Efficient Detection of Unpaired DNA Requires a Member of the Rad54-Like Family of Homologous Recombination Proteins

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ABSTRACT Meiotic silencing by unpaired DNA (MSUD) is a process that detects unpaired regions between homologous chromosomes and silences them for the duration of sexual development. While the phenomenon of MSUD is well recognized, the process that detects unpaired DNA is poorly understood. In this report, we provide two lines of evidence linking unpaired DNA detection to a physical search for DNA homology. First, we have found that a putative SNF2-family protein (SAD-6) is required for efficient MSUD in *Neurospora crassa*. SAD-6 is closely related to Rad54, a protein known to facilitate key steps in the repair of double-strand breaks by homologous recombination. Second, we have successfully masked unpaired DNA by placing identical transgenes at slightly different locations on homologous chromosomes. This masking falls apart when the distance between the transgenes is increased. We propose a model where unpaired DNA detection during MSUD is achieved through a spatially constrained search for DNA homology. The identity of SAD-6 as a Rad54 paralog suggests that this process may be similar to the searching mechanism used during homologous recombination.

MEIOSIS is fundamental to sexual reproduction. During meiosis, chromosomes are replicated, aligned, recombined, and segregated to nuclei that will develop into gametes. Two of these key processes, alignment and recombination, likely require a search for DNA homology between chromosomes (Barzel and Kupiec 2008; Moore and Shaw 2009). Such homology searching is necessary because sexual organisms inherit a copy of each chromosome from each of its parents. These chromosomes, referred to as homologs, must somehow find each other so that alignment, recombination, and segregation can occur.

Although recent research has improved our understanding of homology search mechanisms (Forget and Kowalczykowski 2012; Renkawitz *et al.* 2013), there are many questions that

remain unanswered. The filamentous fungus *Neurospora crassa* may be useful for investigating the unknowns of homology searching because it possesses a genetically tractable phenomenon called meiotic silencing by unpaired DNA (MSUD) (Aramayo and Selker 2013; Billmyre *et al.* 2013). MSUD scans pairs of homologs for segments of DNA that are not accurately paired between them. If improper pairing (*i.e.*, unpairing) is identified, the offending sequences are silenced for the duration of sexual development. For example, if a hypothetical gene called “*gene A*” is on the left arm of one chromosome but on the right arm of its homolog, it will be silenced. The same holds true if *gene A* has been lost from one of the homologs.

A functional MSUD response can be easily detected with alleles that affect ascospore (sexual spore) color or shape. Indeed, MSUD was discovered during studies of *ascospore maturation-1* (*asm-1*), a gene required for the production of pigmented (black) ascospores (Aramayo *et al.* 1996). A cross between an *asm-1*⁺ strain and an *asm-1*^Δ strain produces mostly unpigmented (white) ascospores. This is because MSUD silences the unpaired *asm-1*⁺ allele (Aramayo and Metzenberg 1996; Shiu *et al.* 2001). The efficiency of MSUD can be inferred

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from such heterozygous *asm-1⁺/asm-1^Δ* crosses by determining the ratio of black-to-white ascospores observed within asci (spore sacs) after their extraction from a perithecium (fruiting body). Alternately, because ascospores are forcibly shot to the lid of a crossing plate at the final stage of perithecial development, phenotypic ratios can be determined from ascospore suspensions made by collecting ascospores from a crossing lid.

Any gene that produces an aberrant ascospore phenotype when it is unpaired during meiosis can be used to determine the efficiency of MSUD. In addition to *asm-1⁺*, one of the most common genes used is *r⁺* [also referred to as *Round spore* (Mitchell 1966)]. *r⁺* expression is required for ascospores to develop their normal shape, which is similar to that of an American football. When MSUD is functional during a heterozygous *r⁺/r^Δ* cross, round ascospores are produced instead of football-shaped ascospores because *r⁺* is silenced (Shiu *et al.* 2001).

Genes that are required for MSUD can be identified through genetic screens for mutants that suppress MSUD efficiency. For example, the first MSUD gene was discovered by screening UV-induced mutants for the ability to produce black ascospores in crosses that were unpaired for *asm-1⁺* (Shiu *et al.* 2001). This approach has since been adapted for use with the *N. crassa* knockout collection (Colot *et al.* 2006). Essentially, strains from the collection are put through crosses where *asm-1⁺* or *r⁺* is unpaired during meiosis (Hammond *et al.* 2011a). The production of phenotypically normal ascospores in such crosses suggests that the knockout (*i.e.*, deleted gene) suppresses MSUD, typically because the deleted gene encodes a necessary component of the MSUD machinery.

There are currently eight characterized MSUD genes, seven of which produce proteins that localize in a ring-like pattern around the nucleus. These seven include common RNA interference (RNAi) proteins such as an RNA-directed RNA polymerase called SAD-1 (Shiu *et al.* 2001), an Argonaute protein called SMS-2 (Lee *et al.* 2003), and a Dicer protein called DCL-1 (Alexander *et al.* 2008). The four others are SAD-2, a presumptive scaffold protein (Shiu *et al.* 2006); SAD-3, a putative helicase (Hammond *et al.* 2011a); SAD-4, a protein required for MSUD-specific small RNA generation (Hammond *et al.* 2013a,b); and QIP, an exonuclease (Lee *et al.* 2010; Xiao *et al.* 2010). The eighth characterized MSUD gene encodes a nuclear protein called SAD-5 (Hammond *et al.* 2013b).

A simple working model of MSUD starts with the detection of unpaired DNA by an undetermined nuclear factor (Hammond *et al.* 2011a; Aramayo and Selker 2013). Unpaired DNA detection may then trigger the production of an “aberrant RNA” (aRNA), which could be delivered to a silencing complex stationed outside the nucleus. There, it could be made double-stranded (dsRNA) and processed into small RNAs by SAD-1 and DCL-1. While the hypothetical aRNA and dsRNA of MSUD have yet to be identified, the small RNAs have recently been discovered by deep sequencing (Hammond *et al.* 2013a,b). These small RNAs, referred to as MSUD-associated small interfering RNAs (masiRNAs), are thought to direct silencing of messenger RNAs by complementary base pairing.

MSUD is a remarkable process. It has the ability to identify and silence an unpaired segment of DNA as small as 700 bp between homologous chromosomes that are millions of base pairs long (Lee *et al.* 2004). Unfortunately, the identities of the proteins that mediate unpaired DNA detection have remained elusive. Although SAD-5 localizes within the nucleus, the role it plays in unpaired DNA detection is difficult to predict because it does not possess a characterized domain.

