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BRCA2 Homolog Required for Proficiency in DNA Repair, Recombination, and Genome Stability in *Ustilago maydis*

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Summary

In a screen for DNA repair-defective mutants in the fungus *Ustilago maydis*, a gene encoding a BRCA2 family member, designated here as Brh2, was identified. A *brh2* null allele was found to be defective in allelic recombination, meiosis, and repair of gaps and ionizing radiation damage to the same extent as *rad51*. Frequent marker loss in meiosis and diploid formation suggested that genomic instability was associated with *brh2*. This notion was confirmed by molecular karyotype analysis, which revealed gross chromosomal alterations associated with *brh2*. Yeast two-hybrid analysis indicated interaction between Brh2 and Rad51. Recapitulation in *U. maydis* of defects in DNA repair and genome stability associated with *brh2* means that the BRCA2 gene family is more widespread than previously thought.

Introduction

Germ-line mutations in the *BRCA2* gene confer a predisposition to early-onset breast cancer in humans (Rahman and Stratton, 1998). The gene encodes a large nuclear protein composed of 3418 amino acids but with no obvious sequence similarity to other known proteins and no clues to function apparent from the sequence (Wooster and Stratton, 1995). Accumulating evidence implicates a role for BRCA2 in DNA recombinational repair (Kerr and Ashworth, 2001; Venkitaraman, 2002; Zheng et al., 2000). Mutant cell lines expressing truncated forms of BRCA2 are sensitive to DNA clastogens and are severely impaired in homology-directed repair of a DNA double-strand breaks (Abbott et al., 1998; Kraakman-van der Zwet et al., 2002; Moynahan et al., 2001; Patel et al., 1998; Sharan et al., 1997; Xia et al., 2001). The repair deficiency appears to be mediated through, or by association with Rad51, the bacterial RecA homolog, which is required for homologous pairing and DNA strand exchange during recombinational repair. The proteins physically interact and colocalize to nuclear foci following radiation (Chen et al., 1998a; Marmorstein et al., 1998; Mizuta et al., 1997; Sharan et al., 1997). Interaction with Rad51 requires the BRC elements, which are present in an array of eight repeats spanning the central region of BRCA2 (Chen et al., 1998b; Wong et al., 1997). Expression of DNA fragments encoding individual BRC repeats disrupts the BRCA2-Rad51 complex, conferring a dominant-negative radiation hypersensitivity and an inability to form radiation-induced Rad51 nuclear foci (Chen et al., 1999). Synthetic peptides with sequences corresponding to BRC repeats interfere with formation of nucleoprotein filaments between Rad51 and DNA, resulting in conversion of Rad51 to a form inactive with respect to DNA binding and its function as a recombinational repair protein (Davies et al., 2001). These findings, together with the observations that transport of Rad51 into the nucleus appears to depend upon nuclear localization signals present at the carboxy-terminus of BRCA2 (Sarkisian et al., 2001; Spain et al., 1999), have led to the notion that BRCA2 imposes a two-tiered system over control of repair by governing the subcellular location of Rad51 as well as its recombinational activity (Davies et al., 2001).

BRCA2 has been regarded as being confined to higher animals only, but on the other hand, Rad51 is found highly conserved in structure and dispersed throughout all the living kingdoms (Aravind et al., 1999). Rad51 provides a core DNA sequence recognition activity and strand transfer function essential for homologous pairing, but in baker's yeast and human multiple Rad51, paralogs are present that appear to provide some auxiliary activity in enhancing the central reaction (Sung et al., 2000; Thompson and Schild, 2001). In the yeast-like fungus *Ustilago maydis*, a distant relative of baker's yeast, there is a binary system composed of the Rad51 ortholog (Ferguson et al., 1997) and Rec2, an evolutionarily divergent Rad51 paralog (Rubin et al., 1994), unusual in having an inherent homologous pairing and DNA strand transfer activity (Bennett and Holloman, 2001). As *U. maydis* is extremely resistant to killing by ionizing radiation (Holliday, 1971; Resnick, 1978), we have been interested in investigating how resistance is promoted by Rad51 and Rec2. These gene products appear to have nonredundant roles in repair (Ferguson et al., 1997), since mutation in either structural gene results in radiation sensitivity, yet both have similar biochemical activities (our unpublished data; Bennett and Holloman, 2001). The unusual activity, size, and structure of Rec2 would seem to make it a distant outlier in the spectrum of Rad51 paralogs. Thus it could provide a perspective for elucidating homologous pairing mechanisms different from that offered by the Rad51 paradigm.

Our studies have suggested an intimate interplay between Rec2 and Rad51. Analysis of a *rec2* allele with dominant-negative properties implicated the N-terminal region of Rec2 as a protein domain likely to interfere with components of genetic pathways dedicated to stabilizing the genome (Kojic et al., 2001). By yeast two-hybrid screening, we determined one such Rec2-interacting partner as Rad51. As an alternative approach to identify interacting genes, we hunted for mutants with a DNA repair phenotype and altered cellular morphology characteristic of the dominant-negative *rec2* allele. One mutant identified was a new *rad51* null allele. In addition, as described in this paper, we report isolation of a mu-

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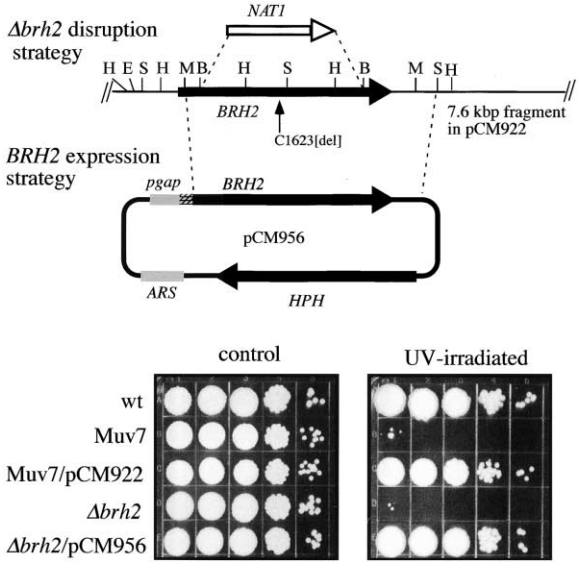

