

1 **Srs2 helicase prevents the formation of toxic DNA damage during late**  
2 **prophase I of yeast meiosis**

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11 *Running title:* A novel role of Srs2 during meiosis

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28

1 **Abstract**

2

3 Proper repair of double-strand breaks (DSBs) is key to ensure proper  
4 chromosome segregation. In this study, we found that the deletion of the *SRS2*  
5 gene, which encodes a DNA helicase necessary for the control of homologous  
6 recombination, induces aberrant chromosome segregation during budding yeast  
7 meiosis. This abnormal chromosome segregation in *srs2* cells accompanies the  
8 formation of a novel DNA damage induced during late meiotic prophase-I. The  
9 damage may contain long stretches of single-stranded DNAs (ssDNAs), which  
10 lead to aggregate formation of a ssDNA binding protein, RPA, and a RecA  
11 homolog, Rad51, as well as other recombination proteins inside of the nuclei.  
12 The Rad51 aggregate formation in the *srs2* mutant depends on the initiation of  
13 meiotic recombination and occurs in the absence of chromosome segregation.  
14 Importantly, as an early recombination intermediate, we detected a thin bridge of  
15 Rad51 between two Rad51 foci or among the foci in the *srs2* mutant, which is  
16 rarely seen in wild type. These might be cytological manifestation of the  
17 connection of two DSB ends and multi-invasion. The DNA damage with Rad51  
18 aggregates in the *srs2* mutant is passed through anaphase-I and -II, suggesting  
19 the absence of DNA damage-induced cell-cycle arrest after the pachytene stage.  
20 We propose that Srs2 helicase resolves early protein-DNA recombination  
21 intermediates to suppress the formation of aberrant lethal DNA damage during  
22 late prophase-I.

23 (220 words)

24

25 **Key words:** Srs2, Rad51, Dmc1, meiotic recombination

26

27

## 1 Introduction

2

3 In sexually reproducing organisms, meiosis, a specialized form of cell division,  
4 produces haploid gametes from diploid germ cells. Following DNA replication,  
5 reciprocal recombination takes place to connect the homologous chromosomes  
6 and to generate genetic diversity of gametes. With arm cohesion, the connection  
7 between the chromosomes, which is cytologically visualized as chiasma, is  
8 essential for faithful chromosome segregation during meiosis I by antagonizing  
9 the pulling force by spindle microtubules to create tension (Petronczki et al.  
10 2003).

11 Meiotic recombination is initiated by the generation of DNA  
12 double-strand breaks (DSBs) by a meiosis-specific topoisomerase-like protein,  
13 Spo11, at recombination hotspots (Keeney et al. 1997). Subsequently, the end  
14 of DSBs is quickly resected to produce 3'-overhanging single-stranded DNAs  
15 (ssDNAs). Replication protein A (RPA) binds to the ssDNAs, followed by the  
16 loading of Rad51, a homolog of bacterial RecA (Shinohara et al. 1992), with the  
17 assistance of auxiliary proteins, such as Rad52, Rad55-Rad57 and  
18 Pys3-Csm2-Shu1-Shu2 (a.k.a. Shu) (New et al. 1998; Sasanuma et al. 2013b;  
19 Shinohara and Ogawa 1998; Sung 1997). Rad51 filaments on ssDNA are active  
20 protein machinery for DNA homology search and strand exchange (Ogawa et al.  
21 1993; Sung 1994). Rad51 filament activity is helped by Rad54, which belongs to  
22 the SNF2/SWI2 DNA helicase family (Shinohara et al. 1997b).

23 Whereas Rad51 is sufficient for the homolog search in recombination  
24 during mitosis, meiosis requires a meiosis-specific RecA homolog, Dmc1, for the  
25 recombination (Bishop et al. 1992). Dmc1 is essential for homology  
26 search/strand exchange in inter-homolog recombination during meiosis while  
27 Rad51 plays an auxiliary role by assisting Dmc1 assembly (Bishop 1994; Cloud  
28 et al. 2012; Shinohara et al. 1997a). Indeed, the Rad51 activity for inter-sister  
29 recombination during meiosis is suppressed by the action of a meiosis-specific  
30 Rad51 inhibitor, Hed1 (Tsubouchi and Roeder 2004). Like Rad51, Dmc1 forms a  
31 nucleo-protein filament on ssDNAs to catalyze the strand invasion of the DNA  
32 into its homologous duplex DNA for the formation of an intermediate,  
33 D(displacement)-loop (Hong et al. 2001).

1           In D-loop, DNA synthesis occurs from 3'-end of invading strand as a  
2 primer. When the synthesized DNA strand is ejected from the D-loop (Allers and  
3 Lichten 2001; Hunter and Kleckner 2001), the ejected synthesized ssDNA is  
4 able to anneal with the complementary ssDNA in the other end of the DSB.  
5 Annealing induces the second DNA synthesis to complete the recombination by  
6 producing non-crossovers. This pathway is called synthesis-dependent strand  
7 annealing (SDSA) (Allers and Lichten 2001). On the other hand, when the newly  
8 synthesized DNA is stably bound to the D-loop, ongoing DNA synthesis can  
9 extend a D-loop with a large displaced ssDNA, which is able to anneal with  
10 ssDNA on the opposite DSB ends. Additional processing of the intermediates  
11 leads to the formation of double-Holliday junction (dHJ) (Schwacha and Kleckner  
12 1994). dHJs are specifically resolved into crossovers. Importantly, meiotic  
13 recombination is tightly coupled with chromosome morphogenesis such as the  
14 formation of the synaptonemal complex (SC), a meiosis-specific zipper-like  
15 chromosome structure, which juxtaposes homologous chromosomes in near  
16 vicinity (Cahoon and Hawley 2016).

17           Srs2 is a 3'-to-5' SF1 helicase related to bacterial UvrD helicase (Rong  
18 et al. 1991). Srs2 protein has some distinct functional domains: 3'-5' DNA  
19 helicase domain, Rad51-interaction domain, and also SUMO- and  
20 PCNA-binding domains in the C-terminus (Marini and Krejci 2010). Genetic  
21 analyses showed positive and negative roles of Srs2 in the recombination  
22 (Marini and Krejci 2010). Biochemical studies have demonstrated that purified  
23 Srs2 protein can dislodge Rad51 filament on ssDNAs and dramatically inhibits  
24 Rad51-joint molecules via direct interaction with Rad51 *in vitro* (Krejci et al.  
25 2003; Veaute et al. 2003). This biochemical activity of Srs2 supports the idea of  
26 Srs2 function as an anti-recombinase. The Rad51-dismantling activity of Srs2 is  
27 confirmed by *in vivo* analysis (Sasanuma et al. 2013a).

28           Deletion of *SRS2* gene shows different kinds of genetic interaction with  
29 mutants deficient in DNA transaction. The *srs2Δ* is synthetic lethal with a  
30 mutation of the *SGS1*, encoding a RecQ-type DNA helicase. By forming a  
31 complex with Top3 and Rmi1, Sgs1 is known to dissolve the dHJ structure into  
32 noncrossovers (Cejka et al. 2010; Wu and Hickson 2003). Moreover, the  
33 *srs2Δ* is synthetic lethal with the deletion of the *RAD54* (Klein 2001), suggesting

1 the role of Srs2 in a late stage of the recombination such as the post-invasion  
2 step in the recombination. This lethality is thought to be caused by a fatal defect  
3 in the resolution of toxic intermediates in the recombination process. This is  
4 supported by the fact that the deletion of *RAD51* can suppress the lethality of  
5 *srs2Δ sgs1Δ* and *srs2Δ rad54Δ* mutants (Gangloff et al. 2000; Schild 1995).

6 During mitosis, crossovers should be suppressed when DNA damage is  
7 spontaneously introduced, because the crossover between homologous  
8 chromosomes and sister chromatids results in the loss of heterozygosity. In  
9 contrast, as described above, meiotic recombination must give rise to at least  
10 one essential crossover per chromosome, which is fostered by a group of  
11 proteins called ZMM (Zip-, Msh-, Mer) (Shinohara et al. 2008). Previous genetic  
12 studies showed a role of Srs2 in meiosis (Palladino and Klein 1992; Sasanuma  
13 et al. 2013b). However, the molecular defects associated with *srs2* deletion in  
14 meiosis have not been described in detail. Therefore, it remains elusive how  
15 Srs2 regulates meiotic recombination.

16 In this study, we analyzed the role of Srs2 helicase in meiotic  
17 recombination, particularly looking at dynamics of its interacting partner, Rad51.  
18 We found that, in the absence of Srs2, abnormal DNA damage associated with  
19 Rad51 aggregation accumulates during late prophase-I, after the completion of  
20 meiotic recombination. The formation of this DNA damage in the *srs2* requires  
21 meiotic DSB formation, but is independent of chromosome segregation. We also  
22 detected thin line-staining of Rad51 connecting between two adjacent Rad51  
23 foci in early prophase in the absence of Srs2, which is rarely seen in the wild  
24 type. We propose that Srs2 protects chromosomes in late meiotic prophase-I  
25 from accumulation of abnormal DNA damage by properly coupling the  
26 completion of meiotic recombination with chromosome morphogenesis.

27

## 1 **Materials and Methods**

2

### 3 **Yeast strains and medium conditions**

4 All yeast strains used in this article are isogenic derivatives of SK1 and listed in  
5 Table S1. *pCLB2-SGS1* and *RAD54-RFB* strains were a gift by Dr. Neil Hunter  
6 and Dr. Andreas Hochwagen, respectively. Mediums and culture conditions  
7 regarding meiosis are described in (Sasanuma et al. 2008).

