Novel Proteins Required for Meiotic Silencing by Unpaired DNA and siRNA Generation in *Neurospora crassa*

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ABSTRACT During meiosis in the filamentous fungus *Neurospora crassa*, unpaired genes are identified and silenced by a process known as meiotic silencing by unpaired DNA (MSUD). Previous work has uncovered six proteins required for MSUD, all of which are also essential for meiotic progression. Additionally, they all localize in the perinuclear region, suggesting that it is a center of MSUD activity. Nevertheless, at least a subset of MSUD proteins must be present inside the nucleus, as unpaired DNA recognition undoubtedly takes place there. In this study, we identified and characterized two new proteins required for MSUD, namely SAD-4 and SAD-5. Both are previously uncharacterized proteins specific to Ascomycetes, with SAD-4 having a range that spans several fungal classes and SAD-5 seemingly restricted to a single order. Both genes appear to be predominantly expressed in the sexual phase, as molecular study combined with analysis of publicly available mRNA-seq datasets failed to detect significant expression of them in the vegetative tissue. SAD-4, like all known MSUD proteins, localizes in the perinuclear region of the meiotic cell. SAD-5, on the other hand, is found in the nucleus (as the first of its kind). Both proteins are unique compared to previously identified MSUD proteins in that neither is required for sexual sporulation. This homozygous-fertile phenotype uncouples MSUD from sexual development and allows us to demonstrate that both SAD-4 and SAD-5 are important for the production of masiRNAs, which are the small RNA molecules associated with meiotic silencing.

EUKARYOTIC genomes are protected from viruses and transposons by a variety of defenses, many of which are based on RNA interference (RNAi). In a typical RNA silencing process, a double-stranded RNA is cleaved into small RNAs of 21–25 nt by an RNase III enzyme known as Dicer (Chang *et al.* 2012). An Argonaute-containing complex incorporates these small RNA species and uses them to guide transcriptional or post-transcriptional gene silencing.

Neurospora crassa, a filamentous fungus, is protected by at least two RNA silencing processes. The first process, called quelling (Romano and Macino 1992), defends the *N. crassa* genome from repetitive elements such as transposons (Nolan *et al.* 2005). The quelling machinery may also play an important role in rDNA stability and DNA damage response (Cecere and Cogoni 2009; Lee *et al.* 2009). The second defense process, known as meiotic silencing by unpaired DNA (MSUD) (Shiu *et al.* 2001), works specifically in meiotic cells and silences genes that are not paired between homologous chromosomes (Kelly and Aramayo 2007; Chang *et al.* 2012). Because parental genomes are likely to have differentially located transposons, MSUD is well suited to protect an organism from their amplification during meiosis.

In *N. crassa*, meiosis and sexual spore (ascospore) formation take place in specialized sac cells (asci). During homolog pairing, MSUD scans for the presence of unpaired DNA. If such unpaired DNA is detected, MSUD will silence the expression of this and all homologous copies. For example, if an extra copy of *Ascospore maturation-1* (*asm-1*$^{+}$) (Aramayo and Metzenberg 1996) is placed at an ectopic location in one parent but not the other, all copies of this gene (paired or unpaired) are silenced during sexual development, resulting in the production of white and inviable ascospores (Shiu

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et al. 2001). MSUD appears to be a robust mechanism and additional genes have been successfully used as reporting markers for its activity. These include *actin* (act^+) and *β-tubulin* (bml^R), whose unpairings result in the abortion of most asci, and *Round spore* (r^+), whose unpairing leads to the production of round ascospores (instead of spindle-shaped ones).

After an unpaired DNA is detected, the working model of MSUD holds that an aberrant RNA (aRNA) is transcribed from the unpaired region. This aRNA is then transported to the perinuclear region, where it encounters at least six known MSUD proteins (five of which are related to other RNAi processes). These include SAD-1, an RNA-directed RNA polymerase thought to turn an aRNA into a double-stranded RNA (dsRNA) (Shiu and Metzenberg 2002); SAD-3, a helicase that may help SAD-1 form dsRNAs (Hammond *et al.* 2011a); DCL-1, a Dicer protein that cleaves a dsRNA into small interfering RNAs (siRNAs) (Alexander *et al.* 2008); QIP, an exonuclease that processes siRNAs into single strands (Lee *et al.* 2010a; Xiao *et al.* 2010); SMS-2, an Argonaute protein that uses siRNAs to target complementary mRNAs (Lee *et al.* 2003); and SAD-2, which is the only uncommon RNA silencing protein listed here and may serve as a scaffold for other MSUD proteins in the perinuclear region (Shiu *et al.* 2006). All six of these proteins colocalize in the nuclear periphery, suggesting that they are members of a silencing complex.

While significant progress has been made in deciphering the MSUD processes outside of the nucleus, little is known about what happens inside of it. And although every reported MSUD protein is known to be required for both sexual development and silencing, their exact relationship remains an enigma. In this study, we have identified two novel components of the MSUD machinery and filled in some of the gaps in our knowledge of this unique silencing mechanism.

Materials and Methods

Fungal manipulation and genotypic information

Standard *Neurospora* protocols were used throughout this work (http://www.fgsc.net/Neurospora/NeurosporaProtocolGuide.htm). Fertilization of designated female (*fl*) strains and ascospore quantification were performed as described (Hammond *et al.* 2011a). Strain names and genotypes are listed in Table 1. Genetic markers and knockouts used in this study are originated 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).

Plasmid and strain construction

Oligonucleotide primers used in this study are listed in Supporting Information, Table S1. For construction of *his-3*$^+$::*rfp-sad-4*, the predicted *sad-4* (*NCU01591.5*) coding region was amplified and inserted into the N-terminal red fluorescent protein (RFP) tagging plasmid pMF334 (Freitag and Selker 2005). *gfp-sad-5*::*hph* was constructed with double-joint polymerase chain reactions (DJ-PCR), essentially as described (Hammond *et al.* 2011b). The complementation test described by Shiu *et al.* (2006) (*e.g.*, *sad-2-gfp* rescues the barren phenotype of a *sad-2*–null cross) is not possible here because neither *sad-4* nor *sad-5* is required for fertility. Nevertheless, the two aforementioned fluorescent proteins are not known to accumulate where SAD-4 and SAD-5 are localized.

Table 1 Strains used in this study

Strain	Genotype
F1-05	*fl a*
F2-01	*fl A*
F2-14	*fl a*
F2-25	*rid his-3*$^+$::*asm-1*$^+$; *fl*; *asm-1*$^\Delta$::*mtr*$^+$ *A*
F2-27	*rid r*$^\Delta$::*hph*; *fl a*
F2-29	*rid r*$^\Delta$::*hph*; *fl A*
F2-35	*his-3*::*act*$^+$; *fl A*
F2-36	*his-3*::*bml*^{R}; *fl A*
F2-37	*his-3*::*act*$^+$; *fl a*
F2-38	*his-3*::*bml*^{R}; *fl a*
F3-24	*rid his-3*$^+$::*asm-1*$^+$; *fl*; *asm-1*$^\Delta$::*hph a*
F5-23	*fl A*
F5-32	*sad-4*$^\Delta$::*hph fl A*
F5-33	*sad-4*$^\Delta$::*hph fl A*
F5-35	*fl a*
F5-36	*fl*; *sad-5*$^\Delta$::*hph a*
F5-37	*fl*; *sad-5*$^\Delta$::*hph a*
F5-38	*r*$^\Delta$::*hph*; *sad-4*$^\Delta$::*hph fl A*
F5-39	*r*$^\Delta$::*hph*; *fl A*
P3-07	Oak Ridge wild type (WT) *A*
P3-08	Oak Ridge wild type (WT) *a*
P3-25	*mep sad-1*$^\Delta$::*hph a*
P8-18	*mep sad-1*$^\Delta$::*hph A*
P11-43	*sad-4*$^\Delta$::*hph a*
P11-46	*sad-4*$^\Delta$::*hph A*
P12-01	*r*$^\Delta$::*hph A*
P12-02	*r*$^\Delta$::*hph a*
P13-22	*rid his-3*$^+$::*rfp-sad-4*; *sad-4*$^\Delta$::*hph a*
P15-14	*rid his-3*; *mus-52*$^\Delta$::*bar*; *gfp-sms-2*::*hph A*
P17-57	*sad-5*$^\Delta$::*hph A*
P17-58	*sad-5*$^\Delta$::*hph a*
P17-59	*r*$^\Delta$::*hph*; *sad-4*$^\Delta$::*hph a*
P17-60	*r*$^\Delta$::*hph*; *sad-4*$^\Delta$::*hph a*
P17-61	*sad-4*$^\Delta$::*hph A*
P17-62	*sad-4*$^\Delta$::*hph a*
P17-63	*sad-4*$^\Delta$::*hph a*
P17-64	*a*
P17-65	*A*
P17-66	*sad-5*$^\Delta$::*hph A*
P17-67	*sad-5*$^\Delta$::*hph a*
P17-68	*a*
P17-69	*sad-5*$^\Delta$::*hph A*
P17-70	*r*$^\Delta$::*hph*; *sad-5*$^\Delta$::*hph A*
P17-71	*r*$^\Delta$::*hph*; *sad-5*$^\Delta$::*hph A*
P18-55	*rid his-3*$^+$::*rfp-sad-2*; *gfp-sad-5 a*
P18-57	*rid his-3*$^+$::*rfp-sad-2*; *gfp-sad-5*; *inv sad-2*^{RIP} *A*

