Localization of recombination proteins and Srs2 reveals anti-recombinase function in vivo

Rebecca C. Burgess,[1,6] Michael Lisby,[2] Veronika Altmannova,[3] Lumir Krejci,[3,4] Patrick Sung,[5] and Rodney Rothstein[6]

[1]Department of Biological Sciences, Columbia University, New York, NY 10027
[2]Department of Molecular Biology, University of Copenhagen, DK-2200 Copenhagen N, Denmark
[3]National Center for Biomolecular Research, and [4]Department of Biology, Masaryk University, 625 00 Brno, Czech Republic
[5]Molecular Biophysics and Biochemistry, Yale University School of Medicine, New Haven, CT 06520
[6]Department of Genetics and Development, Columbia University Medical Center, New York, NY 10032

Homologous recombination (HR), although an important DNA repair mechanism, is dangerous to the cell if improperly regulated. The Srs2 "anti-recombinase" restricts HR by disassembling the Rad51 nucleoprotein filament, an intermediate preceding the exchange of homologous DNA strands. Here, we cytologically characterize Srs2 function in vivo and describe a novel mechanism for regulating the initiation of HR. We find that Srs2 is recruited separately to replication and repair centers and identify the genetic requirements for recruitment. In the absence of Srs2 activity, Rad51 foci accumulate, and surprisingly, can form in the absence of Rad52 mediation. However, these Rad51 foci do not represent repair-proficient filaments, as determined by recombination assays. Antagonistic roles for Rad52 and Srs2 in Rad51 filament formation are also observed in vitro. Furthermore, we provide evidence that Srs2 removes Rad51 indiscriminately from DNA, while the Rad52 protein coordinates appropriate filament reformation. This constant breakdown and rebuilding of filaments may act as a stringent quality control mechanism during HR.

Introduction

Homologous recombination (HR) is a high-fidelity repair process that is critical for genome maintenance. Disruption or misregulation of HR functions can lead to loss of heterozygosity (LOH), chromosome loss, genome rearrangements, or other deleterious events that can ultimately lead to carcinogenesis (Hoeijmakers, 2001; for review see Agarwal et al., 2006; Wyman and Kanaar, 2006; Reliene et al., 2007). Proper regulation of recombination processes is therefore as important for the maintenance of genome integrity as the repair process itself. HR is stimulated by double-strand DNA breaks (DSBs), which can occur spontaneously through normal DNA metabolism, or occur in a programmed fashion during processes such as mating-type switching (in yeast), immunoglobin gene rearrangements (in vertebrates), and meiotic recombination. In addition, HR also processes DSBs from exogenous insults such as ionizing radiation or chemotherapeutic drugs. The current model of DSB repair is largely based on studies from the budding yeast *Saccharomyces cerevisiae* (for review see Krogh and Symington, 2004). In this model, the DSB end is processed by nucleases to create 3′ single-stranded DNA tails, which are immediately coated by replication protein A (RPA), the eukaryotic single-strand DNA binding protein. A critical step of the HR mechanism involves formation of the Rad51 nucleoprotein filament on this ssDNA. However, formation of Rad51 filaments is inhibited by the presence of RPA and needs to be mediated by Rad52 protein, which through direct physical interaction nucleates Rad51 on RPA-coated ssDNA (Sung, 1997a; New et al., 1998; Sugiyama and Kowalczykowski, 2002). The filament can be also mediated or stabilized by the Rad55/57 heterodimer (Sung, 1997b; Fortin and Symington, 2002). Once the Rad51 nucleoprotein filament is formed, it can invade a homologous donor sequence to form a displacement loop (D-loop) structure between the donor double-stranded DNA (dsDNA) and the invading single-stranded DNA (ssDNA). After D-loop formation, new DNA synthesis can occur using the invading strand as a primer. The Swi/Snf-like

Correspondence to Rodney Rothstein: rothstein@cancercenter.columbia.edu

Abbreviations used in this paper: DSB, double-strand break; dsDNA, double-stranded DNA; HR, homologous recombination; HU, hydroxyurea; IR, ionizing radiation; PCNA, proliferating cell nuclear antigen; RPA, replication protein A; ssDNA, single-stranded DNA.

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protein Rad54 directly interacts with Rad51 and is thought to function in multiple steps of HR. Rad54 stimulates DNA strand exchange by Rad51 and extension of the heteroduplex DNA formed by strand invasion (Petukhova et al., 1998; Solinger et al., 2001; Mazina and Mazin, 2004). It also has an ability to translocate along dsDNA, an activity important for its reported roles in disassembling Rad51 nucleoprotein filaments, remodeling chromatin, and migrating branched recombination intermediates (Van Komen et al., 2000; Solinger et al., 2002; Alexeev et al., 2003; Jaskelioff et al., 2003).

The ordered recruitment of recombination factors can be visualized cytologically, as recombination and checkpoint proteins relocalize to discrete foci at DSB sites (Lisby et al., 2001). Recombination protein foci exhibit genetic dependencies that are consistent with reported protein–protein interactions; for instance, Rad54 focus formation requires Rad51, whereas formation of Rad51 foci requires Rad52 (Lisby et al., 2004). HR foci form in response to induction of DSBs generated by exogenous factors, as well as spontaneously during S phase in the absence of such damage. This suggests that HR factors are recruited to replication forks for recombination-mediated repair and restart when fork progression is impaired due to spontaneous damage (Michel et al., 2001; Limoli et al., 2002; Sogo et al., 2002). Recent work also suggests that ssDNA structures can contribute to spontaneous HR (Fabre et al., 2002; Lettier et al., 2006; Mozlin et al., 2008). However, at present, the nature of DNA lesions that lead to spontaneous HR foci is unknown and it remains possible that a majority of short-lived spontaneous foci are assembled in error and subsequently removed (Lisby et al., 2003). This disassembly may allow more appropriate repair pathways to act, such as the post-replicative repair pathways, also referred to as DNA damage tolerance pathways (Eppink et al., 2006; for review see Andersen et al., 2008). Therefore, a logical point for the regulation of HR is the Rad51 nucleoprotein filament before its commitment to strand invasion.

A prominent protein that destabilizes the Rad51 filament is Srs2, a 3′-to-5′ helicase with functional similarities to bacterial UvrD, Rep, and PcrA and to mammalian Fbh1 (Aboussekhra et al., 1989; Krejci et al., 2003; Veaute et al., 2003; Chiolo et al., 2007; Kohzaki et al., 2007). Deletion of *SRS2* causes increased levels of recombination (Aguilera and Klein, 1988) and synthetic interactions with deletions of genes involved in genetic recombination including *RAD54* and the RecQ helicase homologue *SGS1* (Lee et al., 1999). These lethalities can be suppressed by inhibiting early recombination steps, indicating that the loss of viability stems from the accumulation of toxic recombination intermediates (Gangloff et al., 2000; Klein, 2001). Between these genetic interactions and the in vitro observation that Srs2 can disrupt Rad51 recombinase filaments, Srs2 has earned the epithet "anti-recombinase."

The *SRS2* gene was also identified as a suppressor of the sensitivity of *rad6Δ* and *rad18Δ* post-replicative repair mutants to DNA damaging agents (Lawrence and Christensen, 1979; Aboussekhra et al., 1989; Friedl et al., 2001). These and other genetic interactions (Klein, 2001), as well as the anti-recombinase activity of Srs2, led to the hypothesis that Srs2 contributes to the channeling of lesions from HR to other repair pathways, such as post-replicative repair, which depends on *RAD5* or *RAD6* (Aboussekhra et al., 1989; Schiestl et al., 1990). This regulatory role of Srs2 has been further elucidated through novel interactions between Srs2 and replication proteins, such as the Pol∂ subunit Pol32, and importantly, its interaction with the processivity clamp, proliferating cell nuclear antigen (PCNA) (Huang et al., 2000; Papouli et al., 2005; Pfander et al., 2005). Yeast PCNA, a homotrimer encoded by the *POL30* gene, is a substrate for both mono- and polyubiquitination after DNA damage. These two modifications have been proposed to act as a switch between different branches of post-replicative repair (Hoege et al., 2002). PCNA is also sumoylated, but specifically in S phase (Hoege et al., 2002), and Srs2 has been shown to interact preferentially with sumoylated PCNA. Importantly, strains with mutant PCNA that cannot be sumoylated behave similarly to *srs2Δ* strains (Papouli et al., 2005; Pfander et al., 2005). Currently, it is thought that Srs2 is recruited to replication forks by $PCNA^{SUMO}$ to inhibit recombination during replication.

In this study, we examine Srs2 activity in vivo using the cell biological and genetic tools available in yeast to further understand the cellular role of Srs2 at replication forks and in the global regulation of HR. We describe the localization of Srs2 to replication foci and to HR foci, and find that recruitment to these two cellular processes occurs independently. Although the absence of Srs2 increases the recruitment of HR factors to forks during S phase, stalled replication forks are not a major substrate for HR focus formation in *srs2Δ*, whereas collapsed forks are. We also describe a decrease in the requirement for Rad52 during Rad51 focus formation in the absence of Srs2. However, these Rad51 foci do not represent structures that are active for HR. Finally, we show data supporting the hypothesis that the role of Srs2 is to remove Rad51 filaments, regardless of whether they are appropriate or inappropriate for HR, and that Rad52 directs the reformation of appropriate filaments, effectively eliminating potentially toxic HR intermediates.

