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Note

An Argonaute-Like Protein Is Required for Meiotic Silencing

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ABSTRACT

We demonstrate the involvement of ***s****uppressor of* ***m****eiotic* ***s****ilencing-****2*** (*sms-2*$^+$), a Neurospora gene coding for an Argonaute-like protein, in meiotic silencing and normal sexual development.

DURING meiosis, chromosomes "sense" each other through a process called meiotic transvection (Aramayo and Metzenberg 1996), which was discovered by studying the ***As****cospore* ***m****aturation-****1*** (*Asm-1*) locus in *Neurospora crassa* (Aramayo and Metzenberg 1996; Aramayo *et al.* 1996). The presence of unpaired DNA was proposed to activate RNA silencing (Shiu *et al.* 2001) on the basis of the demonstration that mutations in an *R*NA-*d*ependent *R*NA *p*olymerase (RdRP) gene called ***S****uppressor of* ***a****scus* ***d****ominance-****1*** (*Sad-1*) eliminate the ascus dominance of unpaired DNA from *Asm-1* and other genes (Shiu *et al.* 2001). Scanning of the Neurospora genome revealed the existence of a paralog for ***q****uelling* ***de****ficient-2* (*qde-2*), which we call ***S****uppressor of* ***m****eiotic* ***s****ilencing-****2*** (*Sms-2*). The involvement of *qde-2* and of the *Sad-1* paralog [***q****uelling* ***de****ficient-****1*** (*qde-1*)] in the haploid vegetative RNA-silencing pathway called quelling has been firmly established (Cogoni and Macino 1997; Catalanotto *et al.* 2000, 2002). The identification of *Sms-2* as a second gene related to *qde-2* suggested its potential involvement in the meiotic silencing pathway. This is particularly attractive because SMS-2 belongs to the functionally novel, but highly conserved eukaryotic Argonaute protein family (Figure 1). We postulated that if *Sms-2* is involved in meiotic silencing, *Sms-2* loss-of-function mutants should suppress the ascus-dominant phenotype of unpaired *Asm-1* DNA.

To test this hypothesis, we constructed two different null alleles of *Sms-2* and tested their ability to suppress meiotic silencing. *sms-2*$^+$ was mutagenized using *r*epeat *i*nduced *p*oint mutation (RIP), generating two probable null alleles that we called *Sms-2*^{RIP2} and *Sms-2*^{RIP88}. RIP occurs prior to meiosis when a sequence in Neurospora is present in more than one copy in the haploid genome (Selker 1990). The *Sms-2*^{RIP2} allele was generated by duplicating a 2374-bp DNA region corresponding only to the coding region of the gene, whereas the *Sms-2*^{RIP88} was generated by duplicating the entire 5534-bp DNA region encompassing the upstream, coding, and downstream regions of *Sms-2*. In both cases the regions were duplicated by integration at the ***his****tidine-****3*** (*his-3*) locus in linkage group I (LG I). Sequencing of these alleles revealed, among many GC-to-AT transition mutations typical of RIP (see Figure S1, electronic supplementary material at http://www.genetics.org/supplemental), a CAG-to-TAG mutation changing a glutamine to a stop signal at position 118, thus potentially allowing the synthesis of a short truncated polypeptide from the *Sms-2*^{RIP2} allele and an ATG-to-ATA mutation in the translation start codon of *Sms-2*^{RIP88}, thus blocking the synthesis of a complete polypeptide by this allele. As expected, these two alleles differ in the number of transition mutations present along their DNA regions. Most of the mutations present in *Sms-2*^{RIP2} map only to the coding region of the gene. In contrast, the mutations present in *Sms-2*^{RIP88} are distributed along the promoter, coding, and downstream regions of the allele.

The resulting *Sms-2* mutants have no obvious defects during vegetative growth or asexual sporulation. In contrast, homozygous *Sms-2*^{RIP2}/*Sms-2*^{RIP2} or *Sms-2*^{RIP88}/*Sms-2*^{RIP88} crosses and heterozygous *Sms-2*^{RIP2}/*Sms-2*^{RIP88} crosses are completely barren, as was demonstrated in homozygous crosses between *Sad-1* loss-of-function alleles (Shiu *et al.* 2001). Similarly to *Sad-1*, heterozygous *sms-2*$^+$/*Sms-2*^{RIP2} and *sms-2*$^+$/*Sms-2*^{RIP88} crosses were fertile and produced normal ascospores (Table 1, crosses 1–5; Table 2; Table S2, electronic supplementary material at http://www.genetics.org/supplemental/).

The role of Argonautes in RNA silencing is not completely understood and may differ in the different systems studied (Carmell *et al.* 2002). In Drosophila,

Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under accession nos. AF500110, AF508210, AF508211, and AF508212.