Photography and microscopy

For photography of perithecia (fruiting bodies) and asci, a Canon PowerShot S3 IS camera was employed (in

Table 2 ***sad-4*$^{\Delta}$ and *sad-5*$^{\Delta}$ act as semidominant suppressors of MSUD**

Experiment 1	::*act*$^{+}$ (F2-35)	::*bml*^{R} (F2-36)	*asm-1*$^{\Delta}$ (F2-25) (%)	*r*$^{\Delta}$ (F2-29) (%)
WT (P3-08)	36.0×10^3	0.6×10^3	2.7	0.8
sad-4$^{\Delta}$ (P11-43)	173.2×10^3	55.9×10^3	61.6	52.4
sad-1$^{\Delta}$ (P3-25)	285.0×10^3	107.4×10^3	81.0	99.0
	(spores)	(spores)	(black)	(football)
Experiment 2	**::*act*$^{+}$ (F2-37)**	**::*bml*^{R} (F2-38)**	***asm-1*$^{\Delta}$ (F3-24) (%)**	***r*$^{\Delta}$ (F2-27) (%)**
WT (P3-07)	28.8×10^3	0.6×10^3	0.7	0.0
sad-5$^{\Delta}$ (P17-57)	118.9×10^3	12.0×10^3	15.9	0.4
sad-1$^{\Delta}$ (P8-18)	342.9×10^3	398.7×10^3	83.4	98.7
	(spores)	(spores)	(black)	(football)

Each MSUD tester (::*act*$^{+}$, ::*bml*^{R}, *asm-1*$^{\Delta}$, or *r*$^{\Delta}$) is designed to cause the unpairing of a reporter gene during meiosis. When MSUD is proficient (*i.e.*, tester × WT), these unpairings lead to the reduced production of black American football (spindle)-shaped ascospores. When MSUD is suppressed, the phenotypes can be partially (*e.g.*, tester × *sad-4*$^{\Delta}$/*5*$^{\Delta}$) or near-fully (*e.g.*, tester × *sad-1*$^{\Delta}$) restored to normal, depending on the strength of the suppressor.

combination with a VanGuard 1274ZH or 1231CM microscope). For fluorescent microscopy, Zeiss LSM710 and Olympus BX61 were used. Perithecial sample preparation and GFP/RFP/DAPI visualization were essentially as described (Alexander *et al.* 2008; Xiao *et al.* 2010).

Sequence analysis

Accession or genome database numbers for sequences used in this study are listed in Table S2. The protein sequences of SAD-4 (*NCU01591.5*) and SAD-5 (*NCU06147.5*) are available from version 10 of the *N. crassa* genome database (Galagan *et al.* 2003; http://www.broadinstitute.org/annotation/genome/neurospora/MultiHome.html). These were used to predict their molecular weights with the Compute pI/Mw tool (http://web.expasy.org/compute_pi/) and to search the National Center for Biotechnology Information (NCBI) Conserved Domain Database (CDD v.3.03) (Marchler-Bauer *et al.* 2010) for known domains. To identify putative homologs, these sequences were used to search the NCBI protein database (nr) with BLASTP 2.2.26+ and nucleotide collection (nr/nt) with TBLASTN 2.2.26+ (Altschul *et al.* 1997). *Neurospora discreta* homologs were identified and obtained from the Department of Energy (DOE) Joint Genome Institute website (http://genome.jgi.doe.gov/). To identify conserved amino acids among the SAD-4 or SAD-5 homologs, alignments were created with Clustal W (Thompson *et al.* 1994) in BioEdit (Hall 1999). Similarity was determined using the Blosum62 similarity matrix.

Phylogenetic trees

SAD-4 and SAD-5 phylogenetic trees were constructed from Clustal W-based alignments of amino acid sequences (Table S2) with MEGA5 (Tamura *et al.* 2011) using the following parameters: (1) neighbor joining (Saitou and Nei 1987), (2) bootstrapping: 1000 replicates (Felsenstein 1985), (3) p-distance substitution (Nei and Kumar 2000), and (4) gap elimination. Taxonomic classifications were obtained from the NCBI taxonomy database (http://www.ncbi.nlm.nih.gov/taxonomy).

Small RNA sequencing and analysis

Crosses were performed on *Neurospora* crossing medium (Westergaard and Mitchell 1947) overlaid with a single layer of Miracloth (Calbiochem, La Jolla, CA). Five days after fertilization, perithecia were scraped from the Miracloth with a razor blade, weighed, and frozen in liquid nitrogen. A total RNA sample was purified from ~0.8 g of fungal tissue with TRIzol (Invitrogen, Carlsbad, CA), and small RNAs were purified by polyacrylamide gel electrophoresis (PAGE). Small RNA library preparation and sequencing was performed by the University of Missouri DNA Core. Essentially, the TruSeq Small RNA Sample Preparation kit (Illumina, San Diego) was used to prepare indexed small RNA libraries from PAGE-purified small RNAs (15–35 nt). Four libraries were combined and sequenced by the Illumina's HiSeq 2000 sequencing system, and the raw reads were divided into separate files based on their index. These data are available through the NCBI Sequence Read Archive (SRA) database (SRX244308, SRX244469, SRX244665, and SRX244676).

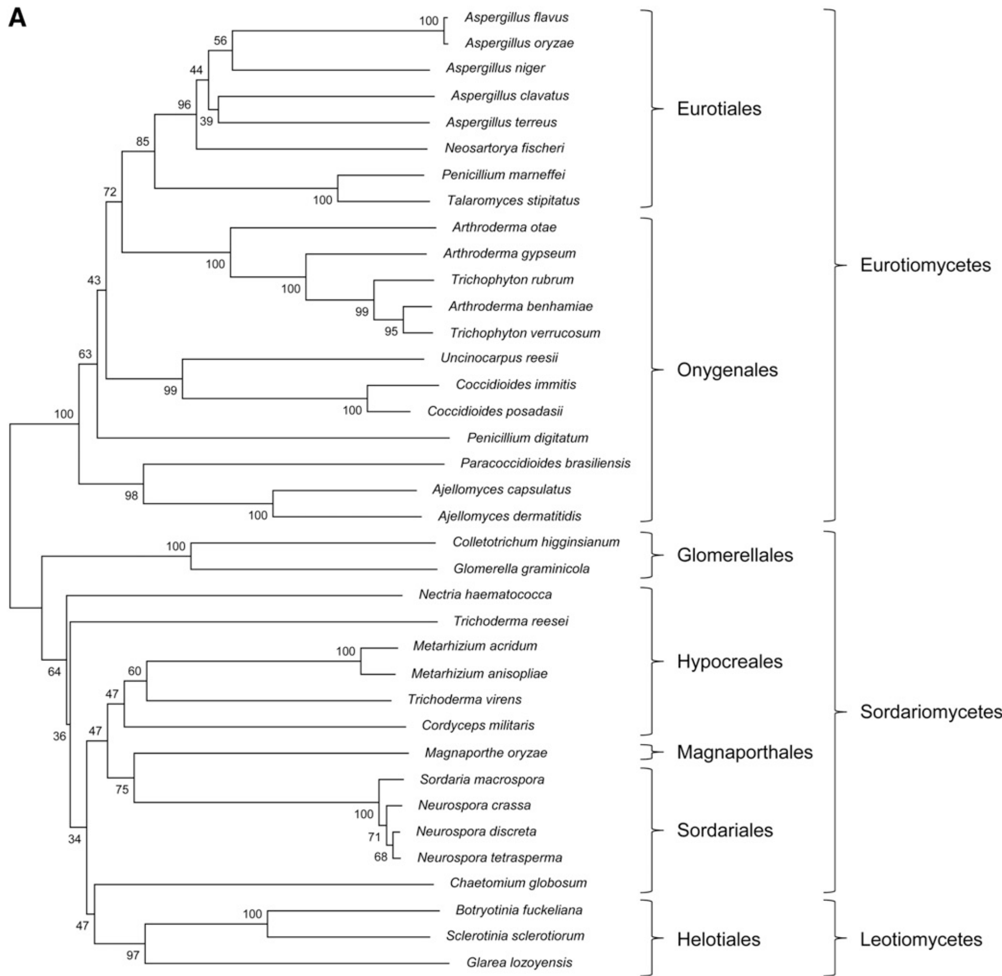
The 3′ adapter sequences were trimmed from the small RNA reads with Cutadapt v.1.0 (Martin 2011). Reads ≥14 nt were aligned to the *N. crassa* genome (Galagan *et al.* 2003). Alignments were performed with Bowtie v.0.12.7 (Langmead *et al.* 2009) and only 18–30 nt reads with no mismatches to the reference genome were included in our final analysis. An overview of the sequencing and alignment data are provided in Table S3.

