MOLECULAR DISSECTION OF THE SPORE KILLER ELEMENTS IN NEUROSPORA

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Doctor of Philosophy

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by

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The undersigned, appointed by the Dean of the Graduate School, have examined the

dissertation entitled

MOLECULAR DISSECTION OF THE SPORE KILLER ELEMENTS IN

NEUROSPORA

Presented by David G. Rehard

a candidate for the degree of Doctor of Philosophy

and hereby certify that, in their opinion, it is worthy of acceptance.

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Dr. Patrick Shiu, Chair

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Dr. Elizabeth Bryda

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Dr. David Setzer

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List of Abbreviations

aRNA   aberrant RNA

dsRNA   double-stranded RNA

GFP   green fluorescent protein

mRNA   messenger RNA

ORF   open reading frame

rasiRNA   repeat-associated small interfering RNA

RdRp   RNA-directed RNA Polymerase

RIP   Repeat Induced Point-mutation

RISC   RNA-Induced Silencing Complex

RNAi   RNA interference

siRNA   small interfering RNA

WG   Westergaard

Abstract

The role of selfish genetic elements in shaping the evolution of genomes (and subsequently species) has been a great subject of scientific interest for much of the last century. These selfish elements act at both the genetic and cellular level to insure that they are propagated within the genome, species, or both. Meiotic drive elements, which increase in number by destroying allelic counterparts, have been implicated in the extinctions of whole species and in the evolution of RNAi-mediated genome defense mechanisms. In fungi, several meiotic drive systems are known but not well understood. In the experiments described below, we attempt to characterize and differentiate the Spore killer (*Sk*) meiotic drive systems of the genus *Neurospora* as well as examine the defensive mechanisms that have arisen to prevent the spread of these and other selfish genetic elements.

The Neurospora Spore killer meiotic drive elements are believed to resemble other classic drive systems in their composition of two loci, a killer locus that creates drive for itself and a resistance locus that protects the killer from self-killing. The majority of my research herein has been a combined effort with Dr. Thomas Hammond on the mapping and characterization of the resistance locus in the Spore killer systems.

We began by utilizing the recently created *Neurospora* gene knockout library to create a series of 3-point testcrosses to map the location of a natural resistance gene in an

r(Sk-2) strain. Testcross analysis narrowed the gene candidate field to just six genes. Next, knockout mutants were created for the remaining genes. Crosses of these mutants to *Sk* revealed that only one of the genes, *NCU09151*, confers resistance to *Sk-2*. We therefore identify *NCU09151* as *resistant to Spore killer* 2 [*rsk$^{r(Sk-2)}$*].

Next, we showed that when *rsk$^{r(Sk-2)}$* is inserted into a sensitive strain, resistance to *Sk-2* is granted to that strain, further solidifying the notion that *NCU09151* is the resistant gene in the *r(Sk-2)* strains. Based on our crossing data, we also hypothesized that *Sk-2* must also use *NCU09151* for its own resistance. Indeed, deletion of *NCU09151* in *Sk-2* lead to self-killing in a cross to a sensitive strain while introduction of *NCU09151^{Sk-2}* to a sensitive strain granted resistance to *Sk-2*.

After the resistance gene had been identified in the *Sk-2* system, we then asked whether the *Sk-3* system utilized the same gene for resistance. Gene knockout and transformation studies similar to those done on the *Sk-2* system revealed that *Sk-3* and *r(Sk-3)* strains also utilized the *NCU09151* locus for resistance [*rsk^{Sk-3}*, *rsk$^{r(Sk-3)}$*].

Sequence analysis of *rsk* from both *Sk-2* and *Sk-3* revealed unique indel patterns specific to each Spore killer, solidifying the hypothesis that *Sk-2* and *Sk-3* are unique drive systems. This analysis, along with the observations from our prior experiments, allowed us to propose a killer-neutralization model to explain how the killer molecule and the resistant protein might function in the two Spore killer systems.

Having identified the resistant component in the Neurospora Spore killer systems, we then wanted to tackle the question of how the drive systems are able to suppress meiotic silencing by unpaired DNA (MSUD). One possibility is that the Spore killers might harbor a dominant mutant gene that disrupts the MSUD system, thereby allowing

unpaired genes to be expressed throughout meiosis. *sad-p*, a gene known to be required for proper MSUD is found within the Spore killer recombination block and is thus a prime candidate. Here we report that *sad-p*^{Sk-2} is not a hypermorphic suppressor of MSUD.

