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Role of the *Schizosaccharomyces pombe* F-Box DNA Helicase in Processing Recombination Intermediates

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In an effort to identify novel genes involved in recombination repair, we isolated fission yeast *Schizosaccharomyces pombe* mutants sensitive to methyl methanesulfonate (MMS) and a synthetic lethal with *rad2*. A gene that complements such mutations was isolated from the *S. pombe* genomic library, and subsequent analysis identified it as the *fbh1* gene encoding the F-box DNA helicase, which is conserved in mammals but not conserved in *Saccharomyces cerevisiae*. An *fbh1* deletion mutant is moderately sensitive to UV, MMS, and γ rays. The *rhp51* (*RAD51* ortholog) mutation is epistatic to *fbh1*. *fbh1* is essential for viability in stationary-phase cells and in the absence of either Srs2 or Rqh1 DNA helicase. In each case, lethality is suppressed by deletion of the recombination gene *rhp57*. These results suggested that *fbh1* acts downstream of *rhp51* and *rhp57*. Following UV irradiation or entry into the stationary phase, nuclear chromosomal domains of the *fbh1*Δ mutant shrank, and accumulation of some recombination intermediates was suggested by pulsed-field gel electrophoresis. Focus formation of Fbh1 protein was induced by treatment that damages DNA. Thus, the F-box DNA helicase appears to process toxic recombination intermediates, the formation of which is dependent on the function of Rhp51.

Homologous recombination not only shuffles genetic information upon sexual reproduction but also repairs damaged DNA by use of the homologous information. Furthermore, it can regenerate replication forks when they become stalled or collapsed.

Molecular mechanisms of homologous recombination in eukaryotes have been most extensively studied in the budding yeast *Saccharomyces cerevisiae* (reviewed in references3, 27, 42, 46, and 48). In this yeast, the MRX (Mre11 Rad50 Xrs2) complex is required for the processing of double-strand break ends to generate 3′-protruding ends. The resulting single-strand regions are coated by single-strand-binding protein RPA (replication protein A). Rad52 stimulates loading of Rad51 on RPA-coated single-strand DNA to form Rad51 nucleoprotein filament. A complex of Rad55 and Rad57, which are Rad51 paralogs, is also implicated in the assembly and stabilization of Rad51 nucleoprotein filament. Rad51 nucleoprotein filament searches homologous sequences and catalyzes the exchange of strands to form a heteroduplex joint called a D loop. Rad54 facilitates D-loop formation by remodeling chromatin structures. The annealed 3′ ends are then used as primers for repair DNA synthesis. The resulting junction molecules are resolved either by dissociation of the crossed strands or by cutting of the junction point. The Rad52 group proteins (Rad50, Rad51, Rad52, Rad54, Rad55, Rad57, Mre11, and Xrs2) are conserved throughout eukaryotes, indicating a conservation of the molecular mechanisms pertaining to homologous recombination.

In addition to the aforementioned recombination factors, DNA helicases Srs2 and Sgs1 have been implicated to be involved in the regulation of homologous recombination (reviewed in reference 6). Srs2 dissociates the Rad51 protein from nucleoprotein filament to suppress toxic recombination intermediates (26, 53). Although Srs2 is conserved in fungi, no apparent Srs2 ortholog has been found in higher eukaryotes. Sgs1 is homologous to *Escherichia coli* RecQ, *Schizosaccharomyces pombe* Rqh1, and mammalian WRN, BLM, and RTS helicases. These RecQ family helicases have been implicated to play roles in recombination at various stages such as recombination initiation, reversal or prevention of fork regression, and resolution of recombination intermediates (reviewed in reference 4). In *S. cerevisiae*, the *srs2 sgs1* double mutant is severely impaired in growth (28). This growth defect can be overcome by *rad51* mutation (15), indicating that recombination initiated by the Rad51 protein is toxic in the *srs2 sgs1* background.

Replication forks become stalled when they encounters obstacles such as chemically modified bases, pyrimidine dimers that are generated by UV irradiation, proteins tightly associated with DNA, or certain DNA tertiary structures (reviewed in reference 11). The stalled replication forks are overridden by translesion polymerases, regressed to bypass the lesion by

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TABLE 1. *S. pombe* strains used in this study

Strain	Genotype	Source or reference
B54	*smt-0 rhp51::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	Y. Tsutsui
B63	*smt-0 rhp57::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	Y. Tsutsui
TE767	*h*$^-$ *rqh1::ura4 ura4-D18*	T. Enoch, reference (40)
MP110	*h*$^-$ *leu1-32 ura4-D18*	This study
MP111	*h*$^+$ *leu1-32 ura4-D18*	This study
MPM53	*h*$^+$ *leu1-32 ura4-D18 fbh1-1*	This study
MPM73	*h*$^+$ *leu1-32 ura4-D18 fbh1-2*	This study
MPF1	*h*$^+$ *leu1-32 ura4-D18 fbh1::LEU2*	This study
MPF2	*h*$^-$ *leu1-32 ura4-D18 fbh1::LEU2*	This study
MPF3	*h*$^+$ *leu1-32 ura4-D18 fbh1::LEU2*	This study
MPF21	*h*$^+$ *fbh1::LEU2 rhp57::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF22	*h*$^+$ *fbh1::LEU2 rhp57::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF23	*h*$^+$ *fbh1::LEU2 rhp57::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF24	*h*$^+$ *fbh1::LEU2 rhp51::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF25	*smt-0 fbh1::LEU2 rhp51::his3*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF41	*h*$^-$ *srs2::Kan*^{r} *ura4-D18 leu1-32*	This study
MPF42	*h*$^-$ *srs2::Kan*^{r} *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF43	*h*$^-$ *rqh1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF44	*h*$^+$ *rqh1::ura4*$^+$ *ura4-D18 leu1-32 his3-D1 arg3-D1*	This study
MPF51	*h*$^+$ *leu1-32 ura4-D18 fbh1*int::pREP42-EGFPN-*fbh1*$^+$	This study
MPF52	*h*$^+$ *leu1-32 ura4-D18 rhp51::his3*$^+$ *fbh1*int::pREP42-EGFPN-*fbh1*$^+$	This study

template switching or reinitiated in a manner dependent on homologous recombination (reviewed in references 6, 11, and 31–33). Replication forks collapse when they encounter a DNA nick or gap, and the nascent fork is regenerated at the site by homologous recombination (24).

In the fission yeast *Schizosaccharomyces pombe*, all of the above-mentioned *RAD52* group genes, in addition to *srs2* and the *recQ* homolog *rqh1*, are conserved and have been implicated to be involved in recombination repair (9, 14, 30, 39, 40, 49, 51).

