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# The involvement of Srs2 in post-replication repair and homologous recombination in fission yeast

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## ABSTRACT

**Homologous recombination is important for the repair of double-strand breaks and daughter strand gaps, and also helps restart stalled and collapsed replication forks. However, sometimes recombination is inappropriate and can have deleterious consequences. To temper recombination, cells have employed DNA helicases that unwind joint DNA molecules and/or dissociate recombinases from DNA. Budding yeast Srs2 is one such helicase. It can act by dissociating Rad51 nucleoprotein filaments, and is required for channelling DNA lesions to the post-replication repair (PRR) pathway. Here we have investigated the role of Srs2 in controlling recombination in fission yeast. Similar to budding yeast, deletion of fission yeast *srs2* results in hypersensitivity to a range of DNA damaging agents, *rhp51*-dependent hyper-recombination and synthetic sickness when combined with *rqh1*$^-$ that is suppressed by deleting *rhp51*, *rhp55* or *rhp57*. Epistasis analysis indicates that Srs2 and the structure-specific endonuclease Mus81–Eme1 function in a sub-pathway of PRR for the tolerance/repair of UV-induced damage. However, unlike in *Saccharomyces cerevisiae*, Srs2 is not required for channelling lesions to the PRR pathway in *Schizosaccharomyces pombe*. In addition to acting as an antirecombinase, we also show that Srs2 can aid the recombinational repair of camptothecin-induced collapsed replication forks, independently of PRR.**

## INTRODUCTION

Replication forks that encounter bulky lesions in DNA, such as pyrimidine dimers induced by ultraviolet (UV) light, are impeded (1). Repair of UV-induced damage is typically by nucleotide excision repair (NER) (2). However, the presence of a replication fork adjacent to a UV-induced lesion may hinder NER. One potential solution to this is fork regression, which involves the unwinding of nascent DNA strands and re-annealing of parental DNA strands (1). Once the fork is out of the way NER may gain access to the lesion and repair it. The replication fork then has to be reset and the replisome reassembled. Enzymes that drive homologous recombination (HR) can play key roles in both the regression and re-establishment of a replication fork (1). Even if such a mechanism of fork regression coupled to NER operates *in vivo*, it is clear that cells do not depend solely upon this. Alternative strategies for coping with fork-blocking lesions involve their tolerance rather than their immediate repair. Such mechanisms include: (i) translesion synthesis (TLS), which uses error-prone and error-free DNA polymerases that can replicate past UV photoproducts (reviewed in 3,4); (ii) polymerase 'skipping' of the damaged section of the template, which generates a lesion-containing single-strand gap that is later filled in using either HR or TLS (5–7); and (iii) template switching for the polymerase to bypass the lesion, which can be mediated by HR (8). The importance of these mechanisms is underscored by the inability of chicken DT40 cells disabled for HR and TLS to proliferate (9). What factors determine whether a HR-based mechanism is used in preference to TLS are unclear. Certainly there are potential disadvantages with both mechanisms. TLS can be mutagenic, and HR occasionally occurs between allelic or ectopic sequences resulting in loss of heterozygosity and genome rearrangements, respectively.

TLS falls under the general umbrella of the post-replication repair (PRR) pathway (4). In the budding yeast *Saccharomyces cerevisiae* PRR consists of at least three sub-pathways, two error-free pathways mediated by *RAD5* and *POL30*, respectively, and one error-prone pathway dependent on Polζ (4). Overall control of PRR depends on a ubiquitin-conjugating enzyme Rad6 and a RING finger protein called Rad18 that binds to DNA (4,5). In *S.cerevisiae*, one factor that is instrumental in directing repair from HR to PRR at stalled forks and/or lesion-containing single-strand gaps is the Srs2 DNA helicase. This has been inferred from three main observations: (i) mutation of *SRS2* suppresses the UV sensitivity of *rad6*, *rad18*, *rad5* and *pol30–46* mutants (10–13); (ii) *srs2* suppression of PRR mutant UV sensitivity is dependent on the *RAD52* epistasis group of recombination genes (10,11,14); and (iii) mutating *SRS2* results in hyper-recombination (14).

The *RAD52* epistasis group of proteins includes Rad51, Rad52, Rad54, Rad55, Rad57 and Rad59 (reviewed in 15). Rad51 is a homologue of the archetypal recombinase RecA from bacteria. It binds to single-stranded DNA to form a right-handed helical nucleoprotein filament in which pairing and strand exchange between homologous DNA molecules occurs. Filament assembly is variously aided by RPA, Rad52 and the

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Rad55–Rad57 heterodimer (15). Srs2 seems to direct repair from HR to PRR by dissociating Rad51 nucleoprotein filaments (16,17). This appears to be important for preventing toxic recombination since a diploid *srs2* mutant's sensitivity to UV light is suppressed by semi-dominant mutations in *RAD51* (18). Dissociation of Rad51 filaments may also help Srs2 to suppress the formation of crossovers during the repair of DNA double-strand breaks (DSBs) by directing repair to a synthesis-dependent strand annealing (SDSA) mechanism (19). Srs2's ability to dislodge Rad51 from DNA appears to be especially important when the nucleoprotein filament is aberrant or disabled by mutation. For example, in the absence of Rad54, which promotes Rad51-mediated strand invasion and D-loop formation (20), Srs2 becomes essential for viability (21). Presumably, Rad51 filaments that are rendered useless in the absence of Rad54 block other repair processes if Srs2 does not remove them (16). Srs2 is also important for viability in the absence of the RecQ family DNA helicase Sgs1 (22). Sgs1, like Srs2, has an anti-recombinogenic role, and the poor viability of an *srs2 sgs1* double mutant is rescued by deleting *rad51* or *rad52*, suggesting that both helicases process toxic recombination intermediates (22).

Srs2 is structurally related to the bacterial UvrD and Rep helicases, and although no obvious human homologue has yet been found, an orthologue in the fission yeast *Schizosaccharomyces pombe* has been identified (23,24). The *S.pombe srs2* mutant shares a number of phenotypes with the *S.cerevisiae srs2* mutant, including hypersensitivity to DNA damaging agents, hyper-recombination and strong genetic interactions with *rqh1* and *rhp54* (homologues of *SGS1* and *RAD54*, respectively). In this paper, we present further genetic analyses of *S.pombe* Srs2. Like *S.cerevisiae* Srs2, we show that *S.pombe* Srs2 acts as an anti-recombinase, which counters Rhp51-dependent recombination. In addition, we show that, as in *S.cerevisiae*, Srs2 functions in the PRR pathway for the tolerance/repair of UV damage, which interestingly in *S.pombe* also appears to involve the structure-specific endonuclease Mus81–Eme1. However, unlike in *S.cerevisiae*, lesions appear to be channelled efficiently to the PRR pathway without Srs2 in *S.pombe*. In addition to functioning in the PRR pathway, we also provide evidence that Srs2 functions to promote the recombinational repair of collapsed replication forks. Possible roles for Srs2 in repairing collapsed replication forks are discussed.

## MATERIALS AND METHODS

### Media and genetic methods

Procedures and media used for the routine growth and maintenance of *S.pombe* are described by Moreno *et al*. (25). The complete medium was yeast extract with supplements (YES), the minimal media was Edinburgh minimal medium (EMM) with supplements added where appropriate. Thiamine was used at 4 μM (1.35 μg/ml) where needed. To measure the number of ade$^+$ prototrophs in recombination assays YE medium lacking adenine, and containing 200 μg/ml guanine, to prevent uptake of residual adenine, was used.

