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Mutational analysis of Brh2 reveals requirements for compensating mediator functions

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SUMMARY

Brh2, a member of the BRCA2 family of proteins, governs homologous recombination in the fungus *Ustilago maydis* through interaction with Rad51. Brh2 serves at an early step in homologous recombination to mediate Rad51 nucleoprotein filament formation and also has the capability to function at a later step in recombination through its inherent DNA annealing activity. Rec2, a Rad51 paralog, and Rad52, are additional components of the homologous recombination system, but the absence of either is less critical than Brh2 for operational activity. Here we tested a variety of mutant forms of Brh2 for activity in recombinational repair as measured by DNA repair proficiency. We found that a mutant of Brh2 deleted of the non-canonical DNA-binding domain within the N-terminal region is dependent upon the presence of Rad52 for DNA-repair activity. We also determined that a motif first identified in human BRCA2 as important in binding DMC1 also contributes to DNA repair proficiency and cooperates with the BRC element in Rad51 binding.

INTRODUCTION

Repairing damaged DNA by homologous recombination is a universal mechanism for restoring integrity and maintaining stability of the genetic material. In an emerging view, a DNA molecule damaged by a double-strand break or containing a single-stranded gap as a consequence of a replication problem can be channeled through the homologous recombination system for repair (Budzowska & Kanaar, 2009, Li & Heyer, 2008, Mimitou & Symington, 2009). Central in the homologous recombination system is the pairing step in which there is recognition of sequence homology and DNA strand invasion. In eukaryotes Rad51 catalyzes this step (San Filippo *et al.*, 2008). Reaction begins with loading of Rad51 onto single-stranded DNA where it polymerizes to form a nucleoprotein filament. This latter constitutes the active principle in the search for sequence homology and DNA strand invasion. When a homologous sequence is located, the reaction proceeds with formation of a D-loop joint molecule in which the invading single strand becomes base-paired with its complementary strand in the duplex and the identical sequence strand is displaced. Replication protein A (RPA) bound to single-stranded DNA prevents Rad51 from gaining access and so inhibits strand invasion. This inhibition can be overcome by the action of mediators that promote Rad51 filament assembly and stabilization on the DNA with concomitant displacement of RPA (Sung & Klein, 2006).

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Rad52, which is pivotal for all homologous recombination events in the yeast *Saccharomyces cerevisiae* (Krogh & Symington, 2004, Symington, 2002), plays important roles at two distinct stages in the process. First, it provides mediator function, interacting directly with both RPA and Rad51 to enable Rad51 to gain access to single-stranded DNA coated with RPA (New *et al.*, 1998, Shinohara & Ogawa, 1998, Sung, 1997a). The N-terminal region of Rad52 is conserved from yeast to human. It self-associates to form a multimeric ring structure circumvallated with a groove of basic residues forming a DNA-binding domain (Kagawa *et al.*, 2002, Singleton *et al.*, 2002). The medial region of the protein is not well-conserved, but in yeast associates with RPA bound to DNA (Seong *et al.*, 2008). The C-terminal third of the protein is also poorly conserved, and in yeast has been found to harbor the Rad51-interacting domain (Milne & Weaver, 1993, Shinohara *et al.*, 1992) and another DNA-binding domain structurally unrelated to that in the N-terminal region (Seong et al., 2008). In addition to its role of mediator in strand invasion, Rad52 protein exhibits a powerful DNA annealing activity (Mortensen *et al.*, 1996) that is thought to be important in repair of DNA double-strand breaks when the second broken DNA end must be captured and rejoined with the first to restore integrity of the molecule (Lao *et al.*, 2008, Shi *et al.*, 2009, McIlwraith & West, 2008, Nimonkar *et al.*, 2009). Rad51 paralogs, which are proteins related in structure to Rad51, are also implicated as mediators of Rad51 filament assembly because they also help overcome the inhibition to Rad51-catalyzed strand exchange imposed by RPA (Fortin & Symington, 2002, Sung, 1997b, Gasior *et al.*, 1998). Accumulating evidence suggests that the Rad51 paralogs function in nucleation, extension or stabilization of the Rad51 nucleoprotein filament. Support for the role of Rad52 and the paralogs as mediators of Rad51 filament assembly is supported by cytological and chromatin immunoprecipitation (ChIP) studies which show that Rad51 localization to the sites of DSBs *in vivo* is abolished or else diminished in their absence (Fung *et al.*, 2009, Miyazaki *et al.*, 2004, Sugawara *et al.*, 2003, Wolner *et al.*, 2003).

In eukaryotes other than *S. cerevisiae* and related ascomycete fungi Rad52 appears to be relegated to only marginal service in homologous recombination [see (Kojic *et al.*, 2008) and references herein]. Instead, the functions of mediating Rad51 filament formation and performing second end capture through DNA annealing are provided by the BRCA2 family of proteins in addition to the Rad51 paralogs (San Filippo et al., 2008). In the basidiomycete fungus *Ustilago maydis* the BRCA2 homolog Brh2 is necessary for resistance to radiation and chemical genotoxins, proficiency in recombination, and successful meiosis (Kojic *et al.*, 2002). Brh2 interacts with Rad51 through the single BRC element located in the N-terminal region of the protein and through a second and less well-defined region, the CRE, located at the extreme C-terminus (Kojic *et al.*, 2006, Zhou *et al.*, 2007). These N-terminal and C-terminal Rad51-interaction sites participate with each other in some as yet unknown way to direct proper Rad51 filament assembly. The process depends on Dss1, a small versatile intrinsically disordered protein that physically interacts with Brh2 through a region corresponding to the DSS1/DNA-binding domain (DBD) in mammalian BRCA2 (Kojic *et al.*, 2003, Zhou *et al.*, 2009b). The mammalian BRCA2 DBD domain consists of a tandem array of three OB (oligonucleotide/oligosaccharide-binding) folds and a helix-rich domain (HD) that is laced to the adjacent OB1 fold by intertwining with DSS1 (Yang *et al.*, 2002). Sequence alignment with BRCA2 indicates that a region corresponding to part of the helix-rich domain and the adjacent OB1 and OB2 folds is conserved in the C-terminal region of Brh2, but OB3 is absent (unpublished observations). While the C-terminal region of Brh2 harbors a predicted DNA-binding domain of tandem OB modules, the N-terminal region of Brh2 contains an empirically determined DNA-binding domain (NBD) adjacent to the BRC motif (Zhou *et al.*, 2009a). Expression of the N-terminal region containing the NBD and BRC complements the radiation sensitivity not only of the *brh2* mutant but also the *dss1* mutant (Kojic *et al.*, 2005). Furthermore, expression of the N-terminal fragment restores Rad51 focus formation and allelic recombination proficiency to an even higher level than in

wild type cells (Kojic et al., 2005). Conversely, expression of the C-terminal region harboring the canonical DNA-binding domain is without effect in complementation.

