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BRCA2: One Small Step for DNA Repair, One Giant Protein Purified

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DNA damage, malfunctions in DNA repair, and genomic instability are processes that intersect at the crossroads of carcinogenesis. Underscoring the importance of DNA repair in breast and ovarian tumorigenesis is the familial inherited cancer predisposition gene *BRCA2*. The role of BRCA2 in DNA double-strand break repair was first revealed based on its interaction with RAD51, a central player in homologous recombination. The RAD51 protein forms a nucleoprotein filament on single-stranded DNA, invades a DNA duplex, and initiates a search for homology. Once a homologous DNA sequence is found, the DNA is used as a template for the high-fidelity repair of the DNA break. Many of the biochemical features that allow BRCA2 to choreograph the activities of RAD51 have been elucidated and include: targeting RAD51 to single-stranded DNA while inhibiting binding to dsDNA, reducing the ATPase activity of RAD51, and facilitating the displacement of the single-strand DNA binding protein, Replication Protein A. These reinforcing activities of BRCA2 culminate in the correct positioning of RAD51 onto a processed DNA double-strand break and initiate its faithful repair by homologous recombination. In this review, I will address current biochemical data concerning the BRCA2 protein and highlight unanswered questions regarding BRCA2 function in homologous recombination and cancer.

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†Abbreviations: BRCA2, breast cancer susceptibility gene 2; DSB, double-strand break; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; HR, homologous recombination; RPA, replication protein A; DBD, DNA binding domain; OB, oligonucleotide/oligosaccharide binding; RAD51AP1, RAD51-associated protein 1; PARP, poly ADP-ribose polymerase; DSS1, deleted in split foot/hand disease; PALB2, partner and localizer of BRCA2; DMC1, disrupted meiotic cDNA1; siRNA, short interfering RNA; NLS, nuclear localization signal; FANCD2, fanconi anemia group D2; ES, embryonic stem; BAC, bacterial artificial chromosome.

Keywords: BRCA2, DNA repair, homologous recombination, RAD51, breast cancer, ovarian cancer

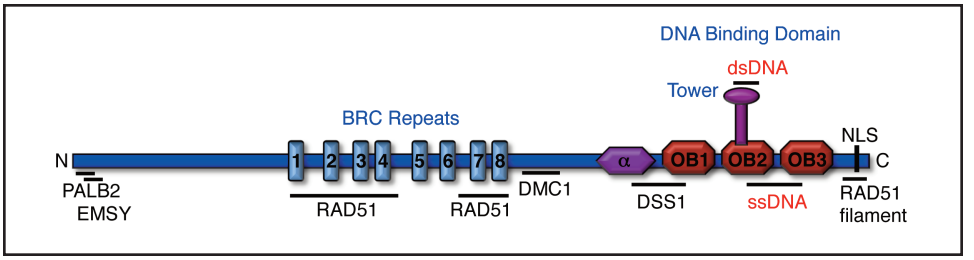

Figure 1. Schematic of the 3,418 amino acid BRCA2 protein. Protein partners known to interact with BRCA2 are depicted beneath the protein in black. PALB2 = partner and localizer of BRCA2, DSS1 = deleted in split foot/hand disease, DMC1 = disrupted meiotic cDNA1, α = alpha-helices region, OB = oligonucleotide/oligosaccharide binding domain, NLS = nuclear localization signals.

INTRODUCTION

The mapping and identification of breast cancer susceptibility gene 2 (*BRCA2*†) quickly followed the identification of the first breast cancer susceptibility gene, *BRCA1*, in 1990 by Mary Claire King [1,2]. Germline mutations in either *BRCA1* or *BRCA2* predispose an individual to increased risk for breast, ovarian, and other epithelial cancers. While both proteins participate in homologous recombination (HR) repair of DNA double-strand breaks (DSBs), the mechanistic functions of BRCA2 have become increasingly clear, while those of BRCA1 remain poorly defined. The link between BRCA2 and homologous recombination was first recognized as an interaction between several BRC repeats located in the midsection of BRCA2 with RAD51 (Figure 1). RAD51 plays a key function in catalyzing homologous recombination. After a DNA DSB occurs, the broken DNA ends are resected by nucleases, resulting in 3' single-stranded (ssDNA) tails. These 3' ssDNA tails are immediately coated and stabilized by the ssDNA binding protein, replication protein A (RPA). RAD51 must then displace RPA to form a nucleoprotein filament on ssDNA and mediate strand invasion into a DNA duplex template to initiate the search for homology [3-6]. Tumor cells isolated from patients lacking BRCA2 function suffer from genomic instability, a loss of DNA damage-induced RAD51 foci (an *in vivo* marker of RAD51 filament formation), and are extremely sensitive to crosslinking agents such as mitomycin C and cisplatin [7-12].