I. Introduction

I.1. *Neurospora crassa*

I.1.i. A brief history

Neurospora crassa was first extensively studied and characterized in 1927 by Bernard Dodge and Cornelius Shear (Perkins 1992). Their work would eventually inspire George Beadle and Barbara McClintock to utilize the species as a model organism for genetic research. George Beadle, along with his colleague Edward Tatum, studied biochemical pathways in *N. crassa*. Their research eventually led to the groundbreaking One-gene One-enzyme theory for which they received the Nobel Prize in 1958. Barbara McClintock, who would also go on the win a Nobel Prize (not for work on *N. crassa*), contributed to the success of *N. crassa* as a model organism when she first identified the seven chromosomes of the species and described meiosis and post-meiotic mitoses in the fungus. Many and more scientists studying *N. crassa* have contributed a great deal of information to the scientific community over the past century and many still do so today.

I.1.ii. Biology of *Neurospora*

Neurospora crassa is a haploid filamentous fungus that is commonly found growing on recently burned, dead organic substrates. It grows on the surface of these charred substrates where it forms a network of hyphae (filamentous cells) called a mycelium. The hyphae contain incomplete crosswalls that allow water, nutrients, and even organelles (including nuclei) to move freely throughout the mycelial network (Roca

et al. 2005). *N. crassa* is easily identified in the wild by the bright orange color of its asexual cells (conidia) (Figure I.1).

Neurospora crassa belongs to the fungal phylum *Ascomycota*. Members of this phylum bear their sexual spores (ascospores) within an "ascus" (Greek for "sac"). The species is typically found in tropical and subtropical regions, but is also known to exist in places as far north as Korea and as far south as Australia (Turner, 2001). It has both asexual and sexual cycles.

In 2003 the genome of *Neurospora crassa* was sequenced and published for public use. Sequencing revealed that the seven *N. crassa* chromosomes contained some 40 megabases worth of DNA with about 10,000 predicted open reading frames (Galagan *et al.* 2003). Publication of the genome has resulted in a resurgence of the use of *N. crassa* as a genetic model organism and has provided unique insights into the workings of fungal species.

I.1.iii. Life cycle

Shown in Figure I.2 are the asexual and sexual cycles of *Neurospora crassa*. The asexual macroconidiation pathway produces millions of multinucleated, clonally identical macrocondia through the constriction of aerial hyphae (Springer 1993). Another asexual pathway, the microconidiation pathway (not pictured), produces small amounts of uninucleate asexual spores that burst through the cell wall of normal mycelial hyphae rather than aerial hyphae.

Figure I.1. *Neurospora crassa*
Pictured above is a wild-type culture of *N. crassa* in its vegetative cycle. A mat of mycelia covers the circular agar plate and on the edges aerial hyphae produce orange conidia. (Picture courtesy of Logan Decker)
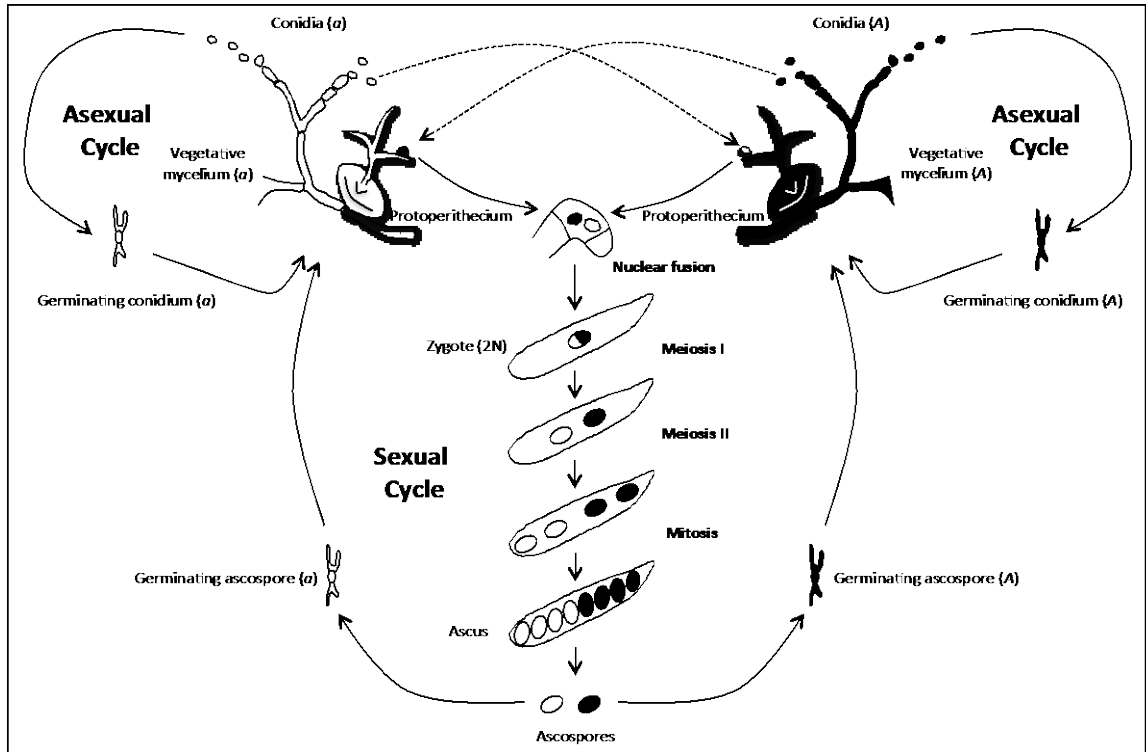
Figure I.2. The life cycle of *Neurospora crassa*
Depicted above are the two mating types of *N. crassa*, *a* (white) and *A* (black). Both mating types are capable of reproducing sexually or asexually depending on the availability of nutrients and presence of conidia from the opposite mating type. Both mating types are also capable of acting as either the male or female during sexual reproduction. The sexual cycle produces zygotes, the only diploid stage in the *N. crassa* lifecycle. The asexual cycle produces conidia that are capable of germinating genetically identical fungal colonies.

