The Human F-Box DNA Helicase FBH1 Faces *Saccharomyces cerevisiae* Srs2 and Postreplication Repair Pathway Roles[▽]

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The *Saccharomyces cerevisiae* Srs2 UvrD DNA helicase controls genome integrity by preventing unscheduled recombination events. While Srs2 orthologues have been identified in prokaryotic and lower eukaryotic organisms, human orthologues of Srs2 have not been described so far. We found that the human F-box DNA helicase hFBH1 suppresses specific recombination defects of *S. cerevisiae* srs2 mutants, consistent with the finding that the helicase domain of hFBH1 is highly conserved with that of Srs2. Surprisingly, hFBH1 in the absence of SRS2 also suppresses the DNA damage sensitivity caused by inactivation of postreplication repair-dependent functions leading to PCNA ubiquitylation. The F-box domain of hFBH1, which is not present in Srs2, is crucial for hFBH1 functions in substituting for Srs2 and postreplication repair factors. Furthermore, our findings indicate that an intact F-box domain, acting as an SCF ubiquitin ligase, is required for the DNA damage-induced degradation of hFBH1 itself. Overall, our findings suggest that the hFBH1 helicase is a functional human orthologue of budding yeast Srs2 that also possesses self-regulation properties necessary to execute its recombination functions.

DNA lesions occur frequently in living cells as a result of spontaneous events or external insults. Growing evidence suggests that the selection of the appropriate DNA repair pathway to deal with broken DNA molecules is crucial for preventing genome instability.

The *Saccharomyces cerevisiae* Srs2 protein is a 3'-5' DNA helicase (40) structurally and functionally related to bacterial UvrD (2, 51). It is thought that Srs2 plays a key role in influencing the choice between homologous recombination (HR) and postreplication repair (PRR) pathways, both of which are required to counteract the accumulation of gaps during DNA replication (5, 45). A body of evidence suggests that Srs2 inhibits HR at an early step (1, 7, 11, 20, 29, 39, 44), acting as a DNA translocase that disassembles the Rad51 nucleofilament (26, 52). Accordingly, srs2Δ mutants show hyperactivation of spontaneous recombination events (41), and unrestrained HR is the source of cell death in srs2 mutants when other factors operating at later stages in recombination are also inactivated. This is the case for Sgs1 (14) and Rad54 (23) helicases, which are involved in the resolution of mature recombination intermediates and in promoting D-loop formation and/or stabilization (47, 55), respectively.

Current models indicate that Srs2 inhibits HR and channels DNA lesions towards the PRR pathway. This model is mainly supported by the observation that the DNA damage sensitivity of PRR mutants can be rescued by SRS2 inactivation in the presence of a functional HR pathway (5, 45). The PRR pathway seems to be required to tolerate rather than immediately repair the DNA damage, using both specialized translesion synthesis DNA polymerases and a less-characterized error-free repair branch that is thought to involve a recombination-dependent replication mechanism, such as template switching (5). The PRR pathway is promoted by two diverse protein complexes that contain ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes and that modify PCNA on lysine (K) 164. In particular, the Rad6 (E2)-Rad18 (E3) complex mono-ubiquitylates PCNA on K164, while Rad5 (E3), along with the heterodimeric Mms2-Ubc13 (E2 variant) enzyme, subsequently attaches polyubiquitin chains via K63 to the mono-ubiquitylated K164 residue (18). Polyubiquitin chains linked by K63 isopeptide bonds, in contrast to those assembled in K48 conformation, do not mark the modified proteins for degradation but, rather, signal for other cellular transactions (37). The K164 residue of PCNA can also be sumoylated by a Ubc9/Siz1-dependent pathway (18). Recent evidence obtained with budding yeast indicates that the modification status of PCNA is crucial in determining the PRR subpathway that will be engaged in front of a lesion. In particular, the Srs2 antirecombinogenic function is enhanced by its physical interaction with sumoylated PCNA (34, 36), sumoylation and mono-ubiquitylation of PCNA contribute to spontaneous mutagenesis mediated by translesion DNA polymerases (46), and the polyubiquitylation of PCNA might promote the error-free PRR subpathway (18). In spite of the suggestion that sumoylated

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PCNA, by recruiting Srs2 at replication forks, would play a key role in preventing HR during S phase (34, 36), recent studies failed to detect the accumulation of recombination intermediates at damaged replication forks in *srs2* or even PCNA mutants, in which sumoylation and/or ubiquitylation is abrogated (4, 29). On the other hand, recombination intermediates broadly accumulate at damaged forks in *sgs1* or *ubc9/mms21* mutants (4, 29), suggesting that the control of HR during replication is far more complicated than the sole regulation of Srs2. Moreover, a number of *srs2* recombination phenotypes cannot easily be explained by considering Srs2 only as a protein that counteracts Rad51-mediated strand invasion. Rather, many genetic findings suggest that Srs2 operates at diverse levels in recombination, downstream of the strand invasion step (39), and possibly in promoting double-strand-break (DSB) repair by HR (3, 19, 20, 35).