In an effort to identify further factors involved in recombination repair, we isolated fission yeast *S. pombe* mutants hypersensitive to methanesulfonate (MMS) and synthetic lethal with a *rad2* mutation (50). *rad2* encodes flap endonuclease which removes the 5′ terminus of the Okazaki fragment (41). Mutants in flap endonuclease require recombination for survival in various organisms (24, 47, 54). In these mutants, Okazaki fragments often remain unjoined. If replication forks encounter such nonrejoined sites, they are thought to generate double-strand break (DSB) ends, and homologous recombination is required to repair the DSB ends to maintain survival (24). Thus, synthetic lethal mutants with *rad2* can be expected to be defective in the regeneration of collapsed replication forks by recombination. Following the screening of such mutants, we identified *rhp57* (50), *rad32* (unpublished), *nbs1* (51), *rad60* (36), and *rad62* (35). In this study, we describe the isolation and characterization of mutants of the *fbh1* gene encoding the F-box DNA helicase. The Fbh1 protein was independently identified through purification of a novel *S. pombe* DNA helicase by Park and colleagues (43). They showed that human Fbh1 forms an SCF ubiquitin ligase complex (22, 23). However, the role of Fbh1 in vivo remains unknown. Here, we characterized the function of the *fbh1* gene in *S. pombe*. The results showed that Fbh1 functions in recombination repair on the Rhp51 (*S. pombe* Rad51 ortholog) pathway downstream of Rhp51 and plays a role in processing recombination intermediates.

MATERIALS AND METHODS

***S. pombe* media, methods, and strains.** *S. pombe* cells were grown in YES or EMM medium (34), and standard genetic and molecular procedures were employed as described previously (34). The *S. pombe* strains used in this study are listed in Table 1. Sensitivity of *S. pombe* cells to γ rays and UV irradiation was analyzed as previously described (36). To determine MMS sensitivity, cells were incubated with MMS in YES medium, appropriately diluted following the specified incubation time, and then spread on YES plates. The number of colonies was scored following incubation for 3 to 5 days at 30°C.

Cloning of the *fbh1* gene that complements the *fbh1-1* and *fbh1-2* mutations. *fbh1-1* and *fbh1-2* cells were transformed with the *S. pombe* genomic library (5) constructed using vector pUR19 and spread on EMM plates containing leucine (200 μg/ml) and MMS (0.004%). Transformants were examined for plasmid-dependent MMS resistance. Plasmids that complemented the MMS sensitivity of the *fbh1-1* or *fbh1-2* cells were isolated, transformed into *E. coli* DH5α, and subsequently recovered from the transformants.

Disruption of the *fbh1* gene. The chromosomal *fbh1* gene was disrupted by a method employing two PCR steps as previously described (25). The region upstream of the *fbh1* coding region was amplified using the primers MVF2 (5′-ACACAAAAAGTAATAGAGTC-3′) and MVD-5 (5′-GTCGTGACTGGGAAAACCCTGGCGTTACCCATAACTAAGAATTTGCTGAC-3′). The region downstream of the *fbh1* coding region was amplified using the primers MVD-3 (5′-TCCTGTGTGAAATTGTTATCCGCTCACAATTAGAAACTATTTGATTTGTT-3′) and MVR3 (5′-TGAAATCATCTTTATGATG-3′). The resulting two fragments, in addition to the primers MVF2 and MVR3, were used to amplify the *LEU2* sequence of pJJ282 (20) to generate the fragment for disruption. This fragment was used to transform the haploid *S. pombe* strain MP111, and a transformant with the appropriate disruption was verified by PCR.

Pulsed-field gel electrophoresis. Pulsed-field gel electrophoresis was carried out as previously described (36), except that a 0.5% Megabase agarose (Bio-Rad, Hercules, Calif.) gel and 1× TAE buffer (40 mM Tris-acetate and 1 mM EDTA) were used and run for 60 h at a 120° angle.

Indirect immunofluorescent staining of Rhp51. *S. pombe* cells were fixed and stained for Rhp51 as previously described (8), except that cells were fixed using 3.7% formaldehyde for 30 min. The primary antibody consisted of a rabbit polyclonal antibody raised against recombinant Rhp51 protein expressed in *E. coli* and diluted 500-fold. The secondary antibody was goat anti-rabbit immunoglobulin G conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, Oreg.) and diluted 1,000-fold.

Expression of the EGFP-Fbh1 fusion protein in *S. pombe* cells. *fbh1* cDNA was cloned into the plasmid pREP42 EGFP N (13) to express enhanced green fluorescent protein (EGFP) fusion protein under control of the medium-strength *nmt1* promoter. The resulting plasmid was linearized at the unique NheI site within the *fbh1* cDNA and introduced into the *fbh1* locus of the *S. pombe*

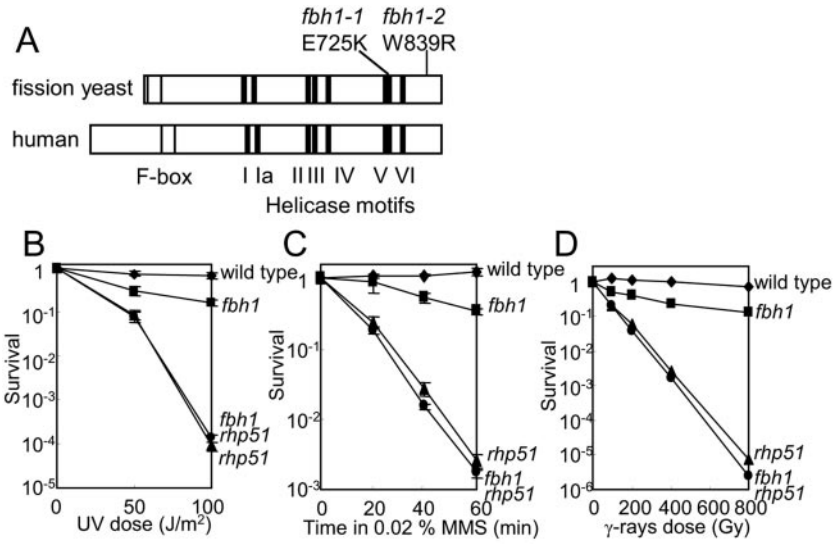

FIG. 1. Identification of the F-box DNA helicase as a factor implicated in recombination repair. (A) Schematic presentation of the distribution of the F-box motif and seven helicase motifs in fission yeast Fbh1 and human Fbh1 proteins, including the *fbh1-1* and *fbh1-2* mutation sites. (B to D) Epistasis between *fbh1* and *rhp51*. Survival curves of wild-type (MP111), *fbh1*Δ (MPF3), *rhp51*Δ (B54), and *fbh1*Δ *rhp51*Δ (MPF25) cells exposed to UV irradiation (B), MMS (0.02%) (C), and γ rays (D) are shown.

genome. The EGFP-Fbh1 fusion protein was expressed by culturing the cells in EMM medium with the appropriate supplements in the absence of thiamine. Cells were fixed with 70% ethanol and observed by fluorescence microscopy.