The sexual cycle is induced when a nitrogen-starved fungus produces female reproductive structures called protoperithecia. When a conidium (asexual spore) of the opposite mating type comes into contact with the protoperithecium, fertilization occurs and leads to the only diploid stage in the life cycle of the fungus. Two rounds of meiosis and a post-meiotic mitosis eventually result in an ascus containing eight haploid ascospores, four of each mating type (*A* or *a*) (Springer 1993).

I.2. RNA interference (RNAi)

I.2.i. Overview

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism that is conserved throughout eukaryotes. Many different kinds of RNAi pathways exist, but a good portion of them utilizes small interfering RNAs (siRNAs), 20-30 nucleotides long, to regulate cellular processes like transcription, chromosome segregation, and translation (Dang *et al.* 2011). The RNAi mechanisms also play important roles in defending cells against selfish genetic elements like transposons and viral DNA (Matzke and Birchler 2005).

I.2.ii. Functional components

There are three common components found in most RNAi systems: Argonaute, Dicer, and RNA-dependent RNA polymerase (RdRp). In a typical RNAi system, aberrant RNA transcripts are recognized and converted into dsRNA by a RdRp. Dicer then binds to and subsequently cleaves the dsRNA into 20-30nt long siRNAs. Then, the double-stranded siRNAs bind to Argonaute where they are converted into single-stranded siRNA molecules. These single-stranded siRNA molecules guide Argonaute, and proteins that

associate with it (collectively known as the RISC, or RNA-induced silencing complex), to homologous mRNA and degrades them. It is important to note that Argonaute and Dicer are both required for RNAi while RdRp has been shown to not be necessary in some systems (Matzke and Birchler, 2005).

I.2.iii. RNAi systems in *Neurospora crassa*

RNAi mechanisms play a key role in protecting the genomic integrity of an organism from selfish genetic elements like viral DNA, transposons, and meiotic drive elements. Because *N. crassa* is a haploid organism, it is more vulnerable to attack and subsequent loss of fitness from these kinds of elements. For these reasons *N. crassa* has three silencing mechanisms that work to suppress selfish genetic elements; Repeat-induced point mutations (RIP), Meiotic Silencing by Unpaired DNA (MSUD), and Quelling (Figure I.3). For a comprehensive review of the three silencing systems please refer to Dang *et al* 2011.

I.2.iv. Meiotic Silencing by Unpaired DNA

Meiotic Silencing by Unpaired DNA (MSUD) is a post-transcriptional RNAi mechanism that silences the expression of unpaired genes during the sexual cycle of *N. crassa* (Shiu *et al.* 2001). During prophase I of meiosis, homologous chromosomes are paired and subsequently compared to each other. Any unpaired gene(s) is recognized and silenced throughout the sexual cycle.

The initial recognition mechanism of complex DNA (in the form of unpaired DNA during homologous pairing) is still unknown. The current model suggests that unpaired DNA might cause a looping out or hairpin-like structure of DNA (Figure I.4).