### Strains and plasmids

The *S.pombe* strains used in this study are listed in Table 1. The *srs2*Δ*ura4*$^+$ mutant contains a replacement of the entire *srs2* open reading frame by a *ura4* marker. It was made by first creating an *in vitro* deletion construct consisting of ~1 kb regions of genomic DNA that flank the *srs2* open-reading frame, which were amplified by PCR, cloned either side of a *ura4* marker to make the plasmid pMW454. The cloned genomic DNA in pMW454 was sequenced to confirm that no mutations had been introduced during the PCR. To delete *srs2 in vivo*, the *ura4* marker with flanking genomic fragments was excised from pMW454 and used to transform a diploid wild-type strain to *ura*$^+$ by one-step gene disruption (26). Stable Ura$^+$ transformants were screened for genuine replacement of *srs2* with *ura4* by genomic Southern blot analysis. *rhp51* was amplified by PCR from a genomic template and cloned as a NdeI–BamHI fragment downstream of the *nmt* promoter in pREP41. Nucleotide sequencing of the cloned *rhp51* gene confirmed that no mutations had been introduced during the PCR.

### Spot assays

Exponentially growing cell cultures were adjusted to a density of $1 \times 10^7$ cells/ml, serially diluted by a factor of 10 down to $1 \times 10^4$ cells/ml, and 10 μl of each dilution spotted onto YES plates, which contained hydroxyurea (HU), methyl methanesulfonate (MMS) or camptothecin (CPT), or were subsequently UV irradiated using a Stratalinker (Stratagene) as indicated. Plates were incubated at 30°C typically for 3–5 days before being photographed. All spot assays were repeated at least once to ensure that results were reproducible.

### Quantitative UV survival assays

Exponentially growing cells were plated to YES in duplicate and UV irradiated. Plates were grown at 30°C for 5–7 days before counting. All data points represent the mean values from at least two independent experiments.

### Recombination assays

Mitotic recombination was assayed using strains containing an intrachromosomal recombination substrate consisting of a non-tandem direct repeat of *ade6* heteroalleles flanking a functional *his3* gene (Fig. 1C). Spontaneous and UV-induced recombinant frequencies were measured as described by Osman *et al.* (27). Recombination frequencies are mean values from at least three independent assays, and in each assay five independent colonies were tested. Two-sample *t*-tests were used to determine the statistical significance of differences in recombination frequencies between given strains.

### Viability testing

Cultures of exponentially growing cells were counted and a known number of cells were plated to YES plates in duplicate. Plates were counted after growth at 30°C for 5 days for control strains and 10 days for the *mus81 rhp54* double mutant strain. Data are mean values from three independent experiments.

**Table 1.** *Schizosaccharomyces pombe* strains

| Strain | Genotype | Reference |
|---|---|---|
| MCW23 | *leu*1-32, *ura*4-D18, *his*3-D1, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | Laboratory stock |
| MCW42 | *leu*1-32, *ura*4-D18, *his*3-D1, h+ | Laboratory stock |
| MCW121 | *rhp*54Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | Muris *et al.* (52) |
| MCW149 | *rqh*1Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | Stewart *et al.* (53) |
| MCW162 | *rad*8Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | Doe *et al.* (49) |
| MCW431 | *rhp*55Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | Laboratory stock |
| MCW449 | *leu*1-32, *ura*4-D18, *ade*6-704, h+ | Laboratory stock |
| MCW460 | *srs*2Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | This work |
| MCW473 | *srs*2Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | This work |
| MCW547 | *srs*2Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | This work |
| MCW595 | *rhp*6Δ*ura*4+, *leu*1-32, *ura*4-D18, *lys*1-131, h– | Reynolds *et al.* (54) |
| MCW680 | *srs*2Δ*ura*4+, *rad*8Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | This work |
| MCW744 | *mus*81Δ*kan*R, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | Laboratory stock |
| MCW745 | *mus*81Δ*kan*R, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | Laboratory stock |
| MCW747 | *srs*2Δ*ura*4+, *mus*81Δ*kan*R, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | This work |
| MCW832 | *rqh1*Δ*ura*4+, *rhp*55Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | This work |
| MCW897 | *mus*81Δ*kan*R, *rhp*54Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | Laboratory stock |
| MCW928 | *rqh1*Δ*kan*R, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | To be described elsewhere |
| MCW1043 | *srs*2Δ*kan*R, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | Maftahi *et al.* (23) |
| MCW1049 | *rhp*55Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | Khasanov *et al.* (32) |
| MCW1086 | *srs*2Δ*ura*4+, *rhp*55Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | This work |
| MCW1088 | *rhp*51Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | To be described elsewhere |
| MCW1097 | *srs*2Δ*ura*4+, *rqh1*Δ*kan*R, *rhp*51D*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4 | This work |
| MCW1099 | *srs*2Δ*ura*4+, *rhp*51Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | This work |
| MCW1162 | *rhp*51Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | Laboratory stock |
| MCW1137 | *mus*81Δ*kan*R, *rhp*6Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1, *lys*1-131 | This work |
| MCW1269 | *rhp*18Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | Verkade *et al.* (48) |
| MCW1270 | *srs*2Δ*ura*4+, *rhp*18Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | This work |
| MCW1271 | *mus*81Δ*kan*R, *rhp*18Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | This work |
| MCW1272 | *mus*81Δ*kan*R, *rad*8Δ*ura*4+, *leu*1-32, *ura*4-D18, *ade*6-704, h+ | This work |
| MCW1273 | *srs*2Δ*kan*R, *rhp*6Δ*ura*4+, *leu*1-32, *ura*4-D18, *his*3-D1 | This work |
| MCW1276 | *srs*2Δ*ura*4+, *rhp*55Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | This work |
| MCW1278 | *rhp*57Δ*LEU*2+, *leu*1-32, *ura*4-D18, *his*3-D1, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | Laboratory stock |
| MCW1279 | *srs*2Δ*ura*4+, *rhp*51Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | This work |
| MCW1280 | *srs*2Δ*ura*4+, *rhp*57Δ*LEU*2+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, *ade*6-M375 int::pUC8/*his*3+/*ade*6-L469, h+ | This work |
| MCW1282 | *srs*2Δ*ura*4+, *rqh1*Δ*kan*R, *rhp*55Δ*arg*3+, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | This work |
| MCW1283 | *srs*2Δ*ura*4+, *rqh1*Δ*kan*R, *rhp*57Δ*LEU*2+, *leu*1-32, *ura*4-D18, *his*3-D1, h+ | This work |
| MCW1284 | *srs*2Δ*ura*4+, *rqh1*Δ*kan*R, *leu*1-32, *ura*4-D18, *his*3-D1, *arg*3-D4, h+ | This work |

## RESULTS

### DNA damage sensitivity and hyper-recombination of *srs2*Δ

The identification of the homologue of the *S.cerevisiae* Srs2 DNA helicase in *S.pombe* has previously been reported by two groups (23,24). We had independently identified the same candidate Srs2 homologue by a BLAST search of the *S.pombe* database, and had made a null mutant strain by deleting the entire *srs2* open reading frame and replacing it with a *ura4*+ marker. Consistent with the published data we found that the *srs2*Δ strain is viable and grows normally as a haploid indicating that *srs2* is not an essential gene.

There are conflicting reports concerning the sensitivity of *S.pombe srs2* mutant strains to DNA damage. In one report, *srs2*Δ is shown to have a mild hypersensitivity to UV light, the ribonucleotide reductase inhibitor, HU, the alkylating agent, MMS, and the radiomimetic, bleomycin (24). However, in a second report, *srs2*Δ was shown to have the same sensitivity as a wild-type strain to UV and HU (23). To assess the sensitivity of our *srs2*Δ mutant strain we spotted dilutions of wild-type and *srs2*Δ cells onto nutrient agar plates that were either subsequently exposed to UV or contained HU, MMS or the topoisomerase I (Top1) poison CPT (Fig. 1A). The *srs2*Δ strain is much more sensitive to HU, MMS and CPT than the wild-type strain, and slightly more sensitive to UV. The increase in UV sensitivity was confirmed by a quantitative UV survival experiment (Fig. 1B). These results corroborate the data from Wang *et al.* (24) and show that Srs2 is required for aiding survival following treatment with agents that damage DNA and cause replication fork arrest. Moreover, the hypersensitivity to CPT indicates that Srs2 plays a role in the repair of collapsed replication forks. CPT collapses forks by inhibiting the religation step during Top1's reaction cycle. This leads to the formation of persistent single-strand nicks at which DNA polymerase run-off can occur, which creates single-end breaks (SEBs) (28,29).