Results

Srs2 localizes to recombination and replication foci in vivo

One of the functions of Srs2 is thought to be restricting recombination to specific times and to particular cellular locations. To investigate the anti-recombinase function of Srs2 during unperturbed cell growth, we monitored the localization of Srs2 foci by simultaneously analyzing the location of these foci relative to a DSB repair protein (Rad52-RFP) and a replication protein (CFP-Pol30: yeast PCNA; Kitamura et al., 2006). We find that YFP-Srs2 localizes to a subset of spontaneous Rad52 foci that likely mark sites of ongoing recombination (Fig. 1 A). In cells with small buds, which correspond to S phase of the cell cycle, Srs2 forms multiple small foci in the nucleus that do not colocalize with Rad52 recombination foci; however, they do colocalize with Pol30 (Fig. 1 A, bottom left cell), suggesting that Srs2 foci are localized to the site of replication forks.

Recent work has shown that sumoylated PCNA is important for recruiting Srs2 to the replication fork (Papouli et al., 2005; Pfander et al., 2005). We asked whether YFP-Srs2 forms

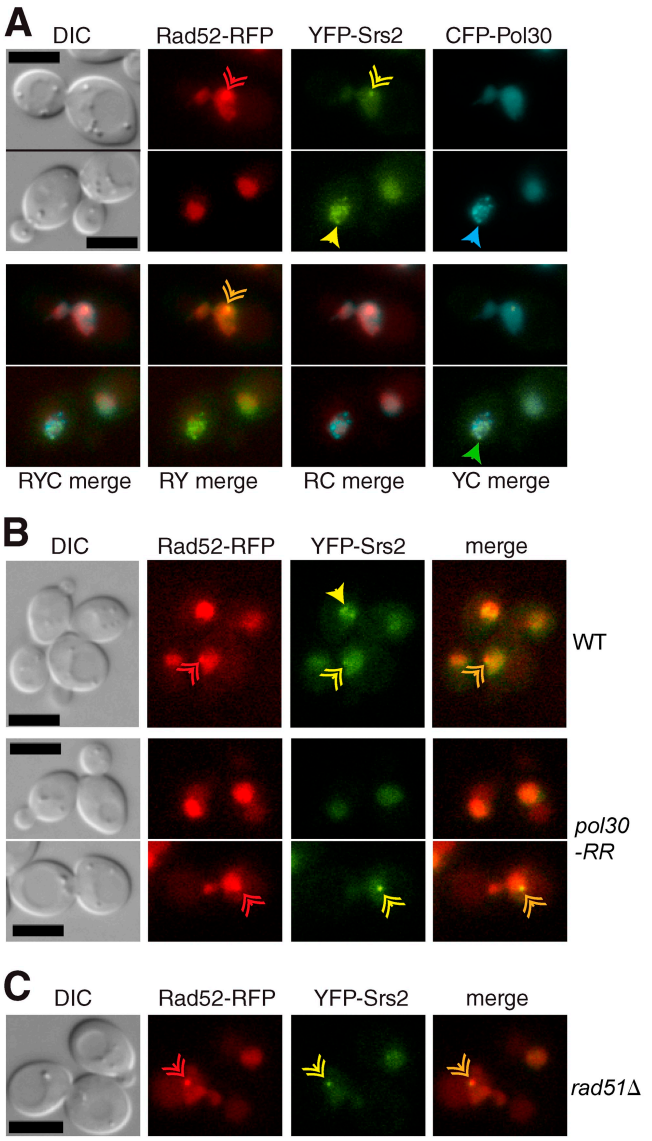

Figure 1. **Srs2 localizes to recombination foci and replication foci with different genetic requirements.** (A) Srs2 foci colocalize with Rad52 recombination foci and with Pol30 (PCNA) replication foci. Colocalization of Rad52-RFP and YFP-Srs2 is marked by the orange barbed arrowhead (RY merge panel); colocalization of YFP-Srs2 and CFP-Pol30 can be seen in the S phase cell on the bottom left (marked with the green solid arrow in the YC merge panel). Images are composites of eleven 0.3-µm Z-stacks to show focal structures in all nuclei in the field. (B) Srs2 forms multiple small S phase foci (solid arrowheads) in the wild-type, but these are not seen in *pol30-RR* (bottom). Srs2 is recruited to Rad52 foci (barbed arrows) in both the wild-type and the *pol30-RR* mutant (B), and in *rad51Δ* (C). Images were taken on the same day and contrasted identically for comparison. Single Z-planes are shown. Bars, 5 µm.

foci at the replication fork in a nonsumoylated PCNA mutant (*pol30-RR*). Indeed, we did not detect the multiple S phase Srs2 foci in the *pol30-RR* mutant, or in the absence of the SUMO E3 ligase responsible for PCNA sumoylation, Siz1 (Pfander et al., 2005; Fig. 1 B; Fig. S1 B). In addition, Srs2 S phase foci were not detectable in a YFP-tagged Srs2 mutant that lacks the consensus SUMO-interaction motif (*srs2-ΔSIM*, Ulrich, 2007), which is defective in its PCNA interaction (Fig. S1, B–D). In contrast, although the formation of S phase Srs2 foci is drastically decreased in the *pol30-RR* mutant, the frequency of Srs2 at recombination foci is unaffected (Fig. 1 B, bottom; Fig. S1 B). Importantly, these results indicate that recruitment of Srs2 to DNA replication forks or DNA repair centers occurs independently of one another, consistent with recently described separation-of-function *srs2* mutants (Le Breton et al., 2008). In fact, the inability of Srs2 to localize to replication forks in the *pol30-RR* mutant results in an increased number of HR foci that themselves can nonetheless recruit Srs2 (Fig. 1 B; Fig. S1 B and Fig. S2 B).

To our surprise, Srs2 still localizes to HR foci in the absence of Rad51 (Fig. 1 C; Fig. S1 B), suggesting the interaction of Srs2 with Rad51 (Krejci et al., 2003) is not the only requirement for recruitment to foci. There is, however, a partial requirement for the E3 SUMO ligase Siz1 in Srs2 recruitment to HR foci (Fig. S1 B), but it does not require Rad52 and Rad59, two recombination proteins that are known to be sumoylated (unpublished data; Sacher et al., 2006; Burgess et al., 2007). To test whether Srs2 interaction with sumoylated proteins was required for recruitment to HR foci, we determined the localization of the srs2-ΔSIM mutant, which lacks the SUMO interaction motif. This mutant localizes as wild-type to recombination foci (Fig. S1 B), showing that Srs2 interactions with sumoylated proteins are dispensable for recruitment to recombination foci. Altogether, these results show that Srs2 localizes to recombination and replication foci independently during normal cell growth, and that different sumoylation events may modulate its recruitment or retention.

Recombination foci accumulate in the absence of Srs2 activity

Although Srs2 localizes to HR foci, the absence of this protein results in increased recombination and stabilization of Rad51 filaments (Aboussekhra et al., 1992; Fung et al., 2006). To examine this relationship in vivo, we visualized spontaneous recombination foci by using YFP- and CFP-tagged versions of the Rad51 and Rad54 proteins in the absence of Srs2. Compared with wild-type cells, *srs2Δ* strains show a three- to fourfold increase in the number of budded cells that contain a Rad51 or Rad54 focus (Fig. 2 A–C; Fig. S3 A). The increased incidence of Rad51 and Rad54 foci in the S/G2/M population can be reversed to wild-type levels by expression of *SRS2* on a plasmid (unpublished data). These data support the notion of an in vivo anti-recombinase activity for Srs2 that mirrors the in vitro activities and fits with its hyper-recombination phenotype (Aguilera and Klein, 1988; Krejci et al., 2003; Veaute et al., 2003). For further study, Rad54 was used as an indicator of Rad51 localization because the Rad54-YFP fusion protein is fully functional, unlike the partially functional Rad51 fusion protein (Lisby et al., 2004), and because Rad54 foci mirror Rad51 foci. Particularly, Rad51 and Rad54 increase similarly in the absence of Srs2 (Fig. 2 A), Rad54 colocalizes with Rad51 over 95% of the time (Fig. 2 A; Fig. S2, A and B), and Rad51 is required for Rad54 focus formation, whether Srs2 is present or absent (Fig. 2 B; Fig. S2 B; Lisby et al., 2004). Furthermore, the percentage of Rad51 and Rad54 colocalization is similar in the presence or absence of Srs2 (Fig. 2 A), indicating that even in *srs2Δ* cells, Rad54 foci closely mimic Rad51 foci.