BRCA2 IS A MEDIATOR OF HR

Why is BRCA2 necessary? The initial clue that BRCA2 and RAD51 directly interact led to the hypothesis that these two proteins work together to promote HR. Similar to its prokaryotic orthologue, RecA, RAD51 was known to form a helical nucleoprotein filament, essentially wrapping itself around ssDNA [13,14]. This protein-DNA structure facilitates strand invasion into a duplex donor template, usually the sister chromatid, and promotes DNA pairing between homologous sequences. While the exact mechanics of this complex reaction remain to be determined, this is an area of intense investigation [15]. In order to form the RAD51 nucleoprotein filament, several parameters must be in place to ensure correct targeting of multiple RAD51 proteins to the ssDNA substrate. Left to its own devices, RAD51 has a propensity to bind dsDNA [16,17]. The binding of RAD51 to dsDNA is inhibitory for DNA strand exchange *in vitro* and, by implication, would corrupt the initial stages of HR *in vivo*. BRCA2 prevents RAD51 from binding dsDNA and instead targets it to the correct substrate, ssDNA [18]. Secondly, RAD51 cannot, by itself, displace the single-strand DNA binding protein, RPA. The need for recombination mediator proteins to overcome the kinetic and thermodynamic barriers imposed by ssDNA binding proteins has been well-established in simpler eukaryotes and prokaryotes [19]. By bringing several RAD51 proteins as its "cargo" to the ss/dsDNA junction, BRCA2 facilitates RAD51 filament formation by

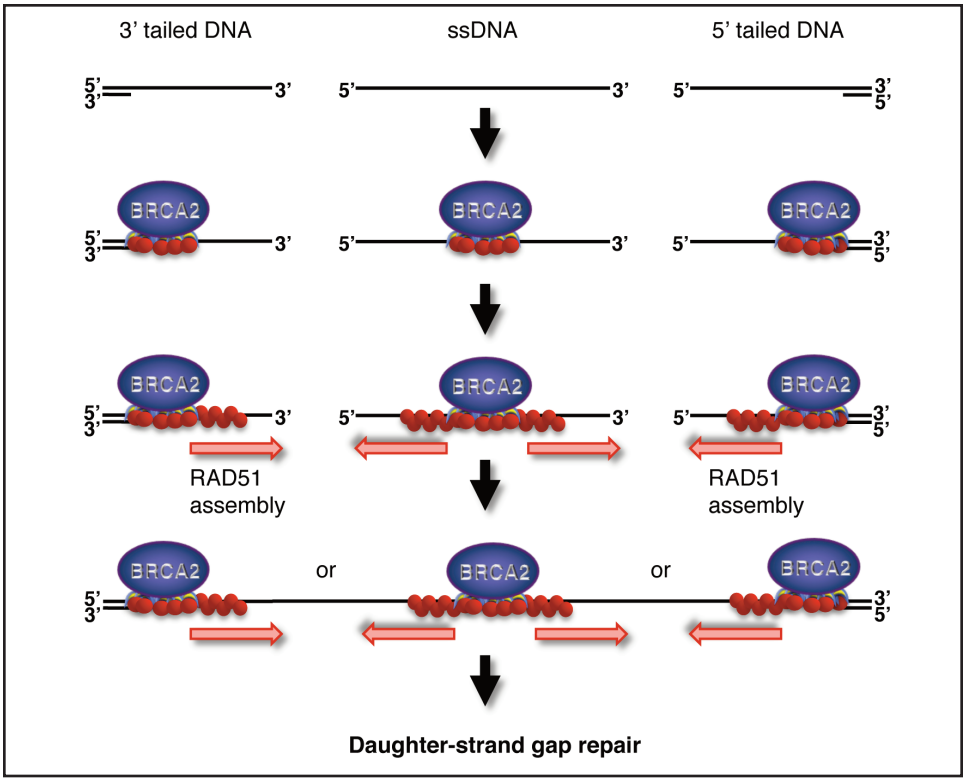

Figure 2. BRCA2 stimulates RAD51-mediated DNA strand exchange on 3' tailed DNA, 5' tailed DNA, and single-stranded DNA (ssDNA). The results imply that BRCA2 can mediate RAD51 filament formation in either the 3' or 5' polarity.

