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Unraveling the mechanism of BRCA2 in homologous recombination

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Abstract

BRCA2 is the product of a breast cancer susceptibility gene in human and the founding member of an emerging family of proteins present throughout the eukaryotic domain that serve in homologous recombination. The function of BRCA2 in recombination is to control RAD51, a protein that catalyzes homologous pairing and DNA strand exchange. By physically interacting with both RAD51 and single-stranded DNA, BRCA2 mediates delivery of RAD51 preferentially to sites of ssDNA exposed as a result of DNA damage or replication problems. Through its action, BRCA2 helps restore and maintain integrity of the genome. This Review highlights recent studies on BRCA2 and its orthologs that have begun to illuminate the molecular mechanisms by which these proteins control homologous recombination.

Repairing damaged DNA by homologous recombination (HR) is a universal mechanism for restoring integrity and maintaining stability of the genetic material. In the emerging view, a damaged DNA molecule sustaining a double-strand break (DSB) as a result of a genotoxic insult, or containing a single-stranded gap as a result of a replication mishap can be channeled through the HR system for repair¹⁻³. Branches in this system, namely DSB repair and post-replication repair or single-stranded DNA (ssDNA) gap repair, converge at a common step following the generation of ssDNA with homologous pairing and strand invasion (Figure 1). ssDNA exposed by processing the end(s) of broken duplexes or introduced as an internal gap becomes the prime substrate required for the HR machinery. The single-stranded region invades a homologous DNA sequence to form a D-loop intermediate, which can be extended by DNA synthesis and acted upon further, so that missing nucleotides are filled in and broken strands are rejoined. A wealth of information is available about HR, but knowledge of crucial steps in the pathways is often incomplete or lacking. Deeper understanding of key steps has both theoretical and practical significance, since defects in the HR system compromise the capacity for maintaining genome integrity, and can lead to chromosome loss, imbalance, and rearrangements, the initiating events for tumorigenic transformation. This Review focuses on molecular mechanistic aspects of BRCA2 and its orthologs in HR and reflects the author's perspective. The reader can find detailed overviews and alternative perspectives on BRCA2 roles in HR, centrosome copy-number control, genomic instability and cancer pathogenesis in several recent authoritative reviews^{2,4-7}.

Rad51 activity and regulation in HR

The homologous pairing and strand invasion step in eukaryotes is catalyzed by Rad51 (see Box 1), which performs the search for DNA sequence homology after polymerizing on the exposed ssDNA^{2,5,8}. This reaction proceeds by formation of a D-loop joint molecule

composed of ssDNA and the target duplex. Strand invasion is modulated by a number of factors with positive or negative influences. The ssDNA binding protein RPA (Replication Protein A) inhibits the strand invasion activity of Rad51 by preventing it from associating with exposed ssDNA. This inhibition can be overcome by the addition of a mediator protein, such as Rad52 in the budding yeast *Saccharomyces cerevisiae*⁵. Rad52 interacts directly with both RPA and Rad51 and mediates Rad51 filament assembly by delivering Rad51 to ssDNA, where Rad51 binds cooperatively to DNA, displacing RPA. Rad52 appears to be also required at a step after D-loop formation, linking together the ends of the broken DNA molecule through its potent DNA annealing activity⁹⁻¹².

In *S. cerevisiae*, Rad52 is pivotal for all types of HR, resistance to ionizing radiation and repair of DSBs¹³. Oddly enough, Rad52 is structurally conserved and widely present throughout the eukaryotic domain of life, yet seems to have a crucial role in recombination only in the ascomycota, the fungal phylum that includes model organisms *S. cerevisiae*, *Schizosaccharomyces pombe*, *Candida albicans* and *Neurospora crassa* (Box 2). In the vertebrate systems of chicken and mouse, Rad52 absence or deficiency confers no obvious phenotype in growth, survival after DNA damage or meiosis, and only very minor defects in recombination¹⁴⁻¹⁶. On the other hand, absent altogether from budding yeast but critical in metazoans for recombination, resistance to radiation-induced damage, DNA genotoxins and genomic stability is the BRCA2 family of proteins, whose founding member is the product of a hereditary breast and ovarian cancer susceptibility gene in human¹⁷ and the subject of this review. How BRCA2 emerged as a tumor suppressor and then came to be realized as a central component of the HR system is a remarkable story of discovery.