Quelling

A conidia

A mycelium

outgrowth

outgrowth

a mycelium

fertilization

germinated ascospores

a conidia

a trichogyne

a protoperithecium

A

a ascus

proliferation of nuclei

peritecithium / perithecium

ascogenous hypha with A and a nuclei

conjugate division

diploid nucleus

meiosis and mitosis

nuclear fusion (karyogamy)

MSUD   **RIP**
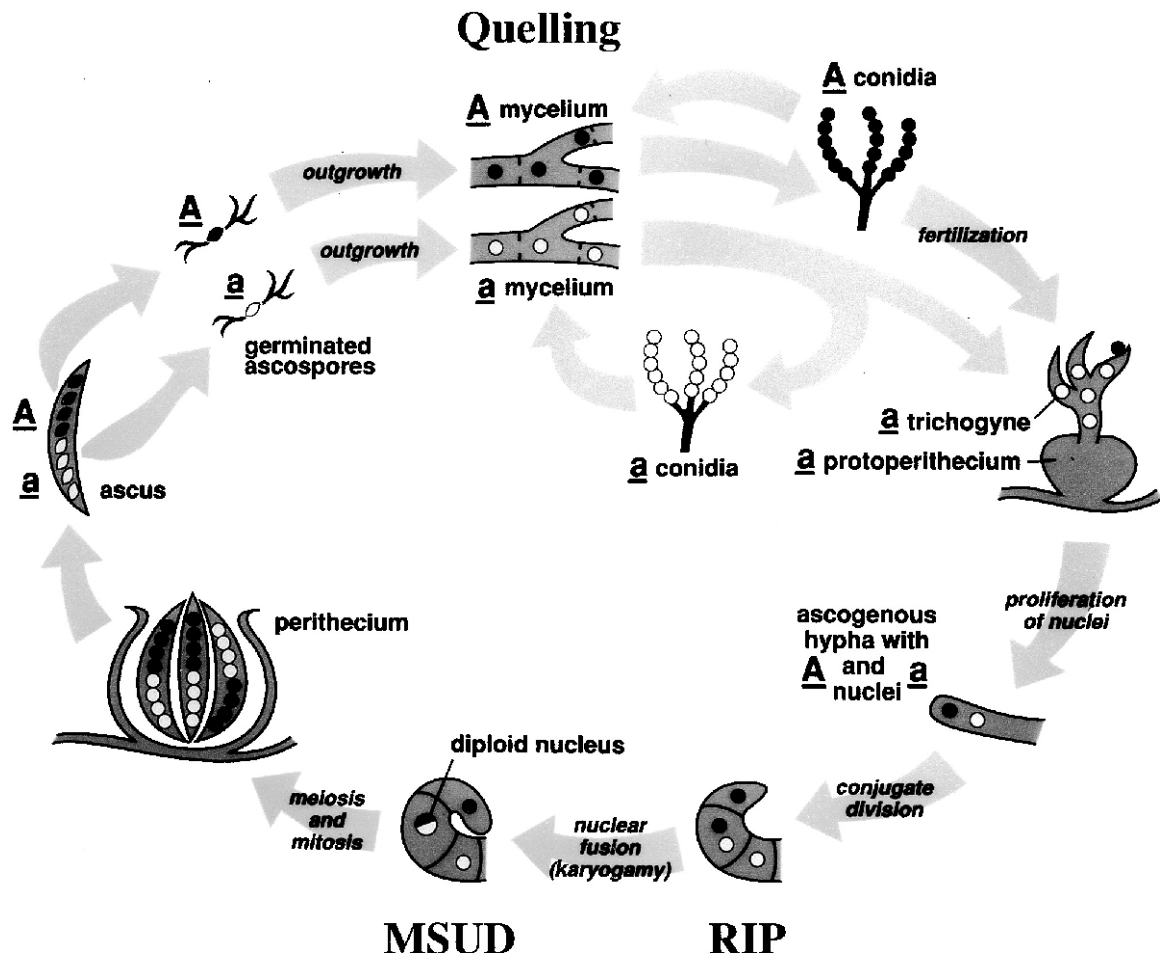
Figure I.3. The RNAi pathways of *Neurospora crassa*
To date, there are three distinct RNAi pathways known in *N. crassa*: Quelling, Repeat-Induced Point mutations (RIP), and Meiotic Silencing by Unpaired DNA (MSUD). Each of the three pathways is active during discrete time periods of the fungal lifecycle. Quelling is active during the vegetative phase. Its machinery silences the expression of genes present in tandem copies by targeting their mRNAs for degradation (Cogonoi and Macino, 1999). RIP is active before karyogamy and its machinery also scans the genome for genes that are present in multiple copies. When duplicated genes are identified, they are mutated via C to T transitions (Galagan and Selker 2004). RIP is the only pathway of the three that creates permanent sequence changes. MSUD is active during meiosis, after karyogamy. MSUD works to post-transcriptionally silence genes that are devoid of a pairing partner during the diploid stage of meiosis. Diagram reproduced from Shiu *et al.* (2001).