*srs2* mutant strains of both *S.cerevisiae and S.pombe* exhibit hyper-recombination (14,24). In *S.pombe* this has been assessed previously by measuring inter-homologue recombination in an *srs2*Δ diploid strain (24). To see whether *srs2*Δ affects inter/intra-chromatid recombination we used strains containing a direct repeat of *ade6*– heteralleles and measured the frequency of Ade+ recombinants (Fig. 1C and D). A His+ marker between the repeats enabled us to distinguish recombinants that had lost (deletion types) or retained (conversion types) the intervening DNA between the repeats

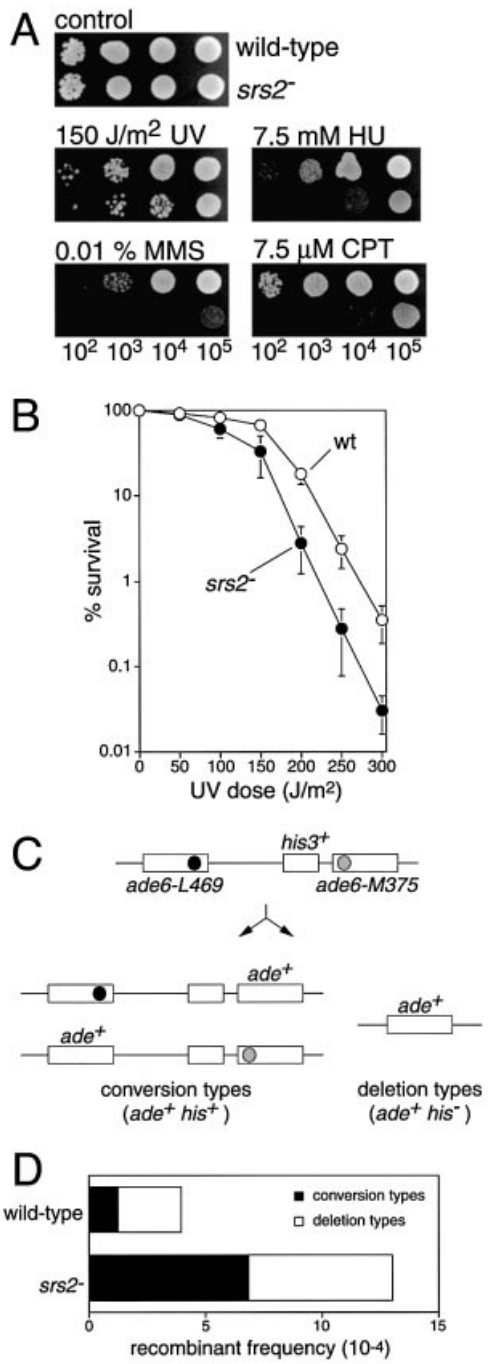


**Figure 1.** Phenotypes of the *srs2* deletion mutation strain. (**A**) Spot assay comparing wild-type (MCW42) and *srs2*Δ (MCW473) strains for sensitivity to UV, HU, MMS and CPT. Plates were incubated at 30°C for 3 days before being photographed. (**B**) UV survival curves of wild-type (MCW42) and *srs2*Δ (MCW473) strains. (**C**) Schematic of the recombination substrate and recombinant products. Solid and grey circles represent the *ade6-L469* and *ade6-M375* mutations, respectively. (**D**) Comparison of the spontaneous recombinant frequencies of wild-type (MCW23) and *srs2*Δ (MCW547) strains.

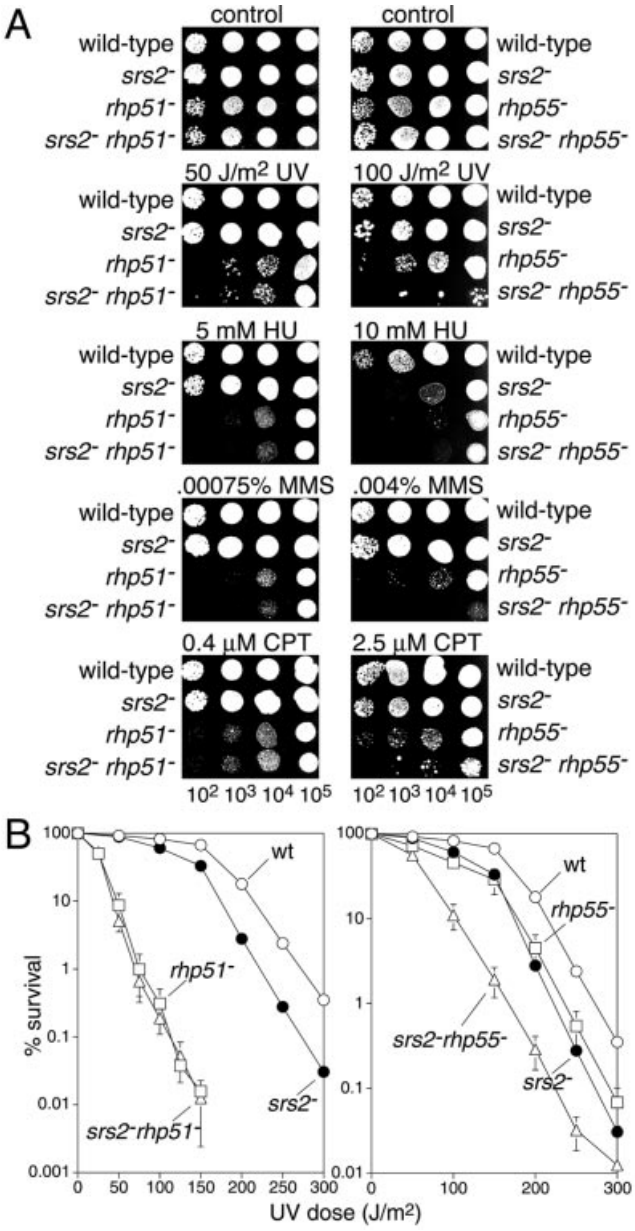


**Figure 2.** Epistasis analysis of *srs2*, *rhp51* and *rhp55* mutants. (**A**) Spot assays comparing wild-type (MCW42), *srs2*Δ (MCW473), *rhp51*Δ (MCW1088), *rhp55*Δ (MCW1049), *srs2*Δ *rhp51*Δ (MCW1099) and *srs2*Δ *rhp55*Δ (MCW1276) strains for sensitivity to UV, HU, MMS and CPT. Plates were incubated at 30°C for 4 days before being photographed. (**B**) UV survival curves of the same strains as in (A). Colonies were grown at 30°C for 5–7 days before being counted. Error bars represent standard deviations about the mean.

(Fig. 1C). In wild-type cells the average frequency of Ade+ recombinants is ~3.5 × $10^{-4}$ of which ~70% are deletion types and ~30% are conversion types. In the *srs2*Δ strain the frequency of Ade+ recombinants is ~3-fold higher than in the wild type and ~50% of these recombinants are conversion types. These data show that Srs2 acts to limit inter/intra-chromatid recombination, particularly that which gives rise to conversion types.