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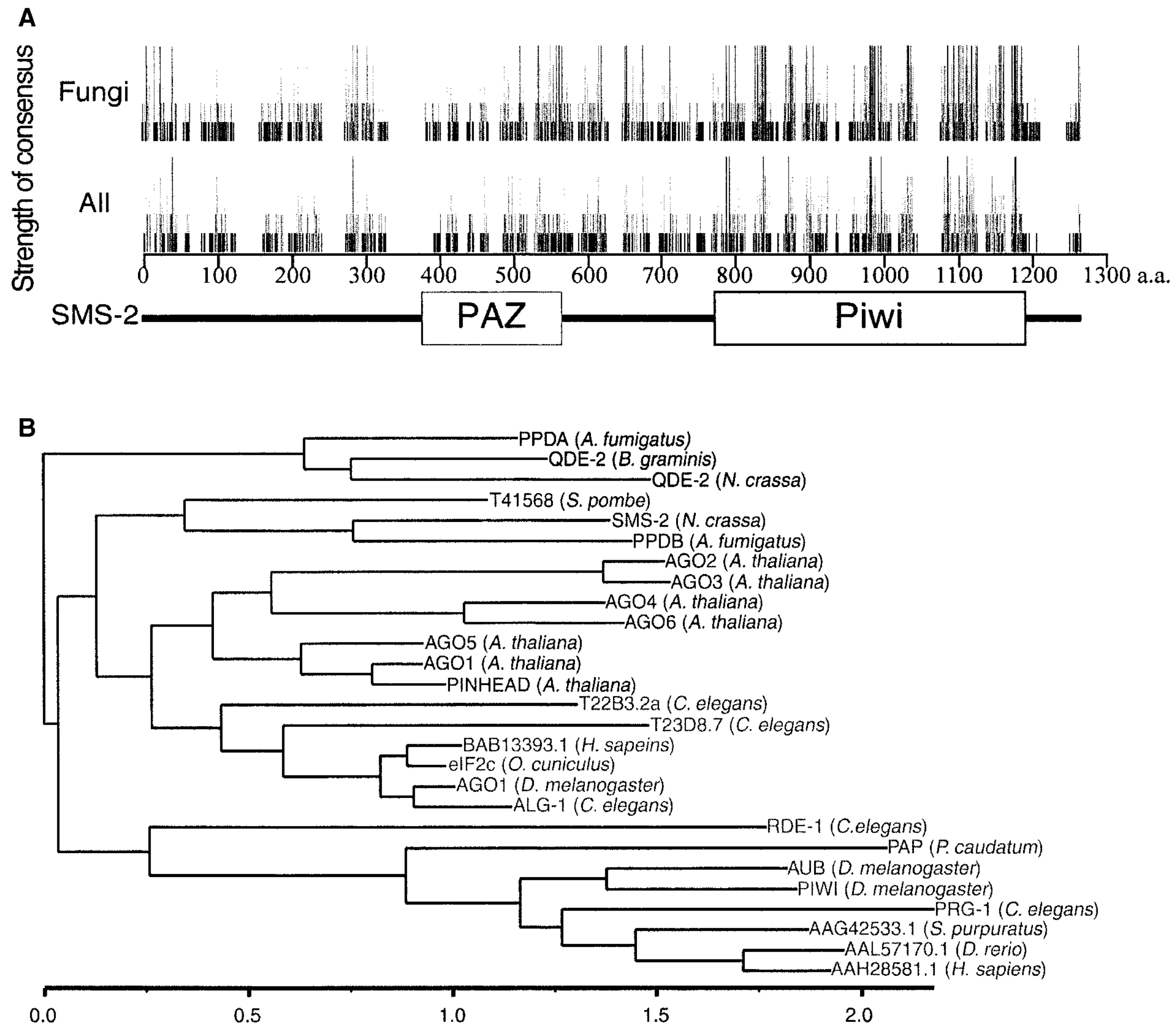

Figure 1.—Alignment of SMS-2-like proteins. (A) Histogram representation of the T-Coffee alignment (Notredame *et al.* 2000) of SMS-2-related proteins. Histograms showing the consensus strength across all aligned proteins (All) and among only the six fungal proteins (Fungi) are shown (see Table S3, electronic supplementary material at http://www.genetics.org/supplemental/for a complete description of the proteins, organisms, and identifiers). The strength of the alignment consensus as determined by MegAlign (DNASTAR, Madison, WI) using the PAM250 matrix correlates with the height of the bars. The tallest bars represent absolutely conserved positions in the alignment. The histograms were scaled to the alignment consensus sequence. Diagrammed below the histogram is the aligned SMS-2 protein with the PAZ and Piwi domains as determined by SMART (http://smart.embl-heidelberg.de/; Schultz *et al.* 1998; Letunic *et al.* 2002; see full alignment in Figure S2, electronic supplementary material at http://www.genetics.org/supplemental/). (B) A Bayesian phylogenetic tree of SMS-2-related proteins created by MrBayes v2.01 (Huelsenbeck and Ronquist 2001; Huelsenbeck *et al.* 2001) using the alignment represented in A. Branch lengths are scaled below the tree (see Figure S3, electronic supplementary material at http://www.genetics.org/supplemental/ for branch lengths and posterior probability values for the clades). Note that the fungal proteins cluster into two clearly distinguishable clades that we argue represent vegetative (quelling) and developmental (meiotic silencing) pathways in fungi (Galagan *et al.* 2003). Intriguingly, the animal proteins also cluster into two distinguishable clades, perhaps signifying two functional classes of Argonaute proteins in animals.

