Suppression of the DNA repair defects of BRCA2-deficient cells with heterologous protein fusions

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The BRCA2 tumor suppressor plays an important role in the repair of DNA damage by homologous recombination, also termed homology-directed repair (HDR). Human BRCA2 is 3,418 aa and is composed of several domains. The central part of the protein contains multiple copies of a motif that binds the Rad51 recombinase (the BRC repeat), and the C terminus contains domains that have structural similarity to domains in the ssDNA-binding protein replication protein A (RPA). To gain insight into the role of BRCA2 in the repair of DNA damage, we fused a single (BRC3, BRC4) or multiple BRC motifs to the large RPA subunit. Expression of any of these protein fusions in *Brca2* mutant cells substantially improved HDR while suppressing mutagenic repair. A fusion containing a Rad52 ssDNA-binding domain also was active in HDR. Mutations that reduced ssDNA or Rad51 binding impaired the ability of the fusion proteins to function in HDR. The high level of spontaneous chromosomal aberrations in *Brca2* mutant cells was largely suppressed by the BRC-RPA fusion proteins, supporting the notion that the primary role of BRCA2 in maintaining genomic integrity is in HDR, specifically to deliver Rad51 to ssDNA. The fusion proteins also restored Rad51 focus formation and cellular survival in response to DNA damaging agents. Because as little as 2% of BRCA2 fused to RPA is sufficient to suppress cellular defects found in *Brca2*-mutant mammalian cells, these results provide insight into the recently discovered diversity of BRCA2 domain structures in different organisms.

double-strand break | mammalian cells | Rad51 | homologous recombination | BRC repeat

Loss of the *BRCA2* tumor suppressor predisposes adult mutation carriers to breast and ovarian cancer (1) and children with biallelic mutations to multiple types of malignancies, including brain and kidney tumors and leukemia (2–4). *BRCA2* also plays a critical role in the mouse during early embryonic development (5) and during meiosis (6) and is critically important in cells for the maintenance of genomic integrity (7). The BRCA2 protein provides an important function in promoting homology-directed repair (HDR) of damaged DNA in cells (8, 9), presumably through its interaction with the Rad51 recombinase (10, 11), which may underlie its role in tumor suppression and development.

Mammalian BRCA2 proteins are >3,300 aa in length and contain several functional domains (Fig. 1*a*) (7). Although the role of the N-terminal third of the protein is uncertain, the central ≈1,000 aa of BRCA2 contain eight BRC repeats that bind Rad51 (10). In their normal context, the constellation of BRC repeats presumably promotes HDR; however, cellular expression of individual BRC repeats recapitulates phenotypes of *Brca2* mutant cells, including reduced HDR, implying that by themselves they interfere with Rad51 function (12–14). C-terminal to the BRC repeats is a region that binds ssDNA (15, 16). This region consists of four globular domains, including two oligonucleotide/oligosaccharide-binding (OB) folds, which have close structural homology to two OB folds in replication protein A (RPA) 70, the largest subunit of the ssDNA-binding protein RPA (15), and an unusual tower domain appended to one of the globular domains (16). The extreme C terminus of BRCA2 contains an additional Rad51-binding motif (11), which is distinct from the BRC repeats, and which has been shown recently to undergo regulated Rad51 binding in response to CDK phosphorylation (17).

Although initially identified in mammalian cells, BRCA2 orthologs have been identified more recently in diverse organisms, including *Ustilago maydis* (18), *Caenorhabditis elegans* (19), and *Arabidopsis thaliana* (20). However, BRCA2 orthologs in different species have widely diverse sizes. For example, in contrast to the >3,300-aa vertebrate BRCA2 proteins, *U. maydis* Brh2 is 1,075 aa (18) and *C. elegans* BRC-2 is 394 aa (19). The nearly 10-fold size range of BRCA2 orthologs is due to highly variable and poorly conserved N-terminal sequences, variation in the number of BRC repeats, and domain differences in the DNA-binding region. Despite this variation, all BRCA2 orthologs have at least one BRC repeat capable of binding Rad51 and apparently at least one domain capable of binding ssDNA. In this report, we sought to determine whether BRCA2 function in HDR in mammalian cells could be contained essentially within these two identified activities, that of Rad51 and ssDNA binding, by using a heterologous ssDNA-binding protein fused to BRC motifs. We found that these heterologous fusion peptides, which contain as little as 2% of BRCA2, restored HDR to *BRCA2* mutant cells, while concomitantly suppressing genetic instability.

Results

BRC-RPA Fusion Proteins Increase HDR in *Brca2* Mutant Cells but Not Wild-Type Cells. To test whether a heterologous ssDNA-binding protein could effectively substitute for the BRCA2 ssDNA-binding domain in HDR, we fused human RPA70 to one or several BRC repeats from BRCA2. For the single BRC repeat, we used the 70-aa BRC3 (Fig. 1*b*), which interferes with HDR when expressed on its own (14, 21). As a control, we also fused RPA70 to BRC3Δ, which is identical to BRC3 except for a 7-aa deletion that abrogates Rad51 binding (22). In addition, we fused larger segments of BRCA2 containing multiple repeats, in particular BRC1-2 and BRC1-4 (Fig. 1*b*). Expression vectors for the BRC-RPA fusion proteins and

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Abbreviations: DSB, double-strand break; HDR, homology-directed repair; IR, ionizing radiation; MMC, mitomycin C; OB, oligonucleotide/oligosaccharide-binding; RPA, replication protein A.