BRCA2 and the link to HR

Affected individuals with mutation in the *BRCA2* gene have a dominant heritable predisposition to breast, ovarian and other types of cancers. Tumorigenesis is associated with loss of heterozygosity of the non-mutated allele. The gene encoding BRCA2 was identified by linkage analysis and positional mapping based on the inheritance of chromosomal markers in large families with numerous kindreds afflicted with cancers. When *BRCA2* was first identified, there was great excitement that the Herculean effort of mapping and cloning the gene might pay off by illuminating the biological role of BRCA2^{18,19}. However, early hope was unfulfilled when not a single defining motif or sequence similarity to a known protein was evident within its 3418 amino acids that might offer some clue to function. The only notable feature evident was the presence of eight repetitive sequences, named the BRC repeat, consisting of strings of about 30 amino acids spaced at intervals in the medial region of the sequence^{20,21}.

Yeast two-hybrid methodology yielded the first provocative evidence establishing a connection between BRCA2 and the HR system. Using a short stretch of residues from the extreme C-terminus of mouse BRCA2 as bait in a screen, Bradley and coworkers identified RAD51 as an interacting partner²². And, in the reciprocal configuration, RAD51 could capture a C-terminal fragment of BRCA2²³ of about 300 residues. Since homozygous *Rad51* mutant mouse embryos were sensitive to ionizing radiation²⁴, reminiscent of HR-defective mutants in simple eukaryotes, homozygous *Brca2*-mutant cells were expected to exhibit similar sensitivity if the BRCA2-RAD51 interaction were functionally significant *in vivo*. Indeed this was the case: although disruption of the *Brca2* gene resulted in lethality in embryonic stem cells and developmental failure in early embryos²⁵, as in the case of *Rad51*²⁶, trophoblast cells and inner cell mass outgrowth derived from early embryos could be obtained and these were extremely sensitive to ionizing radiation²². Thus, the association of BRCA2 with RAD51, plus the similar sensitivity of *Rad51*- and *Brca2*-deficient cells to radiation, suggested a possible role for BRCA2 as a cofactor for RAD51 in HR.

Follow-up analyses revealed additional interactions between RAD51 and the BRC motifs^{27,28} (see Figure 2a). A hierarchy in the capacity for interaction of individual BRC elements with RAD51 suggested that the BRCs were not functionally equivalent. Moreover, single point mutations within an individual BRC motif, some of which associated with familial early-onset cancer, were sufficient to disrupt interaction with RAD51²⁹. Cells overexpressing a BRC motif exhibited hypersensitivity to ionizing radiation and failed to support formation of RAD51 subnuclear foci in response to DNA damage, strongly supporting the idea that interaction between BRCA2 and RAD51 is a critical aspect of BRCA2 function.

With the successful establishment of *Brca2*-disrupted cells in culture, the direct role of BRCA2 in DNA repair could be assessed. Mouse strains were generated with targeted disruptions in *Brca2* to truncate the protein anywhere from about half its normal size to almost full length³⁰⁻³². These alleles resulted in an attenuated phenotype compared to the full deletion, and viable embryos occasionally survived. Depending on the particular disruption, the mouse embryonic fibroblasts obtained were highly sensitive to DNA damage by ultraviolet light (UV), methylmethanesulfonate (MMS), ionizing radiation, or crosslinking agents, showed progressive failure in proliferation, were defective in supporting subnuclear localization of RAD51 into foci following DNA damage, and exhibited spontaneous accumulation of chromosomal abnormalities including breaks, aberrant chromatid exchanges and gross rearrangements^{33,34}. These properties were reminiscent of other inherited cancer predisposition diseases such as Bloom's syndrome³⁵ and highly indicative of a role for BRCA2 in repairing DNA and maintaining genomic integrity.