Measurement of cell length of cells expressing EGFP-Fbh1. Cells expressing EGFP-Fbh1 were treated with 0.1% MMS in EMM medium containing leucine (200 μg/ml) for 1 h, washed twice with EMM2, and then fixed with 70% ethanol. Cells were then observed by fluorescence microscopy, and consecutive focal planes of 1-μm distances were photographed. The cell length was measured directly from the photographs, and cells that carried foci in any of the focal planes were scored as positive for foci.

RESULTS

Cloning of the *fbh1* gene that complements *S. pombe* DNA repair-deficient mutations. The *S. pombe* genomic library was screened for complementation of the MMS-sensitive phenotype of the two mutants previously isolated (50). Identical plasmid clones carrying exactly the same fragment of an *S. pombe* genomic region were obtained independently from the two mutants, and they carried a single open reading frame, SPBC336.01 (*fbh1*). Therefore, the two mutants will be referred to as *fbh1-1* and *fbh1-2*, respectively. *fbh1* encodes a polypeptide comprised of 878 amino acids containing at its N terminus the F-box motif which is known to interact with the Skp1 protein to form an SCF ubiquitin ligase (E3) complex (12) and seven helicase motifs of the superfamily 1 helicases (16) (Fig. 1A).

The *fbh1* region of the *fbh1-1* mutant was recovered utilizing the eviction method (54), and the region obtained carried a single G-to-A nucleotide change, in effect altering the GAA codon for glutamic acid 725 to an AAA codon for lysine. This glutamic acid 725 residue corresponds to a conserved residue within the helicase motif V (Fig. 1A), indicating that helicase activity is important for *fbh1* function in vivo. The *fbh1* region of the *fbh1-2* mutant was amplified by PCR, and the nucleotide sequence was determined. It possessed a single T-to-C nucleotide change, in effect altering the TGG codon for tryptophan 839 near the C terminus to a CGG codon for arginine (Fig. 1A), suggesting that the C-terminal region is important for *fbh1* function in vivo.

The *fbh1*Δ mutant is sensitive to MMS and a synthetic lethal with *rad2*Δ. The *fbh1* gene of the *S. pombe* haploid strain MP111 was disrupted by replacing the entire coding region of the *fbh1* gene with the *LEU2* marker. The resulting *fbh1*Δ strain showed an MMS-sensitive phenotype. The *fbh1*Δ strain was crossed with a wild-type strain and subjected to tetrad analysis. The segregants showed 2+:2− segregation for leucine prototroph and MMS-sensitive phenotypes, where both phenotypes always cosegregated, indicating that the *fbh1*Δ cells are viable and MMS sensitive. The *fbh1*Δ strain was crossed with a *rad2*Δ strain, and the resulting strain was subjected to tetrad analysis. Among the 10 tetrads dissected, no Leu^+ Ura^+ viable segregants were obtained, indicating that the *fbh1*Δ *rad2*Δ double mutant is lethal. Generation time and plating efficiency of *fbh1* cells were 3.7 h and 39%, respectively, while in wild-type cells, they were 2.4 h and 96%, respectively, on YES medium at 30°C. Spontaneous recombination frequency between direct repeats was not affected by the *fbh1* mutation (data not shown).

***fbh1* works on the *rhp51* pathway for recombination repair.** *fbh1*Δ cells were more sensitive to UV irradiation, MMS, and γ rays than wild-type cells (Fig. 1B to D). Homologous recombination represents a major pathway for the repair of radiation-induced DSBs in *S. pombe*. *rhp51*, the *S. pombe* ortholog of *S. cerevisiae RAD51* (19, 37), plays a central role in this process (39). Therefore, the relationship between *fbh1* and *rhp51* was examined. As shown in Fig. 1B to D, the *rhp51*Δ mutant was more sensitive to UV irradiation, MMS, and γ rays than the *fbh1*Δ mutant. The *fbh1*Δ *rhp51*Δ double mutant and the *rhp51*Δ single mutant showed similar sensitivity to UV irradiation, MMS, and γ rays (Fig. 1B to D). These results indicate that *rhp51* is epistatic to *fbh1* with respect to DNA

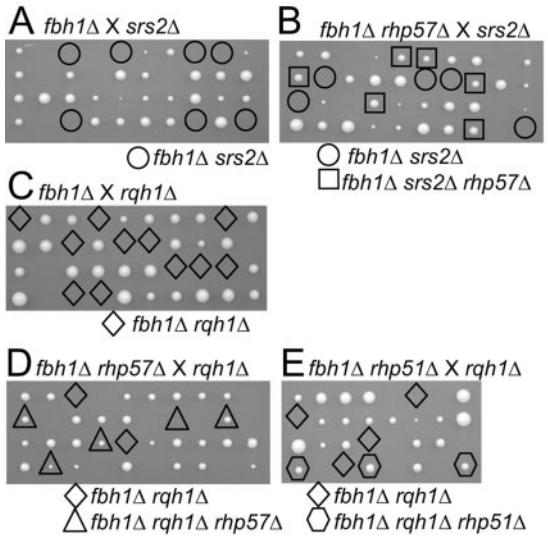

FIG. 2. Lethality of *fbh1*Δ *srs2*Δ and *fbh1*Δ *rqh1*Δ mutants that is suppressed by loss of recombination genes. Spores of crosses between *fbh1*Δ (MPF3) and *srs2*Δ (MPF41) (A), *fbh1*Δ *rhp57*Δ (MPF21) and *srs2*Δ (MPF42) (B), *fbh1*Δ (MPF3) and *rqh1*Δ (MPF43) (C), *fbh1*Δ *rhp57*Δ (MPF22) and *rqh1*Δ (MPF43) (D), or *fbh1*Δ *rhp51*Δ (MPF25) and *rqh1*Δ (MPF44) (E) were subjected to tetrad analysis. Genotypes of inviable segregants were predicted by assuming Mendelian inheritance.

repair and that *fbh1* works in the *rhp51* pathway related to recombination repair.