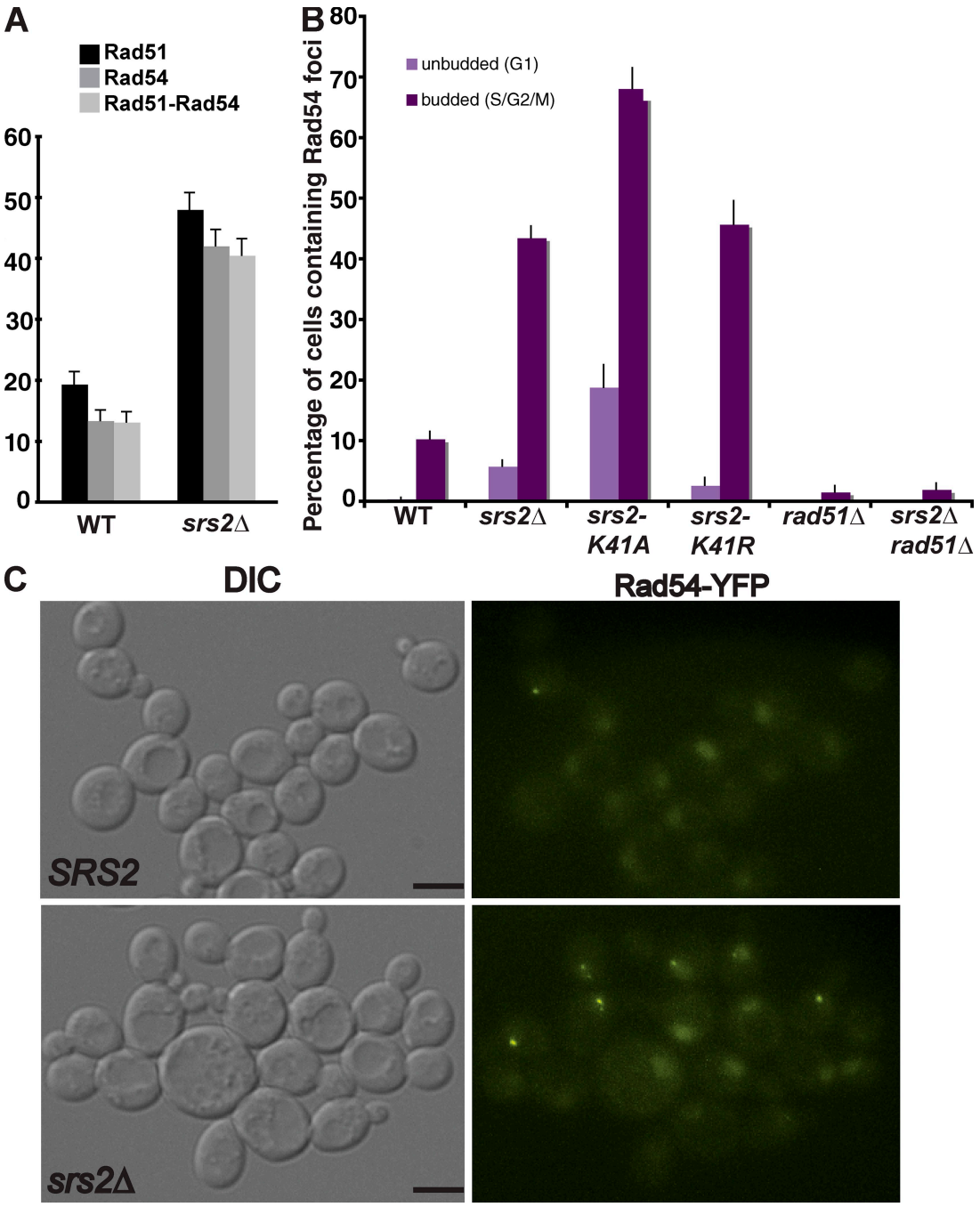

Figure 2. **Srs2 suppresses formation of spontaneous recombination foci.** (A) Rad51 and Rad54 foci increase similarly in the absence of Srs2. (B) Quantification of Rad54 focus frequency from images similar to those shown in C. Cells with Rad54 foci are increased fourfold over wild type in the *srs2Δ* strains, and even further increased in the *srs2-K41A* helicase mutant. The increased focus frequency in *srs2Δ* requires the presence of Rad51. Error bars depict binomial standard error, significance ($P < 0.05$) was determined using the χ^2 test, $n > 300$ cells per strain. (C) Rad54 foci form more frequently in *srs2Δ* cells than in wild type. Maximum intensity projection images of Rad54-YFP are shown in *SRS2* (top panels) and *srs2Δ* cells (bottom panels). Bar, 5 µm.

Because Srs2 requires its helicase activity for Rad51 nucleofilament disruption in vitro (Krejci et al., 2004), we asked whether mutations in conserved helicase domains required for ATP binding or hydrolysis would also lead to increased Rad54 foci. Similar to *srs2Δ*, strains expressing a helicase-defective *SRS2* that can bind but not hydrolyze ATP (*srs2-K41R*) form more Rad54 foci (Fig. 2 B). Interestingly, strains expressing a helicase-defective form of *SRS2* that can neither bind nor hydrolyze ATP (*srs2-K41A*) exhibit even more Rad54 foci than *srs2Δ* (Fig. 2 B). These observations imply that the defective protein is not just incapable of removing Rad51 filaments, as shown in the in vitro studies (Krejci et al., 2004), but that srs2-K41A mutant protein may actively block another repair pathway, or create a poisonous complex resulting in further accumulation of HR intermediates. Surprisingly, the srs2-K41A protein forms fewer replication and recombination foci than wild-type Srs2 protein (unpublished data), suggesting that the process blocked by this mutant protein occurs either before Srs2 focus formation or,

alternatively, that the residence time of this protein is shorter in the absence of ATP binding and hydrolysis. Collectively, these results show that Srs2 helicase activity is necessary to suppress the accumulation of recombination foci.

To determine whether the elevated levels of HR foci observed in *srs2Δ* are due to an increase in the duration or incidence of HR foci, time-lapse microscopy was used to follow the formation of Rad54 foci during unperturbed cell growth. Duration of Rad54 foci did not significantly change between the wild-type and *srs2Δ* strains (Fig. S2 C), showing that, in the absence of Srs2, the increased number of foci is not due to these foci lasting longer. On the other hand, wild-type cells form Rad54 foci in ∼30% of S phases, whereas in *srs2Δ* cells, Rad54 foci formed in 75% of S phases, indicating that HR foci form more frequently in the absence of Srs2.

Spontaneous recombination foci in *srs2Δ* are not solely due to unscheduled recombination at the replication fork

Due to the substantial increase in spontaneous HR foci in *srs2Δ,* we expected that, in the absence of Srs2, recombination factors could accumulate at the replication fork. To further probe the nature of these supernumerary HR foci in *srs2Δ*, we determined the percentage of Rad54 foci that colocalized with a replication fork–associated protein, Rfa1, the large subunit of the single-strand binding protein RPA (Brill and Stillman, 1991). In S phase, Rfa1 localizes to small speckled foci that associate with replication centers and colocalize with PCNA (Fig. S3 B; Dimitrova et al., 1999). RPA also binds to ssDNA that may be engaged in recombinational repair processes (Alani et al., 1992; Barlow et al., 2008). Importantly, Rfa1 foci colocalize with HR foci and are required for Rad52 focus formation (Lisby et al., 2004). However, it is important to note that although most HR foci show an associated Rfa1 signal, the converse is not true; that is, the majority of Rfa1 foci are not associated with HR proteins, and appear as S phase replication foci (Fig. S3 B). In fact, Rfa1 foci engaged in HR appear different from the small speckled Rfa1 replication foci; rather, they appear more similar to HR foci, as larger single foci, and are more often found in G2/M cells. To differentiate between these two types of Rfa1 foci, we use bud size (S phase indicated by small- to medium-sized buds, G2/M indicated by large buds) and Rfa1 focus morphology (speckled vs. single). This distinction allows us to use Rfa1 as a dual HR and replication marker.

In the absence of Srs2, Rad54 still appears as large single foci, but significantly more of these Rad54 foci colocalize with small speckled S phase RPA foci (twofold over wild-type, Fig. 3 A), suggesting that some of the increased HR foci represent recruitment to replication forks in *srs2Δ*. A twofold increase in Rad54 localization to replication forks in *srs2Δ* is also seen when Mcm2 is used as a replication marker. Because Mcm2 disappears from the nucleus during S phase, this analysis was restricted to early S phase cells while Mcm2 foci were still visible (Fig. S3, C and D; Yan et al., 1993). In G2 and M phases, when RPA foci reflect ssDNA regions undergoing repair, RPA-colocalizing Rad54 foci occur six times more frequently in the *srs2Δ* than in wild-type strains (Fig. 3 A). However, in both S phase and G2/M *srs2Δ* cells, a significant percentage of the total Rad54 foci were present at sites with no detectable RPA localization (20–30%). Perhaps, in the absence of Srs2 activity, some HR foci reflect events where recombination proteins are inappropriately recruited to dsDNA sites or represent sites where all RPA was evicted upon formation of a Rad51-ssDNA nucleoprotein filament. Nevertheless, the Rad54 foci that do colocalize with RPA S phase foci in the absence of Srs2 may indicate an inability to control HR at replication forks.

Because we could visualize the HR machinery being recruited more often to replication centers in the absence of Srs2, we wondered if this recruitment was specific to stalled or collapsed replication forks. Replication fork stalling occurs when a fork encounters a damaged template or one that is difficult to traverse. Alternatively, stalling can be artificially induced by depleting dNTP pools through addition of the drug hydroxyurea (HU). Exposing wild-type cells to HU does not induce the formation of Rad52 foci (Lisby et al., 2004). Likewise, the frequency of Rad54 foci in S phase does not increase after HU treatment, either in wild-type cells or in an *srs2Δ* background (Fig. 3 B). Therefore, although a larger fraction of replication forks spontaneously recruit the recombination machinery in *srs2Δ* (Fig. 3 A), it is not the stalling of replication forks, per se, that recruits HR factors in the absence of Srs2 (Fig. 3 B).