The role of BRCA2 in homology-directed recombinational repair was then tested by direct measurement of HR. The experimental system employed was human CAPAN-1 cells, a pancreatic carcinoma line with one *BRCA2* allele lost and the other with a frameshift mutation resulting in a large C-terminal truncation³⁶. CAPAN-1 cells are sensitive to DNA damage and do not support RAD51 foci formation in response to radiation^{37,38}. The Jasin laboratory used an integrated recombination reporter, consisting of two direct repeats of sequences encoding green fluorescent protein (GFP), each bearing different inactivating mutations³⁹. One repeat contained a recognition site for an inducible rare-cutting endonuclease; cleavage at that site created a DSB; repair by HR using the other repeat as a template generated a functional allele that could be readily detected by flow cytometry analysis of a cell population. It was determined that CAPAN-1 cells were defective in homology-directed DSB repair, thus establishing the connection with BRCA2. In a different approach, the Powell laboratory used two mutant versions of a drug-selectable gene, one of which was integrated into the CAPAN-1 genome and the other ectopically introduced by transient transfection⁴⁰; reintroduction of the BRCA2 gene by transient transfection resulted in elevated recombination, implying a causal relationship.

Sequence comparison of newly completed genomes revealed that BRCA2-related proteins were not confined to mammals^{41,42}, and confirmatory evidence for a role for these proteins in HR came from the fungus *Ustilago maydis*, an experimental system developed over previous decades for mechanistic studies on recombination and repair. In a screen for DNA-repair defective mutants, a gene encoding a BRCA2 ortholog named *Brh2* was identified⁴³. The *brh2* null allele was found to be as sensitive to DNA damage and as defective in mitotic and meiotic recombination as the *rad51* mutant. This finding established that the BRCA2 paradigm extended beyond the realm of vertebrates and conversely suggested that conclusions gleaned from mechanistic studies on BRCA2-related proteins from other taxa could be generalized.

BRCA2's DNA-binding potential

Determination of the crystal structure of a ~800 residue C-terminal domain of BRCA2 marked the beginning of a new phase in understanding the molecular mechanism of BRCA2 action because it revealed the capacity for binding DNA ⁴⁴. The C-terminal region is the most conserved portion of BRCA2 across metazoan, plant and fungal orthologs ⁴¹. Production of the C-terminal polypeptide was made possible by co-expression with DSS1, a small acidic protein previously identified as a BRCA2-interacting partner in a yeast two-hybrid screen ⁴⁵, and which was found to be necessary to stabilize and maintain the solubility of the overexpressed C-terminal polypeptide. Structural information was obtained from crystals of the mouse and rat proteins, both of which contained bound DSS1, and also from the mouse protein in complex with ssDNA and DSS1 ⁴⁴.

The structure revealed five domains, four of them globular and packed successively in a linear array (Figure 2b). The first domain comprises a helix-rich region; it is followed by three oligosaccharide/oligonucleotide binding (OB)-folds, modules present in well-known single-strand DNA-binding proteins of prokaryotes (SSB) and eukaryotes (RPA) involved in many DNA replication and repair transactions ⁴⁶. The fifth domain contains two anti-parallel helices protruding from the core structure. DSS1 binds BRCA2 primarily through hydrophobic interactions and through acidic residues with basic grooves in the helical domain and the neighboring OB1 hydrophobic interactions. DNA binds to the opposite face of the molecule, primarily with OB2 and to a lesser extent OB3, through a groove characteristic of the OB fold single-strand binding site. The two long helices are anchored on OB2 and emerge to form a tower topped by a three-helix bundle superimposable on the DNA-binding domain of a site-specific recombinase, suggesting the possibility of an additional mode of binding to duplex DNA through the tower. Direct biochemical assays with oligonucleotides confirmed the ability of the BRCA2 DBD (DSS1/DNA binding domain) complex to bind preferentially ssDNA compared to dsDNA ⁴⁷. In light of the established interaction between the BRC motifs and RAD51 and together with BRCA2 DBD DNA-binding potential and preference for single-strands, it was proposed ⁴⁴ that BRCA2 could facilitate recruitment of RAD51 to sites of processed DSBs requiring repair and enhance RAD51-promoted strand invasion.

