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Sws1 is a conserved regulator of homologous recombination in eukaryotic cells

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Rad52-dependent homologous recombination (HR) is regulated by the antirecombinase activities of Srs2 and Rqh1/Sgs1 DNA helicases in fission yeast and budding yeast. Functional analysis of Srs2 in *Schizosaccharomyces pombe* led us to the discovery of Sws1, a novel HR protein with a SWIM-type Zn finger. Inactivation of Sws1 suppresses the genotoxic sensitivity of *srs2*Δ and *rqh1*Δ mutants and rescues the inviability of *srs2*Δ *rqh1*Δ cells. Sws1 functions at an early step of recombination in a prorecombinogenic complex with Rlp1 and Rdl1, two RecA-like proteins that are most closely related to the human Rad51 paralogs XRCC2 and RAD51D, respectively. This finding indicates that the XRCC2–RAD51D complex is conserved in lower eukaryotes. A SWS1 homolog exists in human cells. It associates with RAD51D and ablating its expression reduces the number of RAD51 foci. These studies unveil a conserved pathway for the initiation and control of HR in eukaryotic cells.

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Introduction

Homologous recombination (HR) is a universal process of error-free DNA repair (Paques and Haber, 1999; Sung *et al*, 2003; West, 2003; Krogh and Symington, 2004). Its defining feature is the use of homologous sequences as a template for repairing damaged DNA. HR is most commonly studied in the context of double-strand breaks (DSBs), but it can also be initiated by single-strand gaps that occur during DNA replication or in certain types of DNA repair, such as base excision repair or trans-lesion synthesis (Swanson *et al*, 1999). In the budding yeast *Saccharomyces cerevisiae* and fission yeast *Schizosaccharomyces pombe*, HR is under the control of the RAD52 epistasis group of proteins, whose members are conserved in eukaryotes (Sung *et al*, 2003; Krogh and Symington, 2004). Formation of a Rad51 nucleoprotein filament, a process dependent on Rad52 and mediated by the Rad51 paralogs, is a key step in HR (Sung, 1997a; Sugawara *et al*, 2003; Lisby *et al*, 2004). The Rad51 paralogs act primarily as heterodimers or larger multimeric complexes (Masson *et al*, 2001). For example, budding yeast Rad55 and Rad57 form a complex (Sung, 1997b), as do the equivalent proteins in fission yeast, Rhp55 and Rhp57 (Tsutsui *et al*, 2001). Multicellular eukaryotes have an additional Rad51 paralog subcomplex, consisting of XRCC2 and RAD51D (Braybrooke *et al*, 2000; Masson *et al*, 2001), that has not been found in yeast species.

Although HR is essential for the preservation of genome integrity, there are pathological situations in which excessive HR can destabilize the genome and cause cell death. One example involves an *S. cerevisiae* mutant that lacks two DNA helicases: the Sgs1 RecQ-type DNA helicase and Srs2. The inviability of this mutant can be suppressed by inactivating HR genes (Gangloff *et al*, 2000). Conservation of an antirecombinase activity of Sgs1 homologs is indicated by the hyper-recombination phenotype of fission yeast *rqh1*Δ cells and the effects of mutations of three of the five human RecQ homologs, BLM, WRN and RECQL4, which cause the cancer-prone syndromes Bloom, Werner and Rothmund–Thomson, respectively. All these syndromes are associated with genomic instability that arises from inappropriate recombination (Hickson, 2003).

The RecQ-like DNA helicases and Srs2 homologs are thought to act as antirecombinases by different mechanisms. In the case of a RecQ-like helicase complexed with a topoisomerase, *in vitro* studies have shown that this complex can dissolve a DNA substrate containing two Holliday junctions (HJs) into a single noncrossover product (Wu and Hickson, 2003). RecQ-like helicases might also unwind dsDNA structures that are HR intermediates, such as D-loops (van Brabant *et al*, 2000). In the case of Srs2, *in vitro* studies have shown it can disassemble the Rad51 nucleoprotein filament (Krejci *et al*, 2003; Veaute *et al*, 2003). Interestingly, inclusion of the single-strand DNA binding protein RPA in these assays has a synergistic effect that can be partially overcome by addition of Rad52. These data suggest that Srs2 and Rad52 have antagonistic effects in controlling HR.

The C-terminal noncatalytic domain of *S. cerevisiae* Srs2 mediates its interaction with Rad51 (Krejci *et al*, 2003). Curiously, this domain is apparently not conserved in *S. pombe* Srs2, even though the antirecombinogenic activities of the Srs2 homologs are conserved (Gangloff *et al*, 2000; Klein, 2001; Doe and Whitby, 2004). To better understand the interplay of pro and antirecombinogenic activities, we undertook a screen for proteins that interact with Srs2 in fission yeast. As described here, this screen led to identification of Sws1, a protein that forms a prorecombinogenic complex with XRCC2 and RAD51D-like proteins. This complex is

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conserved in humans, and it may be related to the Shu group of proteins recently discovered in budding yeast (Shor *et al*, 2005). These studies establish the existence of a conserved pathway for initiation and control of HR in eukaryotic cells.

Results

Sws1 interacts with Srs2

We performed a yeast two-hybrid screen with the C-terminal 187 amino acids of *S. pombe* Srs2, which is the region responsible for its interaction with Rhp51 (our unpublished results), the Rad51 homolog in fission yeast. One of the hits was SPBC11B10.06, a gene listed as a sequence orphan with a SWIM domain (Figure 1A). A SWIM domain is an uncharacterized Zn-finger-like motif found in several classes of proteins, including some members of the SWI2/SNF2 family of ATPases and the MuDR plant transposases (Makarova *et al*, 2002). SPBC11B10.06 was given the name Sws1 (SWIM domain-containing and Srs2-interacting protein 1).

Typical of DNA repair proteins, GFP-tagged Sws1 localized to the nucleoplasm and was excluded from the nucleolus (Supplementary Figure 1A). This pattern was unchanged throughout the cell cycle. Sws1-GFP did not form discrete nuclear foci when cells were exposed to genotoxic agents (data not shown). The nuclear signal of Sws1-GFP was eliminated by *in situ* detergent extraction (Supplementary Figure 1B), indicating that Sws1 does not associate tightly with chromatin (Noguchi *et al*, 2003).

Analysis of *sws1*Δ cells revealed no obvious growth or morphological defects. They were not generally sensitive to genotoxic agents such as ionizing radiation (IR), ultraviolet (UV) light, camptothecin (CPT) or hydroxyurea (HU), but they were sensitive to 0.02% methylmethane sulphonate (MMS) (Figure 1D and Supplementary Figure 2). This finding provided the first hint that Sws1 was involved in DNA repair.

*Sws1 inactivation suppresses srs2*Δ *and rqh1*Δ

Genetic epistasis studies were performed to investigate the functional relationship between Sws1 and Srs2. CPT sensitivity was examined because *srs2*Δ mutants are particularly sensitive to this genotoxic agent (Doe and Whitby, 2004). Surprisingly, we found that *sws1*Δ suppressed the *srs2*Δ CPT sensitive phenotype (Figure 2A). This result was unanticipated because Srs2 is thought to play an active role in promoting the repair of CPT-induced DNA damage, as opposed to preventing the formation of toxic recombination intermediates (Fabre *et al*, 2002; Doe and Whitby, 2004).

This result prompted an analysis of genetic interactions involving Sws1 and Rqh1. The HU and UV sensitivity of *rqh1*Δ cells is thought to arise from the formation of toxic recombination intermediates (Laursen *et al*, 2003; Doe and Whitby, 2004; Hope *et al*, 2005). Remarkably, the *sws1*Δ mutation not only suppressed the HU and UV sensitivity of *rqh1*Δ cells, it also suppressed their sensitivity to IR, CPT and low doses of MMS (Figure 2B).