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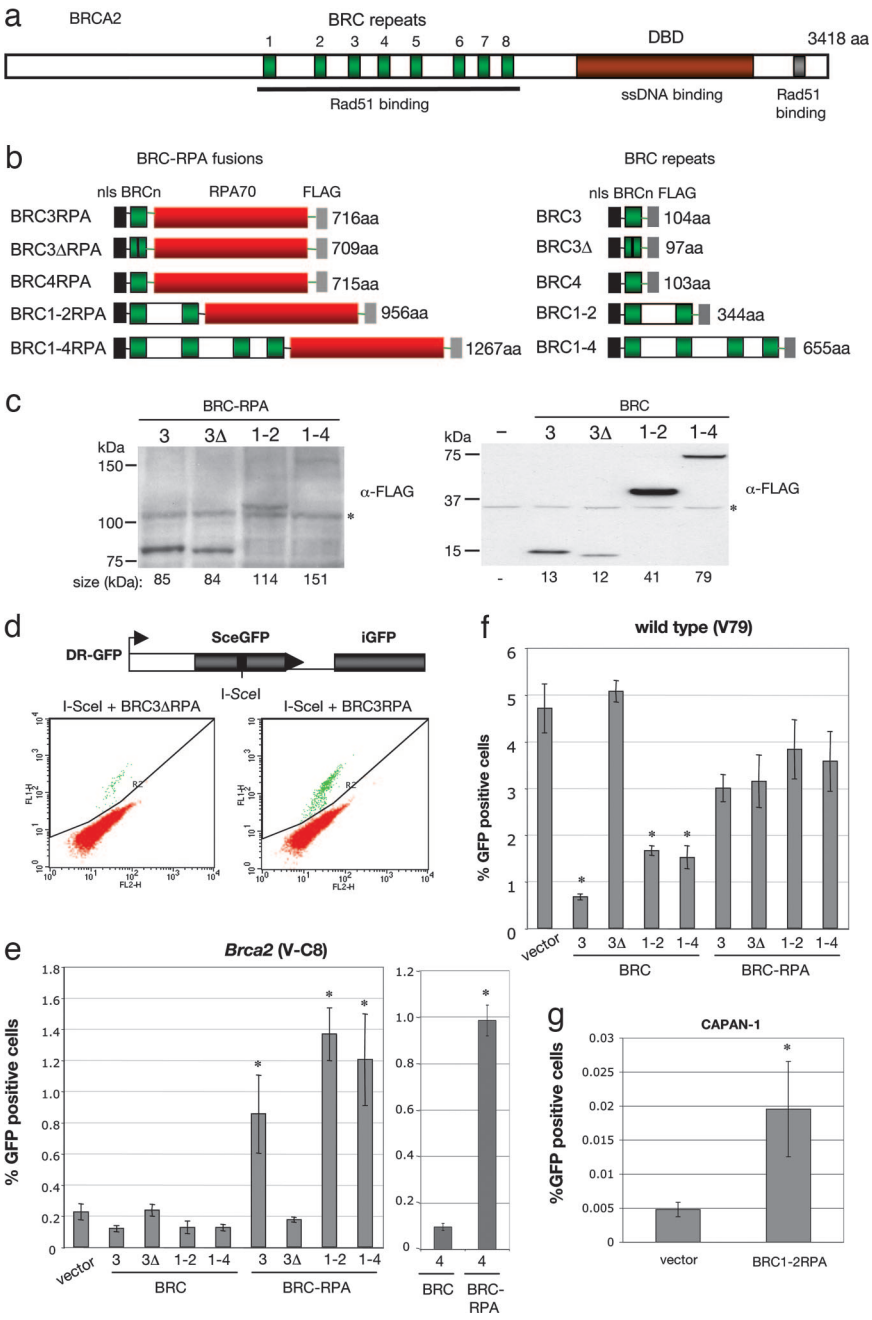

