCC1937 cell lines served as BRCA1-proficient controls. The experiment was performed in quadruplicate. Error bars indicate SEM.
Therapy resistance of mouse and human BRCA1 inherited breast cancer cells is not explained by genetic reversion or loss of 53BP1. Given that BRCA1/2-deficient cell lines and ovarian tumors can become resistant to platinum compounds and olaparib through genetic reversion of the BRCA1/2 mutation (57–60), we first checked whether the Brca1185stop mutation was still present in platinum-resistant KB1(185stop)P tumors. Previously, we did not find any evidence for secondary Brca1 mutations in therapy-resistant KB1C61GP tumors (43). Similarly, Sanger sequencing and melting-curve genotyping revealed that all cisplatin-resistant KB1(185stop)P tumors had retained the Brca1 185stop mutation (Supplemental Figure 4A and data not shown). On the basis of these results, we conclude that the observed platinum resistance in KB1(185stop)P mammary tumors is not caused by genetic reversion of the Brca1 185stop mutation. Furthermore, we were able to derive several cisplatin-resistant clones of the BRCA1-mutated human breast cancer cell line SUM1315MO2, all of which also retained the BRCA1185delAG mutation (Supplemental Figure 4, B–D). In the absence of genetic reversion, loss of 53BP1 may also decrease the sensitivity of BRCA1-deficient mouse mammary tumors to olaparib and cisplatin (61). RING-less BRCA1 expression in mouse and human BRCA1185delAG tumor cells. Since the response of KB1(185stop)P mouse mammary tumors to olaparib and cisplatin is similar to that of BRCA1-proficient KP tumors, we analyzed BRCA1 protein expression in KB1(185stop)P and KB1P tumors. A BRCA1-proficient KP tumor was used as a positive control (right panel). A BRCA1-deficient KB1(5382stop)P and KB1P tumors have decreased expression of BRCA1 protein (left panel). The asterisk in the far right panel indicates another KB1(185stop)P mouse mammary tumor. Expression of POLII was used as a loading control, and the positions of full-length (FL) and RING-less (∆RING) BRCA1 and protein size markers are indicated. mBRCA1, murine BRCA1. (A) BRCA1 protein expression in the BRCA1185delAG-mutant SUM1315MO2 human breast cancer cell line. As controls, SUM1315MO2 cells stably complemented with WT BRCA1 (WT) were depleted of endogenous BRCA1 by a BRCA1 3′-UTR–targeting shRNA (sh1) or depleted of both endogenous and ectopic BRCA1 using an shRNA targeting BRCA1 exon 11 (sh2). shNT, NT shRNA. Expression of POLII was used as a loading control, and the positions of full-length and RING-less BRCA1 and protein size markers are indicated. hBRCA1, human BRCA1. (B) Ribosome profiling of KB1(185stop)P mammary tumor cell lines indicated a translation start at methionine 90. Ribosome footprint profile along Brca1 exons 2 (containing methionine 1) and 6 (containing methionines 90 and 99) of 2 different KB1(185stop)P mouse mammary tumor cell lines (lines 280 and 350) and a KP control. Methionines are indicated in green. Harr., harringtonine; M1, methionine 1; M90, methionine 90. (C) Schematic representations of BRCA1 peptides identified by mass spectrometry in Brca1 WT and KB1(185stop)P mouse mammary tumor cells.
We therefore checked BRCA1 protein expression levels in the SUM1315MO2 human breast cancer cell line, which carries the BRCA1<sup>185delAG</sup> mutation. Remarkably, in BRCA1<sup>185delAG</sup>-mutant SUM1315MO2 cells, BRCA1 immunoblotting also detected a large protein that could be completely depleted using BRCA1-specific shRNAs (Figure 5B). In contrast to the truncated BRCA1 formed by KB1<i>185stop</i>P tumor cells, the BRCA1 protein expressed by SUM1315MO2 cells was clearly smaller than full-length WT BRCA1, indicating the absence of a substantial part of the protein. In addition, we could not detect the truncated protein with a BRCA1 Ab that binds the extreme N-terminus of BRCA1 (aa 1-304; Supplemental Figure 5A). Together, these findings show that the BRCA1<sup>185delAG</sup> mutation can lead to production of a mutant BRCA1-ΔRING protein, which is probably devoid of its N-terminal RING domain (aa 1-109).

Whether the truncated BRCA1 variant expressed by KB1<i>185stop</i>P tumor also lacks the RING domain cannot be deduced from the minimal size difference observed with full-length BRCA1. In the absence of Abs of sufficient quality to distinguish between full-length and RING-less mouse BRCA1 protein, we performed ribosome profiling of cell lines derived from treatment-naïve KB1<i>185stop</i>P and Brca1 WT KP mouse mammary tumors. We used harringtonine treatment to enrich for ribosomes at sites of translation initiation (63) and observed increased use of internal methionine 90 in KB1<i>185stop</i>P mouse mammary tumor cells compared with KP cells (Figure 5C). Since these data suggest that the nearly full-length BRCA1 protein expressed in KB1<i>185stop</i>P mouse mammary tumors also lacks the RING domain, mass spectrometric experiments were performed to verify the loss of the RING domain. BRCA1 protein was immunoprecipitated using an Ab raised against aa 1328-1812 (64) and analyzed after size selection by denaturing polyacrylamide gel electrophoresis. Whereas BRCA1-RING (aa 1-109) peptides were readily identified in WT BRCA1-expressing mouse tumor cells, they were not found in 2 different cell lines derived from KB1<i>185stop</i>P tumors (Figure 5D, Supplemental Figure 5B, and Supplemental Table 4). These data are compatible with the expression of RING-less BRCA1 in KB1<i>185stop</i>P cells. Given that even in WT BRCA1–expressing cells, not all possible RING peptides could be detected by mass spectrometry, the precise composition of BRCA1-ΔRING variants could not be determined (Figure 5D and Supplemental Figure 5B). However, analysis of the most N-terminal peptide identified supports a translation start at methionine 90, as suggested by ribosome profiling.