Here, we report the identification of SAD-6, a protein required for full MSUD functionality. Like SAD-5, SAD-6 is a nuclear MSUD protein, which raises the possibility that it is directly involved in unpaired DNA detection. More importantly, SAD-6 possesses a well-characterized domain that places it within a protein family that includes *Saccharomyces cerevisiae* Rad54. Enzymes from this family participate in the search for DNA homology during double-strand break repair by homologous recombination. We thus designed a new technique called “unpaired DNA masking” to investigate the (possibly related) search process that occurs during MSUD.

Materials and Methods

Strain information and culture conditions

All strains used in this study are listed in Table 1. Vogel's minimal medium (VMM) (Vogel 1956) was used for vegetative cultures and synthetic crossing medium (SCM) (Westergaard and Mitchell 1947) was used for sexual crosses. While crosses and experiments were performed at room temperature, strain propagation was occasionally performed at 28°. Standard *Neurospora* techniques were used for all experiments unless otherwise indicated (Davis and De Serres 1970). Genetic markers and knockouts were obtained from the Fungal Genetics Stock Center (FGSC) (McCluskey *et al.* 2010) and the *Neurospora* Functional Genomics group (Colot *et al.* 2006), and their descriptions can be found in the e-Compendium (http://www.bioinformatics.leeds.ac.uk/~gen6ar/newgenelist/genes/gene_list.htm). Linear growth assays were performed in race tubes as previously described (Perkins and Pollard 1986).

Transformations and transformation vectors

Transformations were performed by electroporation of washed conidia (asexual spores) as described by Margolin *et al.* (1997), except that conidia were separated from the majority of mycelial (vegetative) fragments by filtering through a 100-μm nylon filter (Steriflip; Millipore, Billerica, MA). The transformation vectors for the targeted insertion of ectopic fragments of *r⁺* (*r^{ef}*) were created by double-joint PCR (DJ-PCR) (Yu *et al.* 2004). DJ-PCR was also used to construct the vectors for tagging the native *sad-6* gene at its 5' end with green fluorescent protein (*gfp*) (Hammond *et al.* 2011b) and the native *spo76* gene at its 5' end with mCherryNC (Castro-Longoria *et al.* 2010). A hygromycin resistance marker was used in all transformation vectors (Carroll *et al.* 1994). The *gfp-sad-5* and *gfp-sms-2* tags were described previously (Hammond *et al.* 2011b, 2013b). The sequences of the oligonucleotide

Table 1 Strains used in this study

Strain name	Genotype
F2-01	<i>fl A</i>
F2-26	<i>rid; fl a</i>
F2-27	<i>rid r^Δ::hph; fl a</i>
F3-24	<i>rid his-3⁺::asm-1; fl; asm-1^Δ::hph a</i>
ISU 3036	<i>rid; fl; sad-2^Δ::hph A</i>
ISU 3037	<i>rid; fl; sad-2^Δ::hph a</i>
ISU 3111	<i>sad-6^Δ::hph A</i>
ISU 3112	<i>sad-6^Δ::hph a</i>
ISU 3113	<i>fl; sad-6^Δ::hph A</i>
ISU 3114	<i>rid his-3; mus-52^Δ::bar; VIII::r^{ef4}-hph A</i>
ISU 3115	<i>rid; fl; VIII::r^{ef4}-hph a</i>
ISU 3116	<i>rid; fl; mus-52^Δ::bar; VIII::r^{ef2}-hph a</i>
ISU 3117	<i>rid his-3; VIII::r^{ef2}-hph A</i>
ISU 3118	<i>rid his-3; mus-52^Δ::bar; VIII::r^{ef1}-hph A</i>
ISU 3119	<i>rid his-3; mus-52^Δ::bar; VIII::r^{ef3}-hph A</i>
ISU 3121	<i>rid his-3; gfp-sad-6-hph a</i>
ISU 3122	<i>rid his-3; gfp-sad-5-hph A</i>
ISU 3123	<i>rid; mus-52^Δ::bar mCherryNC-spo76::hph a</i>
ISU 3124	<i>rid his-3; mus-52^Δ::bar; VIII::r^{ef5}-hph A</i>
ISU 3127	<i>rid; fl; VIII::r^{ef5}-hph a</i>
ISU 3141 ^a	<i>rid his-3; VIII::r^{ef6}-hph A</i>
ISU 3143 ^a	<i>rid; fl; VIII::r^{ef1}-hph a</i>
ISU 3154 ^b	<i>r^Δ::hph; sad-6^Δ::hph A</i>
P3-07	Oak Ridge wild type (WT) A
P6-07	<i>rid A</i>
P8-01	<i>sad-2^Δ::hph A</i>
P8-43	<i>rid his-3; mus-52^Δ::bar A</i>
P9-42	Oak Ridge wild type (WT) a
P10-15	<i>rid his-3 A</i>
P12-01	<i>r^Δ::hph A</i>
P15-22	<i>rid his-3; mus-52^Δ::bar; gfp-sms-2::hph A</i>

All strains used in this study are descendants of lines 74-OR23-1VA (FGSC 2489) and 74-ORS-6a (FGSC 4200) (Perkins 2004).

^a The allele for *mus-52* was not determined (*mus-52⁺* or *mus-52^Δ::bar*).

^b The allele for *rid* was not determined (*rid⁺* or *rid*).

primers used to construct all transformation vectors are listed and described in [Supporting Information, Table S1](#).

MSUD suppression assays

MSUD efficiency was quantified with unidirectional or bi-directional crosses. The general method used for unidirectional crosses, where conidia from a “male” strain are used to fertilize protoperithecia (mating structures) of a “female” strain, is depicted in Figure 1, A–D. Detailed methods have been previously described (Hammond *et al.* 2011a). The general method used for bidirectional crosses, where both parents act as males and females, is depicted in Figure 1, E–H. For the bidirectional crosses, ascospores were collected from the lids of the crossing plates 27 days postinoculation for analysis by low-magnification (100×) light microscopy. At least three replicates were performed for each cross.

RNA sequencing and analysis

We produced two independent RNA-seq datasets for this study. One was produced from strains with a *rid⁺* background (F2-01 × P9-42), while the other was produced from strains with a *rid* background (F2-26 × P6-07). *rid* stands for *repeat-induced point mutation (RIP) defective* (Freitag *et al.* 2002).