Figure 1. *BRH2* Disruption and Expression

A restriction map of the cloned 7.6 kbp genomic DNA fragment with Muv7(*brh2-1*)-complementing activity is depicted. The *BRH2* open reading frame is shown as the black bar with arrow indicating orientation. The following restriction enzyme sites are shown: B (*Bam*HI), E (*Eco*RI), H (*Hin*dIII), M (*Mlu*I), and S (*Sph*I). The 2.6 kbp *Bam*HI fragment was replaced by the *NAT1* cassette expressing Nst^R in disrupting the gene. *BRH2* is expressed under the *gap* promoter (gray box) in pCM956, which contains an autonomously replicating sequence (*ARS*) and *HPH* gene expressing Hyg^R. The small hatched box indicates a synthetic linker used to introduce an *Nde*I site at the start codon. Survival after irradiating serially diluted samples (increasing dilution from left to right) with 120 J/m^2 of UV light was determined for the indicated strains—wild-type (wt) (UCM350), Muv7 (UCM549), Δ*brh2* (UCM565).

tant defective in a BRCA2-related gene. We describe genetic experiments linking its action with *rad51* and *rec2*. These findings indicate that BRCA2 function is unlikely to be a specialized attribute limited only to genome stability and repair systems of higher animals but is apt to be much more widespread than previously thought. Our work also raises the possibility that insight into BRCA2 function might be attained through molecular genetic analysis of DNA repair in *U. maydis*.

Results

Isolation of a BRCA2 Homolog

From a screen for DNA repair mutants phenotypically similar to the dominant-negative *rec2-197*allele (e.g., Kojic et al., 2001), we isolated an allele of *rad51* and a mutant Muv7, sensitive to methylmethanesulfonate and UV (i.e., Muv phenotype, *M*MS and *UV* sensitive) and elevated in spontaneous mutator activity. We cloned the gene defective in Muv7 by complementation using a genomic DNA library and isolated a 7.6 kbp DNA fragment with full complementing activity in the self-replicating plasmid pCM922. From the DNA sequence, a single large uninterrupted open reading frame was identified. Several lines of investigation (see Figure 1) established that this open reading frame did indeed correspond to the gene defective in the Muv7 mutant. First, the open reading frame was subcloned and placed under control of a heterologous promoter. When this construct was reintroduced into Muv7 on the self-replicating plasmid pCM956, the mutant was fully complemented. Second, a deletion mutant we created by eliminating essentially the entire open reading frame from the endogenous locus exhibited a phenotype identical to Muv7. Third, sequence analysis of the cloned open reading frame from the Muv7 mutant revealed a frame shift mutation (deletion of a single C residue at position 1623 relative to the start of the open reading frame). Thus, the cloned gene corresponds to that defective in the Muv7 mutant.

The open reading frame identified is predicted to encode a protein of 1075 amino acids with no overall sequence alignment with any known DNA repair gene. However, within the sequence are regions that align locally with conserved domains of BRCA2, the protein encoded by the human breast cancer susceptibility gene (Figure 2). The open reading frame has 24% identity over a stretch of 540 amino acids with the 1136 amino acid residue hypothetical protein F7A7.150 from *Arabidopsis thaliana*, annotated as BRCA2-related in GenBank, and a related hypothetical protein BAB64792.1 from *Oryza sativa*. One segment contains a BRC motif, an element mediating Rad51 interaction that is found iterated eight times in BRCA2 (Bignell et al., 1997; Bork et al., 1996). The alignment shown includes the most highly related BRC elements from among the vertebrate BRCA2 orthologs as well as from the Arabidopsis and Oryza hypothetical proteins. Although four BRC repeats were evident upon inspection of the Arabidopsis protein, only a single BRC element was found in the *U. maydis* sequence. Pairwise analysis using BLAST or LALIGN turned up no other sequence with the characteristic FxTASGK motif that is a hallmark of the mammalian BRC (Bignell et al., 1997). Another more extensive region in the C terminus shares 38% identity with BRCA2 residues 2626-2832, and corresponds to a *C*-terminal *c*onserved *r*egion (designated here as CCR) in the vertebrate BRCA2 orthologs and the related plant hypothetical proteins (e.g., human and chicken BRCA2s and Arabidopsis hypothetical protein pictured in Figure 2). This conservation in sequence similarity suggests a common function. A single putative nuclear localization signal (NLS) at the extreme carboxy terminus of the protein is comparable in position to the three NLSs at the carboxy terminus of BRCA2. Given the sequence similarities of the *U. maydis* protein with BRCA2 and its role in DNA repair and genome stability (see below), we refer to it as Brh2 (BRCA2 homolog).

BRH2 Is Required for Recombination Proficiency

We examined recombination proficiency in *brh2-2* disruption strains using two different assays. First, we measured gap repair using a plasmid-based transformation system. The assay measures Leu^+ prototroph formation in *leu1-1* derivative strains after introduction of a self-replicating plasmid containing the cloned *LEU1* gene inactivated by deletion of 0.7 kbp from the coding sequence (Ferguson and Holloman, 1996). In recombination-proficient strains, the great majority of Leu^+ recombinants arise