8

### 9 **Antibodies and chemicals**

10 The primary anti-sera were used as following concentrations; anti-Rad51  
11 (guinea pig, 1/500), anti-Dmc1 (rabbit, 1/500), anti-Rad52 (rabbit, 1/300),  
12 anti-Rfa2 (rabbit, 1/500), anti-Zip1 (rabbit, 1/500), anti-Red1 (rabbit, 1/500),  
13 Anit-Hed1 (rabbit, 1/200) and anti-Mei5 (rabbit, 1/500) for cytology. Anti-Hed1  
14 serum from rabbit was prepared for denatured Hed1 protein purified from *E. coli*.  
15 Anit-Nop1(mouse) is from Encor Biotech (MCA28-F2).  $\alpha$ -tubulin is monoclonal  
16 antibody of rat that can recognize alpha subunit (AbD Serotec/BioRad,  
17 MCA77G). The second antibodies for staining were Alexa-fluor 488 (Goat) and  
18 594 (Goat) IgG used at a 1/2000 dilution (Molecular Probes).

19 Rapamycin (LC-Laboratories, R-5000) and benomyl (methyl  
20 1-[butylcarbamoil]-2-benzimidazolecarbamate; Sigma Aldrich, PCode  
21 1002355429) were dissolved in DMSO at a concentration of 1 mM and 30 mg/ml,  
22 respectively.

23

### 24 **Immuno-staining**

25 Chromosome spreads were prepared using the Lipsol method as described  
26 previously (Shinohara et al. 2000; Shinohara et al. 2003). Immunostaining was  
27 conducted as described (Shinohara et al. 2000). Stained samples were  
28 observed using an epi-fluorescence microscope (BX51; Olympus, Japan) with a  
29 100X objective (NA1.3). Images were captured by CCD camera (CoolSNAP;  
30 Roper, USA), and afterwards processed using IP lab and/or iVision (Sillicon,  
31 USA), and Photoshop (Adobe, USA) software tools.

32

### 33 **SIM imaging**

1 The structured illumination microscopy was carried out using super  
2 resolution-structured illumination (SR-SIM) microscope (Elyra S.1 [Zeiss],  
3 Plan-Apochromat 63x/1.4 NA objective lens, EM-CCD camera [iXon 885; Andor  
4 Technology], and ZEN Blue 2010D software [Zeiss]) at Friedrich Miescher  
5 Institute for Biomedical Research, Switzerland. Image processing was  
6 performed with Zen software (Zeiss, Germany), NIH image J and Photoshop.

7

### 8 **Whole cell staining**

9 Cells were fixed with 1/10 volume of 37% formaldehyde (Wako) and treated with  
10 10  $\mu$ g/ml Zymolyase 100T (Seikagaku) for 1.5 h. Cells were placed to the poly  
11 L-lysine (Sigma) coated slides and then fixed with cold 100% methanol, cold  
12 100% acetone and cold 1X PBS. Slides were used for immuno-staining.

13

### 14 **Western Blotting**

15 Western blotting was performed for cell lysates extracted by TCA method. After  
16 being harvested and washed twice with 20% TCA, cells were roughly disrupted  
17 by Yasui Kikai (Yasui Kikai Co Ltd, Japan). Protein precipitation recovered by  
18 centrifuge at 1600 *g* for 5min was suspended in SDS-PAGE sample buffer  
19 adjusting to pH8.8 and then boiled for 95°C, 2min.

20

### 21 **Southern Blotting**

22 Southern blotting analysis was performed with the same procedure as in  
23 (Storlazzi et al. 1995). Genomic DNA prepared was digested with both *Mlu*I and  
24 *Xho*I (for crossover/non-crossover, upper panels) and *Pst*I (for meiotic DSB,  
25 lower panels). Probes for Southern blotting were Probe “155” for  
26 crossover/non-crossover and Probe 291 for DSB detection as described in  
27 (Storlazzi et al. 1995). Image gauge software (Fujifilm Co. Ltd., Japan) was used  
28 for quantification for bands of R1, R3 and DSB I.

29

### 30 **Pulsed-field gel electrophoresis**

31 For pulsed-field gel electrophoresis (PFGE), chromosomal DNA was prepared in  
32 agarose plugs as described in (Bani Ismail et al. 2014) and run at 14 °C in a

1 CHEF DR-III apparatus (BioRad) using the field 6V/cm at a 120° angle.  
2 Switching times followed a ramp from 15.1 to 25.1 seconds. Durations of  
3 electrophoresis were 41 h for chromosome III.

4

#### 5 **Statistics**

6 Means ± S.D values are shown. Graphs were prepared using and Microsoft  
7 Excel and GraphPad Prism 7. Datasets were compared using the Mann-Whitney  
8 U-test.  $\chi^2$ -test was used for proportion.

9



## 1 **Results**

2

### 3 ***SRS2* deletion markedly decreased spore viability**

4 As reported previously (Palladino and Klein 1992; Sasanuma et al. 2013a), the  
5 *srs2* deletion mutant exhibits reduced spore viability of 36.8%, indicating a  
6 critical role of this helicase for meiosis (Fig. S1A). This marked reduction of the  
7 spore viability is somehow unexpected given a negative role of this helicase in  
8 recombination.

9 We also confirmed the kinetics of meiotic progression in *srs2Δ* strains  
10 by DAPI staining. In the wild-type strain, meiosis I started at 5 h after incubation  
11 with sporulation medium (SPM) and was sequentially followed by meiosis II.  
12 Finally, ~90% of the wild-type cells completed MII at around 8 h (Fig. 1A). In the  
13 *srs2Δ* mutant, the appearance of cells undergoing MI was delayed by ~2 h and  
14 ~75% of cells finished MII at 14 h (Fig. 1A). A similar delay was observed for a  
15 *srs2* mutant in a different strain background previously (Palladino and Klein  
16 1992). This indicates a defect during prophase-I in the *srs2* mutant. In *srs2* cells  
17 after sporulation; e.g. 12 h, we often detected fragmented DAPI bodies in a  
18 cell/spore (Fig. 1B), indicating a defect in chromosome segregation during the  
19 mutant meiosis.

20

### 21 **The *srs2Δ* mutant showed a defect in meiotic DSB repair**

22 We analyzed meiotic recombination defects the *srs2* deletion mutant in more  
23 detail. First, we checked the repair of meiotic DSBs in the mutant by Southern  
24 blotting. DSB formation was monitored at the *HIS4::LEU2* locus, an artificial  
25 meiotic recombination hotspot in chromosome III (Fig. S1B)(Cao et al. 1990). In  
26 wild type, DSB frequencies reached its maximum value at 3 hours of meiosis  
27 (~10% of total signals) and then decreased gradually (Fig. S1C, D). The *srs2Δ*  
28 accumulates DSB at higher levels (~20%) with more hyper-resection than wild  
29 type and delays the disappearance by ~ 2h. (Fig. S1D), indicating that Srs2 is  
30 required for efficient meiotic DSB repair. We also checked the formation of two  
31 recombinant species, crossover (CO) and non-crossover (NCO) at the same  
32 locus. The *srs2Δ* reduces both CO and NCO to 52% and 64% of the wild-type  
33 levels (at 6 h; Fig. S1C, D), respectively. These show that Srs2 is necessary for

1 efficient formation of meiotic recombinants. This is consistent with previous  
2 return-to-growth experiment showing delayed recombinant prototroph formation  
3 in the *srs2Δ* mutant (Palladino and Klein 1992).

4 During meiotic prophase, homologous chromosomes are tightly  
5 coupled with the formation of the synaptonemal complex (SC), a zipper-like  
6 chromosome structure linking two homologous chromosomes. Zip1 is a  
7 component of the central region of SC, which serves as a marker for synapsis  
8 (Sym et al. 1993). A defect in meiotic recombination results in defective SC  
9 formation. We checked the SC formation in the *srs2Δ* mutant by  
10 immuno-staining analysis of Zip1 on chromosome spreads as well as a  
11 meiosis-specific cohesin component, Rec8 (Fig. S1E). We classified three  
12 categories according to Zip1 staining; Dotty Zip1 (Class I), partially extended  
13 (Class II) and fully-elongated (Class III), which roughly correspond with leptotene,  
14 zygotene and pachytene stages, respectively. In wild type, ~66% of nuclei  
15 contained full-elongate Zip1 lines at 4 h and Zip1 signal gradually disappeared  
16 from chromosomes. In *srs2Δ* strains, although Zip1 focus-positive nuclei  
17 exceeded 80% at 4 h, the proportion of cells with fully-elongated Zip1 was  
18 significantly reduced to 13 and 26% at 4 and 5 h, respectively (Fig. S1F).  
19 Consistent with this, the proportion of polycomplexes (PCs), which are an  
20 aggregate of Zip1, dramatically increased; ~60% of the *srs2Δ* nuclei contained  
21 PCs at 4 h (Fig. S1G). SCs disassembled more slowly in the mutant than wild  
22 type, consistent with delayed meiotic DSB repair (Fig. S1F).

23

#### 24 **The *srs2Δ* mutant accumulated aggregates of Rad51 during late** 25 **meiotic-prophase I**

26 Immuno-staining analysis of chromosome spreads can detect recombination  
27 proteins such as Rad51 and Dmc1 on the spreads as a focus, which marks a  
28 site of ongoing recombination (Bishop 1994). Previous study indicated that the  
29 number of Rad51 foci on chromosome spreads in the *srs2Δ* mutant at 4 h  
30 incubation of SPM is slightly reduced compared to those in wild-type (Sasanuma  
31 et al. 2013b). We performed kinetic analysis of Rad51 and Dmc1 focus formation.  
32 In wild-type cells, dotty signals of both Rad51 and Dmc1 peaked at 4 h of  
33 meiosis (Figs. 1C and S2A). The appearance of Rad51 foci in cells lacking Srs2

1 is slightly delayed, and the disappearance of the foci is delayed relative to  
2 wild-type cells (Fig. 1D), consistent with delayed DSB repair in the mutant.

3 Interestingly, after disappearance of Rad51 foci, we observed  
4 reappearance of Rad51 staining with a unique structure after 5 h incubation in  
5 the *srs2Δ* mutant (Figs. 1C and S2A). This staining shows clustering of  
6 beads-in-line of Rad51 foci, in which 1-5 bright aggregates of Rad51 are  
7 connected with each other through thin threads containing Rad51 as well as  
8 much simple big aggregation of Rad51 (referred to as Rad51 aggregates) (Fig.  
9 1C). The formation of Rad51 aggregates reach a plateau at 6 h, slightly  
10 decreases thereafter, but some cells at 10 or 12 h contained Rad51 aggregates  
11 (Fig. 1D), when most of *srs2* mutant cells finished MII (Fig. 1A). At 6 and 12 h, 56  
12 and 40 percent of cells contained aggregates of Rad51, respectively (Fig. 1D,  
13 bottom).