The single recognizable Rad51 paralog in *U. maydis* is Rec2. In the *rec2* mutant, subnuclear relocalization of Rad51 into foci following DNA damage is greatly curtailed, resistance to radiation is diminished, and recombination proficiency is compromised (Kojic et al., 2006). The DNA repair and recombination deficiencies of *rec2*, however, can be largely suppressed by expression of Brh2 under a strong promoter (Kojic et al., 2006). On the other hand, elevated expression of Rad51 is not sufficient to suppress *rec2* (Kojic et al., 2006). These observations suggest that Rad51 is not limiting and that the loss of Rec2 function can be compensated for by a counterbalance in Rad51 mediator activity. One way of conceptualizing this idea is to suppose that both Brh2 and Rec2 provide a means for stabilizing the Rad51 filament. Thus, in the absence of Rec2, an excess of Brh2 can substitute to provide stabilizing function. This notion of mediator counterbalancing was reinforced by evidence showing that Rec2 and Rad52 appear partially overlapping in function. In a screen for mutants exhibiting synthetic loss of fitness with *rad52* following DNA damage, *rec2* was identified (Kojic et al., 2008). Given that the phenotypes of *rad51, brh2*, and *dss1* are by all accounts equally severe in mitotic cells and are more extreme than *rec2* and certainly more than *rad52*, we visualize a model in which Rad51, Brh2, and Dss1 constitute the core axis of the homologous recombination system while Rec2 and Rad52 serve in an auxiliary capacity. We hypothesize that loss of function of any one of the critical core components would be severely detrimental to recombination activity, but loss of one of the auxiliary components would have less consequence because another auxiliary factor could partially compensate for its function. However, with loss of more than one of the auxiliary components, the system would become non-functional.

Here we were interested in learning more about the relationship between Brh2, Rad52, and Rec2 mediators and took the approach of exploring how Brh2 impaired by certain mutations could be supported by Rad52 or Rec2. Our rationale was that by introducing particular mutations into Brh2, we might be able to identify functional regions with genetic interactions dedicated to Rad52 or Rec2.

RESULTS

Linker scanning mutagenesis of Brh2

We performed a Tn7 transposon-mediated mutagenesis procedure of the Brh2 coding sequence as a means for introducing five-codon insertions into the gene and mapped about 200 transposition insertion events. Unfortunately, the insertion events were biased and so we were unable to obtain a library with randomly spaced insertions. In any event, we chose representatives along the gene to verify by DNA sequencing, and from these culled out a sub-set that contained five-codon inserts in-frame with the Brh2 coding sequence (Fig. 1A). These were cloned into a *U. maydis* expression vector, introduced into *brh2, brh2 rad52*, or *rec2* mutant strains and tested for activity in complementation (or suppression) of DNA repair deficiency by means of survival after DNA damage.

In *U. maydis* survival of cells after irradiation with UV light is highly dependent on the homologous recombination system (Holloman *et al.*, 2007). Therefore, we monitored complementation of UV sensitivity as a measure of recombinational repair activity (Fig. 1B). The single insertion mutation identified with a profound effect on Brh2 function in terms of capacity to complement the *brh2* mutant was Tn146, which introduced the pentamer tract VFKNG following residue 871, apparently within the presumed OB2 region. None of the other events were associated with a strong phenotype, suggesting that Brh2 is fairly plastic in ability to tolerate small insertion tracts.

Tolerance to these codon insertions was particularly surprising in the case of Tn52, because this insertion targets the BRC locus that serves as a critical Rad51-interaction interface. It has been established that two different tetrameric motifs within the BRC element are necessary for interaction with Rad51 (Pellegrini *et al.*, 2002, Rajendra & Venkitaraman, 2010). These are thought to mimic elements at the interface between Rad51 protomers important for polymerization. In Brh2 these correspond to residues 294–297, FQTG, and 315–319, LMMQ, Insertion of Tn52 residues LFKNG after residue 309 is in between the motifs but is apparently accommodated without adverse effect in complementing the *brh2* mutant. When tested for activity in the *brh2 rad52* double mutant, Tn52 was marginally reduced in activity, as was Tn105 or Tn16, these latter two being inserted in a region linking the NBD and helix-rich domain. In the *rec2* mutant, however, it was evident that all three of these insertions, Tn52, Tn105, and Tn16, had a profound effect on the phenotype. That these Brh2 variants were not capable of supporting *rec2* suppression indicates the insertions do disturb mediator activity, but in a manner ordinarily masked in the presence of Rec2. Thus, *rec2* suppression provides an extremely stringent measure of Brh2 functional integrity.

Deletion analysis of the N-terminal DNA binding domain

We reported previously that an N-terminal fragment of Brh2 (1-551 amino acids) harboring the BRC element, but deleted of the entire C-terminal OB-fold-containing DNA-binding domain was active in complementing the DNA repair and recombination deficiency of the *brh2* mutant (Kojic et al., 2005). An explanation for this activity was that another DNA-binding region was likely present within the N-terminal fragment, and indeed, it was demonstrated by in vitro DNA-binding determinations that the N-terminal fragment bound DNA tightly (Zhou et al., 2009a). In studies testing polypeptides derived from the N-terminal region for activity, it was found that the region with DNA-binding activity could be localized to a fragment spanning residues 359–551. No known DNA-binding motif was evident within this sequence, but it was noted to be rich in basic residues, suggesting that the interaction with DNA might be mediated by ionic bonds. To assess the functional contribution of this N-terminal DNA binding domain (NBD) to Brh2 functional activity in vivo, we tested various constructs of Brh2 in which the region was removed or else replaced by a heterologous DNA binding domain (Fig. 2A).

In previous work we referred to the biologically active N-terminal fragment of Brh2 as $Brh2^{NT}$, but to be precise and consistent in terminology with regard to other truncated forms of Brh2, we refer to this same fragment as Brh2ΔC551 to indicate this is Brh2 deleted of C-terminal residues distal to position 551. As shown (Fig. 2B) this fragment can substantially complement the UV sensitivity of both *brh2* and *brh2 rad52*, but unlike the full-length Brh2, is not effective in suppressing *rec2*. Deletion of NBD (Brh2ΔC362) from the N-terminal Brh2 fragment completely abolishes activity in complementing *brh2* or *brh2 rad52*. Since we had shown in earlier work that mutation of critical residues F294A T296A in the Rad51-interacting BRC element greatly reduces Brh2 DNA repair activity (Zhou et al., 2007), we conclude that the functionally active Brh2ΔC551 fragment requires both Rad51-interacting and DNA-binding modules. We were curious, however, whether the requirement for the DNA-binding function could be met by substituting a heterologous binding module. We found that fusing the BRC-containing region of Brh2 with the DNA-binding domain comprised of the tandem OB-A and OB-B folds from the RPA70 subunit (Brh2ΔC362::OB-AB) formed a chimeric molecule that was highly effective in complementing both *brh2* and *brh2 rad52*. Again, no activity in suppressing *rec2* was evident (Fig. 2B). These results suggest that coupling a BRC module with a DNA-binding module forms an artificial Brh2 that can effectively substitute for least some basic Brh2 functions, provided Rad51 paralogs are active.