Fig. 1. Transient expression of BRC-RPA fusion proteins increases HDR in *Brca2* mutant cells. (*a*) Human BRCA2. BRCA2 has a central region containing eight BRC repeats which bind Rad51. C-terminal to the BRC repeats is a region of higher conservation that encompasses a DNA-binding domain (DBD). A distinct Rad51-binding motif is found at the C terminus. (*b*) BRC-RPA fusion proteins and BRC repeat peptides. RPA70 refers to the entire human RPA70-coding sequence. BRC3Δ contains a 7-aa deletion that abrogates Rad51 binding. A nuclear localization signal (nls) is present at the N terminus and a FLAG epitope tag at the C terminus. (*c*) Western blot analysis of *Brca2* mutant V-C8 hamster cells transiently expressing the BRC-RPA fusion proteins and BRC repeat peptides. Sizes of the expressed peptides are as expected. Asterisks denote a background immunoreactive band. (*d*) Flow cytometric analysis of V-C8 cells demonstrates increased HDR after transient expression of the BRC3RPA fusion protein as compared with BRC3ΔRPA. V-C8 cells containing a chromosomal DR-GFP reporter were cotransfected with expression vectors for the I-SceI endonuclease and the indicated BRC-RPA fusion protein. Cleavage of the DR-GFP reporter at the I-SceI site *in vivo* at the *SceGFP* gene and repair by HDR directed by the downstream *iGFP* repeat results in GFP-positive cells. (*e*) HDR is increased in V-C8 cells with transient expression of the BRC-RPA fusion proteins but not with the BRC repeat peptides. Asterisks indicate a statistically significant difference from transfection with the empty expression vector by using an unpaired *t* test (BRC3RPA, $P = 0.013$; BRC1–2RPA, $P = 0.0004$; BRC1–4RPA, $P = 0.0048$; BRC4RPA, $P < 0.0001$) (see also Fig. 4*a*). (*f*) HDR in wild-type V79 hamster cells with expression of the BRC peptides or the BRC-RPA fusion proteins. Asterisks indicate a statistically significant difference from transfection with the empty expression vector (BRC3, $P = 0.0002$; BRC1-2, $P = 0.0006$; BRC1-4, $P = 0.0007$) (see also Fig. 4*b*). (*g*) HDR in CAPAN-1 cells is increased by transient expression of BRC1-2RPA. The human pancreatic adenocarcinoma cell line CAPAN-1 carries a 6174delT mutation on one allele of *BRCA2* with loss of the other wild-type *BRCA2* allele. Asterisk indicates a statistically significant difference in HDR between transfection of the BRC1-2RPA expression vector and the empty expression vector ($P < 0.05$). Error bars in *e–g* indicate 1 SD from the mean. Results are derived from three independent transfections for each sample ($n = 3$).

the BRC repeats were transiently transfected into the *Brca2* mutant V-C8 hamster cell line (23). By Western blot analysis, each of the proteins was detected at the expected size, although the smaller fusion proteins appeared to be better expressed than the larger ones (Fig. 1*c*).

To assess HDR, we used the DR-GFP reporter (Fig. 1*d*). With this reporter, HDR is detected when a double-strand break (DSB) introduced into the chromosome by the I-SceI endonuclease is repaired by HDR to give rise to GFP-positive cells (24). HDR was reduced by >20-fold in *Brca2* mutant V-C8 cells relative to wild-type V79 hamster cells (compare vector control in Fig. 1 *e* and *f*). Strikingly, transient expression of the BRC-RPA proteins substantially increased HDR in the V-C8 cells (Fig. 1*e*). The increased HDR was estimated to be 4- to 6-fold, varying somewhat between the different BRC-RPA proteins, although there was not a direct correlation between HDR level and the number of BRC repeats in the fusion proteins. Improved HDR depended on the BRC-RPA proteins having an intact Rad51-binding motif in the BRC repeat, because BRC3ΔRPA expression did not increase HDR. The presence of RPA70 also was critical, because the BRC repeats themselves did not improve HDR. The increased HDR observed in the *Brca2* mutant cells with BRC-RPA expression was not due to the proteins conferring a general hyperrecombination phenotype to cells, because HDR was not increased in wild-type V79 hamster cells (Fig. 1*f*). Rather, the BRC-RPA fusion proteins, including BRC3ΔRPA, reduced HDR somewhat in wild-type cells, although not as strongly as the BRC repeats (Fig. 1*f*). It is possible that the fusion proteins interfere with endogenous RPA function.

The eight BRC repeats share only a core consensus sequence and appear to bind Rad51 with different affinities (10, 22, 25).