To test whether resistance in KB1<i>185stop</i>P tumors was due to increased expression of BRCA1-ΔRING, we analyzed Brca1 mRNA and BRCA1-ΔRING protein levels. Although we found significantly increased Brca1 mRNA levels in most platinum-resistant KB1<i>185stop</i>P tumors compared with levels in untreated tumors (Supplemental Figure 5C), we did not find a consistent concomitant increase in RING-less BRCA1 protein levels (Supplemental Figure 5D). Moreover, expression of RING-less BRCA1 protein was also not significantly increased in SUM1315MO2 clones that had been selected for increased resistance to cisplatin (Supplemental Figure 5A). These findings imply that upregulation of RING-less protein expression is not strictly required for BRCA1<sup>185delAG</sup>-mutant tumor cells to become resistant to platinum therapy. Nevertheless, expression of RING-less BRCA1 distinguishes KB1<i>185stop</i>P tumors from KB1P tumors and most likely explains the poor response to HRD-targeted therapy.

DNA damage response in mouse and human BRCA1<sup>185delAG</sup> tumor cells. The absence of genetic reversion of Brca1 or loss of 53BP1 in platinum-resistant KB1<i>185stop</i>P tumors suggests that RING-less BRCA1 protein has residual activity in the cellular response to DNA DSBs. In line with our observation that KB1<i>185stop</i>P tumors showed some response to PARP1 inhibition, treatment with olaparib resulted in a DNA-damage increase similar to that seen in KB1P tumors (Figure 6A). However, we also found that the response to olaparib was eventually poor compared with that of KB1P tumors that were completely devoid of BRCA1 protein expression. To investigate whether RING-less BRCA1 protein has activity in DNA DSB repair via HR, we compared the ability to form RAD51 irradiation-induced foci (IRIF) in short-term tumor cell cultures derived from KB1<i>185stop</i>P, KB1P, and BRCA1-proficient KP tumors. As shown previously (43), we could readily detect RAD51 IRIF in short-term cultures of HR-proficient KP tumor cells, but not in HR-deficient KB1P tumor cells (Figure 6, B and C). KB1<i>185stop</i>P tumor cells were also able to form RAD51 IRIF (Figure 6, B and C). Despite the statistically significant difference, the data from the short-term ex vivo cell-culture experiments were quite variable. To gain further insight into the DNA damage response of KB1<i>185stop</i>P tumors, we continued our analysis in mouse mammary tumor–derived cell lines. In agreement with the loss of BRCA1 function we observed, KB1<i>185stop</i>P cell lines had a defect in DNA end resection, as replication protein A (RPA) accumulation at DSBs was impaired (Figure 6D). Indeed, KB1<i>185stop</i>P cell lines showed an initial deficiency in the repair of olaparib-induced DNA damage that was similar to that in KB1P cells (Figure 6E). Nevertheless, KB1<i>185stop</i> tumor cell lines showed RAD51 IRIF formation (Supplemental Figure 6A). In addition, human SUM1315MO2 breast cancer cells — carrying the BRCA1<sup>185delAG</sup> mutation — were also capable of forming RAD51 IRIF (Supplemental Figure 6B). Thus, both mouse and human BRCA1<sup>185delAG</sup> tumor cells display HR activity in response to DNA damage, which could be the result of BRCA1-ΔRING expression.

To study whether the RING-less BRCA1 protein is functional in the DNA damage response, we checked for colocalization of BRCA1 with RAD51 after γ irradiation in cell lines derived from BRCA1-proficient, BRCA1-deficient, and Brca1<sup>185stop</sup> mouse mammary tumors. While massive BRCA1/RAD51 colocalization was observed in BRCA1-proficient tumor cells, Brca1<sup>185stop</sup> tumor cells showed fewer and smaller BRCA1/RAD51 IRIF (Supplemental Figure 6A and data not shown). In contrast, BRCA1-deficient KB1P B11 cells showed no BRCA1/RAD51 IRIF at all (Supplemental Figure 6A). These data suggest that the RING-less BRCA1 protein is partially functional in the response to DNA damage. This may explain why human and mouse BRCA1<sup>185delAG</sup> tumor cells rapidly develop resistance to therapy that targets their HRD.