providing a core nucleus of RAD51 proteins. Additional free RAD51 can then interact and extend the filament from this nucleus, stabilized by BRCA2, while simultaneously displacing RPA from the ssDNA [20]. Thus, by stimulating and accelerating RAD51 filament formation, BRCA2 falls into the class of recombination mediator proteins that catalyze the initial steps of HR.

PURIFICATION OF THE FULL-LENGTH BRCA2 PROTEIN

The purification of full-length human BRCA2 was a formidable challenge due to its size, low expression, and propensity to degrade very quickly. These obstacles have been overcome, and soon we will be able to fully appreciate and understand the detailed functions of this large and complex protein [20-22]. While key insights into the function of BRCA2 in HR had been gleaned from studies on fragments of mammalian BRCA2 and orthologues such as *U. maydis* Brh2 and *C. elegans* BRC-2, purification of the full-length BRCA2 protein revealed unanticipated functions that had previously been unattainable [23-28]. Prior studies from the *U. maydis* orthologue, Brh2, predicted that human BRCA2 would have a strict specificity for binding a junction between ssDNA and dsDNA [24]. This ss/dsDNA substrate would be generated *in vivo* following resection of a DSB and would make a logical "landing pad" for BRCA2 to target the loading of RAD51 onto ssDNA [24]. Surprisingly, while purified human BRCA2 possessed a clear preference for ssDNA over dsDNA, the affinity for a junction was modest compared to its affinity for ssDNA [20]. Furthermore, the ability of BRCA2 to stimulate DNA strand exchange, an *in vitro* assay for HR, was enhanced only two-fold when given a DNA substrate containing an ss/dsDNA junction versus a substrate containing only ssDNA [20]. These results open

the possibility that BRCA2 can not only bind at the junction between ssDNA and dsDNA, but can also bind at multiple locations along the ssDNA to facilitate RAD51 loading (Figure 2). This scenario could allow BRCA2 to augment RAD51 nucleoprotein filament formation on ssDNA, for example, during daughter-strand gap repair.

The finding that BRCA2 can stimulate DNA strand exchange in either the 3' or 5' polarity stands in stark contrast to its orthologues, Brh2 (*U. maydis*) and RecFOR (*E. coli*), which have a clear preference for loading Rad51/RecA in the 5' to 3' direction [24,29]. This flexibility in activity may reflect RAD51's intrinsic ability to nucleate on ssDNA in either polarity, and BRCA2 simply provides a scaffold upon which RAD51 can reach a critical mass and nucleate the filament in either direction. In the case where BRCA2 does encounter a junction with dsDNA, it is likely that RAD51 filament formation is oriented toward the ssDNA region, while filament formation toward the dsDNA region is inhibited, as our previous data suggests [18,20]. As opposed to the bacterial orthologues, RecA and RecFOR, which can vigorously and directionally nucleate a filament, mammalian RAD51 and BRCA2 may require additional help from other recombination mediators, such as RAD51AP1 or the RAD51 paralogs, to assist in stabilizing or determining directionality of the RAD51 filament [30-33]. The existence of multiple recombination mediators in mammals may underlie the more complex regulation of RAD51 in higher organisms due to differences in genomic DNA sequence, chromatin structure, and/or alternative recombination pathways involving RAD51-dependent strand invasion.