Argonaute2 has been isolated as part of the RNA-inducing silencing complex (RISC; *i.e.*, acting at the effector cycle, Figure 2; Hammond *et al.* 2001; Hannon 2002). In *Caenorhabditis elegans*, RDE-1 has been demonstrated to interact with RDE-4, a double-stranded RNA (dsRNA)-binding protein, and also with Dicer, an endonuclease (*i.e.*, acting in the initiation step, Figure 2; Hannon 2002; Tabara *et al.* 2002). In Neurospora, testing the involvement of *sms-2*$^{+}$ in meiotic silencing is not straightforward. The meiotic lethality observed in crosses involving homozygous loss-of-function *Sms-2* alleles complicates the conceptually simple experiment of assaying meiotic silencing in the complete absence of *sms-2*$^{+}$. The best-known experiments that can be done are to assay the degree of meiotic silencing in heterozygous *sms-2*$^{+}$/*Sms-2*^{RIP2} or *sms-2*$^{+}$/*Sms-2*^{RIP88} crosses (Figure 3).

TABLE 1

SMS-2 is required for meiotic silencing in *N. crassa*

Cross number[a]	Relevant genotype[b] LG I *Sad-1*	LG I *mat*	LG I *his-3*	LG V *Asm-1*	LG VII *Sms-2*	Parents	Total ascospores examined[c]	Mature ascospores (%)[c]	Observations	Suppression of silencing?
1	*sad-1*$^+$	*A*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR05A	Thousands	95–99	Control	NA[d]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$;	*asm-1*$^+$;	*sms-2*$^+$	RANCR50A				
2	*sad-1*$^+$	*A*	*his-3*$^+$;	*asm-1*$^+$;	*sms-2*$^+$	RANCR49A	Thousands	95–99	Control	NA[d]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR06A				
3	*sad-1*$^+$	*A*	*his-3*;	*asm-1*$^+$;	*Sms-2*^{RIP2}	MMNCR02A	Thousands	95–99	Control	NA[d]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR06A				
4	*sad-1*$^+$	*A*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR05A	Thousands	95–99	Control	NA[d]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*Sms-2*^{RIP2}	MMNCR03A				
5	*sad-1*$^+$	*A*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR05A	Thousands	95–99	Control	NA[d]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*Sms-2*^{RIP88}	DLNCR88A				
6	*sad-1*$^+$	*A*	*his-3*;	*asm-1*$^+$;	*Sms-2*^{RIP2}	MMNCR02A	872	17.9	Experimental, RIP[e]	Yes[f]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				
7	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT34A	1122	30.0	Experimental, RIP[e]	Yes[f]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*Sms-2*^{RIP2}	MMNCR03A				
8	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT34A	924	23.2	Experimental, RIP[e]	Yes[f]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*Sms-2*^{RIP88}	DLNCR88A				
9	*sad-1*$^+$	*A*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR05A	Thousands	0.3	Control, RIP[e]	No[f]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				
10	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT34A	Thousands	0.3	Control, RIP[e]	No[f]
	×					×				
	sad-1$^+$	*a*	*his-3*;	*asm-1*$^+$;	*sms-2*$^+$	RANCR06A				
11	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*sms-2*$^+$	KYNCT03A	308	51.3	Experimental, RIP[e]	Yes[g]
	×					×				
	Sad-1^{RIP64}	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	DLNCR72A				
12	*Sad-1*^{RIP64}	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*sms-2*$^+$	DLNCR69A	92	48.9	Experimental, RIP[e]	Yes[g]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				

(*continued*)

TABLE 1

(Continued)