Aberrant [(a)RNA] is transcribed from this unpaired DNA and the RdRp, SAD-1, detects the transcript at the perinuclear region (Shiu *et al.*, 2006). SAD-1 replicates the single stranded aRNA into dsRNA through its RNA polymerase function. The dsRNA is then diced into small siRNA fragments (21-25bp long) by the Dicer protein, DCL-1 (Alexander *et al.* 2008). These double stranded siRNA fragments are then loaded onto the Argonaute protein SMS-2 (Lee *et al.* 2003) where they are converted into single strands (Xiao *et al.*, 2010). These single stranded siRNAs (Hammond *et al.* 2013) are then used to specifically degrade homologous mRNA transcripts thereby silencing the expression of the unpaired gene during the sexual cycle.

Other proteins important for MSUD have also been identified; a helicase (SAD-3; Hammond *et al.* 2011), and two novel proteins with no known functional domains (SAD-4 and SAD-5; Hammond *et al*. 2013).

I.3. Meiotic drive elements

I.3.i. Definition and general features

Mendel's First Law, the Law of Segregation, states that for any trait, each parent passes on one of their alleles for that trait, at random, with the equal likelihood that any of their two alleles will be passed on. Meiotic drive elements are defined as a class of "cheating" alleles that are recovered in excess of their Mendelian proportions during meiosis (Figure I.5). This distortion can be as little as 51:49 or as large as 1:0 (Lyttle 1993). Meiotic drive elements have been extensively studied in the plant, fungal, and animal kingdoms. Some of the most well studied meiotic drive systems include *t*-
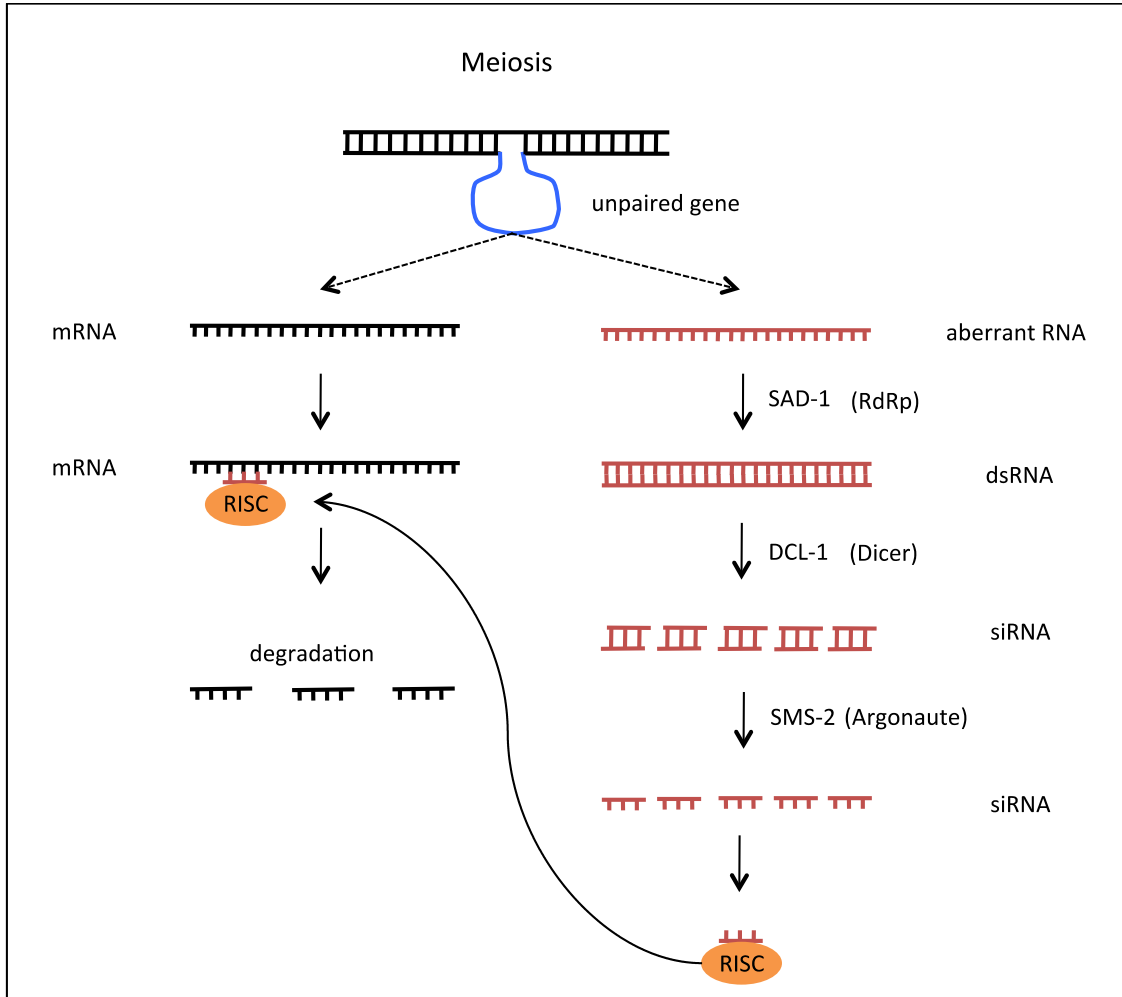
Figure I.4. Meiotic Silencing by Unpaired DNA
In *Neurospora crassa*, expressed genes that are unpaired during meiosis produce both messenger RNA (mRNA) and aberrant RNA (aRNA). Both RNA molecules are processed and then exit the nucleus. The mRNA makes its way to the ribosomes (to begin translation) while the aRNA is met outside the nucleus by SAD-1 where the RNA processing begins.

