Homologous Recombination via Synthesis-Dependent Strand Annealing in Yeast Requires the Irc20 and Srs2 DNA Helicases

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ABSTRACT Synthesis-dependent strand-annealing (SDSA)-mediated homologous recombination replaces the sequence around a DNA double-strand break (DSB) with a copy of a homologous DNA template, while maintaining the original configuration of the flanking regions. In somatic cells at the 4n stage, Holliday-junction-mediated homologous recombination and nonhomologous end joining (NHEJ) cause crossovers (CO) between homologous chromosomes and deletions, respectively, resulting in loss of heterozygosity (LOH) upon cell division. However, the SDSA pathway prevents DSB-induced LOH. We developed a novel yeast DSB-repair assay with two discontinuous templates, set on different chromosomes, to determine the genetic requirements for somatic SDSA and precise end joining. At first we used our *in vivo* assay to verify that the *Srs2* helicase promotes SDSA and prevents imprecise end joining. Genetic analyses indicated that a new DNA/RNA helicase gene, *Irc20*, is in the SDSA pathway involving *SRS2*. An *irc20* knockout inhibited both SDSA and CO and suppressed the *srs2* knockout-induced crossover enhancement, the *mre11* knockout-induced inhibition of SDSA, CO, and NHEJ, and the *mre11*-induced hypersensitivities to DNA scissions. We propose that *Irc20* and *Mre11* functionally interact in the early steps of DSB repair and that *Srs2* acts on the D-loops to lead to SDSA and to prevent crossover.

DDOUBLE-strand DNA breaks (DSBs) are generated by exposure to ionizing radiation or chemical compounds, such as topoisomerase inhibitors and apoptosis inducers, reactive oxygen species (ROS) generated as by-products of oxidative respiration, transposition of transposable elements, and by the actions of restriction enzymes or meiosis-specific endonucleases, such as the *Spo11* complex and intron-homing nucleases. DSBs are repaired through various recombination-dependent pathways (Figure 1), and recombination deficiencies cause genome instability and carcinogenesis.

Holliday-junction-mediated homologous recombination (Figure 1A) is a type of homologous recombination that produces either crossover or noncrossover products. These products are generated on the basis of the resolution of the double-Holliday

structure (Resnick and Martin 1976; Szostak *et al.* 1983). It was proposed that the noncrossover products (B5) are generated from a double-Holliday structure (A4), with reverse migration of the Holliday junctions without cleavage (Wu and Hickson 2003).

Synthesis-dependent strand-annealing (SDSA)-mediated homologous recombination (Figure 1B) is the other type of homologous recombination, which produces only noncrossover products that are not associated with a flanking crossover. In this pathway, a D-loop, formed with a single-strand tail from the terminus of a DSB (B2), migrates with associated DNA synthesis primed at the 3' termini of the invading single-strand tail, but without forming Holliday junctions. These reactions are followed by the annealing of the newly synthesized strands dissociated from the template DNA with the other termini of the DSB (Nassif *et al.* 1994). This pathway was postulated on the basis of homologous recombination studies in phage T4 (Mueller *et al.* 1996), *Saccharomyces cerevisiae* (McGill *et al.* 1989; Nelson *et al.* 1996; Pâques *et al.* 1998), *Ustilago maydis* (Ferguson and Holloman 1996), and *Drosophila melanogaster* (Nassif *et al.* 1994; Adams *et al.* 2003).

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The two homologous recombination pathways described above share common steps up to the D-loop formation of a single-strand tail, from a DSB with a homologous double-strand DNA as the template for DSB repair.

Nonhomologous end joining (NHEJ) (Figure 1C) religates DSB termini lacking extensive homologous sequences and generates imprecise or precise end-joining products, according to the features of the DSB termini. This pathway mainly depends on Ku70 (Boulton and Jackson 1996b), Ku80 (Boulton and Jackson 1996a), and Lig4 (Schär *et al.* 1997; Ramos *et al.* 1998). The alternative NHEJ pathway is independent of Ku and Lig4, but depends on Rad52 (Hegde and Klein 2000).

When a DSB is generated between the centromere and a gene in somatic cells heterozygous for the gene, the Holliday-junction-mediated homologous recombination at S/G2 (4n) and the imprecise end joining at G1/S (2n) or S/G2 (4n) frequently produce a crossover between homologous chromosomes and long deletions, respectively, and then cause loss-of-heterozygosity (LOH) of the alleles upon cell division. In contrast, the SDSA pathway leads only to noncrossover products that are free of deletions around the DSB sites and thus prevents DSB-induced LOH in somatic cells. LOH is a major cause of carcinogenesis (Bignon 2004). Thus, it is important to study the SDSA-mediated homologous recombination pathway to clarify the underlying mechanisms that maintain heterozygosity and genome stability.

Various mitotic or meiotic genetic events, which can be explained by the SDSA model, have been reported from yeast studies. For instance, most of the quite frequent expansions and contractions in a tandem array of 375-bp units, which were induced by a DSB, were not associated with crossovers (Pâques *et al.* 1998). In another SDSA-based mechanism, new sequences were created by template switching between *Kluyveromyces lactis* URA3 and *S. cerevisiae* URA3 and were mostly independent of mismatch repair (Hicks *et al.* 2010). His⁺ and Leu⁺ markers placed on opposite sites of a DSB site were converted, without the conversion of an intervening heterologous insertion surrounded by a few hundred-base duplication on the donor chromatid. This was explained by the annealing of two strands newly synthesized on the few hundred-base duplication, resulting in the loss of the intervening marker (McMahill *et al.* 2007). In many of the meiotic recombination events initiated at the HIS4 recombination hotspot, the heteroduplex formation occurs on only one side of the DSB site and then often produces noncrossovers (Merker *et al.* 2003).

These genetic observations support the presence of the SDSA mechanism in vegetative and meiotic yeast cells. The presence of SDSA in yeast was demonstrated by a triparental system, in which the gapped *leu2* plasmid and two donor templates on two different chromosomes were used (Pâques *et al.* 1998). However, the genetic requirements for the SDSA pathway, and especially the DNA helicases that are likely to determine the SDSA pathway (Figure 1B) or the Holliday junction-mediated pathway (Figure 1A), remain to

be identified. *SRS2* encodes a DNA helicase (Rong and Klein 1993). Mutants with defective *srs2* show a hyperrecombination phenotype (Aguilera and Klein 1988) and increased mitotic crossovers, which rarely occur in the wild-type cells (Ira *et al.* 2003). These results suggested that the *Srs2* helicase functions negatively in somatic homologous recombination. The negative function is explained by the observation that *Srs2* disrupted *in vitro* Rad51 filaments formed on single-strand DNA and inhibited the DNA strand exchange mediated by Rad51 (Veaute 2003). A synthetic D-loop, formed from double-strand DNA with a homologous 3'-single-strand tailed double-strand DNA was efficiently dissociated by the *Srs2* DNA helicase (Dupaigne *et al.* 2008). This observation favors a model in which *Srs2* promotes the migration of a D-loop, accompanied with DNA synthesis initiated at the 3' terminus of the invading single-strand tail in the SDSA pathway (Dupaigne *et al.* 2008) (Figure 1, B3). Thus, it is critical to elucidate whether the *Srs2* helicase inhibits D-loop formation or acts after the D-loop formation, to determine whether the SDSA pathway or the Holliday junction-mediated pathway is utilized in somatic cells.

In this study, we developed a new yeast genetic assay involving a triparental system, consisting of the gapped *ura3* plasmid and two donor templates on two different chromosomes, to determine the genetic requirements either for SDSA-mediated noncrossover, as distinguished from those for noncrossover through a double-Holliday intermediate, or for the NHEJ pathway. Using this assay, we showed that the elimination of the *Srs2* helicase decreased SDSA production, but increased imprecise end joining. We then tested *Irc20*, which is a new putative DNA/RNA helicase classified into the *Srs2* subgroup by a phylogenetic analysis. On the basis of our studies on the effects of the inactivation of *Srs2* and *Irc20* within various genetic backgrounds, we discuss the roles of these two DNA helicases in SDSA-mediated homologous recombination.

Materials and Methods

Yeast strains and media

The *S. cerevisiae* strains used in this study are listed in Table 1. YPD (1% yeast extract, 2% peptone, and 2% glucose) was used as the complete medium, and SD (2% glucose or 2% galactose, 0.67% Bacto-yeast nitrogen base, and appropriate supplements) was used to select cells carrying selective markers. Yeast transformation was performed using a lithium acetate-based protocol (Gietz *et al.* 1995) for the construction of knockout strains, the SDSA/NHEJ assay, and the targeted integration (crossover) assay.