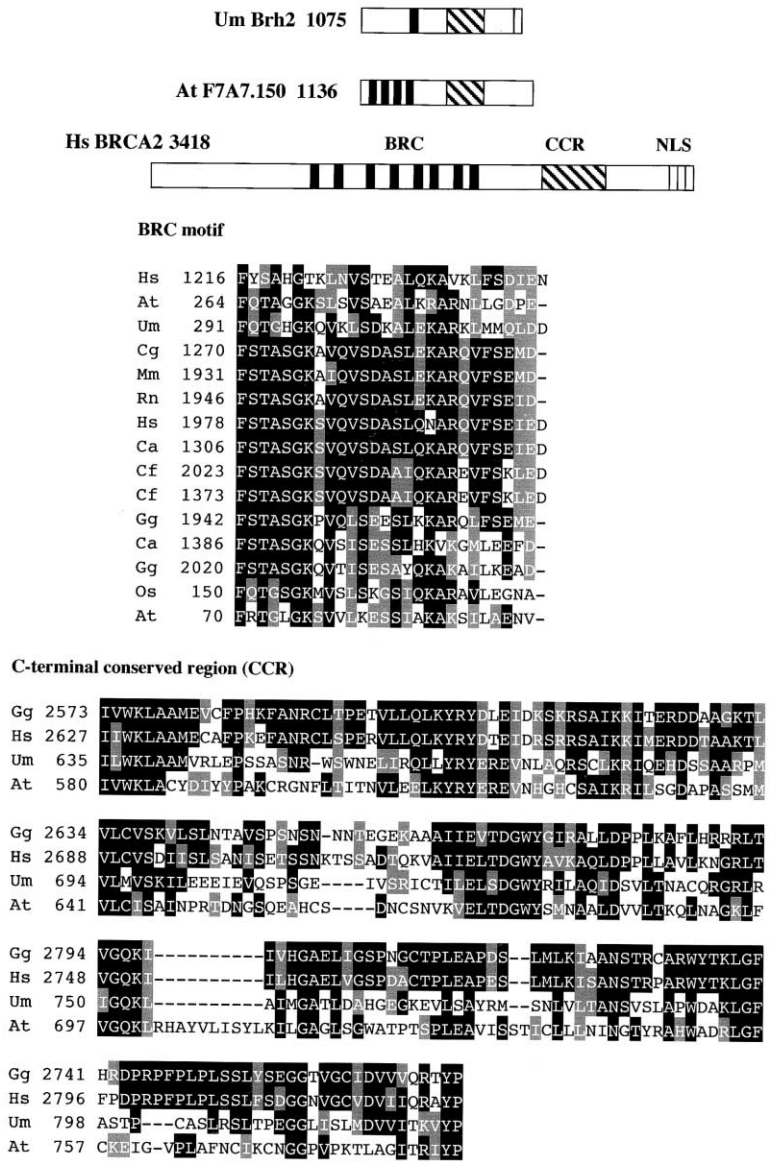

Figure 2. Comparison of Brh2 with BRCA2

Schematic representation of Brh2 protein in scale with human BRCA2 and *Arabidopsis* hypothetical protein F7A7.150 is shown at the top. The BRC repeats are indicated as black bars in BRCA2 and F7A7.150, and the single bar in Brh2. The single presumed (NLS) nuclear localization signal (PPRKVR, residues 969–974) is indicated at the extreme C terminus of Brh2 as compared with the three NLSs of BRCA2 (thin lines). The hatched box indicates the C-terminal conserved region (CCR). Alignment of the Brh2 BRC sequences was performed by pairwise BLAST (http://www.ncbi.nlm.nih.gov/blast/) and LALIGN (http://www.ch.embnet.org/software/LALIGN_form.html) analysis using the *U. maydis* BRC region as query to search each eukaryotic genome indicated. Matches with identities >30% were then aligned in multiple format using ClustalW (http://searchlauncher.bcm.tmc.edu/cgi-bin/multi-align/multi-align.html) and ordered using BOXSHADE (http://www.ch.embnet.org/software/BOX_form.html). For simplicity, the C-terminal conserved region (CCR) alignment includes a single representative BRCA2 from mammals (human), nonmammalian vertebrates (chicken), and plants (Arabidopsis). Black boxes with white letters indicate identity, while gray boxes with white letters indicate similarity. Abbreviations and database identifiers are as follows: At (*Arabidopsis thaliana* F7A7.150 hypothetical protein, GI 11357826); Ca (*Cercopithecus aethiops* BRCA2, GI 1523876); Cf (*Canis familiaris* BRCA2, GI 20502478); Cg (*Cricetulus griseus* BRCA2, GI 11277003); Gg (*Gallus gallus* BRCA2, GI 19568157); Hs (*Homo sapiens* BRCA2, GI 1177438); Mm (*Mus musculus* BRCA2, GI 20850160); Os (*Oryza sativa*, similar to *A. thaliana* F7A7.150, GI 15528750); Rn (*Rattus norvegicus* BRCA2, GI 2443441); and Um (*Ustilago maydis* Brh2, GenBank accession AY124376).

by repair of the 0.7 kbp gap in the plasmid-containing Δ*leu1* allele. Only a minor fraction of recombinants results from plasmid-directed gene conversion of the endogenous *leu1-1* allele. Previously, using this system, we found that in both *rec2* and *rad51*, the frequency of gap repair was reduced by a factor of 100-fold or more (Ferguson et al., 1997). Likewise, we find here that in *brh2*, the frequency of gap repair was also reduced by about a factor of 100 (Table 1). Thus, recombinational repair of double-stranded DNA breaks is defective in the absence of *BRH2* function. Unfortunately, to date we have been unable to obtain a *brh2 rad51* double mutant (see below), but in *brh2 rec2* there appeared to be no additive deficiency suggesting an epistatic relationship between the two genes.