Upon entering mitosis after treatment with HU, *rqh1*Δ cells display a characteristic 'cut' phenotype in which unsegregated chromosomes are bisected by the cell division plate. This phenotype is thought to arise from unresolved covalent linkages of sister chromatids that have occurred through HR (Stewart *et al*, 1997; Doe *et al*, 2000). We reasoned that, if Sws1 promotes these HR events, the suppression of the HU sensitivity of *rqh1*Δ cells by *sws1*Δ should be accompanied by a reduction of the 'cut' phenotype. Indeed, upon release from an HU arrest, the frequency of 'cut' phenotypes was reduced ~50% in *rqh1*Δ *sws1*Δ cells relative to *rqh1*Δ cells (Figure 2C). A similar effect was seen by combining the *srs2*Δ and *sws1*Δ mutations (Figure 2C). From these results we conclude that Sws1 promotes the generation of the toxic HR intermediates in *rqh1*Δ and *srs2*Δ cells.

These genetic interactions could be explained if Sws1 inhibits the antirecombinogenic activities of both Srs2 and Rqh1. To test this model, we determined whether *sws1*Δ rescued the extreme sickness of a *rqh1*Δ *srs2*Δ double mutant (Lee *et al*, 1999; Wang *et al*, 2001). Remarkably, *sws1*Δ almost completely rescued the poor growth of *rqh1*Δ *srs2*Δ cells (Figure 3A). It also rescued their poor plating efficiency, restoring it from 5 to 44% compared to wild type. The specificity of this rescue was confirmed by controlling *sws1*$^+$ expression from the thiamine (vitamin B_1)-repressible *nmt1* promoter in *sws1*Δ *rqh1*Δ *srs2*Δ cells (Figure 3B). These findings showed that Sws1 can function independently of both Rqh1 and Srs2.

Rqh1 is essential for viability in strains that lack the structure-specific endonucleases Mus81–Eme1 or Slx1–Slx4 (Boddy *et al*, 2001; Coulon *et al*, 2004), and in strains that

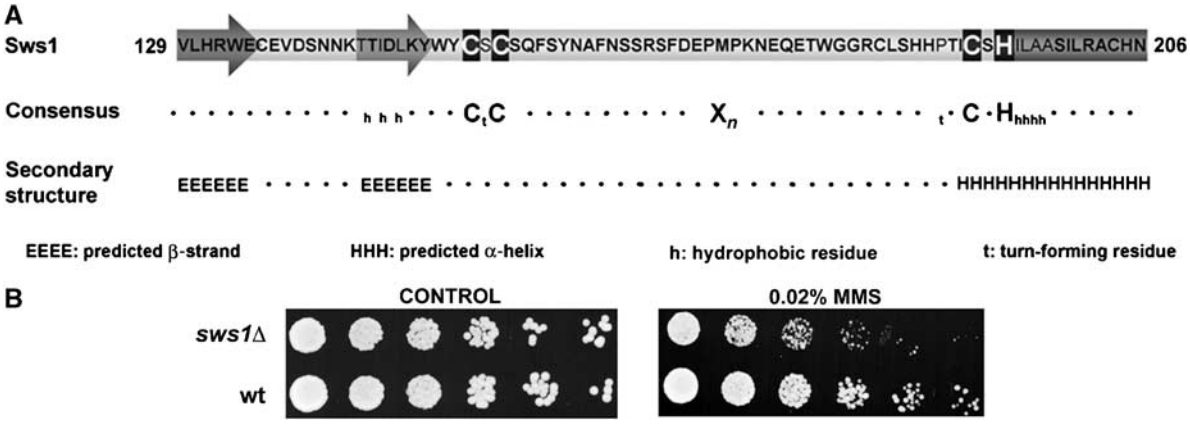


Figure 1 SWIM domain of Sws1 and MMS sensitivity of *sws1*Δ mutants. (**A**) SWIM domain of Sws1. The potential metal-chelating residues defined by Makarova *et al* (2002) are shown. The predicted α-helix and β-strands are highlighted. The secondary structure prediction was obtained using the Jpred program. (**B**) DNA damage survival assay. Four-fold serial dilutions of wt (PR109) and *sws1*Δ (VM3723) cells were plated on YES media and exposed to 0.02% MMS. Photographs were taken after 4 days at 32°C.

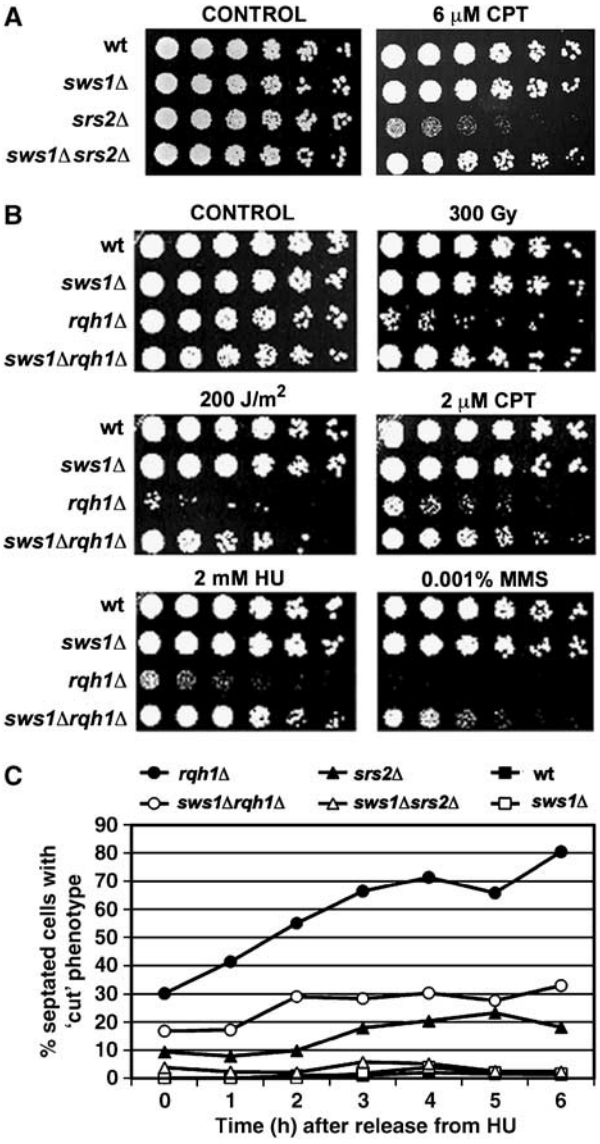


Figure 2 Effect of *sws1*$^+$ deletion on survival of *srs2*Δ and *rqh1*Δ mutants. (**A**) Spot assay of wt (PR109), *sws1*Δ (VM3723), *srs2*Δ (VM3724) and *sws1*Δ *srs2*Δ (VM3721) strains. Four-fold serial dilutions of each strain were plated on YES or YES supplemented with 6 μM camptothecin. Photographs were taken after 4 days at 32°C. (**B**) DNA damage sensitivity of wt (PR109), *sws1*Δ (VM3723), *rqh1*Δ (SC3250), and *sws1*Δ *rqh1*Δ (VM3722) cells. Four-fold serial dilutions were plated onto YES plates and treated with the indicated DNA damaging agents. Photographs were taken after 4 days at 32°C. (**C**) Percentages of dividing cells with 'cut' phenotype observed in wt (PR109), *sws1*Δ (VM3723), *srs2*Δ (VM3724), *rqh1*Δ (SC3250), *sws1*Δ *srs2*Δ (VM3721) and *sws1*Δ *rqh1*Δ (VM3722) strains at the indicated time points after release from 5-h-incubation in YES supplemented with 12 mM HU.

lack the Swi1–Swi3 replication fork protection complex (Noguchi *et al*, 2003; Coulon *et al*, 2004). The interactions involving Swi1–Swi3 complex, but not those involving Mus81–Eme1 and Slx1–Slx4 complexes, are rescued by mutations that inactivate HR proteins (e.g. Rad22^{Rad52}, Rhp51^{Rad51} and Rhp55^{Rad55}). As summarized in Figure 3C, *sws1*Δ had a pattern of genetic interactions that matched those of *rad22*Δ, *rhp51*Δ and *rhp55*Δ mutations. These findings further supported the conclusion that Sws1 has a prorecombinogenic activity that acts in concert with the other known recombination proteins in fission yeast.

