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PLoS genetics (2011), 7(7)

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The Epistatic Relationship between BRCA2 and the Other RAD51 Mediators in Homologous Recombination

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Abstract

RAD51 recombinase polymerizes at the site of double-strand breaks (DSBs) where it performs DSB repair. The loss of RAD51 causes extensive chromosomal breaks, leading to apoptosis. The polymerization of RAD51 is regulated by a number of RAD51 mediators, such as BRCA1, BRCA2, RAD52, SFR1, SWS1, and the five RAD51 paralogs, including XRCC3. Here we show that brca2-null mutant cells were able to proliferate, indicating that RAD51 can perform DSB repair in the absence of BRCA2. We disrupted the BRCA1, RAD52, SFR1, SWS1, and XRCC3 genes in the brca2-null cells. All the resulting double-mutant cells displayed a phenotype that was very similar to that of the brca2-null cells. We suggest that BRCA2 might thus serve as a platform to recruit various RAD51 mediators at the appropriate position at the DNA–damage site.

Introduction

Homologous recombination (HR) maintains genome integrity by accurately repairing double-strand breaks (DSBs) that arise during the mitotic cell cycle or are induced by radiotherapy [1,2]. HR also plays an important role in releasing the replication forks that stall at damaged template DNA strands [3,4]. Thus, effective HR makes tumor cells tolerant to the chemotherapeutic agents that damage DNA and stall replicative DNA polymerases. Such chemotherapeutic agents include cis-diaminedichloroplatinum(II) (cisplatin), camptothecin, and poly(ADP-ribose) polymerase (PARP) inhibitors, including olaparib (AstraZeneca). Cisplatin is a crosslinking agent that generates intra- and inter-strand cross-links and thereby stalls replicative DNA polymerases. Camptothecin inhibits the ligation of single-strand breaks (SSBs) that are formed during the normal functioning of topoisomerase I. Resulting unrepaird SSBs are converted to DSBs upon replication. Similarly, PARP inhibitors interfere with SSB repair [5]. Since HR plays a major role in repairing DNA lesions generated by camptothecin, cisplatin, and PARP inhibitors [6], measuring HR efficiency in individual malignant tumors may help predict the efficacy of these chemotherapeutic treatment for each tumor [7–9].

HR-dependent DSB repair is accomplished by the following step-wise reactions [10]. DSBs are processed by the Mre11/Rad50/Nbs1 complex and the CtIP, Exo1 and DNA2 nucleases to develop 3’ single-strand DNA (ssDNA) tails [11–17]. RAD51, an essential recombinase, polymerizes on these ssDNAs, leading to the formation of nucleoprotein filaments. These filaments undergo homology search and subsequent invasion into homologous duplex DNA to form a D-loop structure, where they serve as a primer for DNA synthesis [18,19]. After the extended end is displaced from the D-loop, it anneals to its partner-end to complete DSB repair. We know that RAD51 plays a key role in HR in vertebrate cells, as inactivation of RAD51 results in the accumulation of chromosomal breaks in mitotic cells and inhibits the completion of even a single cell cycle [1,2]. The polymerization of RAD51 at damage sites is strictly regulated by a number of accessory factors (hereafter called RAD51 mediators), including the five RAD51 paralogs, SWS1, RAD52, SFR1, BRCA1, BRCA2, and PALB2 [3,20–27]. The functional relationships of these RAD51 mediators are poorly understood, because cells deficient in multiple RAD51 mediators have not been established.

BRCA2 was originally identified as a tumor suppressor, as germline mutation of the BRCA2 gene results in a high risk of developing breast, ovarian, pancreatic, prostatic, and male breast cancer [3,20,28,29]. BRCA2 is recruited to processed DSBs, and facilitates the assembly of RAD51 at the single-strand tail. The middle of the BRCA2 protein has eight BRC repeats that encode a truncated form of BRCA2. Cells derived from brca2tr mice and brca2tr DT40 cells are able to...
Mutations in BRCA1 and BRCA2 predispose hereditary breast and ovarian cancer. Such mutations sensitize to chemotherapeutic agents, including camptothecin, cisplatin, and poly(ADP-ribose) polymerase (PARP) inhibitor, since RAD51 mediators including both BRCA proteins promote repair of DNA lesions induced by these drugs. Little is known of functional relationship among RAD51, BRCA2, and other RAD51 mediators, because no brca2-null cells were available. Furthermore, the phenotype of sws1 mutants has not been documented. We here disrupted every known RAD51 mediator and analyzed the phenotype of the resulting mutants in both BRCA2-deficient and -proficient backgrounds. The understanding of the function of individual RAD51 mediators and their functional interactions will contribute to the accurate prediction of anti-cancer therapy efficacy.