In a second assay, recombination in diploids heteroallelic at the *nar1* (nitrate reductase) locus was measured. In this system, reconstruction of an intact *NAR1* gene by recombination between *nar1* heteroalleles enables growth on nitrate as a source of nitrogen. Thus, colony formation on nitrate minimal medium is a measure of recombination proficiency. In wild-type spontaneous recombination between *nar1-1* and *nar1-6* occurred at a frequency of 2×10^{-6} and could be strongly induced by DNA damage resulting from UV irradiation (Table 1) in accord with previous reports (Holliday, 1967). In contrast, the level was reduced at least 100-fold in the *brh2* homozygous diploid and no induction of recombination was apparent after DNA damage by a dose of UV low enough to cause only a 2-fold drop in viability. This result shows the level of recombination in *brh2* after DNA damage is 2000-fold below the level observed in a strain with a functional *BRH2* gene. The situation in a *rad51* diploid mirrored that observed in *brh2*. The spontaneous level of allelic recombination was reduced below the level of detection, and there was no observable induction after UV irradiation. This extreme loss of recombination proficiency in *brh2* and *rad51* stands in

Table 1. Recombination Deficiency in DNA Repair Defective Strains

Genotype[a]	Gap Repair Leu$^+$ Frequency[b], ($\times 10^{-2}$)	Nar$^+$ Recombinants per 10^6 Viable Cells[c]	
		Spontaneous	UV Induced
+	2.3	2.1 ± 0.4	22 ± 4
rec2	0.033	1.7 ± 0.3	1.4 ± 0.4
rad51	0.018	<0.01	<0.01
brh2	0.020	<0.01	<0.01
brh2 rec2	0.030		

[a] Gap repair was performed in haploids and allelic recombination, in homozygous diploid strains.
[b] For gap repair, Leu$^+$ transformation with the following strains was determined: UCM5 (+), UCM342 (*rad51*), UCM568 (*rec2*), UCM573 (*brh2*), and UCM574 (*brh2 rec2*). Determinations were standardized against variation in protoplast competency and DNA uptake by comparison with the transformation frequency of pCM216 (Ferguson and Holloman, 1996). Gap repair frequency is defined as the ratio of Leu$^+$ recombinants per microgram pCM291 DNA to Leu$^+$ transformants per microgram pCM216 DNA. The results presented are an average of two independent determinations.
[c] Allelic recombination was determined in diploid strains which contained *nar1-1/nar1-6* heteroalleles. Cells were plated on nitrate minimal medium to enable selection of Nar$^+$ recombinants. Strains were as follows: UCM33 (+/+), UCM123 (*rec2/rec2*), UCM585 (*rad51/rad51*), and UCM577 (*brh2/brh2*). Cells were irradiated with a UV dose of 7.8 J/m^2. The average of five to seven samples is presented.

contrast to *rec2* in which there is little effect on spontaneous recombination, but no damage-induced recombination (Table 1) (Holliday, 1967).

Effects of *brh2* Deficiency on Meiosis

Teliospores are highly differentiated diploid cells formed after mating of compatible haploid parents (Banuett and Herskowitz, 1996). These spores are competent to undergo meiosis but are arrested at a premeiotic phase and held in a dormant state. When plated on rich medium, the teliospores germinate forming a metabasidium, or promycelium, into which the premeiotic nucleus migrates (O'Donnell and McLaughlin, 1984). Reductional and equational nuclear divisions take place within the promycelium, followed by septation, and budding off of the yeast-like haploid progeny. Thus, generation of viable progeny from teliospores measures successful completion of meiosis. Earlier it had been established that recombination proficiency is necessary for teliospore germination as no meiotic products were produced in crosses homozygous for *rec2-1* (Holliday, 1967). Promycelia are actually formed from *rec2-1/rec2-1* homozygous teliospores, but meiosis aborts with the failure of subsequent nuclear divisions. We observed that teliospores obtained from *rec2*/+, *brh2*/+, or *rad51*/+ heterozygous crosses yielded viable meiotic progeny at a frequency in excess of 80% (data not shown). However, there was complete failure to produce viable colonies ($<1 \times 10^{-5}$) from teliospores obtained from any of the *rec2/rec2*, *brh2/brh2*, or *rad51/rad51* homozygous crosses (data not shown). Thus, *BRH2,* like *RAD51* and *REC2,* is absolutely required for meiosis, and by inference, meiotic recombination. It was interesting to note that unlike the situation with *rec2/rec2* homozygous teliospores in which promycelia are formed, there was absence of germination ($<1 \times 10^{-5}$) of both *brh2/brh2* and *rad51/rad51* homozygous teliospores (Figure 3A). This observation clearly implies that the temporal action of both *BRH2* and *RAD51* is similar to, but different from, *REC2*.

While *brh2*/+ or *rad51*/+ heterozygous crosses yield viable progeny, we noted nonetheless that meiotic chromosome segregation from these crosses was abnormal. As an expeditious assay for diploidy in *U. maydis*, and by extension, failure in reductional division during meiosis, one can monitor meiotic progeny for heterozygosity at the mating type loci (Figure 3B). When strains are heterozygous at both the *a* and *b* loci, they form white, fuzzy colonies (fuz+ phenotype) on rich media containing charcoal (Banuett and Herskowitz, 1989). Thus, by simply plating meiotic progeny on charcoal medium and noting the frequency of fuz+ colonies, one can gain a quantitative measure of nondisjunction of the *a* and *b* chromosomes, which presumably reflects the larger picture of total chromosome missegregation. In wild-type crosses, 0.5% to 3% of meiotic progeny were fuz+, in line with previous estimates of the frequency of diploids after germination of teliospores (Holliday, 1961). Chromosome segregation also appeared normal in crosses with the *rec2-1* null allele, although, as previously reported (Kojic et al., 2001), the dominant-negative *rec2-197* allele disturbs meiosis. In contrast, null alleles of both *brh2* and *rad51* disturb chromosome segregation, and in the *brh2* × *rad51* heterozygous cross, the disturbance even appears additive. Thus, it seems that the dosage of both *BRH2* and *RAD51* is important in ensuring proper disjunction during meiosis.