Sws1 controls an early step of HR

Rad52 has a crucial early role in HR, displacing replication protein A (RPA) and recruiting Rad51 to single-stranded DNA, leading to the formation of the Rad51 nucleoprotein filament (Sung, 1997a; Sugawara *et al*, 2003; Lisby *et al*, 2004). If Sws1 controls an early step of HR, recruitment of Rad22 (the Rad52 homolog) to DNA damage sites should be reduced in *sws1*Δ cells. Rad22-YFP forms bright nuclear foci at sites of DNA damage (Du *et al*, 2003); therefore, we monitored the effect of *sws1*Δ on formation of spontaneous Rad22-YFP foci in wild type, *rqh1*Δ and *srs2*Δ backgrounds. As shown in Figure 4A, the number of nuclei containing Rad22-YFP foci was substantially elevated in *rqh1*Δ and *srs2*Δ mutants relative to wild type. In all of these genetic backgrounds, *sws1*Δ reduced the number of spontaneous Rad22-YFP foci without affecting Rad22-YFP abundance (Figure 4A and data not shown).

To formally address whether Sws1 promotes HR, we measured the rates of recombination between direct repeats of *ade6*$^-$ heteroalleles that are separated by the *ura4*$^+$ gene. This assay can distinguish between two classes of recombination events: deletion types (*ade6*$^+$ *ura4*$^-$) and conversion types (*ade6*$^+$ *ura4*$^+$) (Osman *et al*, 1996). Rad22 is required for both types, whereas Rhp51, Rhp55 and Rhp57 are only required for conversion types. It should be noted that the overall rate of recombination is actually increased in the absence of Rhp51, Rhp55 and Rhp57, with all events being Rad22-dependent deletion types (Doe and Whitby, 2004; Doe *et al*, 2004). As shown in Figure 4B, *sws1*Δ reduced the frequency of recombination events by about half in both *rqh1*$^+$ and *rqh1*Δ cells, with both conversion and deletion types decreased. Taken together with suppression of *rqh1*Δ DNA damage sensitivity by *sws1*Δ (Figure 2), these findings show that Sws1 controls an early step of HR.

Sws1 is related to S. cerevisiae Shu2

Before proceeding further, we decided to re-examine whether Sws1 is conserved in eukaryotes. As noted above, the only recognizable sequence motif in Sws1 is the SWIM domain (Figure 1A), which consists of a $CxCx_nCxH$ motif located downstream of two predicted β strands and followed by an α-helix (Makarova *et al*, 2002). To ascertain the functional significance of the SWIM domain, the first cysteine of the $CxCx_nCxH$ motif was replaced by a serine residue (C152S). This mutation is predicted to ablate metal chelation. The *sws1-C152S* mutation caused MMS sensitivity and suppressed the DNA damage sensitivity of *rqh1*Δ cells, behaving identically to *sws1*Δ (Figure 5A). We confirmed that Sws1-C152S-GFP localized to the nucleus with the same pattern as the wild-type protein (Supplementary Figure 1C).

Having demonstrated the importance of the SWIM domain, we performed iterative PSI-BLAST searches with a bias towards hits containing the SWIM motif $CxCx_nCxH$. Highly divergent homologs of Sws1 were found in most eukaryotes, with an x_n variable between 15 (human) and 39 (*Candida albicans*) (Figure 5C). The existence of an Sws1-like gene in the yeast *C. albicans* prompted a closer examination of the *S. cerevisiae* genome. Using relaxed criteria, we found a *S. cerevisiae* Sws1-related gene that has a SWIM motif with an unusually long insert ($x_n = 59$) (Figure 5B and C). This gene was named Shu2 for 'Suppressor of Sgs1 HU sensitivity' (Shor *et al*, 2005). As the name indicates, *shu2*Δ suppresses the HU sensitivity of mutants defective in Sgs1,

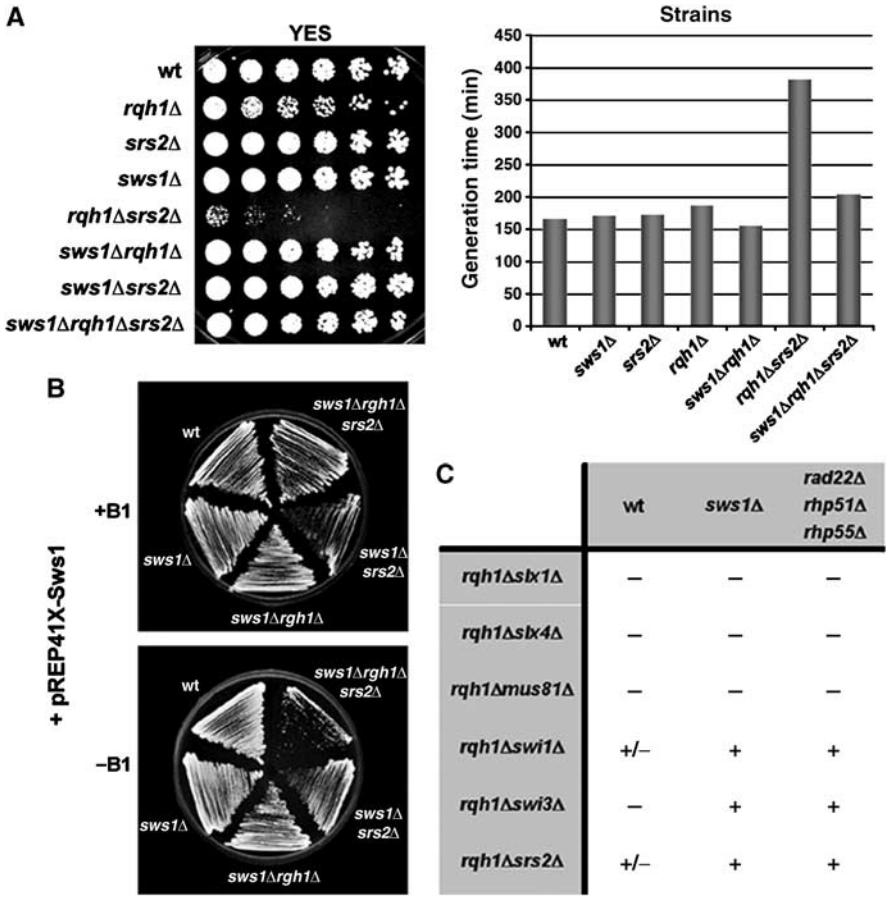


Figure 3 Deletion of *sws1*$^+$ rescues the slow growth of *srs2*Δ *rqh1*Δ double mutants. (**A**) Spot assay of wt (PR109), *rqh1*Δ (SC3250), *srs2*Δ (VM3724), *sws1*Δ (VM3723), *rqh1*Δ *srs2*Δ (VM3756), *sws1*Δ *rqh1*Δ (VM3722) *sws1*Δ *srs2*Δ (VM3721) and *sws1*Δ *rqh1*Δ *srs2*Δ (VM3720) strains. Four-fold serial dilutions of each strain were plated on YES. Photographs were taken after 4 days at 32°C. The generation times of wt and some of the mutants used in the spot assay is shown. Cell growth of cultures incubated at 32°C in liquid YES was monitored by measuring optical density at 600 nm. (**B**) Specificity of the rescue. wt (PR109), *sws1*Δ (VM3723), *sws1*Δ *rqh1*Δ (VM3722), *sws1*Δ *srs2*Δ (VM3757) and *sws1*Δ *rqh1*Δ *srs2*Δ (VM3735) were transformed with a pREP41x-Sws1 vector and plated on EMM plates with (for overexpression of Sws1) or without thiamine (repression). When *sws1*$^+$ is overproduced, *sws1*Δ *rqh1*Δ *srs2*Δ (VM3735) cells return to the same rates of slow growth as *srs2*Δ *rqh1*Δ (VM3756) cells. Photographs were taken after 5 days at 32°C. (**C**) Comparison between the subsets of double mutants rescued by *sws1*Δ and the already characterized recombination genes *rad22*Δ, *rhp51*Δ and *rhp55*Δ. Spores derived from the corresponding crosses were plated on YES and the resulting colonies screened by PCR. Crosses giving rise to triple mutants are shown as '+'. Crosses in which it was not possible to recover any triple mutant, or synthetic lethal combinations appeared as '−'. '+/−' indicates synthetic slow growth.

the homolog of Rqh1. Like *sws1*Δ cells, *shu2*Δ mutants are also sensitive to MMS and they have reduced Rad52 foci (Shor *et al*, 2005). The implication, therefore, is that Sws1 and Shu2 are highly divergent homologs.

