

Chromosome segment duplications in *Neurospora crassa*: barren crosses beget fertile science

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Studies on *Neurospora* chromosome segment duplications (*Dps*) performed since the publication of Perkins's comprehensive review in 1997 form the focus of this article. We present a brief summary of Perkins's seminal work on chromosome rearrangements, specifically, the identification of insertional and quasiterminal translocations that can segregate *Dp* progeny when crossed with normal sequence strains (*i.e.*, $T \times N$). We describe the genome defense process called meiotic silencing by unpaired DNA that renders *Dp*-heterozygous crosses (*i.e.*, $Dp \times N$) barren, which provides a basis for identifying *Dps*, and discuss whether other processes also might contribute to the barren phenotype of $Dp \times N$ and $Dp \times Dp$ crosses. We then turn to studies suggesting that large *Dps* (*i.e.*, >300 kbp) can allow smaller gene-sized duplications to escape another genome defense process called repeat-induced point mutation (RIP), possibly by titration of the RIP machinery. Finally, we assess whether in natural populations dominant RIP suppressor *Dps* provide an "RIP-free" niche for evolution of new genes following the duplication of existing genes.

Keywords: genome defense, meiotic silencing by unpaired DNA, *Neurospora* chromosome rearrangements, repeat-induced point mutation, translocations and duplications

Chromosome segment duplications in *Neurospora*

In 1997, Perkins published a "tour-de-force" review article that described everything then known about 355 chromosome rearrangements in *Neurospora crassa*.⁽¹⁾ These included 262 reciprocal translocations, 30 insertional translocations, 31 "quasiterminal" translocations, 25 complex translocations, 6 pericentric inversions, and 1 intrachromosomal transposition. In reciprocal translocations, the terminal segment of two chromosomes is reciprocally interchanged, whereas in insertional translocations, a segment from a donor chromosome is transferred to a recipient chromosome without any

reciprocal exchange (Fig. 1). Quasiterminal translocations move a distal segment of the donor chromosome to the tip region of another chromosome, distal to any essential gene. The breakpoint on the donor chromosome is presumably capped with a tip from the recipient chromosome, thus making the quasiterminal translocation formally equivalent to a reciprocal translocation (Fig. 1). Intrachromosomal transpositions are essentially insertional translocations in which a single chromosome is both the "donor" and "recipient." Insertional and quasiterminal translocations were deemed to be especially noteworthy, because their heterozygous crosses with normal sequence strains (*i.e.*, $T \times N$) can generate a duplication (*Dp*) of the translocated segment (Fig. 2). Depending on the translocation, the duplicated segment can be hundreds of kilobase pairs (kbp) in size and can include many genes.⁽¹⁾ Following Perkins,⁽¹⁾ the term "duplication" (*Dp*) will be used to designate either a chromosome segment that is present as two non-tandem copies or a strain that contains such a segment. An individual *N. crassa* strain corresponds to the vegetative mycelium produced by the germination of a single ascospore.⁽²⁾ Strains can be of one of two mating types, *mating type A* (*mat A*) or *mating type a* (*mat a*). In a cross, *mat A* and *mat a* individuals come into contact and form fruiting bodies called perithecia (Fig. 3). Within the perithecia, fusion of *mat A* and *mat a* nuclei results in the formation of diploid zygote nuclei that immediately undergo meiosis. The four haploid products of meiosis (a tetrad) stay together in a sac called an ascus. The meiotic products undergo a further mitotic division to produce an octad of ascospores within each ascus. The progeny ascospores are forcibly ejected from the perithecia and can be collected as an unordered octad on an agar surface (additional details of the life cycle can be found at <http://www.fgsc.net/Neurospora/neurospora.html>). *Dp* strains are recognizable by the fact that their crosses with the wild type (*i.e.*, $Dp \times N$) are characteristically barren.⁽¹⁾ Barren crosses make normal looking perithecia but produce exceptionally few progeny ascospores. The barrenness of *Dp*-heterozygous crosses is now known to be caused, at least in part, by a presumably RNAi-based "genome defense" process called meiotic silencing by unpaired DNA (MSUD, or simply "meiotic silencing").⁽³⁾

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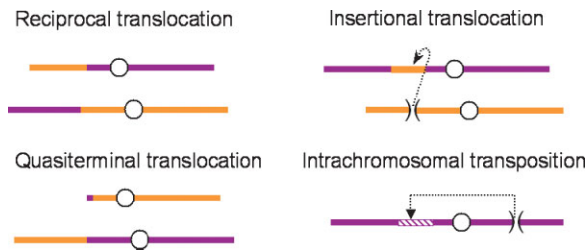


Figure 1. Translocation chromosomes. In the reciprocal translocation the terminal segments of two chromosomes (one purple, the other orange) are reciprocally interchanged, whereas in the insertional translocation, a segment from the orange (donor) chromosome is transferred to the purple (recipient) chromosome without any reciprocal exchange. In a quasiterminal translocation a distal segment of the donor (orange) chromosome is moved to the tip region of the purple chromosome, distal to any essential gene. Presumably the breakpoint on the orange chromosome is capped with the tip from the purple chromosome. The intrachromosomal transposition is essentially an insertional translocation with the same chromosome being both the “donor” and “recipient.”

This article focuses on the studies done on *Dps* since the publication of Perkins’s 1997 *magnum opus*. After briefly summarizing Perkins’s seminal work on the chromosome rearrangements, we describe how meiotic silencing contributes to the barren phenotype of *Dp*-heterozygous crosses. Evidence is presented that suggests that meiotic silencing of *Dp*-borne genes might not be as complete as, say, that of a gene unpaired by an ascus-dominant mutation. We also assess whether other processes contribute to the barrenness of *Dp*-heterozygous crosses. In addition, we summarize

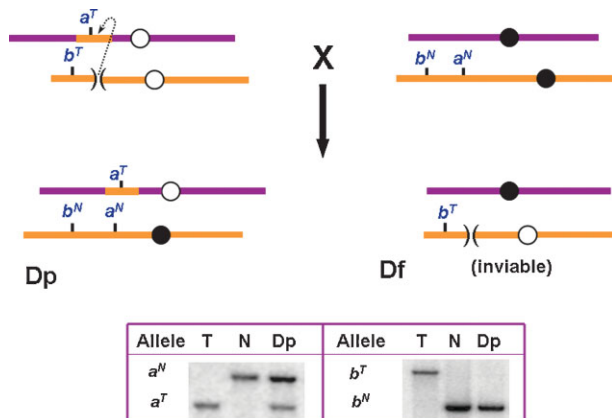


Figure 2. Bracketing of *Dp* breakpoints between covered (locus *a*) and uncovered (locus *b*) RFLPs. The translocation strain is usually of the standard OR genetic background, defined by the RFLP alleles *a^T* and *b^T*, whereas the normal sequence strain has the wild-isolate (W) genetic background, defined by the alleles *a^N* and *b^N*. *Dp* strains, identified by the barrenness of *Dp*-heterozygous crosses, possess both alleles of the covered locus *a* (*a^T* and *a^N*), but only the *b^N* allele of the uncovered locus *b*, complementary *Df* (deficiency) progeny possess the *b^T* allele and are deleted for the *a* locus. *Df* progeny are generally inviable.