Results

Loss of SFR1 or SWS1 has a limited impact on DNA-damage responses

To analyze SFR1 and SWS1, we disrupted the SFR1 and SWS1 genes in DT40 cells (Figure 1A–1D). Table 1 summarizes the selection marker genes we used to disrupt genes in this study. The resulting sfr1 and sws1 mutant clones proliferated with nearly normal kinetics (Figure 2) and exhibited an increase in cellular sensitivity to cisplatin. The sfr1 mutant was sensitive also to camptothecin and olaparib (Figure 3). Both mutants showed a slight but significant decrease in ionizing-radiation-induced RAD51 focus formation (Figure 4). We conclude that SFR1 and SWS1 indeed work as RAD51 mediators, though their contribution to HR-dependent repair is less significant than that of BRCA1, BRCA2, and the RAD51 paralogs.

The brca2-null mutant is capable of proliferating

To create brca2-null cells, we generated compound heterozygous mutant cells (hereafter called BRCA2−/−/con1 cells) (Figure 1E). The whole coding sequence was deleted in the minus (−) allele of the −/− conditional-null allele-1 (−/−/con1) genotype of the BRCA2−/−/con1 cells (Coding sequence deletion allele in Figure 1F). We conditionally deleted the con1 allele of the BRCA2−/−/con1 cells by adding tamoxifen, which activated the chimeric Cre recombinase [28] and thereby eliminated the promoter and coding sequences, including exons 1 and 2 of the con1 BRCA2 allelic gene (BRCA2 conditional-null allele-1 in Figure 1F). At day two of continuous tamoxifen treatment, the vast majority of cells lost the intact BRCA2 gene in the conditional allele, and a substantial fraction of cells began to die. To our surprise, we were able to reproducibly establish clonally expanding cells wherein the conditional BRCA2 allele was deleted (BRCA2−/− cells, hereafter called brca2null cells) from 10 to 20% of the tamoxifen-treated populations. We verified deletion of the BRCA2 allele in the established clones by Southern-blot (Figure 1G) and western-blot (Figure 1H) analysis. The ability of the brca2-deleted cells to proliferate is in marked contrast to the immediate cell death observed in rad51-deleted cells [1]. The plateauing efficiency of the brca2-null clones was around 20%, which is significantly lower than that of the wild-type (100%) and brca2tr (60%) cells [33].

One obvious concern with this experiment was that expression of the N-terminal-truncated BRCA2 protein in the Cre-mediated deletion lines could allow for residual function. We therefore created a second version of the conditionally inactivated BRCA2 allele, wherein sequences spanning from the promoter to intron 12 could be eliminated by induction of Cre (BRCA2 conditional-null allele-2 in Figure S1). We exposed the resulting compound heterozygous mutant cells to tamoxifen and confirmed reproducible establishment of BRCA2-deleted clones (Text S1). This second brca2 conditional-null allele supported proliferation with generation times very similar to those of the first version of the brca2-null cells (data not shown). The more extensively deleted brca2 clones showed a phenotype indistinguishable from that of the smaller deletion clones, indicating that both deletions confer the null phenotype.