### Genetic interactions between *srs2* and *rhp51*, *rhp54*, *rhp55* and *rhp57*

Both genetic and biochemical analyses indicate that a prime function of Srs2 in *S.cerevisiae* is to dissociate Rad51

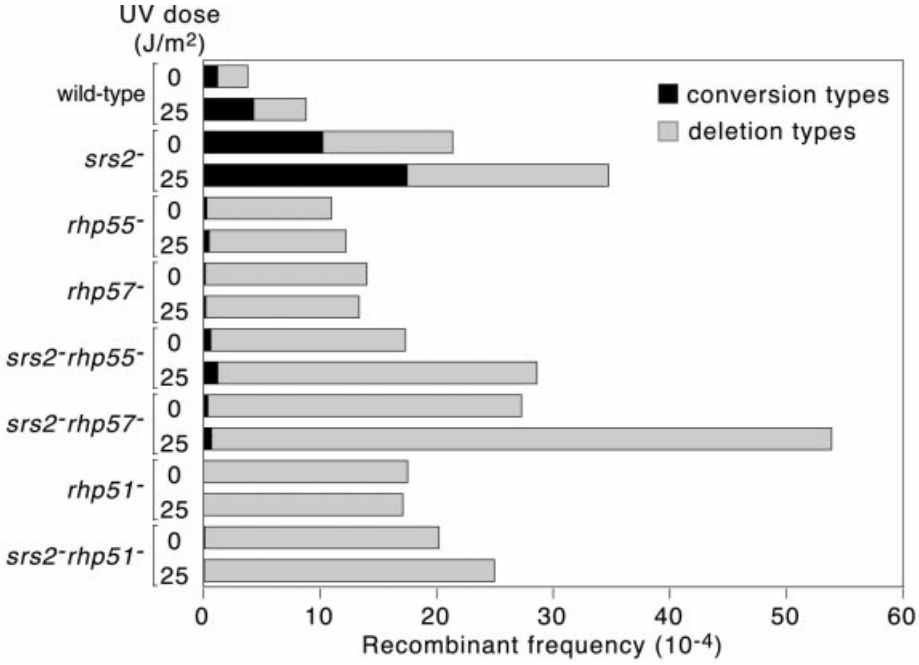


**Figure 3.** Bar chart showing the effect of *rhp55*, *rhp57* and *rhp51* mutations on the hyper-recombination of an *srs2*Δ mutant strain. Spontaneous and UV-induced Ade+ recombinant frequencies were measured using strains containing the recombination substrate shown in Figure 1C. The strains used were MCW547 (*srs2*Δ), MCW431 (*rhp55*Δ), MCW1278 (*rhp57*Δ), MCW1086 (*srs2*Δ *rhp55*Δ), MCW1280 (*srs2*Δ *rhp57*Δ), MCW1162 (*rhp51*Δ) and MCW1279 (*srs2*Δ *rhp51*Δ). The mean frequencies ($\times 10^{-4}$) of conversion types (ct) and deletion types (dt) (± standard deviation) are as follows: 1.2 ± 0.5 (ct) and 2.6 ± 0.8 (dt) (wild-type, no UV); 4.3 ± 2.1 (ct) and 4.5 ± 2.2 (dt) (wild-type, 25 J/m$^2$ UV); 10.2 ± 4.0 (ct) and 11.1 ± 4.7 (dt) (*srs2*Δ, no UV); 17.5 ± 4.6 (ct) and 17.3 ± 8.5 (dt) (*srs2*Δ, 25 J/m$^2$ UV); 0.27 ± 0.19 (ct) and 10.7 ± 3.5 (dt) (*rhp55*Δ, no UV); 0.48 ± 0.43 (ct) and 11.7 ± 3.5 (dt) (*rhp55*Δ, 25 J/m$^2$ UV); 0.16 ± 0.10 (ct) and 13.8 ± 5.5 (dt) (*rhp57*Δ, no UV); 0.19 ± 0.15 (ct) and 13.2 ± 6.6 (dt) (*rhp57*Δ, 25 J/m$^2$ UV); 0.63 ± 0.50 (ct) and 16.7 ± 7.8 (dt) (*srs2*Δ *rhp55*Δ, no UV); 1.2 ± 1.3 (ct) and 27.4 ± 13.6 (dt) (*srs2*Δ *rhp55*Δ, 25 J/m$^2$ UV); 0.38 ± 0.30 (ct) and 26.9 ± 10.4 (dt) (*srs2*Δ *rhp57*Δ, no UV); 0.68 ± 0.86 (ct) and 53.2 ± 23.8 (dt) (*srs2*Δ *rhp57*Δ, 25 J/m$^2$ UV); 0.05 ± 0.05 (ct) and 17.5 ± 5.8 (dt) (*rhp51*Δ, no UV); 0.06 ± 0.11 (ct) and 17.1 ± 7.9 (dt) (*rhp51*Δ, 25 J/m$^2$ UV); 0.10 ± 0.20 (ct) and 20.1 ± 8.1 (dt) (*srs2*Δ *rhp51*Δ, no UV); 0.06 ± 0.10 (ct) and 24.9 ± 10.5 (dt) (*srs2*Δ *rhp51*Δ, 25 J/m$^2$ UV).

nucleoprotein filaments, thereby channelling potential recombination substrates down other pathways of repair. In the absence of Srs2, Rad54, which promotes Rad51-mediated strand invasion, becomes essential (21). Presumably, unproductive Rad51 nucleoprotein filaments fail to be dislodged and therefore block other means of lesion repair/tolerance, resulting in loss of viability (16). This idea is consistent with the fact that the lethality of a *sgs1 rad54* double mutant is suppressed by mutations in *RAD51*, *RAD52*, *RAD55* and *RAD57* (30,31). In *S.pombe* the homologue of Rad54 is called Rhp54 and is likewise essential in the absence of Srs2 (23). The unviability of an *srs2 rhp54* strain is suppressed by deleting *rhp51* (*S.pombe RAD51*), consistent with the idea that Srs2 is required to process Rhp51 nucleoprotein filaments (23). During our studies of *srs2* we have independently confirmed these findings (data not shown). We also looked to see what affect *srs2*Δ would have on the sensitivity of a *rhp51* mutant strain to UV, HU, MMS and CPT (Fig. 2). The sensitivities of the *srs2*Δ *rhp51*Δ strain are indistinguishable from those of a *rhp51*Δ strain (Fig. 2A and B). This is consistent with the idea that Srs2 is only required to process Rhp51 nucleoprotein filaments. However, it should be noted that a *rhp51* single mutant is considerably more sensitive to UV, HU, MMS and CPT than an *srs2* single mutant, so any additive increase in sensitivity could go unnoticed in our assays. In *S.cerevisiae* the Rad55–Rad57 heterodimer is believed to promote the formation and stability of the Rad51 nucleoprotein filament (15). In the absence of the homologues of these proteins in *S.pombe* (Rhp55 and Rhp57, respectively) Rhp51-dependent recombination is ablated (our unpublished data). However, a *rhp51*Δ mutant is more sensitive to DNA damaging agents than either *rhp55*Δ or *rhp57*Δ mutants, which indicates that Rhp51 can still promote repair/tolerance in the absence of Rhp55–Rhp57 (32,33). Based on this we wondered whether deleting *srs2* would improve the stability of the Rhp51 nucleoprotein filament in the absence of Rhp55–Rhp57 enabling it to more efficiently promote DNA repair. This would be reminiscent of the way that *srs2* suppresses certain *RAD51* and *RAD52* non-null mutant alleles in *S.cerevisiae* (34). However, we found that an *srs2*Δ *rhp55*Δ strain is in fact more sensitive to UV, MMS and CPT, although not HU, than its component single mutant strains (Fig. 2). For UV the increase in sensitivity is approximately additive (Fig. 2A and B), whereas for MMS and CPT it appears to be more than additive (Fig. 2A). These data suggest that there may be a greater need for Srs2 to dislodge Rhp51 in the absence of Rhp55–Rhp57. Perhaps without Rhp55–Rhp57 Rhp51 forms more non-functional or aberrant nucleoprotein filaments that need to be processed by Srs2.