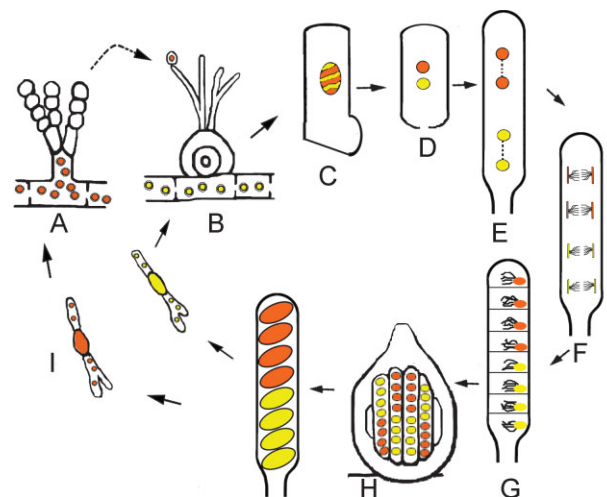


Figure 3. *Neurospora crassa* life cycle (adapted from drawing by T. Griffiths at <http://www.fgsc.net/Neurospora/sectionB2.htm>). Ascospores of the two mating types *mat A* and *mat a*, distinguished here by different colored nuclei, germinate (I) and produce vegetative hyphae that can differentiate into asexual spores (conidia, A) and female structures called protoperithecia (B). Fertilization of protoperithecia by conidia of the opposite mating type (dotted arrow) triggers differentiation of protoperithecia into fruiting bodies called perithecia (C–H). Inside a perithecium many dikaryotic ascogenous cells are formed in which the *mat A* and *mat a* nuclei eventually fuse and produce diploid nuclei (C). Each diploid nucleus immediately undergoes meiosis (D, E). The four haploid products of meiosis stay together in a sac called an ascus. The four meiotic products undergo a further mitotic division (F), and form an octad of eight ascospores within each ascus, four of each mating type (G). Ascospores are forcibly ejected from the “beak” of the perithecium. Conidia can germinate and produce vegetative hyphae like those produced by the ascospores.

studies suggesting that the presence of large *Dps* in a cross enables smaller gene-sized duplications to escape another genome defense process called repeat-induced point mutation (RIP), possibly by titration of the RIP machinery. Finally, we discuss the potential significance of *Dps* as dominant suppressors of RIP in natural populations.

Perkins’ characterization of chromosome rearrangements

Perkins characterized each of the 355 rearrangements by visual inspection of ascospores in unordered octads from *Translocation* × *Normal* (*T* × *N*) crosses. Five classes of octads are possible based on the number of black (B, viable, non-deficiency) and white (W, inviable, deficiency) ascospores per ascus, 8B:0W; 6B:2W; 4B:4W; 2B:6W; and 0B:8W. From the observed proportions of these different octad types Perkins inferred the kind of rearrangement and the position of its breaks relative to the centromere. Isosequential crosses (*i.e.*, *N* × *N* or *T* × *T*) produce 95–98% 8:0 octads, whereas if *T*