BRCA2 contributes to genome maintenance to a lesser extent than does RAD51

We assessed the proliferative properties of brca2-null clones by monitoring their growth curve (Figure 2A and 2B) and cell cycle (Figure 2C). Wild-type cells doubled every 8 hours and increased in cell number by 64 times over 48 hours. The brca2-null cells increased by 17 times over 48 hours (Figure 2A), calculated as a
Figure 1. Gene disruption of the SFR1, SWS1, and BRCA2 loci. (A) Schematic representation of the endogenous SFR1 locus and gene-disruption constructs carrying the puro or bsr selection marker gene. The solid boxes represent exons, and numbers right above boxes represent exon numbers. Relevant BamHI and EcoRI sites are indicated. (B) Southern-blot analysis of genomic DNA digested by both BamHI and EcoRI was performed using the probe DNA shown in (A). Positions of hybridizing fragments of wild-type (WT) and targeted loci are indicated. (C) Schematic representation of the endogenous SWS1 locus and gene-disruption constructs carrying the puro or his selection marker gene. Relevant EcoRV and NotI sites are indicated. (D) Southern-blot analysis of genomic DNA digested by EcoRV and NotI was performed with the probe DNA shown in (C). (E) Experimental methods to generate BRCA22/2 and RAD51mediator2/2/BRCA22/2 cells. We generated BRCA22/2 cells from conditional mutant BRCA22/con1 cells. In the minus allele of the BRCA22/con1 cells, the whole coding sequence is deleted (hereafter called the coding sequence deletion allele). The structures of the conditional-null allele-1 (con1) is shown in (F). Treatment of BRCA22/con1 cells with 4-OH tamoxifen (TAM) led to the generation of BRCA22/-. To generate RAD51mediator2/2/BRCA22/2 cells, we disrupted one of the RAD51mediator genes in BRCA22/con1 cells. Exposure of the resulting RAD51mediator2/2/BRCA22/2 cells to TAM led to the generation of RAD51mediator2/2/BRCA22/-. (F) Schematic representation of BRCA2 conditional-null allele and the brca2-null allele wherein the whole coding sequences are deleted. The conditional-null allele-1 (con1) shown on top was described previously [33]; the structure of the coding sequence deletion allele is shown in the second row. Treatment of the BRCA22/con1 cells with TAM causes deletion of the promoter and initiation codon. The relevant EcoRI sites in the conditional-null allele-1, the relevant Xbal sites in the coding sequence deletion allele, and the position of the probes used in the Southern-blot analysis (G) are indicated. The solid boxes and arrowheads represent the exons and loxP signals, respectively. (G) Southern-blot analysis of the conditional allele (left) and the other coding sequence deletion (right) allele (right) in BRCA22/con1 cells with (+) or without (−) TAM treatment. Southern-blot analysis of EcoRI or XbaI-digested genomic DNA was performed with the probe DNA shown in (F). (H) Western-blot analysis to verify the loss of BRCA2 protein in BRCA22/- cells derived from BRCA22/con1 cells. doi:10.1371/journal.pgen.1002148.g001
Figure 2. Decreased cellular proliferation in brca2-null cells. (A) Growth curve for cells of the indicated genotype. (B) The relative rate of cell growth per 8 hours (a single cell cycle for wild-type cells) plotted for cells carrying the indicated genotypes. Each value represents the averaged results from three separate experiments. Error bars represent standard deviation. (C) Cell-cycle distribution of brca2-null cells that were pulse-labeled with BrdU for 10 minutes and subsequently stained with FITC-conjugated anti-BrdU antibody (Y-axis, log scale) and propidium iodide (PI) (X-axis, linear scale). The upper gate indicates cells incorporating BrdU (S phase), the lower middle gate indicates G1 cells, and the lower-right gate indicates G2/M cells. The sub G1 fraction (lower-left gate) indicates dead cells. The number in each gate indicates the percentage of gated events. doi:10.1371/journal.pgen.1002148.g002
brca2-null cells displayed a stronger phenotype than the brca1, brca2tr, or rad51 paralog mutant clones

We analyzed cellular tolerance to camptothecin, cisplatin, and olaparib by measuring cellular survival at 72 hours (7–9 cell cycles) after continuous exposure to these agents in a liquid medium. We did not use the conventional colony-formation assay for this analysis, because the plating efficiency of the brca2-null cells was only 20%, 5-fold lower than that of wild-type cells. Figure 3A presents an example of cellular sensitivity to camptothecin, a DNA-damaging agent. Subsequent figures illustrate the sensitivity of each mutant, assessed by LC50 values, i.e., the dose that reduces cell survival to 50% relative to the LC50 value of wild-type cells, which is defined as 100% (Figure 3B–3E).

In the cellular-survival analysis, the brca2-null cells showed an increased sensitivity to camptothecin (Figure 3B), cisplatin (Figure 3C), and olaparib (Figure 3D and 3E). Moreover, sensitivity to cisplatin and olaparib was higher with the brca2-null cells than for any of the other HR mutant cells, including the brca1, rad52, rad54, and xrc3 clones (Figure 3). We therefore conclude that BRCA2 plays a more important role in HR-dependent repair than do the other RAD51 mediators, including BRCA1, RAD52, RAD54, the RAD51 paralog, SFR1, and SWS1.

The less prominent phenotype of the brca2tr cells compared to the brca2-null cells indicates that the BRCA2 BRCT3-truncated protein retains significant residual HR function. Although brca1 cells were less sensitive to cisplatin and olaparib than were brca2-null cells, the brca1-null cells exhibited a slightly higher sensitivity to camptothecin than did the brca2-null cells (Figure 3A). The greater contribution of BRCA1 to cellular tolerance to camptothecin might be attributable to the role played by BRCA1 in DNA-damage responses other than HR, such as collaborative action with CtIP to eliminate covalently bound oligo-peptides from DSBs [15].

We next measured the frequency of HR-dependent repair of I-SceI-mediated DSBs in a recombination substrate, SCneo, inserted into the OVALBUMIN locus [44,45] (Table 3). The frequency of HR-dependent DSB repair was calculated as the number of neomycin-resistant (neo+) colonies relative to the number of plated cells. The frequency of HR in the brca2-null, brca2tr, and brca1 cells was decreased by 1.5×10^−5, 1.5×10^−2, and 4.5×10^−5-fold, respectively, compared with wild-type cells. We conclude that the brca2-null cells retain residual HR activity, which may account for their viability even in the complete absence of BRCA2.

Since BRCA2 promotes the loading of RAD51 at damage sites, we measured RAD51 focus formation at 3 hours after ionizing radiation. The number of RAD51 foci was reduced but not eliminated in the brca1 and brca2tr clones, compared with wild-type cells (Figure 4). These findings are consistent with previous observations [33,46]. By contrast, we hardly detected any RAD51 focus formation in the brca2-null cells. In conclusion, the BRCA2 protein plays a key role in the efficient recruitment of RAD51 to DNA-damage sites, but is not essential for every HR reaction.