In the absence of checkpoint proteins, such as the Mec1 kinase, stalling replication forks can lead to disengagement of the replisome, resulting in fork collapse along with the formation of DSBs (Lopes et al., 2001; Tercero and Diffley, 2001; Sogo et al., 2002; Pellicioli and Foiani, 2005; Trenz et al., 2006). To determine if the absence of the checkpoint protein Mec1 would trigger an elevated recruitment of HR proteins to DNA replication forks, cells were analyzed for Rad54 foci in the presence or absence of *SRS2* in a *mec1Δ* strain where *SML1* was also deleted in order to suppress *mec1Δ* lethality. Both in the presence (*mec1Δ*, Fig. 3 B) or absence of Srs2 (*srs2Δ mec1Δ*), conditions that lead to replication fork collapse (HU treatment) strongly induce formation of Rad54 foci and the morphology of these foci changes, from single, large foci to multiple speckled foci. Collectively, these results suggest that, in the absence of Srs2, HR foci form more frequently at multiple sites within the genome, including replication forks but also potentially at dsDNA sites. In addition, during replication fork stalling, Srs2 is not the only obstacle restricting the recruitment of the HR machinery, and large-scale recruitment of recombination factors in *srs2Δ* is inhibited as long as replication fork integrity is maintained.

Decreased requirement for Rad52 in Rad51 focus formation in *srs2Δ*

Because the induction of Rad51 and Rad54 recombination foci after DNA damage requires Rad52 (Lisby et al., 2004), we asked about the role of Srs2 in this dependency. Rad52 colocalizes with ∼60% of spontaneous Rad54 foci, whereas in the absence of Srs2, Rad52 colocalization with Rad54 is reduced to ∼30% (unpublished data). Moreover, spontaneous Rad51 and Rad54 foci can be seen in an *srs2Δ rad52Δ* double mutant at significantly higher levels than in the *rad52*Δ mutant alone (Fig. 4 A; Fig. S2 C). These results show that there is a decreased requirement for

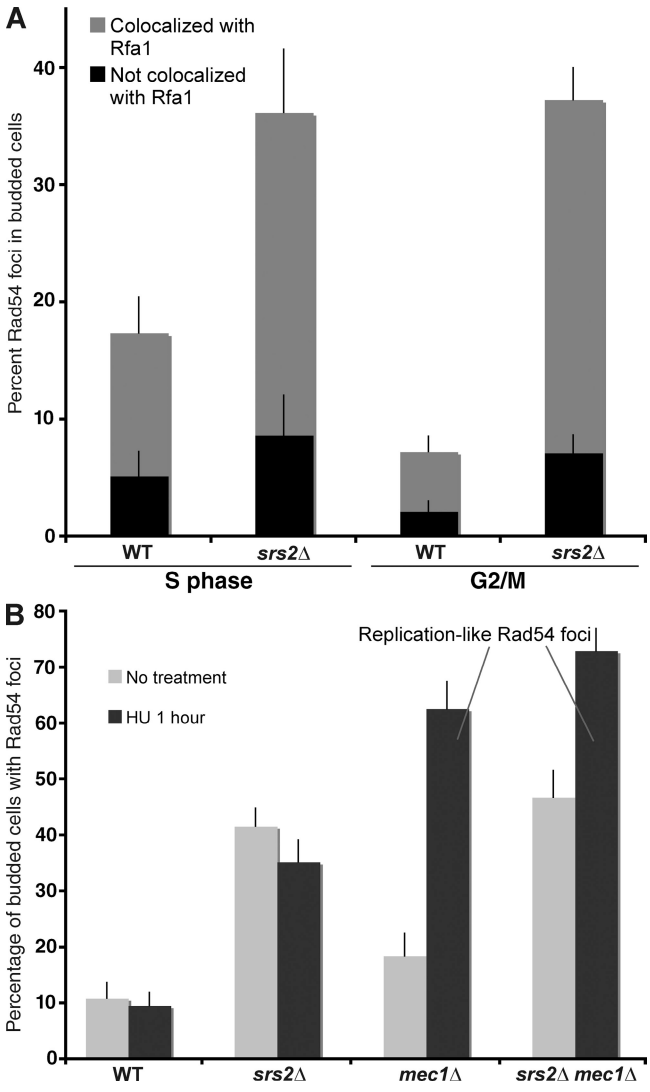

Figure 3. **Supernumerary recombination foci in *srs2Δ* only partially reflect accumulation at stalled replication forks or sites containing ssDNA.** (A) Percentage of Rad54 foci that colocalize with Rfa1 increases in *srs2Δ* cells. The height of the bar represents the total frequency of Rad54 foci in S phase (small- to medium-sized buds, small speckled Rfa1 foci) or G2/M phase cells (large budded cells, large single Rfa1 foci). The black portion of the bar depicts the percentage of Rad54 foci that do not colocalize with an Rfa1 focus, and the gray portion of the bar represents the percentage of Rad54 foci that colocalize with an Rfa1 focus. (B) Rad54 foci in *srs2Δ* are not stimulated by stalling replication forks, but do accumulate in response to fork collapse in the absence of Mec1, and form foci that resemble replication foci. Replication fork stalling/collapse was induced by the addition of 200 mM HU, and the frequency of cells with Rad54 foci was scored before treatment (light gray) and 1 h after treatment (dark gray). Cells with *mec1Δ* are made viable by the simultaneous deletion of *SML1*. Error bars represent binomial standard error, significance ($P < 0.01$) was determined using the χ^2 test, n = 100–200 budded cells for each condition.

Rad52 in the formation of both Rad51 and Rad54 foci in an *srs2Δ* background.

Because spontaneous DNA damage occurs at a low frequency, we asked whether induction of DSBs using ionizing radiation (IR) would affect the ability of Rad51 and Rad54 to form foci when *RAD52* and/or *SRS2* were disrupted. To test the genetic dependencies of focus formation, we monitored the number of Rad51 and Rad54 foci in wild-type, *srs2Δ*, and *rad52Δ* single or double mutants up to 7 h after IR (Fig. 4 B). Unfortunately, we could not easily monitor Rad54 foci after DNA damage in these strains because Rad54-YFP protein levels are dramatically elevated in *rad52Δ* mutants (Fig. S2 C). Although Rad54 foci can be detected and analyzed in unperturbed *rad52Δ* and *srs2Δ rad52Δ* cells, DNA damage treatment renders Rad54 foci indiscernible in *rad52Δ* or *srs2Δ rad52Δ* double mutants. Unlike Rad54, Rad51 levels are not elevated in *rad52Δ*, so foci can be clearly observed after induction of ~2 DSBs/cell with gamma irradiation. Wild-type and *srs2*Δ cells exhibit a similar rapid assembly of Rad51 foci after irradiation, followed by a disassembly phase in wild-type cells. Disassembly is delayed in the *srs2Δ* strains, reflecting the known recovery defect of *srs2Δ* (Vaze et al., 2002). No induction of Rad51 foci above background was observed in the *rad52Δ* strain, as expected from previous work (Lisby et al., 2004). Intriguingly, we observed a slow but steady induction of Rad51 foci after IR in the *srs2Δ rad52Δ* double mutant, indicating that Rad51 is recruited to DSBs, albeit more slowly (Fig. 4 B). These results suggest that Srs2 antagonizes Rad52 in Rad51 focus formation after DNA damage.

Rad52 antagonizes Srs2 in Rad51 filament formation in vitro

We next examined the known recombination mediators, Rad52 and Rad55–Rad57, for their ability to overcome the inhibitory effect of Srs2 on Rad51-mediated DNA strand exchange in vitro. These mediators were incorporated either singly or in combination into DNA strand exchange reactions with or without Srs2 at the time of RPA addition (Krejci et al., 2002). As shown in Fig. 4 C, the addition of Rad52 is sufficient to overcome the Srs2 inhibition, probably by facilitating reassembly of Rad51 filament on an RPA-coated ssDNA template. On the other hand, the Rad55/57 heterodimer does not significantly increase Rad51-mediated strand exchange in the presence of Srs2, when used alone or together with Rad52 protein (Fig. 4 C). Interestingly, when wild-type Rad52 protein is replaced by a *rad52* mutant that is defective in Rad51 interaction (*rad52Δ409-412*; Krejci et al., 2002), Srs2 can still inhibit the strand exchange reaction (Fig. S4 A), underscoring the need for Rad52–Rad51 interactions in overcoming Srs2 inhibition. Neither the Rad52 mediator nor Rad55/57 has any effect on the strand exchange reaction in the absence of Srs2 (Fig. S4 B; Sung, 1997b; Song and Sung, 2000). These biochemical results confirm the in vivo evidence for antagonistic roles of Srs2 and Rad52, and moreover, show that Rad52 plays a specific role in opposing Srs2 during Rad51 filament formation that is separate from other mediators.