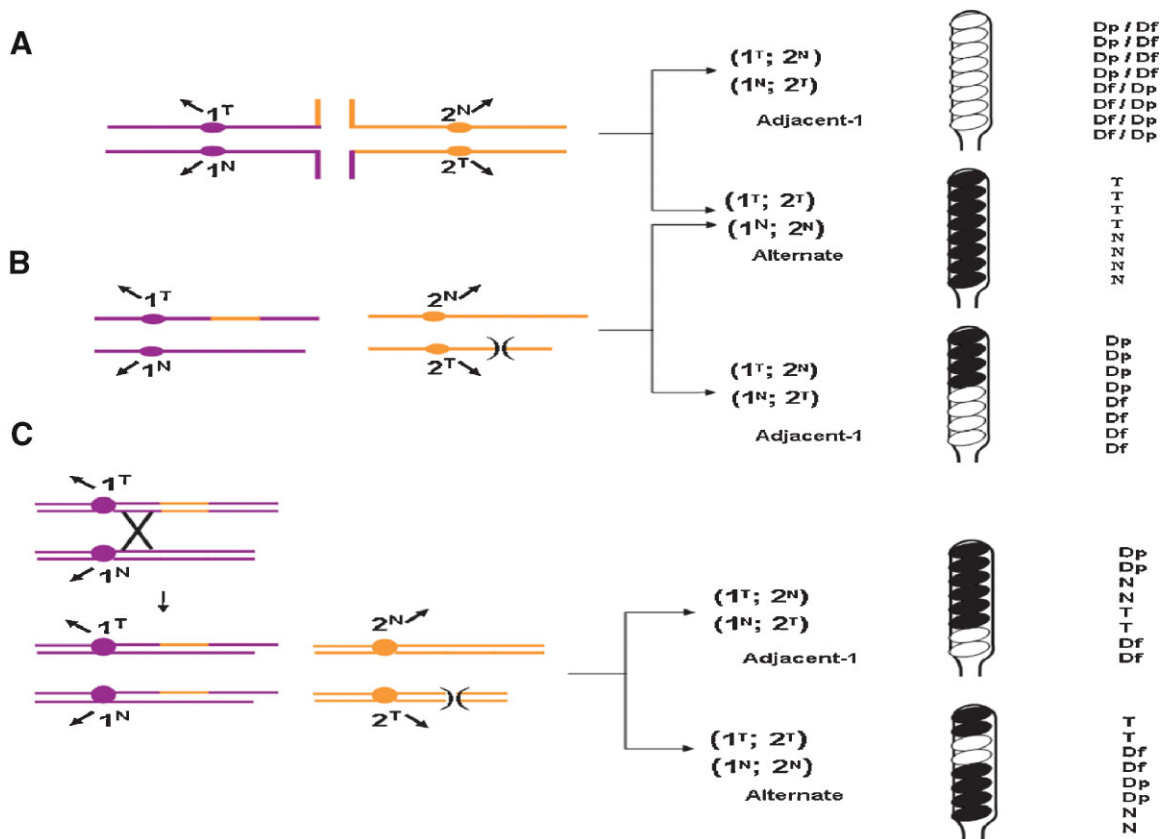


Figure 4. Distinguishing reciprocal and insertional translocations. **A:** Ascus types from the cross *Reciprocal Translocation* × *Normal Sequence*. Alternate segregation without crossover produces 8B:0W asci bearing eight viable black ascospores with the parental genetic constitution. Adjacent 1 segregation without crossover produces 0B:8W asci bearing eight inviable white ascospores with complementary duplication/deficiency classes. **B:** Ascus types from *Insertional Translocation* × *Normal Sequence*. Alternate segregation without crossover produces 8B:0W asci with eight ascospores of the parental genetic constitution. Adjacent 1 segregation without crossover produces 4B:4W asci bearing four viable duplication (*Dp*) ascospores and four inviable deficiency (*Df*) ascospores. In both (A) and (B), alternate and adjacent 1 segregation are equally probable, therefore obtaining 8:0 = 0:8 is diagnostic of a reciprocal translocation, and 8:0 = 4:4, of an insertional translocation. **C:** Production of 6B:2W asci following crossover between the centromere and an insertional translocation. The proportion of 6:2 asci from *Insertional Translocation* × *Normal Sequence* provides an estimate of the interstitial crossover frequency. The frequency of 4B:4W asci from *Reciprocal Translocation* × *Normal Sequence*, in a like manner, provides an estimate of interstitial crossover frequency. In crosses with normal sequence, quasiterminal translocations behave similar to insertional translocations.

is a reciprocal translocation, $T \times N$ crosses make equal numbers of 8:0 and 0:8 octads (Fig. 4). The former result from “alternate” segregation (which generates the parental types), whereas the latter are from “adjacent 1” segregation (which generates complementary duplication/deficiency types). Any 4:4 signals a crossover between the centromere and the translocation breakpoint, and no 6:2 and 2:6 are expected. If T is an insertional or quasiterminal translocation, then 8:0 = 4:4 (Fig. 4), whereas 6:2 signal interstitial crossover, and 0:8 and 2:6 are rare. This is because the “recipient” chromosome from the T parent and the N homolog of the “donor” chromosome move to the same pole in adjacent 1 segregation and generate a duplication of the translocated segment, and only the complementary “deficiency” segregants are inviable (Fig. 4). The largest viable *Neurospora* deficiency recorded thus far is a 77 078-bp deletion from linkage group

(LG) IV of the mutant strain *overaccumulator of carotenoids* (*ovc*), which includes 21 predicted ORFs.⁽⁴⁾

Perkins also obtained *Dp* segregants from intercrosses between reciprocal translocations, or between pericentric inversions, whose breakpoints were appropriately positioned in the same two chromosome arms or in the same arm.⁽¹⁾ Such intercrosses were potentially useful for obtaining interbreakpoint duplications of segments not recoverable with the kit of insertional and quasiterminal translocations. In practice, however, since most mutations are usually recovered on an N chromosome, mapping them onto the genome sequence is more conventionally achieved with *Dps* from $T \times N$ crosses (see for example⁽⁵⁾). If the N parent used is a wild-isolated strain, then one can localize the *Dp* breakpoints with precision by testing linked RFLPs between the T and N strains for coverage by the duplication.⁽⁶⁾ This method (Fig. 2,

see also Box 1), is not as easily extendable to *Dps* derived from intercrosses between reciprocal translocations and pericentric inversions, since both the parental rearrangement strains share the standard laboratory Oak Ridge (OR) background. Other applications of *Dps* have included examination of dosage effects and identification of genes that determine vegetative incompatibility.⁽¹⁾

Sizing *Dps*.

Several wild-isolated (*W*) *N. crassa* strains are available from the Fungal Genetics Stock Center (<http://www.fgsc.net/cat/FGSCcat11.html>) and can be used as the *N* parent in *T* × *N* crosses. The *Dps* breakpoints can be localized by testing the resultant *Dp* progeny strains for coverage of linked RFLPs between the parental *T* and *W* strains.⁽⁶⁾ *Dp* progeny contain both the *T* and *W* alleles of any RFLPs that lie within the translocated (*i.e.*, duplicated) segment, but only the *W* allele of RFLPs outside this segment (Fig. 2). Almost any *N. crassa* genomic segment can now be amplified by PCR using oligonucleotide primers designed using the genome sequence (<http://www.fgsc.net/Neurospora/neuros.htm>), and the amplified DNA can be searched for restriction site polymorphisms within the amplicon (PCR-based RFLPs) or used to probe for RFLPs *via* Southern hybridization. First, the *Dp* strain is verified to contain both the *T* and *W* alleles of an RFLP marker located within (or close to) a known covered gene. Then additional RFLP markers at distances of, say, ~100 kbp from the initial duplicated RFLP are tested in a similar manner. The presence in the *Dp* of both the *T* and *W* alleles of the new RFLP indicates that the *Dp* extends to that RFLP, but if the *Dp* exhibits only one allele (*W*, in the absence of crossovers) then it does not extend to the RFLP. In this way the duplication breakpoints can be localized to intervals bracketed by a pair of covered and uncovered RFLPs that are at most ~100 kbp apart. By iterating this step with additional RFLPs that sub-divide this interval into progressively narrower intervals, the breakpoints can be localized to segments bracketed by RFLPs only about 3–5 kbp apart. More precise localizations can then be achieved by testing oligonucleotides from within the bracketed interval in PCR with *T* strain DNA as template. If the primers yield an amplified product, it follows that they do not bracket the breakpoint. But if the primers fail to amplify from *T*, but do so from OR, it would suggest that they bracket the translocation breakpoint. Determining the extent of individual *Dps* on the genome sequence with such unprecedented precision creates a powerful asset for duplication mapping studies. All the *N. crassa* *Dp* breakpoints localized to date are summarized in Table 1.