Strain construction

All of the knockout alleles used in this study were constructed by the one-step gene-disruption method (Rothstein 1983). For the preparation of knockout DNA fragments, we used the strategy of short flanking homology PCR (Capozzo *et al.* 2000). The primers used for the knockouts are listed in Supporting

Table 1 Description of *S. cerevisiae* strains used

Strain	Genotype	Source or PCR primers ^a used for strain constructions
W303α	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5</i>	Thomas and Rothstein (1989)
KTTY146	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 rad52Δ::KAN</i>	PRI301/PRI302 for <i>rad52Δ::KAN</i>
KTTY181	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 srs2Δ::TRP1</i>	PRI314/PRI315 for <i>srs2Δ::TRP1</i>
KITTM48	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 irc20Δ::HYG</i>	PRI726/PRI727 for <i>irc20Δ::HYG</i>
KITTM50	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 srs2Δ::TRP1 irc20Δ::HYG</i>	PRI726/PRI727 for <i>irc20Δ::HYG</i>
KTTY106	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 mre11Δ::KAN</i>	PRI243/PRI244 for <i>mre11Δ::KAN</i>
KITTM121	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 mre11Δ::TRP</i>	PRI449/PRI450 for <i>mre11Δ::TRP</i>
KITTM84	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 irc20Δ::HYG mre11Δ::KAN</i>	PRI243/PRI244 for <i>mre11Δ::KAN</i>
KITTM125	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 irc20Δ::HYG mre11Δ::TRP</i>	PRI449/PRI450 for <i>mre11Δ::TRP</i>
KTTY97	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 exo1Δ::KAN</i>	PRI155/PRI156 for <i>exo1Δ::KAN</i>
KITTM71	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 exo1Δ::KAN irc20Δ::HYG</i>	PRI726/PRI727 for <i>irc20Δ::HYG</i>
KITTM111	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 exo1Δ::KAN mre11Δ::TRP</i>	PRI449/PRI450 for <i>mre11Δ::TRP</i>
KITTM99	<i>MATα leu2-3, 112 trp1-1 ura3-1 his3-11, 15 ade2-1 can1-100 RAD5 exo1Δ::KAN irc20Δ::HYG mre11Δ::TRP</i>	PRI449/PRI450 for <i>mre11Δ::TRP</i>
KTTY84	<i>MATα leu2-3, 112 trp1-1 URA3 his3-11, 15 ade2-1 can1-100 RAD5</i>	Materials & Methods
KTTY86	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI245/PRI249 for <i>5'Δ::(<i>HIS3</i>)-<i>ura3</i></i>
KTTY90	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI231/PRI23 for <i>ura3-3'Δ</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1</i>	
KTTY120	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI175/PRI176 for <i>lig4Δ::KAN</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>lig4Δ::KAN</i></i>	
KTTY142	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI301/PRI302 for <i>rad52Δ::KAN</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>rad52Δ::KAN</i></i>	
KTTY176	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI314/PRI315 for <i>srs2Δ::TRP1</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>srs2Δ::TRP1</i></i>	
KITTM36	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI301/PRI302 for <i>rad52Δ::HYG</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>lig4Δ::KAN rad52Δ::HYG</i></i>	
KITTM38	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI726/PRI727 for <i>irc20Δ::HYG</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>irc20Δ::HYG</i></i>	
KITTM51	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI726/PRI727 for <i>irc20Δ::HYG</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>srs2Δ::TRP1 irc20Δ::HYG</i></i>	
KITTM95	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI243/PRI244 for <i>mre11Δ::KAN</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>mre11Δ::KAN</i></i>	
KITTM97	<i>MATα leu2-3, 112 trp1-1 5'Δ::(<i>HIS3</i>)-<i>ura3</i> his3-11, 15 ade2-1 can1-100 RAD5</i>	PRI243/PRI244 for <i>mre11Δ::KAN</i>
	<i>AUR1-C(pUCori-Amp^R-<i>ura3-3'Δ</i>)-AUR1 <i>irc20Δ::HYG mre11Δ::KAN</i></i>	

^a Sequences of primers are shown in Table S2.

Information, Table S2. The knockout fragment with the kanamycin-resistant marker was created by PCR amplification of the KanMX4 module, using a primer pair containing a 5' portion homologous to the gene to be disrupted and a 3' portion homologous to the multiple cloning sites (MCS) of the plasmid pFA6a-KanMX4, which was introduced into competent cells. YPD plates with 200 μg/ml of G418 (Invitrogen, CA) were used to select the clones bearing *geneΔ::KAN*. The knockout fragment with a hygromycin-resistant marker was created by PCR amplification, using the primer pair containing the 5' portion homologous to the gene to be disrupted and the 3' portion homologous to the MCS of the plasmid pAG34-HYG, which was introduced into competent cells. YPD plates with 200 μg/ml of hygromycin B (Invitrogen, CA) were used to select the clones bearing *geneΔ::HYG*. The knockout fragment with the *TRP1* marker was created by PCR amplification, using the primer pair containing the 5'

portion homologous to the gene to be disrupted and the 3' portion homologous to the *TRP1* gene derived from the plasmid pAS2-1, and was used for transformation into Trp⁺ clones, which carried *geneΔ::TRP1*.

SDSA/NHEJ assay

5'Δ-ura3 (one template allele, residing at the *ura3* locus on chromosome V; Figure 2A): At first, the *ura3-1* allele of the W303 strain was reverted to Ura⁺, by transformation with the *URA3* fragment encompassing base pairs 2734 to 3841 in the sequence of the pYES2 plasmid, to construct the strain KTTY84. To construct the strain with a deletion at the 5' end of this *URA3* locus, two primers, PRI245 (with the homology arm for the upstream region of the *URA3* locus) and PRI249 (with the homology arm for the internal region of the *URA3* gene), were designed to create a deletion from the promoter to the 39th codon and to introduce the *HIS3* fragment

derived from the T334 strain. The fragment of 5'Δ-*ura3* with the *HIS3* marker was used to transform the *URA3* locus of KTY84 into 5'Δ(*:HIS3*)-*ura3*, to construct the strain KTY86. This structure was confirmed by PCR with the PRI240 (downstream of the *URA3* locus) and PRI245 (upstream of the *URA3* locus) primers, which produced the 1848-bp fragment.

ura3-3'Δ (the other template allele, integrated into the *AUR1* locus on chromosome XI; Figure 2A): To construct the strain with the ectopic *ura3* allele in which the 3' end was deleted, the region from the promoter to the 139th codon of the *URA3* gene, derived from the pYES2 plasmid, was amplified with two primers, PRI231 (with a *KpnI* site tag) and PRI232 (with an *XbaI* site tag). The 684-bp fragment of *ura3-3'Δ* was ligated into the *KpnI* and *XbaI* sites of the MCS of the pAUR101 plasmid, which was used for targeted integration. The *StuI*-cleaved pAUR101::*ura3-3'Δ* plasmid was introduced into the *AUR1* locus to construct the strain KTY90, with *AUR1-ura3-3'Δ-Amp^R-pUCori-AUR1C*. This structure was confirmed by PCR with the primer pair PRI231 and PRI232, which produced the 684-bp fragment of *ura3-3'Δ*, and with another primer pair, PRI766 (at the 5' end of *AUR1* gene) and PRI769 (at the multiple cloning site of pAUR101), which produced the 3613-bp fragment including the *AUR1-C* segment.

ura3-intΔiscl (the recipient allele with the internal deletion of the *URA3* gene, sealed with an *I-SceI* site, residing within the plasmid with the *LEU2* marker; Figure 2A): The pAUR101 plasmid was first cleaved with the *HindIII* restriction enzyme and was self-ligated, to create the 3162-bp plasmid pUCori-Amp^R-MCS, lacking the *AUR1-C* gene. The 1173-bp *KpnI/XbaI* fragment containing the *URA3* gene derived from the pYES2 plasmid, obtained from the amplification by PRI231 (with a *KpnI* site tag) and PRI273 (with an *XbaI* site tag), was inserted into the *KpnI* and *XbaI* sites of this plasmid, to create the plasmid pUCori-Amp^R-*URA3*. To introduce the CEN6/ARSH4 sequence into this plasmid, the 554-bp *SphI/XbaI* fragment, including CEN6/ARSH4 derived from the plasmid pYC6/CT, was amplified with the PRI274 (with an *SphI* site tag) and PRI275 (with an *XbaI* site tag) primers and was inserted into the *SphI* and *XbaI* sites of the plasmid pUCori-Amp^R-*URA3*, to create pUCori-Amp^R-*URA3-CEN6/ARSH4*. To add the *LEU2* transformation marker to this plasmid, the 1951-bp *SphI* fragment, including the *LEU2* gene derived from the plasmid pRS315, was amplified with the PRI271 and PRI278 primers (both with an *SphI* site tag) and was inserted into the *SphI* site of this plasmid, to create pUCori-Amp^R-*URA3-CEN6/ARSH4-LEU2*. To enlarge the 3'-end homology of the recipient *ura3* allele, the 1580-bp fragment, from the *StuI* site within *URA3* to the downstream region of *URA3*, was amplified by PRI276 and PRI277 (with an *XbaI* site tag), and the 1501-bp *StuI/XbaI* fragment was replaced with the 473-bp *StuI/XbaI* region of the plasmid pUCori-Amp^R-*URA3-CEN6/ARSH4-LEU2*. To create the gap of the 458-bp *PstI/StuI* region in the recipient *ura3* allele, this plasmid was cleaved by the *PstI* and *StuI* restriction enzymes and treated with T4 DNA polymerase, and

then the *NruI* linker (CTCG|CGAG) was inserted. Finally, the sequence of the *I-SceI* site (5'-TAGGGATAA|CAGGGTAAT/3'-ATCCC|TATTGTCCCATTA) was introduced into the *NruI* site, to create the pSDSA/NHEJ plasmid. This assay plasmid has 1492-bp of 3'-end homology and 3365-bp of 5'-end homology.