Rad51 foci formed in the absence of Rad52 likely represent filaments that are defective for recombination

Because Rad52-independent Rad51 foci form at lower frequency and with delayed kinetics compared with those in a *RAD52* proficient strain, we wondered whether these foci reflect active recombination filaments in vivo. To address this issue, we asked if the *srs2Δ rad52Δ* double mutant was better able to repair and recover from DSBs than the *rad52Δ* alone. We measured the fraction of cell survival after increasing doses of IR and found that an *srs2Δ rad52Δ*

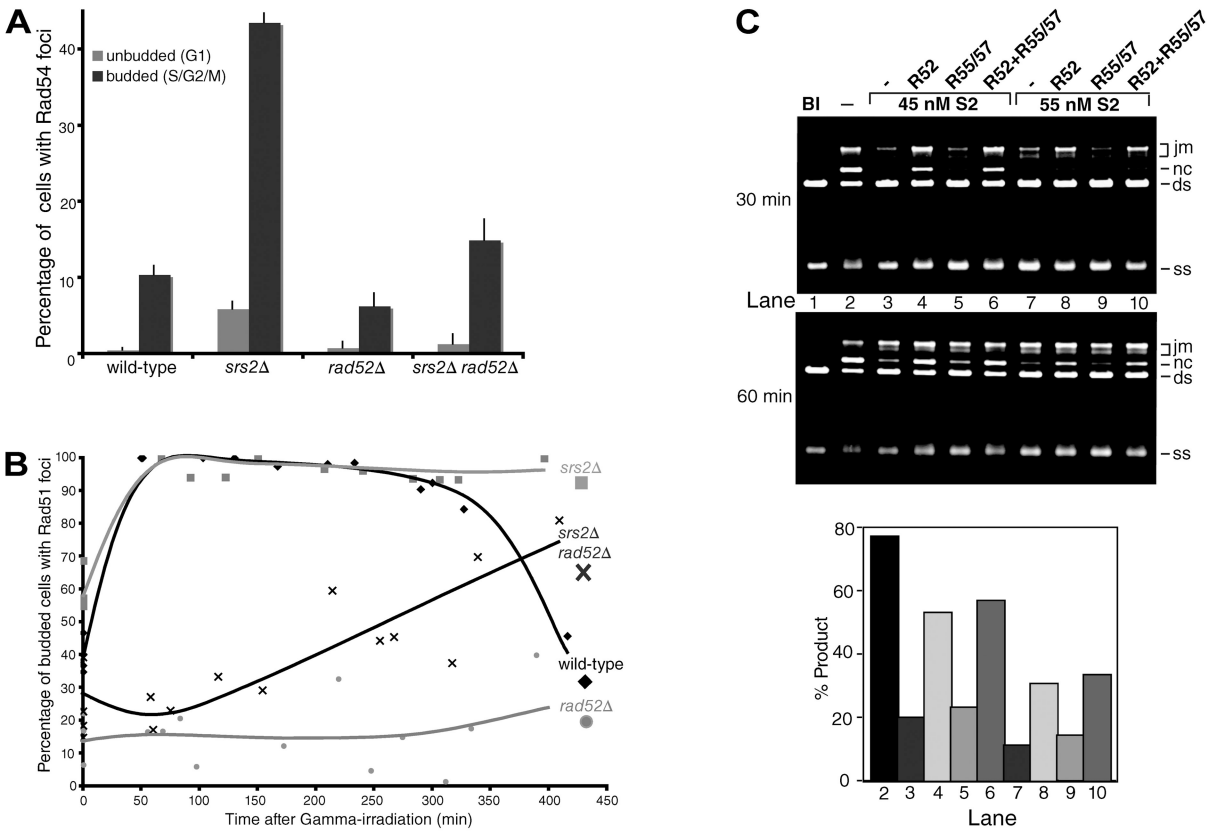

Figure 4. **Srs2 antagonizes Rad52 during Rad51 focus formation.** (A) Spontaneous Rad54 foci can form in the combined absence of Srs2 and Rad52. The bar graph depicts the frequency of cells with Rad54 foci in unbudded (light gray) and in budded cell (black) populations, $n > 300$ cells per strain. Error bars show binomial standard error, and significance ($P < 0.05$) was determined using the χ^2 test. (B) Ionizing radiation-induced Rad51 foci are able to form in *srs2Δ rad52Δ*. YFP-Rad51–expressing cells of indicated genotype were grown asynchronously to mid-log phase and irradiated (40 Gy), and aliquots were taken at various time points for microscopic analysis. The scatterplot shows the frequency of Rad51 focus-positive budded cells at exact time points from three independent experiments. Curves show the best-fit line for Rad51 focus induction of each genotype (wild type, black diamond; *srs2Δ*, gray square; *rad52Δ*, gray circle; *srs2Δ rad52Δ*, black X). (C) Rad52, but not Rad55–Rad57, can overcome Srs2 inhibition of Rad51-mediated strand exchange. Rad52 protein (R52) and the Rad55–Rad57 heterodimer (R55/57) were added together to DNA strand exchange reactions with or without Srs2 (S2), as indicated. Aliquots of the reactions were withdrawn at 30 and 60 min and analyzed. The results from the 60-min time point are presented in the histogram to the right. A reaction mixture that contained the DNA substrates but no protein, designated as BI, was also analyzed in lane 1. The percent product represents the amount of input duplex DNA (ds) that had been converted into joint molecules (jm) and nicked circular (nc) duplex. Input single-stranded DNA (ss) and duplex DNA (ds) are from the bacteriophage ϕX174, and are 5,384 nucleotides in length.

double mutant does not survive increasing doses of radiation better than a *rad52Δ* single mutant (Fig. 5 A). These results suggest that Rad51 foci formed in the *srs2Δ rad52Δ* double mutant are either unable to perform proficient repair of the induced DSB or cannot properly complete repair and resume normal cell growth.

Because *srs2Δ* cells have cell cycle recovery defects (Vaze et al., 2002), the possibility remained that the decreased survival of *srs2Δ rad52Δ* represented a cell cycle defect preventing cell cycle reentry and further cell growth even if the DSB had been repaired proficiently. Thus, we examined whether DSB repair products could be formed in the *srs2Δ rad52Δ* double mutants using the well-defined DSB repair assay at the *MAT* locus (Sugawara and Haber, 2006). After induction of the *HO* endonuclease, we monitored formation of DSBs (bottom band) and the resulting repair products (middle band) for up to 5 h after HO induction (Fig. 5 B). All strains show HO cutting 1 h after induction, but only wild-type cells and *srs2Δ* cells exhibit repair product formation (Fig. 5 B). In contrast, neither *rad52Δ* nor *srs2Δ rad52Δ* cells contain any detectable repair product, even 5 h after induction of the HO break (Fig. 5 B). These results suggest that the Rad51 foci formed in the *srs2Δ rad52Δ* double mutant do not represent recombination-proficient filaments, presumably due to additional roles for Rad52 and Srs2 in homologous recombination.

The data presented above support the hypothesis that Srs2 and Rad52 play competing roles in the cell, with Rad52 promoting, and Srs2 inhibiting, Rad51 focus formation (Symington and Heyer, 2006). Because the Rad51 foci formed in the absence of Rad52 are not recombination proficient, and Srs2 inhibits spontaneous Rad51 and Rad54 focus formation at many structures throughout the genome, we have suggested the following notion: Srs2 removes Rad51 filaments indiscriminately from ssDNA, while Rad52 acts to reform filaments where and when they are appropriate (Kanaar et al., 2008).

Srs2 inhibits Rad54 focus formation both at DSBs and elsewhere

To test the possibility that Srs2 inhibits Rad51 and Rad54 focus formation indiscriminately, we compared the formation of Rad54

