

Dss1 Interaction with Brh2 as a Regulatory Mechanism for Recombinational Repair[∇]

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Brh2, the BRCA2 ortholog in *Ustilago maydis*, enables recombinational repair of DNA by controlling Rad51 and is in turn regulated by Dss1. Interplay with Rad51 is conducted via the BRC element located in the N-terminal region of the protein and through an unrelated domain, CRE, at the C terminus. Mutation in either BRC or CRE severely reduces functional activity, but repair deficiency of the *brh2* mutant can be complemented by expressing BRC and CRE on different molecules. This intermolecular complementation is dependent upon the presence of Dss1. Brh2 molecules associate through the region overlapping with the Dss1-interacting domain to form at least dimer-sized complexes, which in turn, can be dissociated by Dss1 to monomer. We propose that cooperation between BRC and CRE domains and the Dss1-provoked dissociation of Brh2 complexes are requisite features of Brh2's molecular mechanism.

Accumulating evidence points to a choreographed interaction between Rad51 and BRCA2 as a critical mechanism governing recombinational repair (9, 21, 27, 30). Proper regulation of the repair process requires assembly of Rad51 into its catalytically active form, the nucleoprotein filament. This filament is generated through Rad51's polymerization on single-stranded DNA, a process that appears to be mediated by BRCA2. Biochemical analyses using Brh2, the BRCA2-related protein from *Ustilago maydis*, have demonstrated that it nucleates Rad51 assembly at the site of a double-strand/single-strand DNA junction, the prerequisite structure for recombinational repair arising from resection of a double-strand DNA end to reveal a protruding 3' single-stranded tail (32). However, molecular genetic experimentation with *U. maydis* (15) as well as biochemical studies using peptides modeling BRC elements from the human BRCA2 (6) or a polypeptide containing multiple BRC elements (26) suggest a role for BRCA2 in organization or stabilization of Rad51 filaments.

Regulated assembly of the filament may be balanced on the one hand by interaction of Rad51 with BRC elements and on the other hand by some interplay with a second domain located at the extreme C terminus of BRCA2 (CRE [C-terminal Rad51-interacting element]; see below) (4). The BRC domain comprises eight reiterated sequences of about 30 amino acids each whose structures have been proposed to mimic an element in Rad51 that provides a critical determinant at the polymerization interface, gluing Rad51 molecules into a chain (20, 25). Rad51 interaction with the C-terminal domain appears to be controlled by phosphorylation of a key BRCA2

residue that is targeted by cyclin-dependent kinases (4). Proper Rad51 filament assembly at DNA sites of repair requires a precisely coordinated interplay between BRCA2's Rad51-interacting domains and its DSS1/DNA-binding domain (DBD). The latter (31) consists of a tandem array of OB (oligonucleotide/oligosaccharide-binding) folds, with a double-helical tower emerging from one OB fold topped by a helix-turn-helix, and a helical domain that is laced to the adjacent OB folds by intertwining with DSS1 (14), a small, versatile protein that also serves as a component of the proteasome 19S regulatory particle (5, 17, 28), as a factor dedicated to mRNA nuclear export (29), and as a member of an RNA polymerase II-associated complex mediating snRNA processing (1). The half-life of BRCA2 has been reported to be greatly diminished upon depletion of DSS1 by RNA interference (18), suggesting dynamic instability as a means for regulating BRCA2 gene function.

Evidence for a Rad51-interaction domain located at the extreme C terminus of human BRCA2 was actually obtained before the importance of the BRC elements in Rad51 interaction was realized (19, 24). Overshadowed by investigation of the BRC-Rad51 interplay, this C-terminal domain went largely underappreciated until just recently, when it was recognized that phosphorylation of a critical serine residue by cyclin-dependent kinase–cyclin A and cyclin-dependent kinase–cyclin B might provide a molecular switch tying Rad51 activity to the cell cycle (4). The modification blocks or abrogates BRCA2's C-terminal interaction with Rad51, raising the notion that the two different types of Rad51-interacting domains, BRC and that in the C-terminal region, work in some kind of concerted hand-off mechanism in a cell cycle-dependent manner to transfer Rad51 to DNA sites where nucleoprotein filaments are assembled. Precisely how the BRC repeats and the C-terminal region of BRCA2 bind and release Rad51 at the appropriate time and in response to a particular signal is unknown.

Brh2, the BRCA2 ortholog in *Ustilago maydis*, is a streamlined version of the mammalian protein, exhibiting a similar

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modular arrangement (12). Only a single BRC element is present, and the Dss1/DNA-binding domain is more circumscribed. Two putative nuclear localization signals are present, one medial and the other near the C terminus. The sequence of the Brh2 C-terminal region with the highest similarity to the BRCA2 DBD corresponds to part of the helical domain and extends through the OB1 and OB2 folds. Within this region in the BRCA2 DBD, 40 residues contact DSS1, with 24 being identical in Brh2 and 12 conservatively substituted, suggesting a conserved function of DSS1 in regulating BRCA2 and Brh2. The essential role of Dss1 in the homology-directed repair system of *U. maydis* was interpreted to mean that it serves as an activator of Brh2 (14). Several lines of evidence from more recent studies suggesting that Brh2's C-terminal region provides a regulatory function have linked that role to Dss1 (15). First, Brh2 allele proteins with the entire DBD deleted can partially complement the radiation sensitivity of *brh2* mutant cells but in addition can also partially suppress the radiation sensitivity of *dss1* mutant cells, implying that a negative regulatory function has been removed. Second, DNA damage-induced Rad51 focus formation in *brh2* mutant cells can be restored to nearly normal levels upon expression of the Brh2 N-terminal BRC domain. Third, replacing Brh2's entire C-terminal Dss1/DNA-binding domain with that of RPA70 creates a hybrid that has extremely elevated recombination levels regardless of Dss1 status. This finding received support from studies of a mammalian system in which a fusion protein of BRC elements from human BRCA2 linked to RPA70 suppressed DNA repair defects of BRCA2-deficient cells (22). These findings indicate that the N-terminal BRC domain has an inherent ability to organize Rad51 and to support DNA repair independent of Dss1, and they imply that a negative regulatory function is normally operational in the natural DBD and is subject to governance by Dss1.

In line with the BRCA2 paradigm developed in higher organisms, interaction between the *U. maydis* Brh2 and Rad51 proteins has been established by a combination of genetic and biochemical studies (12, 32). By using a coprecipitation (pull-down) procedure to map Rad51-interacting regions in Brh2, we demonstrated the capability of the N-terminal BRC to bind Rad51 and confirmed the importance of certain canonical residues in the interaction (16). As our previous deletion mapping of Brh2 demonstrated that the removal of 41 residues from the C terminus was enough to abolish the activity of Brh2 in promoting survival after DNA damage (15), we were curious to know if this deletion disrupted a Rad51-binding domain or element (CRE) analogous to that found at the extreme C terminus of BRCA2 and, if so, whether it might be responsible in part for the putative negative regulatory function. Could communication between such dual elements in Brh2 enable a dynamic balancing of Rad51 for proper execution of function? Here we found a strong indication for such a CRE and provide evidence for a mode of intermolecular cooperation between BRC and CRE elements that is controlled by Dss1.

MATERIALS AND METHODS

***U. maydis* strains and genetic methods.** Manipulations of *U. maydis*, culture methods, gene transfer procedures, survival after irradiation, and green fluorescent protein fluorescence microscopy and differential interference contrast imaging of live cells have been described previously (see reference 16 and refer-

ences therein). *U. maydis* strains included UCM565 ($\Delta brh2\ pan1-1\ nar1-6\ alb1$), UCM591 ($\Delta dss1\ met1-2\ nar1-1\ a2b2$), as described previously (12, 14), and UCM637 ($brh2-1\ \Delta dss1\ pan1-1\ nar1-6\ alb1$), *pan*, *met*, *nar*, and *ab* indicate the requirements for pantothenate and methionine, inability to reduce nitrate, and mating loci, respectively. UCM637 was generated by disrupting the *DSS1* gene with a gentamicin resistance cassette in UCM549 ($brh2-1\ pan1-1\ nar1-6\ alb1$) (12) cells harboring the cloned *BRH2* gene on a replicating plasmid and then curing the cells of the plasmid.

Plasmids with a variety of antibiotic resistance markers used for gene disruption and protein expression in *U. maydis* have been described previously (11, 16). These are pUC derivatives containing an *U. maydis* *ARS* gene, a fragment of the glyceraldehyde-3-phosphate dehydrogenase promoter (*gap*) used for driving expression of Brh2 alleles, and a gene expressing resistance to hygromycin (Hyg^R), gentamicin (G418^R), or carboxin (Cbx^R) for use as a selectable marker. Dss1 was expressed from a gene fragment containing the natural promoter (14). Cells transformed with self-replicating plasmids expressing Hyg^R and Cbx^R were cured of plasmid by culturing for a determined number of generations in the absence of drug selection. With this regimen, cells in the culture retaining one of the transforming plasmids but not the other could be determined by testing isolated colonies for drug resistance.

For immunoprecipitation of myc-tagged Brh2 from *U. maydis* UCM565, cultures (50 ml) of cells transformed with a self-replicating Hyg^R plasmid expressing 6Myc-Brh2 under the control of the *gap* promoter were grown to late log phase and cells collected by centrifugation and resuspended in ice-cold 0.5 ml 25 mM Tris-HCl, pH 7.5, 0.5 M NaCl, 1 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol, 0.1% NP-40, and 2% reconstituted complete protease inhibitor cocktail (Roche Applied Science). An equal volume of zirconium beads (BioSpec) was added and the mixture pulsed for three 20-s intervals with intermittent cooling in a Bio101 FastPrep machine (Thermo Electron Corp.) to disintegrate cells. The slurry was decanted and centrifuged in a Beckman TLA100.3 rotor at 40,000 rpm for 20 min in a tabletop ultracentrifuge and 200 μ l of supernatant immediately immunoprecipitated by the addition of polyclonal anti-myc antibodies (Sigma-Aldrich) followed by protein A agarose beads (Roche Applied Science). Beads were collected and washed six times with lysis buffer without protease inhibitors and protein eluted by the addition of glycine buffer, pH 2.5. For Western analysis, anti-myc monoclonal antibody (Sigma-Aldrich) was used to detect myc-tagged Brh2. A loading control of the cell extracts was performed on the cleared lysates by using anti- β -tubulin antibodies (Sigma-Aldrich).

Yeast two-hybrid analysis was performed using standard methodology as described previously (13), measuring β -galactosidase activity according to the guidelines of the Clontech Laboratories yeast protocols handbook PT3024-1 (2a). The Brh2 BglII-HindIII fragment spanning residues 505 to 853 was cloned into pGADT7 and pGBKT7 vectors (Clontech), which were used to transform *Saccharomyces cerevisiae* strain PJ69-4A. Activity was expressed as units of β -galactosidase activity, for which one unit is defined as hydrolysis of 1 nmol o-nitrophenyl-galactoside per min per 10^7 cells at 28°C.

Protein expression, coprecipitation, purification from *Escherichia coli*, and cross-linking. Pull-down analyses were performed using bacterial extracts prepared after expression of maltose binding protein (MBP)-tagged or His-tagged fusion proteins in *E. coli* strain BL21(DE3) (16). As deletions removing up to 213 amino acid residues from the N terminus of Brh2 make no difference in the ability to complement the radiation sensitivity of the *brh2* null mutant (15), we cut into the coding sequence for this nonessential region to expedite cloning of Brh2 truncation alleles and to enhance the solubility of their protein products. For expression in *E. coli*, the allele representing completely functional Brh2 started at residue 106 (see Fig. 1A). Plasmids with MBP fusions used for expression were derivatives of pMAL-C2 vector (New England Biolabs), while those with His-tagged Brh2 or Dss1 fusions as well as untagged Rad51 were derivatives of pET vectors (Novagen). Cell cultures (300 ml) in LB broth with appropriate antibiotics were incubated at 37°C until cells reached a density of 2×10^8 per ml, then induced by the addition of 0.25 mM IPTG (isopropyl- β -D-thiogalactopyranoside), and shifted to 18°C to enhance the solubility of expressed proteins. After 8 to 10 h, cells were collected by centrifugation, resuspended in 10 ml buffer A (25 mM Tris-HCl, pH 7.5, 1 mM DTT, 1 mM EDTA, 10% glycerol, 0.1% NP-40) plus 200 mM NaCl, crushed by two passes through a French pressure cell, and centrifuged at 16,000 rpm in a Sorvall SS34 rotor for 30 min. The supernatant was recovered and used in subsequent procedures, which were all done at 0 to 4°C.