Cross number[a]	Relevant genotype[b] *Sad-1* (LG I)	*mat* (LG I)	*his-3* (LG I)	LG V *Asm-1*	LG VII *Sms-2*	Parents	Total ascospores examined[c]	Mature ascospores (%)[c]	Observations	Suppression of silencing?
13	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*sms-2*$^+$	KYNCT03A	289	0.0	Control, RIP[e]	No[g]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				
14	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT34A	71	53.5	Experimental, RIP[e]	Yes[g]
	×					×				
	Sad-1^{RIP64}	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*sms-2*$^+$	DLNCR70A				
15	*Sad-1*^{RIP64}	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	DLNCR71A	186	52.0	Experimental, RIP[e]	Yes[g]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*sms-2*$^+$	KYNCT05A				
16	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT34A	511	0.1	Control, RIP[e]	No[g]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*sms-2*$^+$	KYNCT05A				
17	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*Sms-2*^{RIP88}	DLNCR126A	541	16.7	Experimental, RIP[e]	Yes[g]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				
18	*Sad-1*^{RIP64}	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*Asm-1*$^{\Delta(3430\text{-}9336)}$;	*Sms-2*^{RIP88}	DLNCR127A	515	48.7	Experimental, RIP[e]	Yes[g]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				
19	*sad-1*$^+$	*A*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT34A	625	36.8	Control, RIP[e]	NA[d]
	×					×				
	sad-1$^+$	*a*	*his-3*$^+$::*Asm-1*$^+$[3430-9336];	*asm-1*$^+$;	*sms-2*$^+$	KYNCT35A				

[a] When necessary, heterokaryons were constructed between the Griffiths' sterile helper strain (Fungal Genetics Stock Center no. 4564) and the required strains. On those occasions, sexual crosses were set up with both parents homokaryotic and also with both parents heterokaryotic. The results of the homokaryotic and the heterokaryotic crosses were equivalent.

[b] Complete genotypes are described in Table 2.

[c] Scoring was done by either inspecting or counting the indicated number of spores. We counted spores present on the lid of the petri dish without any further treatment.

[d] Not applicable (*i.e.*, meiotic silencing was not induced in these crosses).

[e] Reduced percentage of black ascospores in these crosses reflects the vulnerability of one or both parents to RIP of the *Asm-1* gene.

[f] In these crosses, meiotic silencing was induced by unpairing 5906 bp of *Asm-1* DNA (*Asm-1*$^+$[3430-9336]) at the *his-3* locus in LG I in the presence of two paired copies of *asm-1*$^+$ in LG V.

[g] In these crosses, meiotic silencing was induced by unpairing 5906 bp of *Asm-1* DNA (*asm-1*$^+$) at its canonical location in LG V in the presence of two paired copies of *Asm-1* (*Asm-1*$^+$[3430-9336]) in LG I.

TABLE 2

Fungal strains used in this study

Name[a]	Genotype[b,c]	Origin[c]
DLNCR63A	*Sad-1*^{RIP}, *his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Sad-1*[301-3950]; *inl A*	Progeny from RANCT45A × (KYNCT02A + FGSC 4564)
DLNCR64A	*Sad-1*^{RIP64}, *his-3; inl a*	Progeny from DLNCR63A × (KYNCT02A + FGSC 4564)
DLNCR69A	*Sad-1*^{RIP64}, *his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl A*	Progeny from DLNCR64A × KYNCT03A
DLNCR70A	*Sad-1*^{RIP64}; *his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl a*	Progeny from DLNCR64A × KYNCT03A
DLNCR71A	*Sad-1*^{RIP64}; *his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *inl A*	Progeny from DLNCR64A × KYNCT03A
DLNCR72A	*Sad-1*^{RIP64}; *his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *inl a*	Progeny from DLNCR64A × KYNCT03A
DLNCR88A	*his-3; inl; Sms-2*^{RIP88} *a*	Progeny from DLNCT84A × RANCR06A
DLNCR126A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl; Sms-2*^{RIP88} *A*	Progeny from DLNCR88A × DLNCR69A
DLNCR127A	*Sad-1*^{RIP64}, *his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl; Sms-2*^{RIP88} *A*	Progeny from DLNCR88A × DLNCR69A
DLNCT62A	*his-3*::*lpl*$^{\Delta(5192\text{-}6046)}$::*hph*$^{+}$::*tk*$^{+}$; *inl A*	Transformation of RANCR05A with pDLAM073 (Lee *et al.* 2003)
DLNCT84A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Sms-2*$^{+}$[5534-1]; *inl A*	Transformation of DLNCT62A with pDLAM092
FGSC 4564	*ad-3B cyh-1*^{R} *a*^{m1}	FGSC[d]
KYNCT01A	*his-3; Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl A*	Transformation of RANCR05A with pKYAM052
KYNCT02A	*his-3; Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl a*	Transformation of RANCR06A with pKYAM055
KYNCT03A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl A*	Transformation of KYNCT01A with pKYAM011
KYNCT05A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *Asm-1*$^{\Delta(3430\text{-}9336)}$::*hph*$^{+}$::*mcl-1, inl a*	Transformation of KYNCT02A with pKYAM011
KYNCT34A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *inl A*	Transformation of RANCR05A with pKYAM011
KYNCT35A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Asm-1*$^{+}$[9336-3430]; *inl a*	Transformation of RANCR06A with pKYAM011
MMNCR02A	*his-3; inl Sms-2*^{RIP2} *A*	Progeny from RANCT40AΔ35 × RANCR06A
MMNCR03A	*his-3; inl Sms-2*^{RIP2} *a*	Progeny from RANCT40AΔ35 × RANCR06A
RANCR05A	*his-3; inl A*	RANC collection
RANCR06A	*his-3; inl a*	RANC collection
RANCR49A	*fl A*	RANC collection
RANCR50A	*fl a*	RANC collection
RANCT40A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*sms-2*[2095-4468]; *inl A*	Transformation of RANCR05A with pQde-2l
RANCT40AΔ35	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*Sms-2*^{RIP}[2095-4468]; *inl; Sms-2*^{RIP} *A*	Progeny from RANCT40A × KYNCT02A
RANCT45A	*his-3*$^{+}$::*lpl*$^{\Delta(5192\text{-}6046)}$::*sad-1*[301-3950]; *inl A*	Transformation of RANCR05A with pRdRP3