Despite its key role in maintaining genome integrity in yeast, Srs2 has no human orthologues identified so far. However, the Srs2 DNA helicase has been conserved in other fungi, such as *Schizosaccharomyces pombe* and *Neurospora crassa*, and the genetic characterization of null mutants in both organisms showed that the Srs2 orthologues are fundamental players in HR (10, 30, 31, 48, 53). Genetic analysis carried out with fission yeast suggests that Srs2 has overlapping functions in processing recombination intermediates with both Rqh1 and Fbh1 DNA helicases (32, 33). Fbh1 orthologues have been found in mice, chickens, and humans but not in budding yeast (21, 24). In DT40 cells, Fbh1 and BLM proteins cooperate in constraining the extent of sister chromatid exchanges (SCEs) in HR (24). Unusually for DNA unwinding enzymes, Fbh1 DNA helicases possess an F-box motif (22). Studies performed on human cell extracts indicated that human Fbh1 (hFBH1), through the F-box domain, is part of a Skp–Cullin–F-box (SCF) ubiquitin ligase complex; while it has been shown that the E3 SCF complex containing hFBH1 promotes the assembly of polyubiquitin chains, the ubiquitin targets of hFBH1 are still unknown (22).

The aim of our study was to identify a human orthologue for budding yeast Srs2. Here we show that the F-box DNA helicase hFBH1 suppresses specific recombination defects of *S. cerevisiae srs2* mutants. Furthermore, when hFBH1 substitutes for Srs2, the PRR functions required to induce the K164 ubiquitylation of PCNA are dispensable for cell survival upon DNA damage treatment. The F-box domain is essential for hFBH1 functions in substituting for Srs2 and PRR roles, and it controls the DNA damage-induced turnover of hFBH1 by cooperating with yeast SCF components. Overall, our data indicate that hFBH1 represents a bona fide functional orthologue of budding yeast Srs2 that, in addition, during its evolution might have acquired self-regulatory properties necessary to modulate its DNA recombination functions.

MATERIALS AND METHODS

Plasmids, yeast strains, and procedures. All of the yeast strains used in this study are isogenic to the W303 background. Deletion or Myc-tagged strains were obtained by a one-step method. Multiple mutant strains were derived by meiotic segregants. The *srs2::hFBH1* strain was constructed as follows. The *HIS3-MX6* cassette was inserted at the C terminus of the *hFBH1* gene carried by the pIC1 plasmid, a derivative of pAS2-1 (21), thus creating the pIC1-HIS3 plasmid. The plasmid region containing both *hFBH1* and *HIS3* was amplified by PCR, using primers with 5' and 3' tails homologous to the genomic sequences flanking the

genomic *SRS2* locus. The resulting PCR product was used to transform a wild-type (wt) W303 yeast strain. Details about the oligonucleotide sequences are available upon request. *hfbh1-ΔN* and *hfbh1-hd* strains were constructed using the same strategy by amplifying a PCR fragment lacking the F-box motif or a PCR fragment containing a K520A mutation obtained by site-direct mutagenesis of plasmid pIC1-HIS3. The *F-SRS2* strain was constructed by inserting the *hFBH1* F-box domain into the NotI site of the pG35 plasmid (28) to generate the pSAPO1 plasmid. PstI-linearized pSAPO1 plasmid was used to integrate the *F-SRS2* construct at the *SRS2* locus.

Standard genetic analyses were performed according to published procedures (42). An intrachromosomal recombination assay was performed using yeast strains carrying a heteroallelic duplication of *LEU2*, with *URA3* between the *LEU2* genes, as previously described (25). Spot assays were performed by evaluating cellular growth on synthetic complete medium containing adenine at a final concentration of 0.7 g/liter, with or without methyl methanesulfonate (MMS), hydroxyurea (HU), and 4-nitroquinolone-1-oxide (4-NQO; Sigma). All spot tests were repeated at least three times in independent experiments, using different yeast transformants or segregants to ensure the reproducibility of the results. Dose-response killing curves for zeocin (Invitrogen) were determined by plating serial dilutions of exponentially growing cells treated for 1 hour with or without the mutagen; to measure UV light sensitivity, a known number of cells were plated from exponentially growing cultures and exposed to different UV doses. CFU were evaluated after 3 to 5 days at 28°C. All survival curves were repeated three times in independent experiments. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and Western blot procedures were done as previously described (28), using anti-Myc 9E11 (Bio Optica, United Kingdom) and α -tubulin (Oxford Biotechnology, United Kingdom) antibodies. Nocodazole and cycloheximide (Sigma) were used at final concentrations of 10 μ g/ml and 50 μ g/ml, respectively. The protein quantification analysis shown in Fig. 5 was carried out using Image J (NIH) software. The relative amounts of the hFBH1 protein were normalized by using tubulin as a loading control, as calculated in independent experiments.