haplotype in mice, *Segregation Distorter* (*SD*) in flies, and *Spore killer* (*Sk*) in fungi (See Lyttle 1991 for a comprehensive review of all three drive systems).

There are two key components associated with most meiotic drive systems: a drive element (killer) and a resistance factor (Figure I.6). The drive element acts to bias segregation in its favor, while the resistance factor protects the drive element from targeting itself. It is essential that the drive and resistance elements remain in tight linkage. If the drive element were not tightly linked to the resistance factor, the two elements could segregate away from each other which would cause the drive element to kill itself. As such, chromosomal rearrangements, often in the form of inversions, are common to most meiotic drive elements because they block recombination, aiding in the linkage between the killer and resistant elements (Campbell and Turner, 1987).

Many of the known meiotic drive elements can be lumped into two categories based on the end products of meiosis. The most well studied drive elements fall into the category of gamete killers, while the least well-understood group are known as the progeny killers.

The gamete killers work to ensure that gametes containing the meiotic drive element are preferentially used in sexual reproduction, and they can do this with an efficiency of about 90-99% depending on the drive element in question (Trivers 2006). Gamete killers are also sex specific with a far greater tendency for drive elements to occur in males (Zimmering *et al*. 1970).

The progeny killers, on the other hand, work to eliminate essentially every single offspring that does not contain the drive element, with the result being the nearly 100% of the viable progeny from meiosis contain the drive element. Unlike the gamete killers,
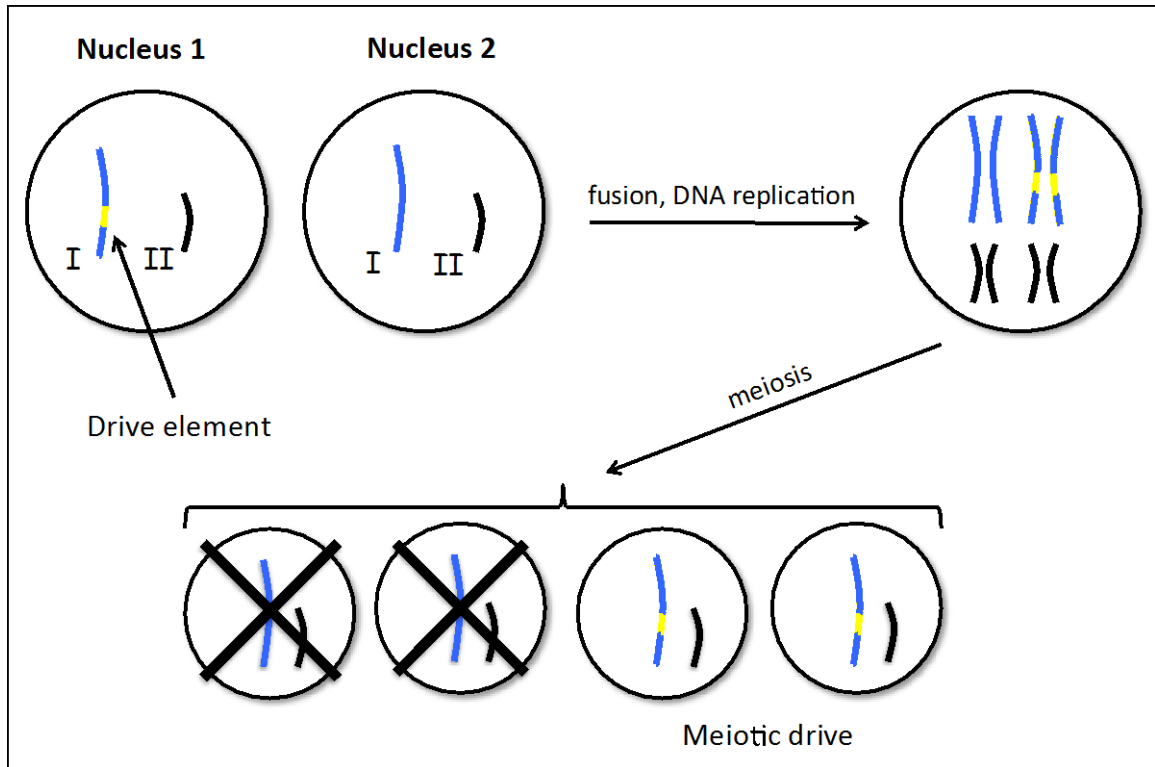
Figure I.5. General mechanism for meiotic drive
Shown above are two nuclei of opposite mating types, each containing two chromosomes. The two nuclei differ only in which version of chromosome I they contain, either with or without a meiotic drive element. If these two nuclei fuse, replicate their DNA, and then undergo meiosis, four daughter cells will result. According to Mendel's Law of Segregation both versions of chromosome I will be represented in equal proportions in the daughter cells, i.e. 1:1. However, because there is a meiotic drive element present, only those daughter cells containing the drive element will be represented in the next generation while those that do not contain the drive element will be killed off. Thus, the meiotic drive element skews Mendel's law in its favor.