Meiotic silencing and the barren phenotype of *Dp*-heterozygous crosses

The first sighting of meiotic silencing was reported in 1996. It was referred to then as “transvection.”⁽⁷⁾ A recent review puts meiotic silencing in context with similar phenomena in other organisms.⁽⁸⁾ Meiotic silencing silences genes that fail to pair with a homolog in meiotic prophase I.^(3,9) Since silencing might involve an RNA-based mechanism, it also causes silencing of any homologs of the unpaired gene, regardless of whether the homologues are themselves paired. For example, a cross heterozygous for an ectopically inserted transgenic copy of the *Ascospore maturation-1* (*Asm-1*) gene, despite being homozygous for the endogenous *asm-1*⁺ allele, nevertheless produces only white inviable ascospores, whereas crosses homozygous for both the ectopic and the endogenous genes produce viable black ascospores.^(3,7,10) In other words, ascospore maturation is blocked by the presence of an “unpaired” *Asm-1* allele.⁽³⁾ Heterozygosity for an ectopic copy of any of several other genes also triggers barrenness if their encoded product is essential during meiosis (*e.g.*, β -tubulin, actin, and histones H3 and H4-1), whereas the corresponding homozygous crosses are fertile.⁽³⁾ Fertility was unaffected if the unpaired gene’s product was not required in meiosis (*e.g.*, *pan-2*⁺, *int1*⁺, *hH1-GFP*).⁽³⁾ Thus, an “ascus-dominant” phenotype ensues only if the silenced gene is essential for normal meiosis and ascospore development.⁽³⁾

Semi-dominant mutant alleles of *Sad-1*, *Sad-2*, and *Sms-2* suppress meiotic silencing (and consequently suppress ascus-dominant mutations), hence their designations *Suppressor of ascus-dominance* (*Sad*) or *Suppressor of meiotic-silencing* (*Sms*).^(3,9,11,12) The semi-dominant mutations suppressed the ascospore maturation defect due to heterozygosity of the *Asm-1* transgene. They also suppressed other “classic” ascus-dominant phenotypes such as those due to the mutations *Round spore* (*R*), *Banana* (*Ban*), and *Peak-Dominant* (*Pk^D*), suggesting that the latter also result from MSUD.^(3,11) *R* × *wild-type* crosses produce 100% round ascospores, whereas *R* × *Sad-1* crosses produce normal-shaped ascospores (Fig. 5). *Pk^D* × *wild-type* crosses develop asci that are swollen sacs of disordered ascospores, whereas *Pk^D* × *Sad-1* crosses make many normal asci. In *Ban* × *wild-type* crosses, all four meiotic products and their mitotic progeny nuclei are contained in one giant banana-shaped ascospore, whereas significantly more asci are eight-spored in *Ban* × *Sad-1* crosses. The *sad-1* gene encodes a putative RNA-dependent RNA polymerase (RdRP),^(3,9) the *sad-2* gene, a protein required for the proper perinuclear localization of the SAD-1 RdRP,⁽¹¹⁾ and the *sms-2* gene, an argonaute-like protein used in meiotic silencing.⁽¹²⁾ The semi-dominant alleles are either complete or partial deletions, or their sequence is so severely altered by RIP that they probably fail to pair with their wild-type homologs. It is speculated that this induces the semi-dominant alleles to make

Table 1. *Dp* breakpoints localized on the *N. crassa* genome sequence.

<i>Dp</i>	LG	Size (kbp)	Proximal break		Distal break		Reference
			Primers	bp/contig	Primers	bp/contig	
1. <i>EB4</i>	VR	140	gctcggtagtctcggaaatca cgccccgatcaacctttacaa	7527/7.13	ggcggcaacatagcattgac caactatgtcctggctccta	6486/7.13	(30)
2. <i>IBj 5</i>	VIL	403	tcccgcittcgtttaataaaaat ggatcaagcgacactcaataatc	3404/7.12	tgtcttacccttcacagaggta tgtagaataagctcatcggagga	2175/7.12	(6)
3. <i>UK14-1</i>	VIR	272	cgccccgttctctgctctct cggggcaggcgaacgagaaga	2960/7.4	ND	–	a
4. <i>AR17</i>	IIIR	352	tcccatcagaggaaaattctgta aacaagcgctgttctacattagg	3480/7.17	catcctgcttgaacattctgta gggcaaaaagatcaaaggagtct	2772/7.17	(6)
5. <i>Z88</i>	VIIR	545	ggcggcgacaagcgactagc cggcgcgtaacacacatagca	2404/7.52	ccgccttctcgttgccctct ccacctccgcccgcactctg	1721/7.23	a
6. <i>Y112M4i</i>	IR	404	ccgcccccaactccatcac ccgggtgtgctggtctcttc	2026/7.9	gcggggatagccaccagtctg cgccggcctcctggatgatgt	8567/7.9	a
7. <i>AR173</i>	IR	260	IR centromeric sequences ^b		tgtgtgtggcctgagatgta gtggtgtggcgaagaggaaa	1628/7.72	a
8. <i>NM177</i>	IIR	691	gcgccaacctttccaattct cggggtggcagtaaacgagtg	2220/7.33	gggggaaagtgggcgataaac ccgccgtagttgcaagatga	1572/7.5	a
9. <i>OY320</i>	VIR	424	cgcgcatgaatgaggtggt ccgggcaggggtcgtatga	2360/7.4	ND		a
10. <i>OY329</i>	VIR	704	tcacatacgacgaagtagacacc ggtgagaggatcaactccttttc	2159/7.4	attatgtagggagggtacgtgt gcaattaattatcctagacgttag	1183/7.4	(6)
11. <i>B362i</i>	IVR	117	ttctggaccagtattgttcggt acatacaccatggagcagagt	15 439/7.47	caagcatcagttcttgtcacac ggaagagacaggtcattcaggat	1965/7.47	(6)
12. <i>R2394</i>	IIL	151	cgccgagtcaagtccatcat atccatccgtccatccatcc	4122/7.8	cggggttgcggggagtctg gcgcgctcgaagacctcaagac	6338/7.8	(30)
13. <i>4540</i>	IR	554	ccgccccgtggtgtgat ggcggggcatcaaacagacaa	1839/7.9	cccgcgcccgaatgatgac ccccacgtcccgcacacacc	2176/7.9	a
14. <i>39311</i>	IL	2592	gcaaggcaaggagaaacaaaga cgcaatgaggagaatggagtca	1501/7.6	ND		a

ND = not determined.

^aThis work.^bEstablished by inverse PCR from sequences adjacent to the distal breakpoint.

an abnormal product (*i.e.*, RNAi) that autogenously silences them. Therefore, the semi-dominant mutations are themselves ascus dominant.^(3,13) Inadequate levels of the SAD-1, SAD-2, and SMS-2 proteins result in an overall suppression of meiotic

silencing.^(3,11,12) Crosses homozygous for *sad-1*, *sad-2*, or *sms-2* mutants are completely infertile.^(3,11,12) This suggests these proteins might have another more general role, probably before meiotic prophase, when the silencing of many loci occurs.

The *Neurospora Spore killer* meiotic drive elements, *Sk-2* and *Sk-3*, can dominantly suppress meiotic silencing.⁽¹⁴⁾ In a heterozygous *Sk* × *wild-type* cross, the *Sk* element causes the death of ascospores that do not inherit it. All mature asci contain four normal-sized, viable black ascospores that have inherited the killer element and four tiny unpigmented and undeveloped inviable ascospores that have not. All spores are killed in the doubly heterozygous *Sk-2* × *Sk-3* cross (mutual killing), whereas no spores are killed in the *Sk*-homozygous crosses, although they also are suppressed for meiotic silencing. Both *Sk-2* and *Sk-3* map to a 30-map unit region that spans the centromere of LG III in which recombination is blocked in the *Sk*-heterozygous crosses.