Synthetic growth defects of the *fbh1*Δ *srs2*Δ and *fbh1*Δ *rqh1*Δ double mutants are suppressed by loss of the recombination gene *rhp57*. Fbh1 helicase belongs to superfamily 1 of helicases that includes Srs2. Therefore, we examined the functional relationship between *fbh1* and *srs2*. The *fbh1*Δ strain was crossed with the *srs2*Δ strain, and the resulting spores were subjected to tetrad analysis. Of the 10 tetrads dissected, no *fbh1*Δ *srs2*Δ segregants gave viable colonies, indicating that the *fbh1*Δ *srs2*Δ double mutants are lethal (Fig. 2A). *rhp57* is the *S. pombe* ortholog of *S. cerevisiae RAD57*, whose product forms a heterodimer with Rad55 and is implicated in the loading of Rad51 to RPA-coated single-strand DNA (46). Rhp57 and Rhp55 play a role in the subpathway of Rhp51 since *rhp57* and *rhp55* mutants are less sensitive to DNA-damaging agents than the *rhp51* mutant (1, 21, 50). The *fbh1*Δ *rhp57*Δ double mutant was crossed with the *srs2*Δ mutant, and the resulting spores were subjected to tetrad analysis. Of the 10 tetrads dissected, six *fbh1*Δ *srs2*Δ *rhp57*Δ triple mutants and no *fbh1*Δ *srs2*Δ double mutants gave viable colonies (Fig. 2B). The size of the *fbh1*Δ *srs2*Δ *rhp57*Δ triple mutant colonies was comparable to that of *rhp57*Δ cells, indicating that the *rhp57*Δ mutation suppressed the lethality of the *fbh1*Δ *srs2*Δ double mutant. We were unable to determine whether *rhp51*Δ restored *srs2*Δ *fbh1*Δ inviability, since crosses between *fbh1*Δ *rhp51*Δ and *srs2*Δ strains produced only a few viable segregants (13 segregants out of 10 tetrads) by tetrad analysis. Low viability of the *rhp51* cells could to some extent contribute to this spore inviability.

In *S. cerevisiae*, the *srs2*Δ *sgs1*Δ double mutants are lethal or severely defective in growth, and the growth defect is suppressed by loss of the *RAD51* gene (15, 28). A similar relationship has been shown in *S. pombe*, where the *srs2*Δ *rqh1*Δ double mutants are severely impaired for growth and the growth defect is suppressed by loss of the *rhp57* or *rhp51* gene (14, 30). The relationship between *fbh1*Δ and *rqh1*Δ was analyzed by crossing an *fbh1*Δ and an *rqh1*Δ strain. Of the 10 tetrads dissected, no *fbh1*Δ *rqh1*Δ segregants gave viable colonies, indicating that the *fbh1*Δ *rqh1*Δ double mutants are lethal (Fig. 2C). The *fbh1*Δ *rhp57*Δ double mutant was crossed with the *rqh1*Δ mutant, and the resulting spores were subjected to tetrad analysis. Of the 10 tetrads dissected, four *fbh1*Δ *rqh1*Δ *rhp57*Δ triple mutants and no *fbh1*Δ *rqh1*Δ double mutants gave viable colonies (Fig. 2D). The size of the *fbh1*Δ *rqh1*Δ *rhp57*Δ triple mutant colonies was comparable to that of *rhp57*Δ cells, indicating that the *rhp57*Δ mutation suppressed the lethality of the *fbh1*Δ *rqh1*Δ double mutant. Colonies from the tetrads of the cross between *fbh1*Δ *rhp51*Δ and *rqh1*Δ gave rise to three *fbh1*Δ *rqh1*Δ *rhp51*Δ triple mutants and no *fbh1*Δ *rqh1*Δ double mutants (Fig. 2E), indicating that the *rhp51* mutation suppressed the lethality of the *fbh1*Δ *rqh1*Δ double mutant. These results indicate that homologous recombination is responsible for cell death in the *fbh1*Δ *srs2*Δ and *fbh1*Δ *rqh1*Δ double mutants and suggest that the three helicase genes *fbh1*, *rqh1*, and *srs2* act downstream of *rhp51* and *rhp57*.

Nuclear chromosomal domain shrinks in the *fbh1*Δ mutant and extends in the *rhp51*Δ and *rhp51*Δ *fbh1*Δ mutants following UV irradiation. The nuclear chromosomal domains of *fbh1*Δ mutant cells were examined by staining with 4′,6′-diamidino-2-phenylindole (DAPI). Six hours after UV irradiation, the nuclear chromosomal domains of approximately half of the *fbh1*Δ cells appeared like a compact sphere and were smaller than the hemispherical nuclear chromosomal domains of wild-type cells (Fig. 3A). Additionally, staining of the nucleolus with ethidium bromide highlighted the difference in nuclear morphology more clearly. In the *fbh1*Δ mutant, the nuclear chromosomal domains and nucleolus (stained less brightly) formed separate spheres, while the nuclear chromosomal domains and nucleolus of wild-type cells constituted the same spheres (Fig. 3B). In contrast to the *fbh1*Δ mutant, the nuclear chromosomal domains of the *rhp51*Δ mutant and the *fbh1*Δ *rhp51*Δ double mutant were more extended and amorphous in contrast to the hemispherical shape of wild-type cells when observed 6 h after UV irradiation (Fig. 3A). Thus, *rhp51*Δ is epistatic to *fbh1*Δ with respect to the morphology of the nuclear chromosomal domain following UV irradiation. This also suggests that *fbh1* functions downstream of *rhp51*.

Recombination intermediates remain unresolved in the *fbh1*Δ mutant after UV irradiation. The difference in nuclear morphology described above suggests a difference in chromosomal structure among the wild-type, *fbh1*Δ, and *rhp51*Δ cells grown after UV irradiation. Therefore, we analyzed chromosomes in these mutants by pulsed-field gel electrophoresis.

The intensity of the three chromosomal bands decreased dramatically 2 h after UV irradiation of wild-type cells, whereas the band intensity remained largely unaltered for at least up to 2 h after UV irradiation of *rhp51*Δ mutants (Fig. 3C). This difference probably reflects the formation of certain recombination intermediates dependent on the function of Rhp51, which migrated poorly into the gel and therefore remained at the position of the loading well. These intermediates