The importance of the Rad51-binding function of the BRC motif is well-supported by a variety of genetic and cell-based studies (see below), but its exact role is more paradoxical, despite the implications from the structural work on the DBD. In studies with *U. maydis* Brh2, it was found that a mutant comprised of the N-terminal half of the protein including the BRC motif, but lacking the entire DBD, was substantially active in DNA repair and recombination ⁴⁸. One potential explanation put forth was that a second DNA-binding binding domain, unrecognizable from sequence analysis, might be present ⁴⁸. Indeed a DNA-binding activity was detected in the isolated N-terminal half of Brh2, which bound ssDNA about twenty times tighter than the isolated DBD ⁴⁹. Thus, in the case of Brh2, the overall DNA-binding activity manifested in vitro by the full-length protein appears attributable almost entirely to the DNA-binding region outside of the DBD.

Related findings were made by the Ashworth laboratory on human BRCA2 ⁵⁰. When CAPAN-1 cells were cultured in the presence of poly ADP-ribose polymerase (PARP) inhibitors, which cause synthetic lethality with BRCA2 deficiency ^{51,52}, occasional resistant clones arose that were active in DSB-induced recombination and proficient in RAD51-focus formation, and that recovered chromosome stability due to reversion of the *BRCA2* mutation ⁵⁰. Among these revertants were new BRCA2 isoforms with intragenic deletions that restored the reading frame but still lacked the entire DBD. In a different study, Jasin and colleagues found that expression of BRCA2 fragments containing just a single or multiple

BRC motifs fused with the ssDNA-binding protein RPA70 substantially improved HR, restored Rad51 focus formation and survival in response to DNA damage, and suppressed the high level of spontaneous chromosome aberrations in BRCA2-deficient cells⁵³. These findings suggest that the DBD is to some extent expendable and that a synthetic and active version of BRCA2 can be assembled by coupling the BRC motif to a generic ssDNA binding module.

BRC and other RAD51-interacting motifs

The BRC motif, comprised of a core sequence of about 30 amino acids, is a key functional element of the BRCA2 class of proteins. Conservation of the BRC motif and its common presence in supergroups across the eukaryotic domain attest to its importance. The motif is reiterated eight times in mammalian BRCA2s but the number of repeats varies widely according to taxa⁴². The function of BRC is to interact with RAD51. Mutations in BRC that abrogate interaction with RAD51 result in loss of activity in supporting DNA repair²⁹. Resolution of the crystal structure of human BRC4 sequence (35 residues) fused with the core region of RAD51 revealed a series of hydrophobic and hydrophilic interactions at the interface⁵⁴, with a β -hairpin structure of BRC binding to the oligomerization surface of RAD51. Further structural analysis of the BRC sequence and competition studies with synthetic peptides revealed the importance of a secondary structural domain within an α -helix context also essential for BRC-RAD51 interaction⁵⁵.

The BRC motif controls the status of the RAD51 filament in a manner to govern DNA strand exchange. An 1127 amino acid region of BRCA2 with all eight BRC repeats was found to promote recruitment of RAD51 to ssDNA while slowing the association of RAD51 with dsDNA^{56,57}. Even the single isolated BRC4 motif was found capable of promoting assembly of RAD51 onto ssDNA, but not dsDNA, and to stimulate DNA strand exchange. BRC4 decreased ATP hydrolysis of RAD51 by stabilizing the fraction of ATP-bound RAD51 on ssDNA⁵⁶. A related mode of stabilization of RAD51 was noted previously by the Mazin laboratory in demonstrating accumulation of the strand-exchange-proficient, ATP-bound form of the RAD51 filament when Ca^{2+} was substituted for Mg^{2+} ⁵⁸. Thus, attenuating ATP hydrolysis of RAD51 when bound to ssDNA maintains the nucleoprotein filament in a form active for strand exchange. This observation is reminiscent of the findings with CeBRC-2, the BRCA2-related protein from *C. elegans*, showing that it reduces ATP hydrolysis of the cognate Rad51⁵⁹. The implication is that BRC motifs enhance the DNA strand exchange activity of RAD51 by reducing ATP hydrolysis. Thus, the emerging evidence supports a model in which the BRC motif differentially regulates RAD51 filament assembly, promoting loading of RAD51 on ssDNA but impeding loading on dsDNA, thus establishing a favorable state for DNA strand exchange^{56,57}.