BRC REPEATS AND DNA-BINDING DOMAIN

The key domains shared among all organisms from which a BRCA2 homologue have been identified include a BRC repeat to bind RAD51 and a DNA-binding domain (DBD) comprised of three oligonucleotide/oligosaccharide binding (OB) folds [23,28,34,35]. These core components allow BRCA2 to bind ssDNA generated at a processed DSB and to load RAD51 onto ssDNA forming the nucleoprotein filament. The variability in the number of BRC repeats among different organisms has remained an enigma (eight in human and mouse, six in chicken, four in arabidopsis, and one in *C. elegans* and *U. maydis*). Despite the unknown reasons for the wide discrepancy in the number of BRC repeats between organisms, all *BRCA2* homologues discovered to date possess a means by which to bind RAD51 and DNA. The two key modules in BRCA2, the BRC repeats and the DBD, are required in *cis* to both stimulate DNA strand exchange *in vitro* and complement brca2 mutant cells *in vivo* [36] (Jensen & Kowalczykowski, unpublished results). In fact, as little as one BRC repeat (BRC4) fused to the OB folds of RPA has the ability to complement HR function in *brca2* mutant hamster cells [36]. The most recent studies have demonstrated that full-length BRCA2 can bind to at least six RAD51 molecules [20,21]. Given the evolutionary conservation of the BRC repeats among different mammalian species, multiple RAD51 binding modules may reflect the need for an enhanced or more efficient cellular response to DSBs in higher organisms. Additionally, more complex levels of regulation in multicellular organisms resulting in specific responses to various forms of DNA damage may necessitate the use of multiple BRC repeats to coordinate the efficient loading and nucleation of RAD51.

Although the amino acid sequences that lie between each BRC repeat are not conserved, their spatial organization is maintained [37]. Given that tumor-associated mutations are found both within the BRC repeats as well as in the interstitial regions, it will be interesting to study the consequences of disabling individual repeats as well as their spatial organization within the context of the full-length BRCA2 protein [37]. A systematic analysis of the contribution each BRC repeat makes toward BRCA2 function may further our understanding of why the number of repeats varies between different

organisms. Moreover, these studies may help to shed light on a surprising result that emerged from an analysis of PARP (poly ADP-ribose polymerase) inhibitor resistant *brca2* mutant cell lines selected under chronic exposure. PARP inhibitors, such as olaparib, take advantage of a concept termed "synthetic lethality," wherein tumors deficient in HR repair are selectively killed, sparing normal, healthy cells [38,39]. *BRCA2* mutant cells are normally exquisitely sensitive to PARP inhibitors. The report revealed secondary mutations in *BRCA2* lacking almost the entire DNA binding domain, and yet these revertant alleles were capable of functionally complementing *brca2* mutant cells [40]. While these particular reversion mutations would imply loss of DNA binding, perhaps other recombination mediators such as PALB2 are able to compensate and facilitate targeting of BRCA2 to sites of DNA damage. A better understanding of how these secondary mutations regained function can now be addressed both biochemically and *in vivo*. The consequences of analyzing specific reversion alleles or sequence variants in *BRCA2* must be regarded with caution, however, as interpretation may depend upon the assay used for complementation. For example, alleles that result in resistance to cross-linking agents as an endpoint may appear to function correctly, but only at the expense of high rates of mutagenesis or error-prone repair, outcomes that may go undetected in a survival assay.

THE TOWER DOMAIN AND DNA BINDING

A feature of BRCA2 that has not been studied in detail is the tower domain. In the Yang et al. crystal structure, the tower domain consists of two long alpha-helices that extend dramatically upward (~ 90 angstroms) from the OB2 domain ending in a three-helix bundle that has been shown in other proteins to mediate binding to dsDNA (see Figure 1) [23]. Many tumor-associated missense mutations are located within the tower domain; however, it is unclear what the functional consequences of these mutations are within the context of the full-length protein. The role of the tower domain may be to recognize the interface between ssDNA and dsDNA, allowing BRCA2 to bind preferentially to this junction. A prediction of this scenario would be a loss of DNA junction specificity in the DNA strand exchange study by Jensen et al [20]. A more provocative hypothesis is that the tower domain allows BRCA2 to capture the dsDNA template (e.g., sister chromatid) and brings it into close proximity such that the ensuing RAD51 filament will be within close range for invasion. In this scenario, loss of the tower domain's ability to bind dsDNA may result in an overall loss of efficiency in the DNA strand exchange reaction. It is now possible to test these scenarios biochemically using the full-length protein to determine if the functional role of the tower domain is dsDNA recognition.