14 Interestingly, this aggregate staining is specific to Rad51, not seen to  
15 Dmc1 (Figs. 1C and S2A). Western blots show that Dmc1 and its mediator Mei5  
16 (Hayase et al. 2004) are still present at MI and MII (Fig. S2B). On the other hand,  
17 like Rad51 foci, we do see the aggregates of Rad52, a mediator of Rad51  
18 (Shinohara and Ogawa 1998), on chromosomes only in the *srs2Δ* mutant, but  
19 not in wild type cells at late times (Fig. S2C, D). We also found that a  
20 Rad51-inhibitor protein, Hed1 (Tsubouchi and Roeder 2006), formed an  
21 aggregate with Rad51 with co-localization (Fig. S2E, F). The kinetics of  
22 appearance of Rad52 and Hed1 aggregates in the *srs2Δ* mutant are similar to  
23 those of Rad51 (Fig. S2D, F).

24 In order to know the nature of the late Rad51 foci/aggregates, we also  
25 studied the localization of RPA (Rfa2, a middle subunit of RPA) at late prophase  
26 I of the *srs2Δ* mutant. Immuno-staining showed that, in addition to early RPA foci  
27 (Fig. 1E, F), like Rad51-aggregates, aggregate staining of Rfa2 re-appeared at  
28 late times of the *srs2* meiosis; e.g. 6-10 h (Fig. 1G). Closer examination reveals  
29 that RPA also exhibits a long-line like staining (Fig. 1F). The kinetics of Rfa2  
30 aggregates in the *srs2* mutant is very similar to that of Rad51 (Fig. 1D, G). Some  
31 RPA lines and aggregates co-localized with Rad51 lines and aggregates (Fig.  
32 1F). This suggests that the formation of ssDNAs during late prophase-I in *srs2*  
33 cells.

1           One possibility is that Rad51 aggregates bind to DNA damage in  
2 ribosomal DNA (rDNA) region, whose segregation defect is often observed in the  
3 recombination defective mutants (Li et al. 2014). We co-stained Rad51 with  
4 anti-Nop1, a marker for an rDNA region (Schimmang et al. 1989). As shown in  
5 Fig. S3A, a single Nop1 signal does not co-localize with late Rad51 aggregates  
6 as well as early Rad51 foci in the *srs2* mutant. This excludes the possibility that  
7 late Rad51 aggregates are induced by abnormal recombination in the rDNA  
8 repeat.

9           In order to know the relationship of Rad51 aggregate formation with  
10 chromosome segregation, we performed whole cell immuno-staining for Rad51  
11 and Dmc1. At early time points, both wild-type and *srs2* $\Delta$  mutant cells showed  
12 punctate staining for both Rad51 and Dmc1 with some background diffuse  
13 staining in a nucleus (Fig. 2A). Rad51-positive nuclei appear at 2 h, peaks at 4 h,  
14 and then disappear in wild-type cells while the positive nuclei peaks at 5 h in the  
15 *srs2* cells (Fig. 2C). Consistent with results for chromosome spreads (Fig. 1C),  
16 the *srs2* $\Delta$  cells start to show a big aggregate of Rad51, but not of Dmc1 in nuclei  
17 from 5 h and this staining reached to plateau at 8 h (Fig. 2C). Rad51 aggregates  
18 in a nucleus often contained thin lines and the number of the aggregate varies up  
19 to 2-5 per a nucleus. Importantly, we could also detect Rad51 aggregates in  
20 *srs2* $\Delta$  cells with two and four big DAPI bodies in a cell, which correspond with  
21 cells finishing MI and MII, respectively (Fig. 2B, D). This suggests that DNA  
22 damage associated with Rad51 aggregates does not induce delay or arrest of  
23 the progression of meiosis. To see the DNA damage checkpoint activation at late  
24 meiosis of the *srs2* cells, we analyzed the phosphorylation status of Hop1, which  
25 is a substrate of Mec1/ATR and Tel1/ATM as a marker of the activation (Carballo  
26 et al. 2008), and found that the *srs2* cells accumulated more phosphorylated  
27 Hop1 at 4 h compared to wild type and showed residual phosphorylation during  
28 late time points (Fig. 2E).

29

### 30 **Rad51-aggregate formation in the *srs2* mutant depends on Spo11**

31 To know the nature of Rad51 aggregates in the *srs2* $\Delta$  mutant, we looked for  
32 genetic requirement of the aggregate formation in the mutant. Rad51-aggregate  
33 formation in *srs2* $\Delta$  cells is dependent on DSB formation, since a catalytic-dead

1 *spo11* mutation, *spo11-Y135F* (Keeney et al. 1997), almost abolishes both early  
2 focus and aggregate of Rad51 staining in the *srs2* $\Delta$  mutant (Fig. S3B). It is likely  
3 that early DSB-related events in the *srs2* $\Delta$  cells may trigger Rad51 aggregates  
4 during late meiosis.

5 In mitosis, the *sgs1* mutation is synthetic lethal with the *srs2* mutation,  
6 indicating a redundant role of these two helicases (Gangloff et al. 2000). Sgs1  
7 helicase, together with Top3 and Rmi1, is known to prevent the formation of the  
8 untangled chromosomes. The absence of Sgs1 results in abnormal meiosis  
9 divisions due to accumulation of un-resolve recombination products involving  
10 multi-chromatids (Jessop and Lichten 2008; Jessop et al. 2006; Oh et al. 2007;  
11 Oh et al. 2008; Tang et al. 2015). In mammals, the lack of Sgs1 ortholog, BLM  
12 helicase, induces anaphase bridges, which are associated with DNA damage  
13 generated during S-phase (Biebricher et al. 2013; Chan et al. 2007). We  
14 examined the late Rad51 aggregate formation in a meiotic-null allele of *sgs1*,  
15 *sgs1-mn* (*CLB2p-SGS1*) (Oh et al. 2007). The *sgs1-mn* forms early Rad51 foci  
16 with delayed disappearance in prophase of MI, but, unlike the *srs2*, the mutant  
17 does not form late Rad51 aggregates (Fig. S3C, D), suggesting that unresolved  
18 recombination intermediates formed in the absence of the Sgs1 do not trigger  
19 Rad51 aggregates formation.

20

## 21 **The effect of Rad54 depletion on the kinetics of Rad51 aggregates in the** 22 ***srs2* mutant**

23 We postulated that some Rad51 aggregates turned over during meiosis and  
24 could expect to stall its dynamics by blocking late stage of the recombination  
25 reaction. We focused on Rad54, which functions at post-assembly stage of  
26 Rad51 (Shinohara et al. 1997b), and tried to examine the effect of *RAD54*  
27 deletion on Rad51 aggregates. However, it is reported that the *rad54* deletion is  
28 synthetically lethal with the *srs2* deletion (Klein 2001; Palladino and Klein 1992;  
29 Schild 1995). To circumvent this, we used Rad54-anchor away system, which  
30 specifically depletes nuclear Rad54 fused with RFB by the addition of the drug  
31 rapamycin (Haruki et al. 2008; Subramanian et al. 2016). The *srs2 RAD54-RFB*  
32 cells grow normally in the absence of rapamycin while the *srs2 RAD54-RFB*  
33 cells grow poorly on the plate containing the drug, confirming synthetic lethality

1 of the *rad54* and *srs2* (Fig. 3A). In order to know the functional relationship  
2 between Rad54 and Srs2 during late meiosis, first, we added rapamycin at 4 h to  
3 *RAD54-RFB* and *srs2 RAD54-RFB* cells and analyzed both spore viability and  
4 Rad51 foci. The *srs2 RAD54-RFB* decreased spore viability to 64% in the  
5 absence of the drug. As reported (Shinohara et al. 1997b), *RAD54-RFB* cells  
6 decreased spore viability to 48% in the presence of the drug. Addition of the  
7 rapamycin also reduced the spore viability of the *srs2 RAD54-RFB* to 24%,  
8 indicating the additive effect of the *srs2* deletion and *RAD54* depletion on spore  
9 viability (Fig. 3B). *RAD54* depletion does not affect delayed MI progression in the  
10 *srs2* deletion (Fig. 3C). As in wild-type cells, *RAD54-RFB* cells showed normal  
11 assembly and disassembly of Rad51 foci in the absence of the drug (Rapa<sup>-</sup>; Fig.  
12 3D, E). However, we found that, from 5 h, one hour after the addition of the drug  
13 (Rapa<sup>+</sup>), a new class of Rad51 staining appeared. This class contains 5-10  
14 brighter foci of Rad51, called “Rad51 clump”, which is distinct from the typical  
15 Rad51 foci and aggregates (Fig. 3D). This Rad51 clamp peaks at 6 h and then  
16 disappears (Fig. 3E), indicating the role of Rad54 in the post Rad51-assembly  
17 stage. In the absence of rapamycin, the *srs2 RAD54-RFB* mutant shows the  
18 similar kinetics for both Rad51 foci and aggregates to the *srs2Δ* mutant. By the  
19 addition of the rapamycin at 4 h, like *RAD54-RFB* cells, the *srs2 RAD54-RFB*  
20 mutant formed Rad51 clamp from 5 h and showed the similar kinetics to that in  
21 the *RAD54-RFB* (Rapa<sup>+</sup>). In addition, Rad51 aggregates appeared at 5 h and  
22 accumulated during further incubation. Rad51 aggregate kinetics in the absence  
23 of *RAD54* (Rapa<sup>+</sup>) is delayed relative to its presence (Rapa<sup>-</sup>) (Fig. 3E). This  
24 result indicates that Rad51 clumps formed without Rad54 are independent of  
25 Srs2. And also, Rad51 aggregate kinetics in the *srs2* cell is independent of  
26 Rad54 function.