RING-less BRCA1 supports DNA repair via HR, resulting in reduced sensitivity of BRCA1<sup>185delAG</sup> tumor cells to PARP1 inhibition and cisplatin. To test whether mutant RING-less BRCA1 protein is indeed functionally important for BRCA1<sup>185delAG</sup> tumor cells, we performed BRCA1-knockdown experiments. Expression of RING-less BRCA1 in BRCA1<sup>185delAG</sup>-mutant SUM1315MO2 cells was markedly reduced by transduction with lentiviruses encod-
able to complement SUM1315MO2 \textit{BRCA1}^{185delAG}-mutant cells depleted of endogenous BRCA1 expression by shRNAi in a clonal growth assay (Figure 7C and Supplemental Figure 7F). These data show that \textit{BRCA1}^{185delAG} cells are dependent on expression of the RING-less BRCA1 protein, possibly through its function in the repair of DNA DSBs.

To further analyze the effects of RING-less BRCA1 expression on treatment response, we used mouse \textit{KB1(185stop)}P tumor cells, which were much less dependent on RING-less BRCA1 for proliferation than were SUM1315MO2 cells (data not shown). \textit{KB1(185stop)}P tumor cell lines showed varying levels of sensitivity to olaparib and cisplatin, which seemed to correlate to some extent with expression levels of RING-less BRCA1 protein (Supplemental Figure 8, A and B). Similar to SUM1315MO2 cells, \textit{KB1(185stop)}P tumor cells also showed decreased RAD51 IRIF formation upon knockdown of \textit{Brca1} expression (Supplemental Figure 8, C and D). As stable suppression of human or mouse BRCA1 expression was not sufficiently efficient for long-term cytotoxicity experiments (data not shown), we used CRISPR/Cas9 mutagenesis in \textit{KB1(185stop)}P tumor cells to obtain

- Figure 6. DNA damage response in mouse and human \textit{BRCA1}^{185delAG} tumor cells. (A) Olaparib induced DNA damage in orthotopically transplanted \textit{KB1(185stop)}P (185stop, \(n = 6\)), \textit{KB1}P (\(n = 7\)), and \textit{KP} (\(n = 6\)) tumors and nontreated controls (\(n = 4\) for each genotype). Statistical significance was calculated using a 2-tailed, unpaired \(t\) test. (B) RAD51 IRIF in \textit{KB1(185stop)}P, \textit{KB1}P, and \textit{KP} tumor cell suspensions. Nuclei (blue) with more than 10 RAD51 foci (red) are indicated with red arrowheads. Red outlined areas highlight single-cell zoom-in (zoom, \(×63\)). Scale bars: 25 \(\mu\)m. (C) Quantification of RAD51 IRIF in \textit{KB1(185stop)}P (\(n = 5\)), \textit{KB1}P (\(n = 10\)), and \textit{KP} (\(n = 7\)) tumors. Percentages were normalized to \textit{KP}. Statistical significance was calculated using a 2-tailed, unpaired \(t\) test. (D) Accumulation of RPA at \(\alpha\) tracks for 2 different \textit{KB1(185stop)}P mouse mammary tumor cell lines (lines 350 and 351) and \textit{KB1}P and \textit{KP} controls. Cells were cultured in normal medium (day 0 [D0]) or treated for 24 hours with 10 \(\mu\)M olaparib and either analyzed directly (D1) or after a 3-day recovery period (D3). Red bars indicate the mean number of 53BP1 foci in at least 120 cells, and data are representative of 2 independent experiments. Statistical significance was calculated using a 2-tailed, unpaired \(t\) test.

- Figure 7. DNA damage response in mouse and human \textit{BRCA1}^{185delAG} tumor cells. (A) Olaparib induced DNA damage in orthotopically transplanted \textit{KB1(185stop)}P (185stop, \(n = 6\)), \textit{KB1}P (\(n = 7\)), and \textit{KP} (\(n = 6\)) tumors and nontreated controls (\(n = 4\) for each genotype). Statistical significance was calculated using a 2-tailed, unpaired \(t\) test. (B) RAD51 IRIF in \textit{KB1(185stop)}P, \textit{KB1}P, and \textit{KP} tumor cell suspensions. Nuclei (blue) with more than 10 RAD51 foci (red) are indicated with red arrowheads. Red outlined areas highlight single-cell zoom-in (zoom, \(×63\)). Scale bars: 25 \(\mu\)m. (C) Quantification of RAD51 IRIF in \textit{KB1(185stop)}P (\(n = 5\)), \textit{KB1}P (\(n = 10\)), and \textit{KP} (\(n = 7\)) tumors. Percentages were normalized to \textit{KP}. Statistical significance was calculated using a 2-tailed, unpaired \(t\) test. (D) Accumulation of RPA at \(\alpha\) tracks for 2 different \textit{KB1(185stop)}P mouse mammary tumor cell lines (lines 350 and 351) and \textit{KB1}P and \textit{KP} controls. Cells were cultured in normal medium (day 0 [D0]) or treated for 24 hours with 10 \(\mu\)M olaparib and either analyzed directly (D1) or after a 3-day recovery period (D3). Red bars indicate the mean number of 53BP1 foci in at least 120 cells, and data are representative of 2 independent experiments. Statistical significance was calculated using a 2-tailed, unpaired \(t\) test.
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BRCA1-BRCT–encoding region of the Brca1 185stop allele (Supplemental Figure 9, C–F).