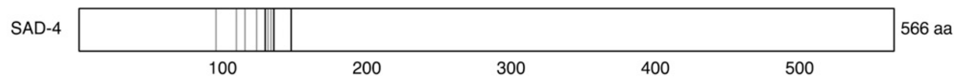
Results

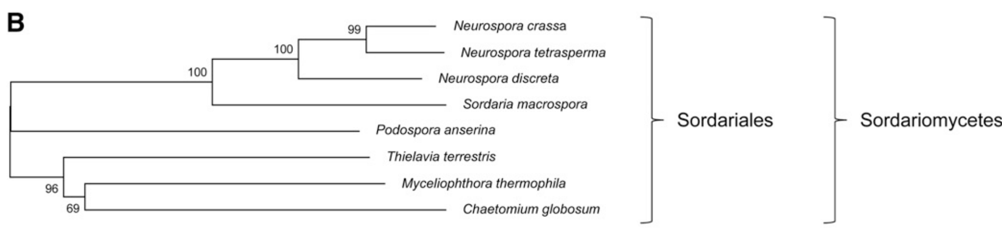
Identification of *sad-4*$^{\Delta}$ and *sad-5*$^{\Delta}$ deletion strains as semidominant MSUD suppressors

Using the high-throughput reverse-genetic screen described by Hammond *et al.* (2011a), we identified four additional strains in the *N. crassa* knockout library (Colot *et al.* 2006) that appeared to be MSUD-deficient. These strains correspond to the putative deletion mutants (in both mating types) of *NCU01591* (FGSC 13237 and 13238) and *NCU06147* (FGSC 17863 and 17864).

To verify that *NCU01591*$^{\Delta}$ and *NCU06147*$^{\Delta}$ truly suppress MSUD, the aforementioned knockout strains were isolated from the library plates and their deletions were confirmed







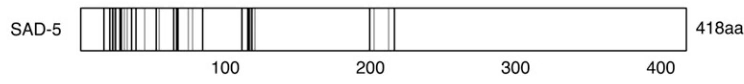


Figure 1 SAD-4 homologs are present in a wide range of Ascomycete fungi, while SAD-5 homologs are restricted to a single order. (A, top) Phylogenetic tree of SAD-4 homologs. Sequence accession numbers are listed in Table S2. Numbers next to branches indicate the percentage of bootstrap support. (Bottom) Conserved amino acids of SAD-4. Strictly conserved residues are solid (identical) and shaded (similar). See Figure S1 for sequence alignment. (B) Phylogenetic tree and conserved amino acids of SAD-5 homologs. The *Thielavia terrestris* SAD-5 sequence is incomplete at both ends. See Figure S2 for sequence alignment.

with standard polymerase chain reaction (PCR) assays. The knockout mutants were then individually tested for their ability to suppress MSUD in crosses with four different MSUD-tester strains, which were designed to create the meiotic unpairing of either *actin* (::*act*$^{+}$), *Ascospore maturation-1* (*asm-1*$^{\Delta}$), β-*tubulin* (::*bml*^{R}), or *Round spore* (r^{Δ}). Unpairing of these genes leads to abnormal sexual phenotypes (lollipop asci, white ascospores, elongated asci, and round ascospores, respectively) unless MSUD is suppressed (Shiu *et al.* 2001). When an *NCU01591*$^{\Delta}$ strain (P11-43) was crossed to the four

Table 3 Expression of RNA silencing genes during the vegetative phase

Function	Gene	Expression (RPKM)
Housekeeping	*actin*	1634.1998
	β-tubulin	685.3888
Quelling	*dcl-2*	3.9767
	qde-1	11.1222
	qde-2	63.7201
	qde-3	5.1974
Quelling/MSUD	*dcl-1*	3.8117
	qip	10.3140
MSUD	*sad-1*	0.0736
	sad-2	0.0088
	sad-3	0.0213
	sad-4	0.1357
	sad-5	0.0123
	sms-2	0.0225

Neurospora vegetative mRNA-seq datasets were obtained from the NCBI SRA database (SRX033295, SRX033369, SRX033410, SRX033477, SRX033487, SRX033498, and SRX037168–037170) and aligned to predicted *N. crassa* transcripts with Bowtie (Langmead *et al.* 2009). RPKM, reads per kilobase of exon model per million mapped reads (Mortazavi *et al.* 2008).

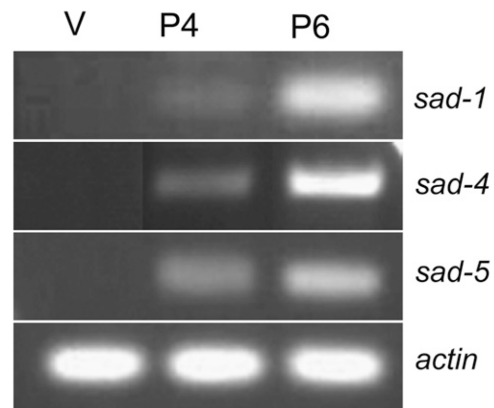


Figure 2 *sad-4* and *sad-5* are expressed in the sexual tissue. *sad-4* and *sad-5* transcripts, like those from *sad-1*, can be detected in the sexual but not vegetative tissue. RT-PCR products from various *sad* genes and *actin* (control) are shown (from top: 250 bp, 1054 bp, 369 bp, and 227 bp). RNAs from vegetative (V; P3-07) and 4/6-day perithecial (P4 and P6; F1-05 × P3-07) preparations were used for the amplification.

testers, MSUD suppression was seen in each case (Table 2). On the other hand, when a similar experiment was performed with an *NCU06147*$^{\Delta}$ strain (P17-57), silencing was suppressed in three of the four test crosses but not the one containing *r*$^{\Delta}$ (Table 2; F2-27 × P17-57). This is reminiscent of the observation by Raju *et al.* (2007), where the MSUD suppressors encoded by *Sk-2* and *Sk-3* also had no effect on *r*$^{\Delta}$. Accordingly, we have assigned the names *suppressor of ascus dominance-4* and *-5* (*sad-4* and *sad-5*) to genes *NCU01591* and *NCU06147*, respectively.

SAD-4 is a novel protein found in three classes of Ascomycete fungi

The translated SAD-4 sequence consists of 566 amino acids with a molecular weight of 60.5 kDa. Although SAD-4 does not contain known functional domains, its homologs can be found among three classes of Ascomycete fungi: Sordariomycetes (*e.g.*, *Neurospora*), Leotiomycetes (*e.g.*, *Sclerotinia*), and Eurotiomycetes (*e.g.*, *Aspergillus*) (Figure 1A). However, SAD-4 is not ubiquitous in these fungal classes as database searches and syntenic analyses failed to reveal similar sequences in *Gibberella zeae*, a Sordariomycete thought to be capable of MSUD (Son *et al.* 2011), and *Aspergillus nidulans*, a Eurotiomycete whose *sad* genes are degenerating (Hammond *et al.* 2008). Other exceptions include the dung-inhabiting *Podospora anserina* as well as the thermophilic biomass-degrading *Thielavia terrestris* and *Myceliophthora thermophila*. This is particularly interesting because although these three lack a SAD-4 sequence, they are among the relatively few fungi that encode a SAD-5 homolog (see below).

The aforementioned SAD-4 homologs appear to be orthologous (separated by speciation) rather than paralogous (separated by duplication). This notion is supported by the syntenic finding that many of the *sad-4*–like sequences were found adjacent to *hsp60*, a highly conserved heat-shock protein-encoding gene present in both prokaryotic and eukaryotic organisms (Johnson *et al.* 1989). These include the *sad-4* homolog identified in *Aspergillus terreus*, which is one of the most distant species from *N. crassa* in our phylogenetic analysis.

Despite their orthologous nature, SAD-4 sequences are quite diverse. For example, while the *N. crassa* and *N. tetrasperma* orthologs are 99% alike, the *N. crassa* and *Trichoderma reesei* orthologs share a similarity level of only 21%. Nevertheless, a highly conserved region (positions 94–147) exists among these SAD-4 orthologs from three different classes of fungi (Figure 1A and Figure S1). The conservation of these amino acids suggests that they could constitute an important functional motif.

SAD-5 is a novel protein restricted to a single order of Sordariomycete fungi

sad-5 encodes a 418-aa (47.8 kDa) polypeptide with no known conserved domains. A search of the NCBI and the *N. discreta* genome databases identified seven SAD-5 homologs (Figure 1B). Syntenic analysis indicated that SAD-5, like SAD-4, is orthologous to its homologs.