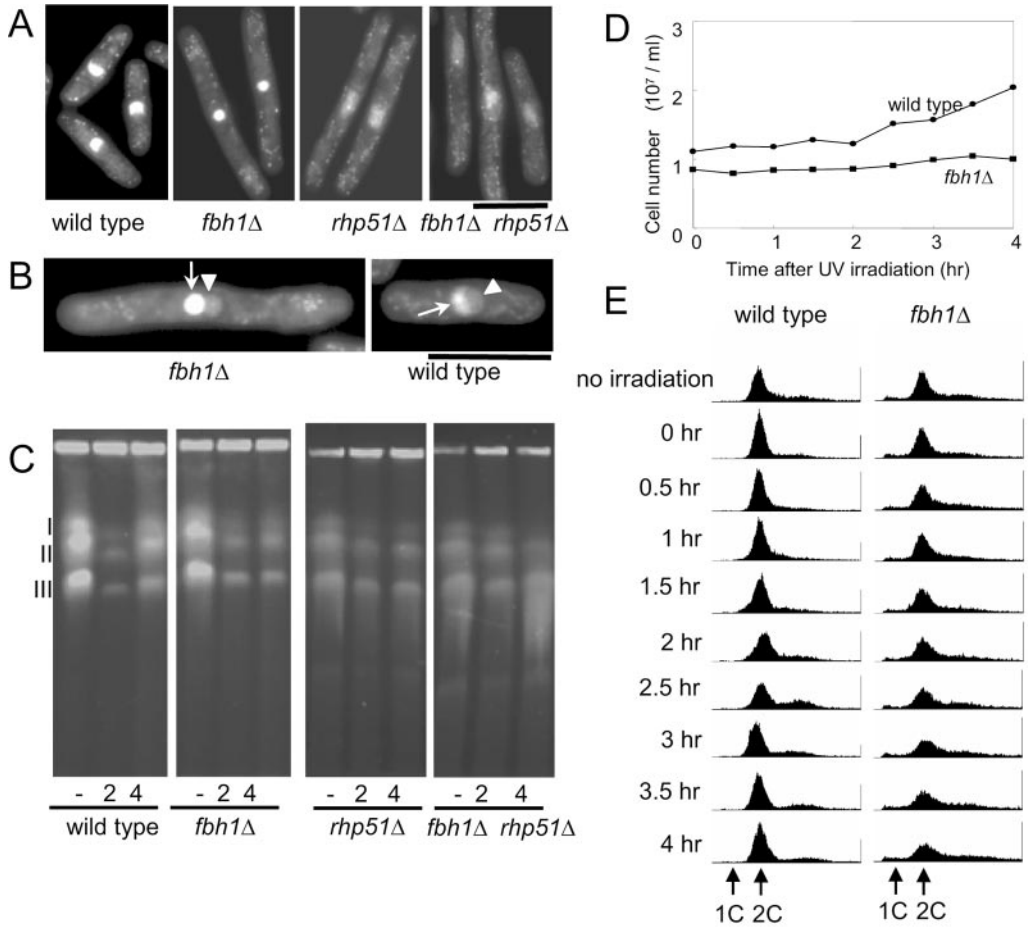

FIG. 3. Nuclear shrinkage and chromosomal aberration of the *fbh1* mutant following UV irradiation. (A) Cells of wild-type (MP111), *fbh1*Δ (MPF3), *rhp51*Δ (B54), and *fbh1*Δ *rhp51*Δ (MPF25) strains were UV irradiated (200 J/m^2), cultured for 6 h at 30°C, fixed with glutaraldehyde (2.5%), stained with DAPI (1 μg/ml), and then photographed using a fluorescence microscope. Representative cells with shrunken or normal nuclei are shown. The scale bar indicates 10 μm. (B) *fbh1*Δ (MPF3) cells or wild-type (MP111) cells UV irradiated and fixed as described above (A) were stained with DAPI (1 μg/ml) and ethidium bromide (10 μg/ml) and then photographed using a fluorescence microscope. The chromosomal region stained with DAPI and nucleolar region (less bright) stained with ethidium bromide are shown by arrows and arrowheads, respectively. The scale bar indicates 10 μm. (C) Wild-type (MPF111), *fbh1*Δ (MPF3), *rhp51*Δ (B54), and *fbh1*Δ *rhp51*Δ (MPF25) cells were UV irradiated (200 J/m^2). Chromosomes of cells before (−) and 2, 4, or 6 h after UV irradiation were analyzed by pulsed-field gel electrophoresis. Chromosomes from 10^8 cells were loaded onto each lane. The three chromosomes of *S. pombe* are indicated by I, II, and III, respectively. (D and E) Wild-type (MP111) or *fbh1*Δ (MPF3) cells were UV irradiated (200 J/m^2) and then cultivated in YES medium at 30°C. Cell numbers were scored at the indicated time after irradiation (D). Cells were fixed in 70% ethanol and processed for fluorescence-activated cell sorter analysis (E) as described previously (45).

that had accumulated in the *fbh1*Δ cells are not likely to be replication intermediates, since the major population of exponentially growing *S. pombe* cells are at G_2 phase (29), and UV irradiation prevents entry of G_2 cells into mitosis (2). Consistent with this, cell number and DNA contents only slightly increased during 2 h after UV irradiation in either wild-type or the *fbh1*Δ strains (Fig. 3D and E). The intensity of the chromosomal bands was recovered 4 h following UV irradiation of wild-type cells to the level of the unirradiated control (Fig. 3C), indicating that the recombination intermediates had been resolved. In contrast, the signals of the three chromosomes was not recovered up to 4 h after UV irradiation in the *fbh1*Δ mutant (Fig. 3C), indicating that recombination intermediates remained unresolved in the *fbh1*Δ mutant. In the *fbh1*Δ *rhp51*Δ mutant, the chromosomal signal pattern was not altered upon incubation after UV irradiation at least up to 2 h and similar to that of the *rhp51*Δ mutant (Fig. 3C), indicating that *rhp51*Δ is epistatic to *fbh1*Δ in this regard. These results suggested that the *fbh1*Δ mutant is defective in the processing of recombination intermediates, the formation of which is dependent on Rhp51 function.

The *fbh1*Δ mutant dies following entry into the stationary phase, and this lethality is suppressed by the *rhp57*Δ mutation. During maintenance of the *fbh1*Δ mutant, we found that the *fbh1*Δ mutant has a defect in growth recovery when the culture in stationary phase was diluted with fresh medium. The growth kinetics of the *fbh1*Δ mutant were therefore examined from log phase to the stationary phase. The *fbh1*Δ mutant ceased growth at a lower cell density than the wild-type strain, i.e., at ca. 4×10^7 cells/ml for *fbh1*Δ and at ca. 1.5×10^8 cells/ml for the wild type (Fig. 4A). When the *fbh1*Δ mutant reached this maximum cell density (time, 16 h [Fig. 4A]), the