Aliquots of supernatant adjusted to contain approximately equal levels of the bait proteins were brought to a final volume of 600 μ l with buffer A, mixed with 100 μ l cross-linked amylose resin (New England Biolabs), and rocked on a platform mixer at 4°C. After 20 min, the slurry was washed six times by repeated centrifugation and decantation using a microcentrifuge, resuspending the resin in

fresh buffer A plus 200 mM NaCl (650 μ l) between spins. Bound protein was then recovered by washing the resin with the same buffer containing 10 mM maltose and analyzed by electrophoresis on polyacrylamide gels containing sodium dodecyl sulfate (SDS) followed by staining with Coomassie brilliant blue or by Western blot hybridization following transfer to a polyvinylidene difluoride (PVDF) membrane. In cases in which the bait protein was His tagged, the same procedure was followed except that cells were crushed in buffer B containing 0.2 M KCl (see below) and Ni²⁺-charged nitrilotriacetate (Ni-NTA) agarose resin (QIAGEN) was used to capture the His-tagged protein. Bound proteins were specifically eluted using buffer B plus 200 mM imidazole.

MBP combined with residues 505 to 1075 of Brh2 (Brh2⁵⁰⁵⁻¹⁰⁷⁵) and His-Brh2⁵⁰⁵⁻¹⁰⁷⁵ were used for size analysis by gel filtration. Cleared lysates from cultures (6 liters) of cells expressing both tagged forms plus His-Dss1 were prepared as described above after resuspending cell pellets in buffer B (25 mM Tris-HCl, pH 7.5, 20 mM imidazole, 1 mM DTT, 10% glycerol, 0.1% NP-40) and either 0.2 M KCl or 0.5 M NaCl and then passed onto a column (1 cm by 5 cm) with Ni-NTA agarose. After being washed with 40 ml of the lysis buffer, bound protein was eluted with the lysis buffer containing 200 mM imidazole. The conductivity of the pooled fractions was adjusted, by diluting with buffer without salt, to be equal to that of a solution of 100 mM KCl and the sample loaded onto a fast-protein liquid chromatography (FPLC) HR5/5 Mono Q column. The column was washed with 10 ml of 25 mM Tris-HCl, pH 7.5, 0.1 M KCl, 1 mM DTT, and 10% glycerol and then eluted with 20 ml of a linear (0.1 to 0.6 M KCl) gradient (see Fig. 4) at 1 ml per min. Appropriate fractions were applied to a column (1 ml) of cross-linked amylose resin (New England Biolabs) and eluted with 10 mM maltose in 0.5-ml fractions. The bulk of the protein eluted in a single fraction, which was applied to a Superose 6 10/300 gel filtration column equilibrated with 25 mM Tris-HCl, pH 7.5, 0.2 M KCl, 1 mM DTT, and 10% glycerol and chromatographed using an AKTA FPLC system (Amersham-Pharmacia). Fractions were analyzed by Western blotting after transfer to a PVDF membrane. Blots were developed using anti-MBP antiserum (New England BioLabs), His-Tag monoclonal antibody (Novagen), and C-myc monoclonal and polyclonal antibodies (Sigma-Aldrich) and/or affinity-purified rabbit polyclonal antibodies to Rad51 or to Brh2⁵³⁹⁻¹⁰⁷⁵ (DBD) coupled with ECL chemiluminescence reagents (Amersham Biosciences) for detection. Polyacrylamide gels were soaked in InVision stain (Invitrogen) to enable detection of His-Dss1.

Protein cross-linking was performed using bis(sulfosuccinimidyl) suberate (BS³; Pierce) by the manufacturer's protocol but at much lower concentrations of BS³. Protein solutions were dialyzed against 20 mM potassium phosphate, pH 7.5, 200 mM KCl, and 10% glycerol to a final concentration of 2.5 μ M as estimated using Bradford reagent (Bio-Rad Laboratories). Reactions were started by the addition of BS³ and allowed to proceed at 22°C for 30 min before quenching with Tris-HCl, pH 8.0, to a final concentration of 100 mM. The protein used in cross-linking was MBP-Brh2¹⁰⁶⁻¹⁰⁷⁵ and was purified in complex with His-Dss1 by a variation of the procedure described above for the MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵ protein. Protein was purified by sequential chromatography steps on Ni-NTA agarose, amylose resin, a Mono Q column, and Superose 6 by FPLC. Rad51 tagged at the N terminus with a hexahistidine leader sequence (His-Rad51), which was used as a control, was purified by Nayef Mazloum of this laboratory by an unpublished procedure that involved sequential chromatography on DEAE-cellulose, Ni-NTA resin, cibacron blue agarose, hydroxyapatite, and Mono Q beads via FPLC. Ovalbumin (Sigma-Aldrich) was used as a negative control.

RESULTS

CRE. The presence of two different Rad51-interacting domains in BRCA2 might well constitute the underpinning of a regulatory mechanism. Therefore, we were curious to explore the possibility of a second Rad51-interacting domain in Brh2. We were unable to identify by using sequence alignment any obvious similarity between the region of mapped interacting residues in BRCA2's C terminus, which interacts with Rad51, and the C-terminal region of Brh2. However, given the results from our own deletion mapping studies indicating the importance of the extreme C-terminal region in Brh2 activity, we suspected there might be a functionally analogous domain. We focused our attention on this region for use as a bait in a coprecipitation or pull-down procedure to test for Rad51 in

teraction. The system was designed to test for the interaction of Rad51 with the extreme C-terminal region of Brh2 (Fig. 1A) and was performed after coexpressing Rad51 with affinity-tagged polypeptides representative of the C terminus of Brh2 in bacteria (16). Following affinity capture, we used antibodies directed to the tag (MBP) or to the Brh2 DBD (Brh2⁵³⁹⁻¹⁰⁷⁵) to identify Brh2 derivatives and then tested the pull-down procedures with antibodies to Rad51 (Fig. 1B). A slight affinity of Rad51 itself for the amylose resin contributed to a low level of Rad51 being carried through in the pull-down assays, but the background amount was small enough to not obscure the authentic interaction between Rad51 and Brh2.

With most of the constructs, the MBP-Brh2 fusions expressed well, although there was some variability, particularly with certain polypeptides representing extremely truncated alleles, such as the Brh2¹⁰⁰⁴⁻¹⁰⁷⁵ allele (Fig. 1B). Also evident to differing degrees depending on the sample was an anti-MBP cross-reacting protein with the same mobility as free MBP itself. We presume this represents the MBP tag liberated from the Brh2 polypeptide fusion as a result of proteolytic cleavage within the spacer sequence coupling the MBP tag to the Brh2 polypeptide. The MBP-Brh2 C-terminal polypeptide fusions were verified by their cross-reaction with anti-Brh2 antibodies which had been raised against Brh2's DBD (Brh2⁵³⁹⁻¹⁰⁷⁵).

A 102-residue polypeptide (Brh2²⁶⁰⁻³⁶¹) harboring the N-terminal BRC element (Fig. 1B) serving as a positive control efficiently pulled down Rad51. As a negative control, a polypeptide which is identical except that the BRC F294 and T296 residues were changed to A was greatly diminished in capturing Rad51, as expected based on previous studies on BRC peptides in which the amino acids essential for Rad51 interaction were defined (2, 3, 20). The polypeptide comprising the C-terminal 72 residues (Brh2¹⁰⁰⁴⁻¹⁰⁷⁵) fused with MBP captured Rad51 when pulled down with amylose beads. This fusion appeared particularly unstable and/or sensitive to proteolytic cleavage, as the bulk of the MBP material ran with a mobility equivalent to that of free MBP. However, it was evident from the blot that the fraction of fusion protein remaining full length bound Rad51 with an efficiency comparable with that of the BRC polypeptide. Two versions of the C-terminal polypeptide, Brh2¹⁰³⁵⁻¹⁰⁷⁵ and Brh2¹⁰⁰⁴⁻¹⁰⁶⁸, from which 31 proximal residues and 7 distal residues were deleted, respectively, were markedly abrogated in interaction with Rad51, implicating residues in those regions as important in the association with Rad51. The allele product with the latter deletion had a severely reduced level of DNA repair activity (Fig. 1C). Furthermore, the same 102-residue polypeptide, but with a W1052A point mutation from an allele product exhibiting sharply reduced survival after UV irradiation, was concomitantly diminished in capacity to associate with Rad51. These pull-down determinations suggest that a domain or element with the capacity to interact with Rad51 is indeed present at the C terminus of Brh2 (referred to as the CRE). To determine whether truncation of the seven C-terminal residues (Brh2¹⁻¹⁰⁶⁸) or the W1052A point mutation affected protein level, we performed Western blot analysis for extracts from cells expressing myc epitope-tagged versions of these Brh2 allele products (Fig. 1D). No substantial decrease in the protein level compared to that of the wild type was detected.

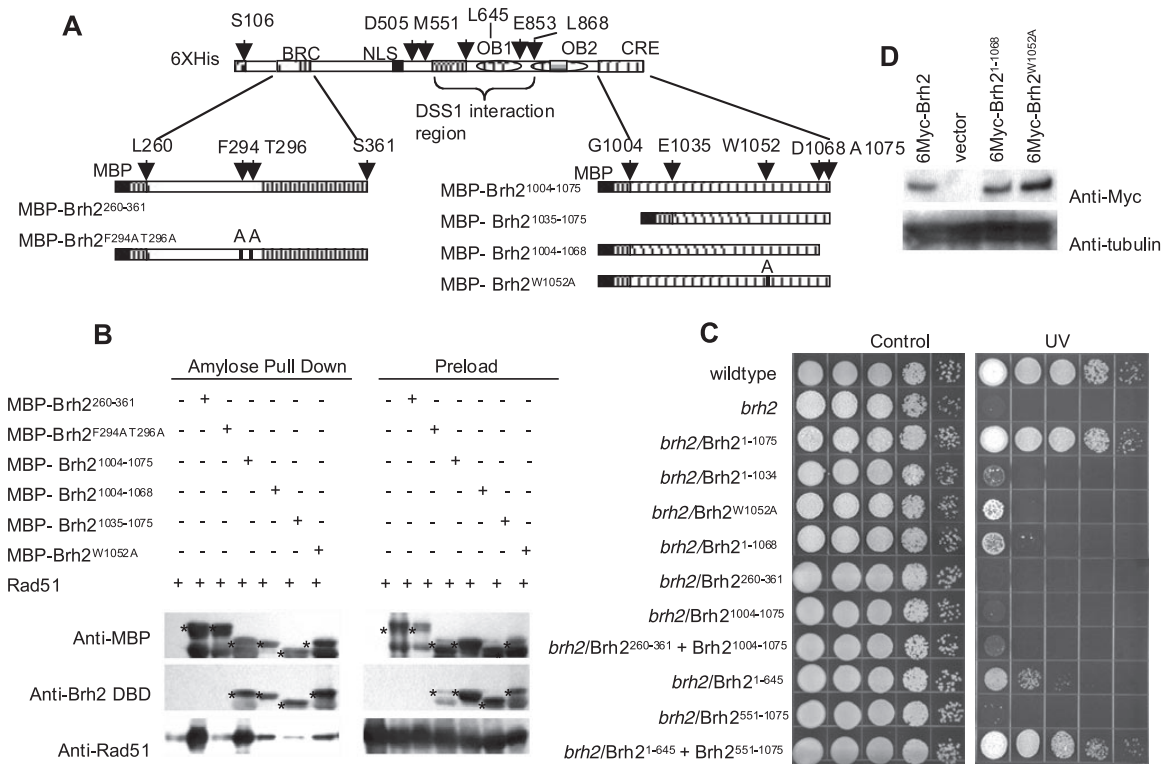


FIG. 1. Mapping the C-terminal Rad51-interacting region, CRE. (A) Brh2 is illustrated schematically with the BRC domain, helical domain, OB1 and OB2 with the inserted tower, and the CRE region regions represented. The boundaries of the truncation mutations or the particular point mutations are shown. The allele product representing completely functional Brh2 started at residue 106 as discussed in Materials and Methods. (B) The indicated fragments were expressed as MBP fusions in *E. coli* in the combinations shown in the matrix. Pull-down assays were performed with cleared cell extracts using amylose beads to capture MBP-tagged proteins. After specific elution of associated proteins from the beads by using maltose-containing buffer, samples were electrophoresed in 10% polyacrylamide gels with sodium dodecyl sulfate and analyzed by Western blotting. MBP-Brh2 fusions are distinguished with asterisks. (C) Survival of *brh2* mutant cells expressing the indicated Brh2 mutant allele products. Serial 10-fold dilutions of cell suspensions were spotted from left to right and plates were irradiated with 120 J/m² UV light. (D) Western blot analysis of 6Myc-tagged Brh2 alleles. Extracts of UCM565 cells expressing the indicated allele products were immunoprecipitated with anti-myc antibodies and protein A agarose. Bound protein was electrophoresed in an SDS-polyacrylamide gel, transferred to membrane, and probed with anti-myc monoclonal antibody to detect tagged Brh2. A loading control using the cell extracts was performed with anti-β-tubulin antibodies.