BRCA2 is required for the effective participation of BRCA1, RAD52, SFR1, SWS1, and XRCC3 in HR

The idea that RAD51 carries out HR even without BRCA2 led us to investigate whether or not other RAD51 mediators substitute for BRCA2 in the promotion of RAD51-dependent HR. To this end, we deleted the BRCA1, RAD52, SFR1, SWS1, and XRCC3 genes in the conditional brca2-null background, then inactivated the BRCA2 gene by treating the cells with tamoxifen (Figure 1E). We also disrupted the RAD54 gene in the conditional brca2-null background (Table 1). The RAD54 protein promotes HR after the assembly of RAD51 at DNA-damage sites [47]. To our surprise, we were able to reproducibly establish all resulting double mutants, although a substantial fraction (~30%) of the brca2-null cells died each cell cycle.

The growth kinetics for the brca1/brca2-null, rad52/brca2-null, sfr1/brca2, sws1/brca2-null, and xrc3/brca2-null double-mutant clones was similar to those of the brca2-null single mutant (Figure 2). Taking the very severe phenotype of brca1 cells into account, the viability of the brca1/brca2-null cells was surprising. The cloning efficiency of the brca1/brca2-null cells was slightly higher than that of the brca2-null single-mutant cells (30% compared to 20%). Accordingly, the number of spontaneous chromosomal aberrations in the brca1/brca2-null cells was consistently slightly lower than that in the brca2-null cells (Table 2). In summary, although the loss of either BRCA1 or BRCA2 greatly increased the number of spontaneous chromosomal breaks, inactivation of BRCA1 in the brca2-null cells resulted in a slight reduction in the severity of the brca2 phenotype. An early study shows rad52/xrc3 double-mutant cells are synthetic lethal and exhibit numerous chromosomal breaks [24], whereas we here found that the brca2/rad52 and brca2/xrc3 double-mutant cells were viable. Thus, the synthetic lethality might be attributable to BRCA2 mediated formation of toxic HR intermediates, because the brca2/xrc3 cells exhibit spontaneously arising isochromatid breaks, where two sisters are broken at the same site due to defective completion of HR [48]. To test this hypothesis, we conditionally inactivated the BRCA2 gene in the rad52/xrc3 cells (Text S1). We found that the inactivation of the BRCA2 gene indeed rescued the rad52/xrc3 cells (Figure S2). This observation indicates that the synthetic lethality of the rad52/xrc3

Figure 3. Cellular tolerance to camptothecin, cisplatin, and olaparib. (A) Cells of the indicated genotype were exposed to camptothecin for 72 hours, a period during which wild-type cells are able to divide nine times in the absence of exogenous DNA damage. The X-axis represents the concentration of camptothecin and the Y-axis represents the relative number of surviving cells at 72 hours. The vertical dotted lines show LC50 values (the concentration of camptothecin that reduces cellular survival to 50% relative to cellular survival without camptothecin treatment). Relative LC50 values of camptothecin (B), cisplatin (C), and Poly(ADP-ribose) polymerase inhibitor olaparib (D and E) are shown. Values shown are mean ± SD. doi:10.1371/journal.pgen.1002148.g003
cells does not argue against the idea that the functioning of RAD52 and XRCC3 depends on BRCA2. Likewise, the formation of toxic HR intermediates might explain the apparent discrepancy between the viability of rad52/brca2-null DT40 cells and the mortality caused by shRNA mediated depletion of RAD52 in brca2 deficient mammalian cells [49], as the latter cells express a residual amount of RAD52 and truncated BRCA2 proteins perhaps leading to the formation of toxic HR intermediates.

We next measured the sensitivity of the brca1/brca2-null, rad52/brca2-null, rad54/brca2-null, sfs1/brca2-null, sws1/brca2-null, and xrcc3/brca2-null double-mutant clones to camptothecin, cisplatin, and olaparib (Figure 3 and Figure 5). Remarkably, inactivation of

![Figure 4. γ-ray–induced Rad51 subnuclear foci in RAD51-mediator mutant cells.](image-url)
any gene did not increase cellular sensitivity to the three damaging agents by more than two-fold. This observation indicates that the contribution made by BRCA1, the RAD51 paralogs, RAD52, RAD54, SFR1, and SWS1 to HR depends mostly on BRCA2. Interestingly, the loss of BRCA1, SFR1, and SWS1 somewhat increased the cellular tolerance of the \textit{brca2-null} cells to cisplatin. Similarly, the loss of SWS1 increased the cellular tolerance of the \textit{brca2-null} cells to camptothecin and olaparib. This increased tolerance was not accompanied by the upregulation of RAD51 focus formation (data not shown). We therefore suggest that, in the absence of BRCA2, SWS1 has a moderately antagonistic effect on HR-dependent repair. By contrast, the loss of RAD52 and XRCC3 significantly increased the cellular sensitivity of the \textit{brca2-null} cells to olaparib. In summary, BRCA2 is required for all the analyzed RAD51 mediators to function, and the functional relationships between BRCA2 and the other RAD51 mediators in HR-mediated repair differ slightly depending on the type of DNA damage.