In *S.pombe* the generation of conversion-type recombinants depends on Rhp51 together with its accessory proteins Rhp55 and Rhp57 (27) (Fig. 3). If the hyper-recombination of an *srs2* mutant is due to unconstrained Rhp51-dependent recombination then conversion types in an *srs2*Δ strain should be

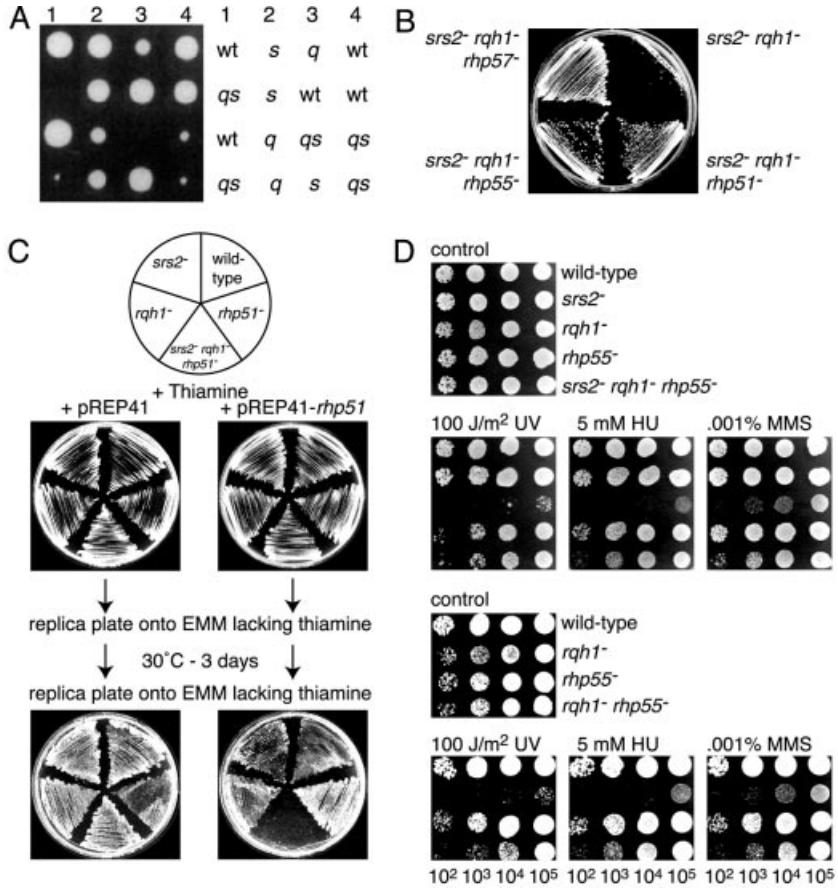


**Figure 4.** The poor viability of an *srs2*Δ *rqh1*Δ double mutant strain is rescued by deletion of *rhp51*, *rhp55* or *rhp57*. (**A**) Tetrad analysis of spores from a cross between *srs2*Δ (MCW473) and *rqh1*Δ (MCW149) mutant strains. Spores were micro-dissected as grids onto YES agar plates and incubated for 5 days at 30°C before being photographed. The growth of spores from four separate asci is shown. The genotype of each spore is shown on the right of the panel. wt, wild-type; s, *srs2*Δ single mutant; q, *rqh1*Δ single mutant; qs, *rqh1*Δ *srs2*Δ double mutant. (**B**) Streak plate showing the relative growth on complete medium of the *srs2*Δ *rqh1*Δ double mutant strain (MCW1284) and the *srs2*Δ *rqh1*Δ *rhp51*Δ (MCW1097), *srs2*Δ *rqh1*Δ *rhp55*Δ (MCW1282) and *srs2*Δ *rqh1*Δ *rhp57*Δ (MCW1283) triple mutant strains. (**C**) Complementing the improved growth of an *srs2*Δ *rqh1*Δ *rhp51*Δ triple mutant strain by expression of *rhp51* from a plasmid. The schematic at the top of the panel shows the order in which strains were streaked onto selective media. The strains carry either the plasmid pREP41 or a derivative of it expressing *rhp51* from a thiamine repressible *nmt* promoter. Strains were initially streaked onto EMM containing thiamine followed by two successive rounds of replica plating onto EMM lacking thiamine. By the second replica plating the *nmt* promoter is fully derepressed and the expression of *rhp51* is therefore switched on. The strains used were MCW42 (wild-type), MCW473 (*srs2*Δ), MCW928 (*rqh1*Δ), MCW1088 (*rhp51*Δ) and MCW1097 (*srs2*Δ *rqh1*Δ *rhp51*Δ). (**D**) Spot assay showing the relative sensitivities of wild-type (MCW42), *srs2*Δ (MCW473), *rqh1*Δ (MCW928), *rhp55*Δ (MCW1049), *rqh1*Δ *rhp55*Δ (MCW832) and *srs2*Δ *rqh1*Δ *rhp55*Δ (MCW1282) strains to UV, HU and MMS.

abolished by deleting either *rhp55* or *rhp57*. To test this we constructed *srs2*Δ *rhp55*Δ and *srs2*Δ *rhp57*Δ strains containing the direct repeat substrate shown in Figure 1C, and measured their Ade$^+$ recombinant frequencies with and without exposure to UV light (Fig. 3). In accord with our prediction, conversion types are largely abolished in *srs2*Δ *rhp55*Δ and *srs2*Δ *rhp57*Δ strains. However, the residual levels of conversion types in these double mutant strains are slightly higher than their respective *rhp55*Δ and *rhp57*Δ single mutant strains. This difference is statistically significant ($P \leqslant 0.03$) and may be due to the residual activity of ill-formed Rhp51 nucleoprotein filaments that would be dissociated in *srs2*$^+$ cells. *rhp55*Δ and *rhp57*Δ mutants, although deficient in conversion type recombination, exhibit ~4- to 5-fold higher frequencies of deletion types compared with wild type. Interestingly the *srs2*Δ *rhp55*Δ and *srs2*Δ *rhp57*Δ strains exhibit statistically significant increased frequencies of deletion type recombinants compared with their respective *rhp55*Δ and *rhp57*Δ single mutant strains ($P \leqslant 0.003$). Furthermore, in both cases, the frequency of these deletion types is induced by UV. In contrast, recombination is not induced by UV in the *rhp55*Δ and *rhp57*Δ single mutant strains. These data indicate that Srs2 acts to block the formation of some spontaneous and all UV-induced deletion types in *rhp55*Δ and *rhp57*Δ mutants. To see whether this reflects Srs2's potential for processing partially active Rhp51 nucleoprotein filaments, we next compared the frequency of recombination of a *rhp51*Δ strain with that of an *srs2*Δ *rhp51*Δ strain (Fig. 3). Like the *rhp55* and *rhp57* single mutant strains, the *rhp51*Δ strain produces hardly any conversion type recombinants, is hyper-recombinant for deletion types and shows no significant increase in recombination following UV irradiation. The *srs2*Δ *rhp51*Δ strain exhibits no significant difference from the *rhp51*Δ strain for the frequency and type of recombinants produced with or without UV. These data indicate that the recombination induced by deletion of *srs2*, in *rhp55*Δ and *rhp57*Δ