To investigate whether disruption of the Brca1 185stop allele also translates to a better treatment response in vivo, we performed orthotopic transplantation experiments in mice. The parental KB1(185stop)P tumor cells and their Brca1 knockdown clone C3 grew into mammary tumors with a similar latency (data not shown), although untreated C3 tumors tended to proliferate at a somewhat slower rate once palpable (Supplemental Figure 10A). Strikingly, we also observed in vivo that inactivation of the Brca1 185stop allele rendered KB1(185stop)P tumor cells much more sensitive to PARP inhibition and cisplatin (Figure 7, G and H, and Supplemental Figure 10B).

Figure 7. BRCA1185delAG tumor cells are dependent on RING-less BRCA1 for proliferation, DNA damage signaling, and treatment response. (A) BRCA1 protein levels after BRCA1 knockdown in BRCA1185delAG-mutant SUM1315MO2 tumor cells. –, no shRNA. Expression of POLII was used as a loading control, and protein size markers are indicated. (B) Quantification of RAD51 IRIF–positive SUM1315MO2 cells after BRCA1 knockdown as in A. The percentage of cells with 10 or more RAD51 foci was evaluated for at least 300 cells per condition. Statistical significance was calculated using Fisher’s exact test, and results are representative of 2 independent experiments. (C) Complementation of SUM1315MO2 cells by stably transduced cDNA constructs expressing WT BRCA1 (WT) or BRCA1 N-terminal truncation variants (M48, M128, or M237). NTF, nontransfected control. Cells were assayed for clonal growth after transduction with the BRCA1 3′-UTR–targeting shRNA (sh1) or the NT shRNA (shNT) control. P = 0.0005 by 2-tailed, unpaired t test for NTF control shNT versus sh1. Significant complementation of proliferation is indicated by ***P < 0.01 or ****P < 0.0001. Results shown are representative of 2 independent experiments. (D) RAD51 IRIF formation in Brca1 WT (KP) cells, the KB1(185stop)P (288) cell line, and its Brca1 deficient subclone C3 (288-C3). Cells were irradiated with 10 Gy, and RAD51 foci formation in S and G2 phases was compared with a nonirradiated control (no IR). Red bars indicate the mean number of foci in at least 116 EdU-positive cells. P value was determined using a 2-tailed, unpaired t test, and data are representative of 2 independent experiments. (E and F) IC50 values of KB1(185stop)P cell line 288 and subclones C1-3 for olaparib (E) and cisplatin (F). Error bars indicate the SD for 3 independent experiments. (G and H) OS of mice transplanted with KB1(185stop)P cell lines 288 and 288-C3 and treated with 100 mg/kg AZD2461 daily for 28 consecutive days (G) or with 6 mg/kg cisplatin on days 0 and 14 (H), or untreated (G). P values were calculated using the log-rank test.

Figure 7. BRCA1185delAG tumor cells are dependent on RING-less BRCA1 for proliferation, DNA damage signaling, and treatment response. (A) BRCA1 protein levels after BRCA1 knockdown in BRCA1185delAG-mutant SUM1315MO2 tumor cells. –, no shRNA. Expression of POLII was used as a loading control, and protein size markers are indicated. (B) Quantification of RAD51 IRIF–positive SUM1315MO2 cells after BRCA1 knockdown as in A. The percentage of cells with 10 or more RAD51 foci was evaluated for at least 300 cells per condition. Statistical significance was calculated using Fisher’s exact test, and results are representative of 2 independent experiments. (C) Complementation of SUM1315MO2 cells by stably transduced cDNA constructs expressing WT BRCA1 (WT) or BRCA1 N-terminal truncation variants (M48, M128, or M237). NTF, nontransfected control. Cells were assayed for clonal growth after transduction with the BRCA1 3′-UTR–targeting shRNA (sh1) or the NT shRNA (shNT) control. P = 0.0005 by 2-tailed, unpaired t test for NTF control shNT versus sh1. Significant complementation of proliferation is indicated by ***P < 0.01 or ****P < 0.0001. Results shown are representative of 2 independent experiments. (D) RAD51 IRIF formation in Brca1 WT (KP) cells, the KB1(185stop)P (288) cell line, and its Brca1 deficient subclone C3 (288-C3). Cells were irradiated with 10 Gy, and RAD51 foci formation in S and G2 phases was compared with a nonirradiated control (no IR). Red bars indicate the mean number of foci in at least 116 EdU-positive cells. P value was determined using a 2-tailed, unpaired t test, and data are representative of 2 independent experiments. (E and F) IC50 values of KB1(185stop)P cell line 288 and subclones C1-3 for olaparib (E) and cisplatin (F). Error bars indicate the SD for 3 independent experiments. (G and H) OS of mice transplanted with KB1(185stop)P cell lines 288 and 288-C3 and treated with 100 mg/kg AZD2461 daily for 28 consecutive days (G) or with 6 mg/kg cisplatin on days 0 and 14 (H), or untreated (G). P values were calculated using the log-rank test.

evidence for the relevance of RING-less BRCA1 in therapy response. We targeted the BRCA1-BRCT-encoding region using a single-guide RNA (sgRNA) specific for exon 21 of Brca1. In 2 of 3 KB1(185stop)P clones analyzed (clones C1 and C3), frameshift mutations in exon 21 of the Brca1 185stop allele resulted in loss of RING-less BRCA1 protein expression (Supplemental Figure 9, A and B). Similar to Brca1 knockdown, these frameshift mutations disrupted RAD51 IRIF formation (Figure 7D and data not shown). In addition, inactivation of RING-less BRCA1 resulted in dramatically increased sensitivity to both olaparib and cisplatin (Figure 7, E and F). These results were confirmed using another sgRNA targeting the Cas9 nuclease to exon 17 in the BRCA1-BRCT-encoding region of the Brca1 185stop allele (Supplemental Figure 9, C–F).