In biochemical studies performed in vitro with purified Brh2 components and oligonucleotide substrates we showed that the DNA-binding activity manifested by the full length Brh2 was attributable almost entirely to the NBD, and that the contribution in binding activity by the OB-fold-containing C-terminal DNA-binding domain was minor by comparison (Zhou et al., 2009a). Based on these findings, our expectation was that Brh2 deleted of the NBD would be inactive in vivo. This, however, was not the case. On the contrary, variant Brh2Δ362-541, which was deleted of the NBD, was quite capable of complementing the *brh2* DNA repair deficiency (Fig. 2B). This observation suggests that the DNA-binding domain within the C-terminal region of Brh2 is active in vivo and is an effective substitute for the NBD. However, unlike the case with full length Brh2, the complementation activity of Brh2Δ362-541 was strongly dependent on the presence of Rad52. Thus, Rad52 appears to supply a function necessary for DNA repair proficiency in the absence of Brh2's NBD domain. Brh2 variants (Brh2Δ362-493, or Brh2Δ493-541) with less extensive deletions of the NBD in which some residues remained were still active in the absence of Rad52, as was the mutant Brh2 KRPR496-499AAAA, altered in a highly basic motif that resembles a nuclear localization signal. As above, in the absence of Rec2 function none of these mutants was active in DNA repair (Fig. 2B).

DMC1-interaction motif PhePP and related sequences

The BRC motif is critical for interaction between proteins of the BRCA2 class and Rad51 (Yu *et al.*, 2003). From the high-resolution crystal structure of a human BRC4-RAD51 core fusion protein, it was proposed that the crucial FHTA motif in the BRC4 element mimics the 86FTTA89 stretch in the N-terminal linker region of RAD51, which mediates contacts between monomers necessary for polymerization, and that the critical F residue functions in the coupling like the spherical donor-end of a ball-and-socket joint (Pellegrini et al., 2002). In a different study, West's laboratory examined interaction of the meiosis-specific RAD51 paralog DMC1 with BRCA2 and identified a conserved element different from the BRC motif that was specific for interaction with DMC1 (Thorslund *et al.*, 2007). This element contains the motif 2406FVPPFK2411 in which the critical F2406 residue plus the P2408 and P2409 residues are necessary for DMC1 interaction. Switching residue P2408 to T is an allowable change, while requirement for the other residues is much less stringent. The element is highly conserved in vertebrate BRCA2s, and is evident in BRCA2 homologues in plants and invertebrates, although is more divergent.

We were curious about the newly identified DMC1-interacting motif, referred to by the West laboratory as the PhePP motif (Thorslund & West, 2007), for three reasons. First, we were intrigued by the possibility of a parallel role between the conserved phenyalanine residue in the PhePP motif and the critical phenyalanine residue in the RAD51-interaction BRC element. Second, the PhePP motif is present in Brh2 as 488FVTPFK493 with the acceptable T in place of the proximal P (Thorslund et al., 2007) and is referred to below as Phe(P/T)P to distinguish the element in *U. maydis* Brh2. It is paradoxical, however, that bioinformatics analysis of the annotated *U. maydis* genome indicates no DMC1 ortholog to be present (Holloman *et al.*, 2008), prompting the question of whether the Phe(P/T)P motif is important for Brh2 functional activity in associating with Rad51. Third, there are two other related sequences, 422FTTP425 and 454FTTP458, in the region of Brh2 lying between the BRC motif (294FQTG297) and the putative Phe(P/T)P core motif 488FVTP491. So, we wondered whether these motifs might represent an array of related Rad51-interacting elements. To gain insight into these issues, we examined the contribution of the Phe(P/T)P motif FVTP and the two FTTP motifs to proficiency of Brh2 in complementing UV sensitivity of the *brh2* mutant (Fig. 3A) by altering the presumptive critical F residue in each motif. Mutation of F to A (F422A, F454A, F488A) within any of the three motifs had little apparent effect on the ability of Brh2 to complement *brh2*, and

neither did the P491A alteration. Furthermore, in combination with the inactivating F294A T296A mutations in the BRC element, F422A and F454A conferred no additional repair deficiency. However, combination of either the F488A or P491A mutation with the BRC motif mutations resulted in a synthetic loss of activity suggesting that the Phe(P/T)P-motif does contribute to Brh2 function in recombinational repair.

Expression of the Brh2ΔC551 fragment in the *brh2* mutant is sufficient to complement the DNA repair deficiency substantially and to restore recombination proficiency (Kojic et al., 2005). Mutation in BRC in the Brh2ΔC551 fragment severely reduces activity. When the Phe(P/T)P motif was altered by introducing the F488A or P491A mutation, complementation activity was markedly reduced (Fig. 3B), even though neither change has discernible effect within the context of the full-length protein (Fig. 3A). On the other hand, F422A and F454A in the context of the Brh2ΔC551 fragment conferred no repair deficiency. These results suggest the possibility that the Phe(P/T)P motif contributes to Brh2 activity, but that Phe(P/T)P function appears masked in the full-length protein. One idea to account for this possibility is that the Phe(P/T)P function is redundant or partially overlapping with another Rad51-interacting domain in the C-terminal part of the protein. Thus, it would be predicted that a phenotype for F488A or P491A mutants would become evident in combination with mutation in the presumed redundant C-terminal Rad51-interacting domain.

We had previously identified a region at the extreme C-terminus of Brh2 (CRE) as capable of binding Rad51 (Zhou et al., 2007). The single point mutation W1052A within a 72-residue peptide encompassing the CRE resulted in substantial abrogation of Rad51-binding. Thus the CRE seemed an obvious candidate as a putative domain functionally redundant with Phe(P/T)P. Therefore, we tested whether mutation in the Phe(P/T)P motif would show a synthetic loss of activity in combination with the W1052A mutation, and indeed it did (Fig. 3C). In summary, it would appear that the Phe(P/T)P element contributes to Brh2-mediated DNA repair function, but the FTTP motifs do not.