Total RNA for RNA-seq was extracted from perithecia and associated vegetative tissue from 6-day-old directional crosses with TRIzol (Life Technologies, Grand Island, NY). The extracts were purified from residual genomic DNA and other potential contaminants with the PureLink RNA Mini Kit (Life Technologies). The RNA samples were then sent to the University of Illinois (Roy J. Carver Biotechnology Center, Urbana–Champaign, IL) where they were treated with the RiboZero Human/Mouse/Rat Kit (Illumina, San Diego) and used to make RNA libraries with a TruSeq Stranded RNA-seq Sample Prep Kit (Illumina). The pooled libraries were then quantified by quantitative PCR (qPCR) and sequenced on one lane for 101 cycles from one end of the cDNA fragments on a HiSeq2500 2000, using the TruSeq SBS sequencing kit (version 3; Illumina). FASTQ files were generated with CASAVA (version 1.8.2; Illumina). The two data files have been deposited in the National Center for Biotechnology Information’s (NCBI) Sequence Read Archive (SRA) (Leinonen *et al.* 2011). The accession numbers are SRR957218 for the *rid⁺* cross and SRR957223 for the *rid* mutant cross.

For comparison of vegetative and sexual transcripts, *N. crassa* vegetative phase RNA-seq datasets SRR90363, SRR90364, and SRR90366 (Ellison *et al.* 2011) were downloaded from the SRA. RNA sequences from the vegetative and sexual datasets were aligned to all predicted *N. crassa* transcripts with Bowtie [version 2-2.1.0 (Langmead and Salzberg 2012)]. Reads per kilobase of exon model per million mapped reads (RPKM) (Mortazavi *et al.* 2008) were calculated with the aid of custom Perl scripts and Microsoft Excel.

Phylogenetic analysis of SAD-6

Amino acid sequences were aligned with Muscle, Version 3.8.31 (Edgar 2004) and a neighbor-joining tree was constructed using the p-distance method and a bootstrap test of 1000 replicates in MEGA5 (Tamura *et al.* 2011).

Results

Deletion of *ncu06190* suppresses MSUD

We recently developed a high-throughput genetic screen for suppressors of MSUD (Hammond *et al.* 2011a). Preliminary data from this screen suggested that strains deleted of gene *ncu06190* are MSUD-deficient. To follow up on these data, we examined *ncu06190^Δ* in a quantitative assay of MSUD suppression.

Crosses between *N. crassa* wild-type strains typically produce black and football-shaped ascospores. However, when MSUD is active, crosses produce nearly 100% white ascospores when *asm-1⁺* is unpaired and nearly 100% round ascospores when *r⁺* is unpaired (Shiu *et al.* 2001). These aberrant phenotypes can be rescued by suppressing MSUD. Figure 1 details the results of MSUD-suppression assays with strains deleted for *ncu06190*. The results indicate that *ncu06190^Δ* is a suppressor of MSUD (Figure 1, I and J) and that the level of suppression is similar to that of two recently

Figure 1 Deletion of *ncu06190* suppresses MSUD. Unidirectional and bidirectional crosses were performed to test whether deletion of *ncu06190* suppresses MSUD. (A–D) The methods used to quantify MSUD efficiency with unidirectional crosses. (A) The female parent was transferred to a 60-mm petri dish containing SCM and cultured for 6 days. (B) The female strain was fertilized at three locations with a suspension of conidia from the male parent. (C) The cultures were incubated at room temperature for 3 weeks, during which perithecia developed and ascospores were shot to the lids of the petri dishes. (D) Ascospores were collected from the lids and suspended in water for analysis by microscopy. (E–H) The methods used to quantify MSUD efficiency with bidirectional crosses. (E) Conidial suspensions of each parent were transferred to the edges of 100-mm petri dishes containing SCM. (F) Parents grew across the petri dishes. (G) Mating and perithecia development occurred predominantly along the middle of the petri dishes. Conidia developed predominantly along the edges of the petri dishes, but this did not interfere with the experiments. (H) Ascospores were collected from the lids of the petri dishes and suspended in water for phenotypic analysis by microscopy when the crosses were 27 days old. A–H are adapted from Harvey *et al.* (2014). (I) Unidirectional crosses of *wt*, *ncu06190 Δ /sad-6 Δ* , and *sad-2 Δ* (males) to *asm-1 Δ* (female) resulted in

2%, 40%, and 68% black ascospores, respectively, demonstrating that MSUD of unpaired *asm-1 $^{+}$* is suppressed by *ncu06190 Δ /sad-6 Δ* . (J) Unidirectional crosses of *wt*, *ncu06190 Δ /sad-6 Δ* , and *sad-2 Δ* (males) to *r $^{\Delta}$* (female) resulted in 0%, 40%, and 98% football-shaped ascospores, respectively, demonstrating that MSUD of unpaired *r $^{+}$* is suppressed by *ncu06190 Δ /sad-6 Δ* . (K) Bidirectional crosses were performed to determine whether MSUD is completely dysfunctional when *ncu06190 $^{+}$ /sad-6 $^{+}$* is missing from both parents. However, the results indicate that MSUD of unpaired *r $^{+}$* is still partially functional when both parents carry the *ncu06190 Δ /sad-6 Δ* allele (abbreviated as *6 Δ*). (I–K) “*wt*” is used to indicate strains that are wild type for all MSUD genes. The error bars depict standard deviation values. (I and J) Strains: *wt* P3-07, *06190 Δ /sad-6 Δ* ISU 3111, *sad-2 Δ* P8-01, *asm-1 Δ* F3-24, and *r $^{\Delta}$* F2-27. (K) Strains: i, P12-01 $\times$ P9-42; ii, P3-07 $\times$ P9-42; iii, ISU 3111 $\times$ ISU 3112; iv, ISU 3154 $\times$ P9-42; and v, ISU 3154 $\times$ ISU 3112.

identified *sad Δ* alleles [*sad-3 Δ* and *sad-4 Δ* (Hammond *et al.* 2011a, 2013b)]. Traditionally, the name “*sad*” is given to an MSUD suppressor because the first one was discovered during studies of strains that suppress ascus dominance (Aramayo and Metzenberg 1996; Shiu *et al.* 2001). Since deletion of *ncu06190* suppresses MSUD, we have renamed it *sad-6* for suppressor of ascus dominance-6.

SAD-6 is a member of the SNF2 family of chromatin remodeling proteins

The *N. crassa* genome database predicts that *sad-6* encodes an 1870-amino acid polypeptide. A search of NCBI’s conserved domain database with this sequence predicts that SAD-6 has a helicase-like domain (data not shown). This is consistent with a recent survey that has placed SAD-6 in a Rad54-like subgroup of SNF2 helicase-related proteins (Flaus *et al.* 2006).