A RAD51-interacting region at the extreme C-terminus of BRCA2, unrelated to the BRC motif⁶⁰, is highly conserved among the vertebrate BRCA2 proteins but appears absent in other taxa such as plants and microbes. Interaction of this C-terminal motif with RAD51 is cell-cycle regulated and is mediated by cyclin-dependent kinase (CDK) phosphorylation of a key serine residue⁶⁰. Using a peptide corresponding to this C-terminal motif, the interaction with Rad51 was found to be functionally different from the one between Rad51 and a peptide containing the BRC motif. The C-terminal motif could bind RAD51 in multimeric, but not monomeric form, and was initially thought to stabilize RAD51 filaments from the dissociative action of BRC peptides^{61,62}. However, in light of the role of the BRC motif in filament stabilization^{56,57}, the proposed antagonistic action between BRC and the C-terminal motif could be a reflection of limitations in using peptides to model BRCA2 action.

Venkitaraman and colleagues investigated the function of the C-terminal RAD51-interaction motif *in vivo* by characterizing the activity of chicken Brca2 variants with mutations in the cognate CDK target site that abolished or enhanced Rad51 binding⁶³. They observed no DNA repair defect or HR deficiency in DT40 cells expressing Brca2 mutated in the CDK phosphorylation site. Instead, they found that disassembly of Rad51 foci induced by DNA damage was modulated by mutation in the Brca2 C-terminal CDK target site and was coupled with the onset of mitosis. In a different study, the Jasin laboratory found that BRCA2 provides protection against degradation of stalled replication forks⁶⁴. Fork protection specifically required the C-terminal RAD51-interaction motif, which likely stabilizes RAD51 filaments in protecting nascent DNA strands from degradation⁶⁵. In summary, it appears that the C-terminal RAD51-interaction motif is dispensable for recombination and repair proficiency, but it provides a means for replication fork protection and links dissolution of the RAD51 filament to chromosome segregation.

A third region in the medial portion of BRCA2, termed the PhePP motif, was shown by *in vitro* pulldowns with affinity-tagged BRCA2 polypeptides and peptide arrays to bind the meiosis-specific RAD51-related strand exchange protein DMC1⁶⁶. The PhePP motif is highly conserved in vertebrate BRCA2s, is also evident in BRCA2 orthologs in plants and invertebrates, albeit more divergent. Biochemical and genetic studies with *U. maydis* Brh2 have shown that this element contributes to Rad51 binding and radiation resistance⁶⁷.

Lessons from BRCA2 orthologs

The idea that BRCA2 might function as a RAD51 mediator derived from the knowledge of the BRCs' ability to bind RAD51 and the DBD's activity in binding ssDNA, suggesting a capacity to bring these components together⁴⁴. Because purified intact BRCA2 protein was unavailable for biochemical studies until just recently (see below), the mediator hypothesis was first tested using *U. maydis* Brh2 protein. At 1075 residues in length, and with only one BRC motif and a more curtailed DBD domain, Brh2 appears to be a more streamlined version of mammalian BRCA2. Added to Rad51-catalyzed DNA strand exchange reactions, Brh2 could stimulate product formation at substoichiometric amounts relative to Rad51⁶⁸. Brh2 stimulated the ATPase activity of Rad51 on RPA-coated DNA containing a single-stranded gap, and eliminated the lag phase in hydrolysis caused by RPA inhibition of Rad51 nucleation. Furthermore, it promoted Rad51-filament formation on RPA-coated gapped DNA while decreasing the amount of RPA bound. These results were interpreted to mean that Brh2 facilitates Rad51 filament nucleation by recruiting Rad51 to DNA and displacing RPA at the nucleation site, i.e., that Brh2 serves as a Rad51 mediator.

Additional biochemical studies showed that Brh2 has a DNA annealing function and an inherent ability to promote D-loop formation with no requirement for a nucleotide cofactor, in contrast to Rad51^{69,70}. The biological function for such activity is unknown but it suggests a potential role for Brh2 beyond that of simply facilitating loading of Rad51 on ssDNA. One idea is that the annealing activity could serve at a step removed from D-loop formation and help join the two ends of a broken DNA molecule in a second-end capture reaction (e.g., Figure 1, step iv). Evidence supporting a role for Rad52 in second-end capture has been provided by biochemical studies with purified proteins from yeast¹¹ or human⁹⁻¹¹ and by physical analysis of molecular intermediates formed during meiotic recombination in yeast¹². *In vitro* studies revealed that Brh2 could indeed promote second-end capture and that this reaction could take place in the presence of Rad51, a condition found to attenuate the annealing activity of Rad52⁷¹.