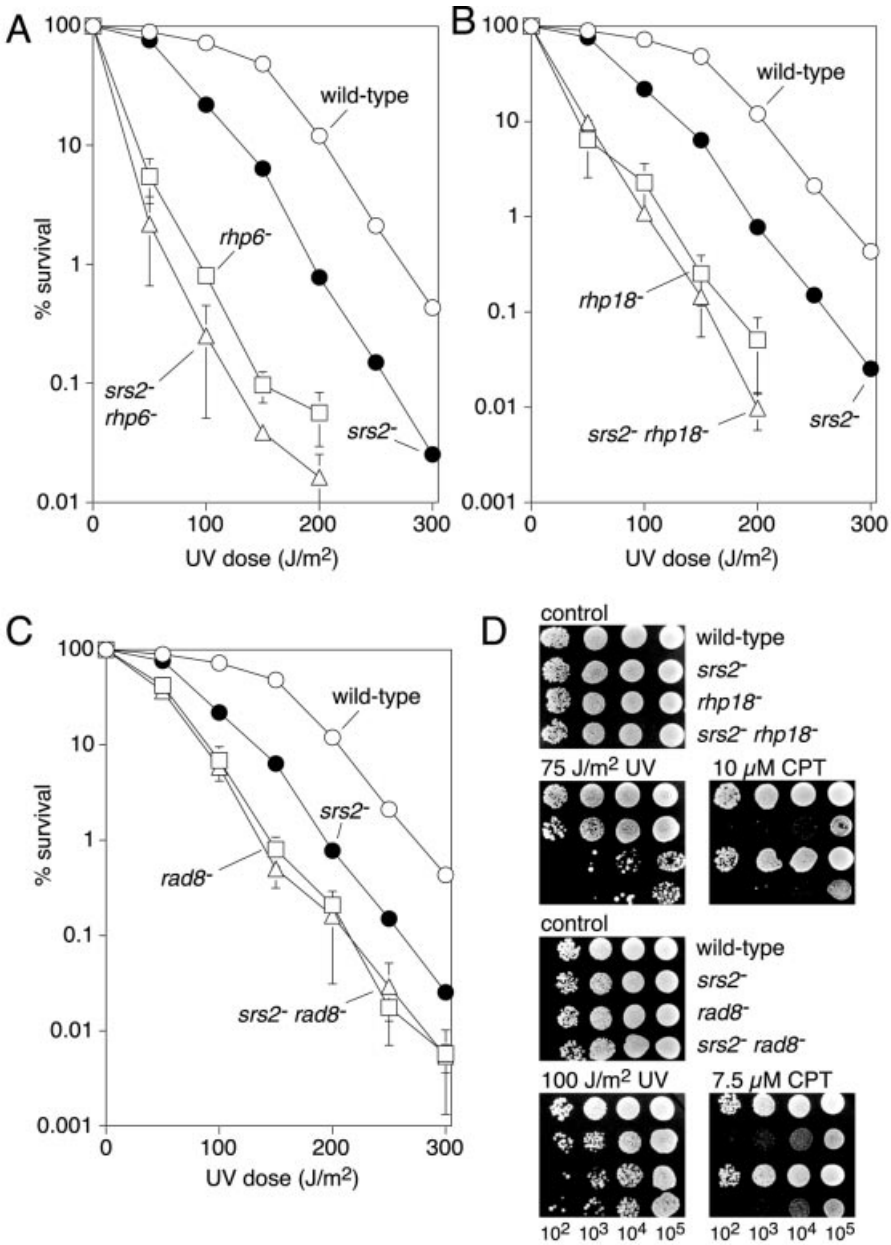


**Figure 5.** Epistasis analysis of *srs2*, *rhp6*, *rhp18* and *rad8* mutants. (**A**–**C**) Survival curves showing the relative sensitivity to UV of wild-type (MCW449), *srs2*Δ (MCW460), *rhp6*Δ (MCW595), *rhp18*Δ (MCW1269), *rad8*Δ (MCW162), *srs2*Δ *rhp6*Δ (MCW1273), *srs2*Δ *rhp18*Δ (MCW1270) and *srs2*Δ *rad8*Δ (MCW680) strains. (**D**) Spot assay showing the relative sensitivities to UV and CPT of wild-type (MCW449), *srs2*Δ (MCW460), *rhp18*Δ (MCW1269), *rad8*Δ (MCW162), *srs2*Δ *rhp18*Δ (MCW1270) and *srs2*Δ *rad8*Δ (MCW680) strains.

backgrounds, depends on *rhp51*, and is consistent with the idea that Srs2 dissociates partially active Rhp51 nucleoprotein filaments.

### Deleting *rhp51*, *rhp55* or *rhp57* suppresses the poor growth of an *srs2 rqh1* double mutant

In *S.cerevisiae* an *srs2 sgs1* double mutant is poorly viable (22). This phenotype is suppressed by deleting members of the *RAD52* epistasis group including *RAD51*, *RAD55* and *RAD57* (22). Similar poor growth/viability is seen with an *srs2 rqh1* double mutant of *S. pombe* (Fig. 4A) (23,24). However, there are conflicting data concerning whether the poor growth of an *srs2 rqh1* strain can be suppressed by blocking recombination. Maftahi *et al.* (23) have shown suppression with deletion of either *rhp51* or *rhp57*, whereas Wang *et al.* (24) concluded that viability is made worse by deleting *rhp51* in an *srs2 rqh1* mutant background. To provide a further independent test of this we constructed three triple mutant strains each containing a deletion of *srs2* and *rqh1* combined with a deletion in either *rhp51*, *rhp55* or *rhp57*. These were then compared with an *srs2*Δ *rqh1*Δ double mutant for growth (Fig. 4B). Each triple mutant grew better than the *srs2*Δ *rqh1*Δ double mutant. In the case of the *srs2*Δ *rqh1*Δ *rhp51*Δ triple mutant we confirmed that suppression was due to the deletion of *rhp51*, rather than

by some other spontaneous mutation, by complementing the improved growth with a plasmid-borne copy of *rhp51* expressed from a thiamine repressible *nmt* promoter (Fig. 4C). These data corroborate the results reported by Maftahi *et al.* (23). The failure of Wang *et al.* (24) to observe suppression of *srs2*Δ *rqh1*Δ poor growth by deletion of *rhp51* may relate to the particular *rhp51*Δ mutant that they used in their studies (23).

### Suppression of *rqh1*Δ DNA damage sensitivity by *rhp55*Δ is independent of Srs2

The hypersensitivity of a *rqh1*Δ mutant to certain DNA damaging agents is suppressed by deleting *rhp51*, *rhp55* or *rhp57* (Fig. 4D, and our unpublished data) (35). As Srs2 appears to control residual Rhp51 activity in *rhp55*Δ and *rhp57*Δ mutants, we looked to see whether it is required for *rhp55*Δ's suppression of *rqh1*Δ's sensitivity to UV, HU and MMS (Fig. 4D). The *srs2*Δ *rqh1*Δ *rhp55*Δ strain behaves like a *rqh1*Δ *rhp55*Δ strain and is noticeably less sensitive to UV, HU and MMS than a *rqh1*Δ single mutant (Fig. 4D). These data suggest that residual Rhp51 activity in the absence of Rhp55 and Srs2 does not necessitate the action of Rqh1.

### Srs2's relationship to the PRR pathway in *S.pombe*

To investigate whether Srs2 is involved in the PRR pathway in *S.pombe* we first compared the UV sensitivities of *srs2*Δ and *rhp6*Δ (homologue of *RAD6*) single mutant strains with that of an *srs2*Δ *rhp6*Δ double mutant (Fig. 5A). The double mutant is only slightly more sensitive to UV than the most sensitive single mutant (*rhp6*) indicating that *srs2* is more or less epistatic with *rhp6* for UV sensitivity. The same analysis was performed with two further PRR genes [*rhp18* (*RAD18* homologue) and *rad8* (*RAD5* homologue)]. In both cases *srs2* is epistatic with the PRR gene for UV sensitivity (Fig. 5B and C). These data indicate that Srs2 functions in the PRR pathway for the tolerance/repair of UV-induced DNA damage. The slight increase in UV sensitivity of the *srs2*Δ *rhp6*Δ double mutant compared with a *rhp6*Δ single mutant may be due to the fact that Rhp6 is likely to have roles separate from PRR (36). Possibly, defects in these non-PRR functions enable some UV-induced damage to be processed by a mechanism that involves Srs2.

We also compared the *srs2* and PRR mutant strains for sensitivity to CPT (Fig. 5D and data not shown). Whereas an *srs2*Δ mutant is hypersensitive to CPT, *rhp6*Δ, *rhp18*Δ and *rad8*Δ mutants are about as sensitive as a wild-type strain. Furthermore, the *srs2*Δ *rhp6*Δ, *srs2*Δ *rhp18*Δ and *srs2*Δ *rad8*Δ double mutant strains exhibit the same sensitivity as an *srs2*Δ single mutant. Together with the data in Figure 2B, these results suggest that Srs2 and Rhp51 function together, independently of the PRR pathway, for the repair of SEBs.