RESULTS

The *S. cerevisiae* Srs2 protein contains seven segments of amino acids that constitute the helicase motifs and are well conserved in all DNA helicases of the UvrD family, including *Escherichia coli* UvrD, *S. pombe* Srs2, and *N. crassa* Srs2 (2, 48, 53). To search for putative human orthologues of *S. cerevisiae* Srs2, the helicase motifs were compared by a BLAST search of the *Homo sapiens* genome. The F-box DNA helicase hFBH1 (21) scored the best positive result in our analysis. As shown in Fig. 1A, sequence alignment of the helicase motifs of hFBH1 with those of *S. cerevisiae* Srs2, *S. pombe* Srs2, and bacterial UvrD reveals significant conservation, which is limited to the helicase domains. In particular, the helicase domains of *S. cerevisiae* Srs2 and hFBH1 possess 19.2% identity and 34.4% similarity at the amino acid level. *S. cerevisiae* Srs2 is the only UvrD member with a long C-terminal tail (Fig. 1B), which has been implicated in protein regulation (8, 26, 36, 40). Conversely, the Fbh1 proteins in *S. pombe*, mice, and humans are characterized by the presence of an F-box domain, located in the N-terminal part, that is absent in Srs2 and in bacterial UvrD helicases (Fig. 1B).

To address whether hFBH1 might represent a functional orthologue of *S. cerevisiae* Srs2, we tested the ability of hFBH1 to suppress specific phenotypes of *srs2Δ* mutants. For this analysis, we replaced the *SRS2* open reading frame at its chromosomal location with the human cDNA encoding the hFBH1 protein. In the resulting *srs2::hFBH1* strain, the yeast *SRS2* promoter, which is responsible for the cell cycle and the DNA damage-induced regulation of the original transcript (16), controls the expression of the *hFBH1* cDNA insert. By using Western blot analysis, we found that the hFBH1 protein is expressed in the yeast strain, although its level is lower than the

FIG. 1. The UvrD helicase family. (A) Amino acid sequence alignments, performed with CLUSTALW, among the seven helicase motifs found in *E. coli* (ec) UvrD, *S. cerevisiae* (sc) Srs2, *S. pombe* (sp) Srs2, *S. pombe* Fbh1, mouse (m) Fbox18, and hFBH1. Identical and conserved amino acids are indicated within gray and white boxes, respectively. (B) Schematic representation of UvrD helicases and conserved motifs.

level of the endogenous Srs2 protein (see Fig. 3C). *srs2Δ* mutants are characterized by hyperactivation of spontaneous recombination (25, 41), and such a deletion is lethal in the absence of *RAD54* (23) or *SGS1* (14), which are both required for the maturation of recombination intermediates. The cell lethality of *srs2 rad54* and *srs2 sgs1* double mutants is suppressed by deleting *RAD51* (14, 23, 44), consistent with the idea that Srs2 dismantles Rad51 nucleofilaments (26, 52). Using a genetic system that allows the analysis of gene conversion and recombination-mediated deletion events occurring between directed repeats, we confirmed previous observations (25) indicating that in *srs2Δ* mutants the rate of gene conversion is enhanced four to five times compared to that of wt cells. We found that the gene conversion rate was reduced in the *srs2::hFBH1* strain compared to that of the *srs2Δ* strain (Fig. 2A), indicating that hFBH1 is able to counteract recombination in the absence of Srs2. We also found, by tetrad analysis, that while *srs2Δ* mutants are synthetically lethal in combination with *rad54Δ* deletion, *srs2::hFBH1 rad54Δ* double mutants are viable (Fig. 2B). We then tested the ability of hFBH1 to prevent cell lethality in *srs2Δ sgs1Δ* double mutants and found that the presence of a wt copy of *SRS2*, carried by a *URA3* plasmid and counterselectable by treatment with 5-fluoroorotic acid, is much less critical for cell survival in *srs2::hFBH1 sgs1Δ* mutants than in *srs2Δ sgs1Δ* mutants (Fig. 2C). *srs2Δ*, but not *sgs1Δ*, mutants require *RAD27* for cell viability (9, 23). *RAD27* encodes a Flap endonuclease implicated in processing of Okazaki fragments during DNA replication, and *rad27* mutants also exhibit synthetic lethality in combination with HR mutants (49). These genetic observations might imply that the DNA lesions generated by the absence of Rad27 are repaired mainly through HR and are not in contrast with the possibility that

Srs2 favors recombinational repair in this context. *rad27Δ* cells were crossed with *srs2Δ* or *srs2::hFBH1* cells, and tetrad analysis revealed that while *srs2Δ rad27Δ* double mutants are synthetically lethal, *srs2::hFBH1 rad27Δ* cells are viable (Fig. 2D). We therefore concluded that hFBH1 can also substitute for Srs2 in processing DNA lesions that spontaneously arise in *rad27Δ* mutants. Taken together, the data in Fig. 2 indicate that hFBH1 suppresses the spontaneous recombination defects occurring in the absence of a functional Srs2 protein.