Phe(P/T)P cooperates with BRC to promote interaction with Rad51

The stoichiometry of BRC-Rad51 is critical for exact conformation and function of nucleoprotein filament. Excess isolated BRC-repeat will disrupt the Rad51 monomer-monomer interface and lock Rad51 in a monomer conformation (Davies *et al.*, 2001), whereas substoichiometric levels of BRC stabilize the Rad51-DNA complex (Carreira *et al.*, 2009) likely by binding to a different surface formed by the adjacent Rad51 monomer (Galkin *et al.*, 2005). Given the above observation that mutation in Phe(P/T)P results in loss of ability of the Brh2ΔC551 fragment to complement the *brh2* mutant, we tested whether this could be due to decreased interaction with Rad51. This was performed by a pulldown procedure using purified Brh2 fragments and Rad51, taking advantage of an affinity tag on the Brh2 fragments to capture complexes (Fig. 4A). Brh2ΔC551 with F294A T296A mutations in the BRC element, or with F488A mutation in Phe(P/T)P exhibited marginal loss in ability to interact with Rad51 compared to Brh2ΔC551. However, Brh2ΔC551 with F294A T296A and F488A mutations manifested a synthetic response and was reduced about three-fold. We also considered the possibility that the Phe(P/T)P motif might contribute to the DNA-binding activity of the NBD (Zhou *et al.*, 2009a), but observed no difference between the activity of Brh2ΔC551 and Brh2ΔC551 F488A in a gel mobility shift assay (data not shown). These finding supports the notion that, besides BRC, the Phe(P/T)P element contributes to Rad51 interaction with Brh2.

We also wished to test whether mutation in Phe(P/T)P would show a synthetic loss of interaction with Rad51 in combination with the W1052A mutation by co-precipitation using purified proteins. Here it was necessary to perform the analysis with Brh2 derivatives

deleted of the BRC. We constructed an N-terminal truncation of Brh2 deleted of residues 1–359 (Brh2ΔN359), thereby removing the BRC motif, and attempted to purify the mutant derivatives as heterotypic complexes with Dss1. Unfortunately, we were not able to obtain the variant with the W1052A mutation most likely because the complex with Dss1 was less stable. To circumvent the problem we performed pulldowns directly from cleared extracts of *E. coli* cells co-expressing MBP-tagged Brh2ΔN359 derivatives and Rad51, then visualized and quantified the relative levels of Rad51 after western blotting. The F488A W1052A double mutant derivative of Brh2ΔN359 was reduced in Rad51-binding efficiency (Fig. 4B), while the single mutant derivatives appeared no different from the wild type.

Phe(P/T)P is required for rec2 suppression

We showed before that Rec2 physically interacted with Brh2, and found that mutation in the BRC element led to decreased interaction (Kojic et al., 2006). At present we have no physical-interaction domain-mapping data with resolution fine enough to reveal whether Rec2 also interacts with Brh2 through the Phe(P/T)P or FTTP motifs. However, as noted above Brh2 and Rec2 interact genetically, and the ability of ectopically expressed Brh2 to suppress the UV sensitivity of the *rec2* mutant is a stringent metric of Brh2 functional integrity. Therefore, we tested the F-to-A mutations of the Phe(P/T)P-motif and two FTTP-motifs for activity in *rec2* suppression. Phe(P/T)P mutation F488A reduced the activity of Brh2 in *rec2* suppression down to a level comparable to that of the BRC-motif F294A T296A mutant, as did also the P491A mutation (Fig. 5A). The single F422A and F454A mutations did not change the ability of Brh2 to suppress *rec2*, although the double mutant combination F422A F454A was marginally abrogated in activity. Double mutant combinations of F422A or F454A with F488A were reduced in activity to the same extent as the BRC motif mutant.

The *rec2* mutant used in these studies is a null allele resulting from a deletion of the promoter and part of the open reading frame and there is no evidence that any truncated protein product is produced from the remnant of the coding sequence (Kojic *et al.*, 2001). Given the physical interaction between Brh2 and Rec2, we were curious whether the Brh2-F488A mutant protein could restore DNA repair activity in a *rec2* allele that was expressing Rec2 protein that was functionally inactive. This issue was approached with use of Rec2 mutants K257R and K257A, altered in the Walker A box ATP binding loop of the RecA-motif, as these changes are generally presumed to cause loss in ATP hydrolysis or loss in ATP binding, respectively. Previously it had been established that Rec2-K257R retained functional repair and recombination activity, but Rec2-K257A did not (Rubin *et al.*, 1994). However, repair deficiency of *rec2* expressing Rec2-K257A was not suppressed by Brh2-K488A (Fig. 5B). Therefore, we conclude that compensation for the DNA repair defect of *rec2* cannot be achieved by expression of the Brh2-K488A variant when an inactive form of Rec2 is present.

Because we had previously noted that expression of the Brh2ΔC551 fragment in the *rec2* mutant was sufficient to suppress gamma ray sensitivity but not UV sensitivity (Zhou et al., 2009a), we tested the contribution of the BRC, Phe(P/T)P, and FTTP mutations in the context of the Brh2ΔC551 fragment (Fig. 5C). As in the case of the full-length protein, Phe(P/T)P mutations F488A and P491A reduced the activity of Brh2ΔC551 in *rec2* suppression down to a level approaching that caused by the F29A T296A mutations in the BRC motif. The single F454A mutation was marginally reduced in activity, while the F422A mutation had little effect on the ability of Brh2ΔC551 to suppress *rec2*.

Compensatory action of Rad52 domains in Brh2 NBD function

Analysis of Rad52 in yeast has revealed a modular domain arrangement in which a well-defined N-terminal region comprises a DNA-binding domain that functions in second-end capture in DSB repair, while the medial and C-terminal regions contribute to mediator function and harbor sites for interaction with RPA, Rad51, and an additional DNA binding. As the Brh2 variant deleted of the NBD (Brh2Δ362-541) was strongly dependent on the presence of Rad52 in complementing the UV sensitivity of *brh2*, we asked if the requirement for Rad52 could be met by expressing fragments containing only the conserved N-terminal DNA-binding domain (Rad52 DBD), or the C-terminal region distal to the DBD (Fig. 6). Each region by itself appeared capable of restoring repair function to a degree, but neither region could fully compensate for the Rad52 requirement.