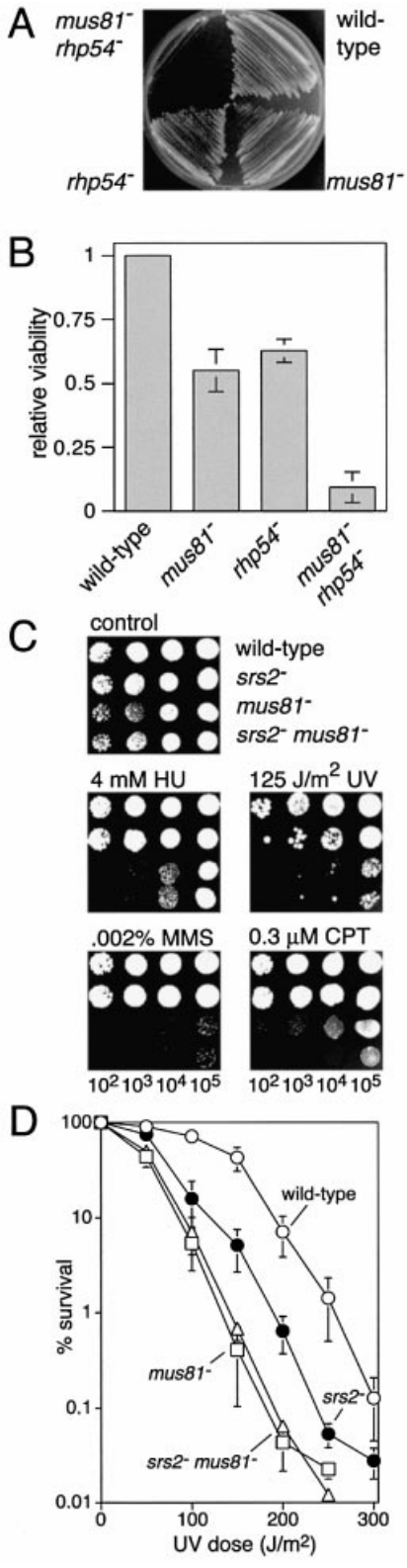


**Figure 6.** Genetic interactions between *mus81* and *rhp54*, and *mus81* and *srs2*. (**A**) Streak plate showing the relative growth on complete medium of the *mus81*Δ *rhp54*Δ double mutant strain (MCW897) compared with wild-type (MCW42), *mus81*Δ (MCW745) and *rhp54*Δ (MCW121) control strains. The plate was photographed after 4 days growth at 30°C. (**B**) Histogram showing the relative viability compared with wild-type (MCW42) of *mus81*Δ (MCW745), *rhp54*Δ (MCW121) and *mus81*Δ *rhp54*Δ (MCW897) strains on complete medium. (**C**) Spot assay showing the relative sensitivities of wild-type (MCW449), *srs2*Δ (MCW460), *mus81*Δ (MCW744) and *srs2*Δ *mus81*Δ (MCW747) strains to UV, HU, MMS and CPT. (**D**) Survival curves showing the relative sensitivity to UV of wild-type (MCW449), *srs2*Δ (MCW460), *mus81*Δ (MCW744) and *srs2*Δ *mus81*Δ (MCW747) strains.

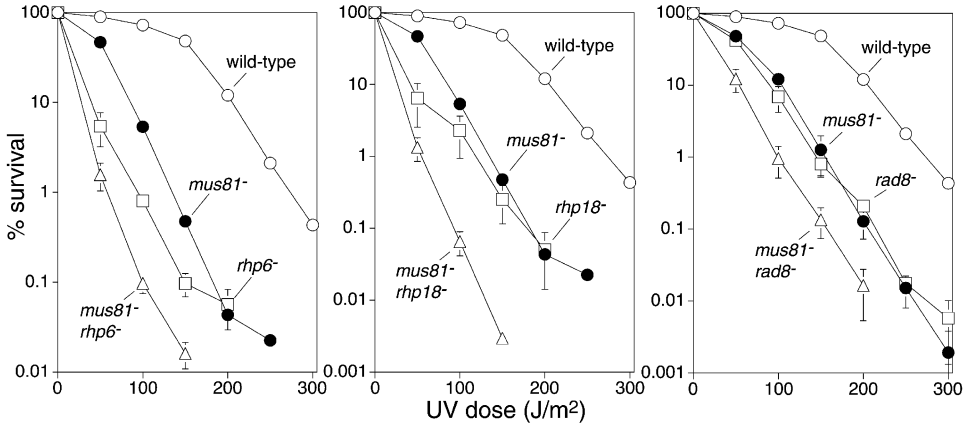


**Figure 7.** Epistasis analysis of the *mus81* mutant with *rhp6*, *rhp18* and *rad8* mutants for UV sensitivity. The strains used were MCW449 (wild-type), MCW744 (*mus81*Δ), MCW595 (*rhp6*Δ), MCW1269 (*rhp18*Δ), MCW162 (*rad8*Δ), MCW1137 (*mus81*Δ *rhp6*Δ), MCW1271 (*mus81*Δ *rhp18*Δ) and MCW1272 (*mus81*Δ *rad8*Δ).

### Genetic interactions between *srs2* and *mus81*

Mus81–Eme1 is a heterodimeric endonuclease that appears to be required for the cleavage of a range of different DNA junctions that are formed during HR and the regression of stalled replication forks (37–41). Similar to *srs2*, *mus81* has strong genetic interactions with both *rqh1*and *rhp54*, with the *mus81 rqh1* double mutant being unviable (38,42), and the *mus81 rhp54* double mutant exhibiting severely reduced viability (Fig. 6A and B). To investigate the genetic interaction between *srs2* and *mus81* we constructed a double mutant strain and compared it with wild-type, and *srs2* and *mus81* single mutant strains for growth and sensitivity to HU, UV, MMS and CPT. The *srs2*Δ *mus81*Δ double mutant grows just as well as a *mus81*Δ single mutant (data not shown). It is also indistinguishable from a *mus81*Δ strain for sensitivity to HU, UV and MMS (Fig. 6C and D). In the case of HU and MMS, the *mus81* mutant is considerably more sensitive than the *srs2* mutant, and this makes it difficult to tell whether deletion of *srs2* adds to the sensitivity of the *mus81*Δ strain or not. However, in the case of UV the difference in sensitivity between the single mutants is not so marked, enabling us to conclude that *srs2* and *mus81* are epistatic for UV sensitivity. This suggests that they function in the same pathway for the repair/tolerance of UV-induced damage. In contrast, the *srs2*Δ *mus81*Δ double mutant is noticeably more sensitive to CPT than either of the single mutants (Fig. 6C). The increase in sensitivity appears to be synergistic, suggesting that Mus81 and Srs2 promote the repair of SEBs by separate but overlapping pathways.

### Mus81 and the PRR pathway

From the data in Figures 5 and 6 it is evident that *srs2* is epistatic with both *mus81* and the PRR pathway genes for UV sensitivity. It was therefore important to establish the genetic relationship between *mus81* and the PRR pathway. Double mutants of *mus81*Δ with *rhp6*Δ, *rhp18*Δ and *rad8*Δ were made and compared with their respective single mutant strains for sensitivity to UV (Fig. 7). Each double mutant is more sensitive than either of its relative single mutant strains, and the increase is no more than additive. These data show that both Mus81 and the PRR pathway can function independently of each other to promote the repair/tolerance of UV-induced damage. However, the fact that they share in common an epistatic interaction with *srs2* suggests that there is a sub-pathway of PRR that depends on both Srs2 and Mus81.

## DISCUSSION

In *S.cerevisiae* a key function of the Srs2 DNA helicase is to prevent or limit potentially deleterious recombination. This is a function that Srs2 shares with the RecQ family DNA helicase Sgs1, as judged by the severe growth defects of an *srs2 sgs1* double mutant, which can be suppressed by blocking recombination at a presynaptic stage. These features of Srs2 behaviour are conserved in *S.pombe* as reported here and by Maftahi *et al.* (23). However, as discussed below, Srs2 is less critical for the PRR pathway in *S.pombe* than it is in *S.cerevisiae*. We also highlight below the genetic interactions between *srs2* and *mus81*, and Srs2's role in promoting the recombinational repair of SEBs.