Genomic Instability Associated with *brh2*

We observed frequent loss of heterozygosity (LOH) during formation of *brh2* heterozygous diploids. In *U. maydis*, diploids can be synthesized by fusing compatible haploids with forcing auxotrophic markers followed by selection on minimal medium. In the absence of the required nutritional supplements, auxotrophic haploid parents fail to survive while diploids grow readily. Diploids heterozygous at both mating type loci can be distinguished by the fuzz-forming ability on charcoal medium as described above. Thus, LOH at one of the mating type loci can be readily measured by loss of fuz+ activity. When appropriately marked but otherwise wild-type strains are mated, about 5% of diploids become fuz−. However, when *brh2* strains were mated with appropriately marked testers, LOH at the mating type loci approached 50%. This was all the more remarkable given that *brh2* appeared dominant with respect to the observed elevated LOH. In light of the considerable evidence for chromosome rearrangements and aberra-

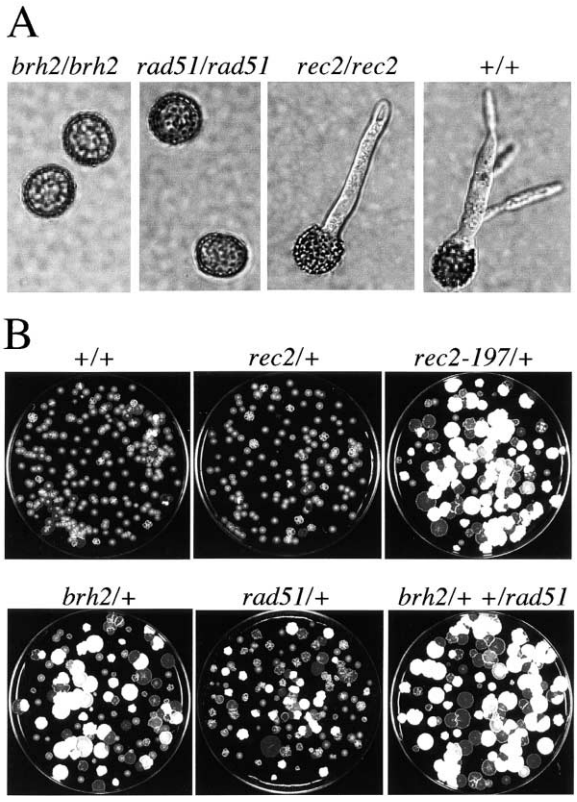

Figure 3. Meiotic Analysis

(A) Teliospore germination. Cultures (1 ml) containing YEPS medium supplemented with ampicillin (50 μg/ml) and tetracycline (15 μg./ml) were inoculated with ~10^6 teliospores and swirled gently at 32°C. Teliospores from the following matings were analyzed: +/+ (UCM33); *rec2/rec2* (UCM123); *rad51/rad51* (UCM342 × UCM548); and *brh2/brh2* (UCM577). After 16 hr, aliquots were removed and teliospores were viewed under the microscope (see photomicrogaphs) and counted using a hemocytometer to determined formation of germ tubes (teliospore germination). Separately, aliquots containing either 10^3 or 10^5 teliospores were spread on solid medium, bathed in ether vapor to kill any asporidial cells, and the plates incubated for 5 days to allow complete colony formation.

(B) Chromosome nondisjunction measured by mating-type heterozygosity in meiotic progeny. Teliospores from the indicated crosses were germinated on plates containing YEPS medium at 30°C for 24 hr. Plates were washed with 1 ml water and scraped with a glass spreader to harvest germinating cells. Cell suspensions were vortexed thoroughly to disaggregate any clumps, the cell density was determined, and ~100 cells were spread on mating medium to enable fuzz formation of meiotic progeny heterozygous at the mating-type loci. Teliospores from the following matings were analyzed: +/+ (UCM350 × UCM5), *rec2*/+ (UCM482 × UCM5), *rec2-197*/+ (UCM164 × UCM350), *brh2*/+ (UCM565 × UCM5); *rad51*/+ (UCM342 × UCM231); and *brh2*/+ +/*rad51* (UCM565 × UCM342).

tions associated with BRCA2 deficiency in mammalian cells (Kraakman-van der Zwet et al., 2002; Patel et al., 1998; Yu et al., 2000), we were alerted to the possibility of some form of genomic instability as the underlying cause for the high LOH frequency. To examine the idea by physical means that there was some disturbance in genome stability associated with *brh2,* we performed molecular karyotype analysis using pulsed field gel electrophoresis focusing in particular on *brh2* heterozygous diploids.

U. maydis has about 20 chromosomes ranging in size

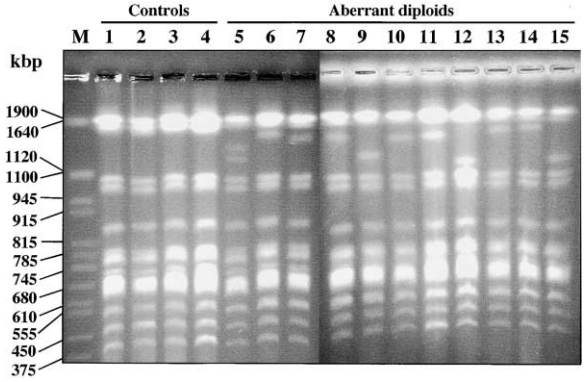

Figure 4. Molecular Karyotype Analysis

Chromosome-size DNA molecules were prepared from protoplasted cells and analyzed by CHEF gel electrophoresis. M, *S. cerevisiae* chromosome markers (BioRad Laboratories). Controls are as follows: lane, wild-type (UCM5); lane2, *brh2-2* (UCM565); lane 3, wild-type (UCM350); and lane 4, wild-type diploid (UCM33). Lanes 5–15 show individual *brh2-2*/+ diploids obtained from mating UCM565 × UCM350. The 11 shown were chosen at random from a larger set prescreened for loss of mating type heterozygosity. Additional bands representing aberrant chromosomes can be seen in the region of the gel corresponding to a size range of 1120–1900 kbp.

from approximately 350 kbp to around 2–3 Mbp (Kinscherf and Leong, 1988). Unfortunately, not all the chromosomes resolve well due to closeness in size. Nevertheless, under a single set of electrophoresis conditions, one can distinguish about a dozen individual chromosomes and chromosome clusters by CHEF gel electrophoresis (Tsukuda et al., 1988). We analyzed a subset of the diploids obtained from mating *brh2-2* (UCM565) with wild-type (UCM5) which had lost mating type heterozygosity. By CHEF analysis of 17 individual diploids that had suffered LOH, gross chromosomal alterations were apparent in 14 of them as evidenced by aberrant banding patterns (Figure 4). Eleven representatives of the fourteen total are shown (three appearing identical to those already present are not shown). For controls we examined haploids as well as diploids obtained from mating wild-type strains (13 total) and from mating *rec2-1* with wild-type (4 total). All appeared identical to the representative examples in Figure 4 (lanes 1–4) and thus free of chromosome aberrations. These results indicate that the genomic instability suggested by the high frequency of LOH in *brh2* matings appears associated with gross chromosomal alterations.