To prepare competent cells of KTY90 and its mutant derivatives (Table 1) which have 5'Δ-*ura3* and *ura3-3'Δ*, a culture was grown in liquid SD media containing 1 μg/ml 5-fluoroorotic acid monohydrate (5-FOA, Wako, Osaka, Japan), which prevented the generation of Ura⁺ cells by homologous recombination between two *ura3* alleles. A portion of the preculture was added to new liquid medium, to an initial OD of 0.05. The liquid culture was incubated at 30° to mid-log phase (OD, 0.5~0.6), and then the cells were collected and suspended in a lithium acetate solution. Aliquots (0.1 ml) of the cell suspension were placed into 1.5-ml microtubes and were frozen slowly at -80°. This slow-freezing procedure generated cells that were mostly viable after mild thawing on ice for 60 min or less, while in contrast, the use of either solid carbon dioxide or liquid nitrogen caused severe cell death. The *I-SceI* cut plasmid DNA (1 μg) was mixed with 0.1 ml of the thawed competent cell suspension, and then appropriate treatments in accordance with the lithium acetate method were performed. After an incubation in 1 ml of nonselective SD medium at 30° for 2 hr, the cell suspension was washed and plated onto selective media. Cells transformed with the SDSA products were selected for Ura⁺ Leu⁺, and cells transformed with the NHEJ products were selected for 5FOA^R Leu⁺. After an incubation at 30° for 3 days, the transformed colonies were scored, and the number of transformant colonies per microgram was determined as the transformation efficiency. The same amount (1 μg) of the uncut plasmid was concurrently transformed in the same manner as the *I-SceI* cut plasmid. The Leu⁺ transformant colonies were selected and scored, and the number of transformant colonies per microgram was determined as the transformation competency. The SDSA frequency was calculated by dividing the Ura⁺ Leu⁺ transformation efficiency with the *I-SceI* cut plasmid by the transformation competency with the uncut plasmid. The NHEJ frequency was calculated by dividing the 5FOA^R Leu⁺ transformation efficiency with the *I-SceI* cut plasmid by the transformation competency with the uncut plasmid. For the structural analysis of the NHEJ products, whole DNA was extracted from each of the 5-FOA^R Leu⁺ colonies. Each *ura3* plasmid DNA was amplified by the PRI231 (at the 5' end of *URA3* gene) and PRI273 (at the 3' end of *URA3* gene) primers with the whole DNA samples and was digested with *I-SceI* nuclease. The *I-SceI*-sensitive clones were judged as precise end-joining products, and the *I-SceI*-resistant clones were sequenced.

Targeted integration (crossover) assay

This assay was performed as previously reported (Yamana *et al.* 2005). pAUR101 (6687 bp; Takara, Kyoto, Japan), which bears the dominant *AUR1-C* allele conferring resistance to Aureobasidin A (Aur^R), does not possess either a centromere

(CEN) or an autonomous replication site (ARS) (Hashida-Okado *et al.* 1998). The base substitution producing the dominance is at 4522 within the pAUR101 sequence (AB012282; Hashida-Okado *et al.* 1998). The blunt-ended cleavages by the *MscI* and *StuI* restriction enzymes occur at 3231 and 4143 of pAUR101, respectively, and each recognition site resides within the *AUR1-C* allele. This plasmid DNA was cleaved by the *MscI* enzyme or the *StuI* enzyme and was introduced into W303 α and its mutant derivatives. The resultant Aur^R transformants with *AUR1-(pUCori-Amp^R)-AUR1-C* were scored as targeted integrants. The pRS315-Aur^R plasmid (8599 bp; Yamana *et al.* 2005), an *AUR1-C* derivative of the pRS315 plasmid (Sikorski and Hieter 1989), was used to determine the transformation competency and to normalize the differences in DNA uptake capacity among preparations of competent cells. Competent cells of W303 α and its mutant derivatives were prepared and transformed in the same manner as for the SDSA/NHEJ assay. The *StuI* cut pAUR101 DNA (1 μ g) and 1.28 μ g of uncut pRS315-Aur^R DNA (the same molar amount as the *StuI* cut DNA) were used for a set of transformations. SD plates with 0.4 μ g/ml of Aureobasidin A (Takara, Kyoto, Japan) were used for the selection. The targeted integration frequency (percentage) by crossover events with associated gene conversion was calculated by dividing the Aur^R transformation efficiency with 1 μ g of the *MscI* cut or *StuI* cut pAUR101 DNA by the Aur^R transformation efficiency (competency) with 1.28 μ g of the uncut pRS315-Aur^R plasmid.

To detect the targeted integration (crossover) events associated with longer gene conversion, we prepared the plasmid pAUR101-*I-SceI*, constructed by inserting an 18-mer bearing the *I-SceI* recognition sequence into the *StuI* site. This 18-mer insertion inhibits the function of the *AUR1-C* gene products. The plasmid DNA linearized by *MscI* digestion results in products conferring the Aur^R phenotype when gene conversion occurs beyond the 18-mer insertion, which is 912 bp away from the DSB end, to regain the *StuI* sequence.

Measurement of sensitivity to DNA scissions

Sensitivity to *EcoRI* expression: The strains to be tested, carrying the plasmid pGAL1::*EcoRI* (*URA3*), which bears the *EcoRI* restriction gene expressed by the *GAL1* promoter (Lewis *et al.* 1998), were grown to mid-log phase (OD, 0.2~0.4) in minimal medium containing 2% glucose. The cells were washed twice to remove the glucose, diluted, and spread onto minimal plates with 2% glucose or 2% galactose. The plates were incubated for 4 days at 30°, and then the number of viable colonies was counted. The surviving fraction was calculated by dividing the colony-forming units on the plates with 2% galactose by those on plates with 2% glucose. For the spot assay, 10 μ l portions of a suspension of serial 10-fold dilutions from a mid-log phase culture with the indicated genotype were spotted onto minimal plates containing 2% glucose or 2% galactose.

Sensitivity to bleomycin: Bleomycin (Zeocin; InvivoGen, Carlsbad, CA) was supplemented within solid media. The strains

to be tested were grown to mid-log phase (OD, 0.2~0.4) in YPD medium. The cells were diluted and spread onto YPD plates with or without bleomycin. The plates were incubated for 3 days at 30°, and then the number of viable colonies was counted. The surviving fraction was calculated by dividing the colony-forming units on the plates with the reagent by those on plates without the reagent. For the spot assay, 10 μ l portions of a suspension of serial 10-fold dilutions from a mid-log phase culture with the indicated genotype were spotted onto YPD plates containing the indicated concentration of a DNA scission reagent, such as bleomycin, methyl methanesulfonic acid, (MMS; ICN Biomedicals, OH), camptothecin (CPT; Wako, Osaka, Japan), and hydroxyurea (HU; Wako, Osaka, Japan).

Results

SDSA/NHEJ assay system

SDSA-mediated recombination, which generates noncrossovers, progresses via the following steps: D-loop formation between a single-strand tail from a processed DSB terminus and the homologous double-strand DNA (Figure 1, A1B1–A2B2), priming of DNA repair synthesis at the 3' end in the D-loop (A2B2–B3), dissociation of the newly synthesized strand from the double-strand DNA-template (B3–B4), and annealing of the new strand with the complementary strand of the other processed DSB end (B4–B5). To detect the noncrossover products formed by SDSA and to distinguish them from those formed by other pathways, we developed a yeast SDSA assay, consisting of two discontinuous templates on different chromosomes and a linearized recipient plasmid DNA containing a gene disrupted by a DSB. The disrupted gene will be repaired for activation by copying both templates, followed by annealing of the complementary single-strand copies (Figure 2A). In this two-separate-template system, no double-Holliday structure is formed from the two discontinuous templates on different chromosomes, and the noncrossover products are generated solely by SDSA (Figures 1 and 2). This assay consists of three different *ura3* alleles: (i) 5' Δ -*ura3*, one template allele with a 5'-end deletion of *URA3*, set at its locus on chromosome V; (ii) *ura3-3'* Δ , the other template allele with a 3'-end deletion of *URA3*, integrated into the *aur1* locus on chromosome XI; and (iii) *ura3-int Δ isceI*, the recipient allele with the gap sealed with an *I-SceI* site, carried on the plasmid with the *LEU2* marker. Transformation of the *I-SceI* cut recipient plasmid would generate Ura⁺ Leu⁺ colonies as the products of SDSA (Figure 2B), and 5FOA^R Leu⁺ colonies as the DSB repair products by precise or imprecise NHEJ (Figure 2C). This assay has advantages over the existing assays consisting of either a template allele with direct repeats or a single-mutant template allele, since these do not distinguish the SDSA-mediated noncrossover products from those formed through double-Holliday intermediates.