27

### 28 **Rad51 aggregate in the *srs2* mutant is dependent of pachytene exit but is** 29 **independent of the onset of meiosis I**

30 Rad51 aggregates in the *srs2Δ* mutant are formed at late times during  
31 prophase-I. To know the relationship between the focus formation and the  
32 progression of meiosis, we first analyzed the Rad51 aggregate formation in the  
33 *srs2Δ* with the *ndt80* mutation, which induces pachytene arrest due to the

1 inability to express genes necessary for exit from mid pachytene stage (Xu et al.  
2 1995). Staining of chromosome spreads in *ndt80* cells reveal accumulation of  
3 cells with Rad51 foci (Figs. 4A and S3E), which is induced by persistent DSB  
4 formation during pachytene arrest by the *ndt80* (Carballo et al. 2013). At later  
5 times, the *ndt80* mutant showed the reduced number of Rad51 foci compared to  
6 early time points (Fig. S3E). However, Rad51 foci seemed to turn over less  
7 efficiently in the *ndt80* mutant (Fig. S3E, F). Little Rad51 aggregate formation  
8 was seen in *srs2 ndt80* cells arrested at mid-pachytene both on chromosome  
9 spreads and in whole cells (Figs. 4A and S3E). This indicates that the formation  
10 of Rad51 aggregates in the *srs2* mutant depends on Ndt80, thus after the exit of  
11 mid-pachytene stage.

12 When the kinetics of Rad51 aggregate formation in the *srs2* mutant was  
13 compared to kinetics of meiosis I entry, Rad51 aggregate in the *srs2* mutant  
14 appear 1 h earlier than the entry into meiosis I (Fig. 1D). To confirm this, we  
15 blocked the microtubule dynamics by treating cells with a benomyl, a  
16 microtubule depolymerization drug. As shown previously (Hochwagen et al.  
17 2005), the addition of benomyl to yeast meiosis at 4 h prior to the formation of  
18 the aggregates, largely suppressed the entry of meiosis I, thus the onset of  
19 anaphase-I, in both wild-type and *srs2* cells (Fig. 5A). The treatment with  
20 benomyl does not affect Rad51-focus kinetics in both wild-type and *srs2* mutant  
21 (Fig. 5A, C). Moreover, the *srs2* cells formed Rad51 aggregates in the presence  
22 of benomyl with similar kinetics in its absence (mock treatment with DMSO) (Fig.  
23 5C). This indicates that Rad51-aggregate formation in the *srs2* mutant occurs in  
24 the absence of microtubule dynamics, thus chromosome segregation,  
25 suggesting that Rad51 aggregate formation in *srs2* mutants is associated with  
26 an event during late prophase-I, not with events during the metaphase-I or  
27 anaphase-I.

28

### 29 **Rad51 aggregates in the *srs2* mutant appear when SC is disassembled**

30 In order to confirm that Rad51-aggregate formation in the *srs2* is independent of  
31 the onset of anaphase I, we used a meiosis-specific null mutant of the *CDC20*,  
32 which encodes an activator of Anaphase promoting complex/cyclosome  
33 (APC/C), the *cdc20-mn* (*CLB2p-CDC20*). As reported previously (Lee and Amon

1 2003), the *cdc20-mn* shows an arrest at the onset of anaphase I. In the  
2 *cdc20-mn*, Rad51 foci appear and disappear like in wild-type control. As  
3 expected from the results with benomyl, the Rad51-aggregate formation occurs  
4 after the disappearance of Rad51 foci in the *srs2 cdc20-mn* double mutant as in  
5 the *srs2* mutant (Fig. 5D, E). This supports the notion that Rad51-aggregate  
6 formation in *srs2* mutant is independent of the entry into anaphase-I, thus  
7 chromosome segregation.

8 The relationship between the formation of Rad51 aggregates and late  
9 meiotic prophase I such as SC disassembly was compared by immuno-staining  
10 of Rad51 with Zip1 (Fig. S4A). After the pachytene exit, the central region of SCs  
11 is dismantled as seen in the loss of Zip1-line signals from chromosomes (Sym et  
12 al. 1993). The *srs2* cells containing Rad51 aggregates were almost negative for  
13 Zip1 lines (Fig. S4A).

14 We also performed the staining of Red1, which is a component of  
15 chromosome axes (Smith and Roeder 1997). Most cells with Rad51 foci at 3-5 h  
16 are almost positive for Red1 staining in both *cdc20-mn* and *srs2 cdc20-mn* cells  
17 (Fig. 5D, E). In contrast, *srs2 cdc20-mn* cells with Rad51 aggregates were  
18 negative for Red1 signal. These indicate that Rad51 aggregate formation in the  
19 *srs2* occurs after or during disassembly of Red1-axes. This is confirmed in the  
20 background of wild type too (Fig. S4B, C).

21 We confirmed this by staining of Rec8, a kleisin subunit of cohesin (Klein  
22 et al. 1999). At late time points such as 6 h, Rec8 showed dotted staining  
23 compared to 5 h (Challa et al. 2019), when most of Rec8 show line staining.  
24 Rec8 line positive spreads contained Rad51 foci (Fig. S4D, E). In *srs2* cells with  
25 or without *cdc20-mn*, Rad51 aggregates are predominantly seen in cells with  
26 Rec8-dots (Fig. S4D, E).

27

### 28 **The *srs2* mutant accumulated bridge staining of Rad51 between two** 29 **recombination foci during early prophase-I**

30 During our staining analysis, we noticed that the *srs2* cells show very unique thin  
31 line staining of Rad51 during early prophase such as 4 h (Fig. 4B). The thin  
32 Rad51-line in *srs2* cells is connected from one Rad51 focus to the other  
33 focus/foci, which we refer to as “Rad51 bridge”. At least one clear Rad51 bridge



1 between two Rad51 foci were observed at ~40% frequency of *srs2* spreads at 4  
2 h (middle graph of Fig. 4C). A few Rad51 bridges were seen in wild type. We  
3 also found the Rad51-bridge staining among more than three Rad51 foci in *srs2*  
4 cells, but not in wild type (right graph of Fig. 4C). Careful examination of Rad51  
5 foci in the wild type often detected a Rad51 focus with “single tail (or whisker)”  
6 (left graph of Figure 4C). The number of Rad51 tail from a single Rad51 focus is  
7 almost one. There is few focus with more than 2 tails. When measured the  
8 length of the bridge between two foci, we found both wild-type and the *srs2* cells  
9 show similar distribution of the lengths (Fig. 4D). These results indicate that *Srs2*  
10 suppresses the formation of Rad51 bridges. Indeed, the *srs2* cells increased the  
11 frequency of the Rad51 bridges and more connections among more than two  
12 foci relative to the wild type (Fig. 4C).

13 We then used super-resolution microscopy to analyze Rad51 localization  
14 on meiotic chromosomes at high resolution. A structural illumination microscope  
15 (SIM) was used to determine Rad51 localization in wild type and *srs2* cells at 4h  
16 (Fig 4E). As shown above, in the *srs2* mutant, we detected both Rad51 bridges  
17 and tails more than in wild type. The wild type the *srs2* mutant shows Rad51 foci  
18 with tail/bridge at a frequency of  $15.4\pm 4.2\%$  ( $n=18$ ) and  $54.3\pm 8.5\%$  ( $n=20$ ),  
19 respectively.

20 The average length of the bridge is  $\sim 0.4 \mu\text{m}$  (Fig. 4D). If the bridge is  
21 postulated to consist of a single Rad51 filament on the ssDNA, which is  
22 extended 2-fold relative to the B-form DNA (Ogawa et al. 1993), we can  
23 calculate the bridge contains  $\sim 600$  nt ( $400 \text{ nm}/2 \times 3.3/10.5$ ). This might be a  
24 range of reasonable estimate ssDNA length at a single DSB site with  $\sim 900$  nt  
25 (Mimitou et al. 2017; Zakharyevich et al. 2010). Rad51 bridge described here  
26 might be similar to the staining of “ultra fine bridge” seen in anaphase of damage  
27 mammalian cells (Chan and Hickson 2011) (see Discussion). The formation of  
28 Rad51 thin bridges in early prophase-I of the *srs2* cells suggests entanglement  
29 of recombination intermediates.

30

31 **Little chromosome breaks are formed in the *srs2* mutant during late**  
32 **prophase-I**

33 In order to detect chromosomal breaks, we tried to analyze chromosome status

1 by pulse field gel electrophoresis (PFGE). At 3, 4 h time points in both wild-type  
2 and *srs2* mutant cells, chromosomal band becomes a smear due to the  
3 introduction of DSBs (Fig. S5). While the smear pattern disappeared at 5 h in  
4 wild type, the *srs2* mutant showed persistent smear bands by 5 h and then  
5 disappeared. Interestingly, we again detected smear bands at 10 and 12 h when  
6 most of the *srs2* diploid makes spores, indicating the formation of DSBs in the  
7 *srs2* spores. The smear bands were barely observed in wild type spore. More  
8 importantly, the *srs2* cells did not show breaks at 5-8 h, when Rad51 aggregates  
9 are induced.

10

## 11 **Discussion**

12

### 13 **Rad51 bridges and Rad51 aggregates in the *srs2* mutant.**

14 The *srs2* $\Delta$  mutant shows decreased levels of CO and NCO relative to the  
15 wild-type, indicating a positive role of Srs2 in meiotic recombination  
16 (pro-recombination role). This weak defect in the recombination is consistent  
17 with delayed DSB repair (delayed disassembly of Rad51 foci) as well as  
18 defective SC formation in the mutant. Our studies also showed that the *srs2*  
19 mutant is partially defective in a step after the DSB processing. However, this  
20 “weak” defect in the recombination cannot explain reduced spore viability of the  
21 mutant, since the mutants with 50% reduction of CO show high spore viability;  
22 e.g. *spo11*, *xrs2*, *msh4/5* hypomorphic mutants (Martini et al. 2006; Nishant et al.  
23 2010; Shima et al. 2005). Consistent with low spore viability of the mutant, we  
24 and others detected abnormal chromosome segregation in *srs2* meiosis,  
25 suggesting the presence of DNA abnormality in the mutant.