γ-Ray Sensitivity

To define the relationship among Brh2, Rad51, and Rec2 in more detail, we examined the sensitivity of the mutants to γ-radiation. The *brh2-2* null allele was extremely sensitive to γ-radiation and appeared identical to the *rad51-1* null allele in dose response (Figure 5). It was slightly more sensitive by comparison to the *rec2* null, but the *brh2 rec2* double mutant was no more sensitive than the *brh2* single mutant, although it grows more slowly than either single mutant. This lack of additivity in γ-radiation sensitivity shows an epistatic relationship between *brh2* and *rec2*. Unfortunately we have been unable to obtain a *brh2 rad51* double mutant for testing

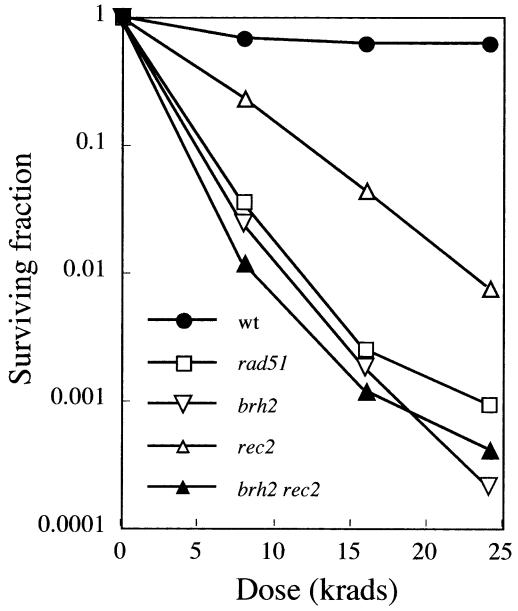

Figure 5. Sensitivity to Ionizing Radiation

The haploid strains with the indicated DNA repair mutation were grown to late log phase, washed in water, resuspended at a density of 10^7 cells per milliliter, and irradiated with γ-rays. Survival was determined by counting colonies visible after incubation for 5 days. Strains are as follows: wt (UCM350), *rad51-1* (UCM342), *brh2-2* (UCM565), *rec2-53* (UCM568), and *brh2-2 rec2-53* (UCM574).

to complete the picture, but previously we found epistasis of *rad51* with *rec2* (Ferguson et al., 1997). Therefore, it seems likely that all three genes function in the same genetic pathway in the repair of ionizing radiation damage.

Brh2/Rad51 Interaction

We used yeast two-hybrid methodology to test for Brh2 interaction with Rad51. Analysis was complicated by toxicity caused by expression of either the *BRH2* gene or the *RAD51* gene. Presumably the *RAD51* toxic effect is due to a dominant-negative interaction between a heterologous Rad51 and components of the *S. cerevisiae* recombinational repair system as noted by others (Donovan et al., 1994) and cited in (van den Bosch et al., 2002) and possibly the same is true with *BRH2*. A Rad51 N-terminal deletion lacking 26 residues was found not to be poisonous when expressed as a Gal4-activation-domain fusion, so we chose to test it for Brh2 interaction using the standard β-galactosidase assay. When this truncated *RAD51* gene was coexpressed with *BRH2*, there was strong interaction as measured by high levels of β-galactosidase activity (Table 2). We also tested whether there was interaction between Brh2 and Rec2, but unfortunately, we encountered the additional complication that there was significant Gal4-transactivator activity when the *REC2* gene alone was expressed. Coimmunoprecipitation analysis should be able to resolve whether there is interaction with Rec2. In summary, there appears to be strong physical interaction between Brh2 and Rad51. These findings extend the BRCA2/Rad51-interaction paradigm beyond the realm of vertebrates.

Discussion

Three primary conclusions can be drawn from the present work. First, a *BRCA2*-related gene has been discovered in a simple, genetically amenable lower eukaryote. Second, the gene, *BRH2*, is required for proficiency in repair and recombination and serves in maintaining genome stability in mitosis and meiosis. Third, *BRH2* along with *RAD51* and *REC2* are members of a pathway of genes identified in *U. maydis* dedicated to recombinational repair.

Investigations in mammalian systems point to an intimate connection between BRCA2 and Rad51. Similarly, in our studies we find a close association between Brh2 and Rad51 functions. Phenotypically the *brh2* and *rad51* mutants are virtually indistinguishable. Both exhibit almost the same sensitivity to UV and ionizing radiation, both are epistatic with *rec2* (i.e., see Ferguson et al., 1997), both are defective to the same extent in repair of double-strand gaps and in allelic recombination, both appear blocked at the same step in meiosis, and in the heterozygous state, both disturb meiotic chromosome segregation. In this latter feature, *brh2* and *rad51* differ from the *rec2* null allele, which has no effect on chromosome disjunction in heterozygous crosses. However, the dominant-negative *rec2-197* allele (Kojic et al., 2001) causes a similar high level of chromosome nondisjunction, suggesting an intersection of all three of these gene functions at a common step.