An additional activity noted in studies with Brh2 was an ability to promote a template switch. During DNA replication HR is needed for repair of single-strand gaps left in the

wake of replication past a blocking lesion. Homologous pairing of the single-stranded region with the undamaged sister chromatid would form a D-loop that would serve as template for DNA synthesis to fill in the unreplicated gap. Some small degree of branch migration would be required to reposition the 3' end of the strand flanking the gap so that it could switch templates and prime synthesis across the displaced strand of the D-loop (e.g., Figure 1, step iii-a). In a model strand invasion reaction catalyzed by Rad51, evidence was obtained that Brh2 could promote such a template switch to enable DNA synthesis to extend past a blocking lesion⁷². The mechanism underlying this switch is not clear but it seems likely that Brh2 exerts an effect on DNA structure such that the duplex region at a single-strand/double-strand junction is partially opened in the presence of a homologous DNA molecule to allow template switching. The annealing activity of Brh2 and its binding preference to D-loop DNA as well as to Holliday junctions coupled with its limited strand exchange activity likely provide it with capability to promote template switching.

In summary, the contribution of Brh2 to recombinational repair might be multifactorial. Brh2 appears to have the capacity not only for protein-protein interactions important in the process, but it also enables DNA transactions through its annealing and binding activities. Whether these functions are maintained throughout the BRCA2 family of proteins remains an open question.

BRCA2 functional activities

Progress in analyzing the mechanism of action of the full length human BRCA2 has been slow due to technical difficulties in isolating the protein intact and in sufficient amounts for experimentation. Recently, a breakthrough in purification has been achieved by three groups, each using a different approach. The Kowalczykowski laboratory produced the protein from cultured immortalized human embryonic kidney cells, after expressing the gene under transient conditions from a strong constitutive promoter⁷³. The West laboratory obtained the protein from HeLa cells carrying a human bacterial artificial chromosome with the gene embedded in a locus with the normal upstream and downstream regulatory elements⁷⁴. The Heyer laboratory produced the protein from yeast after overexpressing the gene from a multicopy plasmid under control of a strong inducible promoter⁷⁵. All three preparations were active in stimulating DNA strand exchange by RAD51, and those from the Kowalczykowski and Heyer studies were active in enabling RAD51 to bind RPA-coated ssDNA, thus confirming the mediator hypothesis.

In addition, all three laboratories reported new findings likely to contribute to mechanistic understanding. Heyer's laboratory found that addition of DSS1 stimulated BRCA2-mediated binding of RAD51 to RPA-coated ssDNA⁷⁵. Using the electron microscope to visualize shadow-casted protein, West and collaborators found two distinct particle sizes which they proposed to be monomeric and dimeric forms of BRCA2⁷⁴. Kowalczykowski's laboratory reported that BRCA2 has a strong preference for ssDNA versus dsDNA, that approximately six RAD51 proteins could be bound per BRCA2, in agreement with the Heyer report⁷⁵, and that BRCA2 inhibited DNA-dependent ATP hydrolysis of RAD51. This inhibition of TP hydrolysis was in agreement with findings using BRC peptides⁵⁶ and with studies on the worm ortholog CeBRC-2⁵⁹. The Kowalczykowski study went on to show using functional strand exchange assays that partially duplex DNA molecules with single-stranded tails were better substrates than single-strands alone, but there was no preference for 5' or 3' tails, in contrast to the 3'-tail preference noted in the case of Brh2⁶⁸.