### The involvement of Srs2 and Mus81 in a sub-pathway of PRR

In *S.cerevisiae*, deletion of *srs2* suppresses the UV sensitivity of *rad6*, *rad18* and *rad5* mutants (10–13). This has been taken as evidence that Srs2 is required to channel DNA lesions into the PRR pathway. In *S.pombe*, mutation of *srs2* exerts no suppression on the UV sensitivity of *rhp6*$^{-}$, *rhp18*$^{-}$ or *rad8*$^{-}$ strains. Therefore, in contrast to *S.cerevisiae*, Srs2 does not appear to be essential for channelling DNA lesions to the PRR pathway in *S.pombe*. This may be because PRR proteins are more able to compete with HR proteins for access to lesion-containing DNAs. In this regard it is worth noting that in *S.pombe*, PRR proteins can work upstream of HR proteins, at least in some situations. Rhp18, for example, is active throughout the cell cycle in response to UV-induced damage, and in $G_2$, Rhp51 foci formed in response to UV are partially dependent on Rhp18 (35). Another possibility for why Srs2 appears to be less critical for PRR in *S.pombe* than in *S.cerevisiae* is redundancy of function with Rqh1. In *S.cerevisiae*, over-expression of Sgs1 can partially suppress the need for Srs2 (43). Perhaps in *S.pombe* endogenous levels

of Rqh1 are more able to compensate for the lack of Srs2 than they are in *S.cerevisiae*. In this regard it is interesting to contemplate the possibility that in humans, where a homologue of Srs2 has not been identified, the role of Srs2 has been taken on by one of five known RecQ DNA helicases (44).

Even though Srs2 is not required to channel lesions to PRR in *S.pombe*, it still plays some role in the PRR response to UV-induced damage, as judged by its epistatic relationship to *rhp6*, *rhp18* and *rad8* for UV sensitivity. Interestingly, this sub-pathway of PRR also appears to depend on Mus81, based on the epistatic relationship between *srs2* and *mus81* for UV sensitivity. In *S.cerevisiae* it is thought that Srs2 channels lesions to the PRR pathway by dissociating Rad51 nucleoprotein filaments. If the same is true in *S.pombe*, what possible role could Mus81–Eme1 have in this pathway? Mus81–Eme1 is a structure-specific endonuclease, which can potentially cleave a variety of DNA junctions *in vivo* (37–41). These include the aberrant structures that can form when replication forks are blocked, the D-loops that are formed by strand invasion reactions, and 3′ flaps that can form during the repair of DSBs and single-strand gaps by SDSA. It is difficult to see how any of these known activities of Mus81–Eme1 could collude with Srs2 to dislodge Rhp51 from DNA and direct repair to the PRR pathway. Possibly Srs2 only acts after strand invasion, and although *in vitro* it is unable to dissociate an intact D-loop (17), perhaps it can do so if the base of the D-loop is first nicked by Mus81–Eme1 (40). Alternatively, as suggested by Fabre and co-workers, Srs2 and Mus81–Eme1 could work together in a SDSA-type mechanism for the repair of lesion-containing gaps (45). In this model, Rhp51 catalyses strand invasion from the damaged DNA into an intact sister chromatid to form a D-loop. The 3′ end of the invading strand primes DNA synthesis enabling recovery of the missing genetic information. Srs2 then dissociates the D-loop so that the invading strand re-anneals to the lesion-containing strand. Over-replication will result in the formation of a 3′ flap, which could be removed by Mus81–Eme1. It is unclear what role PRR proteins could play in this mechanism. One possibility is that when Mus81–Eme1 removes the 3′ flap it generates a small single-stranded gap (39,40). Conceivably, PRR proteins could be involved in the repair of this gap.

### Do the shared genetic interactions of *srs2* and *mus81* point to a common pathway?

Srs2 and Mus81 appear to work in a common pathway responding to UV-induced damage. However, the synergistic increase in CPT sensitivity of a *mus81 srs2* double mutant, compared with *mus81* and *srs2* single mutants, indicates that Mus81 and Srs2 also function in independent pathways for the repair of SEBs. Interestingly, both *mus81* and *srs2* have strong genetic interactions with *rqh1* and *rhp54*. However, these common interactions are not necessarily indicative of Mus81 and Srs2 functioning in the same pathway. As shown here and elsewhere, the poor growth of an *srs2 rqh1* double mutant is suppressed by mutation of *rhp51* (23). In contrast, the unviability of a *mus81 rqh1* double mutant is not rescued by *rhp51* mutation (C. L. Doe, F. Osman, J. Dixon and M. C. Whitby, unpublished data). These data suggest that Rqh1 is needed in an *srs2*$^{-}$ background to process recombination intermediates, whereas in a *mus81*$^{-}$ background it is needed to process other kinds of intermediates that are formed independently of Rhp51. As mentioned earlier, the unviability of an *srs2 rhp54* double mutant may reflect a need for Srs2 to dissociate Rhp51 nucleoprotein filaments that are incompetent for normal strand invasion in the absence of Rhp54 (16). What underlies the poor growth of a *mus81 rhp54* double mutant is not certain. In fact, this interaction was a surprise to us, since in *S.cerevisiae*, *MUS81* and *RAD54* exhibit a purely epistatic relationship and their proteins physically interact (46). One possible explanation for the strong genetic interaction between *mus81* and *rhp54* in *S.pombe* is that they both contribute to the stability of joint molecules formed by Rhp51-mediated strand invasion. Based on the activities of *S.cerevisiae* and human Rad54 *in vitro*, Rhp54 could do this in a number of ways, from dissociating proteins that might impede Rhp51-mediated strand invasion, to altering the supercoiling of the target DNA so that its strands can be readily unpaired for D-loop formation (20). We have recently shown that Mus81–Eme1 can cleave D-loops *in vitro*, and this activity could provide an additional way of helping to stabilize the strand invasion (40). It is worth noting here that Srs2 may also aid joint molecule stability by dissociating Rhp51 from the displaced single strand at a D-loop, thereby preventing potential reversal of strand invasion (17).

### Srs2 and the repair of collapsed replication forks

The hypersensitivity of an *srs2* mutant to CPT indicates that Srs2 promotes the repair of SEBs formed when replication forks collapse at strand nicks in the template DNA. This role for Srs2 must be distinct from PRR because *rhp6*, *rhp18* and *rad8* mutants show no hypersensitivity to CPT. Interestingly, *S.pombe* PRR mutants are hypersensitive to ionizing radiation (47–49). So, although they might not be important for SEB repair, PRR proteins do play a role in the repair of DSBs. This provides evidence for mechanistic differences between SEB and DSB repair.

Srs2 seems to function in the Rhp51-dependent pathway for SEB repair. We suspect that this pathway proceeds via strand invasion from the broken arm of the replication fork back into the intact arm. This generates a D-loop, which may act as a site for replisome reassembly (1). What might Srs2's involvement be here? One possibility, which is mentioned above, is that it could help to stabilize the D-loop by dissociating any Rhp51 that nucleates onto the displaced strand (17). Promoting the recycling of a finite resource of Rhp51 may also aid in the efficient repair of multiple SEBs. Alternatively, Srs2's requirement for SEB repair could reflect its potential involvement in DNA damage checkpoint responses. Srs2 in *S.cerevisiae* is phosphorylated as part of the intra-S checkpoint response to DNA damage, and is required for 'switching off' the checkpoint response after the damage has been repaired (50,51). This might be achieved by dissociating Rad51 and/or checkpoint proteins from the DNA (16). It will be interesting to see whether Srs2 behaves similarly in *S.pombe*, and whether this, or any of the above potential activities, underlies Srs2's involvement in repairing collapsed replication forks.

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