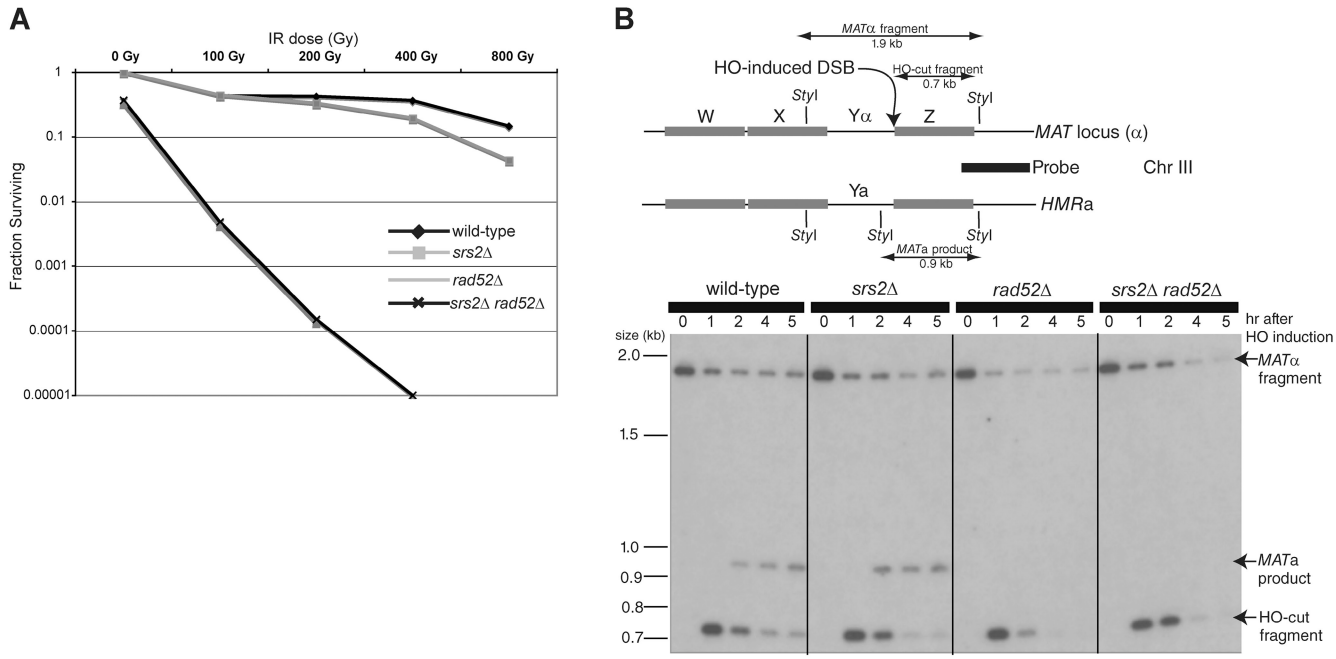

Figure 5. **Rad51 foci formed in *srs2Δ rad52Δ* strains do not represent recombination-proficent Rad51 filaments.** (A) Survival curves of wild-type, *srs2Δ*, *rad52Δ*, and *srs2Δ rad52Δ* in response to increasing doses of gamma irradiation. The *srs2Δ rad52Δ* double mutant is as sensitive to gamma irradiation as the *rad52Δ* single mutant. (B) The *srs2Δ rad52Δ* double mutant does not form recombination products during *MAT* switching. The cartoon depicts the *MAT*α locus and HO cut site on Chromosome III and the location of the probe used for Southern blotting. After cutting with HO endonuclease, the 1.9-kb *MAT*α fragment is cleaved to a 0.7-kb fragment, and recombination with the *HMR*a locus results in the appearance a 0.9-kb fragment. The bottom panel shows a time course of *MAT* switching after pulsed induction of HO for 1 h at time zero. The *MAT*a product is detected only in wild-type and *srs2Δ* cells, but not in either *rad52Δ* or *srs2Δ rad52Δ*.

foci throughout the cell and at a defined fluorescently tagged DSB site in the presence of increasing amounts of Srs2. To titrate Srs2 levels, we used the copper-inducible promoter *CUP1* to drive *SRS2* expression in an *srs2Δ* strain (Fig. 6 A). Basal expression of *SRS2* from the copper promoter reduces the number of Rad54 foci in budded cells by about threefold both throughout the genome and at a site-specific DSB (Fig. 6 B).

To explore the notion that Rad52 may direct the rebuilding of critical filaments, we tested whether the colocalization of Rad52 with Rad54 varied throughout the genome versus at an induced DSB. Fittingly, in the absence of Srs2, Rad52 and Rad54 are often colocalized at an induced DSB, but they colocalize much less frequently elsewhere (Fig. 6 B). Interestingly, with basal Srs2 expression, where Rad54 foci show striking decreases, Rad52 focus levels at the DSB remain unaffected compared with the vector control (Fig. 6 C), suggesting that Rad52 is not removed by Srs2, and remains available to re-recruit downstream factors such as Rad51 and Rad54 at sites that require homologous recombination.

Discussion

The Srs2 anti-recombinase is a vital regulator of recombination activity in the cell. Although many aspects of its function have been described in vitro, we undertook a cell biological approach to examine its intracellular organization and the consequences of disturbing its function in vivo. Using a fluorescently tagged Srs2, we visualized its localization to recombination foci and to replication forks in logarithmically growing cells (Fig. 1). The localization of Srs2 to recombination foci, surprisingly, does not require the presence of Rad51, the very protein it is known to dislodge from DNA and with which it interacts physically (Krejci et al., 2003). Perhaps Srs2 senses the Rad51 filament indirectly through interactions with other members of the recombination machinery, through interactions with single-stranded DNA, or Srs2 is recruited specifically by modified forms of recombination proteins. In fact, we find that Siz1 is partially required for Srs2 recruitment to HR foci (Fig. 1 D), suggesting that sumoylation plays a role in this recruitment. However, in the absence of sumoylated Rad52 and Rad59, Srs2 recruitment is not impaired (unpublished data). Because the *srs2* mutant lacking the SUMO-interaction domain does not show a measurable defect in localization to HR foci (*srs2-ΔSIM*, Fig. S1 B), sumoylation of Srs2 itself may be one of the modifications important for its recruitment to HR foci. Possibly multiple proteins, both sumoylated and unsumoylated, may recruit Srs2 to HR foci. During S phase (cells with small- to mid-size buds), Srs2 appears as multiple small foci colocalizing with PCNA. Formation of these foci requires interaction with sumoylated PCNA because we observe no S phase Srs2 foci in mutants defective in PCNA sumoylation (*pol30-RR*, *siz1Δ*), or by mutating the Srs2 SIM domain (Fig. S1). These results support previous observations that Srs2 interacts preferentially with sumoylated PCNA and there is $PCNA^{SUMO}$-dependent enrichment of Srs2 at replication forks (Papouli et al., 2005). This cited report also showed that recombination proteins are increasingly recruited to replication forks in the absence of Srs2, and to a lesser extent, in the absence of $PCNA^{SUMO}$. Likewise, we observe an increase

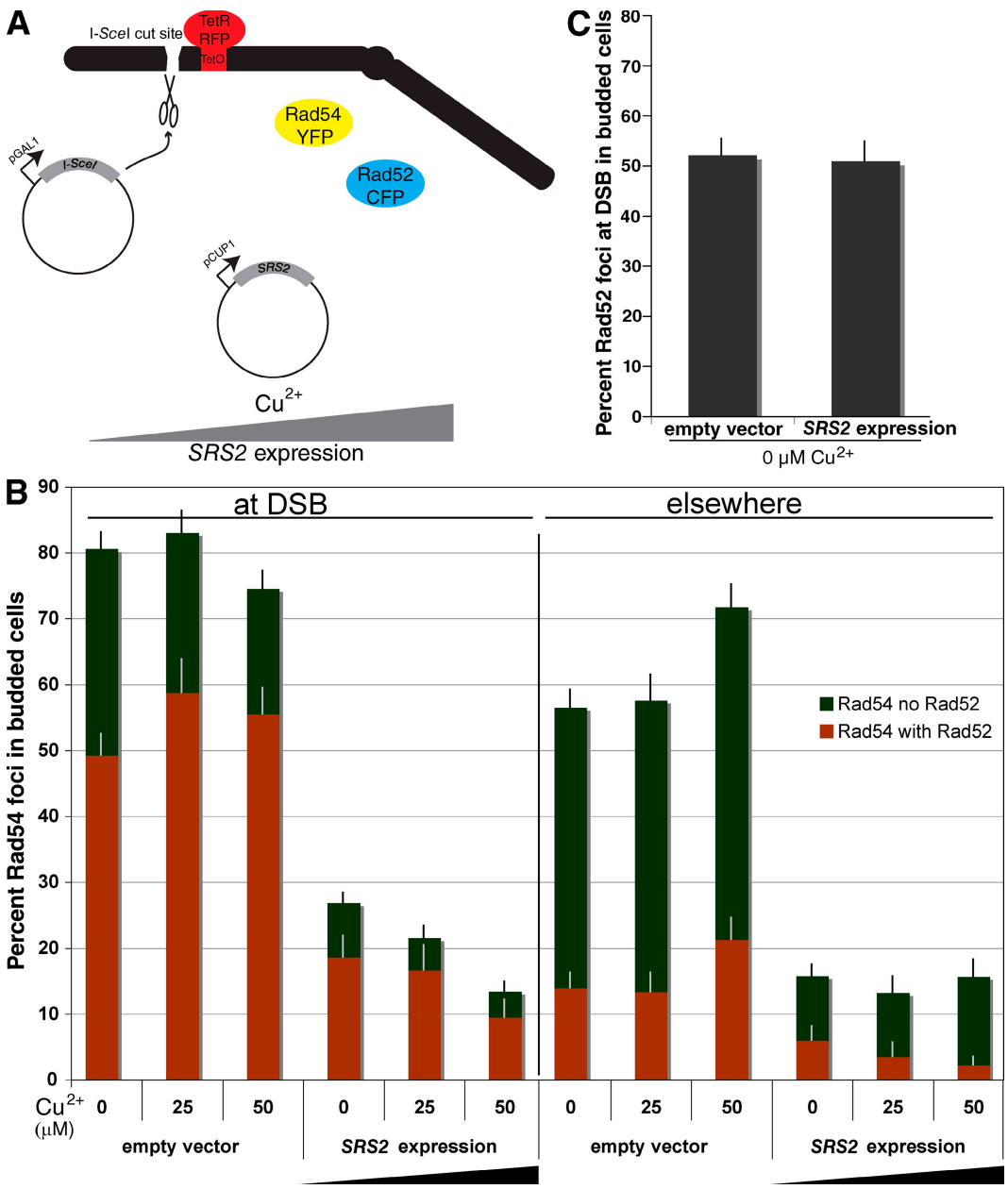

Figure 6. **Srs2 inhibits HR focus formation at both DSBs and elsewhere.** (A) Cartoon depicting experimental scheme: *srs2Δ* strains containing an RFP-marked I-SceI site, Rad54-YFP, Rad52-CFP, and a plasmid expressing the I-SceI endonuclease from a galactose-inducible promoter, was transformed with either an empty vector or a plasmid expressing *SRS2* from a copper-inducible promoter. (B) Both Rad54 foci localizing to DSBs (left) and elsewhere (right) decrease with the expression of Srs2. Expression of Srs2 increases along the X-axis: vector controls represent zero expression of Srs2, whereas the *SRS2* plasmid at 0 μM copper leads to significant amounts of Srs2 expression with further Srs2 expression at 25 and 50 μM copper. Rad54 focus frequency is represented by the total bar height, and the number of Rad52-colocalizing Rad54 foci is depicted by the sienna-colored portion of the bars. (C) The percentage of Rad52 localizing to the DSB does not change with basal Srs2 expression (0 μM copper, compare with significant decrease in Rad54 foci in B). All experiments were performed in triplicate and significance was tested using the χ^2 test ($P < 0.05$). For each time point, 150–300 budded cells were individually examined.

in spontaneous HR foci in the *pol30-RR* mutant, but find that Srs2 recruitment to HR foci is not impaired in this mutant (Figs. S1 and S2). This result suggests that when Srs2 is unable to be recruited to the replication fork directly, HR proteins can assemble there and can later recruit Srs2.