### Discussion

In this study, we established \textit{brca2-null} cells as well as cells deficient in each of the RAD51 mediators. We show that BRCA2 plays a more important role in the promotion of both RAD51 polymerization at DNA-damage sites and HR-dependent repair than does any other RAD51 mediator, including BRCA1, the RAD51 paralogs, RAD52, SFR1, and SWS1. The ability of \textit{brca2-null} cells to proliferate is in marked contrast with the immediate cell death that occurs upon depletion of RAD51 [1]. Therefore, RAD51 is able to perform HR even in the absence of BRCA2. To explore the question of which RAD51 mediators might substitute for BRCA2 in the promotion of RAD51-dependent HR repair, we inactivated the RAD51 mediators in \textit{brca2-null} cells. Loss of any one of the other RAD51 mediators did not further reduce the viability of \textit{brca2-null} cells. In a related study, we also found that the \textit{brca2-null} mutant and the \textit{palb2/brca2-null} double mutant showed the same phenotype with respect to both spontaneous chromosomal aberrations and increased sensitivity to DNA-damaging agents (manuscript in preparation). Thus we conclude that BRCA1, PALB2, the RAD51 paralogs, RAD52, SFR1, and SWS1 all require BRCA2 to contribute to HR.

BRCA2 plays a major role in the recruitment of RAD51 to DNA–damage sites, but is not essential for every HR reaction. Data on \textit{Ustilago maydis} [50] and \textit{Arabidopsis thaliana} [51] suggest that BRCA2 might be essential for RAD51 to function in any HR reaction. However, we here report that RAD51 can form HR products even in \textit{brca2-null} cells, indicating that RAD51 plays a more important role than BRCA2 in HR. This hierarchy between RAD51 and BRCA2 is supported by previous reports of experiments with mice, as \textit{rad51 null} embryos died earlier (~E6.5) than did \textit{brca2 null} (~E8.5) embryos [52,53]. The viability of \textit{brca2-null} DT40 cells is consistent with the clonal expansion of BRCA2-deficient cells derived from mammary epithelial lineage-specific or T cell lineage-specific BRCA2-null-deficient mice [54,55]. Adding to these findings, we here show solid evidence that vertebrate RAD51 is capable of functioning in the absence of BRCA2.

### Table 1. DT40 mutants used in this study.

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<th>Selection marker for gene disruption</th>
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### Table 2. Spontaneous chromosomal aberrations.

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Spontaneous chromosomal aberration of indicated genotypes. Data are the numbers of aberrations per 100 cells. At least 100 mitotic cells were analyzed for each genotype. doi:10.1371/journal.pgen.1002148.0002
Collaboration between BRCA1 and BRCA2 is required for efficient HR
The phenotypic analysis of brca1, brca2-null, and brca1/brca2-null clones, combining with the previous study of rad51-null cells, reveals the functional relationship described as follows. The capability of HR was dramatically diminished when either BRCA1 or BRCA2 was absent, indicating that the collaboration of BRCA2 and BRCA1 is required for efficient HR events. brca2-null cells exhibited more prominent defects in HR than did brca1-null cells, indicating that BRCA2 can function in HR independently of BRCA1. Moreover, BRCA2’s contribution to the repair of cisplatin-induced interstrand crosslinks is more significant than BRCA1, which is likely attributable to the fact that BRCA2, but not BRCA1, functions in the Fanconi anemia repair pathway [56]. BRCA1 has additional functions other than in HR, such as mediating the damage checkpoint and processing DSBs [15,57]. The fact that rad51-null cells have a considerably stronger phenotype than brca2-null cells indicates that RAD51 could still perform HR-dependent repair in brca2-null cells.

The phenotypic similarities between the brca2-null and the brca1/brca2-null clones indicate that BRCA1 contributes to HR by collaborating with BRCA2. Presumably, the two BRCA proteins form a functional unit and collaborate intimately to load RAD51 at DNA-damage sites. This idea is supported by the fact that BRCA1 physically associates with BRCA2 through the PALB2 protein [58]. However, this idea is challenged by recent studies that suggest that BRCA1 plays a role in the resection of DSBs [14,59]. One possible scenario is that the complex formed by BRCA1 and BRCA2 may allow for close collaboration between the BRCA1-dependent resection of DSBs and the subsequent loading of RAD51 on the resulting 3’ overhang. Such an interaction interface might be shared by the E. coli RecBCD complex, which serves as the DSB resection complex and also interacts directly with RecA following chi site recognition [60]. In summary, the phenotypic analysis of brca1, brca2-null, and brca1/brca2-null DT40 clones demonstrates that BRCA1 controls RAD51 in HR, mainly through collaboration with BRCA2.