Sws1 associates with Rad51 paralogs

Genetic and yeast two-hybrid studies have indicated that Shu2 functions in association with three other proteins: Shu1, Psy3 and Csm2 (Huang *et al*, 2003; Lee *et al*, 2005; Shor *et al*, 2005). These proteins have no identifiable motifs, nor do they have homologs except in closely related yeast species (Shor *et al*, 2005). With the aim of identifying highly divergent homologs of Shu1, Psy3 or Csm2, we decided to use multidimensional protein identification technology (MudPIT) to identify proteins that coprecipitate with TAP-tagged Sws1 (Washburn *et al*, 2001). Besides Srs2, this analysis identified only one other protein known to be connected to HR (Supplementary Table II). This protein was Rlp1, a RecA-like protein that is most closely related to the RAD51 paralog XRCC2 (Khasanov *et al*, 2004). The most notable reported phenotype of *rlp1*Δ cells is their sensitivity to MMS, which was consistent with our analysis of *sws1*Δ cells (Figure 1B). Rlp1 has a weak two-hybrid interaction with Rhp57 (Khasanov *et al*, 2004), but Rhp57 was not detected in the MudPIT analysis of Sws1-TAP, nor were Rhp51 or Rhp55. However, upon careful analysis of the other proteins identified by MudPIT (Supplementary Table II), we found that one of them, encoded by SPAC17H9.03c, was related to RAD51 paralogs. Remarkably, it was most similar to vertebrate RAD51D (>45% overall similarity) (Figure 6A and B), homologs of which were heretofore thought to be absent in lower eukaryotes. The discovery of XRCC2 and RAD51 paralogs associated with Sws1 was particularly striking because vertebrate XRCC2 and RAD51D form a functional heterodimeric complex (Braybrooke *et al*, 2000; Masson *et al*, 2001). SPAC17H9.03c was named Rdl1 (Rad51D-like protein 1).

Rlp1 and Rdl1 shared no obvious sequence similarities to Shu1 or Csm2. However, Rdl1 and Psy3 were significantly similar (Figure 6B). Interestingly, this similarity was centered

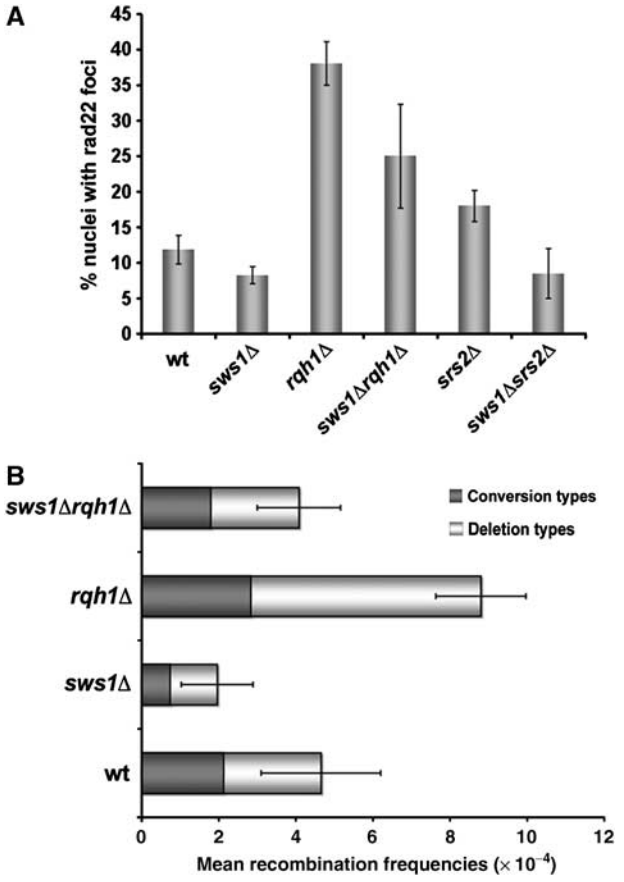


Figure 4 Spontaneous Rad22-YFP foci formation and recombination levels are reduced in *sws1*Δ background. (**A**) Cells containing a genomic copy of Rad22-YFP were grown in YES media at 32°C until mid-log phase and their Rad22-YFP foci were quantified. For each strain more than 300 cells were counted. The average percentage of nuclei containing at least one focus is shown. The strains used in this assay were wt (VM3725), *sws1*Δ (VM3729), *rqh1*Δ (VM3726), *sws1*Δ*rqh1*Δ (VM3730), *srs2*Δ (VM3727), *sws1*Δ*srs2*Δ (VM3728). (**B**) Comparison of the spontaneous recombination frequencies of wt (PS3), *sws1*Δ (VM3731), *rqh1*Δ (VM3732) and *sws1*Δ *rqh1*Δ (VM3736). Strains containing an *ura4*$^+$ marker flanked by two *ade6*$^-$ heteroalleles were used in this analysis. Recombination frequencies are mean values from three independent assays.

in a previously unnoticed Walker B-like motif of Psy3 (expectation value of $<10^{-10}$ predicted by the MACAW program) that was also shared with human RAD51D (expectation value of $<10^{-8}$) and RAD51D proteins from other species (Figure 6B). Importantly, Shu2 and Psy3 interact in yeast two-hybrid assays (Shor *et al*, 2005). These data suggest that Psy3 is a very diverged member of the RAD51D family.

Coprecipitation and immunoblot studies confirmed that Sws1 associates with Rlp1 and Rdl1 *in vivo* and further showed that Rdl1 coprecipitates with Rlp1 (Figure 6C), indicating that the three proteins form a stable complex. We also observed that Rlp1-GFP and Rdl1-GFP were low abundance proteins that colocalized with Sws1-GFP in the chromatin region of the nucleus (Supplementary Figure 1D).

As observed for *sws1*Δ cells, *rdl1*Δ and *rlp1*Δ mutants were viable and were not obviously sensitive to genotoxic agents, except for 0.02% MMS (Figure 6D). The somewhat enhanced MMS sensitivity of *rdl1*Δ and *rlp1*Δ mutants indicated that activities of Rdl1 and Rlp1 in promoting MMS survival were not fully dependent on Sws1, which is unlike the relationship between Shu2 and Psy3 (Shor *et al*, 2005). However, *rdl1*Δ and *rlp1*Δ suppressed the DNA damage sensitivities of *rqh1*Δ cells in a manner that was not additive with the suppressive effect of *sws1*Δ (Figure 6E), indicating that Sws1, Rlp1 and Rdl1 act in the same pathway to promote toxic recombination events in *rqh1*Δ cells.

Formation of Rad22 foci and recombination levels were monitored in *rlp1*Δ and *rdl1*Δ mutants alone and in combination with *rqh1*Δ (Figure 7A and B). In these assays, *rlp1*Δ behaved similarly to *sws1*Δ, reducing the frequency of Rad22-YFP foci and the recombination rates between *ade6*$^-$ heteroalleles (Figure 7). Although *rdl1*Δ and *rlp1*Δ had similar abilities to suppress *rqh1*Δ, they had different effects on the formation of Rad22-YFP foci and recombination rates. In *rqh1*$^+$ cells, the *rdl1*Δ mutation increased the occurrence of spontaneous Rad22-YFP foci (Figure 7A). A similar but stronger effect was seen with the *rhp55*Δ and *rhp57*Δ mutations (Figure 7A). The *rdl1*Δ mutation also increased the rate of recombination between the *ade6*$^-$ heteroalleles, which was largely attributed to an enhanced rate of deletion types. Again, in this assay, the effect of *rdl1Δ* was similar although not as strong as those caused by *rhp55*Δ (Doe and Whitby, 2004) or *rhp57*Δ (Figure 7B). In contrast to *rlp1*Δ, the *rdl1*Δ mutation did not reduce the frequency of Rad22-YFP foci in the *rqh1*Δ background, nor did it reduce the total recombination frequency. However, *rdl1*Δ did accentuate the bias towards deletion type recombinants in the *rqh1*Δ background (Figure 7A and B).