Although there is an increase in the number of spontaneous HR foci in the *pol30-RR* mutant, there is an even larger accumulation of foci in the complete absence of Srs2. This accumulation is partially due to increased recruitment of HR proteins to replication forks (Fig. 2 A; Fig. S3 A; Fig. 3 A). However, because there is no further induction of HR foci in *srs2Δ* after HU addition, cells must retain some mechanism to inhibit HR at stalled replication forks even in the absence of Srs2 (Fig. 3 B). These observations suggest that there is still some restriction on recruitment of recombination proteins to forks other than Srs2, as long as replisome integrity is maintained. However, stalling replication forks in the absence of the Mec1 checkpoint kinase results in collapsed replication forks (Tercero and Diffley, 2001), which then leads to strong induction

of HR foci, both in the presence of Srs2 and even more so in its absence (Fig. 3 B). Together, these data imply that the recruitment of HR factors to intact replication forks is disfavored even in the absence of Srs2. Perhaps there are active mechanisms still in place under these conditions that restrict inappropriate HR. Alternatively, these differences may simply be due to the lower accumulation of ssDNA in the presence of stalled versus collapsed replication forks. In any case, these observations argue only a minor role for Srs2 in reversing HR intermediates at stalled forks.

Several lines of evidence suggest that Srs2 restricts inappropriate HR at multiple sites throughout the genome, and that unscheduled HR at the replication fork is probably not the only cause of the increased Rad51 and Rad54 focus levels in *srs2Δ*. First, colocalization data show that there are a number of Rad54 foci that are neither located at replication forks nor at sites of ssDNA in an *srs2Δ* (as determined by Rfa1 colocalization, Fig. 3 A). Second, levels of Rad54 foci in the *pol30* SUMO mutant (*pol30-RR*), which fails to recruit Srs2 specifically to replication forks, are not as high as in an *srs2Δ* (Fig. S3 A). Third, although *srs2Δ rad54*Δ double mutants are inviable, *pol30-RR rad54Δ* are viable (unpublished data), implying that the general role of Srs2 in recombination is the cause of lethality in *rad54Δ*. Thus, we suggest that Srs2 acts throughout the genome to restrict HR and that only a subset of its function is at replication forks.

An indication that HR foci form inappropriately in the absence of Srs2 comes from the observation that Rad51 and Rad54 foci can form in the absence of Rad52 if Srs2 is also absent. Furthermore, Rad51 and Rad54 colocalize to a similar extent in *srs2Δ rad52Δ* double mutant as they do in wild-type cells, again indicating that these foci reflect similar structures (unpublished data). These foci are likely nonfunctional Rad51 filaments because *srs2Δ rad52Δ* cells are as DNA damage sensitive as the *rad52Δ* single mutant (Fig. 5 A), reflecting the crucial role for Rad52 in additional steps of HR. It is possible that some of these foci may be formed at structures that do not require HR, suggesting that in the absence of negative regulation by Srs2, Rad51 and Rad54 nucleate on sites that are not relevant to HR. Although it is not known whether Rad51 forms foci at dsDNA sites in vivo, Rad51 binds both single- and double-stranded DNA in vitro, with a slightly higher affinity for dsDNA (Shinohara et al., 1992). It is of note that Srs2 dsDNA unwinding activity described by Dupaigne et al. (2008) could act not only to displace Rad51 that is bound to dsDNA in legitimate HR structures, such as D-loops, but also to displace it from illegitimate dsDNA-Rad51 structures. We observe Rad51 foci at a low level in *rad52Δ* cells but, unlike the foci formed in *srs2Δ rad52Δ* double mutants, these aberrant foci are not inducible by gamma irradiation. Perhaps these foci form an aggregate or storage structure, as has been observed for RecA (Renzette et al., 2005), or they are the result of binding to a DNA structure that is not formed by IR, such as dsDNA.

Rad54 stabilizes Rad51 bound to ssDNA, but also removes Rad51 from dsDNA (Kiianitsa et al., 2002; Solinger et al., 2002; Mazin et al., 2003), so it is conceivable that some of the increased Rad54 foci in *srs2Δ* cells may indicate increased Rad54 dsDNA translocase activity in the absence of Srs2. However, the majority of detectable Rad54 foci are associated with Rad51 and ssDNA (96% of Rad54 foci colocalize with Rad51 in wild-type, and nearly 80% of Rad54 foci have detectable RPA association). Therefore, the majority of Rad54 foci are likely the result of binding to Rad51, most often on ssDNA.

The decreased requirement for Rad52 during Rad51 focus formation in *srs2Δ* suggests that Srs2 and Rad52 act antagonistically in the formation of Rad51 filaments. Indeed, addition of Srs2 to the in vitro Rad51-mediated strand exchange reaction inhibits formation of Rad51 filaments, whereas Rad52 protein allows their formation even in the presence of Srs2 (Fig. 4 C). Unlike Rad52, the Rad55/57 heterodimer does not strongly promote in vitro filament formation in the presence of Srs2, suggesting that Rad55/57 has a different or less potent mediator function than Rad52 in driving Rad51 filament formation. Indeed, Rad51 focus formation in the *srs2Δ rad52Δ* double mutant is greatly delayed, suggesting that additional impediments to Rad51 nucleation remain (Fig. 4 B). An obvious candidate is the ssDNA binding protein RPA, which competes for Rad51 binding sites. This competition is relieved by Rad52, which mediates the efficient replacement of RPA by Rad51. However, in an *srs2Δ rad52Δ* double mutant, Rad51 foci can eventually assemble, at the time when the foci in wild-type cells start to disappear. This observation suggests that in the absence of Srs2 activity, Rad51 filaments can be assembled even without Rad52. Despite the fact that Rad51 foci are able to form under these conditions, these foci do not represent recombination-proficient filaments.

Likewise, partially defective *rad52* alleles can be suppressed by overexpression of Rad51 or deletion of Srs2 (Milne and Weaver, 1993; Kaytor et al., 1995; Schild, 1995), suggesting that the role of Rad52 in filament formation may be separable from its other recombination functions. Furthermore, in *Schizosaccharomyces pombe*, the HR defects and damage sensitivity of deletion of *rad22*, its *RAD52* homologue, is completely suppressed by deletion of an Srs2 orthologue, *fbh1* (Osman et al., 2005). All of these observations lead to the conclusion that defects in Rad52 mediator functions can be overcome by making Rad51 nucleation more favorable.

It is well accepted that Srs2 reverses toxic recombination intermediates, but our results show that expression of Srs2 also results in the disassembly of Rad51–Rad54 complexes from an appropriate site for HR, i.e., an I-SceI–induced DSB (Fig. 6 B). Because we are not directly visualizing Rad51, it remained possible that the decrease in Rad54 foci at the DSB was due to decreased recruitment of Rad54 to Rad51 foci caused by Srs2 expression. However, this is not the case because Rad54–Rad51 colocalization is similar both in the presence and absence of Srs2 (Fig. 2 A). Furthermore, because a large percentage of Rad51 foci colocalize with Rad54 foci, it is very likely that we are detecting the dismantling of Rad51 foci by proxy. In fact, even if only the subset of Rad51 foci that are represented by Rad54 foci were showing this dramatic threefold decrease at DSB sites (Fig. 6 B), it still supports the conclusion that Srs2 can dismantle HR complexes at appropriate sites.

As shown in Fig. 6 B, disruption of HR complexes "elsewhere" is nearly maximal at basal Srs2 expression levels, whereas the DSB-localized complexes require increased expression. We also find that Rad52 is preferentially enriched at the DSB-localized Rad54 foci. These data suggest that more Srs2

protein is required to shift the equilibrium to dismantle Rad54 foci at a DSB, due to the Rad52-mediated forward reaction. Although the nature of the foci outside of the I-SceI break site (i.e., "elsewhere") is not precisely known, a portion of these foci are likely spontaneous lesions that are also appropriate sites for HR. However, because Srs2 is well established to reverse toxic HR complexes (Gangloff et al., 2000), in all likelihood a high percentage of these foci are inappropriate.