BRCA2 is required for BRCA1, PALB-2, the RAD51 paralogs, SFR1, and SWS1 to promote HR
Our study reveals that rad52/brca2-null, sfr1/brca2-null, sas1/brca2-null, and xrc3/brca2-null clones exhibit a phenotype very similar to that of brca2-null cells (Figure 5). In a separate study, we confirmed phenotypic similarity between brca2-null and palb2/brca2-null clones (data not shown). We therefore suggest that, like BRCA1, PALB2, the RAD51 paralogs, RAD52, SFR1, and SWS1 are also able to participate in HR, mostly depending on BRCA2. One possible scenario is that BRCA2 is recruited to DNA-damage sites through PALB2 or by directly interacting with the junction between the duplex DNA and the single-strand sequences [61]. BRCA2 might thus serve as a platform to recruit various RAD51 mediators to the appropriate positions of DNA-damage sites (Figure 6).

Applications for clinical research
DT40 is a unique cell line that offers a panel of DNA-repair-deficient isogenic mutants derived from a stable parental line. DT40 cells have several characteristics that affect cellular responses to anti-cancer agents. First, DT40 appears, for unknown reasons, to possess a significantly higher HR efficiency than any mammalian cell line [43]. The efficient HR in DT40 cells is prominent particularly in HR between diverged homologous sequences such as Immunoglobulin V gene diversification [62] and gene targeting, where the selection marker genes of gene-disruption constructs may interfere with HR as heterologous sequences. Second, like many cancer cells, DT40 lacks the functional p53, and as a result has no G1/S damage checkpoint [63]. In addition, 70% of the DT40 cell cycle takes place in the $S$ phase. Thus, DNA damage at any phase of the cell cycle may have a direct impact on DNA replication. These characteristics, specific to DT40, suggest that a defect in DNA repair associated with DNA replication, including HR-mediated DNA repair, may display a more prominent phenotype in DT40 cells than in other cell lines that have a longer G1 phase and/or a normal G1/S checkpoint. Bearing this in mind, DT40 is revealed as a unique and valuable tool and has been used extensively to explore the role of individual HR factors responsible for cancer therapy.

Materials and Methods
Cell culture and DNA transfection
Cells were acquired and cultured as described previously [1,43]. All mutants were isolated from single colonies. DNA transfection
and selection were performed as described previously [43,64]. Details of the cell lines used in this study are shown in Table 1.

Generation of SFR1<sup>–/–</sup> DT40 cells

To disrupt the SFR1 gene, we generated SFR1-puro and SFR1-bsr disruption constructs by combining two genomic PCR products with the puro- or bsr-selection-marker cassette. Genomic DNA sequences were amplified using the 5′-CCCAGTACTGAG-\(\text{GGGGTGCATTGCTTGAGCAG}3′\) and 5′-CCCTAGTTGGAGTATTGG-\(\text{CACATTCATGGCTAAAG}3′\) primers for the upstream arm, and the 5′-GGGTCAAGATACTGGAGAGATGGAGC-\(\text{CCAGGGCTCAAAAC}3′\) and 5′-CCACATCGCACTCAAAAGGGCAGG-\(\text{AGGA}3′\) primers for the downstream arm.

**Figure 5.** Effect of brca2 deletion on sws1-, sfr1-, rad52-, rad54-, xrcc3-, and brca1-deficient cells. Cellular sensitivities to the indicated DNA damaging agents were analyzed using the same method as in Figure 3. The LC₅₀ values are shown in Figure 3B–3E.

doi:10.1371/journal.pgen.1002148.g005
ACG-3' primers for the downstream arm. Amplified PCR products were cloned into pCR2.1-TOPO vector (Invitrogen). The 1.7 kb fragment of the upstream arm was cloned into the KpnI site of pCR2.1 containing the 3.0 kb downstream arm. Marker-gene cassettes were inserted at the BamHI site of the resulting plasmid.

To generate SFR1/2 cells, SFR1-puro and SFR1-bsr disruption constructs linearized with NotI were transfected sequentially by electroporation (Bio-Rad). The genomic DNA of the transfectants was digested with both BamHI and EcoRI, and gene-targeting events were confirmed by Southern blot analysis. The probe was prepared from a PCR-amplification of DT40 genomic DNA using primers 5'-GAACAGCACCACGCAATTCA-3' and 5'-CCTTAGAGTTGCACTCATTGG-3'.

Cloning of SFR1 cDNA

Chicken SFR1 cDNA was isolated by PCR amplification of the primary cDNAs using the 5'-GGGACAGCACTCCAGAGCAATTCA-3' and 5'-GGACAAGCTTGTTGTAATTCCACTATTGAGG-3' primers. The gene bank accession number of the chicken SFR1 gene is XM-001234167.