26 In this study, we described “unusual” DNA damage formed in the  
27 absence of Srs2 helicase during meiosis. This damage is marked with the  
28 association of the recombination protein, Rad51, with a large quantity, which we  
29 refer to as “Rad51 aggregate”. The Rad51 aggregate is not a protein  
30 aggregate since it contains another recombination protein, Rad52, as well as  
31 RPA, but not meiosis-specific recombination proteins such as Dmc1. The  
32 presence of RPA strongly suggests the presence of ssDNAs. Indeed, thin  
33 line-like staining of Rad51 and RPA emanating from the aggregate are often

1 observed.

2         The formation of Rad51 aggregates in *srs2Δ* mutant requires Spo11  
3 catalytic activity, thus DSB formation. On the other hand, kinetic analysis  
4 revealed that Rad51 aggregates in *srs2Δ* mutant appear in late prophase-I after  
5 the disappearance of Spo11-dependent Rad51 foci associated with meiotic  
6 recombination. Rad51 aggregates appear just after the disappearance of  
7 “normal” Rad51 foci. This suggests that the formation of Rad51 aggregates  
8 occur after the completion of DSB repair such as Rad51-mediated strand  
9 invasion. Consistent with this, the *ndt80* mutation, which induces an arrest at  
10 mid-pachytene stage, blocks the aggregate formation in the *srs2Δ* mutant. The  
11 *ndt80* mutant accumulates dHJ as a product of completion of Rad51-dependent  
12 strand invasion (Allers and Lichten 2001), and also shows persistent formation  
13 of Spo11-dependent meiotic DSBs (Carballo et al. 2013). Therefore, persistent  
14 DSBs and dHJs are unlikely to be directly linked with Rad51 aggregate  
15 formation.

16         Mutant analysis shows the formation of Rad51 aggregates in the *srs2Δ*  
17 requires pachytene-exit, but occurs prior to the transition of metaphase-I to  
18 anaphase-I, chromosome segregation. Indeed, Rad51 aggregate formation  
19 occurs even when chromosome segregation was inhibited by the treatment with  
20 a microtubule polymerization inhibitor and the *CDC20* depletion, which delays  
21 and blocks the onset of anaphase-I. These indicate that the aggregate formation  
22 is induced around the disassembly of meiotic chromosome structure; e.g.  
23 diplotene or diakinesis.

24         One possibility to explain Rad51 aggregate formation in the *srs2* mutant  
25 is that, after the exit of Ndt80-execution point, there might be unrepaired DSBs,  
26 which could be repaired by Rad51-dependent pathway (but not Dmc1-pathway)  
27 during late prophase-I. The *srs2* mutant might be specifically defective in this  
28 DSB repair after the pachytene exit. In this pathway, Srs2 may be essential for  
29 Rad51 removal, which may lead to the accumulation of unrepaired ssDNAs.  
30 However, this is unlikely since even DSB ends formed during pachytene are  
31 bound by Dmc1 as well as Rad51. However, the Rad51 aggregates in the *srs2*  
32 mutant do not contain Dmc1 even when Dmc1 protein is present in a cell.

33         Alternatively, Rad51 aggregates and/or its associated DNA damage

1 are formed in two-step process. First, DSB repair in the absence of Srs2 may  
2 result in the formation of aberrant recombination products/intermediates such as  
3 entangled duplexes DNAs (see Fig. 6B). Second, this aberrant  
4 product/intermediate might be converted into DNA damage with Rad51  
5 aggregates in late prophase-I. Consistent with this two-step model, we found a  
6 novel structure called Rad51-bridge (or whisker), thin lines of Rad51 which  
7 connect Rad51 foci. This bridge is seen at early prophase I of the *srs2* mutant  
8 more frequently than in wild type.

9 The presence of Rad51-bridge and -whisker from Rad51 focus  
10 suggests that Rad51 focus is not a simple Rad51 filament, rather may contain a  
11 three-dimensional configuration of Rad51 filament (Fig. 6A). Rad51-bridge line  
12 staining is reminiscent of anaphase bridge or ultra-fine bridge of chromosomes  
13 in mammalian cells (Chan et al. 2007). The formation of anaphase bridges in  
14 mammalian cells is a two-step process. Although these bridges are formed  
15 during M phase with onset of anaphase, the initiation event leading to the bridge  
16 formation occurs during S-phase. These bridges are induced by the treatment of  
17 the cell with DNA replication inhibitor(s) or in the absence of DNA repair protein  
18 such as BLM helicase. The bridges are decorated with repair proteins such as  
19 BLM and RPA, but not Rad51.

20 Rad51 aggregate formation in *srs2* meiosis is clearly different from the  
21 anaphase bridge in the following two aspects. First, the formation is not required  
22 for chromosome segregation. Second, the initiation event should be  
23 Spo11-dependent DSB formation in early prophase-I (meiotic G<sub>2</sub> phase). If the  
24 two-step model as described above is true, the conversion of the aberrant  
25 product/intermediate into the aggregate in *srs2*Δ mutant should occur after  
26 mid-pachytene exit. Given drastic chromosome morphogenesis such as  
27 chromosome compaction and disassembly of the meiosis-specific chromosome  
28 structure, SC, occur during late meiotic prophase-I (Challa et al. 2019), these  
29 events might induce the aberrant DNA damage with Rad51 aggregates.

30 Given that Rad51-bridge in the *srs2* mutant is formed between Rad51  
31 foci, Srs2 might play a role of this kind of Rad51-associated DNA entanglement  
32 between the two DSB sites (Fig. 6B). One likely intermediate is multiple invasion  
33 (Piazza et al. 2017), which are formed by Rad51-mediated strand invasion into

1 multiple loci. Therefore, Srs2 might play a role in resolution of multiple-invasion  
2 by controlling Rad51 filament dynamics using its Rad51-dismantling activity.

3 Bishop and his colleagues show a pair of Rad51 foci during early  
4 meiotic pro-phase I are formed in the two ends of a single DSB site (Brown et al.  
5 2015). Thus, it is likely that the Rad51 bridge we observed is formed between a  
6 pair of Rad51 foci on the two DSB ends. If so, one likely possibility is that the  
7 bridge is a ssDNA between two DSB ends. One way to connect the two DSB  
8 ends is bridged by the annealing of ejected ssDNA from the D-loop after the  
9 DNA synthesis (Fig. 6B). Since the bridge is mainly seen in the absence of Srs2,  
10 we propose that Rad51 dismantling activity of Srs2 promotes the removal of  
11 Rad51 from the rejected ssDNA. Moreover, it is likely that Srs2 also remove  
12 Rad51 in the other end of the DSB during the second end-capture. This idea  
13 could explain the formation of the bridge between two Rad51 foci in the *srs2*, but  
14 not in wild type. In wild-type cells, Srs2 seems to remove Rad51 assembly from  
15 the intermediates for the second end capture. Importantly, genetic analysis of  
16 mitotic recombination in the *srs2* mutant suggest the role of Srs2 to facilitate the  
17 annealing of the newly synthesized strand to second resected ends by removing  
18 Rad51 from the second end (Elango et al. 2017; Ira et al. 2003; Liu et al. 2017;  
19 Mitchel et al. 2013).

20 We still cannot figure out recombination products formed in the  
21 absence of Srs2, which trigger the formation of the Rad51 aggregate. 2D gel  
22 analysis has shown that there is few accumulation of abnormal recombination  
23 intermediate such as multiple dHJs (Lichten/Goldman, accompanying paper).  
24 Thus, multiple dHJs is unlikely. Rather, there might be an entanglement of DNA  
25 strands after the completion of the meiotic recombination (Fig. 6B). This  
26 intermediate seems to be related to a lethal recombination intermediate formed  
27 in the *srs2* mutant during mitosis.

28 In either scenario, our analysis reveals a novel pathway to protect  
29 meiotic cells in late prophase-I from the formation of aberrant DNA damage  
30 induced by Spo11. This repair pathway heavily depends on Srs2 function. For  
31 this function, Srs2 is almost essential for meiosis. We would like to point out that  
32 Rad51 aggregates in the *srs2* mutant is related to lethal recombination  
33 intermediates in mitotic cells with *SRS2* deletion, which is postulated to form

1 through two-step model.

2 Rad51 aggregate-associated DNA damage seems unrepaired during  
3 meiosis. During late prophase-I, there should be sister chromatid or other  
4 recombination partners to repair the damage, this might be due to the presence  
5 of Rad51-inhibitor Hed1, which clearly suppresses Rad51-mediated DNA repair.

6 The result that CHEF-Southern for chromosome III did not detect any  
7 DNA fragmentation at times of Rad51-aggregates in the *srs2Δ* mutant; e.g. 6  
8 and 8 h, implies Rad51 aggregate-associated DNA damage does not contain  
9 DSBs. One simple interpretation is that Rad51 aggregates are on either the  
10 ssDNA gaps or unwound duplex DNAs.

11

## 12 **No activation of DNA damage checkpoint during late prophase I, meiosis I** 13 **and meiosis II**

14 In the absence of Srs2, DNA damage with Rad51 aggregates is formed and  
15 passed into MI and MII with activation of DNA damage checkpoint, which leads  
16 more catastrophic events such as chromosome fragmentation with DSB  
17 formation in spores. This may explain quite a big reduction of spore viability of  
18 the *srs2* diploid with reasonable levels of meiotic recombination.

19 The absence of DNA damage-induced delay in late meiosis-I in the *srs2*  
20 cells is quite different from cell cycle delay induced by the recombination  
21 (pachytene) checkpoint during early prophase-I (MacQueen and Hochwagen  
22 2011; Tsubouchi et al. 2018). In the recombination checkpoint, DSBs and  
23 associated ssDNA activate sensor kinases Tel1(ATM) and Mec1(ATR),  
24 respectively. During meiosis, activated Mec1 and Tel1 induce the activation of a  
25 meiosis-specific kinase, Mek1, Rad53 homolog, by phosphorylating its partner  
26 protein Hop1. High Mek1 activity down-regulates Ndt80 activity, thus, blocking  
27 the exit of mid-pachytene stage. During meiosis, the activation of mitotic DNA  
28 damage downstream kinases, Rad53 and Chk1, is blocked through an unknown  
29 mechanism. At late times in the *srs2* mutant, we did not see prolonged  
30 phosphorylation of Hop1, thus little activation of Mec1 (and Tel1). This strongly  
31 suggests that DNA damage with Rad51 aggregates in the *srs2* is masked by the  
32 checkpoint activation or there is no such mechanism in late G2 phase of meiotic  
33 cells. Alternatively, although not exclusive with the above, Srs2 may function in

1 the activation of the checkpoint during this phase.