To investigate whether disruption of the Brca1 185stop allele also translates to a better treatment response in vivo, we performed orthotopic transplantation experiments in mice. The parental KB1(185stop)P tumor cells and their Brca1-knockout clone C3 grew into mammary tumors with a similar latency (data not shown), although untreated C3 tumors tended to proliferate at a somewhat slower rate once palpable (Supplemental Figure 10A). Strikingly, we also observed in vivo that inactivation of the Brca1 185stop allele rendered KB1(185stop)P tumor cells much more sensitive to PARP inhibition and cisplatin (Figure 7, G and H, and Supplemental Fig-
Figure 8. A RING-less BRCA1-expressing BRCA1<sup>185delAG</sup> PDX model of breast cancer shows poor response to olaparib. (A and B) Western blot analysis of BRCA1 expression in 3 different BRCA1<sup>185delAG</sup> PDX models of breast cancer and BRCA1-deficient (−) and BRCA1-proficient (+) controls, using the BRCA1 Abs MS110 (A; epitope: aa 1–304) and 9010 (B; epitope: aa 305–325). Expression of POLII was used as a loading control, and protein size markers are indicated. (C) Waterfall plot showing the olaparib response of individual PDX mammary tumors after 31 (PDX127, PDX230) or 33 (PDX179) days of treatment (50 mg/kg olaparib, i.p., 5 days/week). (D) Quantification of RAD51 foci in olaparib- or vehicle-treated BRCA1<sup>185delAG</sup> PDX tumors. Percentages of S/G2-phase cells (geminin<sup>+</sup>) with 5 or more RAD51 foci (RAD51<sup>+</sup>) were determined on FFPE tumor samples for each PDX model. Error bars indicate the SD for 3 biological replicates, and P values were determined using a 2-tailed, unpaired t test.

Discussion

We have used genetically engineered mouse models mimicking the 2 most common BRCA1 founder mutations, BRCA1<sup>5382insC</sup> and BRCA1<sup>185delAG</sup>, to study the effects of these mutations on tumor development and therapy response and resistance. While mice carrying the BRCA1<sup>5382insC</sup> or BRCA1<sup>185delAG</sup> mutation develop similar types of mammary carcinomas, BRCA1<sup>5382insC</sup> tumors respond significantly worse to HRD-targeted therapy than do BRCA1<sup>185delAG</sup> tumors and rapidly develop therapy resistance. It has previously been shown that secondary mutations in BRCA1 can mediate resistance to platinum-based chemotherapy in BRCA1<sup>185delAG</sup> ovarian carcinomas (59, 60). However, we could not detect genetic reversion of the BRCA1<sup>185delAG</sup> mutation in any of the platinum-resistant BRCA1<sup>185delAG</sup> tumors.
mouse mammary tumors. Also, in multiple cisplatin-resistant clones of the human BRCA1<sup>185delAG</sup> breast cancer cell line SUM1315MO2, no reversal of the mutation was found. Instead, we noticed that mouse and human BRCA1<sup>185delAG</sup> tumor cells produced RING-less BRCA1 proteins, which are involved in the development of platinum and PARP inhibitor resistance through their activity in the DNA damage response.

A role for RING-less BRCA1 proteins in therapy response and resistance. While it was thus far thought that the BRCA1<sup>185delAG</sup> frameshift mutation only leads to the formation of a small N-terminal protein of 38 aa, we detected nearly full-length, RING-less BRCA1 protein in Brca1<sup>185stop</sup> mouse mammary tumors. This was not merely an artifact of our genetically engineered mouse model, since we could also detect RING-less BRCA1 in human breast cancer cells carrying the BRCA1<sup>185delAG</sup> mutation.