Brh2 intermolecular interaction. With the establishment of two different Rad51-interacting regions in Brh2, the question as to whether there might be some form of collaboration or communication between them necessary to achieve proper functional activity was raised. To explore this notion, we tested whether the expression of both regions, together in the same cell but independently as separate polypeptides, could restore the DNA repair capacity to a mutant devoid of Brh2. First, the two peptides Brh2²⁶⁰⁻³⁶¹ and Brh2¹⁰⁰⁴⁻¹⁰⁷⁵, encompassing the BRC and CRE Rad51-binding elements, were expressed together in the *brh2* mutant, but these had no effect on the alleviation of the UV sensitivity. Next, we tested another pair of severely truncated allele products, but in this case, there was some overlap in the remnant sequences. One allele product (Brh2¹⁻⁶⁴⁵) had 430 C-terminal residues encompassing most of the DNA-binding domain and the CRE deleted, while the other (Brh2⁵⁵¹⁻¹⁰⁷⁵) had 550 N-terminal residues, including BRC, deleted. Expressed individually, each allele exhibited little to no capacity for complementing UV sensitivity of *brh2*, but when expressed together here, they could restore a nearly normal level of UV resistance (Fig. 1C). This resurrection of

DNA repair activity by coexpression of two fragments of Brh2 in *trans*, each bearing a different region interacting with Rad51, is thus consistent with the prediction that there is some form of communication or cooperation between the two regions that interact with Rad51. The trivial but real possibility that the activity was due to reconstruction of the *BRH2* gene by recombination through the overlapping coding sequence was ruled out in more-extensive control experiments described below. The possibility that the complementation activity might in some way be due to an overexpression artifact was also considered but deemed unlikely, since plasmids are maintained at very low copy number in *U. maydis*, only one to two per cell (10), and since the *gap* promoter is from an ordinary house-keeping gene. However, until the promoter of the Brh2 structural gene is characterized and more is known about how the gene is regulated, it would be prudent to bear in mind that expression of the Brh2 alleles in the experimental situation described is out of the ordinary context.

If the intermolecular complementation was truly a result of some cooperation between the two different regions interacting with Rad51, then it would be important to determine

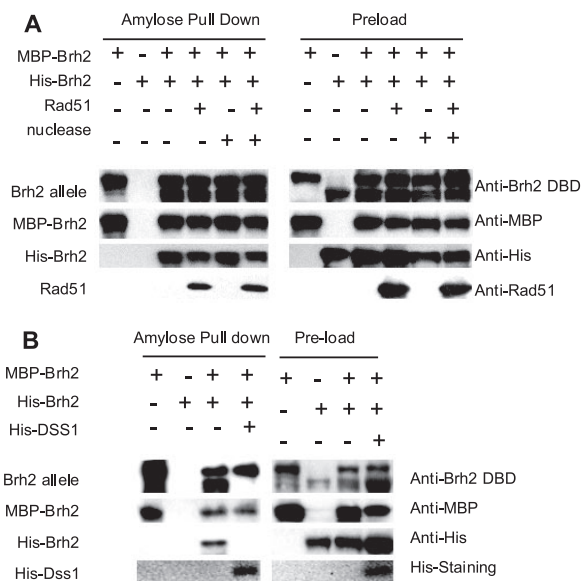


FIG. 2. Determinants of Brh2 association. (A) MBP- and His-Brh2 fusions were coexpressed in the presence or absence of Rad51, and pull-down assays were performed with amylose beads used to capture MBP-Brh2 and associated proteins. The allele product representing completely functional Brh2 started at residue 106 as discussed in Materials and Methods. (B) Aliquots of cell extracts containing 1 mM MgCl₂ were pretreated by incubation with 2 μg per ml DNase I and RNase A at 4°C for 12 h before pull-down assays were performed.

whether the complementation was a result of a direct interaction between N- and C-terminal regions or whether it was an indirect interplay mediated by Rad51. To investigate this issue, we turned to the pull-down procedure to ask simply whether Brh2-Brh2 interaction was dependent or independent of Rad51. As we had previously determined that the removal of up to 213 amino acid residues or the addition of various affinity tags to the N terminus of Brh2 did not diminish the ability to complement the UV sensitivity of the *brh2* null mutant (16), we tested whether MBP-Brh2 could associate with His-tagged Brh2 in the pull-down assay. Therefore, we coexpressed MBP-Brh2 and His-tagged Brh2 in the presence or absence of Rad51, selectively trapped the MBP-Brh2 on amylose beads, and assayed for associated His-Brh2 by Western blotting. Approximately equal levels of MBP-Brh2 and His-Brh2 were coexpressed, and approximately equal amounts were present in the amylose pull-down procedures (Fig. 2A). Capture of MBP-Brh2 by the amylose resin was highly efficient under the conditions utilized, and we estimate that >90% of the input MBP-Brh2 was pulled down. The relative proportions of MBP-Brh2 and His-Brh2 were unchanged in the presence or the absence of Rad51, ruling out Rad51 as a bridge for interaction between Brh2 molecules. A second possibility, that the association was mediated by cobinding to nucleic acids, was deemed unlikely, as there was no change evident upon removal of nucleic acids by DNase and RNase digestion (Fig. 2A). Cellular nucleic acids evident by staining samples run on agarose gels with ethidium bromide disappeared after nuclease treatment, indicating their removal (data not shown). As expected, Rad51 itself was also pulled down by MBP-Brh2. We estimated that, on a molar basis, there was about 5 to 10 times more Rad51

than Brh2 in the extract used in this experiment, based on the relative intensity of the bands as measured by Coomassie blue staining after SDS gel electrophoresis and that no more than 5 to 10% of the total Rad51 present in the preload was present in the pull-down procedure (data not shown). These results provide evidence that Brh2 can associate to form higher-order complexes that must be at least dimer-sized and are consistent with the notion that the intermolecular complementation by the N- and C-terminal regions is a result of physical interaction between those polypeptides. Evidently, Rad51 is not required for Brh2-Brh2 partnering.

To map the Brh2-Brh2 interaction region, we employed a series of fragments spanning the entire length of the protein to construct MBP fusions (Fig. 3A). These were then tested individually in pull-down assays to test which could be captured by the full-length His-Brh2 (Fig. 3B). The Brh2 polypeptides corresponding to the proximal region up to residue 551 and to the distal region beyond residue 838 of the protein were not effective in interacting with His-Brh2. However, polypeptides spanning the medial region from residue 551 to 867 were capable of interacting with His-Brh2. The results from this mapping procedure localized the Brh2-Brh2 interaction region to a stretch of residues encompassing those deduced from sequence alignment with BRCA2 to interact with Dss1.

Curious as to whether Dss1 might actually play some role in the Brh2-Brh2 interaction, we examined its effect in the pull-down assay. For this experiment, we utilized His-tagged Dss1, relying on the tag as a means of monitoring protein expression. The His tag had no more than a slight effect on Dss1's function in DNA repair, as measured by the ability to complement the UV sensitivity of the *dss1* mutant (data not shown). When His-Dss1 was coexpressed with both MBP-Brh2 and His-Brh2, it was captured in the amylose pull-down assay, indicating that it formed a complex with Brh2 as expected. However, little His-Brh2 was evident in the amylose pull-down assay, indicating that it was no longer associated with the MBP-Brh2 (Fig. 2B). These results provide evidence that Dss1 abrogates Brh2's capacity for self-interaction, supporting the notion that this function has a crucial biological significance in Brh2-Dss1 interplay enabling recombinational repair in *U. maydis* cells.

Biochemical evidence for Brh2 intermolecular interaction. As an approach to obtaining physical evidence that Dss1 controls Brh2 self-interaction, we experimented with purified proteins, hoping to find a way to reconstitute a Dss1-responsive system from the individual components. Unfortunately, in the absence of Dss1, Brh2 is prone to aggregation, and we were not able to find conditions under which it would readily take up Dss1 to form a complex. To mitigate the aggregation problem, we utilized an N-terminal truncation allele product, Brh2⁵⁰⁵⁻¹⁰⁷⁵, that had the BRC domain deleted but still retained the Dss1- and self-interaction domains. Although aggregation was still a problem with this allele product when overexpressed in *E. coli*, we discovered conditions in which the reverse of the reconstitution reaction could be observed, i.e., the dissociation reaction. With the Brh2/Dss1 complex as starting material, we noticed that dimeric and higher-order complexes could be generated by judicious adjustment of the salt conditions. Concomitantly, Dss1 dissociated from dimer-sized and higher-order forms and remained bound only with the monomeric form.