From these results we conclude that Sws1 and Rlp1 have fully interdependent functions in controlling recombination, whereas Rdl1 has a more complex set of functions. As discussed below, these data may indicate that Rdl1 functions at two stages of recombination or has locus-specific functions that are not shared by Sws1 and Rlp1.

Sws1 function is conserved in humans

Finally, we examined whether the putative Sws1 homolog in human cells (Figure 5C) has a role in HR. We first determined whether it associates with RAD51D. HeLa cells were transiently transfected with epitope-tagged constructs of SWS1, RAD51D and XRCC2. Both SWS1 and XRCC2 were detected in immunoprecipitates of RAD51D (Figure 8A). This result suggests that, in addition to its sequence similarity to fission yeast Sws1, human SWS1 shares the ability to associate with RAD51D. (We suspect that SWS1 also associates with XRCC2, but technical problems with a crossreacting protein in the immunoblots interfered with this analysis.) SWS1 failed to coprecipitate with coexpressed XRCC3 and RAD51C (our unpublished data), indicating specificity for its interactions with RAD51 paralogs.

We reasoned that if human SWS1 was functionally similar to its homolog in fission yeast, its elimination would reduce the frequency of recombination events represented by RAD51 foci. To test this hypothesis, we used RNAi to knockdown expression of human SWS1 mRNA. Four pSuper-SWS1-RNAi vectors were constructed to target different regions of the SWS1 coding sequence. Two of these vectors successfully suppressed expression of a cotransfected 3HA-SWS1 construct (Figure 8B). HeLa cells transfected with the RNAi vectors were then scored for the presence of two or more RAD51 foci. Ablation of SWS1 expression reduced the

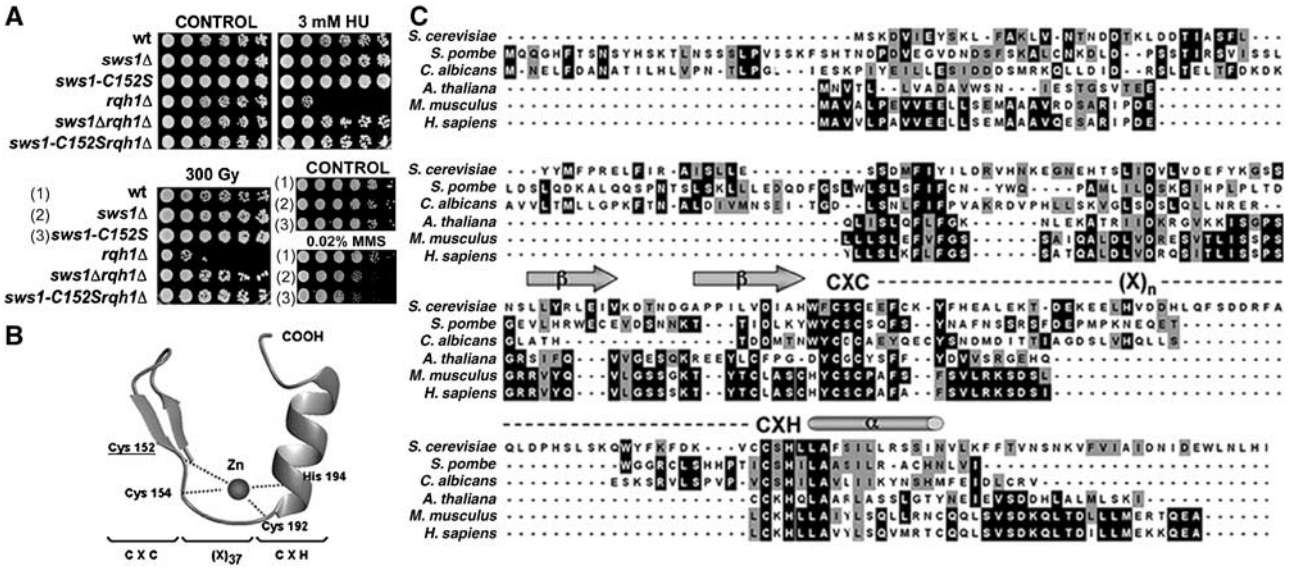


Figure 5 Sws1 is a conserved protein and the SWIM domain is required for its function. (**A**) Four-fold serial dilutions of wt (PR109), *sws1*Δ (VM3723), *sws1-C152S* (VM3734), *rqh1*Δ (SC3250), *sws1*Δ *rqh1*Δ (VM3722) and *sws1-C152S rqh1*Δ (VM3733) were plated on YES plates (control), YES plates supplemented with 3 mM HU or 0.02% MMS, or YES plates that afterwards were treated with 300 Gy of IR. Photographs were taken after 4 days at 32°C. (**B**) Model of the Zn-binding region of the SWIM domain in Sws1. The three cysteine residues and the histidine residue predicted to be involved in Zn chelation are indicated. The cysteine residue (Cys152) mutated in *sws1-C152S* is underlined. (**C**) Alignment of Sws1 and its orthologs from *H. sapiens, M. musculus*, *A. thaliana*, *C. albicans* and *S. cerevisiae*. The conserved CxC(x)$_n$CxH motif and secondary structure elements (upstream β-strands and the downstream α-helix) are shown.

frequency of RAD51 foci positive cells from ~28 to ~14% in undamaged cells (Figure 8B, C and D). SWS1 ablation also had an effect in IR-treated cells, reducing the frequency of RAD51 foci positive cells from ~55 to ~35% (Figure 8C and D). Flow cytometry analyses showed that cell cycle distribution 24, 48 or 72 h after transfection was unaffected by suppression of SWS1 expression, as was the abundance of Rad51 detected by immunoblotting (data not shown).

From these results we conclude that human SWS1 has a prorecombinogenic activity that is likely analogous to the activity of its homologs in fission and budding yeasts.

Discussion

Srs2 DNA helicase is a canonical antirecombinase, negatively modulating recombination by disrupting Rad51 filaments (Krejci *et al*, 2003; Veaute *et al*, 2003). *In vitro*, however, the inclusion of the Rad55–57 complex can overcome the inhibitory effect of Srs2 on Rad51-mediated strand exchange (Krejci *et al*, 2003). Rad52 was also reported to have the same effect (Krejci *et al*, 2003). The latter observation has at least two nonmutually exclusive interpretations. One is that *in vivo* Srs2 mainly targets Rad51-DNA nucleofilaments that are devoid of Rad52 and Rad55–Rad57. Alternatively, Srs2 could target other recombination proteins in addition to Rad51. With this possibility in mind, we conducted a two-hybrid screen with the C-terminal region of fission yeast Srs2. This screen uncovered a previously uncharacterized protein that we have named Sws1. Consistent with our hypothesis, elimination of Sws1 reduces recombination in fission yeast. Moreover, *sws1*Δ suppresses the hyper-recombination phenotypes of *srs2*Δ and *rqh1*Δ mutants and rescues the nearly lethal effects of simultaneously inactivating Srs2 and Rqh1. These striking effects contrast with the absence of IR sensitivity of *sws1*Δ cells. Clearly, Sws1 is not essential for HR events that repair DSBs, but it is culpable for nearly all the toxic recombination structures that form in a *srs2*Δ *rqh1*Δ strain. The MMS sensitivity of *sws1*Δ cells suggests that Sws1 might be involved in channeling a subset of repair events involving oxidative damage to DNA into the HR pathway. From these facts we surmise that Sws1 is a catalyst of a very early step in recombination.

Sws1 shares potentially significant sequence similarity to *S. cerevisiae* Shu2 and mutants that lack these proteins share a number of interesting phenotypes. Most notably, a *shu2*Δ mutation partially suppresses the HU sensitivity of an *sgs1*Δ mutant and reduces the frequency of Rad52 foci (Shor *et al*, 2005). It is likely, therefore, that Sws1 and Shu2 are highly divergent homologs that have analogous functions in catalyzing an early step in recombination.