The semi-dominant *Sad-1*, *Sad-2*, and *Sms-2* alleles and the *Sk* haplotypes could significantly (>100–1000-fold)

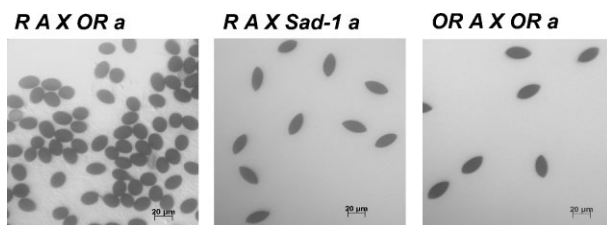


Figure 5. Meiotic silencing and the round spore phenotype. **A:** All ascospores from the cross *R A* × *OR a* are round (or nearly so), as expected for the phenotype of the ascus-dominant *R* (*Round spore*) mutant. The *OR a* strain represents the standard laboratory *OR* background. **B:** Ascospores from the cross *R A* × *Sad-1 a* have the normal spindle shape as expected from suppression of meiotic silencing by *Sad-1*, and are indistinguishable from control ascospores from *OR A* × *OR a* (**C**).

Table 2. Crosses involving strains bearing *Dps*, *R*, and suppressors of meiotic silencing.

Sr. no.	Cross	Phenotype	Productivity
1	<i>Dp(AR17) A</i> × <i>OR a</i>	Barren	10–20 Spores
2	<i>Dp(B362i) A</i> × <i>OR a</i>	Barren	No spores
3	<i>Dp(IBj5) A</i> × <i>OR a</i>	Barren	No spores
4	<i>Dp(AR17) a</i> × <i>Sad-1 A</i>	Fertile	
5	<i>Dp(B362i) A</i> × <i>Sad-1 a</i>	Fertile	
6	<i>Dp(IBj5) A</i> × <i>Sad-1 a</i>	Fertile	
7	<i>Dp(AR17) A</i> × <i>Sad-2 a</i>	Fertile	
8	<i>Dp(B362i) A</i> × <i>Sad-2 a</i>	Fertile	
9	<i>Dp(IBj5) a</i> × <i>Sad-2 A</i>	Fertile	
10	<i>Dp(AR17) a</i> × <i>Sms-2 A</i>	Fertile	
11	<i>Dp(B362i) A</i> × <i>Sms-2 a</i>	Fertile	
12	<i>Dp(IBj5) a</i> × <i>Sms-2 A</i>	Barren	10–20 Spores
13	<i>Dp(UK 3-41) A</i> × <i>OR a</i>	Barren	~50 Spores
14	<i>Dp(UK 3-41) A</i> × <i>Sad-1 a</i>	Fertile	
15	<i>Dp(UK 3-41) A</i> × <i>Sad-2 a</i>	Fertile	
16	<i>Dp(UK 3-41) A</i> × <i>Sms-2 a</i>	Fertile	
17	<i>R A</i> × <i>OR a</i>	Fertile, round	
18	<i>R A</i> × <i>Sad-2 a</i>	Fertile, spindle	
19	<i>R a</i> × <i>Daggaluru A</i>	Fertile, Round	
20	<i>Sad-1; Dp(AR17) a</i> × <i>Dp(AR17) A</i>	Barren	150–250 Spores
21	<i>Sad-1; Dp(B362i) A</i> × <i>Dp(B362i) a</i>	Barren	150–250 Spores
22	<i>Sad-2; Dp(AR17) A</i> × <i>Dp(AR17) a</i>	Barren	150–200 Spores
23	<i>Sad-2; Dp(B362i) A</i> × <i>Dp(B362i) a</i>	Barren	50–200 Spores
24	<i>Sad-2; Dp(IBj5) a</i> × <i>Dp(IBj5) A</i>	Barren	150–250 Spores
25	<i>Sms-2; Dp(AR17) a</i> × <i>Dp(AR17) A</i>	Barren	150–250 Spores
26	<i>Sms-2; Dp(B362i) A</i> × <i>Dp(B362i) a</i>	Barren	50–200 Spores
27	<i>Sms-2; Dp(IBj5) a</i> × <i>Dp(IBj5) A</i>	Barren	No spores
28	<i>R Dp(AR17) a</i> × <i>Dp(AR17) A</i>	Barren, round	20–30 spores
29	<i>R Dp(AR17) a</i> × <i>Dp(AR17); Sad-2 A</i>	Barren, mostly spindle; few round	150–250 Spores

enhance the productivity of *Dp*-heterozygous crosses, thus showing that meiotic silencing contributes to the barren phenotype^(3,11,14) (see also Table 2 entries 1–12). In comparison with *Sad-1* and *Sad-2*, *Sms-2* effected at best only a marginal increase in the productivity of crosses heterozygous for the chromosome segment duplication *Dp(IBj5)* (Table 2, entry 12), possibly reflecting the fact that decreasing the level of the argonaute-like protein is a less effective way to suppress meiotic silencing than *via* reduction or mislocalization of the SAD-1 RdRP.⁽¹³⁾ Presumably, during meiosis of a *Dp*-heterozygous cross, a copy of each *Dp*-borne gene fails to pair properly, thereby silencing all the *Dp*-borne genes, including those essential for the completion of meiosis and ascus development, and this renders the cross barren.

Interestingly, crosses of *Dp(UK3-41)* strains with OR strains of the opposite mating type were barren, whereas their crosses with *Sad-2* strains were fertile (Table 2, entries 13 and 15). This result was noteworthy because *Dp(UK3-41)* covers the *sad-2* locus;^(1,11,15) consequently, in both *Dp(UK3-41)*-heterozygous crosses the *sad-2*⁺ gene must be silenced like any other *Dp*-borne gene, and, if the silencing was complete, their productivity ought not to have differed. One explanation for the difference is that *Dp*-borne genes might not be as completely silenced as, say, a gene unpaired