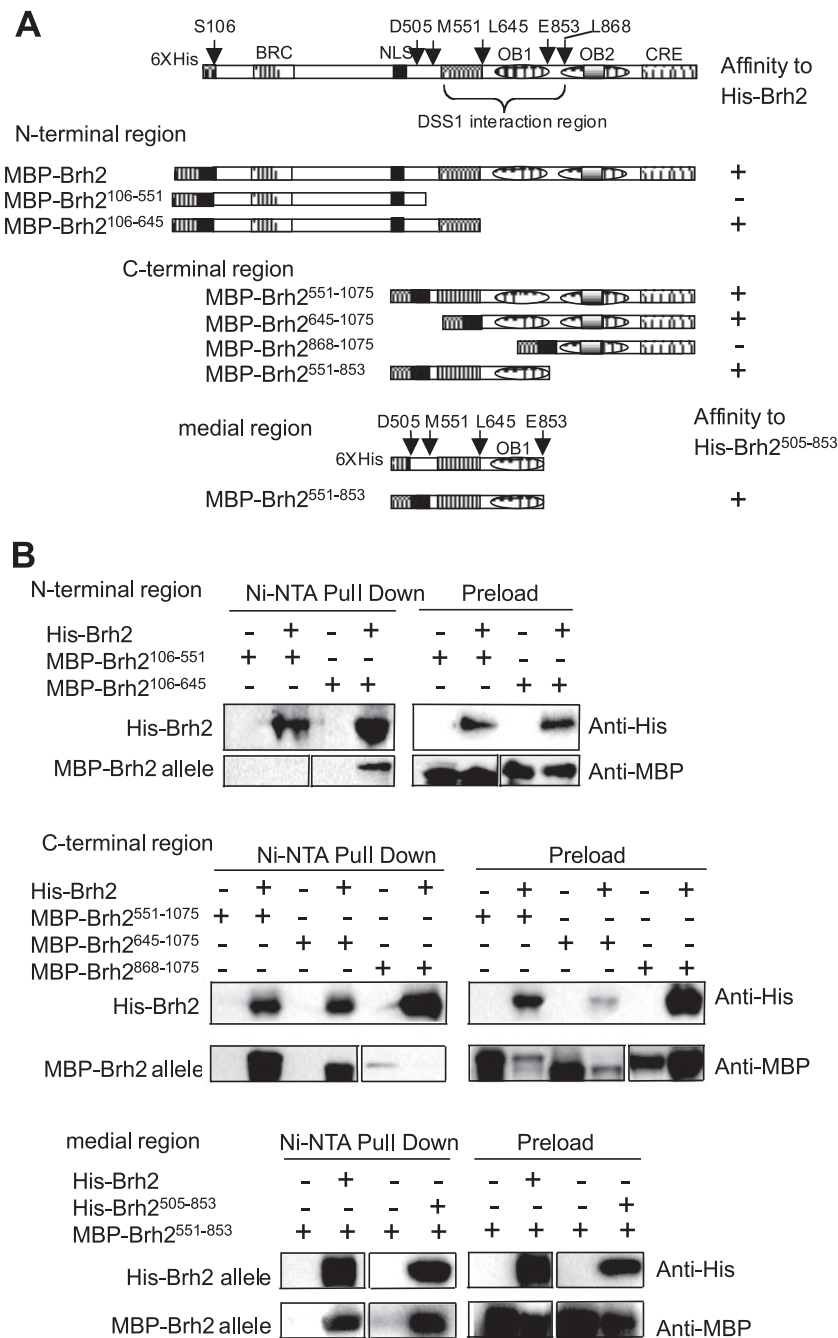


FIG. 3. Pull-down mapping of Brh2 interaction domains. (A) Schematic illustration of MBP-tagged Brh2 fragments used in pull-down assays with His-tagged Brh2 or an internal fragment. A summary of the pull-down results below is presented schematically on the right with pluses and minuses to indicate the relative strengths of association (affinities). The data for interaction of the full-length MBP-Brh2 with His-Brh2 are presented in Fig. 2B. (B) Pull-down assays were performed with Ni-NTA resin to capture the His-tagged bait protein, either His-Brh2 (Brh2¹⁰⁶⁻¹⁰⁷⁵) or His-Brh2⁵⁰⁵⁻⁸⁵³. After the resin processing and elution with 200 mM imidazole, Western blotting was performed using anti-His monoclonal antibody and anti-MBP antiserum. Since the MBP-Brh2 allele products being tested were of different lengths, their mobilities during SDS gel electrophoresis differed. To keep the figure compact and to allow for easy comparison of the levels of protein, lanes with different allele products were spliced from the same autoradiographic film as indicated by the boxes, and the boxes with the bands representing the Brh2 allele products were aligned (indicated as "MBP-Brh2 allele"). It is to be emphasized that the sizes of the Brh2 allele products are different from box to box, even though the bands are aligned as indicated. The sizes of the polypeptides are indicated by the amino acid residues of the MBP-tagged Brh2 allele product. The mapping results presented for the individual N-terminal, C-terminal, or medial regions came from the same autoradiographs under the same exposure. Background intensity varied somewhat due to the position of the bands relative to each other in the autoradiographs.

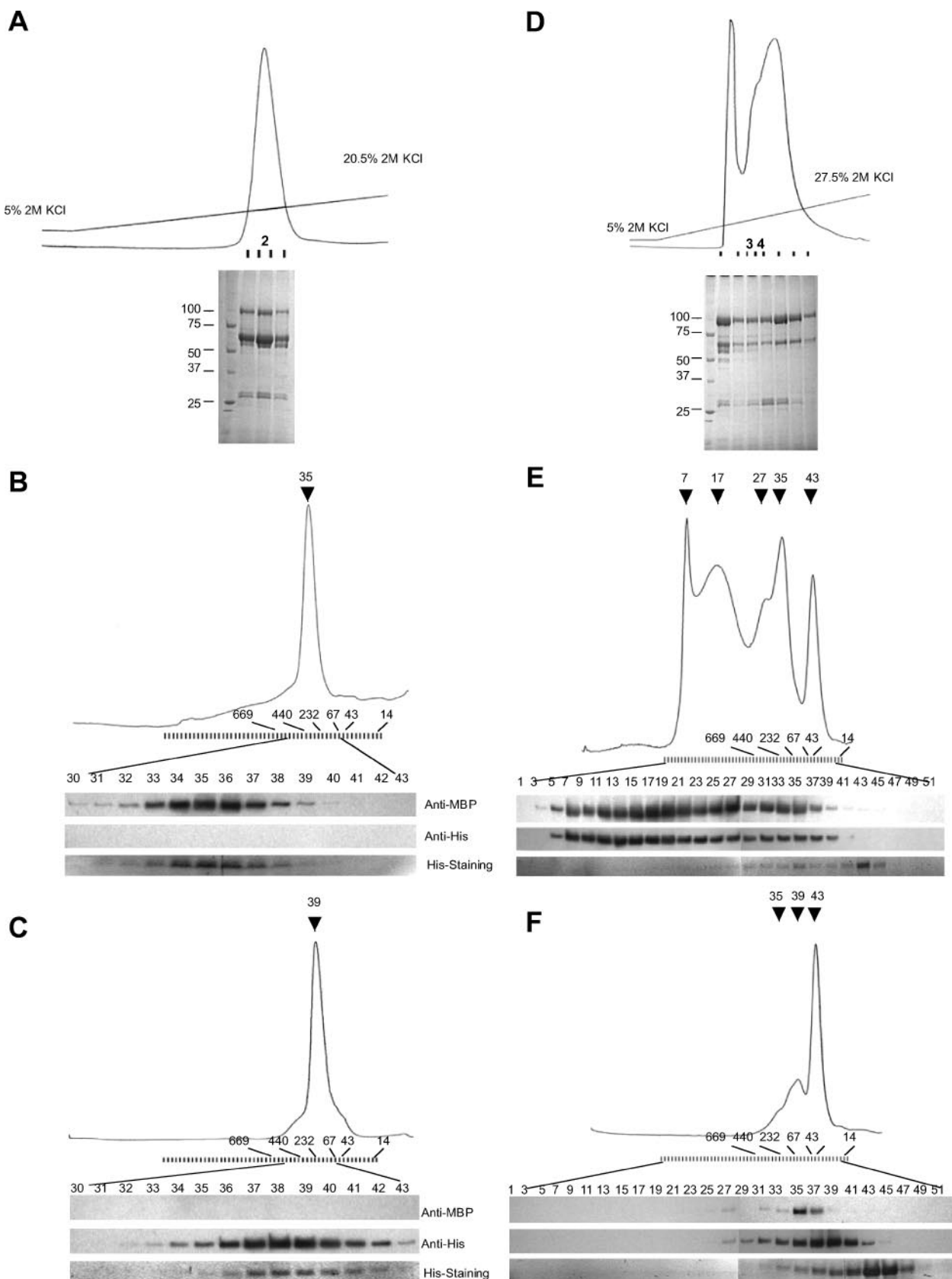


FIG. 4. Gel filtration analysis of Brh2. Complexes of MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵, His-Brh2⁵⁰⁵⁻¹⁰⁷⁵ and His-Dss1 were affinity purified on Ni²⁺-NTA resin. (A) Complexes eluted from Ni²⁺-NTA with imidazole buffer containing 0.2 M KCl were chromatographed on Mono Q beads with the KCl gradient

His- and MBP-tagged versions of the Brh2 allele product were coexpressed with His-Dss1 and affinity purified on immobilized Ni²⁺ resin. In 0.2 M KCl, the Brh2/Dss1 complexes behaved uniformly during chromatography on Mono Q beads and coeluted (Fig. 4A). The peak fractions were then pooled and applied to amylose resin. The bound fraction was stripped off by washing with maltose and was examined by molecular sieve chromatography. A single symmetrical peak containing MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵ and Dss1 but no detectable His-Brh2⁵⁰⁵⁻¹⁰⁷⁵ eluted (fraction 35; Fig. 4B). As the protein complex in this peak included no His-Brh2⁵⁰⁵⁻¹⁰⁷⁵, we conclude this is the monomeric form of Brh2. As noted in previous studies the Brh2¹⁻¹⁰⁷⁵/Dss1 complex runs anomalously during gel filtration due presumably to its nonglobular conformation (supplemental figure S1 in reference 14) and appears much larger than expected. Based on the elution volume noted in the present study, the MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵/Dss1 complex runs with an apparent size of about 260 kDa in contrast to the calculated mass of approximately 122 kDa. Similarly, the fraction not binding to amylose contained His-Brh2⁵⁰⁵⁻¹⁰⁷⁵ and Dss1 (fraction 39; Fig. 4C) and chromatographed in a symmetrical peak corresponding to monomer size but was distinguishable from the MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵/Dss1 complex.

The findings reported above show that in the presence of 0.2 M KCl and Dss1, MBP-tagged and His-tagged Brh2 proteins, which differ slightly in mass, form monomer-sized complexes with Dss1. However, when the tagged Brh2 proteins were affinity purified from the Ni²⁺ resin in 0.5 M NaCl, the results were quite different. Upon chromatography on Mono Q beads, there was no longer elution in a symmetrical peak but rather evidence for fractionation of multiple molecular species (Fig. 4D). After the application of pooled fractions to amylose resin, the bound fraction was found to contain only a small amount of MBP- and His-tagged Brh2⁵⁰⁵⁻¹⁰⁷⁵ that coeluted with associated Dss1, but for the most part, both MBP- and His-tagged Brh2⁵⁰⁵⁻¹⁰⁷⁵ proteins coeluted with little to no associated Dss1 at elution volumes corresponding to dimer-sized and higher-order forms (fractions 27 and below; Fig. 4E). A minor amount of protein that was not bound by the amylose resin contained separate peaks of apparently monomer-sized MBP- and His-tagged Brh2⁵⁰⁵⁻¹⁰⁷⁵ versions with associated Dss1 plus free Dss1 (fraction 43; Fig. 4F). These results suggest that heterotypic monomer-sized Brh2/Dss1 complexes can be dissociated in 0.5 M NaCl, liberating the Dss1, and in so doing, the Brh2

reassociates, forming homotypic complexes the size of dimers size and of higher-order forms.

We performed additional experimentation by independent methods in efforts to obtain supporting evidence for protein interaction. In our view, the best evidence would come from pull-down procedures with *U. maydis* extracts, but we have not succeeded in developing that methodology yet. We have been unsuccessful in finding another suitable affinity tag for Brh2 that could be used in combination with the 6myc-tagged Brh2 due to either poor expression of the tagged protein or else loss of its biological activity resulting from the affinity tag modification.

We turned to using the yeast two-hybrid system by measuring β -galactosidase activity-induced expression of the *lacZ* reporter gene, but this was complicated by an inherent ability of Brh2 to activate transcription when fused to either the Gal4 activation domain (GAD) or the Gal4 DNA-binding domain (GBD). In a survey of various Brh2 fragments, we found that the medial region (residues 505 to 853), which was capable of self-interaction, as noted in the legend to Fig. 3A, strongly activated *lacZ* transcription when fused to GAD (51 units, compared to the value of the positive control with T-antigen and p53 Gal4 fusions, 67 units) but had no effect when fused to GBD (<2 units after subtracting the small amount of activity present in extracts with no vector). However, when these GAD/GBD-Brh2⁵⁰⁵⁻⁸⁵³ fusions were coexpressed, transcriptional activation was reduced to 32 units. We suggest that this repression could be an indication, although an unconventional one, of protein interaction. Such repression of transcriptional activation has been noted by others, for example, with the human BRCA2-interacting protein EMSY (8), and interpreted as evidence for interaction. We considered the possibility that repression is even stronger in the absence of Sem1, the yeast Dss1 ortholog, but found that expression of Brh2 or the Brh2 DBD was toxic to the *sem1* mutant.