Generation of SWS1/2 DT40 cells

To disrupt the SWS1 gene, we generated SWS1 gene-disruption constructs containing the 2.6 kb upstream and the 3.0 kb downstream genomic fragments. The 2.6 kb fragment was PCR-amplified using the 5'-GGGACAGCTTTGCTAATTCCACTATTGAGG-3' and 5'-GGGACAGCTTTGCTAATTCCACTATTGAGG-3' primers. The 3.0 kb fragment was PCR-amplified using the 5'-GGGACAGCTTTGCTAATTCCACTATTGAGG-3' and 5'-GGGACAGCTTTGCTAATTCCACTATTGAGG-3' primers. Note that the underlined sequences denote the recognition sequences in the Gateway system (Invitrogen). Using the MultiSite Gateway system with pENTR-lox-his, pENTR-lox-puro and pDEST-DTA-MLS [65], a floxed his or puro gene was inserted between the upstream and downstream arms on a plasmid carrying a diphtheria toxin A (DT-A) gene, thus yielding the two targeting vectors, SWS1-his/loxP and SWS1-puro/loxP.

To generate SWS1/2 cells, SWS1/2-loxP and SWS1/2-loxP gene-disruption constructs linearized with AscI were transfected sequentially into DT40 cells (Bio-Rad). The genomic DNA of the
transfectants was digested with both EcoRV and NotI, and gene-targeting events were confirmed by Southern-blot analysis. The probe was prepared by PCR-amplification of chicken genomic DNA using the 5'-GCTCGAGGAGAACGACTCT-3' and 5'-GTACGAGAATTCTTTCT-3' primers.

Cloning of SW5 cDNA

The gene bank accession numbers for the human and chicken Sox1 genes are XP-038899 and XP-415841, respectively. RTPCR of DT40 transcripts was done using the 5'-CCGCTCGAGTGA- CATGATGACGCTACGCA-3' and 5'-CCGGATCCATATGACGCTACGCA-3' primers.

Generation of BRCA2-/- DT40 cells

The brc2-null mutant cells were generated as follows (Figure 1). We inserted conditional brc2 heterozygous cells (BRCA2+/−/cont) harboring two loxP signals into the other allele upstream of the promoter and downstream of exon 2. Construction of the BRCA2 conditional-null targeting vector was carried out as described previously [33]. To delete the intact allele of the BRCA2+/−/cont cells, we constructed a targeting vector to delete all exons of the BRCA2 gene. The ~6 kb and ~3.5 kb fragments at the BRCA2 locus [66] were amplified from DT40 genomic DNA by using the 5'-CCGCTCGAGTTTTGATTGTAGATGTGTG-3' and 5'-TTATCGGGCTTTGATGTTCT-3' primers and the 5'-CGGGATCCATATGACGCTACGCA-3' and 5'-GTGAAATTGAAACTGGCGG-3' primers, respectively. Both fragments were cloned into TOPO-pCRXL cloning vector (Invitrogen, Carlsbad, California) to make the topo/6.0 kb and topo/3.5 kb vectors. The ~3.5 kb NotI site in the topo/6.0 kb vector was inserted into the NotI site in the multicloning site of the topo/3.5 kb vector, resulting in the pUpper/Lower vector. Finally, a loxP-flanked puro-resistance cassette was inserted into the BglII site in the multicloning site of the topo/3.5 kb vector, resulting in the pUpper/Lower vector. The resulting targeting construct was transfected into the BRCA2+/−/cont cells followed by selection with puromycin. The genomic DNA of the transfectants was digested with XbaI, and gene-targeting events were confirmed by Southern-blot analysis with a probe that was amplified from genomic DNA using the 5'-ATCCATGTCAGTGTGAGA- CACACTCGACTGACC-3' and 5'-AGATACGACACCTCGGTGAGA- GAAACCAGGTGTG-3' primers. The bands detected by the probe were 8.6 kb from the wild-type allele and 5.2 kb from the targeted allele.

Upon exposure of the BRCA2+/−/cont cells to tamoxifen, an estrogen antagonist, nucleotide sequences, including promoter and coding sequences encoding the initiation codon to the 67th amino acid, were excised by a chimeric Cre recombinase fused to the estrogen-receptor ligand-binding domain [24], leading to the complete disruption of the BRCA2 gene.

Disruption of individual HR genes in BRCA2-/- DT40 cells

To disrupt RAD51 mediator genes in BRCA2-/- DT40 cells, we disrupted each gene in the BRCA2+/−/cont cells (Table 1). We exposed the resulting RAD51 mediator-/−/BRCA2+/−/cont cells to tamoxifen and isolated the RAD51 mediator-/−/BRCA2+/−/cont cells.

Western blot analysis

Western blotting was performed as previously described [33]. The rabbit polyclonal primary antibody, which recognizes the N-terminal 203 amino acids of chicken BRCA2, was diluted 1:100 with blocking buffer. The anti-rabbit IgG HRP conjugated antibody was diluted 1:5000 with blocking buffer.