2

### 3 **Role of Rad54 in late recombination**

4 Upon Rad54 depletion after the assembly of Rad51 on the ssDNA during  
5 meiosis, we found a novel staining of Rad51 called Rad51 clump, which is  
6 different from typical Rad51 foci in wild-type and aggregates in the *srs2* cells.  
7 The presence of Rad51 clumps supports the idea of a role of Rad54 after the  
8 assembly of Rad51 filaments. Moreover, the Rad54-Rad51, not with Dmc1, may  
9 function in the repair of DSBs in late prophase-I. Previous cytological analysis of  
10 the *rad54* deletion does not show the formation of Rad51 clump (Shinohara et al.  
11 2000; Shinohara et al. 1997b). One possibility is that Rad54 depletion may  
12 remove Rad54-associated proteins also from the nuclei. As a result, we do see  
13 clear defect in Rad51 dynamics upon Rad54 depletion in late prophase-I.

14

15 In the accompanying paper, Goldman and his colleagues described the  
16 formation of Rad51 aggregates during *srs2* meiosis.

17

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9 H.S.M.S., Y.F., and L.P. performed all experiments. M.S. provided reagents.  
10 H.S., H.S.M.S., and A.S. analyzed the data. A.S. prepared manuscripts with help  
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30  
31

1 **Figure legends**

2

3 **Figure 1. The *srs2* deletion shows Rad51 aggregates during late**  
4 **prophase-I**

5 A. Meiosis I was analyzed by DAPI staining of the wild type (open circles;  
6 NKY1303/1543) and *srs2* (filled circles; HSY310/315) cells. The number of  
7 DAPI bodies per nucleus was counted in a minimum of 150 DAPI positive  
8 cells at each time point.

9 B. DAPI image of wild-type and *srs2* cells at 12 h.

10 C. Immunostaining analysis of Rad51 (green) and Dmc1 (red) on chromosome  
11 spreads in wild type (NKY1303/1543) and *srs2* (HSY310/315) cells.  
12 Representative images with or without DAPI (blue) dye at 4, 6 and 8 h for wild  
13 type and the *srs2* are shown.

14 D. Kinetics of Rad51 focus-positive cells in various yeast strains. A spread with  
15 the Rad51 foci is defined as a cell with more than five foci. Spreads containing  
16 Rad51 aggregates were also counted. A minimum of 100 cells were analyzed  
17 at each time point. Graphs show kinetics of one representative experiment for  
18 the wild type cells (top; NKY1303/1543), and *srs2* cells (bottom; HSY310/315).  
19 Circles and triangles show spreads with Rad51 foci and aggregate,  
20 respectively.

21 E.F. Immunostaining analysis of a component of RPA, Rfa2 (green) with Rad51  
22 (red) in wild type (NKY1303/1543; top) and *srs2* (HSY310/315; bottom) cells  
23 at 4 h (E) and at 8 h (F). In F, a dashed square is enlarged in the right.

24 G. Kinetics of Rfa2 foci-positive cells in wild type (NKY1303/1543; top) and *srs2*  
25 (HSY310/315; bottom) cells as shown in (B). Circles and triangles show  
26 spreads with Rfa2 foci and aggregates, respectively.

27

28 **Figure 2. Rad51 aggregates in the *srs2* deletion pass through Meiosis I**  
29 **and II.**

30 A, B. Whole cell immunostaining analysis of Rad51 (green) and Dmc1 (red) in  
31 wild type (NKY1303/1543) and *srs2* (HSY310/315) cells at 4 and 8 h in  
32 meiosis. In (B), cells with Rad51 in prophase-I (top), after MI (middle), and  
33 after MII (bottom) are shown.



- 1 C. Kinetics of Rad51 foci-positive cells in wild-type and *srs2* cells. A foci-positive  
2 cell is defined as a cell with more than five foci (closed circles, wild type,  
3 NKY1303/1543; open circles, *srs2* cells, HSY310/315). A minimum of 100  
4 cells were analysed at each time point. Graphs show the mean values with  
5 S.D. from three independent experiments.
- 6 D. Kinetics of *srs2* cells containing Rad51 aggregates prior to MI (blue; one  
7 DAPI body in a cell) after MI (green; two DAPI bodies in a cell) and MII (red;  
8 three or more DAPI bodies in a cell) are shown. Mean values with S.D. from  
9 three independent experiments are shown.
- 10 E. Western blotting analysis of Hop1 phosphorylation during meiosis. Cell  
11 lysates at different time points in meiosis in wild type (NKY1303/1543) and  
12 *srs2* (HSY310/315) cells were probed with anti-Hop and anti-tubulin  
13 antibodies. Phosphorylated Hop1 (shown as “Hop1-P”) shows slower  
14 mobility relative to un-phosphorylated Hop1.

15

16 **Figure 3. Rad54-depletion induces abnormal Rad51 assembly.**

- 17 A. Mitotic plate assay to confirm synthetic lethality of *srs2* *RAD54*-anchor on  
18 YPAD plates containing 1  $\mu$ M rapamycin.
- 19 B. Spore viability of *RAD54-RFB* (H7790/7791) and *RAD54-RFB srs2*  
20 (HYS71/82) cells in the absence (-) and the presence (+) of rapamycin.
- 21 C. Kinetics of MI entry in *RAD54-RFB* (H7790/7791) and *RAD54-RFB srs2*  
22 (HYS71/82) cells in the absence (-) and the presence (+) of rapamycin.  
23 Rapamycin was added at 4 h at a concentration of 1  $\mu$ M. Graphs show the  
24 mean values with S.D. from three independent experiments.
- 25 D. Immunostaining analysis of Rad51 (green) and Dmc1 (red) on chromosome  
26 spreads in *RAD54-RFB* (H7790/7791) and *RAD54-RFB srs2* (HYS71/82)  
27 cells in the absence and the presence of rapamycin. Rapamycin (1  $\mu$ M) was  
28 added at 4 h in meiosis. Representative images of Rad51 staining with or  
29 without the addition of rapamycin are shown.
- 30 E. Kinetics of Rad51 foci-positive cells in *RAD54-RFB* (top green graph;  
31 H7790/7791) and *RAD54-RFB srs2* (bottom red graph; HYS71/82) cells in  
32 the absence (-) or the presence (+) of rapamycin. Rad51 foci (circles),  
33 -clumps (square), and -aggregates (triangles); open symbols (without

1 rapamycin) and closed symbols (addition of the rapamycin at 4 h).

2

3 **Figure 4. Rad51 aggregate forms in the absence of chromosome**  
4 **segregation.**

5 A. Immuno-staining analysis of Rad51 in *ndt80* (HSY596/597) and *srs2 ndt80*  
6 (LPY058/059) cells.

7 B. Rad51-bridges in *srs2* cells at 4h. Rad51 tail and bridge are shown in  
8 arrowheads and arrows, respectively.

9 C. Rad51-tail or bridge is classified into three classes; Rad51 focus with tail  
10 (left), Rad51 bridge between two foci (middle), Rad51 bridge among three or  
11 more foci (right). On each spread, the number of each class per a spread  
12 was counted, and then a count of the spreads in each class is shown. 42  
13 spreads of wild-type (NKY1303/1543) and *srs2* (HSY310/315) cells were  
14 analyzed and counted.

15 D. The length of the Rad51 bridge between two Rad51 foci was measured and  
16 plotted. Three horizontal lines from the top indicate the 75, 50 (median), and  
17 25 percentiles, respectively.  $P=0.39$ ; Mann-Whitney  $U$  test.

18 E. SR-SIM microscopic observation of Rad51 (green) in wild-type  
19 (NKY1303/1543) and *srs2* (HSY310/315) cells. Representative image DAPI  
20 (blue; left) dye and Rad51 (green, middle) is shown. White insets in middle  
21 images are shown in a magnified view at right. The bar indicates 2  $\mu\text{m}$ .

22

23 **Figure 5. Rad51 aggregate forms in the absence of chromosome**  
24 **segregation.**

25 A. Immunostaining analysis of Rad51 (green) in wild type (NKY1303/1543) and  
26 *srs2* (HSY310/315) cells in the presence of benomyl. The benomyl was  
27 added at 4 h at a concentration of 120  $\mu\text{g/ml}$ .

28 B. Kinetics of MI entry in wild type (green) and *srs2* (red) cells in the absence  
29 (open symbols) or the presence (closed symbols) of benomyl.

30 C. Kinetics of Rad51 foci and Rad51 aggregates in wild type (top, green) and  
31 *srs2* (bottom, red) cells in the absence (open) or the presence (closed) of  
32 benomyl. Circles, Rad51 foci; triangles, Rad51 aggregates.

33 D. Immuno-staining analysis of Rad51 and Red1 in *CDC20-mn* (YFY74/77) and

1 *srs2 CDC20-mn* (YFY80/83) cells. The chromosome spreads at 5 and 7 h  
2 were immuno-stained against Rad51 (green) as well as chromosome protein  
3 Red1 (red).

4 E. Kinetics of Rad51 aggregate-positive cells in Red1-positive and -negative  
5 spreads. Rad51-focus and Rad51-aggregate positive spreads were  
6 classified into Red1-negative (open bars) and Red1-positive (closed bars) at  
7 each time point. At each time point, more than 50 spreads were counted.