Rad54 and its homologs have been implicated in nuclear processes, including homologous recombination (Mazin *et al.* 2010; Ceballos and Heyer 2011). Therefore, if SAD-6 has Rad54-like functionality, we would expect to observe it in the nucleus. We tested this hypothesis by tagging SAD-6 with green fluorescent protein (GFP) and examining its localization during meiosis. As predicted, SAD-6 was observed to localize in a diffuse pattern within meiotic nuclei (Figure 2A), similar to that reported for SAD-5 (Figure 2C) (Hammond *et al.* 2013b). To determine that the diffuse localization pattern was not due to a technical artifact, we tagged *N. crassa* SPO76 with mCherryNC and visualized its localization pattern during meiosis. In *Sordaria macrospora*, SPO76 localizes to chromosomal axes during meiosis (Storlazzi *et al.* 2008). Our analysis indicates that SPO76 also localizes to chromosomal axes in *N. crassa* (Figure 2B). More importantly, the contrasting localization patterns for SPO76 and SAD-5/SAD-6

Figure 2 SAD-6 localizes within the nucleus during meiosis. (A and E) SAD-6 displays a diffuse nuclear localization pattern (excluding the nucleolus) within prophase asci (ISU 3036 × ISU 3121). (B and F) SPO76 localizes to chromosomal axes (ISU 3036 × ISU 3123). (C and G) SAD-5 (ISU 3037 × ISU 3122) and (D and H) SMS-2 (ISU 3037 × P15-22) localize within the nuclear and perinuclear regions, respectively, which is consistent with previous findings (Hammond *et al.* 2011b, 2013b). All asci were fixed and prepared for imaging as previously described (Hammond *et al.* 2011b). Images were obtained with a Leica SP2 system. All fluorescent images are shown with original contrast with no σ -curve used. The transmitted light image is shown in grayscale, overlaid with the GFP (green) or mCherryNC (red) image. Bar, 10 μ m.

suggest that the diffuse patterns observed for SAD-5 and SAD-6 are biologically meaningful. We also reexamined a perinuclear protein, SMS-2. As expected, the perinuclear localization pattern of SMS-2 (Figure 2D) (Hammond *et al.* 2011b) contrasted with the nuclear localization patterns of SAD-5 and SAD-6.

The *N. crassa* ortholog of *S. cerevisiae* Rad54 is MUS-25 (Handa *et al.* 2000; Zhang *et al.* 2013). MUS-25 is the only other SNF2-family protein in *N. crassa* predicted to be a member of the Rad54-like group (Flaus *et al.* 2006), and it has been shown to be important for quelling, a vegetative RNA silencing process (Chang *et al.* 2012; Zhang *et al.* 2013). Accordingly, our own search of the *N. crassa* genome with the sequence of SAD-6 identified MUS-25 as the most similar protein of 24 matches (blastp, all *E* values were better than $1e-08$).

To further investigate the relationship between SAD-6 and MUS-25, we created a phylogenetic tree with the sequences of Rad54-like proteins from humans and three non-*Neurospora* model fungi. We also included the sequences for SAD-6 and the five *N. crassa* proteins most similar to it. In this tree, SAD-6 clusters with Rad54-like homologs from other organisms, rather than with other SNF2-family proteins from *N. crassa*. This suggests that SAD-6 and MUS-25 are more closely related to Rad54-like proteins from other organisms than they are to other SNF2 proteins from *N. crassa* (Figure 3).

sad-6 transcript analysis suggests that it has functions outside of meiosis

In a previous study, we used publically available *N. crassa* datasets to examine the transcript levels of MSUD genes under a vegetative culture condition (Hammond *et al.* 2013b). We found that transcripts from MSUD-specific genes are essentially undetectable at this time point in the *N. crassa* life cycle (after 24 hr of vegetative growth). In contrast, transcripts for genes that are shared between MSUD and quelling are easily detected under the same conditions (Hammond *et al.* 2013b).

To determine the expression pattern of *sad-6*, we included it in a similar analysis on three publically available *N. crassa*

vegetative RNA datasets that were different from those analyzed in the previous study. In addition, we produced two new RNA datasets for analysis of transcripts from sexual cultures, *i.e.*, fertilized cultures undergoing meiosis and ascospore development. RPKM values (Mortazavi *et al.* 2008) were then calculated for *sad-6*, *mus-25*, quelling genes, MSUD genes, and two housekeeping genes (Table 2). Transcripts for quelling genes were present at similar levels in vegetative cultures (4.32–155.31 RPKM) and sexual cultures (6.24–247.54 RPKM). In contrast, transcripts for MSUD-specific genes were essentially undetectable in the vegetative RNA datasets (0.00–0.35 RPKM) but were found at relatively high levels in the sexual datasets (9.79–567.76 RPKM). *sad-6* transcripts were at relatively high levels in both vegetative and sexual RNAs (10.93 and 22.61 RPKM), demonstrating that *sad-6*'s expression pattern is more like those of quelling genes than of MSUD-specific genes. Its pattern is also similar to that of *mus-25* (6.56 and 13.51 RPKM), which is known to

Figure 3 Rad54-like proteins from four model fungi and humans. Sequences of Rad54-like proteins from *Homo sapiens*, *S. cerevisiae*, *Schizosaccharomyces pombe*, and *Aspergillus nidulans* (Flaus *et al.* 2006) were downloaded from the NCBI, while the *N. crassa* sequences were downloaded from the *N. crassa* genome database.