opposite an ascus-dominant mutation. In this case, the *Dp*-borne *sad-2*⁺ allele would be incompletely silenced, whereas, based on the analogy with the complete silencing observed for the hH1-GFP transgene in heterozygous crosses,^(3,11) the semi-dominant *Sad-2* mutation probably effects a more complete silencing of *sad-2*⁺. An alternative possibility could be a dosage effect. There are three *sad-2*⁺ copies in the first cross (one of which is unpaired) and only two *sad-2*⁺ copies in the second cross (both of which are unpaired); however, it is difficult to see how an RNAi-based process could have such a sensitive dosage response. Incomplete silencing of *Dp*-borne genes might explain why, on the one hand, heterozygosity for a single ectopic copy of, say, the β -tubulin gene can induce almost complete infertility,⁽³⁾ while on the other hand, although a cross heterozygous for a ~700-kb duplication like *Dp(OY329)* is barren, it nevertheless consistently produces a few ascospores and it is not necessarily less productive than, for example, a cross heterozygous for the much smaller (~117-kb) duplication *Dp(B362i)*.⁽⁶⁾ One way to verify whether *Dp*-borne genes are incompletely silenced would be to engineer a *Dp*-heterozygous cross with three copies of the hH1-GFP transgene,^(16,17) two allelic and one *Dp*-borne; if the asci display nuclear GFP-fluorescence, then the meiotic

silencing induced by the “*Dp*-borne” transgene would be proven to be incomplete.

Do other processes contribute to the barren phenotype?

Prior to the discovery of MSUD, the barrenness of *Dp*-heterozygous crosses was attributed to RIP, another genome defense process of *Neurospora* (and other fungi).⁽¹⁸⁾ RIP occurs in the premeiotic dikaryon that forms following fertilization, prior to the fusion of the *mat A* and *mat a* nuclei, and alters duplicated DNA sequences (in the otherwise haploid genome) by hypermutating G:C base pairs into A:T base pairs and subsequently methylating many of the remaining C residues.⁽¹⁸⁾ The presumed function of RIP is to protect the genome against the proliferation of transposable elements and other repeated DNA sequences.⁽¹⁹⁾ Duplications of more than 400 bp and sharing >85% sequence identity can serve as substrates for the RIP machinery.⁽²⁰⁾ Perkins *et al.*⁽²¹⁾ showed that chromosome segment *Dps* also can be substrates for RIP. Based on this, it was suggested that mutation of *Dp*-borne genes by RIP might contribute to the barrenness of *Dp*-heterozygous crosses (see also Ref⁽²²⁾). It was never made clear why a cross should be rendered barren by RIP-induced mutations occurring in only one nucleus of the premeiotic dikaryon (*i.e.*, the one with the *Dp*). This hypothesis was undermined by the demonstration that *Dp*-heterozygous crosses that were also either heterozygous for a dominant suppressor of RIP, or homozygous for the recessive *RIP-defective* (*rid*) mutation,⁽²³⁾ did not differ in productivity from that of their RIP-competent controls.^(24,25) Ruling out a role for RIP in the barren phenotype did not exclude the involvement of other processes.

One hint that barrenness might involve additional processes came following the discovery that significantly more ascospores were produced in crosses of *Dp* strains with the wild-isolated Dagguluru-1 strain (FGSC #3360).⁽²⁶⁾ However, the Daggaluru-1 strain did not suppress meiotic silencing. We performed the cross Daggaluru-1 *A* × *R a* and found that the progeny ascospores were round rather than spindle-shaped (Table 2, entry 19). Additionally, in the cross Daggaluru-1 *A* × *hH1-GFP a* (heterozygous for a histone H1-green fluorescent protein fusion), the unpaired *hH1-GFP* transgene was not rescued from silencing in meiotic tissues (data not shown). Therefore, productivity enhancement by Dagguluru-1 must not be *via* suppression of meiotic silencing. Instead, the Daggaluru-1 strain might suppress some other process that also contributes to the barren phenotype of *Dp*-heterozygous crosses.

Another hint that meiotic silencing might not provide a complete explanation for the barren phenotype came from

Dp-homozygous crosses. Whereas an ectopic copy of an essential gene induces meiotic silencing-dependent barrenness only in heterozygous, but not in homozygous crosses,⁽³⁾ *Dp*-homozygous crosses were about as barren as the corresponding *Dp*-heterozygous crosses⁽⁶⁾ and references therein). Moreover, *Sad-1* and *Sad-2* failed to significantly enhance the productivity of the *Dp*-homozygous crosses⁽⁶⁾ (see also Table 2, entries 20–27). This raised the possibility that there may be additional processes, distinct from meiotic silencing, that contribute to the barrenness of *Dp*-homozygous crosses, and that, although these processes might not be as pronounced in a *Dp*-heterozygous cross, they might nonetheless contribute to its barren phenotype.

To try and retain a role for meiotic silencing in the barrenness of the *Dp*-homozygous crosses, it was hypothesized⁽²⁵⁾ that the *Dp*-borne genes might switch between allelic and “non-allelic” pairing partners, and that transient unpairing during the switches triggers meiotic silencing, thus rendering the homozygous cross as barren as the heterozygous one. In this model, gene-sized duplications do not switch partners in homozygous crosses or might do so in a way that does not trigger meiotic silencing. It was further hypothesized that the switching might desensitize the detection of unpairing at *sad*⁺, possibly by decreasing signal-to-noise, thus rendering the *Sad-1* and *Sad-2* alleles recessive. If the “desensitization model” were correct, then homozygosis for a *Dp* would, in a like manner, also be expected to suppress other ascus-dominant mutations. We performed the cross *R; Dp(AR17) a* × *Dp(AR17) A* and found that, although it was barren, it nevertheless produced a few ascospores and all were round (Table 2, entry 28). We also crossed the *R; Dp(AR17) a* strains with a *Dp(AR17); Sad-2 A* strain. Although these crosses also were barren, they produced marginally more ascospores and they were overwhelmingly spindle-shaped (Table 2, entry 29). That homozygosis for *Dp(AR17)* failed to suppress both the R phenotype and its suppression by *Sad-2* argued against the desensitization model, but was consistent with the idea that other process might underlie the barrenness of *Dp*-homozygous crosses, and possibly *Dp*-heterozygous ones as well.

In a personal communication to one of us (D. P. K.), the late Metzberg very tentatively suggested a radical interpretation of our finding that heterozygosis for *Sad-1* or *Sad-2* does not relieve the barren condition of *Dp(AR17)*-homozygous crosses. He suggested “that there is not just one, but TWO requirements *vis a vis* pairing of genes if mRNA and then proteins are to be made under their direction. First, they must be paired at pachytene, and second, they must become unpaired at diplotene or soon thereafter. Normally, this would occur in any mating of *N* × *N*. In a mating of *N* × *Dp*, the unpaired segment will normally cause barrenness, but not if meiotic silencing is suppressed by heterozygosis for *Sad-1* or *Sad-2*. After pachytene pairing of homologs is disrupted, the

ectopic duplicated segment, formerly unpaired, would immediately pair with the canonical segment homologous to it so that these segments of DNA are not expressed as the zygote moves out of meiotic prophase 1. However, the other canonical chromatid, with no ectopic partner to usurp it, is expressed normally. Since no cell walls have been laid down at this stage, the products are shared, and meiosis proceeds normally. The contrasting situation is when both parents to the cross are *Dp(AR17)*. As the homologs separate as they leave prophase 1, all the canonical sequences involved in *Dp(AR17)* promptly pair with their ectopic homologs and are inactivated. Hence, the cross is barren." This novel and unconventional idea (typical of Metzberg) might be testable either by cytological examination or by looking for suppressors of this new form of barrenness among mutagenized cultures, or even among existing mutants that are thought to weaken or eliminate pairing at some other stage of meiosis (*e.g.*, *mei-2*).