[a] DLNC, KYNC, MMNC, and RANC indicate strains constructed or provided for this study by Dong W. Lee, Kye-Yong Seong, Malcolm McLaughlin, and Rodolfo Aramayo, respectively.

[b] Allele numbers or designations are: ***ad**enine-**3B*** (*ad-3B*; 12-17-114); *a*^{m1}, a mutant allele of the *a* idiomorph described in Griffiths and DeLange (1978); *Asm-1*; ***cyclo**heximide **r**esistant-**1*** (*cyh-1*^{R}; KH52r); ***fl**uffy* (*fl*; P); *his-3* (1-234-723); ***h**ygromycin B **ph**osphotransferase* (*hph*$^{+}$); *hph*$^{+}$ fused in frame to the herpes simplex virus ***t**hymidine **k**inase* gene, *hph*$^{+}$::*tk*$^{+}$ (Lupton *et al.* 1991); ***in**ositol* (*inl*; 89601); ***l**yso**p**hospho**l**ipase* [*lpl*$^{\Delta(5192\text{-}6046)}$] (see Methods in the electronic supplementary material at http://www.genetics.org/supplemental/ for details); ***m**yosin **c**hain-**l**ike-**1*** (*mcl-1*; see Methods in the electronic supplementary material at http://www.genetics.org/supplemental/ for details); *Sad-1* (RIP64); and *Sms-2* (RIP2 and RIP88).

[c] Description of the different alleles and plasmids is described in Methods in the electronic supplementary material at http://www.genetics.org/supplemental/.

[d] FGSC, Fungal Genetics Stock Center (University of Kansas Medical Center, Kansas City).

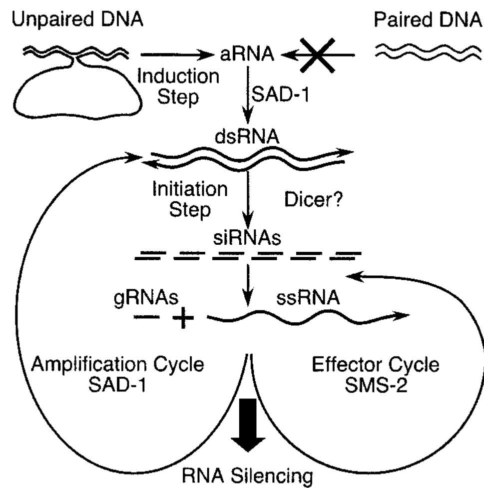

Figure 2.—Proposed meiotic silencing pathway of *N. crassa*: the proposed two steps and two cycles involved in initiating and maintaining meiotic silencing, respectively. Paired DNA does not induce the pathway. Unpaired DNA triggers the induction step, which involves the synthesis of *a*berrant *RNA* (aRNA) and its conversion to dsRNA by the SAD-1 RdRP. The presence of dsRNA triggers the initiation of the meiotic RNA-silencing process, which is composed of the following: the conversion of the dsRNA trigger into siRNAs (initiation step), predicted to occur via a Dicer-like endonuclease; the use of *g*uide *RNA*s (gRNAs) as primers and *s*ingle-*s*tranded *RNA* (ssRNA) as template by SAD-1 RdRP to generate dsRNA (amplification cycle); and the incorporation of the gRNAs generated by both the initiation step and the amplification cycles into the RISC to direct the endonucleolytic cleavage of mRNA or ssRNA (effector cycle).