Table 2 RNA transcript levels for quelling, MSUD, and other genes in *N. crassa* cultures

Gene name	Gene no.	Vegetative transcript levels (RPKM)	Sexual transcript levels (RPKM)
Quelling			
<i>dcl-1</i> ^a	<i>ncu08270</i>	4.32 ± 0.73	31.96 ± 2.33
<i>dcl-2</i>	<i>ncu06766</i>	17.92 ± 18.61	43.13 ± 2.02
<i>qde-1</i>	<i>ncu07534</i>	16.31 ± 2.07	19.90 ± 4.55
<i>qde-2</i>	<i>ncu04730</i>	155.31 ± 155.10	247.54 ± 2.28
<i>qde-3</i>	<i>ncu08598</i>	11.37 ± 1.97	6.24 ± 0.23
<i>qip</i> ^a	<i>ncu00076</i>	25.01 ± 10.95	99.55 ± 3.46
MSUD			
<i>sad-1</i>	<i>ncu02178</i>	0.35 ± 0.20	16.84 ± 2.82
<i>sad-2</i>	<i>ncu04294</i>	0.02 ± 0.03	35.22 ± 2.13
<i>sad-3</i>	<i>ncu09211</i>	0.02 ± 0.03	23.50 ± 3.82
<i>sad-4</i>	<i>ncu01591</i>	0.15 ± 0.05	9.79 ± 2.65
<i>sad-5</i>	<i>ncu06147</i>	0.00 ± 0.00	13.08 ± 1.85
<i>sms-2</i>	<i>ncu09434</i>	0.04 ± 0.07	567.76 ± 63.63
Rad54-like			
<i>mus-25</i>	<i>ncu02348</i>	6.56 ± 0.43	13.51 ± 3.54
<i>sad-6</i>	<i>ncu06190</i>	10.93 ± 0.71	22.61 ± 1.34
Housekeeping			
<i>β-tubulin</i>	<i>ncu04054</i>	1160.22 ± 70.15	131.34 ± 17.73
<i>actin</i>	<i>ncu04173</i>	2274.02 ± 256.81	583.44 ± 56.30

RPKM, reads per kilobase of exon model per million mapped reads.

^a *dcl-1* and *qip* have roles in both MSUD and quelling (Catalanotto *et al.* 2004; Maiti *et al.* 2007; Alexander *et al.* 2008; Lee *et al.* 2010; Xiao *et al.* 2010).

have roles in vegetative and sexual phases of the *N. crassa* life cycle (Handa *et al.* 2000; Zhang *et al.* 2013). Overall, *sad-6*'s expression pattern suggests that it has roles in both vegetative and sexual processes.

Characterization of basic developmental processes in *sad-6*^Δ strains

Deletion or mutation of MSUD genes has not been observed to affect vegetative growth processes, such as growth rate, conidia production, or overall appearance under standard growth conditions (e.g., Hammond *et al.* 2011a, 2013b). We therefore performed assays to determine whether *sad-6*^Δ would be the first *sad* mutant associated with such defects. Like previously characterized *sad*^Δ strains, *sad-6*^Δ appears to have no effect on growth rate or morphological features of *N. crassa* cultures under standard growth conditions (Figure 4, A and B).

With respect to sexual development, many MSUD genes are essential. *sad-1*⁺, for example, is absolutely required for meiosis and crosses homozygous for *sad-1*^Δ are infertile (Shiu *et al.* 2001). However, we have recently found that not all MSUD genes are required for meiosis. An example is *sad-5*⁺, whose deletion from both parents of a cross has no detectable effect on sexual development and ascospore production (Hammond *et al.* 2013b). We thus sought to examine whether *sad-6*⁺ is essential for sexual development. We found that homozygous *sad-6*^Δ crosses complete meiosis and produce abundant levels of ascospores, demonstrating that *sad-6*⁺ is not critical for this part of the *N. crassa* life cycle (Figure S1).

MSUD detects unpaired DNA with a spatially constrained search for DNA homology

To gain new insight into the mechanism of unpaired DNA detection during MSUD, we tested its limits by placing an

ectopic fragment of *r*⁺ (*r*^{ef}) at slightly different locations on chromosome VII in different strains (Table 3; Figure 5, A and B). We then performed a series of crosses between the strains and examined the efficiency of MSUD in each cross. Our findings demonstrate that when two *r*^{ef} are separated by a short distance (4.1 kb) on different homologs, they barely trigger MSUD even though they are at different positions (Figure 5C). However, as the distance between two *r*^{ef} increases, the strength of MSUD also increases (Figure 5C). This suggests that the closer the fragments are to one another, the more likely they are to escape detection by MSUD. Surprisingly, even when fragments are separated by a distance of 37.6 kb, they often escape detection. Various interpretations of these data and how they could result from a SAD-6-mediated homology search are discussed below.

MSUD is partially functional in homozygous *sad-6*^Δ crosses

The fact that *sad-6*^Δ strains can be crossed to one another allowed us to test whether MSUD is completely deficient in crosses where both parents lack *sad-6*⁺. Surprisingly, MSUD was partially functional in such crosses (Figure 1K), suggesting that another protein is functionally redundant with SAD-6 in MSUD.

Discussion

MSUD can be divided into at least two distinct processes: detection of unpaired DNA and its silencing. Since the initial discovery of MSUD in the laboratory of R. L. Metzenberg, much has been learned about the latter process. Essentially, it appears to be mediated by an elaborate silencing complex that stations itself around the perimeter of the nucleus,

Figure 4 *sad-6Δ* is similar to wild type in vegetative growth and production of asexual spores under standard growth conditions. (A) For each strain, 3 μ l of a conidial suspension (1000 conidia per microliter) was inoculated to the center of a 150-mm culture dish. Three dishes were inoculated for each strain. Photographs were taken after growing the cultures for 8 days at room temperature. Only a single representative photograph is shown for each strain. (B) Linear growth assays were performed in triplicate. Most of the error bars, representing the standard deviation, are too small to be seen in the charts. (A) Left, wt P3-07; right, *sad-6Δ* ISU 3111. (B) Left, P3-07 and ISU 3111; right, P9-42 and ISU 3112.

attempting to prevent mRNAs generated by the unpaired DNA from entering the cytoplasm. In contrast, little is known about how MSUD detects unpaired DNA and initiates the silencing process. Here, we have identified a new suppressor of MSUD: *sad-6Δ*. Since it is a nuclear protein, SAD-6 could directly participate in unpaired DNA detection.

The simplest explanation for the MSUD suppression phenotype in *sad-6Δ* heterozygous crosses is that *sad-6+* encodes an MSUD protein and its absence in one parent results in haploinsufficiency. Additionally, by taking away its pairing partner, the *sad-6Δ* allele may cause the *sad-6+* allele to undergo self-silencing (Shiu *et al.* 2006; Kasbekar 2012). For either scenario, haploinsufficiency and/or self-silencing, MSUD suppression most likely results from insufficient levels of SAD-6.