srs2 mutants exhibit hypersensitivity when exposed to a variety of DNA-damaging agents that cause the accumulation of different DNA lesions, including DSBs (45). We used a colony formation assay to test whether hFBH1 was able to rescue the hypersensitivity of *srs2Δ* cells to different mutagens. As shown in Fig. 3A, we found that hFBH1 suppresses the hypersensitivity of *srs2Δ* mutants to MMS, HU, and 4-NQO. Similarly, hFBH1 suppresses *srs2Δ* hypersensitivity to UV light and zeocin, as assessed by treating exponentially growing cells with increasing doses of mutagens (Fig. 3B).

We then dissected the contributions of the helicase and F-box domains to hFBH1 function by creating two specific mutants (Fig. 3C) in which the F-box motif was deleted (*hfbh1-ΔN*) or in which the conserved K within the ATP binding domain, essential for the enzymatic activities of the yeast Srs2 protein (25), was changed to A (*hfbh1-hd*). Both *hfbh1* alleles were replaced at the *SRS2* locus, and using Western blot analysis, we found that the mutated proteins were expressed at least at the same level as wt hFBH1, both under untreated conditions and in response to MMS (Fig. 3C). *srs2::hfbh1-ΔN* and *srs2::hfbh1-hd* strains showed increased sensitivity to drug treatments, resembling that of *srs2Δ* mutants, compared to the *srs2::hFBH1* strain (Fig. 3A). Hence, both the helicase and the

FIG. 2. hFBH1 suppresses *srs2* recombination defects arising from spontaneous DNA lesions. (A) Gene conversion rates were determined for wt, *srs2*Δ, and *srs2::hFBH1* strains. (B) Tetrads obtained from sporulation of diploids heterozygous for *rad54*Δ and *srs2*Δ (left) or *rad54*Δ and *srs2::hFBH1* (right). (C) The indicated strains, containing *SRS2* cloned into a *URA3*⁺ vector, were grown at an equal cellular concentration and sequentially diluted 1:6 before being spotted onto plates without treatment (UNT) or with 5-fluoroorotic acid (5-FOA) to counterselect the plasmid. Cellular growth was evaluated after incubation at 28°C for 3 to 5 days. (D) Tetrads obtained from sporulation of diploids heterozygous for *rad27*Δ and *srs2*Δ (left) or *rad27*Δ and *srs2::hFBH1* (right).

ubiquitin ligase functions of hFBH1 are essential for suppressing the DNA damage sensitivity of *srs2*Δ mutants. Moreover, the DNA damage sensitivities of *rad51*Δ, *srs2*Δ *rad51*Δ, and *srs2::hFBH1* *rad51*Δ strains were comparable, indicating that hFBH1 suppresses *srs2*Δ DNA damage sensitivity only in the presence of a functional HR pathway (Fig. 3A).

It is thought that Srs2 channels DNA lesions into the PRR pathway by preventing HR (5, 45). According to this model,

the elevated DNA damage sensitivities of PRR-deficient mutants can be rescued partially by deleting *SRS2* in the presence of a functional HR pathway (2, 6, 13, 41, 43, 50). We therefore tested whether hFBH1 in the absence of Srs2 could restore the MMS sensitivity of PRR-deficient mutants. As previously shown, we found that the deletion of *SRS2* partially rescued the MMS sensitivities of *rad5*Δ, *rad18*Δ, *rad6*Δ, and *ubc13*Δ mutants (Fig. 4A); surprisingly, we found that *rad5*Δ *srs2::*

FIG. 3. hFBH1 suppresses the hypersensitivity of *srs2* mutants to mutagens. (A) The indicated strains were grown at an equal cellular concentration, sequentially diluted 1:6, and spotted onto plates containing MMS, HU, or 4-NQO at the indicated concentrations. Cellular growth was evaluated after incubation at 28°C for 3 to 5 days. (B) An appropriate number of log-phase cells of wt, *srs2Δ*, and *srs2::hFBH1* yeast strains were plated and exposed to different doses of UV light. Cell survival was then evaluated compared to that of an untreated control. Log-phase cultures of the same strains were incubated with different doses of zeocin for 1 h, and cell survival was then calculated as the plating efficiency with respect to untreated cells. (C) Schematic view of hFBH1, Srs2, and mutants used in this study. Crude protein extracts were prepared from the indicated Myc-tagged strains grown under unperturbed conditions (–) or in the presence of 0.02% MMS for 3 h (+) and were analyzed by Western blotting using antibodies against Myc or α-tubulin as a loading control.