PROTEIN PARTNERS OF BRCA2

Several proteins have been found to bind and potentially regulate BRCA2 function including PALB2 (partner and localizer of BRCA2), EMSY, FANCD2 (fanconi anemia group D2), p53, RAD51, DMC1 (disrupted meiotic cDNA1), and DSS1 (deleted in split foot/hand disease) [41-47]. One of the more fascinating and contentious proteins is a small, acidic 70 amino acid protein, DSS1. The crystal structure of DSS1 bound to the carboxy terminus of BRCA2 revealed an intimate association as several residues of DSS1 tunnel directly through the OB2 and OB3 domains of BRCA2 [23]. Predictions have varied on the role of DSS1 from mimicking a DNA substrate to controlling the monomer/dimer state of BRCA2 [23,48]. Multiple studies of the DSS1 yeast orthologue Sem1 place this protein in the 19S regulatory cap complex of the proteasome [49-51]. Further studies have suggested that in addition to regulating DNA DSB repair, ubiquitin-mediated proteolysis may link mammalian DSS1 to the instability and turnover of BRCA2 [52,53].

Two independent studies utilizing siRNA treatment of human cells to knock down DSS1 expression have revealed dis-

parate results. The first group observed that siRNA-mediated knockdown of DSS1 coincided with loss of BRCA2 expression at the protein level, implying that DSS1 somehow stabilizes the BRCA2 protein [54]. They further demonstrated that cells depleted of DSS1 became sensitive to cross-linking agents (similar to BRCA2 deficiency) and were impaired for HR function as revealed by loss of RAD51 foci and decreased repair in response to induced DSBs. In contrast, a second group, also utilizing siRNA to knock down DSS1, found no effect upon the protein level of BRCA2 [36]. In agreement with the first study, the second group found that siRNA depletion of DSS1 phenocopied knockdown of BRCA2 [55]. As DSS1 has been linked to a function involving the proteasome, it is an attractive hypothesis that the link between BRCA2's instability and its function *in vivo* is regulated by degradation and turnover during the cell cycle [56]. To date, however, there is no evidence for a role of DSS1 and the proteasome in the regulation of BRCA2 turnover [57]. Studies with BRCA2 and DSS1 proteins purified separately and then reconstituted *in vitro* have shown that DSS1 enhances the ability of BRCA2 to load RAD51 onto a gapped DNA substrate [21]. The exact mechanism of how DSS1 achieves this stimulation, and if DSS1 further regulates the stability and higher order state of BRCA2 *in vivo*, requires further exploration.

The majority of studies concerning BRCA2 function have focused on either the BRC repeats or the DNA binding domain. In comparison, the N-terminus of BRCA2 has remained largely unexplored territory. EMSY and PALB2 are two proteins with connections to increased cancer risk and have been found to bind BRCA2 within the first 100 amino acids. EMSY was initially discovered in a yeast two-hybrid screen approach using the N-terminus of BRCA2 as bait, while PALB2 was identified in a BRCA2 chromatin-containing fraction using mass spectrometry [41,42]. Amplification of the *EMSY* gene was found in a significant proportion of sporadic breast and ovarian cancer cases and may implicate the BRCA2 pathway in non-hereditary tumorigenesis [42]. Germline *PALB2* mutations have been associated with an increased risk for breast and pancreatic cancer [58]. PALB2 was discovered to be the basis for the interaction between BRCA2 and BRCA1, as it provides the interaction bridge between these two large proteins [59]. Recently, PALB2 has been shown to stabilize RAD51 filament formation through direct interaction with RAD51, RAD51AP1, and dsDNA [60]. Additionally, a truncated version of BRCA2 was found to work in concert with PALB2 to stimulate RAD51 mediated D-loop formation [61]. The underlying mechanism of how PALB2 stimulates synaptic complex formation is unclear; however, both studies suggest that PALB2 somehow stabilizes formation of the D-loop structure and may be involved in capture of the dsDNA template utilized for strand invasion.