Control experiments without any plasmid showed that Ura⁺ clones in competent cell suspensions of the double-template strain were spontaneously generated at $6.9E-08 \pm 3.0E-9$

($n = 3$), indicating that the spontaneous frequency of Ura⁺ clones in competent cells is sufficiently low to contain them in a population of Ura⁺ transformants possessing SDSA products (Figure 3A).

A quantitative analysis with the SDSA/NHEJ plasmid in the double-template strain revealed that SDSA and NHEJ occurred at 4.7 and 0.99%, respectively (Figure 3, A and B, and Table S1). In a similar triparental system, in which a plasmid with a gapped *leu2* allele and two donor templates on two different chromosomes were used, the frequency of SDSA was reportedly ~0.3% (Pâques *et al.* 1998), suggesting that the difference in the frequency from their system would be due to the homology length at the DSB ends. A molecular analysis of the SDSA products from the wild-type strain, by a PCR analysis of each of the Ura⁺ Leu⁺ colonies and treatments with *Pst*I and *Stu*I (Figure 2B and Figure S1A), revealed that all of them (24/24) had the 458-bp *Pst*I–*Stu*I fragment, indicating that they were SDSA products (Figure S1A). A molecular analysis of each NHEJ product (5FOA^R Leu⁺ colony) from the wild-type strain, by PCR and a treatment with *I*-SceI (Figure 2C and Figure S1B), showed that all of them (144/144) were sensitive to the *I*-SceI endonuclease, indicating that they were precise end-joining products (Figure S1B and Table 2).

Rad52 promotes the SDSA pathway and the Lig4-independent NHEJ pathway

Rad52 plays a central role in homologous recombination and mediates D-loop formation by **Rad51** nucleoprotein filaments between a single-strand tail from a processed DSB end and homologous double-strand DNA (Sung 1997). The **RAD52** gene knockout caused a 2500-fold reduction in SDSA (from 4.7 to 0.0019%; Figure 3, A and B, and Table S1), indicating that SDSA-mediated noncrossover formation requires **Rad52** function. On the other hand, the *rad52* knockout did not affect NHEJ (Figure 3, A and B, and Table S1), indicating that **Rad52** is not required for the major NHEJ pathway.

DNA ligase 4 (**Lig4**, *LIG4* gene product) is known to promote the Ku-dependent NHEJ (Schär *et al.* 1997; Ramos *et al.* 1998). The *LIG4* gene knockout caused a fivefold reduction in NHEJ (from 0.99 to 0.18%), but did not significantly affect SDSA (Figure 3, A and B, and Table S1). These results indicate that the NHEJ detected by this assay is mostly dependent on the main pathway promoted by Ku and **Lig4**, consistent with previous reports (Boulton and Jackson 1996b; Schär *et al.* 1997; Ramos *et al.* 1998). Molecular analyses of the NHEJ products from the *lig4* knockout strain revealed that 6% of the NHEJ products (9/144) were resistant to the *I*-SceI endonuclease, indicating that some products are generated by imprecise NHEJ (Table 2). They appear to be joined with the associated deletion of a few bases (from 1 to 8 bases) within the *I*-SceI recognition sequence (Table 2). Next, we tested whether the remaining NHEJ capacity in the *lig4* mutant is dependent on **Rad52**. The addition of the *rad52* knockout to the *lig4* defective strain caused a 22-fold further reduction in NHEJ (from

Figure 1 DSB repair pathways. (A) Holliday-junction (HJ)-mediated homologous recombination. This pathway involves the resolution of two Holliday junctions. A1: Both ends at the DSB are unwound and exonucleolytically degraded, resulting in the formation of a 3'-single-strand (ss)-tailed DNA. A2: One 3'-ss-tailed DNA invades into the homologous DNA, and a D-loop with a short heteroduplex is formed. A3: The heteroduplex region is expanded in the 5' direction by the strand-exchange reaction, and a Holliday junction is formed. A4: From the 3' end of the heteroduplex, DNA-repair synthesis is primed. Strand exchange occurs at the other end of the DSB, and the second Holliday junction is formed. A5 or A6: One of two types of products, gene conversion or gene conversion associated with crossover, is generated depending on how the two Holliday junctions are resolved. The ends-in gene targeting produces the targeted integrant (A6) through the association of gene conversion with crossover, but does not through gene conversion without crossover (A5). (B) Synthesis-dependent strand-annealing (SDSA)-mediated homologous recombination. This pathway is associated with D-loop migration. B1: This intermediate is identical to A1. B2: This intermediate is identical to A2. B3: Immediately after D-loop formation, repair synthesis is primed from the 3' end of the heteroduplex DNA. While DNA synthesis continues, the rear heteroduplex region is unwound. B4: As DNA synthesis proceeds to the homologous region with the other DSB end, the newly synthesized strand is detached. B5: The detached strand is reannealed to the other ss-tailed region, and the gap is filled. The arrow from A4 to B5 indicates that two heteroduplex molecules linked by a double-Holliday junction (A4) are resolved into separate duplexes (B5), by migrating the two Holliday junctions into the center. (C) Nonhomologous end-joining (NHEJ) pathway. C1: Both ends of a DSB are unwound. C2: Two unwound ends interact with each other through microhomology. C3: Immediately after this interaction, the two ends are ligated. C4: The end-joining product is formed.

0.18 to 0.0083%; Figure 3, A and B, and Table S1). As the reduction of NHEJ by the *rad52* knockout was observed only in the *lig4*-deficient background, the cells may have an alternative NHEJ pathway requiring **Rad52** function, in addition to the *Lig4*-dependent NHEJ pathway.

The Srs2 DNA helicase and the Irc20 putative helicase promote the SDSA pathway

The **Srs2** DNA helicase recognizes synthetic DNA structures that mimic D-loops as substrates and translocates them on

Figure 2 SDSA/NHEJ assay system. (A) The different *ura3* template alleles and the *I-SceI*-cut plasmid bearing the *ura3* recipient allele with a gap. One *ura3* template allele is 5'Δ-*ura3*, in which the region from the promoter to the 39th codon was deleted in the *URA3* locus on the 5th chromosome. The other *ura3* template allele is *ura3*-3'Δ, which is the ectopic allele containing the region from the *URA3* promoter to the 139th codon, located within the *AUR1* locus on the 11th chromosome. These template alleles share 300 bp of internal homology. The *ura3* recipient allele is *ura3*-intΔ*iscI*, which lacks the 458-bp region from the *PstI* site to the *StuI* site. As this gap is sealed with the 18-bp *I-SceI* sequence, *I-SceI* cleavage produces the double-strand gap. The plasmid bearing *ura3*-intΔ*iscI* has *ARSH4*, *CEN6*, and *LEU2* selection marker gene. (B) SDSA products are obtained as Ura⁺ Leu⁺ transformants bearing the plasmid with the *URA3* allele, which is repaired via SDSA using the two templates. The reversion of the 458-bp gap was verified by PCR for the entire region of the plasmid DNA with a pair of primers, PRI271 (solid arrow) and PRI274 (open arrow), and *PstI*/*StuI* digestion (Figure S1A). (C) NHEJ products are obtained as Ura⁻ (5-FOA^R) Leu⁺ transformants bearing the plasmid with an *ura3* allele. The retention or deletion of the *I-SceI* sequence was detected by PCR for the plasmid DNA with the primers PRI271 (solid arrow) and PRI274 (open arrow) or with another pair of primers, PRI231 (green arrow) and PRI277 (gray arrow), *I-SceI* digestion (Figure S1B), and sequence determination (Table 2).

RPA-coated single-strand DNA (Dupaigne *et al.* 2008). We tested the possible functions of *Srs2* in SDSA, using the *in vivo* SDSA/NHEJ assay system. The knockout of *SRS2* caused a fourfold reduction in SDSA (from 4.7 to 1.1%) (Figure 3, A and B, and Table S1), indicating that *Srs2* actively promotes SDSA-mediated noncrossover production, probably by its D-loop processing activity.