hFBH1, *rad18Δ srs2::hFBH1*, and *ubc13Δ srs2::hFBH1* mutants were more resistant to MMS treatment than *rad51Δ srs2Δ*, *rad18Δ srs2Δ*, and *ubc13Δ srs2Δ* mutants, while *rad6Δ srs2::hFBH1* cells were more sensitive to MMS than *rad6Δ srs2Δ* mutants (Fig. 4A). In response to DNA damage, the PRR factors deleted in the above mutants induce the mono- and polyubiquitylation of PCNA at the conserved K164 residue; *srs2Δ* mutants rescue the DNA damage sensitivity of ubiqui-

FIG. 4. hFBH1 and the hybrid F-Srs2 protein suppress the DNA damage sensitivity of PRR mutants. (A and B) The indicated strains were grown at equal cellular concentrations, sequentially diluted 1:6, and spotted onto plates containing MMS at the indicated concentrations. Cellular growth was evaluated after incubation at 28°C for 3 to 5 days.

tylation-defective *pcnaK164R* mutants (18). We found that hFBH1 also rescues the MMS sensitivity of *pcnaK164R* mutants in the absence of Srs2 and, similar to what was found for *rad5Δ*, *rad18Δ*, and *ubc13Δ* mutants, that this rescue is better

than that by deletion of *SRS2* itself, although not to the wt level (Fig. 4A). Thus, ubiquitylation of PCNA at K164 is also dispensable for MMS survival when hFBH1 substitutes for Srs2. Altogether, these findings indicate that hFBH1 compensates

not only for the absence of Srs2 in promoting cell survival upon MMS treatment but also for PRR functions related to PCNA ubiquitylation, although not for those of Rad6. To further characterize this aspect, we asked if the F-box domain of hFBH1 was required for the PRR suppression phenotype. Indeed, we found that *fbh1-ΔN* did not rescue the MMS sensitivity of *rad5Δ* mutants (Fig. 4B). Notably, we found that *hfbh1-hd* mutants behaved similarly to *hfbh1-ΔN* mutants (Fig. 4B), suggesting that a functional hFBH1 helicase domain is also required for *rad5Δ* suppression. Since we cannot rule out that deletion of the F-box domain partially affects the helicase activity of hFBH1 (22), we tested the contribution of the F-box domain to the suppression of MMS sensitivity of PRR-deficient mutants in a different way. We asked if the addition of the hFBH1 F-box domain to the yeast Srs2 protein could be sufficient to relieve the need for PRR factors. To test this hypothesis, we created a hybrid *SRS2* gene carrying the coding region for the F-box domain of hFBH1 (Fig. 3C) and used this chimera to replace endogenous *SRS2* in PRR-deficient backgrounds. We found that the F-box-Srs2 protein (F-Srs2) restored the MMS sensitivity of *rad5Δ* or *rad18Δ* cells to a similar extent to that by hFBH1 (Fig. 4B). Hence, the hybrid F-box Srs2 protein recapitulates hFBH1 properties in suppressing PRR mutants.

We noted that the MMS sensitivity of *rad5Δ srs2::hFBH1rad51Δ* cells was identical to that of *rad5Δ srs2Δ rad51Δ* or *rad5Δ rad51Δ* cells, indicating that the suppression of the DNA damage sensitivity of *rad5Δ* mutants by hFBH1 requires a functional *RAD51* gene (Fig. 4B). Conversely, neither the inactivation of *RAD30* nor that of *REV3*, resulting in ablation of the translesion DNA polymerase η and DNA polymerase ζ , respectively, affected the capability of hFBH1 to suppress the MMS sensitivity of *rad5Δ* mutants in the absence of *SRS2* (data not shown).

Altogether, the data presented in Fig. 4 indicate that the replacement of Srs2 with hFBH1 suppresses PRR mutant repair defects in the presence of an intact HR pathway and that the F-box domain, in association with functional UvrD helicase activity, plays an essential role in this suppression.

In the course of our studies, we noticed that the deletion of the F-box domain in *hfbh1-ΔN* mutants led to an increase of the hFBH1 protein level, while its addition to endogenous Srs2 in F-SRS2 strains correlated with a reduced Srs2 protein level (Fig. 3C). In order to better characterize this aspect, we measured hFBH1 protein stability in yeast strains, using the protein synthesis inhibitor cycloheximide.

Different cultures of a yeast strain carrying a Myc-tagged version of hFBH1 were treated with increasing doses of MMS and then exposed to cycloheximide to prevent further protein synthesis. As shown in Fig. 5A, the hFBH1 protein level abruptly decreased in time upon protein synthesis inhibition, and in particular, the hFBH1 turnover rate increased by raising the dose of MMS, as also judged by quantification analysis. Hence, although our analysis suggests that hFBH1 is subjected to rapid degradation even under unperturbed conditions (data not shown), the DNA damage treatment further stimulated its turnover. In contrast, the turnover of a truncated version of hFBH1 lacking the F-box motif was dramatically impaired in MMS-treated cells (Fig. 5B). Given that hFBH1 interacts with Skp1 through the F-box domain to form an SCF complex in

vitro (22), we asked if yeast SCF factors could be required for hFBH1 protein turnover under our experimental conditions. As shown in Fig. 5B, we found that hFBH1 degradation is indeed prevented by inactivating the Cdc53 or Skp1 SCF component by using temperature-sensitive alleles.