One unexplained result in this study is the finding that MSUD is partially functional in homozygous *sad-6Δ* crosses. One possibility is that an unidentified protein is functionally redundant with SAD-6. An obvious candidate is MUS-25. Unfortunately, MSUD-suppression assays of *mus-25Δ* with unpaired *asm-1+* and *r+* have produced conflicting results; while some experiments suggested no difference in MSUD efficiency, others suggested a slight suppression (our unpublished results). This variability may be related to the aberrant meiosis and low

Table 3 Locations of *r^{ef}* insertions

<i>r^{ef}</i> vector no.	Location
r1	VII: 214,752–214,773
r2	VII: 218,848 –219,011
r3	VII: 232,895 –232,984
r4	VII: 241,034 –241,159
r5	VII: 256,797 –256,822
r6	VII: 1,918,140 –1,918,315

A 4.1-kb *r^{ef}-hph* fusion construct was placed between the indicated positions on chromosome VII. The sequences between the indicated positions were deleted in the process.

fertility of heterozygous *mus-25+/mus-25Δ* crosses. While we have not investigated a *mus-25Δ sad-6Δ* mutant, it seems likely that such a strain would not be more fertile than the *mus-25Δ* single mutant.

SNF2-family proteins are required for efficient homologous recombination in eukaryotes (Flaus *et al.* 2006; Ceballos and Heyer 2011; Hopfner *et al.* 2012). In *N. crassa*, MUS-25 and at least three other SNF2-family proteins have been implicated in homologous recombination (Handa *et al.* 2000; Zhang *et al.* 2013). These are *swr1* (NCU09993), *chd1* (NCU03060), and *isw1* (NCU03875), two of which are included in Figure 3 because of their similarity to SAD-6. Given the connection between SNF2-family proteins and homologous recombination, it is conceivable that SAD-6 may help detect unpaired DNA during MSUD through a homology search process similar to that used during homologous recombination.

Homologous recombination is one of the methods used to repair double-strand breaks in DNA (San Filippo *et al.* 2008; Jasin and Rothstein 2013). During this process, a Rad51-coated single-stranded (ss) molecule of DNA invades a dsDNA template and searches for homology (Forget and Kowalczykowski 2012; Renkawitz *et al.* 2013). A recently proposed model suggests that Rad54 aids this homology search in at least two ways (Wright and Heyer 2014): first, by driving together heteroduplex DNA, which consists of the invading strand and its complement within the original dsDNA template; and second, by dissociating the invading strand if it does not encounter a fully homologous complement. The close relationship between SAD-6 and Rad54 suggests that SAD-6 could promote homology identification in a similar manner.

It is also possible that the strand-invasion-based homology search used for homologous recombination is not related to the unpaired DNA detection process of MSUD. Recent evidence suggests that the homology search for *N. crassa* RIP (discussed below) does not require dsDNA strand breaks (Gladyshev and Kleckner 2014). If a similar (break-independent) process is used to identify homology during MSUD, then perhaps SAD-6's role in MSUD is to remodel chromatin. Accordingly, some SNF2-family members are chromatin remodelers and even Rad54 has been shown to possess the ability to translocate on DNA and slide nucleosomes (Alexeev *et al.* 2003; Amitani *et al.* 2006; Ryan and Owen-Hughes 2011). SAD-6's role in MSUD could thus be to clear DNA of

Figure 5 Unpaired DNA detection in *N. crassa* is spatially constrained. (A) Six locations (r1–r6) on chromosome VII were chosen for the insertion of an ectopic fragment (r^+ ^{ef}) of r^+ . Predicted genes (black boxes) are labeled with their database numbers. Their coding directions are indicated with a “+” (for left to right) and a “–” (for right to left). Distances between the insertion sites are shown below the dashed lines. (B) The components of the r^+ ^{ef} construct are shown. The native r^+ coding region is 3.3 kb long and is found on chromosome I. A 2.3-kb fragment was taken from the 3' end and joined to a hygromycin resistance cassette (*hph*) to create the 4.1-kb r^+ ^{ef}-*hph* fusion construct. Note that each transgenic strain carries, at most, one r^+ ^{ef} insertion. “wt” is used to indicate a strain that does not carry an r^+ ^{ef} insertion. Full genotypes for each strain are listed in Table 1. (C) A series of directional crosses was performed. When a strain carrying an r^+ ^{ef} is crossed to a wt strain, close to 100% round ascospores are observed (left, 1 r^+ ^{ef}, unpaired). This indicates that the r^+ ^{ef} is detected as unpaired in such crosses, and as a result the native r^+ gene on chromosome I is silenced. When a strain carrying an r^+ ^{ef} is crossed to a strain carrying an r^+ ^{ef} at the exact same position, relatively few round ascospores are produced (right, 2 r^+ ^{ef}, paired). This indicates that the r^+ ^{ef} are not detected as unpaired in such crosses. Therefore, the native r^+ on chromosome I is expressed at normal levels. When strains carrying different r^+ ^{ef} are crossed, the percentage of round ascospores is proportional to the distance between the markers (middle, 2 r^+ ^{ef}, unpaired). For example, when two r^+ ^{ef} are separated by a small distance of 4.1 kb, only 5% round ascospores are produced. When two r^+ ^{ef} are separated by 37.6 kb, the level of round ascospores increases to 68% (r2 × r5). This suggests that the closer the r^+ ^{ef} are to one another on the homologs, the less likely they are to be detected as unpaired. These data represent a fraction of the crosses performed to test the relationship between unpaired DNA detection and distance. Please see Table S2 for a complete list of crosses and results. Crosses: 2, F2-26 × ISU 3118; 3, F2-26 × ISU 3117; 17, F2-26 × ISU 3119; 29, F2-26 × ISU 3114; 28, F2-26 × ISU 3124; 53, ISU 3143 × ISU 3141; 18, ISU 3116 × ISU 3124; 20, ISU 3127 × ISU 3119; 12, ISU 3116 × ISU 3119; 32, ISU 3115 × ISU 3118; 33, ISU 3116 × ISU 3114; 6, ISU 3116 × ISU 3118; 22, ISU 3127 × ISU 3124; 38, ISU 3115 × ISU 3114; 11, ISU 3116 × ISU 3117; 39, F2-26 × P10-15.

associated proteins, making the search for unpaired DNA possible by homologous recombination-related or unrelated mechanisms.

In addition to MSUD, *N. crassa* possesses another genome-scanning process called RIP, which occurs just before meiosis and involves a search for repeated sequences (Galagan and Selker 2004). The scanning process of MSUD may not need to be as broad as it is in RIP and other genome-wide scanning processes; that is, it should be necessary only to compare allelic positions between the paired homologs. However, it may not be as straightforward as lining up the homologs and testing the bases “one by one.” In fact, the experimental results reported here have shown that DNA does not have to be perfectly allelic to be considered paired. Unpaired DNA detection thus seems to involve a certain level of chance and the probability of marking homologous DNA fragments as paired decreases as the distance between them increases.