The negative effect of the *srs2* knockout on SDSA production was much smaller than that of the *rad52* knockout. We interpreted this result as suggesting the existence of another DNA helicase that can replace *Srs2* and looked at the putative DNA/RNA helicase *Irc20*, which was classified into the *Srs2* subgroup by a phylogenetic analysis for the helicase-related proteins in *S. cerevisiae* (Shiratori *et al.* 1999), as a candidate for an *Srs2* functional analog. *Irc20* protein is conserved in *Ascomycota*; however, its function is unknown. We tested the effects of its knockout on DSB repair. The *IRC20* knockout caused a fivefold reduction in SDSA (from 4.7 to 0.98%), similar to the *srs2* knockout (Figure 3, A and B, and Table S1), indicating the function of *Irc20* in SDSA. The double knockout of *SRS2* and *IRC20* decreased SDSA as much as each single knockout (Figure 3, A and B, and Table S1), indicating that *Irc20* works in the SDSA pathway that involves the *Srs2* helicase.

Figure 3 Frequencies of SDSA and NHEJ events in *lig4*, *rad52*, *srs2*, and *irc20*-deficient mutants. (A) Leu⁺ transformation efficiencies per microgram with the uncut plasmid are plotted as the transformation competencies of competent cell suspensions (open symbols). Ura⁺ Leu⁺ and 5-FOA^R Leu⁺ transformation efficiencies per microgram with the *I-SceI* cut plasmid are plotted as SDSA progeny (blue symbols) and NHEJ progeny (green symbols), respectively. (B) The normalized SDSA (blue bar) and NHEJ (green bar) frequencies (%) were calculated from the transformation efficiencies (Materials and Methods), and were plotted with the standard deviations (SD) (white lines). The SDSA and the NHEJ frequencies (%) (mean ± SD) and the numbers of transformation of the indicated strains are shown in Table S1. ND indicates that SDSA progeny was not detected. The results from the *t*-test (vs. wild-type) are shown as follows: **, *P* < 0.01; ***, *P* < 0.001 (blue for SDSA; green for NHEJ).

Maintenance of precise NHEJ by DNA helicases

The efficiencies of NHEJ by the knockout strains of *srs2* (1.9%), *irc20* (1.2%), and *srs2 irc20* (1.4%) were slightly higher than that of the wild-type strain (0.99%; Figure 3, A and B, and Table S1). We analyzed the structures of the NHEJ products. Unlike the wild-type strain (144/144), in which all of the NHEJ products were *I-SceI* sensitive, in the *srs2* strain, 18% of the NHEJ products (21/120) were resistant to *I-SceI* (Table 2). In the *irc20* strain, 8% of the NHEJ products (11/132) were resistant to *I-SceI* (Table 2). These results indicate that imprecise NHEJ is enhanced in these DNA helicase mutants and that the NHEJ products lost a few bases (from 1 to 11 bases) within the linker sequence (Table 2). These results suggest that *Srs2* and *Irc20* contribute to the prevention of imprecise NHEJ.

Irc20 works before D-loop processing by Srs2

To investigate the step at which *Irc20* functions, we utilized a targeted integration (crossover) assay, which produces the duplication of *AUR1* and *AUR1-C* as the crossover form, thus conferring Aur^R (Materials and Methods; Yamana *et al.* 2005;

Table 2 Fractions and structures of imprecise NHEJ products

Relevant genotype	Wild type	<i>lig4</i>	<i>srs2</i>	<i>irc20</i>
5FOA ^R Leu ⁺ transformants analyzed:	144	144	120	132
<i>I-SceI</i> resistant products (%)	0 (0)	9 (6.3)	21 (18)	11 (8.3)
<i>CGTAGGG ATA</i> A <i> CAGGGTAATCGA</i> <i>GCATCCC TAT</i> T <i> GTCCCATTAGCT</i>	0	1	3	5
<i>CGTAGGG A</i> TA <i>A CAGGGTAATCGA</i> <i>GCATCCC T</i> AT <i> GTCCCATTAGCT</i>	0	2	2	0
<i>CGTAGGG A</i> TA <i>A CAGGGTAATCGA</i> <i>GCATCCC T</i> AT <i> GTCCCATTAGCT</i>	0	1	1	0
<i>CGTAGGG A</i> TA <i>A CAGGGTAATCGA</i> <i>GCATCCC T</i> AT <i> GTCCCATTAGCT</i>	0	0	3	0
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	1	1	0
<i>CGTAGGG A</i> TA <i>A CAGGGTAATCGA</i> <i>GCATCCC T</i> AT <i> GTCCCATTAGCT</i>	0	0	3	0
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	1	1	0
<i>CGTAGGG A</i> TA <i>A CAGGGTAATCGA</i> <i>GCATCCC T</i> AT <i> GTCCCATTAGCT</i>	0	0	3	0
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	1	0	1
<i>CGTAGGG A</i> TA <i>A CAGGGTAATCGA</i> <i>GCATCCC T</i> AT <i> GTCCCATTAGCT</i>	0	1	2	0
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	2	1	1
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	0	3	1
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	0	0	3
<i>CGTAGGG a</i> taa <i> CAGGGTAATCGA</i> <i>GCATCCC t</i> att <i> GTCCCATTAGCT</i>	0	0	2	0

12 types of *I-SceI* resistant sequences were found and are shown as the 23-bp inserted sequence (italic Courier font), including the *I-SceI* sequence and the portion lost (lower letters with strikethrough) due to imprecise NHEJ. The vertical line within each of the strands indicates the cleavage by the *I-SceI* enzyme. Underlining in the *I-SceI*-resistant sequences indicates the putative base pair annealed due to imprecise NHEJ. The numbers of each type of imprecise NHEJ are shown for each strain.

Figure 4A). The targeted integration occurred with a 4.1% frequency in the wild-type strain and a 9.3% frequency in the *srs2* knockout strain (Figure 4, B and C), thus revealing a 2.3-fold enhancement by the *srs2* knockout, as observed in a previous report (Ira *et al.* 2003). The single knockout of *RAD52* caused a 147-fold reduction in the targeted integration (from 4.1 to 0.028%) (Figure 4, B and C), indicating the strong dependency of the integration on *Rad52*. The *rad51* knockout decreased the targeted integration to a similar extent as the *rad52* knockout (Yamana *et al.* 2005). We tested whether the *irc20* knockout enhanced the targeted integration, as in the case of the *srs2* knockout. However, the single knockout of *IRC20* caused a threefold reduction in the targeted integration (from 4.1 to 1.3%) (Figure 4, B and C). Since the *irc20* knockout reduced both SDSA and crossover, it is likely that *Irc20* functions in the steps before D-loop formation (Figure 1, A2B2). We further tested whether the

irc20 knockout suppressed the enhancement of the cross-over by the *srs2* knockout. The *irc20 srs2* double knockout generated a fourfold reduction in crossover (from 4.1 to 1.1%), as compared to the wild type, which is in accordance with the level observed in the single *irc20* knockout cells (Figure 4, B and C), indicating the complete suppression by *irc20* of *srs2*-induced crossover enhancement. These results suggest that, contrary to our expectations, the *Irc20* putative DNA helicase has a different function from the *Srs2* DNA helicase in the SDSA pathway. Instead, the results support a model in which *Irc20* works before D-loop processing by *Srs2*.

***Irc20* is a novel factor for *Mre11*-directed DSB-end processing**

The *Mre11* protein forms the MRX complex with *Rad50* and *Xrs2* and processes DSB ends to initiate DSB repair by homologous recombination (Usui *et al.* 1998; Moreau *et al.*

Figure 4 Frequency of crossover events in *srs2* and *irc20*-deficient mutants. (A) The *AUR1-C* plasmid DNA is cleaved by the *Stul* enzyme, whose site is 379 bp away from the *AUR1-C* dominant mutation. The resultant 5' end and 3' end share 1909-bp homology and 1622-bp homology with the *AUR1* gene (chromosome XI), respectively. (B) Aur^R transformation efficiencies per microgram with the uncut pRS315-Aur^R plasmid are plotted as the transformation competencies of competent cell suspensions (open symbols). Aur^R transformation efficiencies per microgram with the *Stul* cut plasmid are plotted as the numbers of crossover progeny (solid symbols). (C) The normalized frequencies of crossover events associated with gene conversion (black bar) were calculated from the transformation efficiencies (Materials and Methods), and the means were plotted with the SD (white line). The means ($\pm$ SD) (%) of the wild-type strain (WT) and the indicated knockout strains are as follows: WT 4.1 ($\pm$ 0.29); *rad52* 0.028 ($\pm$ 0.012); *srs2* 9.3 ($\pm$ 0.80); *irc20* 1.3 ($\pm$ 0.21); *srs2 irc20* 1.1 ($\pm$ 0.25). The results from the *t*-test (vs. wild type) are shown as follows: ***, $P < 0.001$.