DMC1 is the meiotic counterpart to RAD51. While DMC1 expression is restricted to meiotic cells, RAD51 is expressed in both meiotic and somatic cells. The interaction between BRCA2 and DMC1 has been pinpointed to a PhePP motif located just downstream of the BRC8 repeat (see Figure 1) [46]. Both RAD51 and DMC1 are found to localize to SPO11-induced DBSs during meiosis; however, it is unclear why a specialized recombinase is necessary for meiotic as opposed to mitotic recombination [62]. Furthermore, it remains to be shown whether the full-length BRCA2 can stimulate DMC1-mediated recombination *in vitro* or whether this process requires both RAD51 and DMC1 together. Evidence for BRCA2's role in meiosis has emerged from studies on mice, wherein BRCA2 was found to localize to synapsed chromosomes in prophase I [63]. BRCA2 expression was also upregulated during spermatogenesis [63]. Importantly, a key study demonstrated that loss of BRCA2 expression in the mouse gonads led to infertility due to failed spermatogenesis [64]. Given the separate binding domains for RAD51 and DMC1, BRCA2 may load both proteins onto SPO11-induced DSBs in a specific orientation, forming a mixed filament with an opti-

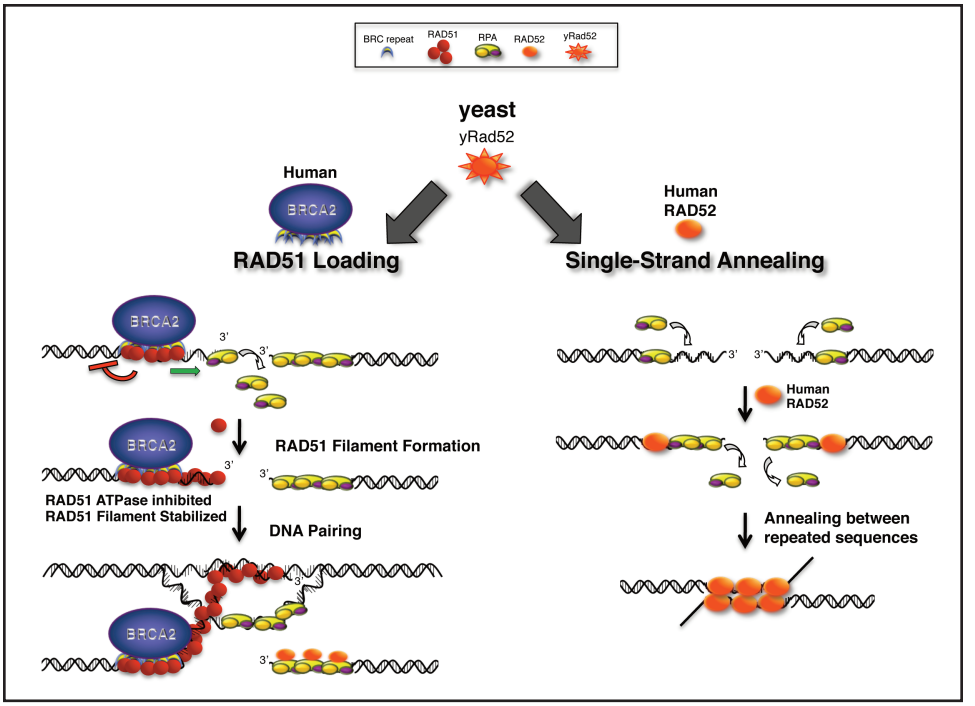

Figure 3. Yeast Rad52 has diverged into two human proteins: BRCA2 and RAD52. BRCA2 provides the RAD51 loading function while human RAD52 can anneal RPA-complexed single-strand DNA.

mal ratio of RAD51:DMC1 facilitating strand invasion and the search for homology. Perhaps the inclusion of DMC1 into the filament allows for tolerance to sequence divergence in the homology search, a feature that would be favored during meiosis when a homologous chromosome is utilized as opposed to the sister chromatid.

EVOLUTIONARY CONSERVATION OF RECOMBINATION MEDIATORS

Rad52 function in yeast (*S. cerevesiae*) is critical for recombination and for the cellular response to most types of DNA damage that result in DSBs [65,66]. By comparison, mammalian cells display little if any phenotypic response to knockout of RAD52 [67]. Prior studies have established that yeast Rad52 can anneal RPA-complexed ssDNA substrates as well as load Rad51 onto RPA-complexed ssDNA to initiate nucleoprotein filament formation [68-70]. Rad52-dependent annealing of ssDNA may play an important role during second-end capture or during break-induced replication repair [71,72]. While both yeast and mammalian cells possess the *Rad52* gene, *BRCA2* is notably missing from yeast.