The Sordariales is one of several orders that fall under the class Sordariomycetes. The failure to identify SAD-5 homologs in any other Sordariomycete order suggests that SAD-5 is specific to the Sordariales. To investigate this possibility more closely, the most likely locations of *sad-5* were identified in four fungi from other Sordariomycete orders by searching their genomes with *sad-5* flanking sequences. Analysis of these regions in *Glomerella graminicola* (of Glomerellales), *Magnaporthe oryzae* (of Magnaporthales), *G. zeae* (of Hypocreales), and *Grosmannia clavigera* (of Ophiostomatales) showed that synteny collapses near the predicted location of *sad-5* in each case, suggesting that these species have indeed lost their *sad-5* orthologs.

Although the SAD-5 orthologs appear to be specific to the Sordariales, a wide range of sequence variation can still be

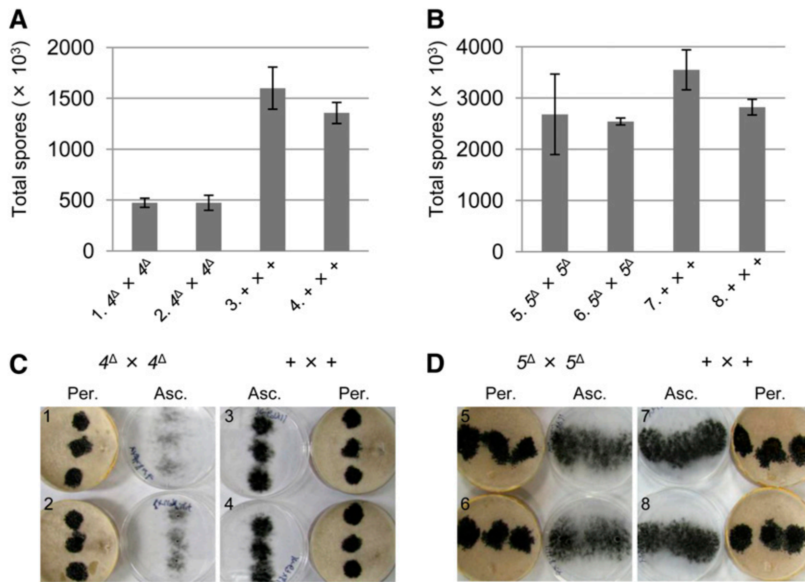


Figure 3 Crosses homozygous for *sad-4*$^{\Delta}$ or *sad-5*$^{\Delta}$ are fertile. (A) Deletion of *sad-4* from both parents reduces, but not prevents, ascospore production. (B) Deletion of *sad-5* from both parents does not affect ascospore production. (C and D) Pictures of crossing plates. Lids have been removed and placed next to the crossing plates to show both perithecial (Per.) and ascospore (Asc.) levels. Note that while *sad-4*–null and wild-type crosses have similar perithecial levels, their ascospore levels are different. Three replicates were performed for each cross, with the error bar representing the standard deviation. *4*$^{\Delta}$ and *5*$^{\Delta}$, deletion of *sad-4* and *sad-5*, respectively. +, wild type at *sad* loci. Cross 1, F5-32 × P17-62. Cross 2, F5-33 × P17-63. Cross 3, F2-01 × P3-08. Cross 4, F2-01 × P17-64. Cross 5, F5-36 × P17-66. Cross 6, F5-37 × P17-69. Cross 7, F2-14 × P3-07. Cross 8, F5-35 × P17-65.

found among them. For example, *N. crassa* SAD-5 is 28% and 90% similar to its counterparts in *T. terrestris* and *N. tetrasperma*, respectively. Despite this variability, we identified a number of consensus amino acids among the SAD-5 sequences, with a clustering of conserved residues toward the N-terminal end (Figure 1B and Figure S2).

sad-4 and sad-5 are expressed during the sexual cycle

Two MSUD proteins, DCL-1 and QIP, are known to have roles in another RNA silencing process known as quelling (Catalanotto *et al.* 2004; Maiti *et al.* 2007). Since quelling, unlike MSUD, is active in the somatic tissue, a gene involved in both processes would need to be expressed in both vegetative and sexual phases of the fungal life cycle. We examined the vegetative levels of *sad-4* and *sad-5* transcripts by two different methods. First, we analyzed several *N. crassa* mRNA-Seq datasets from the NCBI SRA database (Ellison *et al.* 2011). Vegetative expression levels of *sad-4* and *sad-5*, like those found in other MSUD genes, were near the limit of detection in all datasets (reads per kilobase of exon model per million mapped reads, RPKM = 0.01–0.14; Table 3). In contrast, genes required for quelling (whether or not they are also required for MSUD) were expressed at much higher levels (RPKM = 3.81–63.72). These data suggest that *sad-4* and *sad-5* are barely active, if at all, during the vegetative phase. Second, we attempted to detect *sad-4* and *sad-5* mRNAs from vegetative and sexual tissues. In agreement with the database analysis, we were able to amplify *sad-4* and *sad-5* cDNA sequences only from the sexual tissue (Figure 2), demonstrating once again that the expression levels of these genes are very low during the vegetative phase. The low vegetative expression of these *sad* genes predicts that their deletion should have no effect on the vegetative phenotype. Accordingly, morphological analysis and growth assays revealed no significant differences between a *sad-4*$^{\Delta}$ or *sad-5*$^{\Delta}$ mutant and wild type (Figure S3).

Neither SAD-4 nor SAD-5 is essential for sexual development

All known MSUD proteins thus far are required for sexual development. For example, crosses lacking either *dcl-1* or *qip* produce barren perithecia with no asci (Alexander *et al.* 2008; Xiao *et al.* 2010). On the other hand, while *sad-1*–, *sad-2*–, or *sad-3*–null perithecia are also barren, they abort at a later stage, some time after the production of elongated asci (Shiu *et al.* 2001, 2006; Hammond *et al.* 2011a). Predicting that perithecia lacking *sad-4* or *sad-5* would follow one of these two patterns, crosses homozygous for either *sad-4*$^{\Delta}$ or *sad-5*$^{\Delta}$ were performed. Surprisingly, unlike previously characterized MSUD genes, neither *sad-4* nor *sad-5* is required for ascospore production (Figure 3), although the loss of *sad-4* did correlate with a threefold decrease in the total number of shot progeny (Figure 3, A and C).

In an attempt to identify the defect leading to reduced ascospore production in crosses homozygous for *sad-4*$^{\Delta}$, their perithecial development was examined over 13 days. No appreciable differences in perithecial morphology and abundance were observed over this time frame between *sad-4*$^{\Delta}$ and wild-type crosses (Figure S4). This suggests that the sporulation defect may lie within the perithecia. Accordingly, perithecial dissection revealed that crosses homozygous for *sad-4*$^{\Delta}$ have roughly twice the amount of aborted asci (Figure S4). Thus a high ascus abortion rate is the likely cause of reduced ascospore production in a *sad-4*–null cross.

Crosses homozygous for sad-4$^{\Delta}$ *or sad-5*$^{\Delta}$ *are completely deficient in MSUD*

Because all previously characterized MSUD proteins are required for sexual development, a meiotic silencing assay has typically involved a heterozygous cross between deletion strains, with one parent lacking an MSUD gene (*e.g.*, *sad-1*) and the other lacking a gene important for ascospore

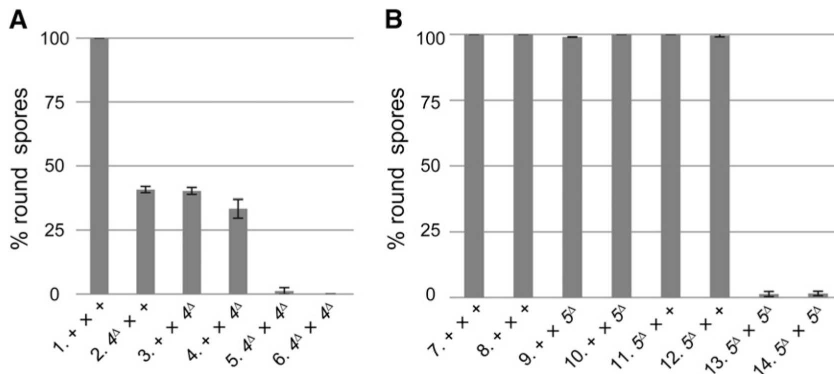


Figure 4 SAD-4 and SAD-5 are essential MSUD proteins. (A) A normal cross produces black American football (spindle)-shaped ascospores. In this study, crosses heterozygous for r^{Δ} were examined. In an MSUD-proficient background, an unpaired r^{+} is silenced and nearly 100% of the progeny are round (cross 1). When both parents are deleted of *sad-4*$^{+}$, progeny are predominantly normal (crosses 5 and 6), suggesting MSUD is suppressed. (B) Similarly, MSUD is suppressed in a *sad-5*–null background (crosses 13 and 14). Unlike *sad-4*$^{\Delta}$ (crosses 2–4), *sad-5*$^{\Delta}$ is not semidominant in a cross (crosses 9–12). Three replicates were performed for each heterozygous r^{Δ} cross, with the error bar representing the standard deviation. WT, wild type at *sad* loci. Cross 1, F2-01 × P12-02. Cross 2, F5-32 × P12-02. Cross 3, F2-01 × P17-59. Cross 4, F2-01 × P17-60. Cross 5, F5-32 × P17-59. Cross 6, F5-33 × P17-60. Cross 7, F2-14 × P12-01. Cross 8, F5-35 × P12-01. Cross 9, F2-14 × P17-70. Cross 10, F5-35 × P17-71. Cross 11, F5-36 × P12-01. Cross 12, F5-37 × P12-01. Cross 13, F5-36 × P17-70. Cross 14, F5-37 × P17-71.