DISCUSSION

Here we examined the effect of various insertions, deletions, and point mutations in Brh2 gene action with the intent of identifying important functional domains in DNA repair function. There are several noteworthy findings. First, Brh2 seems extremely plastic in being able to tolerate a variety of mutations with no apparent loss of DNA repair function as measured by complementation of UV sensitivity of a *brh2* deletion mutant. This plasticity might be inferred from the extreme divergence in structure of members of the BRCA2/Brh2 class of proteins, in which overall size, number of BRC elements, and OB modules varies greatly (Lo *et al.*, 2003, Warren *et al.*, 2002). By comparison, Rad51, the downstream effector of Brh2 is highly conserved in primary sequence throughout the eukaryotic domain of life. However, it could be naive to assume that the plastic or seemingly inert regions of Brh2 and relatives do not contribute to function, because a DNA repair phenotype for a number of apparently silent mutations is indeed manifested upon removal of Rad51 mediators. Rec2 provides a strong contribution ensuring appropriate functional operation of Brh2. In the absence of Rec2, the consequences of even slight alternations in Brh2 activity become apparent or are magnified. Thus, ability to suppress the UV sensitivity of *rec2* is a very sensitive metric of Brh2 functional activity. Divergent regions in Brh2 sequence that at first glance might seem expendable often have functional significance in DNA repair and probably reflect the contexture and intricacies of the specific homologous recombination machine operational in *U. maydis*.

Based on the phenotype of the deletion mutant, Rad52 appears to play no obvious role in the homologous recombination system of *U. maydis* (Kojic et al., 2008). Nevertheless, it compensates for deletion of the N-terminal DNA-binding domain of Brh2. In the absence of Rad52, Brh2 deleted of NBD is defective in DNA repair. This is the first evidence we have obtained that ties Rad52 directly to the central reaction of the homologous pairing system and it suggests that Rad52 is functionally overlapping with the Brh2 NBD. If indeed the case, the question that arises is what mechanistic role could be shared? What would determine the hierarchy in their utilization, and why would redundancy be built into the operating system in the first place?

Brh2 appears to have both early and late functions in repair of DNA double-strand breaks. In addition to its activity mediating assembly of the Rad51 filament (Yang *et al.*, 2005), it also has the ability to function later in the repair process in the capture of the second DNA end (Mazloum & Holloman, 2009). This latter step is required to complete repair of double-strand breaks and appears to proceed through a DNA annealing function inherent in Brh2. Both of these functions have been ascribed to Rad52 in yeast based on extensive genetic, molecular, and biochemical characterization. As mentioned above, *U. maydis* Rad52 is structurally conserved (Kojic et al., 2008) and has an annealing function (Mazloum *et al.*, 2007). In vitro it can also perform the second-end capture reaction as in the case of yeast

Rad52. But the second-end capture activity of *U. maydis* Rad52 is attenuated when the DNA is coated with Rad51. This is not the case with Brh2, *i.e.*, its activity is not attenuated by Rad51 (Mazloum & Holloman, 2009). So, one explanation to account for the Rad52 dependence of Brh2 deleted of the NBD (Brh2 Δ362-541) in DNA repair is that the deletion eliminates Brh2's annealing function in second end capture, loss of which could be compensated for to some extent by Rad52. Based on the yeast model the DNA annealing activity of Rad52 resides in the conserved N-terminal region corresponding to the DNA-binding domain, and the mediator domain and another DNA binding region lie distal. However, since the *U. maydis* N-terminal Rad52 fragment does not completely substitute for the full length Rad52, and since the C-terminal Rad52 fragment can also partially substitute, we cannot eliminate the possibility at this point that Rad52 contributes both annealing and mediator activities in the absence of Brh2 NBD. Why these activities of Rad52 are revealed when Brh2 is crippled is still not clear but is suggestive of a model in which Brh2 serves as the preeminent, preferred component in recombination repair, while Rad52 might ordinarily function in a minor specialized pathway but come into more general play under limited conditions.

Finally, we note that the Phe(P/T)P domain of Brh2, originally identified in mammalian BRCA2 as a site for interplay with the meiosis-specific DMC1, interacts with Rad51 and contributes to the formation and/or maintenance of Brh2/Rad51 complexes and contributes to DNA repair proficiency. After the Brh2 N-terminal BRC element and C-terminal CRE, Phe(P/T)P is a third, independent element mediating binding to Rad51. That Brh2 interacts with Rad51 through multiple points ultimately promoting delivery of Rad51 to DNA, and maintenance of the filament could be interpreted in a mechanistic framework to mean that a hierarchy of protein-protein contact sites facilitates transfer of Rad51 from a primary contact site in Brh2, to secondary sites, then finally to the DNA target enabling formation and stabilizing the Rad51 filament.

EXPERIMENTAL PROCEDURES

U. maydis strains and genetic methods

Manipulations with *U. maydis*, culture methods, gene disruption and transfer procedures, and survival after UV (254 nm) or γ-ray irradiation have been described previously [see (Kojic et al., 2008, Zhou et al., 2007) and references therein]. For survival, exponentially cultures of exponentially growing cells were diluted to 2×10^7 per ml, then aliquots (10 μl) of serial 10-fold dilutions were spotted on solid medium, and irradiated with 254 nm UV (120 J/m^2) or γ-rays (400 Gy). Plates were incubated at 30°C for 2–3 days. The *brh2* and *rad52* genes were disrupted by standard methods with a cassette expressing resistance to nourseothricin (*nst*) or gentamycin (*nph*). Haploid strains utilized for testing survival after irradiation included UCM54 (*rec2-1 pan1-1 nar1-1 a1b1*), UCM350 (*pan1-1 nar 1-6 a1b1*), UCM565 (*Δbrh2 pan1-1 nar1-6 a1b1*), UCM645 (*Δbrh2 Δrad52 pan1-1 nar1-6 a1b1*). *pan*, *nar*, and *ab* indicate auxotrophic requirements for pantothenate, inability to utilize nitrate, and mating type loci, respectively. Self-replicating plasmids expressing genes encoding Brh2 or derivatives driven by the glyceraldehyde 3-phosphate dehydrogenase (*gap*) promoter contained the hygromycin phosphotransferase gene (*hph*) for selection and maintenance. Self-replicating plasmids expressing genes encoding Rec2 and Walker A box mutant derivatives, or else Rad52 and N-terminal or C-terminal fragments contained the *gap* promoter to drive expression and the carboxin resistance (*cbx*) gene for selection.