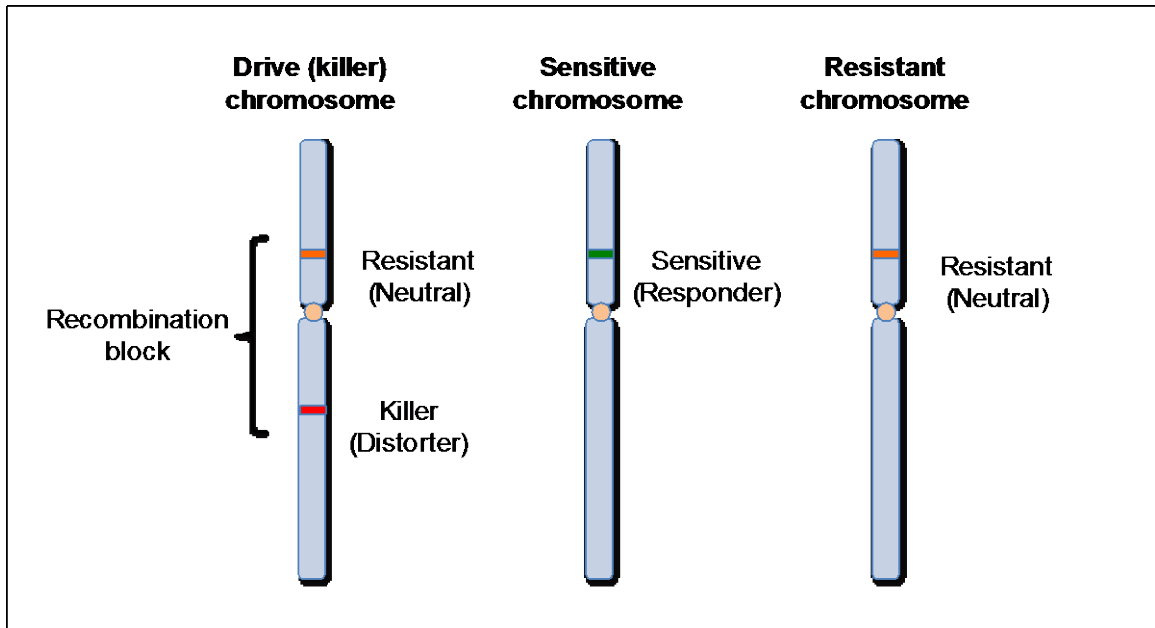
Figure I.6. Chromosomes of meiotic drive systems
Three chromosome types are present in meiotic drive systems; the drive chromosome, which contains the killer and resistant locus as well as a recombination blockage of some sort, a sensitive chromosome which does not contain a resistant or killer allele, and a resistant chromosome that is resistant to the driving effects of the killer but does not contain the killing function.

progeny killers are not sex specific.

Both gamete and progeny killers do share a very important feature; neither presents an observable external phenotype. It is believed that selection pressures are strong enough that the drive elements must reduce any negative effects at the individual level so that the drive element can propagate within the population (Trivers 2006). This makes meiotic drive elements very hard to identify unless the species has a very well characterized genetic line. Many researchers believe that, because of this lack of external phenotype, meiotic drive elements could be abundant in many eukaryotic populations.

I.3.ii. Classic examples of drive

The two most well studied meiotic drive elements are *t*-haplotype in mice and *SD* (*Segregation Distorter*) in flies. Both of these drive elements propagate themselves by impairing or degenerating wild-type sperm in a heterozygous male. Although some of the genetic loci responsible for causing meiotic drive in these two systems have been identified, much of the molecular mechanism for how they create that drive and how they perpetuate within their species' is still unclear. As such, research on *t*-haplotype and *SD* is still ongoing even after more than half a century since their discoveries.

t-haplotype was discovered, purely by coincidence, in 1932 in several strains of laboratory mice. A mutation, *T* (Tail-factor), which causes mice that are carriers (*T*/+) to have short tails, was being examined when it was noticed that certain heterozygous mice had no tail at all. Even more interesting was the observation that males of this tailless phenotype passed the trait on to >50% of their progeny. This appeared to indicate that *T*

was showing meiotic drive. Under further examination, it was discovered that *T* itself is not responsible for creating drive but rather it is linked to a meiotic drive element, *t*-haplotype.