How does the cell distinguish between appropriate and inappropriate sites for reformation of HR complexes? Given the central role for Rad52 in HR and the wide variety of interactions that it displays, we propose that Rad52 is involved in this decision-making process. Accordingly, Rad52 localizes most frequently to the marked DSB and these Rad52 foci are largely unaffected by expression of Srs2 (Fig. 6, B and C). These studies have led us to a new model to explain how Srs2 affects the regulation of recombination in vivo. Srs2 removes Rad51 from DNA indiscriminately, dissociating inappropriate Rad51 filaments, as well as those at appropriate sites. Rad52 guides Rad51 filaments to reform at appropriate locations, effectively channeling the HR machinery into bona fide substrates. In mammalian cells, BRCA2 may serve this same function, as orthologous mediator activities have been ascribed to it (San Filippo et al., 2006; Shivji et al., 2006) and BRCA2 is thought to target hRad51 to sites of damage (Venkitaraman, 2002). We suggest that this mechanism serves as a recombination quality control point through the wanton destruction of recombination complexes followed by directed rebuilding of suitable recombination intermediates. In addition, Srs2 interactions with sumoylated proteins, e.g., PCNA at replication forks, may target its anti-recombinase activity to critical genomic locations, thus ensuring the presence of this quality control mechanism.

Materials and methods

Yeast strains and plasmids

Standard procedures for yeast mating, sporulation, dissection, transformation, and preparation of growth media (Sherman et al., 1983; Sherman, 1991) were used to obtain strains for this study (Table S1).

Live-cell epifluorescence microscopy and analysis

Cells for microscopic analysis were grown to early- to mid-log phase overnight at 23°C in synthetic complete medium plus 100 mg/L additional adenine. Strains harboring plasmids were grown as above, but in selective medium for plasmid maintenance. Cells were harvested by brief centrifugation (3,500 *g*) and resuspended in approximately ten times cell pellet volume of growth media. Immobilization of cells was performed by mixing equal volumes of cell suspension and 1.4% low-melt agarose plus growth medium solution (held at 42°C before mixing) on a glass slide. Slides for time-lapse microscopy were prepared identically, except 1 µm fluorescent beads (Tetraspeck T-7282; Invitrogen) were added to the cell suspension before immobilization to allow software auto-focusing. In addition, for long time-lapse applications, coverslips were sealed with a wax mixture described previously (Lisby et al., 2003). All images were captured at ambient temperature (23–25°C).

Images for Figs. 2–6 were acquired identically as in Lisby et al. (2003), on the microscope setup described therein. Whole images were minimally processed using Openlab (Improvision), maintaining identical linear contrast enhancement settings for images to be compared. Images were false colored and overlaid in Openlab, then transferred to Adobe Photoshop for scaling. Figures were prepared for publication using Adobe Illustrator CS3.

Images for Fig. 1 and Fig. S1 were acquired on a microscope (model DM5500B; Leica) equipped with a Plan-Apochromat 100X 1.46 NA DIC oil immersion objective, an Orca ER-AG CCD camera (Hamamatsu) with Chroma RFP, YFP (HP), and CFP (HP) band-pass filter sets. Images of 11 Z-stacks at 0.3-µm distances were acquired using Volocity software, and were prepared for publication as described above. Exposure times were minimized and light intensity reduced to 10% to prevent photobleaching of the fluorescently labeled cellular proteins.

Fluorescently tagged recombination and replication proteins were expressed from their endogenous chromosomal loci, except in the case of TetI-mRFP1, which was integrated at *iYGL119W* (Lisby et al., 2003). Complementation analysis of *RAD52* epistasis group and checkpoint fusions is described in Lisby et al. (2004). YFP- and CFP-tagged Srs2 and Pol30 were produced with GGPGG linker peptides to the N terminus of both proteins. Fusions were integrated as described elsewhere (Reid et al., 2002). Fluorescent fusions to Srs2 was determined to be functional by viability with either *rad54Δ* or *sgs1Δ* alleles, both of which render *srs2* mutant strains inviable (Lee et al., 1999; Gangloff et al., 2000; Klein, 2001). Tagged Pol30 is partially functional because PCNA is an essential replication factor in yeast, and cells containing this fusion protein as the sole source of Pol30 are viable but slow growing. In addition, *YFP-POL30* strains show some sensitivity to DNA damaging agents and increased Rad52 focus formation (Kitamura et al., 2006), suggesting some defect in PCNA function. *MCM2* was fused to *mRFP1* with a 4-alanine linker at its endogenous locus. Cells containing this tagged protein as the sole source of Mcm2 show no growth defect, suggesting the functionality of this construct because Mcm2 is an essential replication protein in yeast (Yan et al., 1991). *YFP-srs2-ΔSIM* (Δ1170–1174) was made using a cloning-free allele replacement (Erdeniz et al., 1997) into a strain already containing an N-terminal fusion of *SRS2* to *YFP*.

Protein purification and in vitro sumoylation

Rad51, RPA, Srs2, and Rad52 proteins together with the Rad55–Rad57 heterodimer were purified to near homogeneity as described previously (Sung, 1997b; Krejci et al., 2002, 2003). E1 (GST-Aos1/Uba2 complex), E2 (His-Ubc9), and SUMO proteins (His-Smt3) were expressed and purified essentially as described previously (Johnson and Blobel, 1997; Bencsath et al., 2002). Purification of His-Siz1 and His-PCNA proteins was described previously (Burgers and Gerik, 1998; Takahashi et al., 2003). Truncation fragments of Srs2 protein, GST-Srs2 (783–1169) and GST-Srs2 (783–1174), were purified using GTH-Sepharose chromatography as described previously (Seong et al., 2008).

The in vitro sumoylation assay was performed in 20-µl reaction volumes containing 1.3 µg of purified SUMO protein, 1.1 µg of purified E1 protein, 1.0 µg of purified E2 protein, 4.5 µg Siz1 protein, 10 mM ATP, 50 mM Hepes, 100 mM NaCl, 10 mM $MgCl_2$, 0.1 mM DTT, and 5.0 µg of PCNA protein. Reactions were incubated at 30°C for 5 h.

GST pull-down assay

GST-tagged Srs2 fragments (4.8 µg) were incubated with 12.5 µl of reaction mixture from in vitro sumoylation assays with or without ATP in buffer K (20 mM K_2HPO_4, 10% glycerol, 0.5 mM EDTA, 150 mM KCl, 0.01% NP-40, and 1 mM DTT) at 4°C for 30 min. The reactions were mixed with 20 µl GTH-Sepharose (GE Healthcare) and incubated at 4°C for an additional 30 min. After washing the beads twice with 100 µl buffer K, the bound proteins were eluted with 30 µl SDS buffer. The supernatants (S) and SDS eluates (E), 8 µl each, were subjected to Western blotting analysis.

Two-hybrid analysis

RAD51, *POL30* (PCNA), and *UBC9* were cloned into pGAD10 vector containing *GAL4* transcription activation domain, and the resulting plasmids were introduced into the haploid yeast strain PJ69-4a. *SRS2* fragments were cloned into pGBKT7, which bears the *GAL4* DNA-binding domain, and the resulting plasmids were introduced into the haploid strain PJ69-4α. Diploid strains resulting from mating the PJ69-4a and PJ69-4α strains were grown on synthetic media lacking tryptophan and leucine. Individual interactions were examined by replica-plating diploid cells on dropout plates lacking tryptophan, leucine, and adenine.

Strand exchange reaction

Buffer R (35 mM Tris-HCl, pH 7.4, 2.0 mM ATP, 2.5 mM $MgCl_2$, 50 mM KCl, 1 mM DTT, containing an ATP-regenerating system consisting of 20 mM creatine phosphate and 20 mg/ml creatine kinase) was used for the reactions, and all the incubation steps were performed at 37°C. Rad51 (10 µM) was mixed with ϕX circular (30 µM nucleotides) in 30 µl for 5 min, followed by the incorporation of RPA (2 µM) in 1.5 µl and a 3-min incubation. Where applicable, Rad52 protein (1.4 µM) and the Rad55–Rad57 heterodimer (1 µM) were added together to DNA strand exchange reactions with or without Srs2 (45 and 55 nM). The reaction was completed by adding 3 µl 50 mM spermidine hydrochloride and linear ϕX dsDNA (30 µM nucleotides) in

3 µl. Portions (4.5 µl) of the reaction mixtures were taken at the indicated times, deproteinized, and resolved in agarose gels followed by ethidium bromide staining of the DNA species, as described previously. Srs2 was added to the reactions in 0.9 µl at the time of RPA incorporation.

Induction of double-strand breaks and survival curves

Gamma irradiation for microscopic analysis and for damage sensitivity assays was performed as described previously (Lisby et al., 2001). I-SceI endonuclease-mediated DSBs were induced precisely as described in Lisby et al. (2003). For experiments using induction of DSBs while titrating *SRS2* expression from the copper promoter, the I-SceI endonuclease was preinduced in galactose cultures for 2 h before splitting the cultures into aliquots to which various amounts of $CuSO_4$ was added. Cells were harvested for microscopy 2 h after addition of copper, to allow ample time for *SRS2* expression.

Analysis of *MAT* switching intermediates

Southern blotting for recombination intermediates was performed on a positively charged nylon membrane transferred from a 1% agarose gel using alkaline downward capillary transfer. Genomic DNA and *MAT* locus probes were prepared as described previously (Sugawara and Haber, 2006).

Online supplemental material

Table S1: Strains and plasmids used. Fig. S1: Quantification of Srs2 localization to replication foci and to recombination centers in recombination and SUMO mutants. Fig. S2: Rad54 foci mirror those of Rad51, even in the absence of Srs2. Fig. S3: Rad54 focus frequency increases in the absence of Srs2 or PCNA sumoylation, partially due to increased recruitment to replication forks. Fig. S4: Rad51-mediated strand exchange in the presence of Srs2 requires Rad52–Rad51 interactions. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200810055/DC1.

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