Mutant construction

Linker scanning mutagenesis was performed using the GPS-LS system (New England BioLabs) in which Tn7 transposons were introduced by transposition in vitro into a target

pUC19-based plasmid containing a multiple cloning site modified with an oligonucleotide linker to accept the 2646 bp *Mlu*I-*Nsi*I fragment of the Brh2-coding sequence. After passage through *E. coli*, the pUC19 plasmid DNA derivative recovered was cut with *Pme*I and re-ligated to yield a library of Brh2 mutants in which 15 bp insertions with a unique *Pme*I site were introduced in the Brh2 fragment. Insertions were mapped by keying on the *Pme*I site for orientation. Brh2 mutants deleted of fragments of the N-terminal DNA-binding domain were constructed by judicious restriction endonuclease cleavage and re-ligation, taking advantange of the unique *Pme*I sites introduced by transposon linker scanning mutagenesis. All internal deletions and insertions were constructed so that the distal coding region would be translated in frame. Brh2 variants with RPA70 OB-AB modules in place of the entire NBD (residues 362–541) were constructed using codons 141-434 of RPA70 encoding the OB-AB fragment inserted in frame with Brh2 coding sequence. Point mutations in Brh2 were constructed using PCR methodology with a plasmid donor harboring the Brh2 sequence of interest annealed with overlapping oligonucleotides containing the desired mutation spanning the target sequence. Rad52 N-terminal and C-terminal truncations were constructed by cutting the coding sequence (722 codons) in two parts at a unique internal restriction site. $Rad52^{NT}$ comprises residues 1–359. $Rad52^{CT}$ comprises residues 360–722 fused to a short leader sequence (MSSD) that was added so as to incorporate a start codon at the 5′ end of the open reading frame.

Co-precipitation/pulldown analysis

Brh2ΔC551 and variants with point mutations in BRC or or Phe(P/T)P, *i.e.*, F294A T296A, or F488A, or both combinations, were purified from *E. coli* strain BL21 (DE3) (Novae) cells as fusion proteins with an N-terminal MBP-tag and C-terminal hexahistidine (His)-tag as described previously (Zhou et al., 2009a). Rad51 was produced without an affinity tag as described before (Mazloum et al., 2007). Association reactions were performed in 60 μl mixes containing 500 nM Brh2ΔC551 or variant plus 300 nM Rad51, in buffer A (50 mM Tris-HCl, pH 7.5, 200 mM KCl, 1 mM dithiothreitol, 0.01% nonidet NP40, 10% glycerol) at 20° for 30 min. Settled slurry (30 μl) of amylose resin (New England Biolabs) was added and the mixture was held in an ice bath with frequent mixing. After 10 min the resin was collected by brief centrifugation in a microcentrifuge, washed four times with buffer A (0.5 ml each), then bound protein was eluted with buffer A containing 10 mM maltose (50 μl). The eluted sample was analyzed by SDS gel electrophoresis and visualized by staining gels with Simplyblue (Invitrogen). Images were collected with an Odyssey detection platform (LI-COR Biosciences). Relative band intensities were determined using ImageQuaNT software (GE Healthcare) and used to calculate the ratio of Rad51 signal per Brh2ΔC551 signal. To analyze association of Rad51 with Brh2 variants deleted of the BRC, the N-terminal truncation Brh2ΔN359 (deleted of N-terminal residues 1–359) and derivatives with point mutations F488A and/or W1052 were co-expressed in *E. coli* strain BL21(DE3) as fusion proteins with an N-terminal MBP-tag simultaneously with Rad51 as described previously (Zhou *et al.*, 2007). Cleared lysates were prepared in buffer A, mixed with amylose resin and bound protein was eluted by washing the resin with 10 mM maltose, then separated by electrophoresis in an SDS polyacrylamide gel. Following western transfer to polyvinylidene difluoride membrane, the blot was cut in half. Proteins in the top half were visualized after hybridization using anti-MBP antiserum (New England Biolabs) and in the bottom half with affinity purified rabbit polyclonal antibodies to Rad51, coupled with ECL chemiluminescence reagents (GE Healthcare) for detection.

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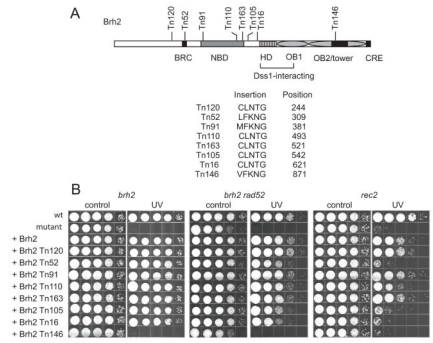

Figure 1.

Transposon scanning mutagenesis. A. Schematic representation of Brh2 with protein elements and domains as follows: BRC -- Rad51 interaction element; NBD -- N-terminal DNA binding domain; HD -- helix rich domain; OB1--OB module; OB2/tower- OB module with tower insert; CRE – Rad51 interaction region. Sites of 15 bp insertions, with corresponding translations, introduced after excision of the indicated transposons are shown. B. Mutant strains expressing the indicated Brh2 were grown to late log phase. Aliquots were adjusted to a density of 2×10^7 cells per ml, spotted on solid medium as serial 10-fold dilutions from left to right, and survival was determined after irradiation with UV at a dose of 120 J/m^2.

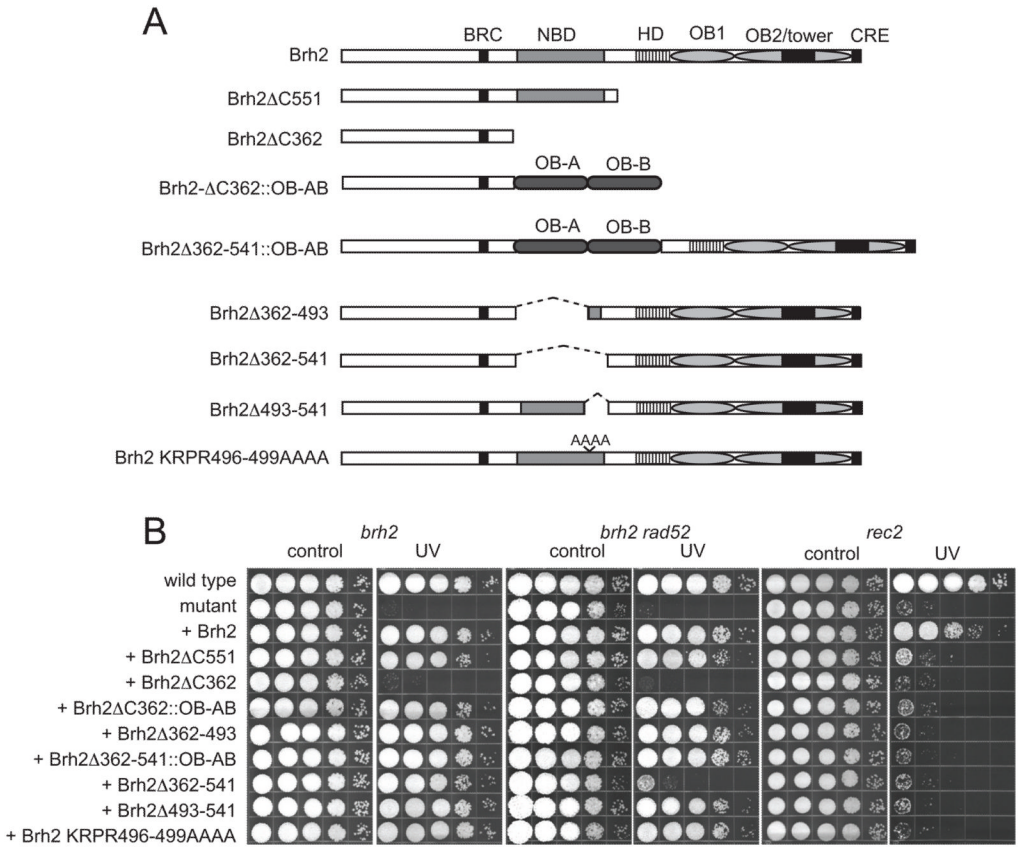

Figure 2.