1999). To investigate the genetic relationship between *MRE11* and *IRC20*, we tested the effect of the *irc20* knockout on SDSA and NHEJ production in the *mre11* knockout strain. The *mre11* single knockout caused a 52-fold reduction in SDSA (from 4.5 to 0.086%) (Figure 5, A and B), indicating that *Mre11* promotes SDSA. The *irc20 mre11* double knockout caused a 10-fold enhancement in SDSA (from 0.086 to 0.85%), as compared with the *mre11* single knockout (Figure 5, A and B), indicating that the *irc20* defect partially suppressed the *mre11*-induced SDSA inhibition. The single

mre11 knockout also caused an 18-fold reduction in NHEJ (from 1.1 to 0.059%) (Figure 5, A and B), consistent with previous reports (Moore and Haber 1996; Lewis *et al.* 1999). The *irc20 mre11* double knockout caused a sixfold enhancement in NHEJ (from 0.059 to 0.33%), as compared with the *mre11* single knockout (Figure 5, A and B), indicating that the *irc20* defect partially suppressed the *mre11*-induced NHEJ inhibition.

We next examined whether the *irc20* single knockout causes hypersensitivity to DNA scission and whether it can suppress the *mre11*-induced hypersensitivity to DNA scission. As shown in Figure 6A, the *irc20* single knockout did not show any sensitivity to *EcoRI* expression; however, the knockout completely suppressed the hypersensitivity of the *mre11* knockout strain. As shown in Figure 6B, the *irc20* single knockout did not show any sensitivity to bleomycin or other DNA scission reagents, such as MMS, HU, and CPT; however, the knockout suppressed the hypersensitivity of the *mre11* knockout strain to these reagents.

We quantitatively examined the effects of the *irc20 exo1* double knockout on the resistance to *EcoRI* expression and bleomycin treatment. The double mutant did not show strong sensitivity to either DNA scission treatment (Figure 7). Foster *et al.* (2011) reported that the elimination of Ku suppressed *mre11* defects, in terms of the sensitivity to DNA scission reagents, and that this suppression was totally dependent on *Exo1* function. To test whether the *irc20* knockout-mediated suppression of *mre11* defects requires *Exo1* function, we quantitatively measured the effects of the *exo1* knockout on the *irc20*-mediated suppression of *EcoRI* resistance and bleomycin resistance (Figure 7). In the presence of *Exo1*, the addition of the *irc20* knockout to the *mre11* mutant background showed a 4.7-fold increase in *EcoRI* resistance, and even in the absence of *Exo1*, it showed a 2.0-fold increase (Figure 7A). Such weak effects of the *exo1* knockout were observed only with a low dose of bleomycin, and not with a higher dose (Figure 7B). These results show that the *irc20*-mediated suppression of *mre11* defects weakly depends on *Exo1* function, suggesting that this suppression is different from the *ku*-mediated suppression, in terms of *Exo1* dependency, and involves other exonucleases or helicases.

We also tested the effect of the *irc20* knockout on the crossover in the *mre11* knockout strain. When the *AUR1-C*-bearing plasmid DNA, linearized by *MscI* digestion, was used to measure crossover (Figure 8A), the single knockout for *MRE11* caused a 153-fold reduction in the crossover (from 4.4 to 0.029%) (Figure 8D). The *irc20 mre11* double knockout showed a 23-fold enhancement in the crossover (from 0.029 to 0.67%), as compared with the *mre11* single knockout (Figure 8D), indicating that the *irc20* defect partially suppresses the *mre11*-induced crossover inhibition. In the absence of *Exo1*, the *irc20 mre11* double knockout showed a fivefold enhancement in the crossover (from 1.2 to 5.8%), as compared with the *mre11* knockout (Figure 8D), indicating that the *irc20* defect-mediated suppression of the *mre11*-induced crossover inhibition partially depends on *Exo1* function.

Figure 5 Frequencies of SDSA and NHEJ events in *mre11*- and *irc20*-deficient mutants. (A) *Leu*⁺ transformation efficiencies per microgram with the uncut plasmid are plotted as the transformation competencies of competent cell suspensions (open symbols). *Ura*⁺ *Leu*⁺ and 5FOA^R *Leu*⁺ transformation efficiencies per microgram with the *I*-SceI cut plasmid are plotted as SDSA progeny (blue symbols) and NHEJ progeny (green symbols), respectively. (B) The normalized SDSA (blue bar) and NHEJ (green bar) frequencies (%) were calculated from the transformation efficiencies (Materials and Methods), and the means were plotted with the SD (white lines). The SDSA and the NHEJ frequencies (%) (mean $\pm$ SD) and the numbers of transformation of the indicated strains are shown in Table S1. The results from the *t*-test (vs. wild type except for vs. *mre11*) are shown as follows: *, *P* < 0.05; ***, *P* < 0.001 (blue for SDSA; green for NHEJ).

Finally, to evaluate whether *Irc20* is involved in the early steps of the pathway where the resection of DSB ends occurs, we tested the effects of *irc20* mutations on the crossover associated with longer-tract (more than 912-bp) gene conversion, using the *MscI*-digested pAUR101-*I*-SceI plasmid DNA (Figure 8B). In this crossover assay, *Aur*^R transformation requires the longer-tract crossover beyond the 18-mer insertion, 912 bp away from the DSB ends. Longer resection of DSB ends or longer branch migration of Holliday junctions is considered to lead to the longer-tract formation. The single knockout of *IRC20* displayed a fourfold increase in the crossover associated with longer conversion (from 2.4 to 9.2%) (Figure 8E), in contrast to the twofold decrease in the crossover associated with non-longer conversion (from 4.4 to 2.0%) (Figure 8D). With regard to the longer-tract crossover production, a similar result was obtained using the single knockout of *Exo1* (Figure 8E). These results suggest that *Irc20* plays a role at an early step, where *Exo1* functions.

Figure 6 Effects of *mre11* and *irc20* mutations on the repair of DNA scission-induced DSBs. Each of the spots is a serial 10-fold dilution from a culture of the strain with the indicated genotype. (A) *EcoRI* expression was induced on minimal plates containing galactose. (B) The DNA scission reagents bleomycin, MMS, HU, and CPT were added at the indicated concentrations to YPD plates.

The *mre11* single knockout caused a 60-fold reduction in the longer-tract crossover (from 2.4 to 0.041%) (Figure 8E). The *irc20 mre11* double knockout generated a 3.6-fold enhancement in the longer tract crossover (from 0.041 to 1.5%), as compared with the *mre11* single knockout (Figure 8E), indicating that the *mre11*-induced inhibition of longer-tract crossover is partially suppressed by the *irc20* defect. In the absence of *Exo1*, the knockouts of *irc20* and *mre11* showed a 2.7-fold increase in the longer-tract crossover (from 0.23 to 0.62%), as compared with the *mre11* knockout (Figure 8E), indicating that the *irc20* defect-mediated suppression of the *mre11*-induced inhibition of longer-tract crossover was not strongly dependent on *Exo1* function.

These results with respect to the two homologous recombination assays, the NHEJ assay and the DNA scission resistance, suggested the functional interaction between *Irc20* and *Mre11*. The role of *Irc20* is discussed later.

Figure 7 Effects of the *exo1* mutation on the *irc20* knockout-induced suppression of *mre11* defects in DSB repair. (A) Repair of *EcoRI*-induced DSBs. The surviving fraction (Materials and Methods) was plotted along with the SD (vertical line). The number (n) of experiments for each strain is as follows: WT (6), *irc20* (4), *mre11* (9), *mre11 irc20* (4), *mre11 exo1* (4), *mre11 irc20 exo1* (5), *exo1* (4), and *irc20 exo1* (4). (B–C) Repair of bleomycin-induced DSBs. The surviving fraction (Materials and Methods) was plotted along with the SD (vertical line). The number (n) of experiments for each strain is as follows: WT (4), *irc20* (4), *mre11* (10), *mre11 irc20* (4), *mre11 exo1* (3), *mre11 irc20 exo1* (6), *exo1* (6), and *irc20 exo1* (6).

Discussion

Roles of *Srs2* in the SDSA pathway

We developed a novel yeast genetic assay to measure SDSA-mediated noncrossover, distinguished from noncrossover through double-Holliday intermediates and crossover. Our quantitative analysis clearly revealed that both *Rad52* and *Srs2* promote SDSA-mediated noncrossover. This result is consistent with the recombination mediator activity of *Rad52* for the *Rad51*-catalyzed D-loop formation between an RPA-coated single-strand tail and a homologous double-strand DNA (Sung 1997). On the other hand, the *Srs2* DNA helicase was suggested to prevent D-loop formation by disrupting the *Rad51* nucleoprotein filament (Veaute *et al.* 2003). In addition, in a recent biochemical study using a synthetic D-loop (Figure 1, A2B2) (Dupaigne *et al.* 2008), the *Srs2* helicase promoted the migration of a D-loop formed by *Rad51* and *Rad52* to stimulate SDSA. Our results showed that the *Srs2* helicase actively stimulates SDSA (Figure 3), prevents crossover (Figure 4), and thus support a model in which *Srs2* acts on a D-loop formed by *Rad51* and *Rad52* to guide it into SDSA and to prevent the formation of a double-Holliday intermediate.