In a final method to detect interaction, we tried chemically cross-linking Brh2 with the bifunctional succinimide ester BS³. In this case, we used MBP-Brh2¹⁰⁶⁻¹⁰⁷⁵ in complex with His-Dss1 that was purified as described for the truncated version MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵ used in the analysis described above. For controls in this experiment, we used ovalbumin, which should not self-associate, as the negative control and His-tagged Rad51, which is known to assemble into higher-order forms through homotypic interaction and would have the additional

as shown. Fractions spanning the peak were collected as shown by the corresponding hatch marks underneath the trace and examined by SDS gel electrophoresis. Relative molecular masses of MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵, His-Brh2⁵⁰⁵⁻¹⁰⁷⁵ and His-Dss1 were approximately 100, 60, and 27 kDa, respectively. (B) Fraction 2 from the Mono Q peak was applied to a column of cross-linked amylose. The bound fraction was eluted with 10 mM maltose and then applied to a Superose 6 column in 0.2 M KCl, calibrated with the size markers thyroglobulin (660 kDa), ferritin (440 kDa), catalase (232 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), and RNase A (14 kDa). Fractions were collected as shown by the corresponding hatch marks underneath the trace, and molecular species were identified by Western blot analysis probing with anti-MBP antiserum and anti-His monoclonal antibody and by directly visualizing His-Dss1 by soaking the gels with InVision stain. (C) The flowthrough from the amylose column was applied to Superose 6 as described above. Fractions were analyzed by Western blotting to detect MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵, His-Brh2⁵⁰⁵⁻¹⁰⁷⁵, and His-Dss1. (D) Complexes eluted from Ni²⁺-NTA with imidazole containing 0.5 M NaCl were chromatographed on Mono Q beads as described above. Fractions spanning the heterogeneous mixture were collected as shown by the corresponding hatch marks and aliquots examined by SDS gel electrophoresis. Fractions 3 and 4 were pooled and applied to amylose resin. The fraction that bound to amylose (E) and the flowthrough (F) were analyzed by gel filtration as described above. MBP-Brh2⁵⁰⁵⁻¹⁰⁷⁵, His-Brh2⁵⁰⁵⁻¹⁰⁷⁵, and His-Dss1 peaked at fractions 35, 39, and 43, respectively. Fraction 27 corresponded to an approximately dimer-sized complex, while fraction 17 was larger than a tetramer. Fraction 7 corresponded to the excluded fraction.

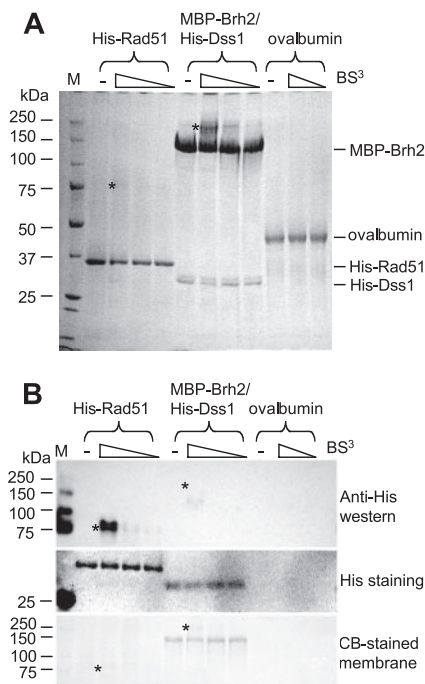


FIG. 5. Chemical cross-linking of Brh2. (A) His-Rad51, MBP-Brh2/His-Dss1, and ovalbumin at 2.5 μ M were held in a reaction mixture containing BS³ as described in Materials and Methods. For His-Rad51 and MBP-Brh2/His-Dss1, reaction mixtures contained no reagent or contained BS³ in decreasing concentrations of 50 μ M, 10 μ M, and 2 μ M, respectively. For ovalbumin, the reaction mixture contained 50 μ M and 10 μ M BS³ only. After the reactions, proteins were separated on an SDS gel with a 5 to 16% gradient of acrylamide. The asterisks indicate bands with the mobility of the expected dimeric form. (B) A parallel gel was cut into two at the position of the 50-kDa marker. The top half was transferred to a PVDF membrane and Western blotted using anti-His monoclonal antibody (top panel). After blotting, the membrane was dipped into Coomassie blue (CB) staining solution for 1 s and then immediately flushed with water and acetic acid/methanol destaining solution to visualize the electrophoretically transferred proteins. The bottom half of the gel was soaked in Invision stain to visualize the His-tagged proteins. The asterisks indicate bands with the mobility of the expected dimeric form.

feature of the His tag as a means for detection in Western blots, as the positive control. Our rationale was that if Brh2 exists in a state of equilibrium between monomeric and dimeric to higher forms, then in cross-linking reactions over an extended reaction period of time, it might be possible to trap the dimeric form of Brh2.

A band with mobility that appeared to be consistent with a dimer form became evident in reactions with 2.5 μ M MBP-Brh2¹⁰⁶⁻¹⁰⁷⁵/His-Dss1 protein when the BS³ level was titrated up to 50 μ M as determined from a Coomassie blue-stained SDS gel (Fig. 5A). At concentrations of 100 μ M and higher, most of the protein ran near the top or else failed to enter the gel altogether (not shown). In reactions with His-Rad51, a protein species with a mobility consistent with that of a dimer-sized protein also became apparent when the reagent concentration was 50 μ M. No dimer formation was apparent in comparable reactions with ovalbumin. These results suggest that Brh2 has an inherent capacity for self-interaction. The gel was cut in two at the position of the 50-kDa marker for further

verification (Fig. 5B). The fast-running His-tagged Rad51 and Dss1 proteins were visualized by staining the bottom half with His tag-specific gel stain. The top half was analyzed by Western blotting using monoclonal antibody to the His tag after transfer to a PVDF membrane. The presence of dimeric Rad51 protein was confirmed. It was interesting that while the stained gel in Fig. 5A indicated that relatively more Brh2 protein was cross-linked than Rad51, there was very little His signal at the position of the dimeric Brh2. However, signal was evident at the approximate mobility of the monomeric MBP-Brh2, suggesting that the His-Dss1 had become cross-linked to the Brh2. That protein was transferred to the PVDF membrane was confirmed by staining with Coomassie blue. The lack of His signal evident at the position of the presumed dimer Brh2 form is consistent with the notion that Dss1 is displaced from Brh2 as Brh2 associates with itself to form a dimeric structure.

Requirements for BRC and CRE communication in trans. With experimental support for two different Rad51-binding domains and evidence that the region of Brh2 that interacts with Dss1 might be critical for intermolecular interaction, we asked whether expressing a pair of mutant Brh2 allele products, each being completely intact across the Dss1-interacting region but one inactivated by deletion of the BRC domain (Brh2³¹⁶⁻¹⁰⁷⁵) and the other by partial deletion of the CRE domain (Brh2¹⁻¹⁰³⁴), could enable DNA repair (Fig. 6A). Expressed separately in the *brh2* mutant, each exhibited greatly reduced complementation activity, as evidenced by the extreme sensitivity to UV irradiation, but when the two were expressed together, there was strong recovery in radiation resistance (Fig. 6B), reinforcing the notion of cooperation or communication between the BRC and CRE domains. When the BRC domain in Brh2¹⁻¹⁰³⁴ was inactivated by point mutations (Brh2^{F294A T296A 1-1034}), complementation with Brh2³¹⁶⁻¹⁰⁷⁵ was lost (Fig. 7B).

That the activity was due to complementation and not recombination between the Brh2 alleles was established by selectively curing the cells with one allele or the other and then demonstrating that complementation activity dropped back to the same low level as in the strain expressing the corresponding original parental allele. As the alleles were expressed ectopically from self-replicating plasmids, the cells were easily cured simply by the omission of drug selection for one plasmid or the other and then propagating the cells for several generations until the unselected plasmid was lost. The experiment was performed in two different formats. In the first, plasmid loss and corresponding complementation activity were monitored in clonal isolates. *brh2* mutant cells expressing Brh2^{ΔBRC} from a plasmid with a Hyg^R marker and Brh2^{ΔCRE} from a plasmid with a Cbx^R marker were propagated for a number of generations in medium with drug selection maintained for either hygromycin or carboxin only. In the absence of selection, about three-fourths of the cells in either case had lost the unselected plasmid at the time of sampling (Fig. 6C). This finding argues that, for the most part, the plasmids present in the cells of the original cotransformant were autonomously maintained and were not recombinants. When two individual cured isolates in each case (Hyg^R Cbx^S, corresponding to the presence of Brh2^{ΔBRC} only, or Hyg^S Cbx^R, corresponding to the presence of Brh2^{ΔCRE} only) chosen at random were tested for UV resistance, the complementing activity had largely disappeared. How-

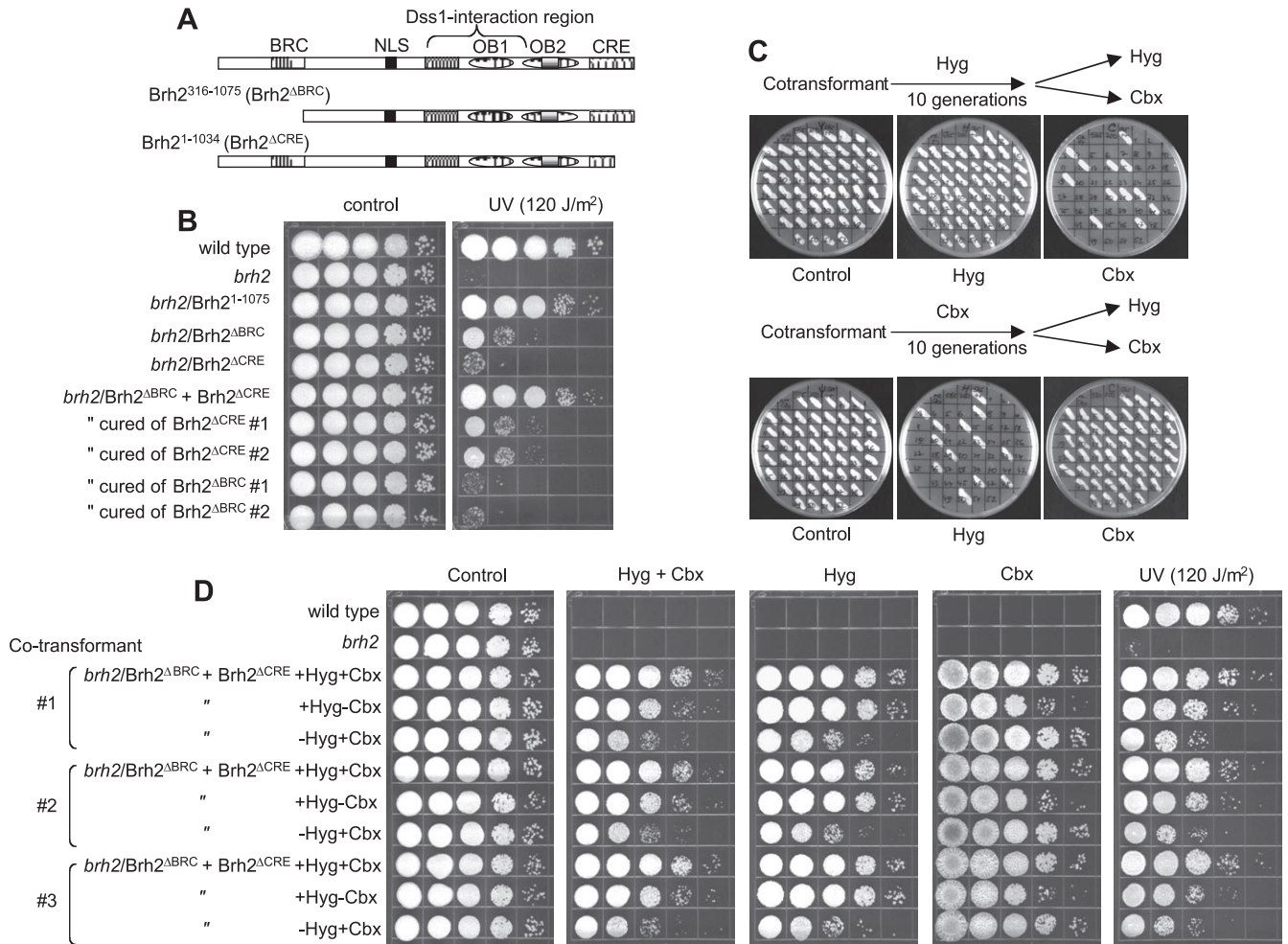


FIG. 6. BRC and CRE communication in *trans* depends upon the maintenance of plasmids expressing each. (A) Schematic illustration of Brh2 allele products with deletions of the BRC or CRE region. (B) Survival of *brh2* mutant cells expressing the indicated Brh2 allele products after irradiation with 120 J/m² UV light is shown. Brh2³¹⁶⁻¹⁰⁷⁵ (Brh2^{ΔBRC}) was expressed from a plasmid with a Hyg^R marker, while the Brh2¹⁻¹⁰³⁴ (Brh2^{ΔCRE}) plasmid carried a Cbx^R marker. Two independent clones of cells cured of one plasmid or the other were tested. (C) For selective loss of Brh2^{ΔBRC} or Brh2^{ΔCRE} measured in individual clones, cells cotransformed with both plasmids were cured of one plasmid or the other by propagation in liquid medium containing either hygromycin or carboxin. After being streaked for isolation on solid medium, 52 single colonies were picked and tested for loss of the unselected marker by sequentially patching onto plates with neither drug or one of the drugs. Across the top of each plate from left to right were three controls, *brh2*, wild type, and *brh2* cotransformant, and then the 52 individuals. Two independent clones that retained one of the parental plasmids but that was cured of the other plasmid were then tested (see panel B) for survival after UV treatment. (D) Population-level loss of plasmid expressing Brh2^{ΔBRC} or Brh2^{ΔCRE}. Three independent cotransformants expressing both Brh2^{ΔBRC} (Hyg^R) and Brh2^{ΔCRE} (Cbx^R) were cultured in liquid medium containing both drugs or one of the two drugs. After growth for 20 generations, aliquots were removed and diluted in a 10-fold serial dilutions, and then samples were plated on solid medium containing the indicated drug or else were irradiated with UV (120 J/m²).

ever, the low levels of activity remaining reverted to that exhibited by expression of those single alleles individually in *brh2*. That is to say the incremental level of activity evident in *brh2* expressing Brh2^{ΔBRC} was similar to that in the isolates obtained from *brh2* cells cotransformed with Brh2^{ΔBRC} and Brh2^{ΔCRE} and then cured of Brh2^{ΔCRE} and vice versa.