Brca1<sup>185stop</sup> tumors did not require secondary Brca1 mutations to become resistant to PARP inhibition or cisplatin, but residual activity of the RING-less BRCA1 protein was already sufficient to withstand these types of targeted therapeutics. This finding extends the conclusions of our previous work with Brca1<sup>C61G</sup> tumors, which expressed a full-length BRCA1 protein containing a missense mutation in the RING domain (43). Our data indicate that RING-less BRCA1 is not completely functional in HR, but that this protein still supports RAD51 IRIF formation. Thus, while insufficient for embryonic survival and tumor suppression, the residual activity of the RING-less BRCA1 protein can contribute to the rapid development of therapy resistance in Brca1<sup>185stop</sup> tumors. Our data suggest that there is no strict requirement for upregulation or stabilization of RING-less BRCA1 protein levels for resistance to occur. In both human and mouse breast cancer cells, RING-less BRCA1 supported RAD51 IRIF formation, demonstrating its functional significance. Moreover, deletion of the Brca1<sup>185stop</sup> allele rendered KB1(185stop)/P tumor cells sensitive to cisplatin and PARP inhibition. To further investigate the potential relevance of our findings for human cancer patients, we analyzed 3 independent BRCA1<sup>185delAG</sup> PDX models of breast cancer for expression of RING-less BRCA1 and response to PARP inhibition. In concordance with the data from our genetically engineered mouse models and the SUM1315MO2 cell line, a PDX model expressing RING-less BRCA1 showed progressive disease in the presence of the PARP inhibitor olaparib. Thus, human tumors expressing RING-less BRCA1 may also be resistant to HRD-targeted therapy. Although we detected no RING-less BRCA1 expression in another PDX model that showed resistance to olaparib, we found no evidence for genetic reversion of the BRCA1<sup>185delAG</sup> mutation in any of the PDX tumors.

Why, then, are secondary BRCA1 mutations still observed in therapy-resistant BRCA1<sup>185delAG</sup> human ovarian carcinomas? One possibility is that these secondary BRCA1 mutations are already present in rare cells of primary carcinomas, possibly because of genomic instability, and subsequently selected under the pressure of chemotherapy. This has been described before for chronic myeloid leukemia, in which BCR-ABL mutations that confer imatinib resistance are already present in a minority of tumor cells before exposure to the drug (65). In addition, the level of RING-less BRCA1 protein in untreated BRCA1<sup>185delAG</sup> human ovarian carcinomas is currently unknown. There could be considerable intertumoral heterogeneity in both the presence (and abundance) of preexisting secondary BRCA1 mutations and the expression levels of RING-less BRCA1 protein. Furthermore, we observed substantial differences among tumors in the levels of RING-less BRCA1 expression, which may also exist between BRCA1<sup>185delAG</sup> breast and ovarian cancers. Genetic reversion might be the preferred mechanism of therapy resistance in tumors with preexisting secondary BRCA1 mutations and weak or no expression of the RING-less BRCA1 protein.

The existence of RING-less BRCA1 proteins in mouse and human BRCA1<sup>185delAG</sup> tumor cells appears to be the result of internal translation reinitiation at downstream start codons, as has previously been observed in transient transfection experiments with human BRCA1 minigenes (66). Translation initiation at methionine 297 of human BRCA1 could also explain why BRCA1<sup>185delAG</sup> mRNA is not degraded by NMD (66, 67). The mutant RING-less BRCA1 protein produced in our genetically engineered Brca1<sup>185stop</sup> mouse model is somewhat larger than its human counterpart, most likely because of usage of the more upstream alternative start codon at methionine 90 in the mouse Brca1 coding sequence.

Differences in therapy response between BRCA1 founder mutations. While patients with BRCA1-mutated ovarian cancer have a better prognosis after platinum-based chemotherapy than do nonmutation carriers (15–18), some heterogeneity in survival appears to exist among carriers of different BRCA1 mutations (16). Survival of ovarian cancer patients carrying the BRCA1<sup>185delAG</sup> mutation has been compared with that of BRCA1<sup>5382insC</sup> patients, but, unfortunately, no conclusions could be drawn because of the small number of patients with a BRCA1<sup>5382insC</sup> mutation (16). Our data indicate that the poor therapy response of Brca1<sup>185stop</sup> mouse mammary tumors is mediated by residual activity of the RING-less BRCA1 protein. Therefore, we predict that RING-less BRCA1 expression may also have consequences for the clinical response of BRCA1<sup>185delAG</sup> patients to DNA-damaging therapy.

Expression of a RING-less BRCA1 protein may not be limited to BRCA1<sup>185delAG</sup> tumors, but could also occur in tumors carrying other truncating mutations in the N-terminus of BRCA1. Our data suggest that the presence of a RING-less BRCA1 protein in these tumors can serve as a marker to predict poor response to treatment with platinum or PARP inhibitors. PARP inhibitors are currently being tested in phase III clinical trials for BRCA1/2-associated breast cancers and approved for advanced BRCA1/2-associated ovarian cancers. Although pathogenic germline mutations in BRCA1 or BRCA2 are thus far the best predictors of PARP inhibitor sensitivity, it will be important to critically evaluate the treatment response of tumors with mutations affecting the N-terminus of BRCA1. This type of analysis will require large numbers of patients carrying specific BRCA1 founder mutations and therefore remains a challenge for the future. Meanwhile, our genetically engineered and PDX mouse models offer ideal platforms for testing hypotheses on therapy response and resistance. Knowledge gained from these models could contribute to more accurate predictions of therapy responses of different BRCA1 mutation carriers, help to prevent ineffective treatment, and lead to earlier development and implementation of alternative therapeutic agents.
Methods