This inexact method of homology searching could be a common characteristic of processes that involve identifying homologous sequences within a genome, which would help explain how such searches are achieved in an energy-efficient and timely manner.

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Supporting Information

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Efficient Detection of Unpaired DNA Requires a Member of the Rad54-Like Family of Homologous Recombination Proteins

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Figure S1 *sad-6^Δ* crosses are homozygous-fertile. Directional crosses were performed by growing the female strain on SCM for 5 days before qualitative transfer of conidial suspensions in water of each male strain to the surface of each female mycelium. Perithecia were allowed to develop for 14 days before dissection. Rosettes of asci were photographed with a VanGuard 1433PHi Compound Microscope with an attached 10 megapixel digital camera (MP1000, AmScope). Strains: (A) F2-01 $\times$ ISU 3112; (B) F2-01 $\times$ P9-42; (C) ISU 3113 $\times$ ISU 3112; (D) ISU 3113 $\times$ P9-42. These data show that *sad-6* is not critical for mating, meiosis, or ascospore production.

Table S1 Oligonucleotide primers used in this study.

Name	Sequence
<i>r^{ef}</i> fragment amplification	
Eco-RSP-F	CAGAATTCAGTCGAGGACAGAACGCAGCA
Eco-RSP-R	TTGAATTCCTGGACCTCTCCGCAGTTTC
<i>hph</i> marker amplification	
APAI-HPH-F	AAGGGCCCAACTGATATTGAAGGAGCAT
APAI-HPH-R	AAGGGCCCAACTGTTCCCGGTCGGCAT
<i>r^{ef}</i> - <i>hph</i> amplification (center fragment for DJ-PCR)	
Rsp-center-A	AGGACAGAACGCAGCAGCAGAGC
Rsp-center-B	ACAGCGAACGAAACCCCTGAAAC
<i>r^{ef1}</i> - <i>hph</i> insertion between <i>ncu09443</i> and <i>ncu09444</i>	
Rsp-040613-C	TAGTGGAGGGGCTTGGGATGGT
Rsp-040613-D	AGAGAAGCTCTGCTGCTGCGTTCTGCTCTGCTGAACGAACACCCCTGCT
Rsp-040613-E	TAACGGGTTTCAGGGGTTTCGTTTCGCTGCTCGTCCACTGATCTTCGCTAGAAATT
Rsp-040613-F	TCACCGCCCGTCCCTACTATCA
Rsp-040613-G	GCCTTGGAAGTGGTATGGTGCT
Rsp-040613-H	GGAGGAGTCGGTTTGCTTTGGTG
<i>r^{ef2}</i> - <i>hph</i> insertion between <i>ncu09444</i> and <i>ncu09445</i>	
Rsp-040613-I	ATGAGGGAGGTGCCGTGTCC
Rsp-040613-J	AGAGAAGCTCTGCTGCTGCGTTCTGCTCTCCATTCTGCCATTTCCCATGC
Rsp-040613-K	TAACGGGTTTCAGGGGTTTCGTTTCGCTGCTCGCACACTTTCTCCACCCATC
Rsp-040613-L	GCAATCCACCTCTGGCATCGAC
Rsp-040613-M	AGCCAATCCTTTACCGACTCCAACA
Rsp-040613-N	GTGGTTCTCGCCGCTTTCAAC
<i>r^{ef3}</i> - <i>hph</i> insertion between <i>ncu09449</i> and <i>ncu09450</i>	
RSP-042613-A	CGAGGGCCGAGTCTGGTGGTTA
RSP-042613-B	AGAGAAGCTCTGCTGCTGCGTTCTGCTCTGCTACTAGCGTTTGCGCGGGACA
RSP-042613-C	TAACGGGTTTCAGGGGTTTCGTTTCGCTGTAGGTGGGAAAGTGTTAGTGGTGGA
RSP-042613-D	GTTGAGGGTCTTGAGGGCGAAG
RSP-042613-E	TCTCACACGTTGCTTCGGCTGT
RSP-042613-F	GAGTTCTGGTTGGCTGGTTGG
<i>r^{ef4}</i> - <i>hph</i> insertion between <i>ncu09451</i> and <i>ncu17161</i>	
RSP-042613-A	AAGTGGGCGTTGAAGGAGGATG
RSP-042613-B	AGAGAAGCTCTGCTGCTGCGTTCTGCTCTCGGAGGTCGGAGACGAGATG
RSP-042613-C	TAACGGGTTTCAGGGGTTTCGTTTCGCTGTCCAAGTCTCCATCCGTTCCATC
RSP-042613-D	TTCATCCAGCAATCCACCACCA
RSP-042613-E	CCTTTCACCTCTACCCAAACGA
RSP-042613-F	AGCGACCATCCCAAACCAACA
<i>r^{ef5}</i> - <i>hph</i> insertion between <i>ncu09455</i> and <i>ncu09456</i>	
RSP-050213-A	CAGACAGTGGTGGGAAGGTGGTC
RSP-050213-B	AGAGAAGCTCTGCTGCTGCGTTCTGCTCTCAGTGCAGGAAATGGAAGGGAGAG
RSP-050213-C	TAACGGGTTTCAGGGGTTTCGTTTCGCTGCTCGGCCATCACGGTCAAAGAAAC
RSP-050213-D	ATGGTGCCGACGCTAAAGGAGA
RSP-050213-E	CGTTCCGTCATTGCGGTATTGC
RSP-050213-F	ACGCAGGGAGGGAGATTGCCTA
<i>r^{ef6}</i> - <i>hph</i> insertion between <i>ncu06068</i> and <i>ncu06067</i>	
RSP-061813Y	AGCTCGTTTGGGTATCAGCAGTCC
RSP-061813Z	AGAGAAGCTCTGCTGCTGCGTTCTGCTCTGGATGCAAGGGCGAGAGTCAA

RSP-061813AA	TAACGGGTTTCAGGGGTTTCGTTCTGCTGTGCGAAACCCTGGAGATAACGGAAG
RSP-061813AB	GGCGTCGGCAACTGAAGGAC
RSP-061813AC	CGTGGGAAGCGAGGTGAGAGG
RSP-061813AD	CGTGGTCTGTGTGTGTGTGGTCTG