development (*e.g.*, *r*). In such a "silencing the silencer" assay, the *sad* gene is itself unpaired and hence self-silenced, leading to the loss of (or decrease in) MSUD activity. *sad-4*$^{\Delta}$ and *sad-5*$^{\Delta}$ did not appear to be strong dominant suppressors of MSUD (as compared to *sad-1*$^{\Delta}$). This is especially true for the r^{Δ} test crosses, where *sad-4*$^{\Delta}$ and *sad-5*$^{\Delta}$ suppress roughly half and none of the MSUD activity, respectively (Table 2). Since the two *sad* genes are dispensable for sexual development, it is possible to assay MSUD suppression in a homozygous *sad*$^{\Delta}$ cross for the first time. In crosses homozygous for *sad-4*$^{\Delta}$ or *sad-5*$^{\Delta}$, the silencing of an unpaired r^{+} appeared completely suppressed, demonstrating that these genes are indeed essential for MSUD (Figure 4).

SAD-4 and SAD-5 localize in the perinuclear and nuclear regions, respectively

All previously characterized MSUD proteins (SAD-1/2/3, DCL-1, QIP, and SMS-2) colocalize around the nucleus during meiosis (Shiu *et al.* 2006; Alexander *et al.* 2008; Xiao *et al.* 2010; Hammond *et al.* 2011a), suggesting that they form an RNA-processing complex. Despite the fact that MSUD must also involve nuclear proteins (*e.g.*, those that detect unpaired DNA), their discovery has remained elusive thus far. To determine the subcellular localization of SAD-4 and SAD-5, they were tagged with RFP and GFP, respectively. Like all known MSUD proteins, SAD-4 is found in the perinuclear region (Figure 5B). Moreover, it colocalizes with SMS-2, a component of the meiotic silencing complex described above (Figure 5D). SAD-5, unlike any MSUD protein reported before it, is localized diffusely in the nucleus (excluding the nucleolus) (Figure 5E).

Deletion of sad-4 or sad-5 correlates with loss of masiRNAs

The involvement of RNA silencing proteins in MSUD, such as Dicer, Argonaute, and RdRP, suggests that this process is mediated by small RNAs. In a related work, we have identified and characterized MSUD-associated small interfering RNAs (masiRNAs) that correlate with the unpairing of r^{+} during meiosis (see Hammond *et al.* 2013, accompanying article in this issue). To determine if *sad-4* or *sad-5* deletion affects the production of *r*-specific masiRNAs, we prepared and sequenced small RNA libraries from perithecia of various crosses. The positive control (*r*-unpaired) cross, consistent with our previous result, produced abundant *r*-specific small RNAs mostly 21–27 nt in length (Figure 6A, blue). In contrast, the negative control (*r*-paired) cross produced a much lower level of *r*-specific small RNAs, which were uniformly distributed and likely mRNA degradation products (Figure 6A, purple). For the experimental (*r*-unpaired) crosses involving homozygous *sad-4* or *sad-5* deletion (Figure 6A, green and orange), the levels and size distributions of *r*-specific small RNAs were similar to the negative control where *r* was not silenced. These data suggest that SAD-4 and SAD-5 function upstream of masiRNA generation in the MSUD pathway.

SAD-4 and SAD-5 are not required for milRNA and disiRNA biogenesis

MicroRNA-like RNAs (milRNAs) and dicer-independent small interfering RNAs (disiRNAs) are small RNA species that originated from hairpin and convergent transcripts, respectively (Lee *et al.* 2010b). Some of these molecules require known RNAi factors (*e.g.*, Dicers and Argonaute) for their production. To determine if *sad-4* and *sad-5* are required for the biogenesis of milRNAs and disiRNAs, we examined their levels in crosses homozygous for *sad-4*$^{\Delta}$ or *sad-5*$^{\Delta}$. We found that milRNA and disiRNA production was qualitatively unaffected when either of these *sad* genes was deleted (Figure 6, B and C). This observation is consistent with the notion that SAD-4 and SAD-5 are possibly specific for masiRNA generation during meiotic silencing.

Discussion

In this study, we have characterized two MSUD proteins that were identified with a recently developed reverse-genetic screen. This screen involves the transferring of conidial (asexual spore) suspensions of knockout strains to various MSUD testers, and *sad-4*$^{\Delta}$/*sad-5*$^{\Delta}$ strains were first identified as candidates in crosses that were unpaired for *asm-1*$^{+}$. *sad-5*$^{\Delta}$,

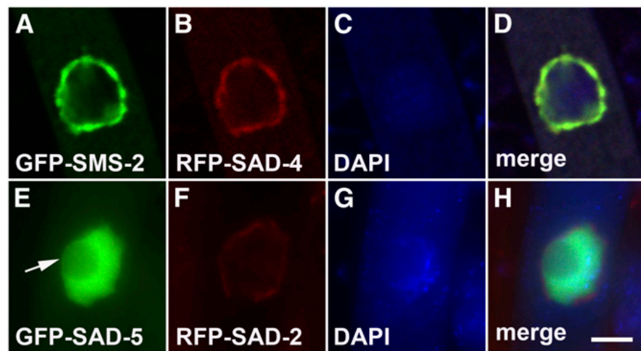


Figure 5 SAD-4 and SAD-5 localize in the perinuclear and nuclear regions, respectively. Micrographs illustrate prophase asci expressing (A–D) *gfp-sms-2* and *rfp-sad-4* (P15-14 × P13-22) and (E–H) *gfp-sad-5* and *rfp-sad-2* (P18-57 × P18-55). SAD-4 colocalizes with SMS-2, a component of the perinuclear MSUD complex that also includes SAD-2. SAD-5 localizes in the nucleus, excluding the nucleolus (arrow). Bar, 5 μm.

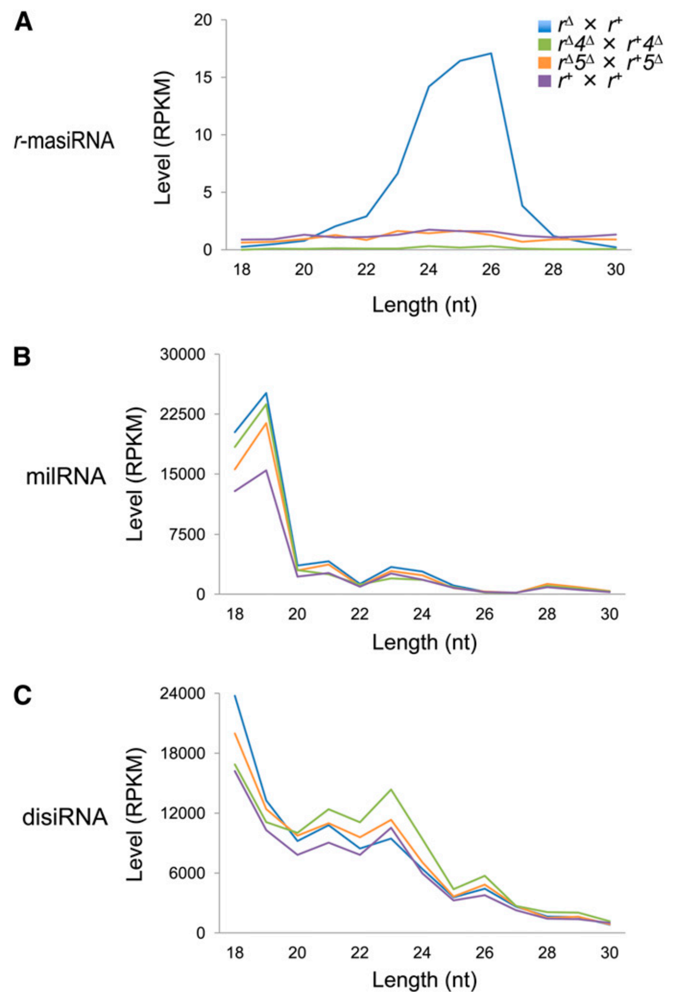


Figure 6 SAD-4 and SAD-5 are required for the generation of masiRNAs. Small RNA (sRNA) levels and lengths were determined for four different crosses. These included three *r*-unpaired crosses that were MSUD-proficient (blue; F5-39 × P3-08), *sad-4*–null (green; F5-38 × P17-62), or *sad-5*–null (orange; F5-36 × P17-71). The fourth cross was the *r*-paired control (purple; F2-01 × P3-08). (A) *r*-specific sRNAs do not accumulate when *sad-4* or *sad-5* is deleted, suggesting that the two genes are involved in masiRNA generation. (B and C) Deletion of either *sad* gene does not qualitatively affect milRNA/disiRNA levels. RNA levels are listed in reads per kilobase of exon model per million mapped reads (RPKM).