Dps and RIP

A gene-sized duplication (typically, <5 kbp) can escape RIP in crosses that are heterozygous for another relatively large (>300 kbp) *Dp*. Crosses involving strains bearing a transgenic 1.2-kbp duplication of the *ergosterol-3* (*erg-3*) gene ordinarily produce RIP-induced *erg-3* mutant progeny at frequencies in the range of 2–25%, but if the cross is also heterozygous for a large *Dp* then the frequency is <0.5%.^(26–30) Ascospores mutant in *erg-3* produce colonies with a distinct morphology on Vogel's-sorbose agar medium that makes them easy to score under a dissection microscope.⁽²⁴⁾ Of 39 duplications screened in this way, 34 were shown to possess this "dominant RIP suppressor" phenotype and only 5 were non-suppressors.⁽³⁰⁾ A minimum size could be estimated for 27 of the suppressor *Dps* and they were all >270 kbp. In striking contrast, three non-suppressor duplications for which a maximum size was estimated were of 100–200 kbp.⁽³⁰⁾ Thus, in general, suppressor *Dps* appear to be larger than non-suppressor *Dps*. Moreover, RIP was also suppressed in crosses heterozygous (or homozygous) for multiple non-suppressor duplications if the combined duplicated segments exceeded 300 kbp.⁽³⁰⁾ These results are consistent with the hypothesis that large duplications titrate out the RIP machinery and that the "equivalence point" is ~300 kbp. Dominant suppression of RIP by large *Dps* was also demonstrated using a small duplication of the *downy* (*dow*) gene located on LG IIIR, about five map units proximal to *erg-3*.⁽⁶⁾ The ~355-kbp chromosome segment duplication, *Dp(AR17)*, covers *dow*, whereas *Dp(dow)* is a much smaller (~1.4 kbp) tagged ectopic duplication of *dow*. The cross *Sad-1; Dp(AR17) × Dp(dow); erg-3* can potentially generate RIP-induced *dow* mutant progeny either by RIP in *Dp(AR17)* or in *Dp(dow)*; if crossovers are disregarded, these mutants are recovered, respectively, among the *erg+* or *erg-3* progeny. We

found the *dow* mutant phenotype in 4/98 *erg+* progeny but 0/86 *erg-3* progeny.⁽⁶⁾ These results suggested that the occurrence of RIP in the large duplication suppresses it in the small duplication. In other work, it was suggested that the suppression of *eas*^{*UCLA191*}-induced recurrent *cya-8* mutation by *Dp(OY329)* and *Dp(S1229)* reflected the dominant suppression of RIP targeted by a presumptive insertion of *cya-8* sequence into the *eas*^{*UCLA191*} allele.⁽³¹⁾

Mutant alleles generated by RIP in a small duplication tend to be more severely altered than those generated by RIP in a large *Dp*. *Dp(D305)* is a large and unstable chromosome segment duplication of distal LG IIIR that includes the *dow* and *erg-3* loci. One RIP-induced *erg-3* mutant was obtained out of 7558 progeny examined from the cross *Dp(D305) × OR*.⁽³²⁾ Southern analysis of *Sau3A* 1-digested genomic DNA from this mutant showed no evidence for cytosine methylation at *erg-3*, suggesting it had suffered only mild RIP.⁽³²⁾ In contrast, the *erg-3* locus was heavily methylated on cytosine residues in 3/3 *erg-3* mutants obtained following RIP in the small *Dp(erg-3)* duplication.⁽³³⁾

A role for Dps in natural populations?

Screens of ~450 wild-isolated strains resulted in the identification of 7 strains as dominant RIP suppressors. Two suppressor strains, Golur-1 (P0334) and Sugartown (P0854) were also barren in crosses, thus hinting that the dominant RIP suppressor phenotype might be due to chromosome segment duplications.⁽²⁹⁾ Three other barren strains identified in these screens did not show the suppressor phenotype [Georgetown-6 (P2622), Batu Ferringi-1 (P2581), and Brabadougou (P4296)], and it is possible these three strains contain duplications <300 kbp. One wild-isolated suppressor strain, Adiopodoumé (FGSC 430), was also the only *Neurospora* strain found containing active copies of the transposable element called *Tad*.⁽³⁴⁾ All the other *Neurospora* strains examined, including those of species other than *N. crassa*, contained only RIP-altered relics of *Tad*.⁽³⁵⁾ Therefore, the dominant RIP suppressor phenotype is likely to have contributed to the survival of *Tad* in the Adiopodoumé strain. RIP suppression by the Adiopodoumé strain was mapped to a variant allele for translesion DNA polymerase ζ .⁽³⁶⁾ Pol ζ is dispensable for RIP,⁽³⁷⁾ but an RIP-suppressive Pol ζ encoded by the Adiopodoumé strain might be well placed to interfere with the assembly of other factors essential for RIP in *Neurospora* (see below for an alternative hypothesis). These findings demonstrate that dominant RIP suppressor wild isolates can provide RIP-free passage to duplicated genes (*i.e.*, *Tad*) through sexual crosses. This raises the possibility that dominant RIP suppressor *Dps* might facilitate the evolution of new genes from duplications of existing genes. Once the duplicate gene acquires a different function, its sequence has probably been sufficiently changed

that it is no longer a substrate for RIP, whereas other “hitchhiking” selfish DNA would be eliminated. A complete *Tad* element is about 7 kbp in size;⁽³⁴⁾ thus, even if the *Adiopodoumé* genome contained ~40 copies of the complete element, it would harbor as much as 280 kbp of duplicated DNA, which is still less than the ~300-kbp “equivalence point” for titration of the RIP machinery. However, it is conceivable that in derivative strains that contained, say, twice as many *Tad* elements, a dominant RIP suppressor phenotype might be triggered by titration and thus become independent of the suppressor Pol ζ .