Construction of the *gfp-sad-6 gfp* tagging vector

NCU06190-E	TTGAAAATGCGAGGATAAGACGAAGA
NCU06190-NGFP1	GCAGCCTGAATGGCGAATGGACGCGCCAAGTGTGAAAGCAATCTGTGTTGGA
NCU06190-NGFP2	CAGGAGCGGGTGCGGGTGCTGGAGCGATGGCCGAACTCAACGAAAATGAACC
NCU06190-F	CCTCTCCAAAGTCCAAGACGACCT
NCU06190-G	TGCCCAACAGATAACGTGACTTCG
NCU06190-H	TTGGCATCGGAAAGAAAGGTGCT

Construction of the *mCherryNC-spo76* tagging vector

NCU00424-E	ACTCGCAAGCAAGGCACTGAA
NCU00424-X1	GCAGCCTGAATGGCGAATGGACGCGCCCTGCGTATGATCTTGAGGACGAG
NCU00424-X2	CAGGAGCGGGTGCGGGTGCTGGAGCGATGGCGCCACGTCGAAGCGCTC
NCU00424-F	TCACTTGGGGTTCCGCTCTTTCT
NCU00424-G	CAAAGGCCCCCATCCAGTACGA
NCU00424-H	TCTTTTCAACCTGCTCTCCCTTG

r^{ef} fragment amplification primers were used to amplify a fragment of the *r^{*}* gene.

hph marker amplification primers were used to amplify the *hph* fragment.

r^{ef}-hph amplification primers. The *r^{ef}* fragment and the *hph* marker were placed next to each other in a plasmid. These primers amplified the *r^{ef}* fragment and the *hph* marker from the plasmid to serve as the center product in DJ-PCR (Yu *et al.* 2004).

r^{ef1-6}-hph insertions: For each set of *r^{ef}-hph* insertion primers, the first two primers were used to amplify the left flank, the middle two primers were used to amplify the right flank, and the last two primers were used for nested amplification of the final vector.

The *gfp-sad-6* and *mCherryNC-spo76* primers were used to make tagging vectors for *sad-6* and *spo76* by DJ-PCR as previously described (Hammond *et al.* 2011b). The center product for *gfp* tagging was obtained from plasmid pTH1117.12 (Hammond *et al.* 2011b). The center product for *mCherryNC* tagging was obtained from plasmid pEBTH1252.3, which is similar to pTH1117.12 except that *gfp* sequences are replaced with *mCherryNC* sequences.

Table S2 Crosses performed for the unpaired DNA masking experiments.

Cross #	Strain names	r^{ef} locations	Round spores (%)	STDEV
1	F2-26 × P10-15	wt × wt	0.00	0.00
2	F2-26 × ISU 3118	wt × r1	0.99	0.00
3	F2-26 × ISU 3117	wt × r2	0.96	0.03
4	ISU 3116 × P10-15	r2 × wt	0.98	0.01
5	ISU 3116 × ISU 3117	r2 × r2	0.00	0.00
6	ISU 3116 × ISU 3118	r2 × r1	0.05	0.01
7	F2-26 × P10-15	wt × wt	0.01	0.00
8	ISU 3116 × P10-15	r2 × wt	0.92	0.06
9	F2-26 × ISU 3117	wt × r2	0.94	0.05
10	F2-26 × ISU 3119	wt × r3	0.87	0.15
11	ISU 3116 × ISU 3117	r2 × r2	0.00	0.00
12	ISU 3116 × ISU 3119	r2 × r3	0.34	0.06
13	ISU 3116 × P10-15	r2 × wt	1.00	0.00
14	ISU 3127 × P10-15	r5 × wt	0.99	0.01
15	F2-26 × ISU 3124	wt × r5	0.97	0.01
16	F2-26 × ISU 3117	wt × r2	0.90	0.05
17	F2-26 × ISU 3119	wt × r3	0.89	0.05
18	ISU 3116 × ISU 3124	r2 × r5	0.68	0.02
19	ISU 3127 × ISU 3117	r5 × r2	0.54	0.04
20	ISU 3127 × ISU 3119	r5 × r3	0.42	0.06
21	ISU 3116 × ISU 3119	r2 × r3	0.19	0.03
22	ISU 3127 × ISU 3124	r5 × r5	0.00	0.00
23	F2-26 × P10-15	wt × wt	0.00	0.00
24	ISU 3116 × ISU 3117	r2 × r2	0.00	0.00
25	ISU 3115 × P10-15	r4 × wt	1.00	0.00
26	ISU 3116 × P10-15	r2 × wt	0.97	0.02
27	F2-26 × ISU 3118	wt × r1	0.98	0.02
28	F2-26 × ISU 3124	wt × r5	0.97	0.04
29	F2-26 × ISU 3114	wt × r4	0.95	0.01
30	F2-26 × ISU 3117	wt × r2	0.80*	0.15
31	ISU 3116 × ISU 3124	r2 × r5	0.67	0.04
32	ISU 3115 × ISU 3118	r4 × r1	0.34	0.07
33	ISU 3116 × ISU 3114	r2 × r4	0.29	0.06
34	ISU 3115 × ISU 3117	r4 × r2	0.21	0.06
35	ISU3115 × ISU 3124	r4 × r5	0.21	0.08
36	ISU 3116 × ISU 3118	r2 × r1	0.05	0.02
37	ISU 3116 × ISU 3117	r2 × r2	0.00	0.01
38	ISU 3115 × ISU 3114	r4 × r4	0.00	0.00
39	F2-26 × P10-15	wt × wt	0.00	0.00
40	F2-26 × P10-15	wt × wt	0.00	0.00

41	F2-26 × ISU 3118	wt × r1	0.98	0.01
42	F2-26 × ISU 3117	wt × r2	0.95	0.03
43	F2-26 × ISU 3119	wt × r3	0.97	0.01
44	F2-26 × ISU 3114	wt × r4	0.99	0.01
45	F2-26 × ISU 3124	wt × r5	0.99	0.01
46	F2-26 × ISU 3141	wt × r6	0.98	0.01
47	ISU 3143 × P10-15	r1 × wt	0.98	0.01
48	ISU 3143 × ISU 3118	r1 × r1	0.00	0.00
49	ISU 3143 × ISU 3117	r1 × r2	0.05	0.00
50	ISU 3143 × ISU 3119	r1 × r3	0.43	0.05
51	ISU 3143 × ISU 3114	r1 × r4	0.49	0.06
52	ISU 3143 × ISU 3124	r1 × r5	0.84	0.07
53	ISU 3143 × ISU 3141	r1 × r6	0.99	0.00

This table is a complete list of crosses performed during the unpaired DNA masking experiments. The average percentage of round spores produced by each cross (in triplicate) and its standard deviation value are indicated. *An outlier believed to be the result of an experimental artifact.