Generation of Brca1<sup>185stop</sup>- and Brca1<sup>5382stop</sup>-mutant mice. Nonchemically modified deoxyribonucleotides (Sigma-Genosys) were used to introduce the Brca1<sup>185stop</sup> (5′-ATCGCAAAATCTTAGATGGC-GATCTTGAATTCAACA-3′) and Brca1<sup>5382stop</sup> (5′-CAAGGCGATC-CAGAGATCCAGCGGGAAAGTGAAGTC-3′) mutations into mESCs. The procedures for the introduction of oligonucleotides into mESCs, selection for G418-resistant colonies, and identification and purification of modified cells have been described previously (44–47). The resulting ESCs were injected into C57BL/6j blastocysts to produce chimeric males, which were mated with FVB females to generate Brca1<sup>185stop</sup> and Brca1<sup>5382stop</sup> mice. Brca1<sup>185stop</sup> and Brca1<sup>5382stop</sup> mice were bred with K14-Cre Brca1<sup>p53<sup>+/−</sup></sup> (KB1P) animals (48) to generate K14-Cre Brca1<sup>5382stop</sup> p53<sup>+/−</sup> [KB1(185stop)P] and K14-Cre Brca1<sup>185stop</sup> p53<sup>+/−</sup> [KB1(5382stop)P] mice. Full details on the generation of the Brca1<sup>185stop</sup> and Brca1<sup>5382stop</sup> alleles and mouse genotyping are provided in the Supplemental Methods.

Embryo isolations. Timed matings were performed between Brca1<sup>185stop</sup> or Brca1<sup>5382stop</sup> heterozygous male and female mice. The impregnated females were sacrificed at various time points after conception, and their uteri were isolated in ice-cold PBS. The embryos were isolated by removing the muscular wall of the uterus, Reichert’s membrane, and the visceral yolk sac. The visceral yolk sac was used for genotyping.

Orthotopic transplantations and drug interventions. Small fragments of mammary tumors from KP, KB1P, KB1(185stop)P, or KB1(5382stop)P mice were transplanted orthotopically into FVB:129/ Ola F1 hybrid female mice as described previously (55). To generate mouse mammary tumors from KB1(185stop)P cell lines, 500,000 cells were transplanted into the fourth right mammary fat pad of 9-week-old female athymic nude RjOri:NMRI-Foxn<sup>+/−</sup>/Foxn<sup>−/−</sup> mice (Janvier Labs) in 50 μl Matrigel (Corning) and PBS (1:1). When the tumor volume exceeded 200 mm<sup>3</sup>, the mice were treated with the maximum tolerated dose (MTD) of cisplatin, olaparib, or AZD2461 (23, 55, 61). To study resistance, animals received additional doses of cisplatin when tumors grew back to a size of 200 mm<sup>3</sup>. Animals were sacrificed when the tumor volume exceeded 1,500 mm<sup>3</sup> or when they became ill from drug toxicity.

The 3 PDX models used in this study were derived from PARP inhibitor-naive BRCA1<sup>5382stop</sup> carriers presenting with hormone receptor-negative, HER2-negative, triple-negative breast cancers (TNBCs). The patient from whom PDX179 was derived had received cisplatin prior to tumor implantation into nude mice. PDX179 and PDX179 were derived from metastatic lesions, and PDX230 was derived from a primary tumor (additional clinical history is provided in the Supplemental Information). Tumors were s.c. implanted into 6-week-old female athymic nude HsdCpb:NMRI-Foxn<sup>+/−</sup>/Foxn<sup>−/−</sup> mice (Envigo). Animals were supplemented with 1 μmol/l 17β-estradiol (Sigma-Aldrich) in their drinking water. Upon xenograft growth, tumor tissue was reimplanted into recipient mice, which were randomized for olaparib or vehicle treatment, starting at tumor volumes between 50 and 500 mm<sup>3</sup>. Treatment consisted of either vehicle (10% v/v DMSO in 10% w/v Kletonse (Roquette Laisa España) [HP-β-CD] in purified, deionized water) or 50 mg/kg olaparib in vehicle, administered i.p. 5 days per week.

Ribosome profiling of tumor cell lines. Ribosome-protected RNA fragments were isolated and described as described before (63), and libraries were sequenced on an Illumina HiSeq 2000 system. A detailed protocol is described in the Supplemental Methods.

Mass spectrometric analysis of mouse RING-less BRCA1 protein. Immunoprecipitated BRCA1 was subjected to nanoflow liquid chromatography-tandem mass spectrometry (LC-MS/MS) on an 1100 Series Capillary LC System (Agilent Technologies) coupled to an LTQ Orbitrap XL Mass Spectrometer (Thermo Fisher Scientific). Detailed information on the experimental procedures is provided in the Supplemental Methods.

Sanger sequencing. Sequencing was done using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). Sequencing was performed on both genomic DNA and cDNA from tumors and spleens. Primer information is provided in the Supplemental Methods.

Array comparative genome hybridization and data analysis. Genomic DNA samples from tumors and spleens were labeled with the NimbleGen Dual-Color DNA Labeling Kit and hybridized to the NimbleGen 12-plex 135K full-genome mouse custom NKI array. Details of the analysis are described in the Supplemental Methods.