Potential function of the Sws1 family in HR

Through an affinity purification scheme we identified two proteins, Rlp1 and Rdl1, in a complex with Sws1. Genetic analyses confirmed that strains defective in these proteins share many phenotypes with *sws1*Δ cells, indicating that they function in a complex with Sws1. Interestingly, these proteins have significant homology to Rad51 paralogs. Rlp1 is most similar to XRCC2 (Khasanov *et al*, 2004), while Rdl1 has the closest homology to RAD51D. Mammalian XRCC2 and RAD51D form a heterodimer that has DNA-stimulated ATPase activity (Braybrooke *et al*, 2000). This activity is thought to require the Walker A and Walker B motifs, implicated in ATP binding and hydrolysis. However, *in vivo* complementation studies suggest that while the Walker A motif in RAD51D is critical for the function of the complex, the Walker A motif of XRCC2 is largely dispensable (O'Regan *et al*, 2001; Gruver *et al*, 2005). These findings may be relevant to the observations that Rlp1 has a Walker A motif but no Walker B motif, and *vice versa* for Rdl1 (as confirmed

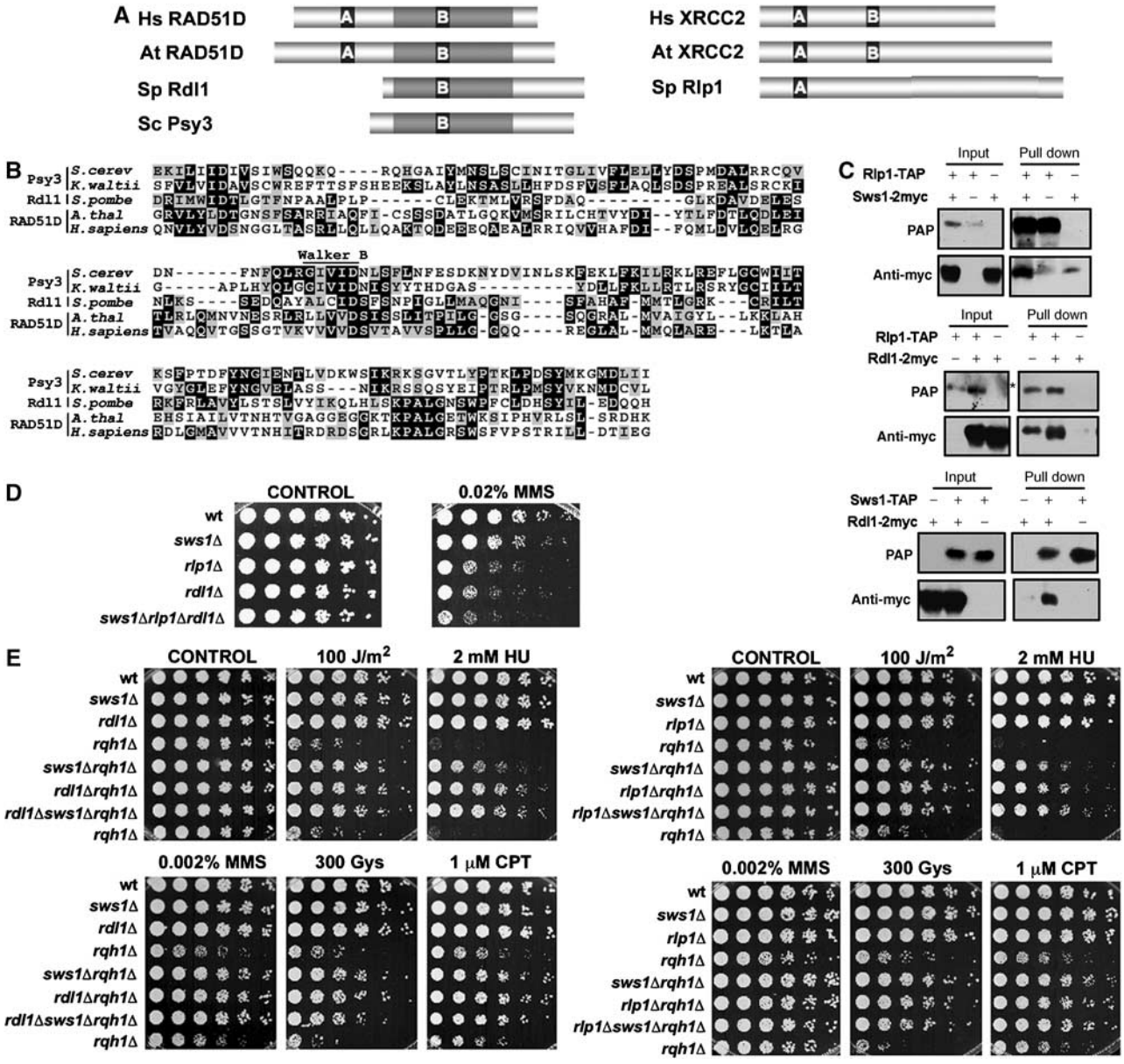


Figure 6 The *S. pombe* Rad51 paralogs, Rlp1 and Rdl1, show physical and genetic interactions with Sws1. (**A**) Schematic representation of the sequences of *S. pombe* Rdl1 and Rlp1 and their orthologs in *H. sapiens* (*Hs*), *A. thaliana* (*At*) and *S. cerevisiae* (*Sc*). A and B represent the Walker A and Walker B domains, respectively. The regions of highest similarity, centered around the Walker A and B domains are highlighted. (**B**) Alignment of the RAD51D family members from *S. cerevisiae*, *K. waltii*, *S. pombe*, *A. thaliana* and *H. sapiens*. The region of similarity between the different proteins (gray-shadowed region from the corresponding proteins in A) is shown. Conserved residues appear highlighted and the position of the Walker B motif is indicated. (**C**) Coimmunoprecipitation of Sws1, Rdl1 and Rlp1. Cells simultaneously transformed with the appropriate Rdl1-, Rlp1- and/or Sws1-expressing vectors were used in the assay (pREP41x for 2myc tags and pREP42x for TAP tags). Cell extracts were obtained after 21 h of incubation in the absence of thiamine. *Indicates the presence of a nonspecific band. (**D**) Spot assay of wt (PR109), *sws1*Δ (VM3723), *rlp1*Δ (VM3741), *rdl1*Δ (VM3744) and *sws1*Δ*rlp1*Δ*rdl1*Δ (VM3755) strains. Four-fold serial dilutions of each strain were plated on YES plates (CONTROL) or YES supplemented with 0.02% MMS. Photographs were taken after 4 days at 32°C. (**E**) Serial dilutions (fourfold) of the indicated strains were plated in YES plates in the presence of different sources of DNA damage. Photographs were taken after 4 days of incubation at 32°C. Strains used in this assays: wt (PR109), *sws1*Δ (VM3723), *rdl1*Δ (VM3744), *rlp1*Δ (VM3741), *rqh1*Δ (SC3250), *sws1*Δ *rqh1*Δ (VM3722), *rlp1*Δ *rqh1*Δ (VM3740), *rdl1*Δ *rqh1*Δ (VM3745), *sws1*Δ *rlp1*Δ *rqh1*Δ (VM3742) and *sws1*Δ *rdl1*Δ *rqh1*Δ (VM3746).

by RT–PCR; Supplementary Figure 3). It is conceivable, therefore, that in the fission yeast Rlp1-Rdl1 complex, a functional ATPase activity depends on the individual contributions of a Walker A and a Walker B motif by the separate subunits. Indeed, our mutational studies have shown that the Walker A and Walker B motifs provided by Rlp1 and Rdl1, respectively, are essential for the function of the complex *in vivo* (Supplementary Figure 4). This situation is reminiscent of the SMC/Rad50 family of ATPases, in which Walker A and Walker B motifs that are separated by huge coiled-coil repeats are juxtaposed in three-dimensional space to form an active ATPase (de Jager *et al*, 2004).

The Rlp1–Rdl1 potential ATPase complex requires the Zn-finger-like SWIM domain of Sws1 for its *in vivo* function.