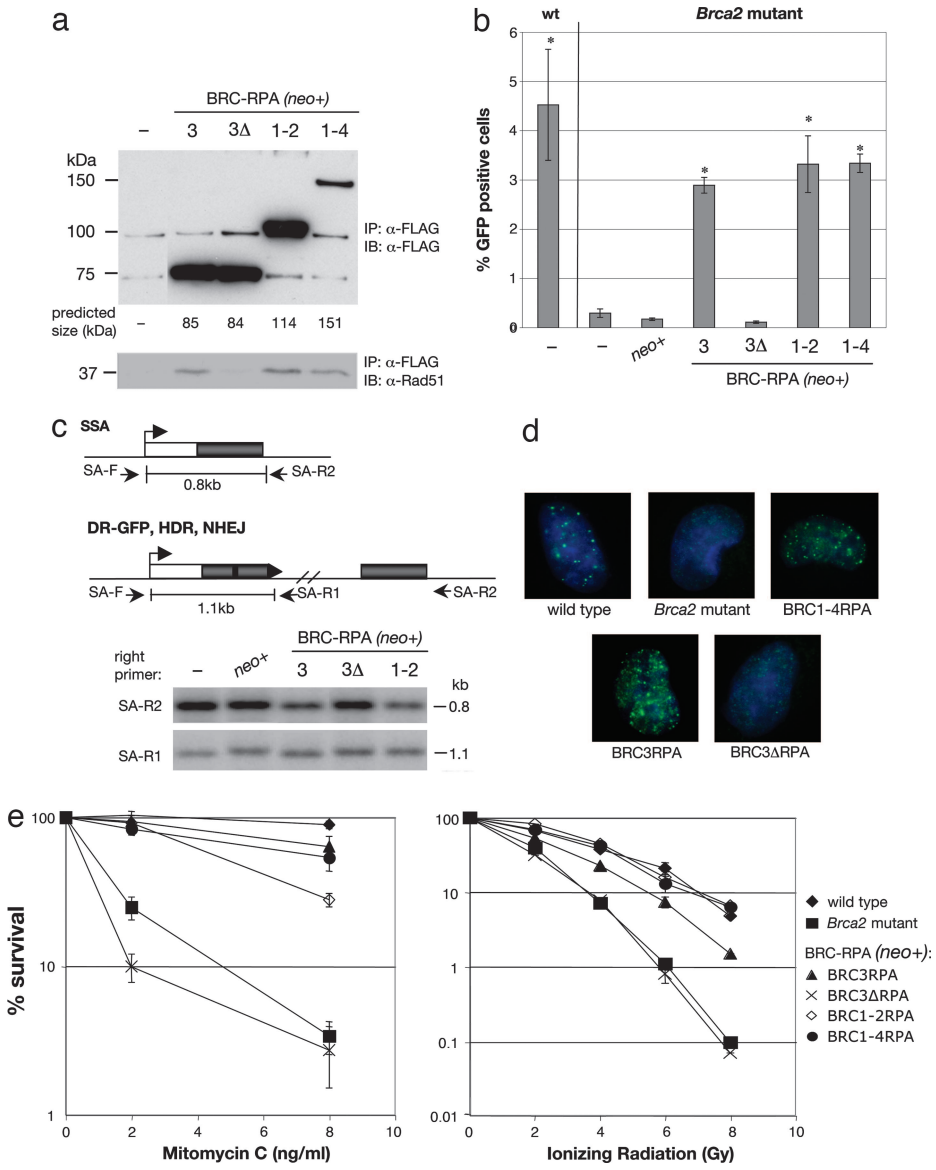

Fig. 2. Correction of the HDR defect and phenotypes associated with impaired HDR in *Brca2* mutant cells by stable expression of the BRC-RPA proteins. (*a*) BRC-RPA expression vectors were cotransfected into V-C8 cells with a neomycin phosphotransferase gene (*neo*+). Immunoprecipitation with anti-FLAG antibody followed by Western blot analysis shows BRC-RPA expression (α-FLAG) and interaction with Rad51 (α-Rad51). (*b*) HDR is increased nearly to wild-type levels in V-C8 cells stably expressing BRC-RPA proteins. Asterisks indicate a statistically significant difference from parental V-C8 cells ($P < 0.0001$; $n = 6$). (*c*) SSA is suppressed in V-C8 cells stably expressing BRC-RPA proteins. The 0.8-kb PCR fragment derived from primers SA-F and SA-R2 specifically detects the SSA repair product, whereas the 1.1-kb PCR fragment from primers SA-F and SA-R1 detects a structurally intact reporter, i.e., from HDR and NHEJ, as well as the parental DR-GFP reporter. See Fig. 5*a* for quantitation. (*d*) Rad51 focus formation in response to DNA-damaging agents is restored in V-C8 cells stably expressing BRC-RPA proteins. Representative wild-type (V79), *Brca2* mutant (V-C8), or *Brca2* mutant cells stably expressing the indicated BRC-RPA peptides are shown after IR. Note that Rad51 is diffusely present on the chromatin of the BRC3RPA and BRC1–4RPA cell lines. Exponentially growing cells were irradiated with 12 Gy of IR and analyzed 7 h later for Rad51 foci. See Fig. 5*b* for quantitation. (*e*) Hypersensitivity of *Brca2* mutant cells to DNA-damaging agents is reduced or eliminated with BRC-RPA expression. Survival was assessed by crystal violet staining after treatment for 24 h (MMC) or by clonogenic survival (IR). The percent survival of cells treated with DNA-damaging agents was computed relative to that of untreated cells, which was set to 100% for each line. Each percentage shown is the mean and error bars represent the SDs. MMC treatments were triplicated except that BRC3RPA and BRC1-2RPA stable cell lines were treated six times. IR treatment for each dose was performed once, except that 6-Gy treatments were quadruplicated.

In addition, BRC3 and BRC4, which have only 30% identity, appear to interact with Rad51 nonequivalently, with BRC3 binding to the N-terminal domain and BRC4 binding to the nucleotide-binding core domain (25, 26). To determine whether BRC3 and BRC4 act differently when fused to RPA70, we constructed a BRC4RPA fusion protein (Fig. 1*b*). Like BRC3RPA, BRC4RPA expression increased HDR in the V-C8 cells (Fig. 1*e*; see also Fig. 4*a*, which is published as supporting information on the PNAS web site). As expected, the isolated BRC4 repeat did not promote HDR in the V-C8 cells (Fig. 1*e*) but did substantially reduce HDR in wild-type cells, as did BRC4RPA to a lesser extent (Fig. 4*b*). These results indicate that when BRC4 is tethered to a ssDNA-binding domain, it is functional to promote HDR.

We also assessed the ability of the BRC-RPA proteins to correct the HDR defect found in the human pancreatic adenocarcinoma cell line CAPAN-1, which expresses a cytoplasmic BRCA2 peptide truncated at BRC7 (27). As with the V-C8 hamster cells, HDR increased in CAPAN-1 cells with expression of the BRC1–2RPA as compared with the vector control (Fig. 1*g*), indicating that the BRC-RPA enhancement of HDR is not specific to the *Brca2* mutant hamster cells.