Flow-cytometric analysis

To measure growth kinetics, cells were counted daily using flow-cytometric analysis, as described previously [7]. To measure cell-cycle distribution, cells (5×10^6) were labeled for 10 minutes with 20 μg/ml 5-bromo-2'-deoxyuridine (BrdU) and subsequently harvested. Harvested cells were fixed and analyzed as previously described [7].

Measurement of cellular sensitivity to camptothecin, cisplatin, and olaparib in liquid culture

To measure cellular survival, cells (1.5×10^3–1.5×10^6) were incubated in 1 ml culture medium per well containing various concentrations of the DNA-damaging agents. At 72 hours, the ATP in the cellular lysates was measured to assess the number of live cells. The camptothecin (TopoGen Inc., Columbus, OH) and olaparib (AstraZeneca) were diluted with DMSO, and the cisplatin (Nihonkayaku, Tokyo, Japan) was diluted with PBS. To measure the sensitivity of the DT40 cell lines to these agents, cells were continuously exposed to various concentrations of the drug and the number of cells was measured at 72 hours. At least three independent experiments were carried out. Sensitivity was calculated by dividing the number of cells treated with the drug by the number of untreated cells [6].

Measurement of ATP to assess cellular sensitivity to DNA damaging agents

To assess cell numbers after treatment with the genotoxic reagents, we measured the amount of ATP in the whole cell lysate [67].

Visualization of RAD51 foci

Cells were harvested at 3 hours after gamma irradiation. Cells were spun onto slides using a Shandon Cytospin 3 centrifuge (Shandon, Pittsburgh, Pa.). Staining and visualization of RAD51 foci were carried out as previously described [34] using rabbit polyclonal antibody, which recognizes human RAD51, and Alexa Fluor 488 goat anti-human IgG antibody at a dilution of 1:1000 (Molecular Probes Inc., Eugene, OR [34]).

Analysis of chromosomal aberrations

Measurement of chromosomal aberrations was performed as described previously [68].

Measurement of HR frequencies for I-SceI-induced DSB repair

Measurement of recombination frequencies for I-SceI-induced DSB repair was performed as described previously [34,44]. Modified SCneo was inserted into the previously described OVALBUMIN locus in wild-type, xrc3, brc1, brc2, and brc2Δ Δ DT40 clones. For transient transfections, 1×10^7 cells were suspended in 0.5 ml of phosphate-buffered saline, mixed with 30 μg of I-SceI expression vector (pCBASce) or pBluescript KS without linearization, and electroporated at 250 V, 960 microfarads. At 24 hours after electroporation, the cells were plated in 96-well plates with or without 2.0 mg/ml neomycin analog (G418). The cells were grown for 7 to 10 days, after which formed colonies were counted. HR frequency was calculated by dividing the number of neomycin-resistant colonies by the number of plated cells.

Statistical analysis

Survival data were log-transformed giving approximate normality. Analysis of covariance (ANCOVA) was used to test for
differences in the linear dose-response curves between wild-type and a series of mutant cells or brca2-null cells and a series of double-knockout mutant cells. Viability of the DT40 cells was estimated using regressing curves. Regression-curve equations were used to calculate LC50 (50% lethal concentration) values. Relative LC50 values were normalized according to the LC50 value of the parental wild-type cells.

Supporting Information

Figure S1 Generation of BRCA2-/- (ver.2) cells. (A) Experimental methods to generate BRCA2-/- (ver.2) cells. (B) Schematic representation of the BRCA2 conditional-null allele-2. The conditional-null allele-2 (conn2) was generated by targeting the his selection-marker gene flanked by two loxp signals (ploxP-his) in intron 12 of the BRCA2 conditional allele 1. Treatment of the BRCA2-/- (conn2) cells causes deletion from the promoter to exon 11, encoding all BRCA2 motifs. The relevant Xhol sites in the conditional-null allele-1 and the position of the probe used in the Southern-blot analysis are indicated. The solid boxes and arrowheads represent the exons and loxp signals, respectively.

Figure S2 Proliferation and spontaneous chromosomal aberrations of brca2/rad52/xrcc3 triple knockout cells. (A) Growth kinetics of the indicated cell cultures in the absence (right) and presence (left) of TAM. Each value represents the averaged results from two separate clones. (B) Cell viability was assessed by flow cytometric analysis of PI uptake and forward scatter (FSC) representing the cell size after tamoxifen (TAM) treatment 4 days. A fixed number of plastic beads was added before flow cytometric analysis to calibrate cell number. Cells falling in the R1 and R2 gates identify dead and viable cells, respectively, and numbers given show their percentages. (C) Spontaneous chromosomal aberrations in cells with indicated genotype after tamoxifen (TAM) treatment 6 days. The data shown in the histogram indicate the types and numbers of chromosomal breaks in 50 analyzed mitotic cells. Two breaks at the same site of both sister chromatids are defined as isochromatid breaks, while breaks at either sister chromatid are chromatid breaks.

References