Clonogenic survival assays. Human and mouse cancer cell lines were plated onto 12-well plates at densities of 15,000 (SUM1315MO2, SUM149PT, HCC1957, and U87; 2891); 6,000 (U20S); and 600–1,000 (KB1(185stop)P) cells per well, respectively. For comparisons of KB1(185stop)P, KP, and KB1P cell lines, 1,000 KB1(185stop)P and KP cells and 3,000 KB1P B11 cells were plated onto 6-well plates. Twelve hours to one day after plating, a range of concentrations of cisplatin (Sigma-Aldrich or Mayne Pharma) in saline or olaparib (AZD2281; Selleck Chemicals or AstraZeneca) in DMSO was added to the cells. Cells were also treated with only saline or DMSO as the “no-drug control.” Cells were allowed to grow in the presence of the drug for 7 days and stained with 0.1% to 1% crystal violet. Clonogenic survival was determined by measuring the absorbance of crystal violet at 590 to 595 nm. Full details on cell culture conditions are provided in the Supplemental Methods.

BRCA1- and Brca1-knockdown experiments. Cells were transduced with plKO-puro shRNA viruses (TRC library clones; Thermo Scientific Open Biosystems) targeting human BRCA1 or mouse Brca1 or an NT control. After puromycin selection, cells were assayed for γ irradiation-induced DNA repair foci or seeded for clonal growth. SUM-1315MO2 cells were incubated with CellTiter-Blue (Promega) or fixed and stained with crystal violet after 2 weeks of clonal growth, and fluorescence or absorbance were measured. Full details are provided in the Supplemental Methods.

CRISPR/Cas9-mediated inactivation of the Brca1<sup>185stop</sup> allele. CRISPR/Cas9 mutagenesis was performed as described previously (68). Briefly, KB1(185stop)P mouse mammary tumor cells were transiently transfected with a modified pX330 CRISPR/Cas9 plasmid (Addgene plasmid 42230) containing a puromycin resistance marker and targeting the BKCT-encoding region of Brca1. After 2 days of puromycin selection (1.8 μg/ml), cells were seeded at clonal density, and colonies were selected for further analyses and experiments. Full details are provided in the Supplemental Methods.

Immunoblotting. Tumor protein lysates were made by using a microhomogenizer and radioimmunoprecipitation assay (RIPA) lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 0.1% SDS, 0.1% deoxycholate, 1% NP40), complemented with 2× Complete Protease Inhibitor Cocktail (Roche) and Pefabloc (Roche; 1 mg/ml). Following homogenization on ice, tumor lysates were kept on ice for 30 minutes. After a
short spin, protein concentrations were determined with the BCA Protein Assay Kit (Pierce, Thermo Fisher Scientific), and samples were prepared for gel electrophoresis. Western blotting was performed as described previously (69).

Immunofluorescence analyses and IHC. Immunofluorescence and IHC were essentially performed as described before (43, 70). For ex vivo analysis of RAD51 foci formation, cells from cryopreserved tumors were grown on glass coverslips for 36 to 48 hours, then irradiated with 10 Gy, and fixed 6 hours later in 2% paraformaldehyde. To quantify RAD51 foci in single tumor cells, 150–200 cells per condition were counted blindly. Cells were scored as RAD51 positive if they had more than 10 RAD51-positive dots per nucleus. For quantification of 53BP1 foci in formalin-fixed, paraffin-embedded (FFPE) sections of olaparib-treated mouse mammary tumors, at least 200 cells were counted using ImageJ software (NIH). Recruitment of RPA to 53BP1-positive DNA DSBs was analyzed using a particle irradiation through the bottom of a Mylar dish, as described by Stap et al. (71). A list of Abs and full procedures for immunohistochemical and immunofluorescence assays are provided in the Supplemental Methods.

aCGH data. aCGH data generated in this study have been deposited in the NCBI’s Gene Expression Omnibus (GEO) database (GEO GSE43997).

Statistics. Statistical analyses were performed using GraphPad Prism 6 (GraphPad Software). Kaplan-Meier survival curves were compared using the log-rank test, contingency tables were compared using Fisher’s exact test, and other data were compared using 2-tailed t tests. A P value of less than 0.05 was considered significant, except when Bonferroni’s correction was applied to correct the significance threshold for multiple testing (P < 0.025 for Figure 6D, Figure 7, B, E, and F, Figure 8D, Supplemental Figure 7B, Supplemental Figure 9, E and F, and P < 0.01 for Supplemental Table 3D).

Study approval. All experiments involving genetically engineered mouse models complied with local and international regulations and ethics guidelines and were approved by the IACUC of the Netherlands Cancer Institute (DEC-NKI). For PDX tumors, patients’ consent for tumor use in animals was obtained under a protocol approved by the Vall d’Hebron Hospital Clinical Investigation Ethics Committee. Mice were maintained and treated in accordance with institutional guidelines, and experiments were approved by the IACUC of the Vall d’Hebron Institute of Research (VHIR Animal Use Committee).

Author contributions

RD, TT, PB, and JJ designed the study. RD, KKD, HvdG, IvdH, IB, CC, DC, MCB, UB, ES, EvdB, EW, MP, FLP, LvD, DHWD, and PB performed experiments. RD, KKD, IB, CC, CK, SK, FLP, RE, DHWD, and PB analyzed data. RD, SR, MvdV, JAAD, DCvG, RA, JB, VS, TT, PB, and JJ supervised experiments. RD, PB, and JJ wrote the manuscript, with input and scientific advice from VS and TT.

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