Single-strand annealing (SSA) is a pathway of repair that can be utilized when DSBs occur between regions of repeated sequences and appears to be RAD52-dependent [66]. Evidence for SSA activity has been garnered from studies on the BRCA2 orthologues, *U. maydis* Brh2 and *C. elegans* C-BRC2 [73-75]. To determine if these activities were conserved in the human BRCA2 protein, we examined the role of human RAD52 and BRCA2 in the process of SSA [20]. We demonstrated that human RAD52 was capable of annealing complementary ssDNA in the presence of RPA, while BRCA2 was unable to perform this function [20]. Conversely, unlike BRCA2, human RAD52 does not catalyze the loading of RAD51 onto ssDNA in the presence of RPA. These key differences reveal that yeast Rad52 function has been separated into two proteins during evolution. Human RAD52 provides an annealing func-

tion between RPA-complexed complementary ssDNA while human BRCA2 directs and mediates RAD51 nucleoprotein filament formation onto RPA coated ssDNA (Figure 3).

SEQUENCE VARIANTS AND TUMOR MISSENSE MUTATIONS

Sequencing of the BRCA2 gene in the high risk or disease population has revealed a complex and varied landscape of mutations scattered throughout the 70 kilobase genomic DNA (breast cancer information core (BIC) (http://research.nhgri.nih.gov/bic/). Those mutations predicted or confirmed to result in a truncated protein product are almost always associated with a negative clinical outcome (BIC database). Mutations and/or polymorphisms found throughout the *BRCA2* genomic DNA consist of in-frame deletions and insertions, missense mutations, and splice-site mutations. The most common mutation, 6174delT, appears 1,087 times at the most recent count (November 2010) in the BIC database. This mutation results in a truncated BRCA2 protein, lacking the DNA binding domain, and is mislocalized to the cytoplasm due to its lack of a nuclear localization signal (NLS) located at the extreme carboxy terminus [76]. This same mutation is found in one of the few human *BRCA2* mutant cancer cell lines available, CAPAN-1, derived from a pancreatic tumor. Because of the NLS location at the carboxy terminus, most truncations in BRCA2 are considered deleterious with the exception of a few isolated cases (e.g., K3326X) [76]. Sequence variants that result in missense mutations require careful analysis. The majority of missense mutations found to date in the BIC database have an unknown classification with respect to clinical importance. The main reason for this shortcoming is the lack of a facile method to determine whether nonsynonymous mutations in the full-length protein impact function. One method, utilizing a mouse knockout ES (embryonic stem) cell line that can be complemented with a BRCA2 human genomic BAC (bacterial artificial chromosome), has addressed some of the unclassified mutations with respect to function by either survival assay or sensitivity to DNA damaging agents [77]. The finding that a subset of tumors resistant or refractory to platinum agents and PARP inhibitors possess intragenic reversions in the *BRCA2* gene will be a vital avenue for future investigation [40,78]. The bulk of these studies were performed in cell culture models undergoing selective pressure with the agents in question, but secondary mutations were also found in a few relapsed patients whose tumors had gained resistance to cisplatin. The alleles described in these two studies consisted mainly of deletions in the BRCA2 protein and as such may reveal dispensable domains or residues within BRCA2. It will be important to determine if *BRCA2* mutant tumors in the context of a larger study will reveal further intragenic reversions targeted to the *BRCA2* alleles resulting in treatment resistance and/or tumor relapse.

CONCLUSIONS

In conclusion, the ability to now purify the full-length BRCA2 protein will allow detailed studies of its domains and various protein partners. As more proteins involved in mammalian HR are purified, conditions can now be optimized to determine how they interact and stimulate (or inhibit) one another using mechanistic classical ensemble biochemistry as well as biophysical single-molecule studies. Given sufficient resources, the ability to obtain detailed structural information on BRCA2 through electron microscopy, small-angle X-ray scattering, or even X-ray crystallography may reveal further unanticipated functions of this important protein. Hopefully, these studies will translate into improved diagnostic procedures or novel therapeutic strategies for the at-risk patient population.

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