like two other MSUD suppressors (Raju *et al.* 2007), does not dominantly suppress r^{Δ}. It is unclear why some sad^{Δ} strains are weaker dominant suppressors of MSUD, although one can speculate that certain *sad* genes may be expressed at a high level or have a long protein half-life, making it harder to silence them. It seems possible that one of these mechanisms (high expression or protein stability) could allow some repetitive elements to escape silencing should they become unpaired. Fortunately for *N. crassa*, there are at least two other surveillance mechanisms (quelling and repeat-induced point mutation) to keep them in check (Catalanotto *et al.* 2006).

SAD-4– and SAD-5–like proteins appear to be fungal specific and have no known motifs. Our report marks the first time they could be associated with a function (*i.e.*, RNA silencing). Perhaps the most interesting aspect of our phylogenetic analysis is that SAD-4 is found in a broader range of fungi relative to SAD-5, with the latter appearing to be specific to a single order of Sordariomycete fungi. One possibility is that SAD-4 has a role in other cellular processes. This is consistent with our finding that although *sad-4* is not absolutely required for sexual sporulation, its deletion from both parents correlates with increased ascus abortion. Additionally, it is interesting that some Sordariomycete fungi encode SAD-5 but not SAD-4, and the only other fungus in which MSUD has been experimentally demonstrated thus far (*G. zeae*) has neither. Perhaps SAD-4 and SAD-5 have mechanistic roles that are relatively species specific. For example, if defense against selfish genetic elements is a major driving force for adaptive change in MSUD, then its mechanism may be subject to the evolutionary arms race typical of host–parasite interactions (Obbard *et al.* 2009). If this is the case, SAD-4 and SAD-5 may represent *N. crassa*'s specific modifications of the MSUD mechanism.

The perinuclear localization of SAD-4 suggests that it may be involved in dsRNA production and/or masiRNA generation. Alternatively, it may assist the Argonaute protein in using masiRNAs to identify/destroy mRNA transcripts. In quelling, the lack of Argonaute (functioning downstream of small RNA generation) leads to the accumulation of siRNAs (Catalanotto *et al.* 2002). The fact that the absence of SAD-4 correlates with the loss of masiRNAs suggests that it must function upstream of their production.

SAD-5 is the first nuclear MSUD protein to be identified. SAD-5's localization makes it tempting to speculate that it may be directly involved in the initial stages of MSUD, *i.e.*, detecting unpaired DNA or producing/transporting the aRNA. Each of these possibilities is supported by the finding that masiRNAs do not accumulate in a *sad-5*–null cross. To decipher the exact roles of SAD-4 and SAD-5 in gene silencing, additional research is needed (*e.g.*, their influence on aRNA production, quelling, expression of other *sad* genes, and silencing of various unpaired loci).

This study demonstrates that masiRNAs are an important part of *N. crassa*'s silencing mechanism and that certain members of the MSUD machinery are indispensable for their production. The detection of masiRNAs (or the lack thereof) allows us to place a protein function upstream or downstream of their generation. In addition, all previously identified MSUD proteins are essential for ascospore production. It has been speculated that perhaps some degree of silencing constitutes a checkpoint in sexual development. The finding that SAD-4 and SAD-5 are not required for sporulation is a breakthrough in our understanding of MSUD, disputing a previous belief that this silencing process is absolutely coupled with sexual development.

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Note added in proof: See Hammond *et al.* 2013 (pp. 279–284) in this issue, for a related work.

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Novel Proteins Required for Meiotic Silencing by Unpaired DNA and siRNA Generation in *Neurospora crassa*

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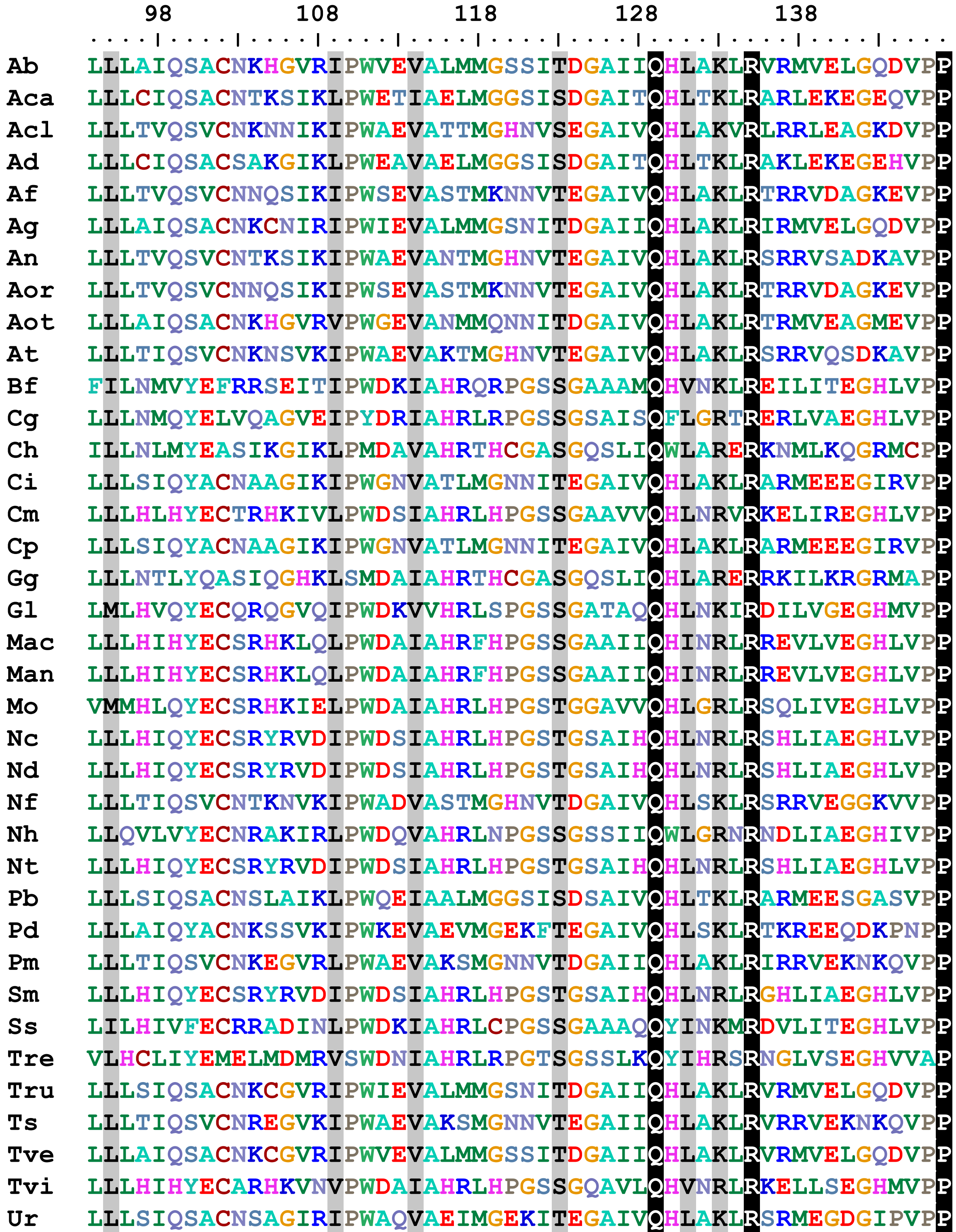

Figure S1 Conserved region in SAD-4 homologs. Positions are relative to *N. crassa* SAD-4. Strain names and sequence accession numbers are listed in Table S2.