On the other hand, RIP is probably not quite as insurmountable an evolutionary “hurdle” as it has been made out to be.⁽¹⁹⁾ All that is necessary for a duplicated gene to persist in the population is for it to have a population advantage in the vegetative phase that equals or exceeds the disadvantage in the sexual phase incurred by RIP. Moreover, *Neurospora* surely undergoes many vegetative doublings between episodes of sexual reproduction. During this phase of growth, of course, RIP does not occur. A very conservative estimate might be 20 doublings or about 48 hours of growth from a single conidium to a small conidiating colony (generating a million nuclei). It even seems possible that as many as 300 doublings could occur, if sexual cycles in the tropics occur only during the cool season (mating in *Neurospora* does not seem to occur at temperatures above 25°C). Now consider RIP. The range of frequency for this process has been from a high of 25% to a low of 2%. Let us assume the worst case: 25% RIP (75% non-RIP) and only 20 doublings between sexual cycles. The vegetative advantage necessary to maintain a gene duplication is then the 20th root of 1.00/0.75, or 1.014. If only 2% RIP occurs and 20 doublings, it is the 20th root of 1.00/0.98, or 1.001. If 300 vegetative doublings intervene between sexual cycles, the corresponding vegetative advantage needed to compensate for the sexual disadvantage of 25% RIP and 2% RIP are, respectively, 1.0096 and 1.000066. Thus, notwithstanding the evidence for *Tad*'s persistence in the *Adiopodoumé* strain, an evolutionary role for dominant RIP suppressors, including *Dps*, still remains to be established.

RIP or RIP-like processes have also been found in a few other fungi including *Podospora anserina*, *Magnaporthe grisea*, *Leptosphaeria maculans*, and *Microbotryum violaceum*, but in an apparently much less severe form than in *Neurospora*.^(38–42) That these fungi also contain active transposable elements^(38,42,43) suggests that “mild” RIP is not an effective genome defense mechanism. In fact, it has been suggested that “mild” RIP might accelerate the evolution of duplicated genes, as for example, in the *het-D/E* family in *P. anserina*.⁽⁴⁴⁾ In this scenario, *Dps* and other dominant RIP suppressors might serve to fine tune RIP between a “mild” form, to accelerate divergence of duplicated genes, and a “severe” form, for genome defense. Intriguingly,

the *upr-1* gene, whose *N. crassa* *Adiopodoumé* allele (*upr-1^{Ad}*) was determined to be an RIP suppressor,⁽³⁶⁾ is located just proximal to the gene *ncu 01953*, the *Neurospora* homolog of the *P. anserina* gene *ami1*. *Podospora* crosses homozygous mutant for *ami1* show a dramatic increase in RIP frequency.⁽⁴⁵⁾ Having two genes with an RIP phenotype sitting “cheek by jowl” in the *N. crassa* genome (~4.5 kbp apart) is probably more than just mere coincidence, and a deeper relationship between their RIP phenotypes might yet be found. For example, it is conceivable that *upr-1^{Ad}* suppresses RIP by altering the local chromatin organization so as to increase expression of the *ami1*-homolog, and the actual *upr-1^{Ad}*-encoded protein itself might not have a role in the RIP suppressive effect.

An RIP-like process, called MIP (methylation-induced premeiotically), was found in the fungus *Ascobolus immerses* (for a review see⁽⁴⁶⁾). Like RIP, MIP causes cytosine methylation of duplicated DNA sequences, but unlike RIP, it does not induce G:C to A:T mutations.⁽⁴⁶⁾ The *masc1* gene encodes the *Ascobolus* ortholog of the *Neurospora* RID protein, and MIP is drastically reduced in crosses heterozygous mutant for *masc1*.⁽⁴⁷⁾ However, whereas homozygous *rid* mutant crosses are fertile, homozygous *masc1* mutant crosses are sterile.⁽⁴⁷⁾ This suggested that either some amount of MIP is required for completion of sexual development or that *masc1* has an essential function in sexual development distinct from its role in MIP. Interestingly, *Aspergillus nidulans* crosses homozygous mutant for *dmtA*, the ortholog of *rid* and *masc1*, also are sterile, even though RIP, MIP, or cytosine methylation have not been observed in this species⁽⁴⁸⁾, but see Ref⁽⁴⁹⁾. More research is needed to understand how the RID protein and its homologs function in genome defense but not in sexual development in *Neurospora*, in sexual development but apparently not in genome defense in *Aspergillus*, and in both processes in *Ascobolus*.

Conclusions

Three major advances have been made in *Neurospora Dp* research since the 1997 review. Foremost was the recognition that meiotic silencing contributes, at least partially, to the barrenness of *Dp*-heterozygous crosses. This finding has had tremendous practical significance because the *Sad-1*, *Sad-2*, *Sms-2*, *Sk-2*, or *Sk-2* suppressors of meiotic silencing can now be used to enhance the productivity of *Dp*-heterozygous crosses, and thereby facilitate the use of *Dps* in routine genetic analysis. Although RIP does not display any detectable contribution to the barren phenotype, the involvement of other processes has not been ruled out. Recently, it was suggested that meiotic silencing may be absent or inefficient in the closely related pseudohomothallic species

N. tetrasperma.⁽¹⁷⁾ Therefore, it might be possible to test whether *Dp*-heterozygous and homozygous crosses are comparably barren in *N. tetrasperma*. Another possibility would be to map the Dagguluru-1 strain's suppressor of barrenness. A third possibility would be to test whether the dominant or semi-dominant MSUD suppressors rescue a "*Dp*-borne" GFP-transgene from meiotic silencing to comparable extents in *Dp*-heterozygous and homozygous crosses.

The second advance in *Neurospora Dp* research came with the demonstration of their susceptibility to RIP together with additional results suggesting that they might titrate out the RIP machinery and thereby act as dominant suppressors of RIP. The discovery that at least one wild-isolated dominant RIP suppressor strain (Sugartown) was also a candidate *Dp* strain, together with the discovery that the only *Neurospora* strain found harboring active transposable elements is also a dominant RIP suppressor provides support to the idea that *Dps* might play hitherto unrecognized yet significant roles in natural populations. The third advance in *Dp* research is the unprecedented precision with which we can now map their breakpoints onto the genome sequence (Box 1). This makes *Dps* yet more powerful genetic tools in the armamentarium of this model organism.⁽²⁾

Acknowledgments: Research in the laboratory of D. P. K. is supported by the Council of Scientific and Industrial Research, India. The Fungal Genetics Stock Center (FGSC) generously waived charges for strains. The FGSC is supported by National Science Foundation grant BIR-9222772. P. K. S., S. V. I., and M. R. were supported by Research Fellowships from the CSIR-UGC. We dedicate this article to the memory of David R. Stadler (1925–2007).

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