In the second format, we monitored plasmid loss and the corresponding loss of UV resistance on a population-wide level. Here, three individual cotransformants expressing Brh2^{ΔBRC} and Brh2^{ΔCRE} as described above were cultured for 20 generations in medium with drug selection for each individual plasmid. Aliquots were removed and then spotted in serial dilutions on plates with hygromycin, carboxin, or both

and also tested for UV resistance. As is evident for each of the three original isolates, there was a close correlation between loss of the unselected marker and loss in UV complementation activity (Fig. 6D) as opposed to the high level of activity evident when selection for both plasmids was maintained. There is a pronounced disproportionate loss of the unselected plasmid depending on which Brh2 allele is maintained. When there is selection for the plasmid expressing Brh2^{ΔBRC}, the plasmid expressing Brh2^{ΔCRE} is lost more slowly than in the opposite situation. This suggests that there is some advantage for the cell to maintain the Brh2 allele product with an intact BRC domain, even though this protein by itself has no UV complementation activity (Fig. 6B). These results also confirm the

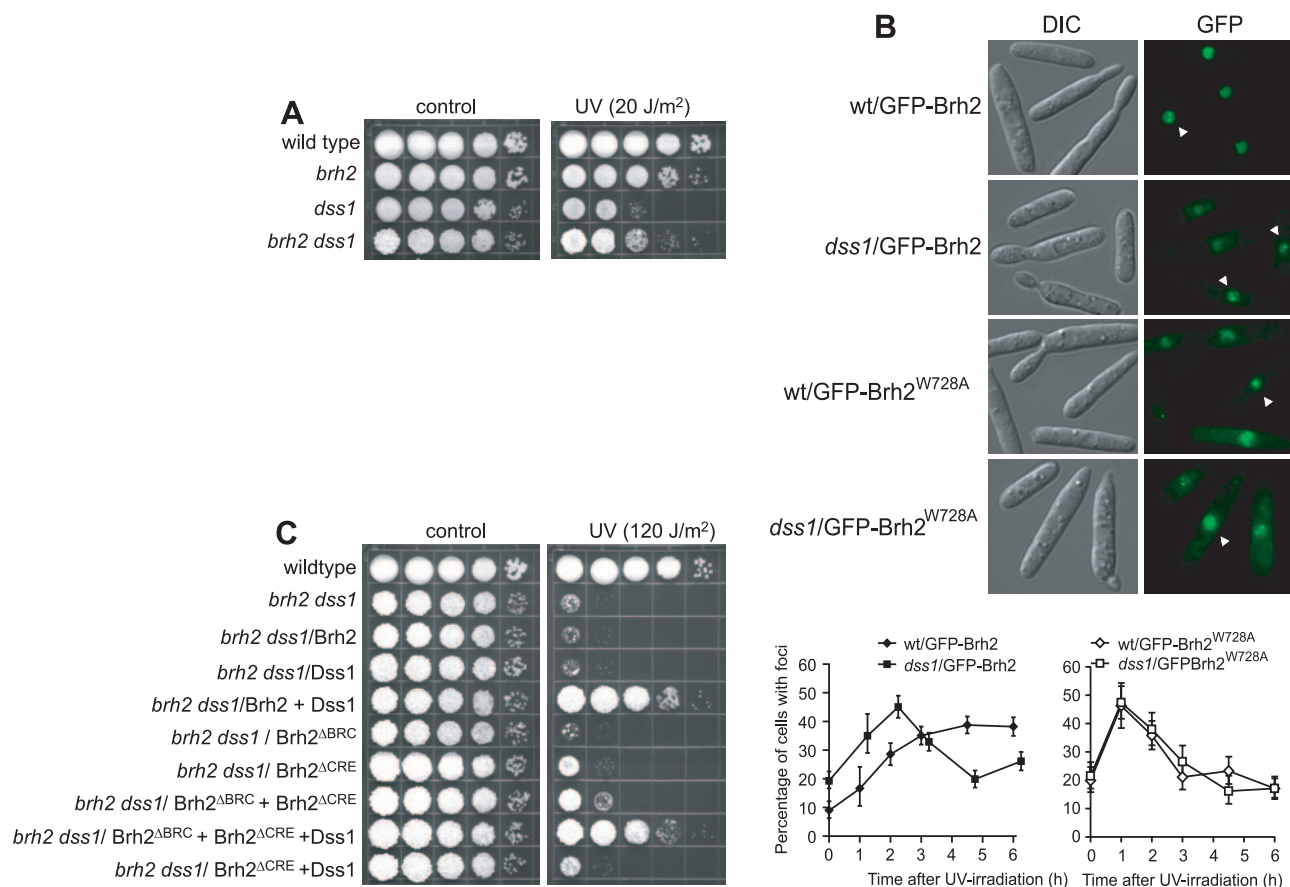


FIG. 7. BRC and CRE communication mediated by Dss1. (A) Survival of *brh2* and *dss1* single- or double-mutant cells at a representative UV dose of 20 J/m². (B, top panel) GFP-Brh2 focus formation 2 h after irradiation with UV (30 J/m²). Cells with representative foci (see arrows) are shown. (B, bottom panel) Following irradiation, aliquots were removed at the indicated times and the percentage of cells with foci was determined after counting approximately 200 cells for each point. wt, wild type. (C) Survival after irradiation with 120 J/m² UV light of *brh2 dss1* double-mutant cells expressing the indicated BRC- and/or CRE-defective Brh2 allele products (Brh2⁵¹⁶⁻¹⁰⁷⁵ and Brh2¹⁻¹⁰³⁴, respectively, as in Fig. 6) with and without Dss1.

notion that in the population on the whole, the UV-complementing activity is a consequence of the presence of two autonomously maintained plasmids, not recombinants. These findings are consistent with the notion that the complementation activity results from an *in trans* interaction involving the BRC and CRE domains. On the other hand, it could be argued that the approach taken in both of these formats is in a sense indirect and that the ideal experiment would be to demonstrate that restored complementation depends upon physical interaction of the two different Brh2 alleles by *in vivo* pull-down procedures. However, as mentioned above, we have not succeeded in developing the methodology for this approach as yet.

The notion of interaction between the two defective Brh2 alleles representing a bona fide manifestation of the natural Brh2 molecular dynamics requires demonstration of the normal mode of regulation. Based on our findings from the pull-down analysis showing that Dss1 governs interaction between Brh2 molecules, Dss1 should also serve as a touchstone for verifying authenticity in genetic control. That is, intermolecular complementation should be subject to regulation by Dss1 such that in its absence, complementation should fail. The determination was performed by expressing the BRC-defective

and CRE-defective Brh2 alleles in a *brh2 dss1* double mutant. This strain was created by disrupting the *DSS1* gene in *brh2-1*, an allele caused by a frameshift mutation that is phenotypically identical to the deletion mutant in radiation sensitivity (12). In previous studies, we found the *dss1* deletion mutant to be more sensitive to killing by UV than the *brh2* null mutant (14). This heightened sensitivity is evident at a low dose of UV (Fig. 7A). The *brh2 dss1* double mutant is not any more sensitive to UV than the *dss1* single mutant, thus arguing in favor of an epistatic relationship between these two genes, as would be expected given that their protein products interact physically to form a complex. However, notably, the double mutant is somewhat suppressed in sensitivity to UV and thus is more similar to the *brh2* single mutant in phenotype. Thus, in the absence of Dss1, expression of Brh2 appears to confer a slightly toxicity, possibly because in that state it is unable to perform normally and so interferes with normal cellular functions. One could easily imagine that with Brh2 in such a form, selective pressure might be mounted in the cell for its removal.

To examine the dynamics of Brh2 in cells lacking Dss1, we monitored green fluorescent protein (GFP)-Brh2 focus formation in living cells after DNA damage. Previously, we estab-

lished that GFP-Brh2 is completely active in complementing the UV sensitivity of the *brh2* mutant (16). The GFP-Brh2 signal in the *dss1* mutant was more diffuse than and not as intense as the wild-type signal (Fig. 7B). However, following UV irradiation of the *dss1* mutant, cells containing GFP-Brh2 foci accumulated to about the same level as those in the case of the wild type, although for the *dss1* mutant, the rates of accumulation and depletion were faster. From these observations, we conclude that Brh2 is produced, imported into the nucleus, and responds appropriately to DNA damage relocating into foci in the absence of Dss1.

In the *brh2 dss1* double mutant, expression of Brh2 or Dss1 separately did not have the ability to complement the UV sensitivity of this mutant, but the expression of both together completely restored the DNA repair capacity (Fig. 7C), reconfirming the authenticity of this strain as a *brh2 dss1* double mutant. But unlike the case with the *brh2* single mutant, there was no improvement in survival for the double mutant upon coexpression of the same BRC-defective and CRE-defective Brh2 alleles as described above, either separately or together. However, when Dss1 was supplied by reintroduction of the gene together with both the BRC-defective and CRE-defective alleles, the intermolecular complementing activity was restored (Fig. 7C), verifying that efficient cooperation between Brh2 molecules is absolutely dependent upon and governed by Dss1. These results are in line with the earlier findings demonstrating an essential role for Dss1 as a positive activator for Brh2 (14). In summary, the results show that critical Brh2 function can be achieved by contribution of BRC and CRE elements present on different molecules through an intermolecular interaction that is mediated by Dss1.

Brh2 point mutation diminishes interaction with Dss1. The experiments described above establish a crucial role for Dss1 in enabling cooperation between BRC and CRE. However, the notion that the key role of Dss1 is achieved through action as a switch causing dissociation of Brh2 complexes, while an intriguing possibility, is less firmly grounded. Unfortunately, gaining more decisive support for this model has been difficult. Pull-down analysis of affinity-tagged proteins directly from *U. maydis* cell extracts has been unsatisfactory due to a variety of problems, including weak signal, protein instability, failure to complement, and interference from cross-reactive substances. Similarly disappointing have been biochemical efforts. In vitro reconstitution of biochemically active Brh2/Dss1 complexes from the purified constituent components has not yet been achieved. Therefore, we turned to genetics as an approach to the problem. Our strategy was to identify a Brh2 mutant unable to switch in response to the action of Dss1.