Overall, these findings indicate that hFBH1 is subject to rapid turnover, which is further stimulated by DNA damage treatment, and that this turnover depends on a functional F-box domain and yeast SCF-dependent degradation machinery.

DISCUSSION

Several human inherited genetic diseases, characterized by genomic instability and cancer predisposition, are due to mutations in genes encoding DNA helicases, a class of enzymes that control fundamental aspects of DNA metabolism, including DNA replication, repair, recombination, and transcription. In budding yeast, the Srs2 DNA helicase plays a crucial role in the maintenance of genome stability by regulating DNA recombination. Srs2 has been conserved structurally and functionally in bacteria and in other fungi, such as *S. pombe* and *N. crassa* (2, 10, 30, 48, 51, 53), yet a human orthologue of Srs2 has never been described, thus leading to the idea that its functions might be sustained by other structurally unrelated DNA helicases in mammalian cells (17).

hFBH1 is highly similar to Srs2 within the helicase domain, but it also carries an F-box motif that self-regulates its turnover. With the aim of searching for putative human orthologues of *S. cerevisiae* Srs2, we compared by BLAST searching just its conserved UvrD helicase domain with the *H. sapiens* genome and identified the F-box DNA helicase hFBH1 as the protein with the best score. Indeed, within the helicase domain, hFBH1 shares a high degree of similarity with all members of the UvrD protein family, including *S. pombe* Srs2 and *E. coli* UvrD. Beyond the helicase domain, hFBH1 shares poor homology with UvrD proteins, and it exclusively contains an F-box domain. Bona fide orthologues of hFBH1 have been identified in *S. pombe*, mouse, and chicken cells (21, 24). Considering the similarities and differences between Srs2 and Fbh1 protein subgroups, they likely constitute a single protein family, resembling the case of RecQ helicases, where the different members share homology mainly within their helicase domains (17). Notably, for *S. pombe*, members of the two subgroups have been described, and genetic data suggest that *fbh1Δ srs2Δ* double mutants are lethal due to unrestrained HR (32, 33). In particular, it has been suggested that *S. pombe* Fbh1 has a role in disassembling Rad51 nucleofilaments, either those formed in the absence of HR mediator proteins (33) or those created after strand invasion (32). Similarly, in DT40 chicken cells, Fbh1, by cooperating with the BLM RecQ helicase, acts in limiting SCEs (24). Thus, in different organisms, Fbh1 functions rely on recombination, and our findings indicate that hFBH1 can functionally substitute for budding yeast Srs2.

As mentioned above, hFBH1 has an F-box domain, a feature not shared with *S. cerevisiae* Srs2. The F-box proteins are the interchangeable recognition subunits of ubiquitin ligase SCF complexes that control protein degradation in different cellular processes (54). In vitro studies indeed indicate that hFBH1 is part of an SCF complex that promotes the formation of ubiqu-

FIG. 5. hFBH1 protein turnover is stimulated by DNA damage and depends on a functional F-box domain and yeast SCF complex. (A) Different cell cultures of an hFBH1 Myc-tagged yeast strain were treated for 3 h at the indicated MMS concentrations. Cycloheximide (chx) was then added to each culture to prevent further protein synthesis. At the indicated times, crude protein extracts were prepared and analyzed by Western blotting using anti-Myc or anti-tubulin antibody as a loading control. Quantification analysis of hFBH1 protein levels is also shown. (B) Log-phase cells of the indicated yeast strains were presynchronized by nocodazole treatment, released at 25°C into 0.02% MMS for 2 h, and then incubated at 37°C for 1.5 h. Cycloheximide was subsequently added, and protein samples were analyzed at the indicated time points as described for panel A. hFBH1 and F-box-truncated mutant proteins are indicated by asterisks. Quantification analysis of hFBH1 protein levels is also shown.

uitin chains, although the targets modified *in vivo* are not known (22). Consistent with findings obtained by studying the *S. pombe* Fbh1 counterpart (32, 33), our data indicate that the F-box domain is necessary for hFBH1 function(s) in substituting for Srs2, and in addition, they raise the possibility that one target of the F-box domain is hFBH1 itself. Indeed, based on data from yeast, hFBH1 is an unstable protein whose turnover

is further stimulated by DNA damage and depends on a functional F-box domain and SCF components.