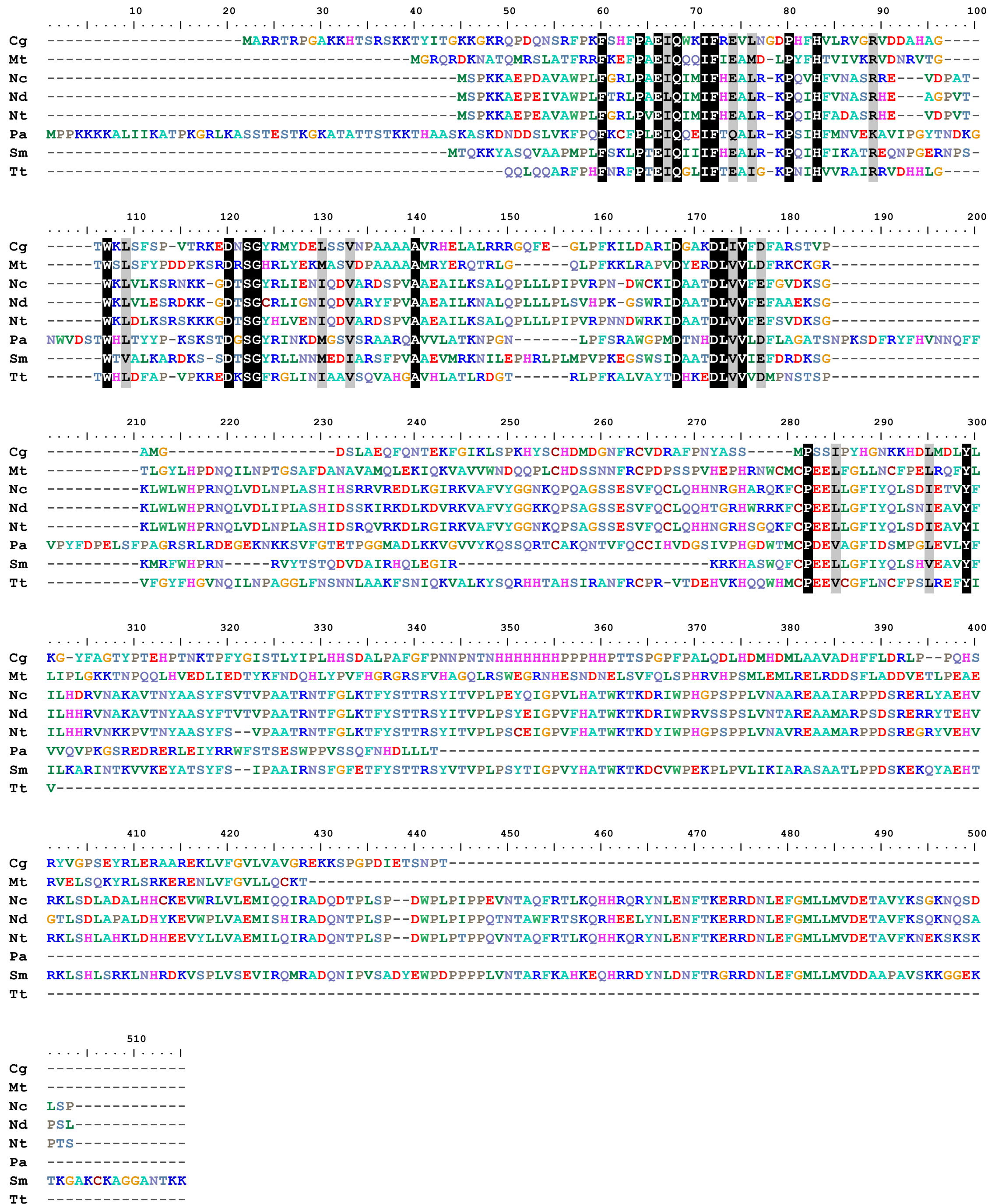

Figure S2 Alignment of SAD-5 homologs. Strain names and sequence accession numbers are listed in Table S2.

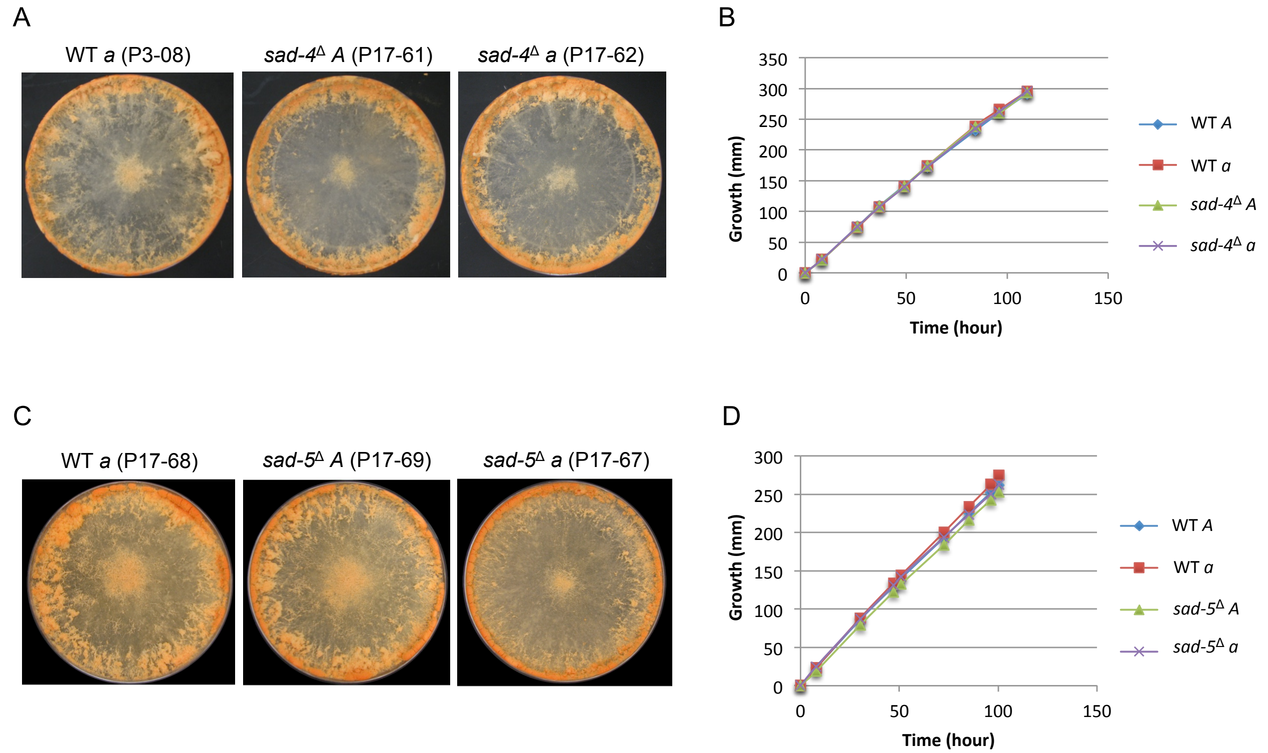

Figure S3 *sad-4* and *sad-5* mutants appear normal during the vegetative phase. *sad-4*$^{\Delta}$ and *sad-5*$^{\Delta}$ strains have normal mycelial morphology (A and C) and growth (B and D).