A Brh2^{W728A} mutant allele product severely defective in survival after DNA damage (Fig. 8A) was identified by an alanine-scanning mutagenesis survey performed at selected sites across the Dss1-interacting region of Brh2. This residue is highly conserved and, according to sequence alignment, corresponds to human residue W2725. Based on the BRCA2 DBD crystal structure (31), W2725 contacts Dss1 by interaction through the carbonyl group of D17 and by hydrophobic interaction with F19. When an MBP-tagged version of Brh2^{W728A} was tested for interaction with His-Brh2^{W728A} by the pull-down assay, it was found to associate just as it did in the case of the wild-type allele product (Fig. 8B). Thus, this single point

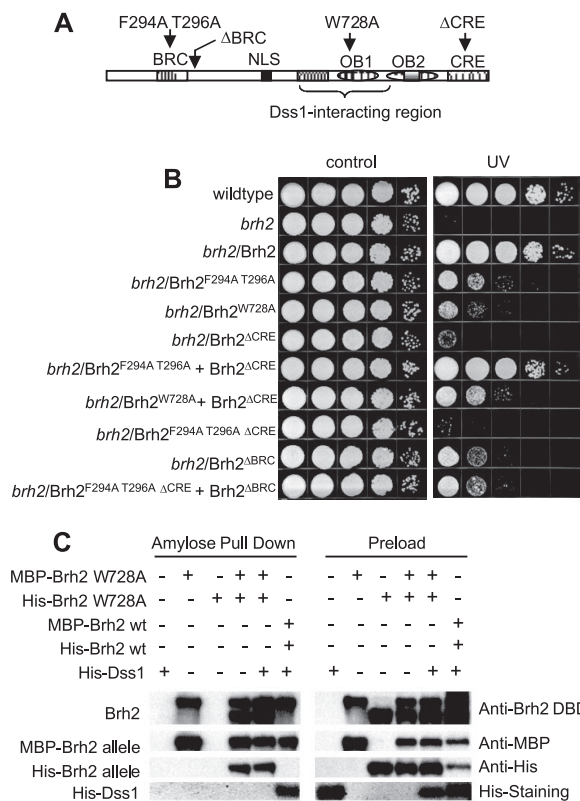


FIG. 8. Brh2 point mutant unresponsive to Dss1. (A) Schematic illustration of Brh2 point mutants and truncations. (B) Survival of *brh2* mutant cells expressing the indicated alleles after irradiation with 120 J/m² UV light. (C) MBP- and His-Brh2 fusions of Brh2^{W728A} and Brh2¹⁰⁶⁻¹⁰⁷⁵ (wt) were coexpressed in *E. coli* in the presence or absence of His-Dss1, and pull-down assays were performed with amylose beads. Western analysis was performed as described for Fig. 2.

mutation has no effect on Brh2's ability to self-associate. However, unlike the case with wild-type Brh2, this allele product was unresponsive to Dss1. There was no dissociation of the Brh2^{W728A} complex induced by Dss1, as evidenced by the unaltered ability of MBP-tagged Brh2^{W728A} to pull down His-Brh2^{W728A} when His-Dss1 was expressed. Furthermore, the ability of Dss1 to interact with Brh2^{W728A} was abolished. This was apparent by the lack of association of His-Dss1 with MBP-tagged Brh2^{W728A} in the pull-down assay compared with the wild-type Brh2 allele product. GFP-Brh2^{W728A} relocated to nuclear foci in response to UV-induced damage suggesting that the protein could be recruited to DNA. However, the kinetics resembled that of the GFP-tagged wild-type protein in the absence of Dss1 (Fig. 7B). When Brh2^{W728A} was coexpressed with the allele product with the CRE deletion in the presence of Dss1, there was little intermolecular complementation evident, compared with the high level achieved by the CRE deletion and BRC point mutation allele products (Fig. 8B). Thus, the Brh2^{W728A} allele product appears to be locked in an impervious state that cannot be recognized by Dss1 and cannot respond to it. These results imply that the association of Dss1 with Brh2 and the concomitant dissociation of higher-order Brh2 complexes constitute linked and interdependent steps in Brh2's pathway of action, again supporting the idea

that a dynamic physical interplay mediated through the Brh2 interaction domain is crucial for the intermolecular complementation activity.

DISCUSSION

Much headway had been made in understanding the roles of the principal components in the BRCA2-dependent recombinational repair pathway, but relatively little is known about the molecular events underlying their interplay. In particular, the mechanism by which Dss1 controls BRCA2 is poorly understood at present. Previously in studies of *U. maydis*, Dss1 was found to be a crucial determinant for Brh2 that enables highly efficient, tightly regulated recombination (15). Here we report findings that illuminate new facets of Brh2 and its molecular interplay with Dss1.

Apart from its proximal Rad51-interacting domain centered on the BRC element, we present evidence here that Brh2 has an unrelated, distal Rad51-interacting domain at its extreme C terminus. Both domains appear essential for Brh2 function, as a mutation in either results in a loss of biological activity. Unlike with the BRC, there is no obvious sequence similarity shared between the latter C-terminal region in Brh2 and mammalian BRCA2's C-terminal Rad51-interacting domain, but the coincidence of their similar locations provokes the idea of a common or analogous function. Recognition of this bimodal domain organization provided the starting point for us to the path for discovering that Brh2 molecules partner through their Dss1-interacting regions to form higher-order complexes at least the size of dimers, that BRC and CRE domains somehow cooperate to promote DNA repair, and that both of these actions are subject to governance by Dss1. A key finding supporting these conclusions was the isolation of the DNA repair-defective Brh2^{W728A} allele product, for which the ability to cooperate with other Brh2 molecules is compromised.

These principal findings furnish an additional layer of reinforcement to the conclusions from earlier work that Dss1 is an essential determinant for Brh2 function. In addition, the results here refine our understanding of the molecular events leading to Brh2 activation and control of Rad51 nucleoprotein filament assembly, while at the same time raising a host of new questions. What molecular events underlie the cooperation between the BRC and CRE domains? How could such a binary domain arrangement prepare Rad51 for service? Might an interdomain handoff of Rad51 involving the BRC and CRE constitute a dynamic intermediary phase in the transfer of Rad51 to DNA? How might the binding of Dss1 lead to activation of Brh2?

In a model that emerges from this work, Dss1 association with the Brh2/Rad51 complex is the key step that triggers the initiation of Rad51 nucleoprotein filament formation. Brh2 is pictured schematically in Fig. 9 with the different Rad51-interacting domains located at either end (BRC and CRE are not specified) and with the self-interacting domain located medially. Brh2 dimerizes by itself through the self-interaction domain. Brh2/Rad51 complexes would form in the absence of Dss1, and in so doing, the bound Rad51 would become properly "certified" or "licensed" through interaction with Brh2's N-terminal and C-terminal domains operating in *trans*. We imagine that Dss1 would promote dissociation of the Brh2/

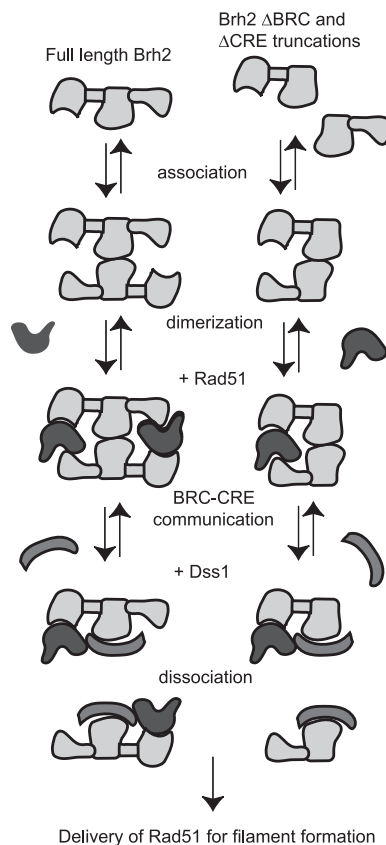


FIG. 9. Dss1 switch model for regulating Brh2. Brh2 shown at the top in light gray has BRC and CRE Rad51-interacting domains located at N and C termini (unspecified). In the middle is the Brh2 self-interacting domain. Brh2 can associate with itself via the medial region and with Rad51 (black helmet shape) through cooperation between BRC and CRE domains in *trans*. Dss1 (dark banana shape) binds to a domain that overlaps with the Brh2 self-interacting region, promoting dissociation of Brh2 and concomitant presentation of Rad51 in a state suitable for initiating filament formation. No information on equilibrium constants or on the disposition of DNA is known.

Rad51 complexes by competing for and interacting with Brh2's self-interacting domain, hence, activating Brh2 and enabling it to deliver the licensed Rad51 for initiating the nucleoprotein filament. Since Brh2 does not require Dss1 for association with Rad51 or for formation of nuclear foci following DNA damage, we imagine that the dimeric Brh2/Rad51 complexes awaiting dissociation by Dss1 as pictured here are already situated at the sites to be repaired.

The model makes no prediction about the number of the Rad51 molecules that must pass through the "hands" of Brh2 for proper nucleoprotein filament initiation or their arrangement within the filament. However, in view of the *in vitro* results demonstrating the ability of Brh2 to recruit Rad51 to a junction of double-stranded and single-stranded DNA facilitating nucleation of the filament, which is then elongated by the pool of free Rad51 (32), it would seem likely that only a small fraction of the total Rad51 population would necessarily be handled by Brh2, but this fraction would constitute a vanguard dedicated to initiating filament formation. The "certification" or "licensing" of those Rad51 molecules by both Brh2's

N-terminal and C-terminal domains is essential for appropriate and precisely regulated recombinational repair.

Conceptualizing Brh2 as a putative antiparallel dimer helps rationalize the BRC-CRE cooperation. Accordingly, two Brh2 molecules with BRC or CRE deleted, respectively, would still be able to couple and then clasp a Rad51 molecule for service in priming filament assembly if their medial domains remain intact (Fig. 7). Based on our gel filtration results showing that a salt-induced switch of Brh2 from a heterotypic monomer formed a complex with Dss1 to a homotypic form consisting of dimer and higher-order forms free of Dss1, we imagine that the reverse reaction comes into play when Rad51 is activated for service. We imagine a dynamic dissociation of Brh2 homotypic complexes driven by Dss1 binding that could be coupled to transfer of Rad51 between the two cooperating Brh2-binding domains and from Brh2 to the nucleoprotein filament. The model illustrates how activation of Brh2 could be impaired not only by the absence of Dss1 but also by mutations in Brh2's Dss1-binding/responding domain (such as Brh2^{W728A}) that abrogate Dss1 association. So far, we have no compelling evidence that can address the disposition of Brh2 with respect to DNA. But, as noted above, Brh2 does relocalize to form subnuclear foci in response to radiation independently of Rad51 and Dss1, suggesting that it can still be recruited to DNA. In this regard, it seems relevant that a human BRCA2 derivative comprised of two BRC repeats fused to the DBD is active in binding DNA in the absence of DSS1 (23). Verification of the model awaits experimentation.

Even though both BRC and CRE bind Rad51 and expression of domains containing BRC and CRE can complement the DNA repair deficiency of the *brh2* mutant, in no way should their functions be construed as somehow equivalent. In light of our previous findings that a fusion of an N-terminal Brh2 fragment containing the BRC with a heterologous DNA-binding domain is able to restore recombinational repair activity to a substantial degree albeit in an uncontrolled manner (15, 16), as well as similar findings on a synthetic fusion of BRC repeats from BRCA2 (22), it would appear that nucleation of the Rad51 filament emanates from the BRC component. The CRE domain in the broader context of the C-terminal region of Brh2 appears to provide some additional regulatory aspect to Rad51 activation such that it is subject to control by Dss1.

We have formulated our model from the standpoint that the specific role of Dss1 is to govern the switch of Brh2 from an inactive form to an active form. While the experimental support for this view is compelling, it is not definitive. An alternative explanation for the role of Dss1 is that it stabilizes Brh2, in essence, establishing its expression by ensuring correct folding of the DBD. It has been shown in the case of mammalian BRCA2 that DBD polypeptides expressed in insect cells in the absence of DSS1 are unstable (31) and that depletion of DSS1 in human cells by RNA interference can result in a loss of BRCA2 by increased degradation (18), although there is disagreement over the latter finding (7). Consequently, it might be argued that in the Brh2 system described here, since polypeptides containing BRC and CRE on separate molecules retain the DBD, they would be prone to degradation in the absence of Dss1. Hence, their cooperation should be dependent on Dss1, as we have documented. However, that does not explain the cooperation itself. Protein stability is one issue, but

the intermolecular complementation is another. It may well be that Dss1 promotes stability of Brh2 molecules, but that does not exclude the additional role of activating Brh2 complexes with Rad51.