Brh2 deletion mapping and domain swapping. A. Schematic representations of Brh2's with various fragments deleted or replaced by DNA binding modules OB-A and OB-B from *U. maydis* RPA70. B. Mutant strains expressing the indicated Brh2 constructs were tested for survival after irradiation with UV at a dose of 120 J/m^2.

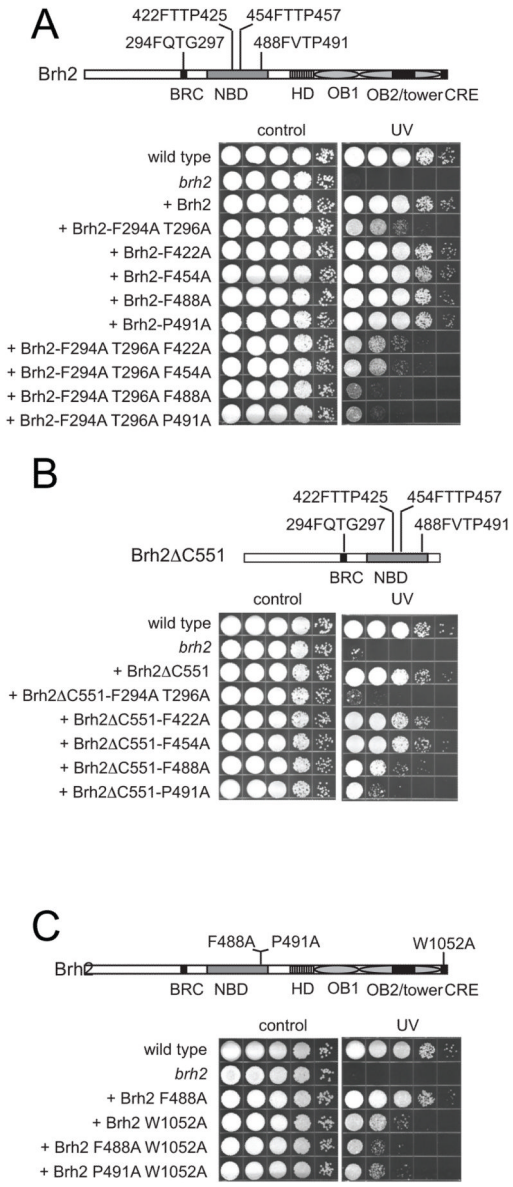

Figure 3.

Contribution of Phe(P/T)P and FTTP motifs to DNA repair activity. A. Schematic representation of Brh2 with mutated residues in BRC, Phe(P/T)P, and FTTP sequence motifs. *brh2* strains expressing the indicated Brh2 point mutation constructs were tested for survival after irradiation with UV at a dose of 120 J/m^2. B. Schematic representation of Brh2ΔC551 with Phe(P/T)P point mutations. *brh2* expressing the indicated Brh2ΔC551 constructs were tested for survival. C. Schematic representation of Brh2 with Phe(P/T)P and CRE point mutations. Constructs were tested for survival.

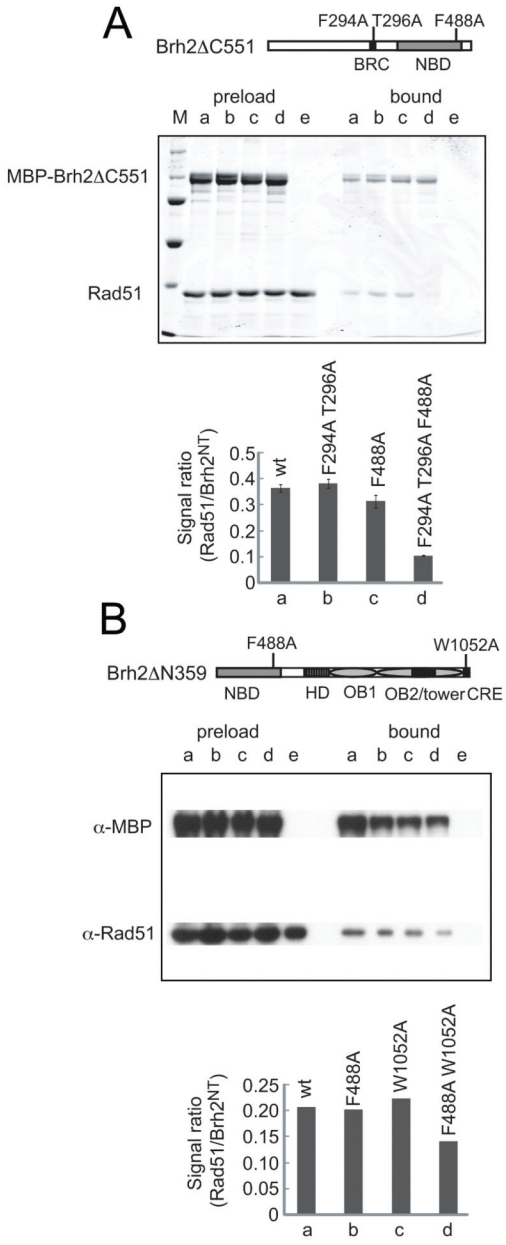

Figure 4.