The roles of *Rad52*, *Srs2*, and *Irc20* in NHEJ

The current genetic assay using the *ura3* internal deletion allele allowed the quantification of NHEJ and the determination of whether precise or imprecise end joining occurred for each event. The single knockout for *RAD52* did not reduce NHEJ. However, the *lig4 rad52* double knockout significantly showed a 22-fold reduction in NHEJ, as compared to the *lig4* single knockout. This indicates that *Rad52* promotes more than 95% of NHEJ events in the absence of the main Ku/*Lig4*-dependent pathway in this assay. Since imprecise end-joining events occurred at only a 6% frequency in *lig4* mutants, *Rad52* mostly promotes precise end joining between *I-SceI*-induced 3'-protruding DSB ends. *Rad52* has potent single-strand DNA annealing activity (Mortensen *et al.* 1996), and this activity may contribute to single-strand annealing between the *I-SceI*-generated 3'-protruding ends. Other ligases and/or annealing proteins might be involved in imprecise end joining involving one or more base pairs (short microhomologies).

Teo and Jackson (1997) showed that *EcoRI*-induced DSB ends are rejoined imprecisely in about half of the end-joining products in *lig4* mutants, in contrast to the results in the current assay. A major difference between these two assay systems is that the *I-SceI*-induced DSB ends in our assay are 3' protruding, whereas the *EcoRI*-induced DSB ends in Teo and Jackson's (1997) assay were 5' protruding. We interpret this difference to mean that a 5'-3' exonuclease might be involved in the processing prior to annealing, which may result in the discrepancy of the end-joining accuracy between 5'-protruding ends and 3'-protruding ends.

The single knockout for *SRS2* or *IRC20* did not reduce NHEJ (Figure 3), but increased the amount of imprecise products in NHEJ (Table 2). The imprecise NHEJ products contained small deletions, as in the case of the *lig4* knockout. These results suggest that *Srs2* and *Irc20* ensure precise end joining in the Ku/*Lig4*-dependent pathway. In another NHEJ assay system, in which the DSB ends lack homology with any chromosomal sequences, the single mutation of *rad52* or *srs2* showed a significant reduction in the NHEJ frequency (Hegde and Klein 2000), suggesting that *Rad52* and *Srs2* promote the NHEJ pathway in parallel with the Ku/*Lig4* pathway, in the absence of homology around the DSB. In the case where the DSB ends share homology with a chromosomal locus, as in our assay system, *Rad52* and *Srs2* predominantly act on the intermediate molecules formed along the homologous recombination pathways, rather than the NHEJ pathway.

Roles of the *Irc20* protein in homologous recombination

IRC20 encodes a putative DNA/RNA helicase, and we expected it to replace the *Srs2* function. The single *irc20* mutation reduced SDSA-mediated noncrossover as much as the single *srs2* mutation. The SDSA in the *srs2 irc20* double mutant was almost the same as that in either single mutant. This indicates that *Irc20* works in the SDSA pathway

Figure 8 Frequency of the crossover events associated with longer-tract gene conversion in *mre11* and *irc20*-deficient mutants. (A) The pAUR101 plasmid DNA was digested by *MscI*. The AUR1-C dominant mutation is 1291 bp away from the DSB end. The resultant 5' and 3' ends share 997-bp and 2534-bp homologies with the AUR1 gene on the 11th chromosome, respectively. The targeted integration by a crossover event produces a tandem duplication of AUR1-C and AUR1 to confer Aur^R. (B) The pAUR101-I-SceI plasmid DNA, possessing the 18-bp (*I-SceI* sequence) insertion at the *StuI* site, digested by *MscI*. This insertion is 912 bp away from the DSB end, and the AUR1-C dominant mutation is 379 bp away from the insertion. The resultant 5' and 3' ends share 997-bp and 2552-bp homologies with the AUR1 gene on the 11th chromosome, respectively. The targeted integration by a crossover event with associated longer gene conversion produces a tandem duplication of AUR1-C and AUR1 to confer Aur^R. (C) Aur^R transformation efficiencies per microgram of the uncut pRS315-Aur^R plasmid are plotted as the transformation competencies of competent cell suspensions (open circles). Aur^R transformation efficiencies per microgram of the *MscI*-cut pAUR101 plasmid are plotted as the numbers of crossover progeny (solid triangles). Aur^R transformation efficiencies per microgram of the *MscI*-cut pAUR101-I-SceI plasmid are plotted as the numbers of crossover progeny (open squares). (D) The normalized crossover frequencies (black bar) were calculated from the transformation efficiencies (Materials and Methods), and the means were plotted along with the SD (white line). The means (±SD) (%) of the wild-type strain (WT) and the indicated knockout strains are as follows: WT 4.4 (±0.32); *irc20* 2.0 (±0.25); *mre11* 0.029 (±0.0030); *mre11 irc20* 0.66 (±0.063); *mre11 exo1* 0.012 (±0.0018); *mre11 irc20 exo1* 0.058 (±0.0058); *exo1* 4.9 (±0.47). (E) The normalized frequencies of crossover associated with longer-tract gene conversion (white bar) were calculated from the transformation efficiencies (Materials and Methods), and the means were plotted along with the SD (black line). The means (±SD) (%) of the wild-type strain (WT) and the indicated knockout strains are as follows: WT 0.024 (±0.0030); *irc20* 0.092 (±0.017); *mre11* 0.0041 (±0.00098); *mre11 irc20* 0.015 (±0.0028); *mre11 exo1* 0.0023 (±0.00086); *mre11 irc20 exo1* 0.0062 (±0.00045); *exo1* 0.069 (±0.0084). The results from the t-test (vs. wild type except for vs. *mre11*) are shown as follows: *, *P* < 0.05; ***, *P* < 0.001.

that involves Srs2. We used crossover assays to elucidate the step in the SDSA pathway where Irc20 functions, considering that SDSA-mediated noncrossover and double-Holliday-mediated crossover share steps up to D-loop formation in DSB repair (Figure 1, A1B1–A2B2). While the *srs2* single mutation enhanced the targeted integration, the *irc20* single mutation reduced the targeted integration and suppressed the *srs2*-induced enhancement of the targeted integration in *srs2 irc20* double-knockout cells. These findings further support a model in which Irc20 works before D-loop formation, and Srs2 processes the D-loop to enhance SDSA and to prevent double-Holliday intermediate formation in the homologous recombination repair of a DSB.

The Mre11–Rad50–Xrs2 (MRX) complex and Exonuclease 1 are involved in DSB end processing, to form the 3'-single-strand DNA tails used for D-loop formation (Moreau et al. 2001; Lewis et al. 2002; Mimitou and Symington 2011). Recent studies in *S. cerevisiae* and *in vitro* have implied that the

MRX complex with Sae2 removes short oligonucleotides from the 5' end of a DSB, to initiate DSB-end resection. Subsequently, the short 3'-single-strand tail is extended in alternative manners, depending on either Exo1 or the Sgs1–Top3–Rmi1 complex with Dna2, to provide a longer 3'-single-strand DNA for D-loop formation (Gravel et al. 2008; Mimitou and Symington 2008; Zhu et al. 2008; Cejka et al. 2010; Nicolette et al. 2010; Shim et al. 2010). We observed that the *irc20* single mutant showed approximately fourfold increase in longer-tract crossover (Figure 8E), as opposed to the effect on shorter-tract crossover (Figure 8D). To our surprise, we found that the knockout of IRC20 suppressed the *mre11* knockout-induced hypersensitivities to DNA scission treatments (Figures 6 and 7) and the *mre11* knockout-induced inhibition of SDSA, NHEJ (Figure 5), and crossovers (Figure 8). This *irc20*-mediated suppression of *mre11* defects was partially dependent on Exo1 function (Figure 7 and Figure 8D), and thus Exo1 and other nucleases would act on DSB ends for resection in the absence

of *Mre11* and *Irc20*. Since the longer-tract crossover was increased by the loss of *Irc20*, and this increase was observed even in the absence of both *Mre11* and *Exo1* (Figure 8E), other nucleases would produce longer 3' tails in the absence of *Irc20*. We interpret that *Irc20* somehow causes the DSB ends to be predominantly directed to *Mre11*, rather than to other exonucleases, to induce the DSB-end resection. It might be important to express the checkpoint function of the MRX complex (Haber 1998). Another explanation for the increased longer-tract crossover in the absence of *Irc20* is that the *Irc20* helicase inhibits the branch migrations of Holliday junctions to produce shorter-tract crossovers.