Stable Expression of BRC-RPA Fusion Proteins in *Brca2* Mutant Cells Increases HDR While Suppressing Mutagenic DSB Repair by Single-Strand Annealing (SSA). To further investigate the repair phenotypes of *Brca2* mutant cells expressing the fusion proteins, each of the BRC-RPA proteins was stably expressed in the V-C8 cells (Fig. 2*a*). Immunoprecipiation by using the FLAG epitope, followed by immunoblotting for Rad51, confirmed that the fusion proteins with intact BRC repeats bound Rad51, whereas the BRC3ΔRPA protein did not (Fig. 2*a*). As with transient expression, HDR was increased in V-C8 cells stably expressing the BRC-RPA proteins (Fig. 2*b*). The overall increase in HDR was 10- to 11-fold, which was greater than that obtained by transient expression of the fusion proteins, such that the absolute level of HDR approached that found in wild-type cells (Fig. 2*b*). Cells that expressed BRC3RPA and BRC1-2RPA had similar levels of the respective fusion protein and also had similar levels of HDR, indicating that one or two BRC repeats fused to RPA70 functioned equivalently. Interestingly, BRC1-4RPA corrected the HDR defect as well as these other two fusions, even though it was expressed at a lower level.

HDR appears to be in competition with a second DSB repair pathway, termed SSA (14, 28). When a DSB occurs between sequence repeats and is resected to ssDNA, annealing of the

complementary ssDNA formed at the repeats leads to SSA (28), such that one repeat and the sequences between the repeats are deleted (Fig. 2*c*). When HDR is impaired as a result of BRCA2 or Rad51 deficiency, SSA levels are elevated (9, 14), presumably because HDR and SSA compete for the same ssDNA intermediates.

To determine whether the BRC-RPA proteins, like BRCA2 itself, suppressed DSB repair by SSA, we used a PCR assay to amplify the SSA deletion product after I-SceI expression (Fig. 2*c*; ref. 29). The level of the SSA-specific product was compared with the level of a control product from a structurally intact DR-GFP reporter. Cell lines stably expressing intact BRC-RPA fusion proteins suppressed SSA compared with parental V-C8 cells or cells expressing BRC3ΔRPA (Fig. 2*c*; see also Fig. 5*a*, which is published as supporting information on the PNAS web site). Thus, intact BRC-RPA fusion proteins acted to restore the balance of SSA and HDR in the *Brca2* mutant cells.

Restored Rad51 Focus Formation and Decreased Sensitivity of *Brca2* Mutant Cells to DNA Damaging Agents by BRC-RPA Expression. The results of the DSB repair assays suggested that BRC-RPA proteins restore other indicators of Rad51 function to *Brca2* mutant cells. In response to DNA damage, Rad51 exhibits a dynamic redistribution within cells, localizing in nuclear foci to sites of DNA damage (30). *Brca2* mutant cells, including the V-C8 cell line (Fig. 2*d*), are defective in DNA damage-induced Rad51 focus formation (13, 23). To determine whether V-C8 cells stably expressing the intact BRC-RPA proteins have recovered the ability to form Rad51 foci after DNA damage induction, cells were treated with ionizing radiation (IR) or the crosslinking agent mitomycin C (MMC). We found that Rad51 focus formation was significantly restored by BRC-RPA expression in response to either agent (Figs. 2*d* and 5*b*). The restoration of Rad51 focus formation depends on the ability of the fusion proteins to bind Rad51, because cells expressing BRC3ΔRPA do not form DNA damage-induced foci.

Interestingly, we noted that in addition to being present in bright foci upon DNA damage, a portion of the Rad51 in V-C8 cells expressing the intact BRC-RPA proteins also was diffusely present in the nucleus, possibly on chromatin (e.g., compare BRC3RPA with wild-type). This result suggested that full-length BRCA2 prevents Rad51 from interacting with undamaged chromatin, supporting a previously proposed role for BRCA2 in regulating Rad51 dynamics (31).

Like other mammalian HDR mutants, *Brca2* mutant cells display an increased sensitivity to DNA damaging agents, especially crosslinking agents (7, 23). We found that the hypersensitivity of V-C8 cells to IR and MMC was greatly reduced or eliminated by expression of the intact BRC-RPA proteins (Fig. 2*e*), indicating that the fusion proteins are proficient in the repair of DNA damage produced by these agents.

Spontaneous Chromosomal Aberrations Are Reduced in *Brca2* Mutant Cells Stably Expressing BRC-RPA Fusion Proteins. *Brca2* mutant cells, including V-C8 cells, exhibit compromised genomic integrity, even in the absence of DNA damaging agents (7, 23). We examined spontaneous chromosomal aberrations in *Brca2* mutant cells stably expressing the BRC-RPA fusion proteins and found that they were substantially reduced (Table 1). Whereas V-C8 cells had a 20-fold increase in aberrations relative to V79 cells, cells expressing the intact BRC-RPA proteins had only a 3- to 4-fold increased level of aberrations. Thus, the BRC-RPA fusion proteins can partially or fully correct many of the cellular phenotypes associated with BRCA2 deficiency, including compromised genomic integrity

Table 1. Spontaneous chromosomal aberrations are reduced in *Brca2* mutant cells stably expressing BRC-RPA fusion proteins

Cell line	No. of cells	Abnormal cells, %	Breaks, %	Chromatid exchanges, %	Total breaks ± SEM, %
V79	150	6	7	0	7 ± 0.7
V-C8	150	73	137	2	141 ± 17
+BRC3RPA	150	17	23	0	23 ± 5
+BRC3ΔRPA	150	65	128	2	132 ± 21
+BRC1-4RPA	150	27	33	0	33 ± 5