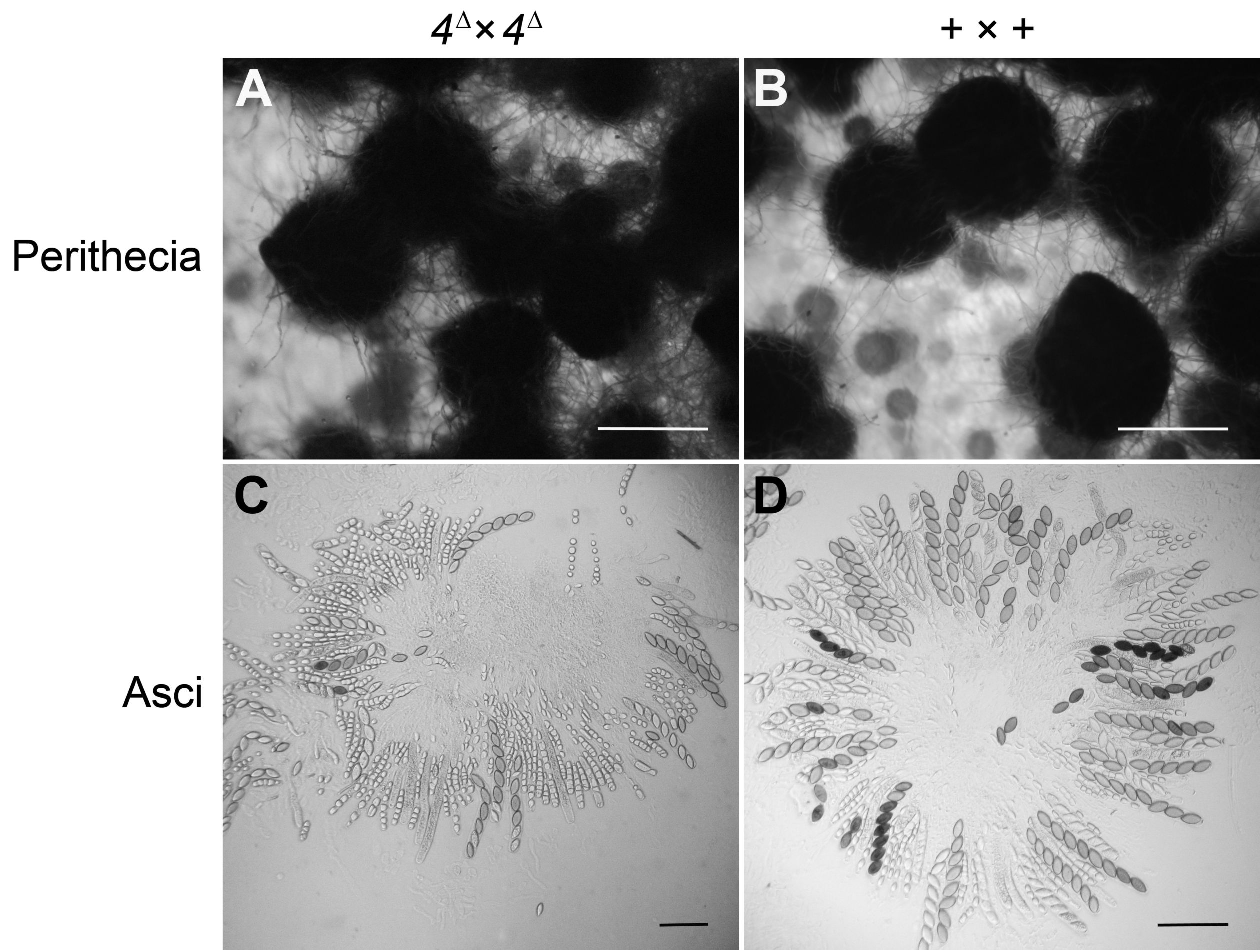

Figure S4 Crosses homozygous for *sad-4*$^{\Delta}$ produce fewer ascospores. (A and B) No differences in perithecial development and abundance were detected between *sad-4*$^{\Delta}$ (F5-32 × P17-62) and control (F5-23 × P3-08) crosses. Bars, 500 µm. (C and D) *sad-4*–null crosses produce more aborted (bubble) asci. Bars, 100 µm.

Table S1 Primers used in this study

Purpose	Primer	Sequence (5' to 3')
sad-4$^{\Delta}$::*hph* confirmation	NCU01591-294825F	TGGCATGAGACTGGCATCTGAG
	NCU01591-299764R	GCAGAAGCGGAAGACATGTCAAG
sad-5$^{\Delta}$::*hph* confirmation	NCU06147-438124F	GACCATGGCCACGACTACAGCTAC
	NCU06147-442142R	ATATGAGACCTGCGCGCTAGCCAAC
rfp-sad-4 construction	NCU01591-296360F*NotI	GTCTAGCGGCCGCGATCATATCAAG
	NCU01591-298299R*SpeI	CCAGACTAGTCTTCCGAATACTAAAATG
gfp-sad-5 construction	NCU06147-E	TCTGCGGAACAATATGAACAACCTG
	NCU06147-F	AGGACAACCCTCCACTCGCAAAG
	NCU06147-G	AGGCTCGAACTCCCGACCTCA
	NCU06147-H	CCGCTTCACGGGCTGCGTTGAC
	NCU06147-NGFP1	GCAGCCTGAATGGCGAATGGACGCGCAGCTGAGACGAGGTGCCGAAAG
	NCU06147-NGFP2	CAGGAGCGGGTGCGGGTGCTGGAGCGATGAGTCCCAAAAAGGCCGAGCCTGAC
sad-1 cDNA amplification	SAD1-4489F	ATGCTCATCTGGCGACAGCAGATG
	SAD1-4796R	TTCTCTTTCATGTCGAATGTTTCCC
sad-4 cDNA amplification	NCU01591-296801F	CTGGTGTAGCCGTTGTGGTTCG
	NCU01591-298048R	GCGTCGAAGGCAGGAACTTGAT
sad-5 cDNA amplification	NCU06147-F2-TH	CGCTTTCGTTTACGGTGGGAAC
	NCU06147-R2-TH	GGCCAGATGCGATCCTTTGTCT
actin cDNA amplification	ACT-507F	CGTTGGTCGTCCCCGTTATCATG
	ACT-811R	TGGGAGCCTCGGTAAGAAGGACG

Table S2 Sequences used in this study

Protein	Abbreviation[1]	Accession #[2]	Strain
SAD-4	Ab	XM_003014454.1	*Arthroderma benhamiae* CBS 112371
	Aca	XM_001539307.1	*Ajellomyces capsulatus* NAm1
	Acl	XM_001267850.1	*Aspergillus clavatus* NRRL 1
	Ad	XM_002623645.1	*Ajellomyces dermatitidis* SLH14081
	Af	XM_002377995.1	*Aspergillus flavus* NRRL3357
	Ag	XM_003173571.1	*Arthroderma gypseum* CBS 118893
	An	XM_001395616.2	*Aspergillus niger* CBS 513.88
	Aor	XM_001826292.2	*Aspergillus oryzae* RIB40
	Aot	XM_002844811.1	*Arthroderma otae* CBS 113480
	At	XM_001218470.1	*Aspergillus terreus* NIH2624
	Bf	CCD55484.1	*Botryotinia fuckeliana*
	Cg	XP_001228162.1	*Chaetomium globosum* CBS 148.51
	Ch	CCF43159.1	*Colletotrichum higginsianum*
	Ci	XM_001242382.1	*Coccidioides immitis* RS
	Cm	EGX93189.1	*Cordyceps militaris* CM01
	Cp	XM003069573.1	*Coccidioides posadasii* C735 delta SOWgp
	Gg	EFQ30009.1	*Glomerella graminicola* M1.001
	Gl	EHK98895.1	*Glarea lozoyensis* 74030
	Mac	EFY84375.1	*Metarhizium acridum* CQMa 102
	Man	EFY96871.1	*Metarhizium anisopliae* ARSEF 23
	Mo	XM_360623.1	*Magnaporthe oryzae* 70-15
	Nc	BK006474	*Neurospora crassa* OR74A
	Nd	102414[3]	*Neurospora discreta* 8579 *mat A*
	Nf	XM_001260425.1	*Neosartorya fischeri* NRRL 181
	Nh	XP_003051566.1	*Nectria haematococca* mpVI 77-13-4
	Nt	EGO56400.1	*Neurospora tetrasperma* FGSC 2508
	Pb	XM_002789947.1	*Paracoccidioides brasiliensis* Pb01
	Pd	EKV11485.1	*Penicillium digitatum* PHI26
	Pm	XM_002149363.1	*Penicillium marneffei* ATCC 18224
	Sm	XP_003350666.1[4]	*Sordaria macrospora* k-hell
	Ss	XP_001597890.1	*Sclerotinia sclerotiorum* 1980
	Tre	EGR52832.1	*Trichoderma reesei* QM6a
	Tru	XM_003235041.1	*Trichophyton rubrum* CBS 118892
	Ts	XM_002484729.1	*Talaromyces stipitatus* ATCC 10500
	Tve	XM_003019899.1	*Trichophyton verrucosum* HKI 0517
	Tvi	EHK24463.1	*Trichoderma virens* Gv29-8
	Ur	XM_002544541.1	*Uncinocarpus reesii* 1704
SAD-5	Cg	XP_001227787.1	*Chaetomium globosum* CBS 148.51
	Mt	XP_003665043.1	*Myceliophthora thermophila* ATCC 42464
	Nc	BK006779	*Neurospora crass*a OR74A
	Nd	62731[3]	*Neurospora discreta* 8579 *mat A*
	Nt	EGO55361.1	*Neurospora tetrasperma* FGSC 2508
	Sm	XP_003344714.1	*Sordaria macrospora* k-hell
	Pa	XP_001905702.1	*Podospora anserina* S *mat*+
	Tt	XP_003654540.1[5]	*Thielavia terrestris* NRRL 8126

[1]Abbreviations are used in Figures S1 and S2. [2]GenBank accession numbers are listed unless otherwise stated. [3]*N. discreta* database at DOE-JGI (http://genome.jgi-psf.org/). [4]A putative intron was removed from its ORF. [5]This sequence is incomplete.

Table S3   Small RNA sequencing and alignment data

	$r^{\Delta} \times r^{+}$	$r^{\Delta}4^{\Delta} \times r^{+}4^{\Delta}$	$r^{\Delta}5^{\Delta} \times r^{+}5^{\Delta}$	$r^{+} \times r^{+}$
Raw reads	58,558,484	31,162,221	31,162,221	36,467,267
≥14 nt after adapter trimming	55,022,756	26,001,036	22,318,233	32,655,370
Homology with the genome	51,641,029	24,578,034	20,920,863	30,650,403
18-30 nt long	33,392,504	15,763,605	14,098,686	20,701,772
No mismatches	30,016,594	14,093,205	12,738,940	18,758,512

Shown are numbers of small RNA reads after each processing step. See Figure 6 for cross information.