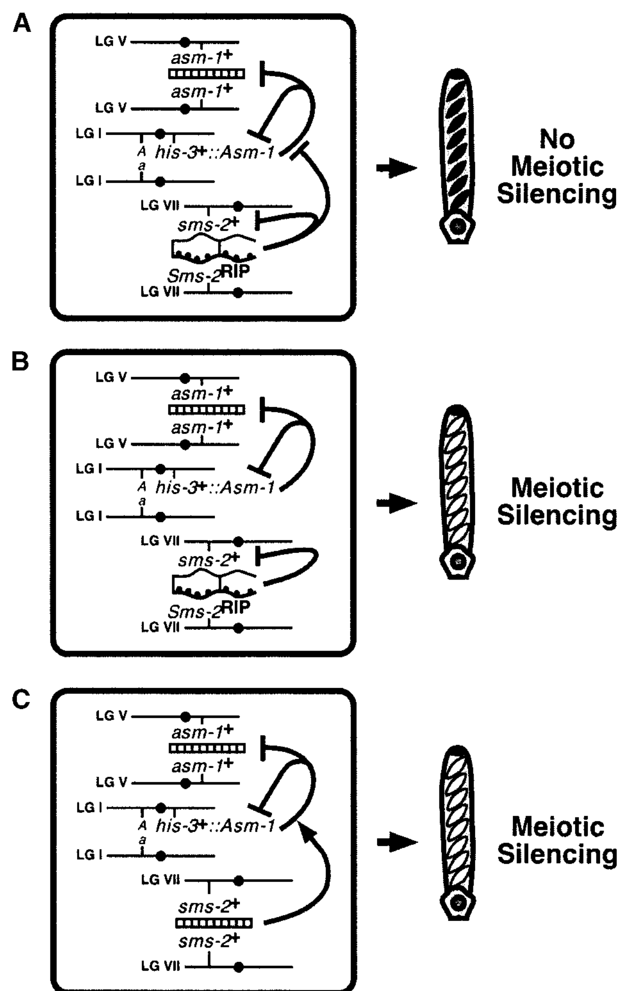

Figure 3.—Testing the role of *sms-2*$^{+}$ in meiotic silencing in a diploid zygote cell. Boxed areas show the pairing of LG I, LG V, and LG VII chromosomes. Both LG V chromosomes carry wild-type alleles of *Asm-1* (*asm-1*$^{+}$). On LG I, one chromosome contains a DNA fragment corresponding to the *Asm-1* region inserted at the *his-3* locus, whereas the other chromosome carries no insert. On LG VII, one chromosome carries a wild-type allele of *Sms-2* (*sms-2*$^{+}$), whereas the other chromosome carries either a mutant allele of *Sms-2* (*Sms-2*^{RIP}; A and B) or a wild-type allele of *Sms-2* (*sms-2*$^{+}$; C). To the right is the schematic representation of a single ascus containing the predicted outcome from the different crosses. Inside these asci, solid ovals and open ovals represent viable and inviable ascospores, respectively.

We predicted this to be possible because, according to the meiotic silencing model, in these crosses the inability of *sms-2*$^{+}$ to pair with *Sms-2*^{RIP2} or with *Sms-2*^{RIP88} presumably induces partial silencing of the *sms-2*$^{+}$ allele itself (Figure 3A). This *cis*-silencing is thus expected to significantly decrease the amounts of *sms-2*$^{+}$ transcript and SMS-2 protein below that which is expected from a single fully functional gene. According to this logic, if there is enough active SMS-2 protein in heterozygous crosses (probably synthesized before the *sms-2*$^{+}$ gene is silenced) to allow meiosis to proceed, but not enough to maintain a fully functional meiotic silencing machinery, we should be able to determine the involvement of *sms-2*$^{+}$ in meiotic silencing.

To test this idea, we set up crosses heterozygous for *Sms-2* (Figure 3A) and induced meiotic silencing by unpairing a copy of the reporter gene *Asm-1*. Silencing of *Asm-1*, whose gene product ASM-1 is required for ascospore maturation, results in white and inviable ascospores. We designed two strains; the first contained either *Sms-2*^{RIP2} or *Sms-2*^{RIP88} at the canonical locus in LG VII (*Sms-2*^{RIP}) in an otherwise wild-type background. The second strain contained *Asm-1* DNA integrated at the *his-3* locus in LG I (*his-3*$^{+}$*::Asm-1*). Both strains contained wild-type *asm-1*$^{+}$ alleles at their normal chromosomal positions in LG V (*asm-1*$^{+}$). Because the unpaired *Asm-1* region present in LG I (*his-3*$^{+}$*::Asm-1*) has no pairing partner on the homologous chromosome, it is expected to trigger meiotic silencing, which, in turn, will silence all unpaired and paired copies of *Asm-1* present in the genome. Under these conditions, if SMS-2 is part of the meiotic silencing machinery, a progeny of black and viable ascospores should be produced by these crosses despite the presence of an unpaired copy of *Asm-1* (Figure 3A). In contrast, if SMS-2 is not part of the meiotic silencing machinery, the unpaired copy of *Asm-1* (*his-3*$^{+}$*::Asm-1*) will silence all *Asm-1* copies present in the genome, resulting in a progeny of white and inviable ascospores (Figure 3B). In any case, control crosses homozy-

gous for $sms\text{-}2^{+}$ are expected to result in the production of a progeny of inviable ascospores (Figure 3C).