Several additional notable findings emerged from the Kowalczykowski study⁷³. First, the investigators observed no physical interaction between BRCA2 and RPA. Since BRCA2 is able to mediate RAD51 loading on RPA-covered DNA, it is puzzling how this is

accomplished in the absence of contact with RPA. Apparently, the interaction between BRCA2 and RAD51 is sufficient to stimulate RAD51 to gain access to RPA-coated DNA. This notion is reinforced by the finding that BRCA2 could also mediate RAD51 loading on DNA coated with bacterial single-strand binding protein SSB. The investigators also found no evidence for DNA strand annealing by BRCA2 in the physiologically relevant situation of saturating RPA and free Mg^{2+} , in contrast to the annealing activities observed with the fungal and worm orthologs^{59,69}, Brh2 and CeBRC-2. It is possible that a functionally analogous annealing activity resides in a separate interacting partner, such as PALB2⁷⁶, a DNA-binding protein that works in concert with BRCA2 and physically interacts with it at the N-terminus⁷⁷. In addition, and again in contrast to the findings made with Brh2 in promoting DNA strand exchange, no specificity for binding to single-strand/double-strand DNA junctions was noted with BRCA2^{73,75}.

Kowalczykowski and colleagues reported that full-length BRCA2 exhibited a strong preference in binding to ssDNA over dsDNA⁷³ in agreement with previous reports on the isolated DBD and BRC3/4-DBD fusion^{44,47}. However, the apparent dissociation constant of the full-length BRCA2 and single-strand DNA, estimated as 10-20 nM in the Kowalczykowski's study⁷³, appears much lower than the K_d of 250 nM reported for the isolated DBD⁴⁴. This discrepancy could reflect differences in reaction conditions and/or substrate composition, or a difference in the DBD itself, in isolation or in the context of the intact protein. The situation is also reminiscent of the observations on the fungal ortholog, where a second, separate higher-affinity DNA-binding domain was identified⁴⁹.

Regardless of speculation here on an additional DNA-binding domain in BRCA2, recent studies suggest that the DNA-binding status of BRCA2 can be modulated through its association with PALB2. This BRCA2 interacting partner is mutated in a subgroup of Fanconi anemia and engenders cancer risk^{78,79}, and was previously shown to associate with chromatin and to mediate BRCA2 association with chromatin⁷⁶. In recent studies with the purified protein, the Sung⁸⁰ and Masson⁷⁷ laboratories have determined that PALB2 has a strong preference for binding D-loop structures, stimulates RAD51-catalyzed D-loop formation, and works synergistically with a truncated BRCA2 chimeric construct termed "piccolo" to promote strand invasion. That the activity of BRCA2 could be augmented through physical interaction with PALB2 suggests a model for conditional regulation.

Concluding remarks

The BRCA2 family of proteins is at the center of a complex system regulating recombinational repair by controlling the recombinase RAD51. Precise control is required to maintain the genome by eliminating DNA breaks and deleterious lesions, as evidenced by the loss of resistance to radiation, genome rearrangements, replication failure, and onset of tumorigenic transformation when HR is disturbed. The primary action of the BRCA2 members appears to be in mediating orderly assembly of RAD51 on ssDNA, the form that is active for homologous pairing and strand invasion. But there appear to be additional means for enhancing the activity of RAD51 through mechanisms that involve redirecting RAD51 from dsDNA and preventing dissociation from ssDNA. Other BRCA2 activities in the DNA dynamics of HR beyond the mediator function are also possible. Understanding how the BRC motifs and DNA-binding region(s) of BRCA2 protein cooperate to deliver RAD51 is a key issue in dissecting the mechanism of action. Fundamental knowledge of BRCA2 action will translate ultimately into deeper understanding of breast cancer etiology, therapy, and prevention.

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Box 1**Rad51**

Central to HR is the search for DNA sequence homology and strand invasion step. Here a ssDNA pairs with a homologous dsDNA to form a D-loop (fig 1). In eukaryotes, the reaction is powered by the RecA homolog Rad51, which forms the active catalytic form for recombination by polymerizing around ssDNA to assemble a nucleoprotein helical filament. ATP is required for this activity. In the ATP-bound form Rad51 extends the DNA structure. ATP hydrolysis to ADP activated by Mg^{2+} results in relief of the extended state and disassembly of the filament. Factors contributing to filament assembly and disassembly thus provide means for regulating HR. ssDNA coated with the single-strand-specific DNA-binding protein RPA is not accessible by Rad51. In such case a mediator is required for enabling Rad51 to nucleate and polymerize along the ssDNA with concomitant RPA dissociation. Stabilization of the Rad51-filament enhanced by mediator interaction further potentiates HR capacity. Rad52 in budding yeast and BRCA2 in taxa beyond yeast function as mediators.