hFBH1 fulfils *S. cerevisiae* Srs2 functions in HR. Despite the presence of the F-box motif, does hFBH1 accomplish the specific Srs2 recombination functions? In fact, our data indicate that hFBH1 suppresses many *srs2*-specific recombination defects, thus indicating that hFBH1 is able to substitute for Srs2

in processing recombination intermediates in different contexts. In particular, it has been suggested that certain *srs2* phenotypes, including synthetic lethality with *rad54* or *sgs1* mutants and UV hypersensitivity, can be ascribed to the accumulation of toxic Rad51 nucleofilaments (1, 14, 23), a hypothesis sustained by the finding that Srs2 displaces Rad51 nucleofilaments in vitro (26, 52). It is not known whether Fbh1 proteins have such DNA translocase activity, but since they are members of the UvrD family, it would not be surprising. In addition, Srs2 is also required for DSB repair by HR (3, 19, 20, 35), a role that might also explain the hypersensitivity of *srs2* mutants to DNA-damaging agents that cause DSBs or their synthetic lethality with *rad27* mutants. In these contexts, Srs2 might act in favoring recombinational repair. It was suggested that the prorecombinational role of Srs2 still reflects its ability to dislodge aberrant or unscheduled Rad51 nucleofilaments that might prevent recombinational repair (1, 33). Otherwise, Srs2 and Fbh1 might also counteract Rad51 nucleofilaments after strand invasion, thus constraining SCE events to the level necessary to repair interrupted DNA molecules. Notably, a role for *S. cerevisiae* Srs2 and even for *S. pombe* Fbh1 downstream of the Rad51 strand invasion step is supported by genetic findings (20, 32, 39). Finally, since both Srs2 and Fbh1 unwind the DNA duplex in vitro as proper DNA helicases (21, 40), they might have a more direct role in vivo in promoting recombination by extending the D loop once strand invasion has occurred (35).

hFBH1 faces error-free PRR functions by the aim of its F box. What is more surprising in our findings is that hFBH1, in the absence of *SRS2*, suppresses the DNA damage sensitivity of PRR mutants, while in contrast, Srs2 causes cell death of PRR mutants (5, 45). As a consequence, hFBH1 and Srs2 have apparently opposite effects on the recombination outcome in this context: while Srs2 is thought to kill PRR mutants by preventing recombination, hFBH1 might rescue PRR mutants from cell lethality by favoring recombination. The requirement of HR for this suppression might support this possibility. Notably, the suppression of PRR mutant cell lethality by hFBH1 cannot be ascribed to the observation that hFBH1 might be present in limiting amounts with respect to Srs2. In fact, hFBH1 suppresses the MMS sensitivity of PRR mutants even more efficiently than that of *srs2Δ* mutants. Our data rather suggest that this suppression entails an active UvrD helicase whose turnover depends on its F-box motif, resulting in an enzyme that efficiently favors recombinational repair.

According to the current model, Srs2 exerts antirecombination functions when it is recruited by sumoylated PCNA (34, 36). The association of Srs2 with sumoylated PCNA depends on its C-terminal tail, and its removal induces a PRR suppression phenotype resembling the one caused by preventing PCNA sumoylation (36). Although the C-terminal domain of Srs2 has not been conserved in hFBH1, our data indicate that the hybrid F-Srs2 protein that retains the SUMO-PCNA binding domain (Fig. 3C) is still able to suppress the MMS sensitivity of PRR mutants. Furthermore, both F-Srs2 and hFBH1 suppress PRR mutants better than mutations that prevent the sumoylation of PCNA, whose phenotypes in this regard resemble those of *srs2Δ* mutants (36). Thus, the presence of the F-box domain rather than the absence of the C-terminal tail

seems to be crucial for the suppression of PRR mutants by hFBH1 or F-Srs2.

Although other possibilities could be envisaged, we favor one interpretation of our data that might suggest a novel functional relationship between Srs2 and PRR factors in *S. cerevisiae*, as follows. In response to DNA damage, Srs2 might act mainly in favoring recombinational repair and PRR factors might enhance this Srs2 prorecombinational activity. In PRR mutants, Srs2 becomes toxic because it is unable to stimulate proper recombination events, and it simultaneously prevents access to DNA lesions of other factors that promote HR in its absence. In this scenario, hFBH1, by the aim of its F-box, fulfils Srs2 functions in HR and the PRR regulatory role in Srs2 activity at the same time.