Progeny of viable ascospores were observed in crosses heterozygous for either $Sms\text{-}2^{RIP2}$ (crosses 6 and 7) or $Sms\text{-}2^{RIP88}$ (cross 8; Table 1). The percentage of mature ascospores observed in experimental crosses *vs.* the percentage observed in control crosses was 17.9% (cross 6) *vs.* 0.3% (cross 9), 30% (cross 7) *vs.* 0.3% (cross 10), and 23.2% (cross 8) *vs.* 0.3% (cross 10), respectively.

The persistence of a fraction of immature white ascospores, in our opinion, is at least partially attributable to RIP (Selker 1990) and/or to an incomplete suppression (remember, these crosses are heterozygous for *Sms-2*). If RIP occurs in a gene that is essential for ascospore maturation, like *Asm-1*, spores carrying only the RIPed allele will not mature (Aramayo and Metzenberg 1996). In addition, if RIP occurs, the crippled silencing machinery present in the zygote of these heterozygous crosses is more capable of silencing the only remaining functional gene than of silencing the three functional genes that are present when RIP does not occur (D. W. Lee and R. Aramayo, unpublished results). Consistent with this interpretation, we observed a direct relationship between the insert size of the *Asm-1* fragments used to induce silencing and the amount of black and viable ascospores produced (data not shown).

These results therefore establish the participation of *Sms-2* in meiotic silencing and are consistent with *qde-2* and *Sms-2* functioning in two different RNA-silencing pathways (*i.e.*, quelling and meiotic silencing, respectively). The biological roles of QDE-2 and SMS-2 proteins are neither redundant nor interchangeable on the basis of the fact that homozygous $Sms\text{-}2^{RIP2}/Sms\text{-}2^{RIP2}$ or $Sms\text{-}2^{RIP88}/Sms\text{-}2^{RIP88}$ crosses and heterozygous $Sms\text{-}2^{RIP2}/Sms\text{-}2^{RIP88}$ crosses, all homozygous for $qde\text{-}2^{+}$, are completely barren and that expression of the $qde\text{-}2^{+}$ coding region under the control of the *Sms-2* promoter does not complement the meiotic barrenness of crosses homozygous for *Sms-2* loss-of-function alleles (R. J. Pratt and R. Aramayo, unpublished results). In addition, homozygous *qde-2* loss-of-function crosses undergo normal meiosis (R. J. Pratt and R. Aramayo, unpublished results). At the DNA level, *Sms-2* is as related to an unrelated gene like *Asm-1* as it is to *qde-2* (data not shown), and at the protein level, SMS-2 is more identical to Argonaute-like proteins from *Homo sapiens* [*e.g.*, 44.4% identity to brain-specific protein KIAA1567 (GenBank accession no. BAB13393.1)] than to QDE-2 (*i.e.*, 37.7%; Figure 1B). On the basis of what we know, however, we still cannot exclude the possibility that genes like *qde-2* play a minor role in meiotic silencing.

In contrast, demonstrating that *Sad-1* and *Sms-2* are both part of the same and only meiotic RNA-silencing pathway is not trivial, due to the nonlinear behavior of RNA-silencing pathways (Hannon 2002). On the basis of the hypothesis that proteins that function together in a pathway or structural complex are likely to evolve in a correlated fashion (Marcotte *et al.* 1999a,b; Pellegrini *et al.* 1999), we argue that *Sad-1* and *Sms-2* are both part of the same silencing pathway. This is because, in phylogenetic trees, SAD-1 and SMS-2 cluster consistently with their corresponding homologs of the single functional pathway observed in *Schizosaccharomyces pombe* (Hall *et al.* 2002; Volpe *et al.* 2002) and Galagan *et al.* (2003).