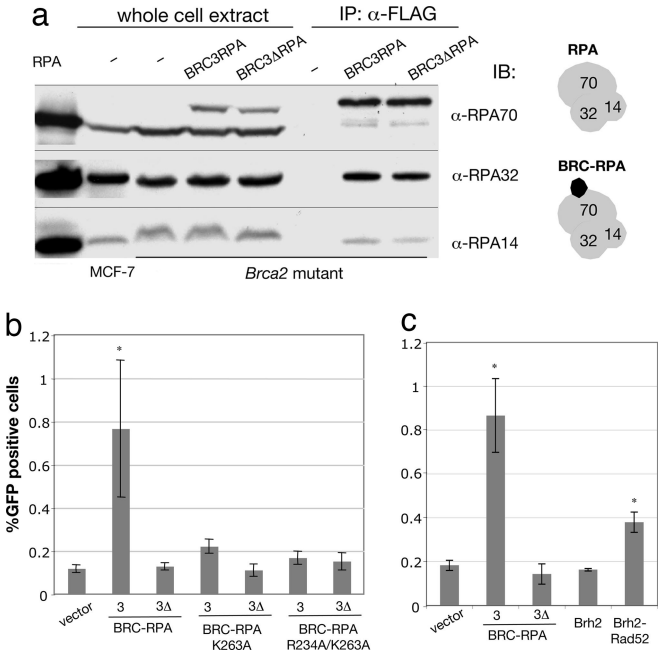

Fig. 3. HDR and ssDNA binding. (*a*) Interaction of BRC-RPA fusion proteins with the other RPA subunits. Whole-cell extracts from cells stably expressing BRC3RPA or BRC3ΔRPA were probed after gel electrophoresis either directly with α-RPA70, RPA32, and RPA14 antibodies or after immunoprecipitation with an α-FLAG antibody. Purified heterotrimeric RPA and whole-cell extracts from MCF-7 cells also are included. The weak band below the BRC-RPA proteins in the IP lanes is likely a degradation product that runs slightly above the position of RPA70 itself. (*b*) RPA mutations that reduce (K263A) or abolish (R234A/K263A) ssDNA-binding interfere with the ability of the BRC-RPA proteins to function in HDR. Asterisk indicates a statistically significant difference for transfection of the wild-type BRC3RPA expression vector compared with the other BRC3RPA expression vectors (K263A, $P = 0.041$ and R234A/K263A, $P = 0.031$; $n = 3$). (*c*) HDR is increased in V-C8 cells with transient expression of a Brh2-Rad52 fusion protein. The Brh2-Rad52 fusion contains the BRC repeat from *U. maydis* Brh2 with surrounding sequences (amino acids 89–551) fused to the conserved ssDNA binding domain of *U. maydis* Rad52 (amino acids 79–314); Brh2 extends from amino acids 89–955 and, hence, is truncated in the DNA-binding domain (18). Asterisks indicate a statistically significant difference from transfection with the empty expression vector (BRC3RPA, $P = 0.0022$ and Brh2-Rad52, $P = 0.0027$; $n = 3$).

BRC-RPA Fusion Proteins Interact with Other RPA Subunits. RPA70 has roles in various cellular processes as part of a heterotrimeric complex (32). We investigated whether the BRC-RPA fusion proteins interact with the other RPA subunits, RPA32 and RPA14. Whole-cell extracts from V-C8 cells stably expressing either BRC3RPA or BRC3ΔRPA were probed with antibodies directed against RPA70, RPA32, or RPA14 after immunoprecipitation with an anti-FLAG antibody (Fig. 3*a*). The BRC-RPA fusion proteins coimmunoprecipitated with RPA32 and with RPA14, suggesting that the BRC-RPA proteins function in HDR as part of a heterotrimeric complex, like RPA70 itself.

HDR Correction and ssDNA-Binding Activity. It is formally possible that the BRC-RPA heterotrimeric complex promotes HDR in

Brca2 mutant cells independent of ssDNA binding. To rule out this possibility, we incorporated RPA70 ssDNA binding mutations into BRC3RPA (Fig. 3*b*). A strong DNA binding mutant (R234A/K263A; 0.9% ssDNA binding affinity; ref. 33) completely abolished the ability of the BRC3RPA peptide to correct the HDR defect in the V-C8 cells, whereas a weak mutant (K263A; 18% ssDNA binding affinity; ref. 33) had residual activity (Fig. 3*b*). Thus, ssDNA binding is required for the BRC-RPA fusions to promote HDR.

Although both RPA and BRCA2 contain OB folds, the number and organization of ssDNA-binding domains in these proteins is quite different (15, 16), raising the possibility that other ssDNA-binding domains could promote HDR when fused to a BRC repeat. We examined a Brh2-Rad52 fusion, which contains a portion of Brh2 (BRC repeat and surrounding sequence) and the Rad52 ssDNA-binding domain, both derived from *U. maydis* (M. Kojic and W. K. Holloman, unpublished results). The Brh2-Rad52 fusion increased HDR ≈2-fold in the V-C8 cells (Fig. 3*c*) while having no effect in wild-type cells (data not shown). Increased HDR in the V-C8 cells depended on the Rad52 ssDNA-binding domain, because a Brh2 peptide had no effect on its own (Fig. 3c). These results demonstrate that a structurally intact ssDNA-binding domain can promote HDR in the *Brca2* mutant cells.