Box 2**Rad52**

Rad52 is crucial for all HR events in the budding yeast *S. cerevisiae*. It provides mediator function, interacting directly with both RPA and Rad51 to enable Rad51 to gain access to ssDNA coated with RPA, and it exhibits a powerful DNA annealing activity thought to be important in second-end capture. However, while widely conserved throughout the eukaryotic domain of life, Rad52 appears to have only a minor role in the HR systems of organisms beyond the fungal phylum ascomycota. A role for Rad52 in systems beyond yeast becomes apparent when HR function is compromised⁸¹. In *U. maydis* the *rad52 brh2* double mutant grows slowly and is more sensitive to DNA damage than the *brh2* single mutant⁸². Through deletion analysis of the *Brh2* structural gene a region was identified that is necessary for survival of the organism after DNA damage, but in a manner dependent on Rad52. This suggests that Rad52 plays some overlapping or compensatory role with *Brh2*⁶⁷. In mouse, deletion of *Rad52* exacerbates survival of *rad54* mutant animals and bone marrow cells after treatment with mitomycin C or ionizing radiation⁸³. Chicken DT40 cells with conditional mutations in both Rad52 and XRCC3, a RAD51 paralog, exhibit extensive chromosomal breaks and synthetic lethality⁸⁴. In human cultured cells lacking BRCA2, there is a synthetic lethal effect on growth and synergistic effects on DSB-induced HR and radiation-induced chromosome abnormalities when Rad52 is depleted⁸⁵. These observations suggest that Rad52 does contribute to recombinational repair.

There has been no report of Rad51-mediator activity associated with non-yeast Rad52 in DNA strand exchange with RPA — and only a single published experiment suggesting to the contrary that human Rad52 lacks mediator activity⁷³. This absence of mediator activity is perplexing given that the yeast and human proteins are highly conserved in structure, and that human Rad52 binds ssDNA strongly, stimulates Rad51-promoted DNA strand exchange⁸⁶, and physically interacts with both Rad51 and RPA⁸⁷, a combination of features that one might expect for a mediator. Perhaps a cryptic mediator function is inherent within Rad52 in non-yeast systems, but a certain undiscovered condition or association with another component might be necessary to reveal such activity.

Figure 1. DNA repair by HR

Schematics are shown illustrating how repair of DNA with a DSB or ssDNA gap could initiate by HR. Both pathways begin with invasion of a ssDNA tract (step i or i-a) into a homologous DNA sequence. In DSB repair the D-loop formed after invasion of the ssDNA tail (step ii) is extended by DNA synthesis (step iii) until it can pair with, or capture, the second end through exposed complementary sequence. The 3' strand of the second end serves as a primer for fill-in synthesis (step iv). Additional processing of the intermediates and several subsequent steps results in a repaired chromosome. In the other pathway, termed post-replication repair, a ssDNA gap generated during DNA replication of a damaged template (step i-a) invades the undamaged homologous sequence present in the sister chromatid (step ii-a). By branch migration through a short tract the 3' end of the broken strand switches templates (step iii-a) and then serves as primer for fill-in synthesis (step iv-a). After additional steps the intermediate is resolved and repair is completed.

Figure 2. BRCA2 organization and DBD domain structure

(a) BRCA2 is shown schematically with protein interaction motifs and domains identified on top. The acronyms are as described in the text. CTRM is C-terminal RAD51-binding motif. The elements are illustrated as gray and black boxes. The DBD is broken down into the helix-rich domain (hatches) and the three OB folds (ovals). The approximate regions of interaction with the various proteins and DNA discussed are shown underneath. (b) The 800 residue DBD is shown schematically with the helix-rich domain (HD) followed by the OB folds. OB2 and OB3 are packed in tandem while OB1 is packed with OB2 in the opposite orientation. The Tower domain emerges from OB2 and has a three-helix bundle (3HB) on top. ssDNA (black squiggle) interacts with OB2 and OB3. DSS1 (gray squiggle) interacts with HD and OB1 on the opposite face of the domain.