The loss of *Irc20* reportedly resulted in an increase in spontaneous *Rad52* foci in diploid cells (Alvaro *et al.* 2007). *Rad52* foci are formed on a 3'-single-strand tail generated by resection, to mediate D-loop formation by *Rad51*. We found that the loss of *Irc20* causes the increase of the crossovers associated with longer-tract conversion (Figure 8E). We interpret these results to mean that in the absence of *Irc20*, other exonucleases would produce longer 3'-single-strand DNA at DSB ends, and then more *Rad52* molecules would bind to the single-strand DNA region containing RPA proteins, resulting in more visible *Rad52* foci.

The meaning of the suppression by DNA helicase defects for nuclease-deficient phenotypes

Previous *Escherichia coli* studies revealed that genetic defects in RecQ helicase suppressed the inhibition of DSB-induced crossover and the hypersensitivity to DNA scissions due to defects in a 5'- to 3'-single-strand DNA exonuclease, RecJ (Kusano *et al.* 1994; Lovett and Sutera-Jr 1995; Handa *et al.* 2009). In addition, a certain point mutation of the *uvrD* gene and the insertion mutation of the *helD* gene, encoding DNA helicase IV, suppressed the negative effects due to the *recJ* mutation (Lovett and Sutera-Jr 1995). The *S. cerevisiae* *RECQ* homolog, *sgs1*, is not another suppressor of the *mre11* defects, because the *mre11 sgs1* double mutant is more sensitive to X-ray irradiation than the single *mre11* mutant (Budd and Campbell 2009). These genetic interactions revealed that a DNA helicase acts on the DNA substrate prior to the nuclease, to create a suitable substrate, and concurrently, to prevent the actions of other nucleases.

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Supporting Information

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Homologous Recombination via Synthesis-Dependent Strand Annealing in Yeast Requires the Irc20 and Srs2 DNA Helicases

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Figure S1 Structures of SDSA products and precise NHEJ products. (A) SDSA products. The reversion of the 458-bp gap was verified by digesting the PCR products from the entire region of the SDSA products with the *Pst*I and *Stu*I restriction enzymes (Fig. 2B). The 458-bp fragment (SDSA segment) generated with the 1.9-kb fragment, consisting of the 3'-*ura3* homologous region and *ARSH4/CEN6*, and the 5.5-kb fragment, consisting of the 5'-*ura3* homologous region and the *LEU2* marker in 24 *Ura*⁺ *Leu*⁺ progeny from the wild-type cells (Lanes 2-25). Lane 1 shows only the 1.9-kb and 5.5-kb fragments, which were generated by digesting the PCR product from the entire region of the uncut substrate plasmid (Fig. 2A). (B) Precise NHEJ products. The precise NHEJ products were verified by cleaving the PCR products from the entire region of the NHEJ products by the *I*-*Sce*I endonuclease (Fig. 2C). The *I*-*Sce*I treatment produced the 1.9-kb and 5.5-kb fragments in all 24 5FOA^R *Leu*⁺ progeny from the wild-type cells (Lanes 2-25). Lane 1 shows the same DNA sample as lane 1 of panel A.

Table S1 Frequencies of SDSA and NHEJ events in recombination-deficient mutants.

Relevant genotype	Transformation (<i>n</i>)	% Frequency of SDSA mean $\pm$ SD (Relative rate to WT)	% Frequency of NHEJ mean $\pm$ SD (Relative rate to WT)
Experiment 1			
WT	11	4.7 $\pm$ 1.3 (1)	0.99 $\pm$ 0.22 (1)
<i>rad52</i>	5	0.0019 $\pm$ 0.0019 (4.0 $\times 10^{-4}$)	0.95 $\pm$ 0.45 (9.6 $\times 10^{-1}$)
<i>lig4</i>	11	3.7 $\pm$ 1.1 (7.9 $\times 10^{-1}$)	0.18 $\pm$ 0.065 (1.8 $\times 10^{-1}$)
<i>lig4 rad52</i>	5	ND	0.0083 $\pm$ 0.0018 (8.4 $\times 10^{-3}$)
<i>srs2</i>	10	1.1 $\pm$ 0.095 (2.3 $\times 10^{-1}$)	1.9 $\pm$ 0.27 (1.9)
<i>irc20</i>	8	0.98 $\pm$ 0.20 (2.1 $\times 10^{-1}$)	1.2 $\pm$ 0.14 (1.3)
<i>srs2 irc20</i>	10	1.2 $\pm$ 0.12 (2.5 $\times 10^{-1}$)	1.4 $\pm$ 0.11 (1.4)
Experiment 2			
WT	4	4.5 $\pm$ 0.27 (1)	1.1 $\pm$ 0.11 (1)
<i>irc20</i>	6	1.2 $\pm$ 0.14 (2.7 $\times 10^{-1}$)	1.0 $\pm$ 0.23 (9.6 $\times 10^{-1}$)
<i>mre11</i>	5	0.086 $\pm$ 0.015 (1.9 $\times 10^{-2}$)	0.058 $\pm$ 0.014 (5.6 $\times 10^{-2}$)
<i>mre11 irc20</i>	5	0.85 $\pm$ 0.11 (1.9 $\times 10^{-1}$)	0.32 $\pm$ 0.071 (3.1 $\times 10^{-1}$)

ND: not detected

Table S2 The list of primers used for knockout or plasmid construction

Primer	Sequences of forward (F) and reverse (R) primers
PRI155	F: GAAAAGAAAAATGCTATGGACTATTTTCAAAAATGTGTCGAC-5'KAN
PRI156	R: ACTTTTGTGCACCCCGATATACTCCTCTGGGATGACCCGGG-3'KAN
PRI175	F: TTTGCGCCTAGTCCAGATTTCAAATGGCTTTGTGAAGAGCTA-5'KAN
PRI176	R: AAGTCTTCTTCAGGCACTTGACAGTTTTCATTAATAGAATG-3'KAN
PRI231	F: ggtaccCGCCGGCGAACGTGGCGAGAATCG
PRI232	R: tctagaCTTCTGCCGCTGCTTCAAACCGC
PRI240	R: CGTCATTATAGAAATCATTACGACCGAG
PRI245	F: CATCAAAGAAGGTTAATGTGGCTGTGGTTTCAGGGTCCATAA-5'HIS3
PRI249	R: GACCTAATGCTTCAACTAACTCCAGTAATTCCTTGGTGGTAC-3'HIS3
PRI243	F: TGATCCAGACACAATAAGGATTTTAATTACTACAGATAATCA-5'KAN
PRI244	R: ATCAAGTACAATATTTTCTTTCTTAGCAAGGAGACTTCC-3'KAN
PRI256	F: CCAAAGTACCAAACGACG
PRI257	R: GCGGTTTGAAGCAGGCGGC
PRI271	F: gcatgcTTTGGCCGAGCGGTCTAAGG
PRI278	R: gcatgcACTCCATCAAATGGTCAGGTC
PRI273	R: tctagaCTCATACTCTTCTTCTTTTCAATGGG
PRI274	F: AAAgcatgcGGTCCTTTTCATCACGTGC
PRI275	R: tctagaATCTCGCCACGTTCCGCGCG
PRI276	F: CGAATGCACACGGTGTGGTG
PRI277	R: tctagaGCACATGGTACGCTGTGGTG
PRI301	F: ATGAATGAAATTATGGATATGGATGAGAAGAAGCCCGTTTC-5'KAN
PRI302	R: TCAAGTAGGCTTGCGTGCATGCAGGGGATTGATCTTTGGTC-3'KAN
PRI314	F: ATAGCAAATGTCGTCGAACAATGATCTTGGTTGCATTTAGT-5'TRP1
PRI315	R: TTTAATTTTGACTTTTTTTTCGCACGTGACAGCTGAGAAAA-3'TRP1
PRI400	F: TTCGGATTCATGCTTATGGG
PRI401	R: TGGAGATGGCTGTTCTAATG
PRI449	F: TGATCCAGACACAATAAGGATTTTAATTACTACAGATAATCA-5'TRP1
PRI450	R: ATCAAGTACAATATTTTCTTTCTTAGCAAGGAGACTTCC-3'TRP1
PRI726	F: CTAAAGGAATGAACTCCAGGAAAGGCCAACTTATTCGACGGTAA-5'loxP-HYG
PRI727	R: ATATTTTAATATATTAGTGTCTGTTTAAAAGTCATACTTCTCTA-3'loxP-HYG
PRI766	F: CCAAAGTACCAAACGACGTTAAGGC
PRI769	R: CAGTGAATTCGAGCTCGGTACC

5'loxP: ataacttcgtataatgtatgctatacgaagttat

3'loxP: ataacttcgtatagcatatacgaagttat

5'HYG (or 5'KAN): GGTAAATTAAGGCGCGCC

3'HYG (or 3'KAN): ATCGATGAATTCGAGCTCG

5'TRP1: CGGCATCAGAGCAGATTG

3'TRP1: CGCATAGATCGGCAAGTGCAC

5'HIS3: CCACCTAGCGGATGACTC

3'HIS3: ATATGATCCGTCGAGTTC

Lower-case letters: the sequence tag cleaved by a restriction enzyme