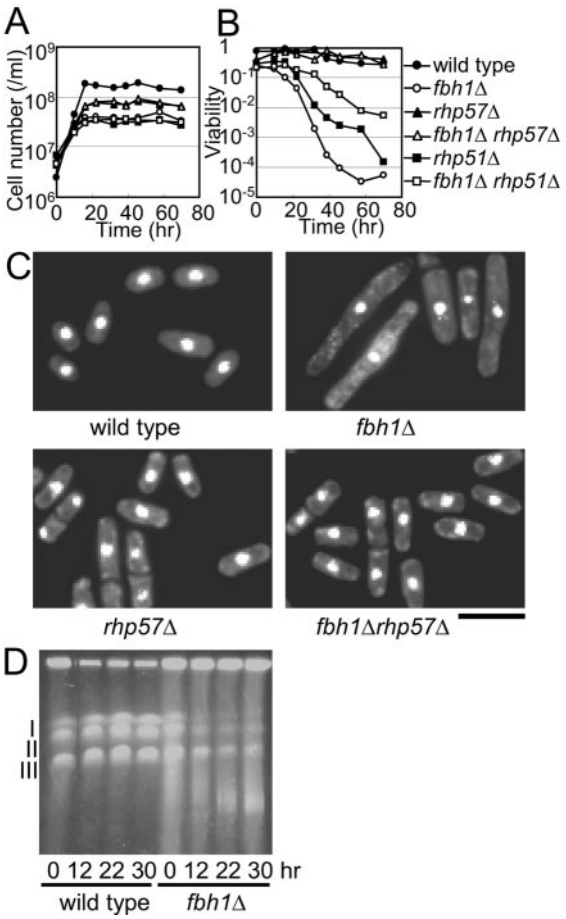

FIG. 4. Cell death, chromosomal aberration, and nuclear shrinkage in the *fbh1* mutant upon entry into the stationary phase. (A, B) Cells of wild-type (MP111), *fbh1*Δ (MPF3), *rhp57*Δ (B63), *fbh1*Δ *rhp57*Δ (MPF21), *rhp51*Δ (B54), and *fbh1*Δ *rhp51*Δ (MPF25) strains were grown in YES medium at 30°C from exponential phase to the stationary phase. Cells were sampled at the indicated time points and analyzed for cell number per milliliter (A) and number of CFU per milliliter. Viability of the cells was determined by dividing the number of CFU by the cell number at each time point (B). (C) Wild-type (MP111), *fbh1*Δ (MPF3), *rhp57*Δ (B63), and *fbh1*Δ *rhp57*Δ (MPF23) strains growing in mid-log phase (around 5×10^6 cells /ml) were cultured for a further 24 h in YES medium at 30°C to saturation, fixed with methanol, stained with DAPI, and then photographed using a fluorescence microscope. The scale bar indicates 10 μm. (D) Wild-type (MP111) or *fbh1*Δ (MPF3) cells at a density of $1 \times 10^7 \sim 2 \times 10^7$ cells/ml were grown in YES medium at 30°C. Cells were sampled at the indicated time points, and their chromosomes were analyzed by pulsed-field gel electrophoresis. Chromosomes of 10^8 cells were loaded onto each lane. The three chromosomes of *S. pombe* are indicated by I, II, and III, respectively.

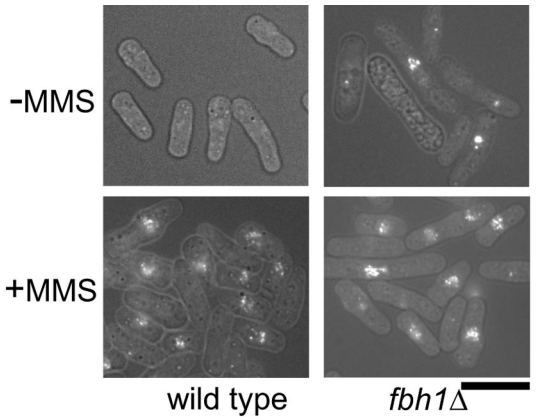

FIG. 5. Rhp51 focus formation in the *fbh1*Δ mutant. Wild-type (MP111) or *fbh1*Δ (MPF3) cells without (−) or with (+) MMS (0.025%) treatment for 1 h were processed for indirect immunofluorescence staining by employing anti-Rhp51 antibody and then photographed using a fluorescence microscope. The scale bar indicates 10 μm.

proportion of viable cells dropped to ca. 10% (time, 16 h [Fig. 4B]) and continued dropping following further incubation. This suggested that the *fbh1*Δ mutant dies at a late growth phase and is thus unable to reach the cell density of wild-type cells at the stationary phase. The viability of the *rhp51*Δ and *fbh1*Δ *rhp51*Δ mutants also decreased upon entry into the stationary phase (Fig. 4B), even though the time course of their cell death was slower than that of the *fbh1*Δ mutant. This indicates that recombination is required for survival of the cells entering into the stationary phase. In contrast to *fbh1*Δ cells, *fbh1*Δ *rhp57*Δ double mutant cells did not die upon entry into the stationary phase (Fig. 4B), indicating that the *rhp57*Δ mutation suppressed the lethal phenotype of the *fbh1*Δ mutant entering the stationary phase. Similar to UV-irradiated *fbh1*Δ cells, *fbh1*Δ cells entering the stationary phase had shrunken nuclear chromosomal domains, and this shrinkage was also suppressed by the *rhp57*Δ mutation (Fig. 4C). Pulsed-field gel electrophoresis showed a dramatic decrease in the band intensities of the three chromosomes as *fbh1*Δ cells entered the stationary phase (Fig. 4D), indicating persistence of the DNA structure representing replication or recombination intermediates. Further incubation resulted in the appearance of a faster-migrating smear signal (22 and 30 h [Fig. 4D]), suggesting that some of the chromosomes eventually became fragmented, probably due to a failure in proper processing of the damaged DNA.

Spontaneous formation of Rhp51 foci in the *fbh1*Δ mutant. Rhp51 forms nuclear foci following DNA damage, and the foci are thought to represent recombination intermediates containing nucleoprotein filaments (8). In an effort to explore the localization of Rhp51 in the *fbh1*Δ mutant, Rhp51 foci were stained by an indirect immunofluorescent method employing anti-Rhp51 polyclonal antibodies. Wild-type and *fbh1*Δ mutant cells were stained either prior to or following treatment with MMS (0.025%) for 1 h (Fig. 5). Rhp51 foci were observed in 35% (44 out of 125) of the *fbh1*Δ mutant cells and in only 6% (7 out of 108) of wild-type cells prior to MMS treatment. This suggests that spontaneously arising Rhp51 nucleoprotein filaments persist longer in *fbh1*Δ cells than in wild-type cells. The proportion of cells carrying Rhp51 foci reached to 68% (89 out of 130) in wild-type and 66% (89 out of 134) in *fbh1*Δ cells following MMS treatment, indicating that *fbh1*Δ cells are proficient in Rhp51 focal induction in response to treatments that damage DNA.