Discussion

We have demonstrated that as little as 2% of BRCA2 fused to the large subunit of the ssDNA-binding protein RPA is sufficient to suppress cellular defects found in *Brca2* mutant mammalian cells. BRC-RPA expression restores precise repair of DSBs by HDR to nearly wild-type levels, concomitantly reducing mutagenic DSB repair by SSA. BRC-RPA expression also promotes DNA damage-induced Rad51 focus formation and resistance to DNA damaging agents and, importantly, suppresses spontaneous chromosome aberrations in the *Brca2* mutant cells. Because the heterologous BRC-RPA fusion proteins are unlikely to substitute for BRCA2 in functions other than HDR (such as transcriptional transactivation) (34), these results emphasize the role of BRCA2 in HDR for maintaining genomic integrity.

An early step in HDR is the exonucleolytic processing of DSBs to produce ssDNA tails, which rapidly become coated with RPA (35). *In vitro*, RPA minimizes secondary structure in ssDNA to promote Rad51 nucleoprotein filament assembly; however, RPA must be displaced by Rad51 with the assistance of mediator proteins for efficient filament assembly. The ability of the BRC-RPA peptides to correct the HDR defect in *Brca2* mutant cells, in a manner dependent on Rad51 and ssDNA binding, implies that the key role of BRCA2 in the cell is to deliver Rad51 to ssDNA.

Although the ability of the BRC-RPA proteins to complement *Brca2* mutants is quite surprising, our results provide insight into the recently discovered plasticity of BRCA2 structures in different organisms (18, 19, 36) and biochemistry on BRCA2 fragments (37). For example, our smallest fusion, BRC3RPA, may be considered analogous to BRCA2 orthologs, which contain a single BRC repeat [*U. maydis* Brh2 (18) and *C. elegans* BRC-2 (19)] or have truncated DNA-binding regions (e.g., *C. elegans* BRC-2, which does not have a tower domain). Because our results indicate that much of mammalian BRCA2 may be dispensable for HDR when substituted with a heterologous ssDNA binding protein, a challenge will be to determine the physiological roles for the remaining portions of this large protein.

Phenotypes of *U. maydis* expressing a Brh2-RPA70 fusion protein were reported recently (38). Unlike the BRC-RPA proteins in mammalian cells, the Brh2-RPA70 fusion caused a hyperrecombination phenotype in *U. maydis.* The Brh2-RPA70 fusion differs from the BRC-RPA proteins in that it includes the entire N-terminal half of the Brh2 protein (551 aa) rather than an isolated BRC repeat or sets of BRC repeats. Therefore, the hyperrecombination phenotype caused by Brh2-RPA70 may point to additional roles for the N terminus in regulating Rad51 function, although it should be noted that this region of the protein is highly diverged among BRCA2 orthologs.

A crystal structure has been solved for one BRC repeat, BRC4, fused to the Rad51 nucleotide-binding core domain (26). In this structure, the fused BRC4 interacts with Rad51 by assuming the same structure as the Rad51 oligomerization motif, thereby preventing the incorporation of Rad51 into nucleoprotein filaments. Because BRC4RPA is proficient at HDR correction, as are other BRC repeats fused to RPA, the inhibitory role that BRC4 has been proposed to have is likely to be a function of its isolation from other parts of BRCA2, namely the DNA-binding domain.

HDR is restored to nearly wild-type levels in the *Brca2* mutant cells by stable expression of the BRC-RPA proteins. The efficient correction of the HDR defect in these cells raises the possibility that these fusion proteins mimic BRCA2, by delivering Rad51 to ssDNA coated with endogenous RPA. Alternatively, the BRC-RPA proteins may simply bypass the cellular requirement for BRCA2, loading Rad51 directly onto ssDNA. A bypass mechanism would not be surprising given the distinctions between RPA and BRCA2. For example, RPA contains six OB folds within its three subunits to bind ssDNA with high affinity (15), whereas BRCA2 contains only three OB folds in its single subunit (16). Moreover, an unusual nonglobular tower domain interrupts one of the BRCA2 OB folds (16) and may act to promote BRCA2 binding to dsDNA–ssDNA junctions (39). Consistent with a bypass mechanism, we found that the structurally distinct ssDNA-binding domain from Rad52 (40, 41) can function in HDR correction in the *Brca2* mutant cells. Besides the DNA-binding domain, other parts of the BRCA2 protein may provide more complex levels of control of BRCA2 function, including the recently identified cell cycle-regulated phosphorylation site that modulates Rad51 binding to the C terminus of BRCA2 (17). Our protein fusions are likely to provide an important approach for delineating roles for these other BRCA2 domains in HDR.

Materials and Methods

Cell Lines and Plasmids. The DR-GFP reporter (24, 42) was integrated into V-C8 hamster cells (23). Several clones with a single copy of DR-GFP were identified by Southern blotting and found to give similar results in HDR assays. One clone was used in the experiments presented here. To create stable cell lines, 5 μg of pMC1neo was cotransfected with 30 μg of the BRC-RPA expression vector, and cells were selected in 1.5 mg/ml G418 (GIBCO) for 2–3 weeks. Clones were screened by Western blotting for expression of the BRC-RPA proteins, and one was used for subsequent manipulations. CAPAN-1 cells with DR-GFP reporter were described in ref. 8.