To study the genetic relationship between *Sad-1* and *Sms-2*, we tested the meiotic behavior of single and double mutants. We generated a null allele of *Sad-1* ($Sad\text{-}1^{RIP64}$) using RIP by duplicating a 2711-bp DNA region corresponding to the promoter and coding region of the gene at the *his-3* locus in LG I (for details see Methods, electronic supplementary material at http://www.gentics.org/supplemental/). As predicted for a loss-of-function allele, homozygous $Sad\text{-}1^{RIP64}/Sad\text{-}1^{RIP64}$ crosses were completely barren, as was found in homozygous crosses between $Sad\text{-}1^{\Delta}$ deletion alleles (Shiu *et al.* 2001; Shiu and Metzenberg 2002). Heterozygous $sad\text{-}1^{+}/Sad\text{-}1^{RIP64}$ crosses produced ascospores, but their number was greatly reduced compared to the number generated in crosses between $sad\text{-}1^{+}$ strains (K. Baker and R. Aramayo, unpublished data). Therefore, as in the case of *Sms-2*, testing the involvement of $sad\text{-}1^{+}$ in meiotic silencing in the complete absence of $sad\text{-}1^{+}$ is not possible, and here again, performing heterozygous crosses is the best known way to go.

We first tested the ability of $Sad\text{-}1^{RIP64}$ to suppress meiotic silencing. For this we constructed strains containing $Sad\text{-}1^{RIP64}$ at the canonical locus in LG I in either an $asm\text{-}1^{+}$ or an $Asm\text{-}1^{\Delta}$ background [$Asm\text{-}1^{\Delta(3430\text{-}9336)}$; Table 1]. In both cases, strains contained wild-type $asm\text{-}1^{+}$ alleles at the ectopic *his-3* chromosomal position in LG I. During meiosis, $asm\text{-}1^{+}$ at its canonical position has no pairing partner due to the presence of the $Asm\text{-}1^{\Delta(3430\text{-}9336)}$ deletion allele in the homologous chromosome. This is expected to trigger silencing, which, in turn, will silence all unpaired and paired copies of *Asm-1* present in the genome. As expected, viable ascospore progeny were observed in crosses heterozygous for $Sad\text{-}1^{RIP64}$ (crosses 11–14, Table 1). The percentage of mature ascospores observed in reciprocal experimental crosses *vs.* the percentage observed in control crosses was 51.3% (cross 11) and 48.9% (cross 12) *vs.* 0% (cross 13) and 53.5% (cross 14) and 52% (cross 15) *vs.* 0.1% (cross 16), respectively. We then determined the fraction of mature ascospores produced by crosses heterozygous for *Sms-2* ($sms\text{-}2^{+}/Sms\text{-}2^{RIP88}$) and *Sad-1, Sms-2* ($sad\text{-}1^{+}/Sad\text{-}1^{RIP64}$, $sms\text{-}2^{+}/Sms\text{-}2^{RIP88}$), to be 16.7% (cross 17) and 48.7% (cross 18; Table 1), respectively. The reduced percentage of mature ascospores observed in crosses between strains, each of them containing duplicated DNA (36.8%, cross 19, Table 1), in our opinion is attributable to RIP and to the consequent induction of meiotic silencing that results from the inability of the $asm\text{-}1^{+}$ allele(s) to pair with their RIPed partners in their homologous chromosomes.

These results are consistent with the idea that *Sad-1*

and *Sms-2* are both necessary but not sufficient for meiotic silencing. They also demonstrate that *Sad-1* is genetically epistatic to *Sms-2*. In addition, under our working meiotic silencing model (Figure 2), it is expected that mutations in *Sad-1* would have a more profound effect than mutations in *Sms-2* in the functioning of the pathway. This is because mutations affecting *Sad-1*, whose gene product is predicted to act at two different stages within the pathway (Figure 2), are expected to stop the silencing reaction at an early stage. In contrast, mutations affecting *Sms-2*, whose gene product is predicted to act in the effector cycle (Figure 2), are not expected to affect the silencing mediated by the SAD-1-RdRP and Dicer in the initiation step (Figure 2). Two Dicer-like genes in the Neurospora genome could serve this function (Galagan *et al.* 2003). This interpretation is consistent with the accumulation of siRNA detected during quelling in *qde-2* loss-of-function mutants (Catalanotto *et al.* 2002).

The connection between the presence of unpaired DNA and RNA silencing during meiosis is tantalizing. Every component of the meiotic silencing pathway identified to date is required for the completion of meiotic prophase (Shiu *et al.* 2001). Current dogma dictates that all RNA-based gene-silencing mechanisms share the objective of protecting the genome from invading transposable elements, but why would a meiotic RNA-silencing pathway be conserved in a genome that has evolved very efficient mechanisms for detecting repeated elements (*e.g.*, RIP)? In our view, the products of the meiotic RNA-silencing machinery either play a previously unrecognized gene regulatory role during meiosis (*e.g.*, via production of micro-RNAs; Ambros 2001; Lagos-Quintana *et al.* 2001; Lau *et al.* 2001; Lee and Ambros 2001) or have evolved as key components of the meiotic chromosome biology (*e.g.*, via formation of heterochromatin; Bernard *et al.* 2001; Allshire 2002; Dernburg and Karpen 2002; Jenuwein 2002; Reinhart and Bartel 2002; Volpe *et al.* 2002).

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