Two additional observations bearing on the issue of stability deserve mention. First, it should be emphasized that the DNA repair-defective Brh2^{W728A} allele product has a compromised ability to cooperate with other Brh2 molecules and does not associate with Dss1 but correctly responds to DNA damage to form nuclear foci, arguing against the notion that the DNA repair deficiency results from a reduction in the level of protein below the threshold necessary to maintain function. These findings also seem at odds with the proposal that DSS1 may be required for the BRCA2-Rad51 complex to become associated with sites of DNA damage (7). Second, the deletion of the *BRH2* gene from *dss1* mutants makes the cells more resistant to radiation, implying that Brh2 is present in the *dss1* mutant cells but that it is somewhat toxic in the absence of Dss1. Under these conditions, it seems logical to suppose that mitigating this toxicity by proteolytic removal of Brh2 might represent one aspect employed by the cell for regulating its usage. The issue warrants careful investigation. Nevertheless, evaluating the function of Dss1 in controlling Brh2's activity solely through the lens of monitoring stability might not adequately clarify more subtle mechanistic features.

Finally, there also exists the broader question of whether or not these findings apply to vertebrate BRCA2. There are numerous close parallels between Brh2 and higher eukaryotic BRCA2 and associated Dss1/DSS1 both structurally in their domain arrangements, and functionally in their roles in DNA repair and recombination. Even fusions of Brh2's BRC domain with RPA70 and BRCA2's BRC elements with RPA70 are both active in complementing the DNA repair and recombination deficiencies caused by the loss of the respective gene functions, attesting to the value of the *U. maydis* system as a vehicle for gathering further insight into BRCA2's molecular mechanism. However, the Dss1-regulated partnering of Brh2 has brought the question of whether the lower and higher eukaryotic proteins work similarly to a more critical plane. The overall organizations of the proteins are similar: two different Rad51-interacting domains separated by the Dss1-interacting region, which is the most highly conserved region of the protein family. Thus, the issue of whether Brh2 and BRCA2 work similarly or not becomes an extremely intriguing one.

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REFERENCES

1. Baillat, D., M. A. Hakimi, A. M. Naar, A. Shilatifard, N. Cooch, and R. Shiekhattar. 2005. Integrator, a multiprotein mediator of small nuclear RNA processing, associates with the C-terminal repeat of RNA polymerase II. *Cell* 123:631-640.
2. Chen, C. F., P. L. Chen, Q. Zhong, Z. D. Sharp, and W. H. Lee. 1999. Expression of BRC repeats in breast cancer cells disrupts the BRCA2-Rad51 complex and leads to radiation hypersensitivity and loss of G(2)/M checkpoint control. *J. Biol. Chem.* 274:32931-32935.

- 2a. Clontech Laboratories. 2001. Yeast protocols handbook. PT3024-1. Clontech Laboratories, Palo Alto, CA.
3. Davies, A. A., J. Y. Masson, M. J. McIlwraith, A. Z. Stasiak, A. Stasiak, A. R. Venkitaraman, and S. C. West. 2001. Role of BRCA2 in control of the RAD51 recombination and DNA repair protein. *Mol. Cell* **7**:273–282.
4. Esashi, F., N. Christ, J. Gannon, Y. Liu, T. Hunt, M. Jasin, and S. C. West. 2005. CDK-dependent phosphorylation of BRCA2 as a regulatory mechanism for recombinational repair. *Nature* **434**:598–604.
5. Funakoshi, M., X. Li, I. Velichutina, M. Hochstrasser, and H. Kobayashi. 2004. Sem1, the yeast ortholog of a human BRCA2-binding protein, is a component of the proteasome regulatory particle that enhances proteasome stability. *J. Cell Sci.* **117**:6447–6454.
6. Galkin, V. E., F. Esashi, X. Yu, S. Yang, S. C. West, and E. H. Egelman. 2005. BRCA2 BRC motifs bind RAD51-DNA filaments. *Proc. Natl. Acad. Sci. USA* **102**:8537–8542.
7. Gudmundsdottir, K., C. J. Lord, D. Witt, A. N. J. Tutt, and A. Ashworth. 2004. DSS1 is required for RAD51 focus formation and genomic stability in mammalian cells. *EMBO Rep.* **5**:989–993.
8. Hughes-Davies, L., D. Huntsman, M. Ruas, F. Fuks, J. Bye, S. F. Chin, J. Milner, L. A. Brown, F. Hsu, B. Gilks, T. Nielsen, M. Schulzer, S. Chia, J. Ragaz, A. Cahn, L. Linger, H. Ozdag, E. Cattaneo, E. S. Jordanova, E. Schuuring, D. S. Yu, A. Venkitaraman, B. Ponder, A. Doherty, S. Aparicio, D. Bentley, C. Theillet, C. P. Ponting, C. Caldas, and T. Kouzarides. 2003. EMSY links the BRCA2 pathway to sporadic breast and ovarian cancer. *Cell* **115**:523–535.
9. Jasin, M. 2002. Homologous repair of DNA damage and tumorigenesis: the BRCA connection. *Oncogene* **21**:8981–8993.
10. Kämper, J., R. Kahmann, M. Bolke, L. J. Ma, T. Brefort, B. J. Saville, F. Banuett, J. W. Kronstad, S. E. Gold, O. Muller, M. H. Perlin, H. A. Wosten, R. de Vries, J. Ruiz-Herrera, C. G. Reynaga-Pena, K. Snetselaar, M. McCann, J. Perez-Martin, M. Feldbrugge, C. W. Basse, G. Steinberg, J. I. Ibeas, W. K. Holloman, P. Guzman, M. Farman, J. E. Stajich, R. Sentandreu, J. M. Gonzalez-Prieto, J. C. Kennell, L. Molina, J. Schirawski, A. Mendoza-Mendoza, D. Greilinger, K. Munch, N. Rossel, M. Scherer, M. Vranes, O. Ladendorff, V. Vincon, U. Fuchs, B. Sandrock, S. Meng, E. C. Ho, M. J. Cahill, K. J. Boyce, J. Klöse, S. J. Klosterman, H. J. Deelstra, L. Ortiz-Castellanos, W. Li, P. Sanchez-Alonso, P. H. Schreiber, I. Hauser-Hahn, M. Vaupel, E. Koopmann, G. Friedrich, H. Voss, T. Schluter, J. Margolis, D. Platt, C. Swimmer, A. Gnirke, F. Chen, V. Vysotskaia, G. Mannhaupt, U. Guldener, M. Munsterkotter, D. Haase, M. Oesterheld, H. W. Mewes, E. W. Mauceli, D. DeCaprio, C. M. Wade, J. Butler, S. Young, D. B. Jaffe, S. Calvo, C. Nusbaum, J. Galagan, and B. W. Birren. 2006. Insights from the genome of the biotrophic fungal plant pathogen *Ustilago maydis*. *Nature* **444**:97–101.
11. Kojic, M., and W. K. Holloman. 2000. Shuttle vectors for genetic manipulations in *Ustilago maydis*. *Can. J. Microbiol.* **46**:333–338.
12. Kojic, M., C. F. Kostrub, A. R. Buchman, and W. K. Holloman. 2002. BRCA2 homolog required for proficiency in DNA repair, recombination, and genome stability in *Ustilago maydis*. *Mol. Cell* **10**:683–691.
13. Kojic, M., C. W. Thompson, and W. K. Holloman. 2001. Disruptions of the *Ustilago maydis* REC2 gene identify a protein domain important in directing recombinational repair of DNA. *Mol. Microbiol.* **40**:1415–1426.
14. Kojic, M., H. Yang, C. F. Kostrub, N. P. Pavletich, and W. K. Holloman. 2003. The BRCA2-interacting protein DSS1 is vital for DNA repair, recombination, and genome stability in *Ustilago maydis*. *Mol. Cell* **12**:1043–1049.
15. Kojic, M., Q. Zhou, M. Lisby, and W. K. Holloman. 2005. Brh2-Dss1 interplay enables properly controlled recombination in *Ustilago maydis*. *Mol. Cell Biol.* **25**:2547–2557.
16. Kojic, M., Q. Zhou, M. Lisby, and W. K. Holloman. 2006. Rec2 interplay with both Brh2 and Rad51 balances recombinational repair in *Ustilago maydis*. *Mol. Cell Biol.* **26**:678–688.
17. Krogan, N. J., M. H. Lam, J. Fillingham, M. C. Keogh, M. Gebbia, J. Li, N. Datta, G. Cagney, S. Buratowski, A. Emili, and J. F. Greenblatt. 2004. Proteasome involvement in the repair of DNA double-strand breaks. *Mol. Cell* **16**:1027–1034.
18. Li, J., Y. Bai, D. E. Wazer, V. Band, and Q. Gao. 2006. DSS1 is required for the stability of BRCA2. *Oncogene* **25**:1186–1194.
19. Mizuta, R., J. M. LaSalle, H. L. Cheng, A. Shinohara, H. Ogawa, N. Cope-land, N. A. Jenkins, M. Lalande, and F. W. Alt. 1997. RAB22 and RAB163/mouse BRCA2: proteins that specifically interact with the RAD51 protein. *Proc. Natl. Acad. Sci. USA* **94**:6927–6932.
20. Pellegrini, L., D. S. Yu, T. Lo, S. Anand, M. Lee, T. L. Blundell, and A. R. Venkitaraman. 2002. Insights into DNA recombination from the structure of a RAD51-BRCA2 complex. *Nature* **420**:287–293.
21. Powell, S. N., and L. A. Kachnic. 2003. Roles of BRCA1 and BRCA2 in homologous recombination, DNA replication fidelity and the cellular response to ionizing radiation. *Oncogene* **22**:5784–5791.
22. Saeki, H., N. Siaud, N. Christ, W. W. Wiegant, P. P. van Buul, M. Han, M. Z. Zdzienicka, J. M. Stark, and M. Jasin. 2006. Suppression of the DNA repair defects of BRCA2-deficient cells with heterologous protein fusions. *Proc. Natl. Acad. Sci. USA* **103**:8768–8773.
23. San Filippo, J., P. Chi, M. G. Sehorn, J. Etchin, L. Krejci, and P. Sung. 2006. Recombination mediator and Rad51 targeting activities of a human BRCA2 polypeptide. *J. Biol. Chem.* **281**:11649–11657.
24. Sharan, S. K., M. Morimatsu, U. Albrecht, D. S. Lim, E. Regel, C. Dinh, A. Sands, G. Eichele, P. Hasty, and A. Bradley. 1997. Embryonic lethality and radiation hypersensitivity mediated by Rad51 in mice lacking Brca2. *Nature* **386**:804–810.
25. Shin, D. S., L. Pellegrini, D. S. Daniels, B. Yelent, L. Craig, D. Bates, D. S. Yu, M. K. Shivji, C. Hitomi, A. S. Arvai, N. Volkmann, H. Tsuruta, T. L. Blundell, A. R. Venkitaraman, and J. A. Tainer. 2003. Full-length archaeal Rad51 structure and mutants: mechanisms for RAD51 assembly and control by BRCA2. *EMBO J.* **22**:4566–4576.
26. Shivji, M. K., O. R. Davies, J. M. Savill, D. L. Bates, L. Pellegrini, and A. R. Venkitaraman. 2006. A region of human BRCA2 containing multiple BRC repeats promotes RAD51-mediated strand exchange. *Nucleic Acids Res.* **34**:4000–4011.
27. Shivji, M. K., and A. R. Venkitaraman. 2004. DNA recombination, chromosomal stability and carcinogenesis: insights into the role of BRCA2. *DNA Repair* **3**:835–843.
28. Sone, T., Y. Saeki, A. Toh-e, and H. Yokosawa. 2004. Sem1p is a novel subunit of the 26 S proteasome from *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**:28807–28816.
29. Thakurta, A. G., G. Gopal, J. H. Yoon, L. Kozar, and R. Dhar. 2005. Homolog of BRCA2-interacting Dss1p and Uap56p link Mlo3p and Rae1p for mRNA export in fission yeast. *EMBO J.* **24**:2512–2523.
30. West, S. C. 2003. Molecular view of recombination proteins and their control. *Nat. Rev. Mol. Cell Biol.* **4**:1–11.
31. Yang, H., P. D. Jeffrey, J. Miller, E. Kinnucan, Y. Sun, N. H. Thoma, N. Zheng, P. L. Chen, W. H. Lee, and N. P. Pavletich. 2002. BRCA2 function in DNA binding and recombination from a BRCA2-DSS1-ssDNA structure. *Science* **297**:1837–1848.
32. Yang, H., Q. Li, J. Fan, W. K. Holloman, and N. P. Pavletich. 2005. The BRCA2 homologue Brh2 nucleates RAD51 filament formation at a dsDNA-ssDNA junction. *Nature* **433**:653–657.