8

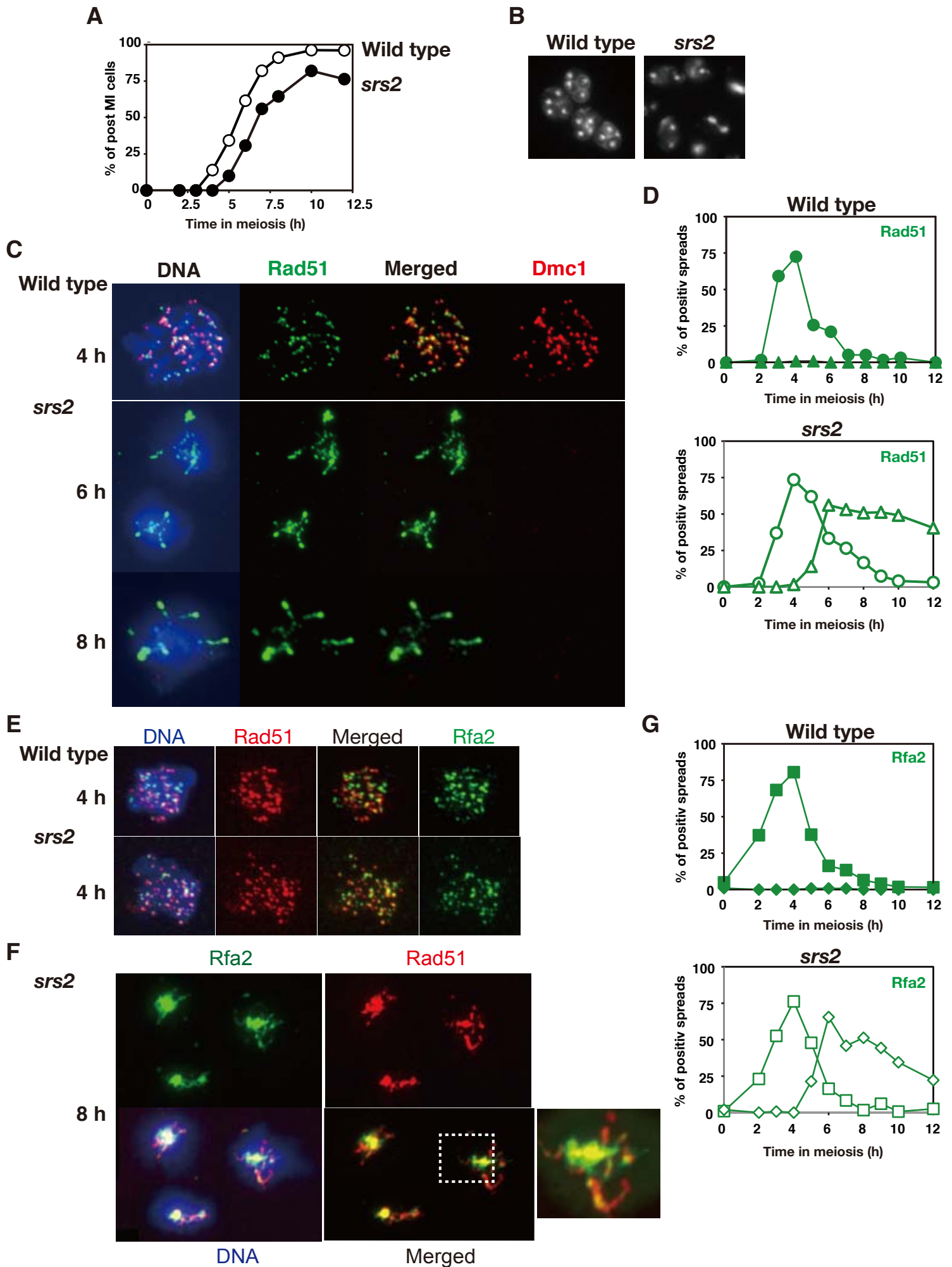
9 **Figure 6. A schematic model of Rad51 bridge and Rad51 aggregate**  
10 **formation in wild type and *srs2* cells.**

11 A. A model of Rad51 focus comprised of the Rad51 filament. Rad51 filament is  
12 accommodated into a three-dimensional structure.

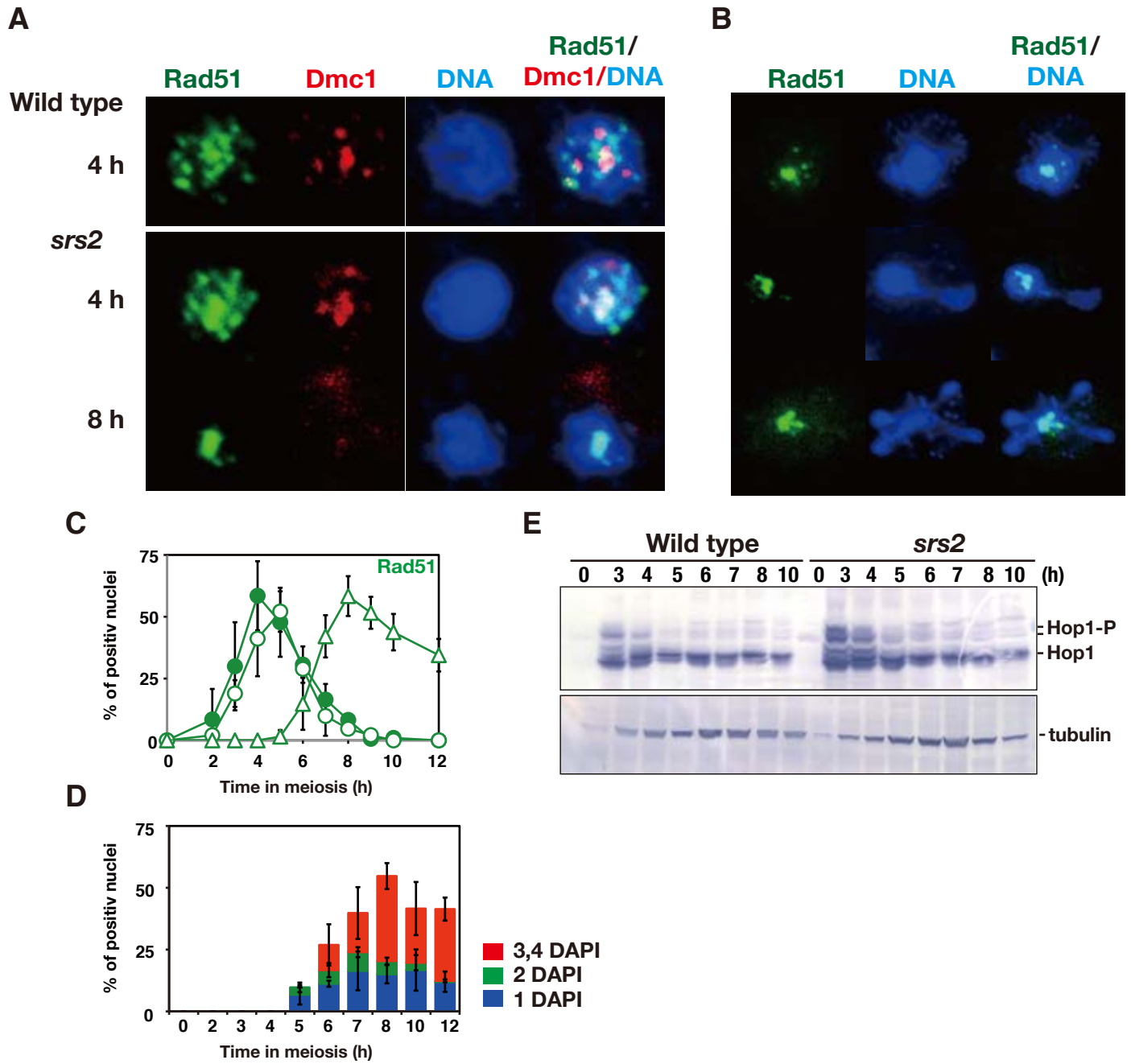
13 B. A recombination pathway with Rad51 focus and filaments is described. In left,  
14 the second end capture by a displaced strand from D-loop, which is mediated  
15 by the Rad51 filament may trigger the disassembly of Rad51 filament (focus)  
16 on the second end, which is promoted by Srs2. Srs2 also prevents the  
17 formation of a multi-invasion intermediate. These abnormal recombination  
18 intermediates are processed into a final product, which may contain a  
19 pathological damage that may lead to the formation of a long ssDNAs with  
20 Rad51 aggregates during late prophase-I.