Fbh1 focus formation in response to DNA damage. In an effort to explore the localization of the Fbh1 protein in cells, we expressed an EGFP-Fbh1 fusion protein in *S. pombe* cells

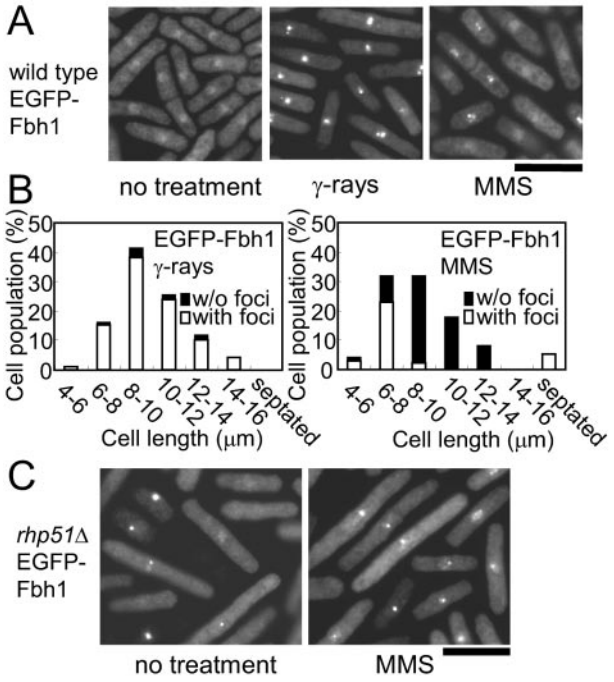

FIG. 6. EGFP-Fbh1 foci induced by DNA-damaging agents or appearing spontaneously in the *rhp51*Δ mutant. (A) Cells expressing the EGFP-Fbh1 fusion protein (MPF51) without treatment, irradiated with γ rays (500 Gy), and cultured for 1 h at 30°C or treated with MMS (0.1%) for 1 h at 30°C were photographed using a fluorescence microscope. The scale bar indicates 10 μm. (B) Cells expressing the EGFP-Fbh1 fusion protein (MPF51) irradiated with γ rays (500 Gy) and cultured for 1 h at 30°C or treated with MMS (0.1%) for 1 h at 30°C were photographed using a fluorescence microscope, and the distribution of cell lengths was determined. Septated cells were classified separately. Open and solid bars indicate cells with or without Fbh1 foci, respectively. (C) *rhp51*Δ cells expressing the EGFP-Fbh1 fusion protein (MPF52) without or with MMS (0.1%) treatment for 1 h were photographed using a fluorescence microscope. The scale bar indicates 10 μm.

and determined its localization by fluorescence microscopy. The EGFP-Fbh1 fusion protein was expressed under the control of the medium-strength version of the modified thiamine-repressible *nmt1* promoter (7). When the expression was repressed by culturing the cells in YES medium containing thiamine, the EGFP signal could not be detected following either DNA-damaging treatments or no treatment. When the expression was derepressed in synthetic medium (EMM2 with appropriate supplements) in the absence of thiamine, a faint EGFP signal was observed throughout the cell body, with the nucleus being slightly brighter than the cytoplasm under normal growth conditions (Fig. 6A). Following a 1-h incubation after irradiation with γ rays at 500 Gy, most of the cells formed nuclear foci (Fig. 6A and B), suggesting that Fbh1 forms nuclear foci in response to DNA strand breakage. Similarly, treatment with MMS (0.1%) for 1 h gave rise to nuclear foci which were restricted essentially to relatively short cells and septated cells (Fig. 6A and B). Given that *S. pombe* cells septate around the S phase (29), this result suggests that Fbh1 foci are formed only in cells that are in S phase during MMS treatment. These results are consistent with the notion that Fbh1 plays a role in the recombination repair of strand breaks and stalled or collapsed replication forks. In the absence of treatments that damage DNA, EGFP-Fbh1 foci were observed in only 2% (8 out of 347) of wild-type cells, while spontaneous EGFP-Fbh1 foci were observed in 41% (93 out of 225) of the *rhp51*Δ mutant cells (Fig. 6C). These results suggest that both Fbh1 and Rhp51 are required to repair spontaneous DNA damage and that the focus formation of them does not require the function of the other.

DISCUSSION

In this study, we presented several pieces of evidence indicating that Fbh1 functions downstream of *rhp51* and *rhp57* and is involved in the processing of certain forms of recombination intermediates. First, the *fbh1*Δ *srs2*Δ and *fbh1*Δ *rqh1*Δ double mutants are lethal, and this lethality is suppressed by the *rhp57*Δ mutation. Second, the nuclear chromosomal domain shrinks in the *fbh1*Δ mutant while it extends in the *rhp51*Δ and *rhp51*Δ *fbh1*Δ mutants following UV irradiation. Third, pulsed-field gel electrophoretic analysis indicates that *fbh1*Δ cells are defective in the processing of certain forms of recombination intermediates formed following UV irradiation and that *rhp51*Δ and *rhp51*Δ *fbh1*Δ cells are defective in the formation. Fourth, the *fbh1*Δ mutant dies at late log phase before entry into the stationary phase, and this lethality is suppressed by the *rhp57*Δ mutation. Both the Srs2 helicase and Rqh1/Sgs1 RecQ-like helicases have been implicated to be involved in the processing of recombination intermediates (4, 6). In fact, the *S. cerevisiae srs2*Δ *sgs1*Δ double mutant is lethal (28), and this lethality is suppressed by the loss of recombination initiation (15). Thus, the absence of Srs2 or Rqh1 may cause an accumulation of spontaneously arising toxic recombination intermediates that require *fbh1* for their resolution. Deleting *rhp51* or *rhp57* would prevent the formation of these toxic intermediates and thereby enable DNA damage to be repaired by an alternative pathway. Fbh1 is a member of the same DNA helicase family as Srs2. This family also includes PcrA, Rep, and UvrD helicases in bacteria. The *Bacillus subtilis pcrA* mutant is lethal, and this lethality is suppressed by *recF*, *recO*, and *recR* mutations (44). Similarly, the *E. coli rep uvrD* double mutant is lethal, and this lethality is also suppressed by *recF*, *recO*, and *recR* mutations (44). This suppression is analogous to the case of the *fbh1*Δ *srs2*Δ double mutant, where suppression of cell death is achieved by the *rhp57* mutation. Thus, assuming functional resemblance among helicases in this family, Fbh1 and Srs2 might share a redundant function that is analogous to the function of Rep, UvrD, or PcrA. Srs2 dissociates Rad51 nucleoprotein filaments (26, 53), and UvrD dissociates RecA nucleoprotein filaments (52). Structural resemblance between these helicases suggests that Fbh1 may dissociate Rhp51 from DNA. Pulsed-field gel electrophoretic analysis indicated a defect in *fbh1*Δ cells for the processing of recombination intermediates. Fbh1 could dissociate Rhp51 from double-strand DNA following the strand exchange reaction, thereby resolving the D-loop structure, instead of dissociating Rhp51 from single-strand DNA. Such a role has been proposed for the Srs2 protein in the synthesis-dependent strand-annealing reaction based on the observation that Srs2 suppresses crossovers (18). As either the *fbh1* or *srs2* single mutant shows sensitivity to DNA-damaging agents, there

would be a difference between reactions carried out by Fbh1 and Srs2. Further investigations will be required to address this possibility.