The mechanistic basis of this disturbance in meiotic chromosome segregation is not known, but we suspect that it is related to the genomic instability associated with diploid formation. Most likely the instability derives from, or is related to, some step during karyogamy. Diploids are not a natural free-living form in the life cycle of *U. maydis* but, as explained above, can be synthesized in the laboratory by selection for prototrophy of complementing auxotrophic markers (Banuett and Herskowitz, 1996). In the normal life cycle, the active diploid state is transient and occurs with initiation of the developmental program leading to teliospore formation. Presumably the series of events accompanying karyogamy and preceding channeling into either the vegetative diploid state or the dormant premeiotic teliospore phase

Table 2. Yeast Two-Hybrid Analysis of Brh2-Rad51 Interaction

Gal4 binding domain	-	Brh2	-	Brh2
Gal4 activation domain	-	-	Rad51	Rad51
Specific activity	0	2.6 $\pm$ 1.2	0.9 $\pm$ 0.1	36 $\pm$ 0.8

Interaction was measured as β-galactosidase activity (nmol o-nitrophenyl-β-D-galactoside hydrolyzed min^{-1} per 10^7 cells). Activity from the combination of empty vectors alone was taken as baseline and subtracted from the experimental determinations. Plasmids utilized were as follows: GBD-Brh2 (pCM960); GAD-Rad51 (pCM971).

Table 3. *U. maydis* Strains

Alias (Haploids)	Genotype[a]	Source
UCM5	*ade1-1 leu1-1 a2b2*	R. Holliday, 87
UCM164	*rec2-197 ade1-1 leu1-1 a2b2*	(Bauchwitz and Holloman, 1990)
UCM179	*rec2-1 ade1-1 leu1-1 a2b2*	This laboratory
UCM231	*nar1-1 pan1-1 pyr3-1 a1b1*	This laboratory
UCM342[b]	*rad51-1hph ade1-1 leu1-1 a2b2*	(Ferguson et al., 1997)
UCM350	*nar1-6 pan1-1 a1b1*	R. Holliday, 91
UCM482[c]	*rec2-53 nar1-6 pan1-1 a1b1*	(Kojic et al., 2001)
UCM520	*met1-2 nar1-1 a2b2*	This laboratory
UCM548[d]	*rad51-2 nar1- pan1-1 a1b1*	This work
UCM549[e]	*brh2-1 nar1-6 pan1-1 a1b1*	This work
UCM565[f]	*brh2-2 nar1-6 pan1-1 a1b1*	This work
UCM568	*rec2-53 ade1-1 leu1-1*	This work
UCM573	*brh2-2 ade1-1 leu1-1*	This work
UCM574	*brh2-2 rec2-53 leu1-1*	This work
UCM575	*brh2 met1-2 nar1-1 a2b2*	This work
UCM583	*rad51-1 nar1-1 ade1-1 leu1-1 a2b2*	This work
(diploids)		
UCM33	*nar1-1/nar1-6 ade1-1/+ met1-2/+ pan1-1/+*	R. Holliday, d66
UCM123	*rec2-1/rec2-1 nar1-1 nar1-6 ade1-1/+ pan1-1/+*	R. Holliday, d29
UCM147	*rec2-1/+ nar1-1/+ ade1-1/+ leu1-1/+ pan1-1/+*	This laboratory
UCM577	*brh2-2/brh2-2 nar1-1/nar1-6 met1-2/+ pan1-1/+*	This work
UCM585	*rad51-1/rad51-2 nar1-1/nar1-6 ade1-1/+ leu1-1/+ pan1-1/+*	This work

[a] *ade*, *leu*, *met*, *pan*, *pyr*, *nar*, and *ab* indicate auxotrophic requirements for adenine, leucine, methionine, pantothenate, and pyrimidine (uracil), inability to reduce nitrate, and mating type loci, respectively.
[b] *rad51* allele disrupted by insertion of *HPH* cassette, (Hyg^R).
[c] *rec2* allele disrupted by insertion of *HPH* cassette, (Hyg^R).
[d] L252P missense *rad51*allele.
[e] Muv7 isolate with *brh2* allele (nucleotide C1623[deleted]).
[f] *brh2* allele disrupted by insertion of *NAT* cassette, (Nst^R)

includes some common steps that involve recombinational repair functions. Perturbation of these functions by an imbalance in Brh2 or Rad51 might then result in improper chromosome maintenance or segregation, and the subsequent daughter cells produced after the first division would then be affected. Once diploids are formed, they appear to remain stable during vegetative growth. It is possible that some physical aspect of the *brh2* or *rad51* parental genome is deranged, and upon karyogamy those chromosomes are recognized as abnormal and missegregate. Perhaps this process bears some similarity to instability of chromosomes in tumor cells. It has been argued that a change in dosage of certain crucial genes may trigger a general loss of accuracy in chromosome segregation at mitosis (Holliday, 1989).

From in vitro analyses it has been found that Rec2 has homologous pairing and strand exchange activity that appears comparable to what has been observed with yeast and human Rad51 (Bennett and Holloman, 2001). However, results from this and previous studies support the idea that Rec2 and Rad51 do not function at the same step in vivo. The germination of a promycelium from *rec2* teliospores, but not *rad51* teliospores, is a clear indication of a difference in gene action. Recombination between homologs is eliminated in mitotic cells by mutation in *RAD51*, while a basal level can still occur in the absence of *REC2*. One possibility that might account for these observations is that *RAD51* is dedicated to interactions between homologs, while *REC2* is more specialized for sister chromatid interactions. It should be possible to test this model by utilizing recombination substrates specific for intrachromosomal events.

In view of the overall compact structure of Brh2 and single BRC element, the *U. maydis* protein might be considered as a minimalist version of BRCA2. However, it is puzzling that the elaboration of BRC repeats does not seem to correlate with overall size. For instance, the similarly compact Arabidopsis (and Oryza) hypothetical protein F7A7.150 has at least four BRC elements that are recognizable by close conservation with the FxTASGK motif. In human it seems clear that the BRC repeats are not functionally redundant since a point mutation in a single BRC element is sufficient to cause loss of function as determined by predisposition to breast cancer (e.g., G1529R or S2071I, Breast Cancer Information Core, http://www.nhgri.nih.gov/Intramural_research/Lab_transfer/Bic/). This is a curious observation given the current view that the association of BRCA2 with Rad51 appears to be mediated through the BRC elements in a 1:1 stoichiometric manner and would seem to rule out the simple model that BRC repeats act independently. Perhaps the multiplicity of BRC repeats in higher eukaryotes goes hand in hand with the elaboration of Rad51 paralogs and reflects a requirement for some specialized interactions involving these components.