But how could PRR genes influence Srs2 duties, and what is the mechanism by which the F-box hFBH1 helicase could substitute for PRR functions? Studies with human cells showed that hFBH1 is a component of the SCF ubiquitin ligase complex (22), and SCF complexes have been implicated in K48 ubiquitylation and in 26S proteasome-dependent degradation of their targets (54). Therefore, one likely possibility is that hFBH1 disruption occurs through an autoubiquitylation mechanism (Fig. 6). Since the F-box domain self-regulates the turnover of hFBH1 and is required for the suppression of PRR mutants, one interesting hypothesis is that the control exerted by the PRR pathway on Srs2 relies on a similar mechanism. Rad6/Rad18 and Rad5/Ubc13 cooperate to assemble ubiquitin via K63 chains, which is not a typical signal for protein degradation (37); thus, it is unlikely that the PRR pathway would directly promote Srs2 disruption. However, in response to DNA damage, the PRR pathway promotes PCNA ubiquitylation (34, 36). Given that hFBH1 also rescues ubiquitin-defective *pcnaK164R* mutants, an intriguing possibility is that one of the essential functions of PRR factors, by inducing PCNA modification, is in turn to influence the stability of Srs2 (Fig. 6). This hypothesis might not be entirely surprising, since it was recently shown that the replication factor Cdt1 is ubiquitylated and degraded, also in response to DNA damage, once it is recruited by PCNA to the DNA template (15). In light of this, we speculate that Srs2 degradation in its natural context is promoted by an SCF complex and that an unidentified endogenous F-box protein in yeast could perform the same F-box-dependent function of hFBH1 (Fig. 6). Therefore, the PRR pathway, by inducing the modification of PCNA, might at the same time influence the capability of an SCF complex to disrupt Srs2 and possibly other factors bound to DNA, maybe by stimulating the recruitment of a putative F-box protein. In this view, hFBH1 does not require certain PRR functions to survive DNA damage because it is able to self-regulate its turnover. Similarly, the proper turnover of endogenous Srs2 in PRR mutants can be reestablished by adding the sole F-box domain of hFBH1 to the helicase.

Our data also suggest that hFBH1 suppresses the MMS sensitivity of PRR mutants, with the exception of *rad6* mutants. Although Rad6 has E2 activity (27), SCF ubiquitin ligases work preferentially with the Cdc34 E2 enzyme (54); in fact, we found that in *rad6* mutants, hFBH1 degradation is not prevented (data not shown). Thus, to explain our suppression data, we favor the hypothesis that Rad6 might control the modification of another target(s) necessary for hFBH1 function in

FIG. 6. Model for SCF-mediated regulation of hFBH1 and Srs2 turnover in response to DNA damage. The F-box domain of hFBH1 forms an SCF ubiquitin ligase that controls hFBH1 DNA damage-induced turnover. In budding yeast under unperturbed conditions, Srs2 is recruited by sumoylated PCNA, and in response to DNA damage, the conserved Rad6/Rad18/Rad5/Ubc13 PRR pathway triggers K63 ubiquitylation of PCNA. PCNA ubiquitylation stimulates the recruitment of a putative SCF complex that, as in the case of the hFBH1 F-box domain, enhances Srs2 turnover. In addition to its role in monoubiquitylating PCNA, Rad6, by promoting the modification of another unknown target(s), aids hFBH1 and Srs2 recombination functions. Increased hFBH1 and Srs2 turnover might lead to the stimulation of recombinational repair of DNA lesions.

favoring recombinational repair. Rad6 cooperates with E3 ligases other than Rad18, and by targeting, for example, histones (27), it might aid hFBH1 in processing recombination intermediates.

Concluding remarks. Genetic analysis performed with fission yeast suggests that the two UvrD helicases Fbh1 and Srs2 have nonredundant functions in recombination (32, 33). An intriguing possibility is that the recombination role(s) performed by the sole Srs2 helicase in budding yeast, regulated by the PRR pathway and possibly by phosphorylation (28), is achieved by distinct polypeptides in fission yeast. Notably, *S. cerevisiae* Srs2, exclusively among UvrD helicases, possesses a long C-terminal tail (Fig. 1B) containing most of the DNA damage-inducible phosphosites (8), whose deletion alone is sufficient to suppress PRR mutants and which is required for the physical interactions with both sumoylated PCNA (36) and Rad51 (26). If the functions of *S. pombe* Srs2 and Fbh1 could be reassumed by a sole PRR-regulated UvrD helicase in *S. cerevisiae*, then this would explain why the genetic relationships between *SRS2* and PRR genes in *S. pombe*, or even in *N.*

crassa, are less consistent with those depicted in *S. cerevisiae*. In fact, while budding yeast *srs2* mutants suppress the DNA damage sensitivity of PRR mutants, *S. pombe* or *N. crassa* *srs2* mutants do not (10, 48). Furthermore, the same hypothesis might justify why the timing of ubiquitylation- and sumoylation-dependent modifications of PCNA, which are crucial for the regulation of Srs2 recombination functions in *S. cerevisiae* (34, 36), has not been conserved equally in *S. pombe* (12). It is interesting that a phylogenetic analysis performed on hemiascomycete yeasts revealed that the *SRS2* locus is duplicated in tandem in certain species, and it is thought that gene duplication might facilitate their rapid evolution toward specialized functions (38).

In conclusion, our data are compatible with the idea that hFBH1 is a bona fide human orthologue of *S. cerevisiae* Srs2 that, thanks to F-box-dependent functions, has evolved to execute its recombination role(s) independently of the PCNA modification status, as instead happens in budding yeast.

It will be a challenge for the future to establish whether

other proteins functionally related to hFBH1 are present in human cells and whether inactivation of hFBH1 might have important implications for human health, like the case for the RecQ helicases BLM, WRN, and RECQ4 (17).

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