This is reminiscent of the bacterial RecFOR ATPase, which contains a Zn-finger motif that is important for its prorecombinogenic function (Lee *et al*, 2004). This analogy may provide clues about how Sws1 promotes HR-dependent error-free repair. The RecFR ATPase has been implicated in the loading of the single-strand annealing protein RecO onto DNA bound with single-strand binding (SSB) protein. The RecFOR complex then catalyzes the loading of the nucleofilament forming protein RecA, which is the bacterial homolog of Rad51 (Morimatsu and Kowalczykowski, 2003; Kidane and Graumann, 2005). Moreover, several *recFOR* mutants suppress the hyper-recombination toxicity associated with loss of *uvrD*, a bacterial Srs2-related helicase (Veaute *et al*, 2005). In view of the role of RecFR in catalyzing the loading of recO onto DNA, and the importance of the ATPase activity and Zn finger in this process, we suggest that the Sws1–Rlp1–Rdl1 complex in fission yeast may fulfill an analogous role in catalyzing the loading of Rad22 onto RPA coated single-stranded DNA (Figure 8E).

This model might also apply for the XRCC2-RAD51D complex in mammalian cells. The lack of RAD51 paralogs or BRCA2 decreases RAD51 foci formation (van Veelen *et al*, 2005). We show here that human SWS1 associates with RAD51D. Consistent with this fact, knockdown of SWS1 reduces the number of cells with RAD51 foci. BRCA2 has been proposed to interact with the RAD51 paralogs through its BRC motifs. It is conceivable that an XRCC2–RAD51D–SWS1 complex acts very early in HR and catalyzes the recruitment of BRCA2 to damage sites. Subsequently, BRCA2 catalyzes the loading of RAD51. These observations are consistent with the model that we have proposed for Sws1 in fission yeast, with the difference being that the human XRCC2–RAD51D–SWS1 complex facilitates the loading of BRCA2, instead of Rad22.

Additionally, we show that Sws1 and Rdl1 are related to *S. cerevisiae* Shu2 and Psy3, respectively. On the basis of these similarities, we propose that Psy3 is in fact a highly divergent homolog of RAD51D and suggest that they function in a complex that possesses ATPase activity. Although not shown here, we have noticed that Shu1 exhibits potentially relevant similarities to the N-terminus of multiple Rad51 paralogs (our unpublished data), thus it is conceivable that Shu1 is an XRCC2/Rlp1-like protein. Mutational and structural studies will be needed to address this question. We cannot speculate on the Csm2 function, as we could not identify any potential homologs in Sws1-associated complexes. In *S. cerevisiae*, two-hybrid studies indicate that Csm2 associates most tightly with Psy3 (Shor *et al*, 2005), therefore, MudPIT analysis of Rdl1-associated complexes is expected to provide more insight.

Potential functions of SWIM domains

The SWIM domain, a proposed module for protein–protein or protein–DNA interactions, is most commonly found in multimodular proteins that have ATP binding domains such as translocases and protein kinases (Makarova *et al*, 2002).

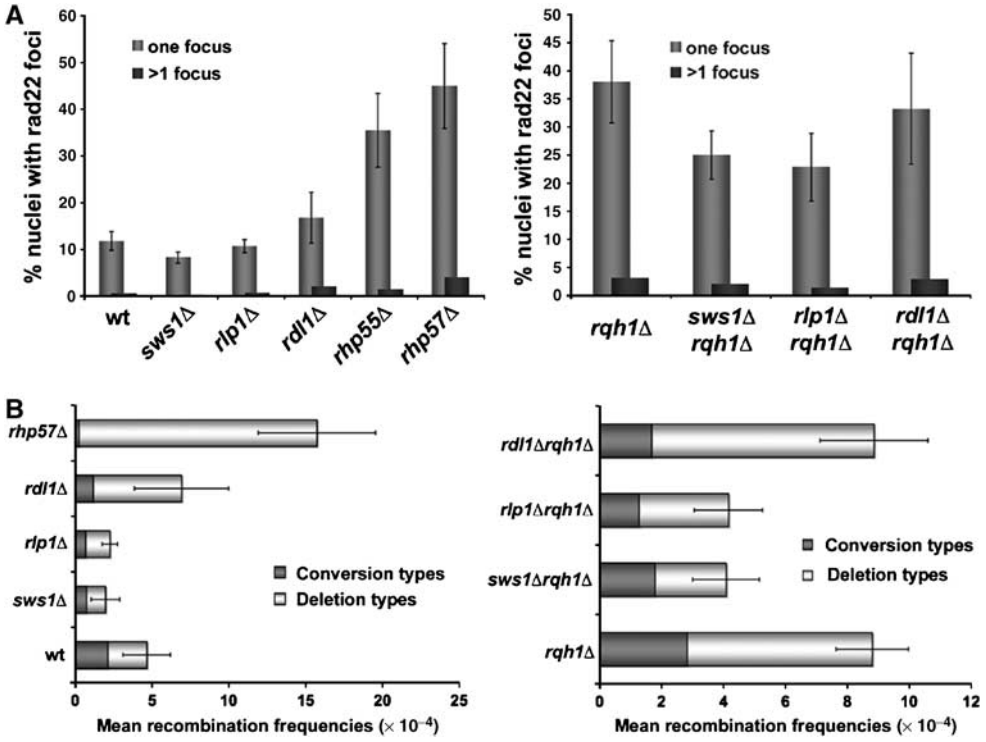
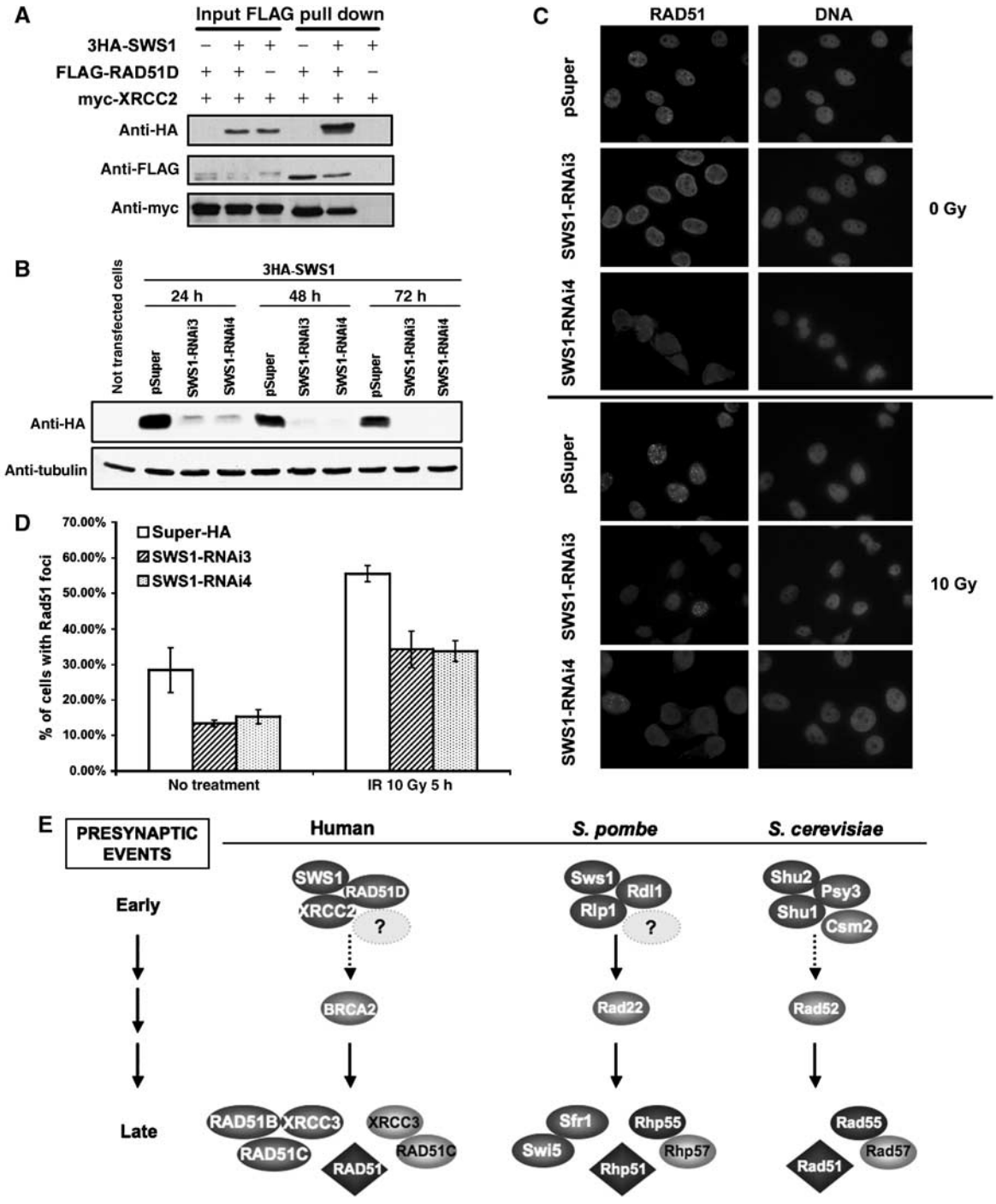