Mutation in Brh2 Phe(P/T)P motif disturbs Rad51 binding. A. Schematic representation shows Brh2ΔC551 with mutated residues in BRC and Phe(P/T)P. Brh2ΔC551 and derivatives were tested for ability to form complexes with Rad51. Pulldowns of MBP-tagged Brh2ΔC551 variants plus associated Rad51 protein were performed as described in the experimental procedures. Left panel- preload samples; right panel-complexes eluted from amylose resin with maltose. Relative levels of Rad51 and Brh2ΔC551 were quantified by comparing the band intensity after staining. Averages of triplicates are presented with the standard error. Variants of Brh2ΔC551 with indicated mutations are as follows: lane a-wild type (wt); b-F294A T296A; c-F488A; d- F294A T296A F488A; e-no Brh2ΔC551 variant. B. Schematic representation shows Brh2ΔN359 with mutated residues F488A in Phe(P/T)P and W1052 in CRE. Pulldowns of MBP-tagged Brh2ΔN359 variants plus associated Rad51 protein from cleared *E. coli* extracts were performed as in experimental procedures. Left

panel- preload samples; right panel-complexes eluted from amylose resin with maltose. Relative levels of Rad51 and Brh2ΔC551 were quantified by comparing the band intensity after ECL development. lane a-wild type (wt); b-F488A; c-W1052A; d-F488A W1052A; e-no Brh2ΔN359 variant.

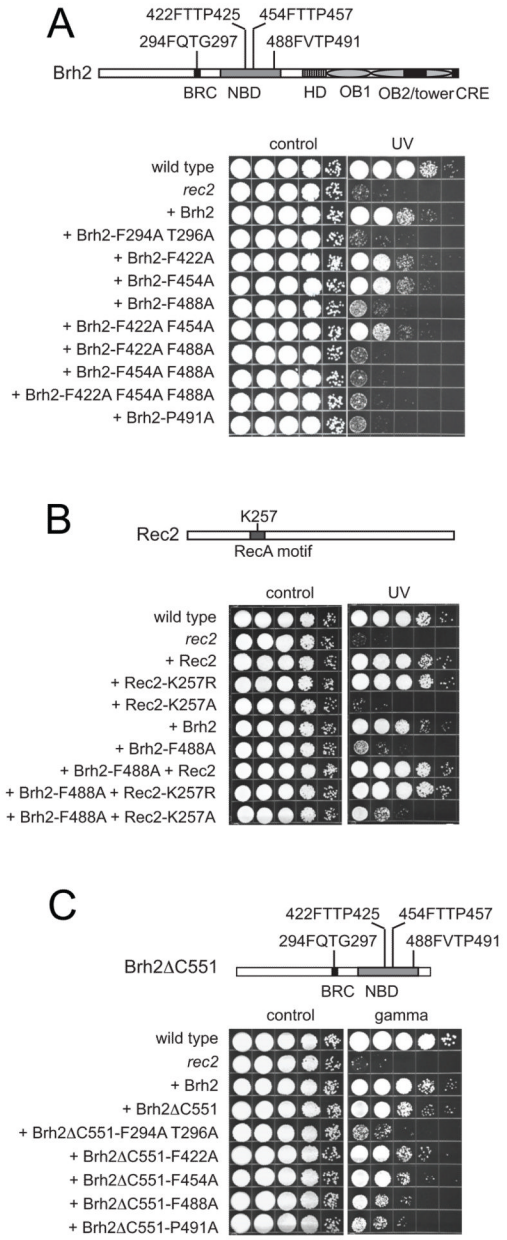

Figure 5.

DNA repair proficiency of Brh2 Phe(P/T)P and FTTP point mutations in the absence of Rec2 function. A. *rec2* strains expressing the indicated Brh2 point mutation constructs were tested for survival after irradiation with UV at a dose of 120 J/m^2. B Schematic representation of Rec2 (781 amino acids) RecA-related sequence and lysine residue K257 within the Walker A motif. *rec2* expressing the indicated Rec2 K257R or K257A mutant constructs or co-expressing the Brh2 F488A Phe(P/T)P motif mutant and Rec2 point mutants were tested for survival. C. *rec2* strains expressing Brh2ΔC551 with the indicated mutations were tested for survival after irradiation with gamma rays at a dose of 400 Gy.

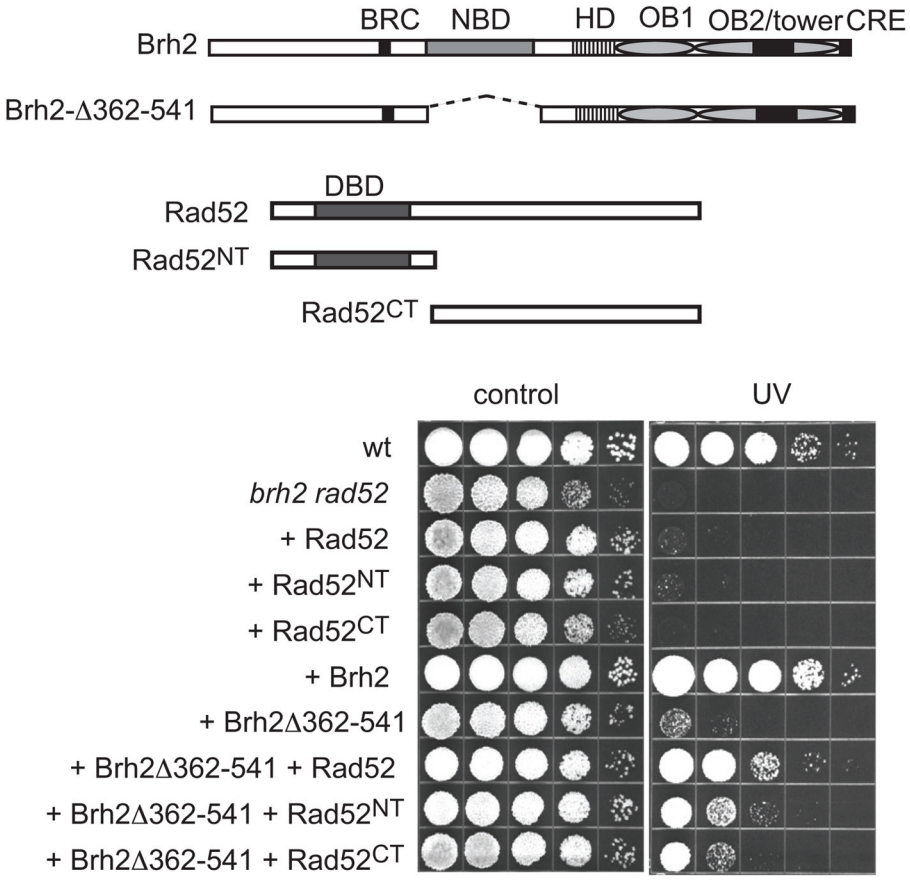

Figure 6.

Contribution of Rad52 domains to DNA repair proficiency of Bhr2 deleted of NBD. A. Schematic showing Brh2 deleted of NBD and Rad52 with its DNA binding domain (DBD) plus N- and C-terminal truncations. B. Survival of *brh2 rad52* mutant strain expressing the indicated constructs after irradiation with UV at a dose of 120 J/m^2.