The *fbh1*Δ cells die when they are entering the stationary phase. As the *fbh1*Δ mutant ceased growth at a lower cell density than the wild-type strain, the cell death seems to have occurred during last several rounds of cell cycles, possibly during S phases. Dramatic physiological changes are assumed to occur at the late log phase. For example, glucose becomes exhausted, and utilization of nonfermentable carbon sources depends on respiration, probably leading to an increase in reactive oxygen species that damage DNA (10). Alternatively, cells may change nucleosomal structures to alter patterns of gene expression, thereby adapting to the stationary phase. Some of the nucleosomal structural changes that modify gene expression patterns may also inhibit replication fork progression. Therefore, it is possible that replication fork progression is more often inhibited during the late log phase prior to the cessation of cell proliferation and that Fbh1 is required to deal with this fork inhibition through its recombination function. Consistent with this notion, stable DNA replication that depends on recombination (24) has been detected in rapidly growing *E. coli* cells at the time of entry into the stationary phase (17). Deletion of *rhp57* suppresses the loss of cell viability in *fbh1*Δ cells entering the stationary phase. As the loading of Rhp51 onto DNA and stabilization of Rhp51 nucleofilament would be compromised in the absence of Rhp57, the accumulation of toxic recombination intermediates would be reduced, negating the need for *fbh1*.

The *fbh1* mutant is less sensitive to DNA-damaging agents than the *rhp51* mutant, but this is not the case for viability upon entry into stationary phase. This lethality upon entry into stationary phase is suppressed by the *rhp57* mutation. As Rhp55-57 is thought to constitute only a part of the Rhp51 pathway (1, 50), it can be explained by assuming that only certain types of recombination intermediates are made by the combination of Rhp51 and Rhp57, and these might be acted on by Fbh1. Replication forks inhibited at late log phase could be mainly acted upon by a combination of Rhp51 and Rhp57, and the absence of Fbh1 could result in failure to resolve recombination intermediates and hence could result in accumulation of toxic intermediates. In the absence of Rhp57, the inhibited forks could be resumed by recombination that does not employ Rhp57, and hence, the absence of Fbh1 would not cause accumulation of toxic recombination intermediates. *rhp51* is epistatic to *fbh1* in survival in DNA-damaging agents, but this is not the case for survival upon entry into stationary phase, and *rhp51* and *fbh1* showed mutual suppression. To explain this, we should consider Rhp51-independent recombination repair, and we leave this issue for further study.

Formation of EGFP-Fbh1 foci in γ-ray-treated cells and MMS-treated S-phase cells is consistent with the hypothesis that Fbh1 functions in the recombination repair of strand breaks and stalled or collapsed replication forks. The EGFP-Fbh1 fusion construct complemented the MMS sensitivity of the *fbh1*Δ cells in the presence of thiamine (data not shown), indicating that the EGFP-Fbh1 fusion protein is functional, and the repressed expression level is sufficient for this complementation. Under this repressed condition, we could not detect the EGFP signal. When cells were cultured in medium in the absence of thiamine to derepress expression, Fbh1 foci were observed in a small percentage of cells. Proportions of the cells with Fbh1 foci increased greatly after DNA-damaging treatments, suggesting that Fbh1 is recruited to the damaged sites to repair them. Under this derepressed condition, expression of the EGFP-Fbh1 fusion protein sensitizes the cells to MMS (data not shown). It could be that overexpression of EGFP-Fbh1 leads to the accumulation of more molecules per single focus, facilitating detection of the fluorescence signal, and the accumulation of too many Fbh1 molecules would be deleterious to normal DNA repair.

The Rhp51 foci were observed in 6% of exponentially growing wild-type cells. The *rhp51*Δ mutant cells grew more slowly than wild-type cells (38). Therefore, Rhp51-dependent recombination is required for repairing DNA damage that spontaneously arises during normal growth. Spontaneous Rhp51 foci were observed in 35% of the *fbh1*Δ cells, and this proportion was much higher than that in wild-type cells. In the absence of Fbh1, the Rhp51 nucleoprotein filament might be formed spontaneously, and further processing of the DNA lesion is defective, leading to the accumulation of recombination intermediates containing Rhp51 nucleofilament. Spontaneous Fbh1 foci were observed in only 2% of wild-type cells, while they were observed in 41% of the *rhp51*Δ cells. In the absence of Rhp51, Fbh1 is localized at the damaged DNA site, even though the DNA lesion is not processed further. Therefore, these results suggest that Rhp51 and Fbh1 are required for the processing of common DNA damage inflicted by endogenous agents.

SCF complexes act as ubiquitin ligases (E3), and the F-box component determines substrate specificity by directly interacting with the substrate at a protein recognition motif outside of the F-box motif (12). Introduction of mutations in the F-box motif sensitized *S. pombe* cells to MMS to a level similar to that of the *fbh1*Δ mutant (unpublished results), indicating that the F box is essential for Fbh1 function. The identity of the substrate of the SCF complex containing Fbh1 remains unknown. One candidate for the substrate is Rhp51, since Rhp51 is degraded when wild-type *S. pombe* cells enter the stationary phase, and this degradation is abolished in *fbh1*Δ cells (unpublished results). However, we could not detect ubiquitinated Rhp51 protein under various conditions, nor could we observe Rhp51 degradation in log-phase cultures. Further analysis is required to precisely delineate the role of the Fbh1-containing SCF complex.

Just as in yeast, RecQ family helicases in humans are required for maintaining genome stability. Indeed, mutations in three RecQ helicases, Blm, Wrn and Rts, result in the cancer predisposition syndromes Bloom, Werner, and Rothmund-Thomson syndromes, respectively (4). We suspect that the human ortholog of Fbh1 (FBH1) will also prove to be important for maintaining genome stability. Intriguingly, no ortholog of Srs2 has been identified in humans. It is possible that FBH1 fulfills the role in humans as yeast Srs2.

An *fbh1* mutation was independently isolated as a suppressor of the *rad22*Δ mutant by Whitby's group. The phenotypes of the *fbh1* mutants were in agreement with ours (41a).

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