Expression vectors for human BRC3 and BRC3Δ (21) were modified to contain a nuclear localization signal and FLAG tag. BRC4, BRC1-2, and BRC1-4 vectors were generated similarly by using cDNAs from human (BRC4, amino acids 1511–1578) and mouse (BRC1-2, amino acids 923-1252; BRC1-4, amino acids 923-1563). Human RPA70 sequences were PCR amplified from p11d-tRPA (43) and cloned C-terminal of the BRC peptide in the respective expression vectors. The integrity of the RPA70-coding sequence was verified by DNA sequencing. RPA70 mutations (33) were introduced into the BRC-RPA vectors by swapping an MfeI/MscI restriction fragment containing the mutations. The RPA70 R234A/R263A mutant (33) also carries a third mutation (N239K), which is inferred to be a silent mutation for DNA binding (M. Wold, personal communication). The Brh2 expression plasmid was constructed by cloning the *Brh2* BamHI fragment, encoding amino acids 89–955, into pCAGGS. To create Brh2-Rad52, Brh2 amino acids 89–551 were fused to *U. maydis* Rad52 amino acids 79–314, which is inferred by sequence conservation to contain the ssDNA-binding domain (M. Kojic and W. K. Holloman, personal communication).

DSB Repair Assays. To measure the repair of an I-SceI-generated DSB, 40 or 50 μg of the I-SceI expression vector pCBASce (44) or the empty pCAGGS vector was mixed with 4–5 $\times$ 10^6 cells suspended in 650 μl of opti-MEM medium (Invitrogen) in a 0.4-cm cuvette, followed by pulsing the cells at 280 V, 1,000 μF for hamster cell lines or 250 V, 950 μF for the CAPAN-1 cell line. For transient expression, 40 or 50 μg of the BRC peptides or BRC-RPA fusion proteins expression vectors were additionally added. To specifically determine the amount of HDR, the percentage of GFP-positive cells was quantified by flow cytometric analysis 2 days after electroporation on a Becton Dickinson FACScan (24). To assay SSA, we used a combined PCR-Southern blotting method (ref. 29; see Fig. 5 legend).

Protein Manipulations. Whole-cell extracts were prepared 24 h after transfection, and total protein was determined by Bio-Rad assays. For Western blot analysis of the BRC peptides, 30–50 μg of lysate was separated by 4–12% Bis-Tris PAGE (Invitrogen). For BRC-RPA fusion proteins, 100 μg of lysate was separated by Tris-glycine 10% PAGE. Immunoprecipitations were performed with 1.2 mg whole-cell extracts and 35 μl of anti-FLAG-M2 agarose affinity gel (A-2220; Sigma) for 4 h at 4°C. Immune complexes were washed with lysis buffer and separated by 4–12% Bis-Tris PAGE for Rad51, RPA32, and RPA14 and by 8% Tris-glycine 10% PAGE for RPA70. Purified RPA was a gift from Jerry Hurwitz (Memorial Sloan–Kettering Cancer Center). Membranes were probed with anti-FLAG-M2 antibody (A-8592; Sigma), anti-Rad51 antibody (ab-1; Calbiochem), anti-RPA70 antibody (ab12320; Abcam, Inc., Cambridge, MA), anti-RPA32 antibody (ab2626; Abcam, Inc.), and anti-RPA14 antibody (Tom Kelly, Memorial Sloan–Kettering Cancer Center). Anti-β-actin antibody (ab8227; Abcam, Inc.) was used to assess protein loading.

MMC and IR Sensitivity Assays. MMC sensitivity was assayed by seeding hamster cells into 12-well plates at 6 $\times$ 10^3 cells per well. After attaching overnight, cells were incubated with media containing MMC at various concentrations for 24 h. After incubation for another 4 days, monolayers were washed once with PBS and fixed for 5 min at room temperature in 10% methanol and 10% acetic acid. Adherent colonies were stained for 5 min at room temperature with 1% crystal violet (Sigma) in methanol. Plates were rinsed in water, and the adsorbed dye was resolubilized with methanol containing 0.1% SDS by gentle agitation for 30 min at room temperature. Dye solution was transferred to 96-well plates and diluted (1:2) in methanol. OD at 595 nm was measured photometrically in a model 3550 microplate reader (Bio-Rad). For quantification, the ODs of each well were normalized to those obtained from untreated cells (100% cell survival) and a well without any cells (0%).

For IR sensitivity assays, hamster cells were seeded in 10-cm plates and exposed to various doses of IR by varying the duration of exposure to a ^{137}Cs source. After 10 days, the clonogenic survival was determined for a given concentration of cells that were plated by dividing the number of colonies on each treated plate by the number of colonies on each untreated plate.

Analysis of Chromosomal Aberrations. Frequencies of spontaneous chromosomal aberrations were determined in exponentially growing cell cultures. Cells were harvested by trypsinization after 2 h of incubation with 1 μg/ml Colcemid and fixed after treatment with hypotonic solution (0.6% sodium citrate) in ethanol-glacial acetic acid (3:1). Air-dried preparations were made and stained with Giemsa.

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