Figure 7 Analysis of the spontaneous rad22-YFP foci formation and recombination levels in different mutant backgrounds. (**A**) Cells expressing Rad22-YFP from its genomic locus were grown in YES media at 32°C until mid-log phase and their Rad22-YFP foci were quantified. For each strain, more than 300 cells were counted. The average percentage of nuclei containing at least one focus is shown (one focus). Average percentages of nuclei containing two or more foci are also indicated (>1 focus). Note that the values of wt, *sws1*Δ and *rqh1*Δ are derived from multiple experiments and are the same as those shown in Figure 4. (**B**) Spontaneous recombination frequencies of wt (PS3), *sws1*Δ (VM3731), *rlp1*Δ (VM3749), *rdl1*Δ (VM3750), *rhp57*Δ (VM3748), *rqh1*Δ (VM3732) *sws1*Δ *rqh1*Δ (VM3736), *rlp1*Δ *rqh1*Δ (VM3753) and *rdl1*Δ *rqh1*Δ (VM3750). Strains containing a non-tandem repeat of *ade6*$^{-}$ heteroalleles flanking a functional *ura4*$^{+}$ gene were used in this study. Recombination frequencies are mean values from three independent assays, and in each assay four independent colonies were tested.



We have shown that the SWIM domain is essential for the prorecombinogenic function of Sws1. Sws1 proteins are unique in being small proteins that have no recognizable motifs other than the SWIM domain. However, as we have shown here, Sws1 proteins form a complex with Rad51 paralogs that have ATPase activity. It is therefore tempting to speculate that SWIM domains are involved in regulating some aspect of ATP binding or hydrolysis.

Additional functions of Rdl1

Rdl1 appears to have additional functions not shared with Sws1 and Rlp1. Human RAD51D might also have XRCC2-independent functions. RAD51D is the only RAD51 paralog known to be present at telomeres or in association with the Bloom helicase (Braybrooke *et al*, 2003; Tarsounas *et al*, 2004). Moreover, RAD51D knockout embryos only survive 10.5 days postconception while XRCC2 knockout mice die perinatally (Deans *et al*, 2000; Smiraldo *et al*, 2005). Biochemical fractionation and yeast two-hybrid studies have indicated the presence of multiple RAD51 paralog complexes: RAD51B–RAD51C, RAD51D–XRCC2, RAD51C–XRCC3, and a larger cocomplex of BCDX2 (Schild *et al*, 2000; Masson *et al*, 2001). Similar to RAD51D, Rdl1 might function within two Rad51 paralog complexes: an Rlp1–Sws1 containing complex that acts at an early stage of recombination, and an Rhp55–Rhp57 containing complex that acts at a later

Figure 8 Conservation of Sws1 function in human cells. (**A**) Physical interaction between 3HA-SWS1, FLAG-RAD51D and myc-XRCC2. HeLa cells were transiently transfected with 3HA-SWS1 and myc-XRCC2 in the presence or absence of FLAG-RAD51D. At 48 h after transfection, lysates and FLAG-immunoprecipitates were probed for the presence of 3HA-SWS1, FLAG-RAD51D and myc-XRCC2. (**B**) Depletion of 3HA-SWS1 by RNAi. Four pSuper-SWS1-RNAi vectors were constructed to target different regions of the SWS1 coding sequence. Two of these vectors (containing SWS1RNAi3 and SWS1RNAi4) successfully suppressed expression of a cotransfected 3HA-SWS1 construct. Western analyses were performed using antiHA antibodies in cell lysates from cells co-transfected with 3HA-SWS1 and pSUPER vectors containing SWS1RNAi3 or SWS1RNAi4. SWS1 protein levels were substantially reduced when RNAi against SWS1 was used, while no loss of SWS1 was seen when cells were transfected with a control vector (pSuper). Tubulin was used as a loading control. (**C, D**) Representative Rad51 foci in cells transfected with control (pSuper-HA, which expressed HA siRNA) or pSuper-SWS1RNAi3 and 4. Untreated cells (0 Gy) and cells exposed to gamma radiation (10 Gy) were fixed 5 h after treatment for immunocytochemical analysis of Rad51. Rad51 foci were visualized by immunofluorescence staining with Abcam 13E4 anti-Rad51 monoclonal antibodies (C, left). DAPI counterstaining for cell nuclei is also shown (C, right). Quantification of Rad51 foci (D) shows a decrease in the number of cells containing at least two Rad51 foci both before and after gamma radiation. This reduction in spontaneous and gamma-induced Rad51 foci is associated with reduced SWS1 expression, as it was observed after transfection with two different constructs that cause reduced 3HA-SWS1 expression but not after transfection with the control RNAi vector (pSUPER-HA), which does not affect the number of HeLa cells showing RAD51 foci. 350 cells were counted for each sample. (**E**) Model for the presynaptic events in HR. Our results support a model in which *S. pombe* Sws1 acts together with Rlp1 and Rdl1 to promote Rad22 recruitment to the sites of DNA damage. The humans SWS1–RAD51D–XRCC2 is proposed to perform a similar function in recruiting BRCA2. We suggest that the Shu2 group of proteins performs an analogous function in *S. cerevisiae*. Psy3 is proposed to be the homolog of Rdl1 and RAD51D. Shu1 may be a very distantly related homolog of Rlp1 and XRCC2. Human and fission yeast proteins related to Csm2 have not yet been found. Other proteins known to be involved in HR are also shown. See text for further details. A color version of this figure is available at *The EMBO Journal* Online.

stage. The finding that potential homologs of RAD51D and XRCC2 exist in fission yeast and possibly budding yeast paves the way for further structural and functional analysis of Rad51 paralogs and their novel interacting partners.

Materials and methods

Strains, plasmids and media

Details of strains, plasmids, media and growth conditions are provided in Supplementary data. A strain list is provided in Supplementary Table I.

In situ chromatin binding assay and microscopy techniques

Log phase cells were harvested and subjected to Triton X-100 extraction to analyze chromatin association of Sws1 as previously described (Noguchi *et al*, 2003). DAPI (4′,6′-diamidino-2-phenylindole) was used at 0.5 μg/ml. For green fluorescent protein (GFP) and yellow fluorescent protein (YFP) visualization, exponentially growing cells were photographed using a Nikon Eclipse E800 microscope equipped with a Photometrix Quantix charge-coupled device camera.

Mass-spectrometry

Sws1-TAP protein was purified from fission yeast cells using a previously described method (Saitoh *et al*, 2002). The resulting peptide mixture was analyzed by multidimensional protein identification technology (MudPIT) as previously described (Boddy *et al*, 2001).

Recombination assays

Mitotic recombination was assayed using strains containing a non-tandem repeat of *ade6* heteroalleles flanking a functional *ura4*$^+$ gene (Osman *et al*, 1996). Further details can be found in the Supplementary data.

SWS1 RNA interference (RNAi)

3HASWS1 was cloned into pCDNA3 (Invitrogen). Two SWS1 19-nucleotide-long regions were selected and cloned into pSuper (nucleotide sequences and further information can be found in the Supplementary data).

Western blot and immunofluorescence analyses

Protocols used for whole-cell extract preparation from *S. pombe* and HeLa cells, as well as for preparation of HeLa cells for immunofluorescence studies are available in the Supplementary data.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online.

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