In light of identification here of a fungal gene and mutant whose properties recapitulate many of the features associated with BRCA2 in mammals, it seems evident that the BRCA2 family is more widespread than has generally been appreciated due to extensive sequence divergence. In this latter regard, it is notable that vertebrate BRCA2 orthologs are unusually divergent. For instance, the mouse and chicken BRCA2 share only 54% and 31% identity, respectively, with human BRCA2. A recent report identifying certain hypothetical gene sequences from mosquitoes and intracellular parasites

with regional similarity to BRCA2 sequences also attests to its extreme divergence (Warren et al., 2002). By contrast, Rad51 is very conserved throughout evolution. The mouse and chicken Rad51 orthologs are virtually identical to human, each differing by only 3 amino acids. *U. maydis* Rad51 is precisely the same size and shares 72% sequence identity with the human protein. The conservation in Rad51 structure could be a consequence of the constraints imposed by the catalytic chemistry of the homologous DNA pairing reaction. The divergence in structure of the cognate BRCA2 members might reflect the diversification and specialization in the regulation of Rad51-mediated DNA damage response by life forms throughout the evolutionary tree.

The emerging view of BRCA2 from mammalian studies is that it functions in direct control of Rad51 by governing nuclear localization and nucleoprotein filament stability (Venkitaraman, 2002). The close parallel between the *brh2* and *rad51* mutant phenotypes and the direct interaction of the proteins raise the possibility of a similar interdependence of function in *U. maydis*, an issue that we plan to investigate more thoroughly. It is of additional interest that there also appears to be interplay between *BRH2* and *REC2*. This latter notion raises the scenario of *BRH2* functioning as a master regulator of the recombinational machinery in *U. maydis*. In a broad sense, the findings reported here expand our framework for viewing the molecular mechanisms of recombination and offer new possibilities for their elucidation. Our findings might also suggest that control of Rad51 function by an enabling partner is likely to be an essential and unifying aspect of homology search mechanisms in eukaryotes. The absence of any apparent BRCA2 homolog in organisms such as *S. cerevisiae* might simply mean that the enabling function is still provided but by an analogous rather than homologous gene.

Experimental Procedures

U. maydis Strains and Methods

Strains used in this study are listed in Table 3. Preparation of media and procedures for diploid construction, meiotic analysis, DNA transformations, gene disruptions, mutator activity, and other manipulations were carried out as described (Fotheringham and Holloman, 1989; Holliday, 1974; Kojic et al., 2001). Semiquantitative UV survival determinations were performed by adjusting parallel cultures to 10^7 cells per milliliter, then spotting 10 μl aliquots of 10-fold serial dilutions onto plates containing solid medium. γ-radiation was performed using a Gammacell 220 ^{60}Co source (Nordion International, Kanata, Ontario, Canada). Gap repair of the *LEU1* gene was determined as described previously using the plasmids pCM216 and pCM291 (Ferguson and Holloman, 1996). Allelic recombination at the *nar1* locus (nitrate reductase) was measured by determining Nar$^+$ prototroph formation (Holliday, 1967). A nourseothricin resistance (Nst^R) cassette *NAT* (Kojic and Holloman, 2000) was used to replace the 2.6 kbp *Bam*HI fragment from within the *BRH2* open reading frame. Plasmid pCM956 is a self-replicating vector expressing Hyg^R which contains the *BRH2* open reading frame under control of the *gap* promoter. Molecular karyotype analysis was performed by contour-clamped homogeneous electric field (CHEF) gel electrophoresis essentially as described previously (Tsukuda et al., 1988). Protoplasts were prepared using Driselase and β-D-glucanase (Interspex Products, Inc., Foster City, CA) in place of Novozyme 234 (Ovechkina et al., 1999). Agarose gels (1%) were run using a CHEF-DR II drive module (BioRad Laboratories, Hercules CA) at 14°C at 200 V for 17 hr with a pulse interval of 60 s and then switched to a pulse interval of 90 s for 9 hr.

Mutant Screen and Gene Isolation

Exponentially growing cells of haploid strain UCM350 were spread on solid medium and irradiated with 254 nm ultraviolet light (UV) to a survival frequency of 5%–10%. Approximately 46,000 mutagenized colonies were screened visually after 3–5 days of growth at 32°C for aberrations in colony morphology. Ten candidates found to be sensitive to UV and Muv2 (*rad51-2*, strain designation UCM548) and Muv7 (*brh2-1*, strain designation UCM549) exhibited UV and MMS survival kinetics comparable to *rec2-1* and *rad51-1* (Ferguson et al., 1997). Gene cloning was performed by complementation using a genomic DNA library prepared in the self-replicating vector pCM54 by methods described previously (Tsukuda et al., 1989). Plasmid pCM922 (*BRH2*) complemented the DNA repair deficiency of the Muv7 mutant (*brh2-1*) and contained a DNA fragment of 7.6 kbp. The *brh2-1* allele was isolated by PCR from UCM549 genomic DNA.

Yeast Two-Hybrid Methodology

Yeast two-hybrid analysis was performed as described before (Kojic et al., 2001) following cotransformation of *Saccharomyces cerevisiae* strain PJ69-4A (*MAT***a** *trp1-901 leu2-3,112 ura3-52 his3-200 gal4*Δ *gal80*Δ *LYS2::GAL1-HIS3 GAL2-ADE2 met2::GAL7-lacZ*) with pACT2 and pAS2-1 (Clontech Laboratories, Palo Alto, CA) derivatives expressing the Gal4-activating domain (GAD) and Gal4 DNA binding domain (GBD), respectively. For Rad51, vector combinations were designed to measure interaction between a GBD-Brh2 fusion (pCM960) and a GAD-Rad51 derivative deleted of the 26 N-terminal amino acid residues (pCM971).

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