21

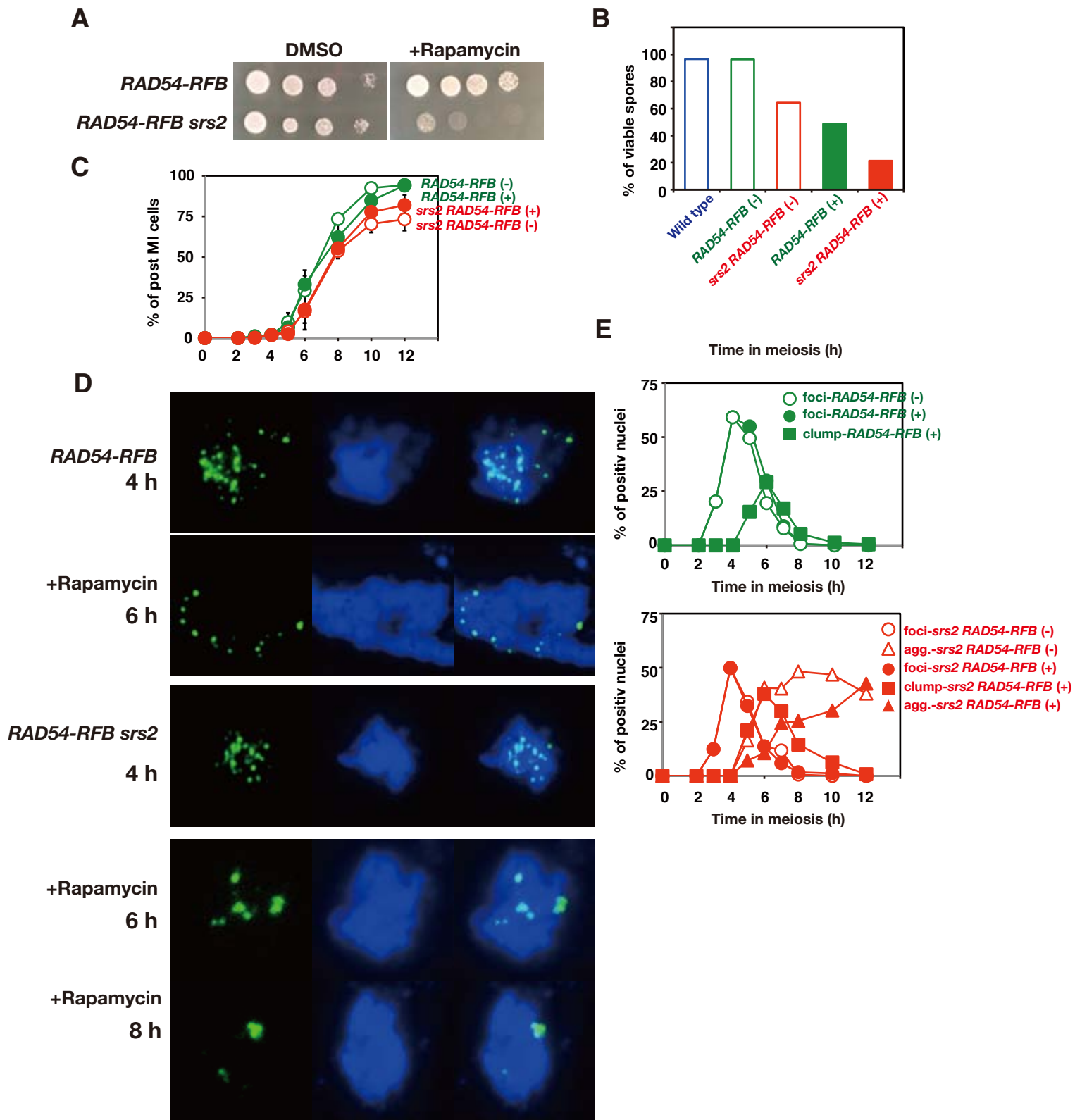
# Figure 1. Sasanuma/Sakurai



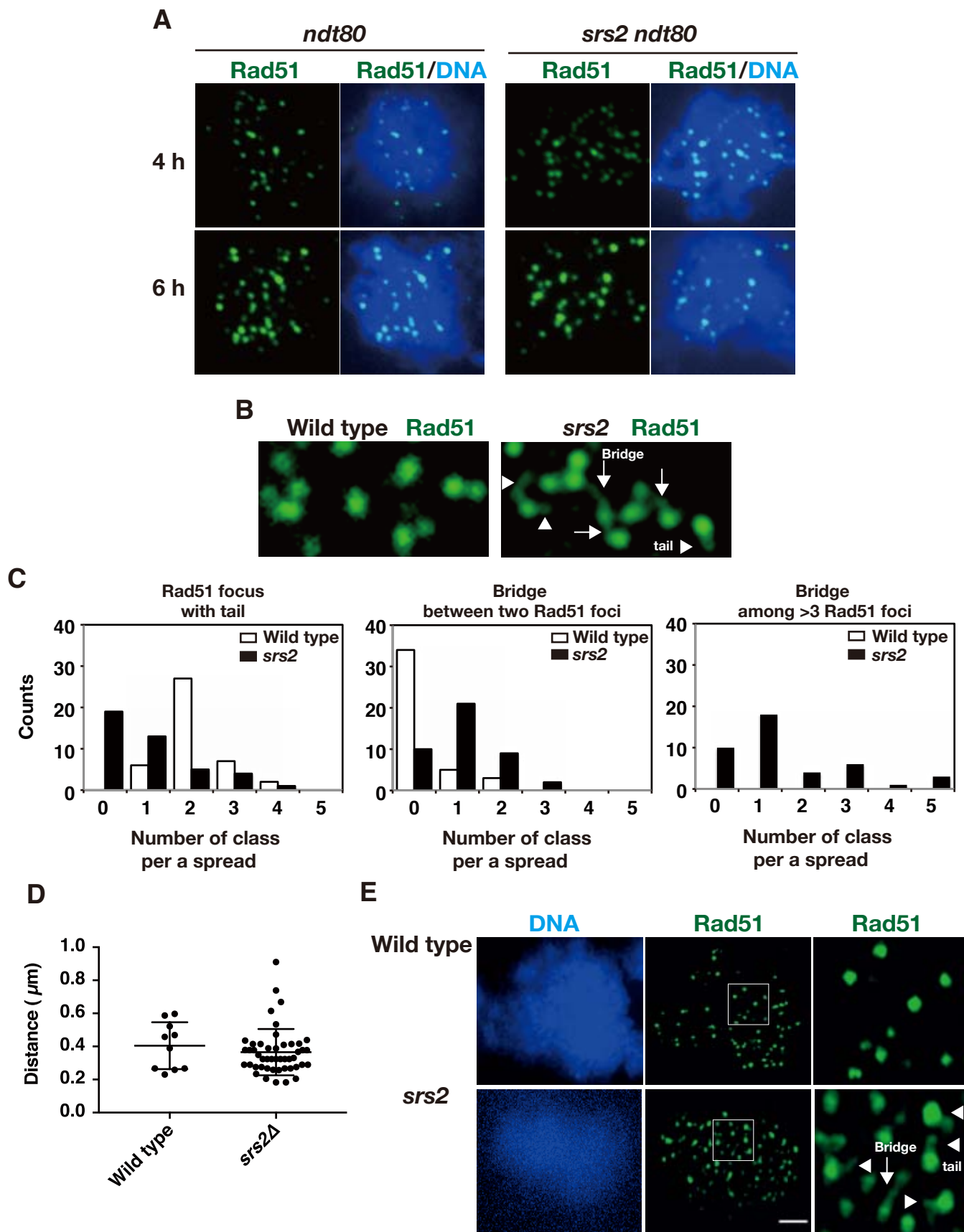
## Figure 2. Sasanuma/Sakurai



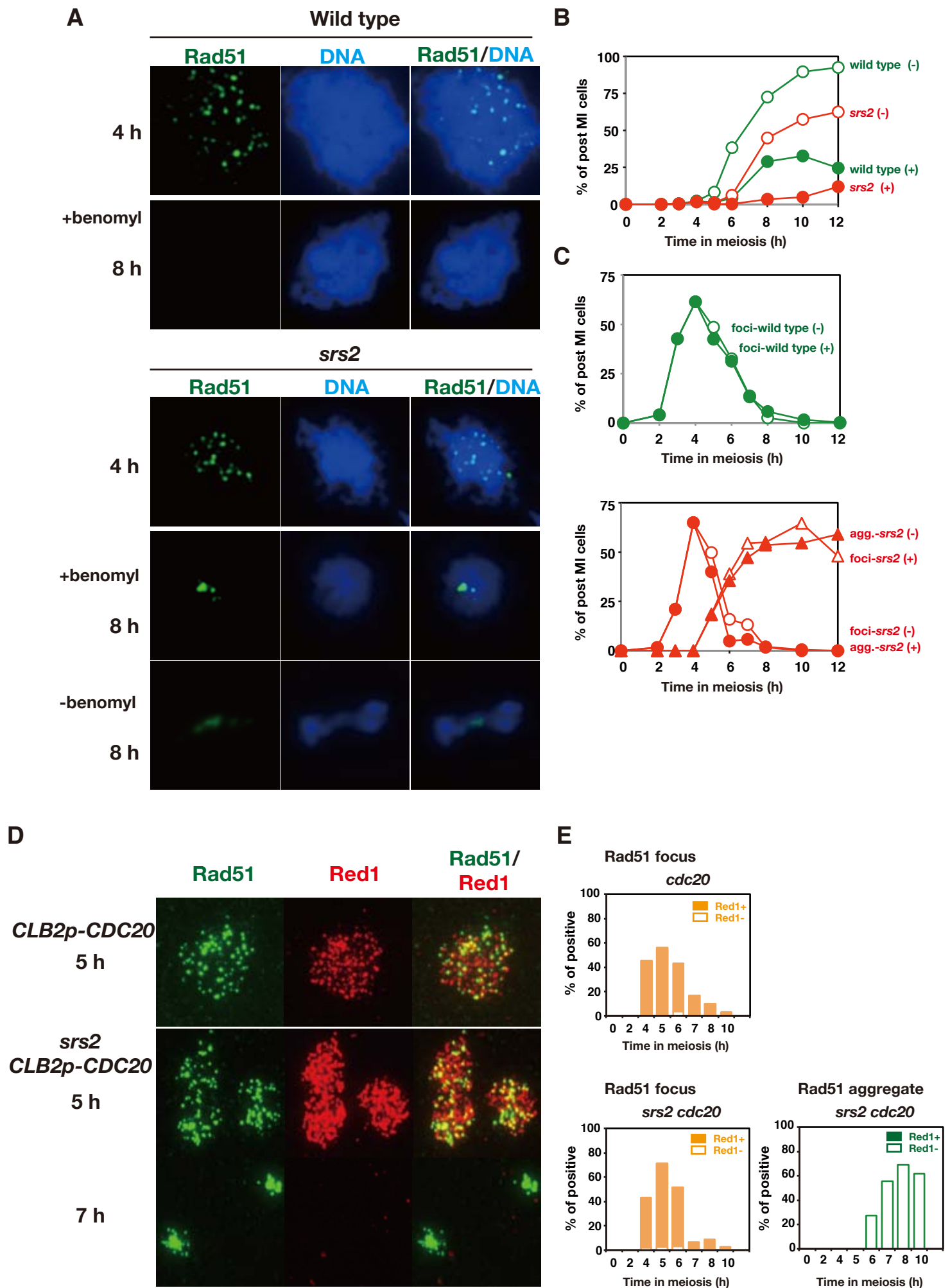
### Figure 3. Sasanuma/Sakurai



## Figure 4. Sasanuma/Sakurai



## Figure 5. Sasanuma/Sakurai





## Figure 6. Sasanuma/Sakurai