The *t*-haplotype meiotic drive element is located near the centromere on chromosome 17 of three subspecies of *Mus musculus*; *M. m. domesticus*, *M.m. castaneus*, and *M.m. musculus* (Silver 1993). The drive element is 30-40Mb in size and encompasses ~1.2% of the mouse genome (Lyttle 1991). Four chromosomal inversions are associated with *t*-haplotype (Figure I.7). The inversions link the distorter and target loci and prevent recombination between them.

Three distorter genes are believed to drive *t*-haplotype, denoted *Tcd1* (O'Neill and Artzt 1995), *Tcd2* (Samont *et al.* 2002), and *Tcd3* (Patel-King *et al.* 1997). *Tcd1* and *Tcd3* are theorized to be the genes *Tctex1* and *Tctex3* (respectively), which encode dynein light chains that are known to be present in mouse sperm flagellum. *Tcd2*, on the other hand, is believed to be the gene *Dnahc8*, a dynein heavy chain gene. Mutations in this gene are known to cause an effect on both flagellar formation and movement.

Each of the three candidate distorter genes is associated with one of the chromosomal inversions found in *t*-haplotype (Figure I.7). Interestingly, each of the distorter loci creates an additive effect on the transmission of the *t*-haplotype (Lyon 1984). When *Tcd1* is the only distorter gene present, *t*-haplotype actually shows drag rather than drive with *t*-haplotype being transmitted to only 40% of the progeny. When *Tcd2* and *Tcd3* are both present, transmission of the haplotype jumps to 65%. And when all 3 of the distorter loci are present (the most common instance of the drive element in wild populations), transmission reaches 99%.
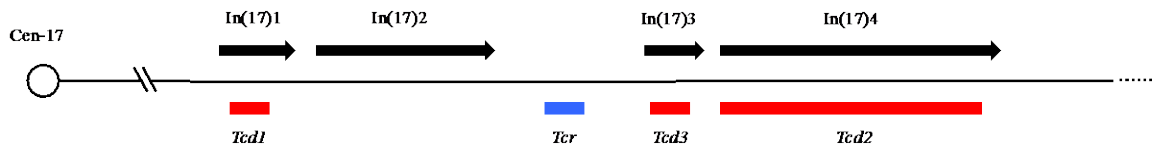
Figure I.7. Depiction of the mouse *t*-haplotype chromosome
t-haplotype is inherited as an entire 30-40Mb region of the right arm of chromosome 17 in *Mus musculus*. Four non-overlapping chromosomal inversions prevent recombination in this region: In(17)1 – In(17)4. The three distorter genes lie within the chromosomal inversions; *Tcd1* in In(17)1, *Tcd2* in In(17)4, and *Tcd3* in In(17)3. The responder gene, *Tcr*, is located in the small area between In(17)2 and In(17)3. Map is not to scale. Modified from Lyon 2003.

The three distorter genes act in trans to affect a responding locus known as *Tcr*. How they act upon the locus is still undetermined. *Tcr* itself is believed to be a fusion of two genes: the 5' end of a sperm motility kinase gene (*Smok*) and a ribosome S6 kinase gene (*Rps6ka2*) that is missing its 5' end (Herrmann *et al.* 1999). Tcr^{Wt} and Tcr^t are both expressed post-meiotically and exhibit kinase activity with only one identified difference: kinase activity in Tcr^t is 10% that of Tcr^{Wt}.

What is known about the function of *t*-haplotype is that it affects gametes, specifically those not containing it. It has been demonstrated that sperm carrying the wild type Chromosome 17 from a heterozygous (*t*/+) male are unable to fertilize the female's egg, therefore resulting in only the *t*-bearing sperm fertilizing the egg and being represented in the next generation (Lyttle 1991). The wild type and *t* sperm cells are created in equal numbers but the wild-type sperm is nonfunctional meaning it is unable to bind to the egg (Zimmering 1970). The end result being that only sperm carrying *t*-haplotype are able to fertilize the egg.

For a comprehensive review of *t*-haplotype, please refer to Mary Lyon's 2003 review entitled "Transmission Ratio Distortion in Mice."

Segregation Distorter (*SD*) was discovered in the fly species *Drosophila melanogaster* in the late 1950s. A graduate student, while attempting to solve a population genetics problem involving the low segregation frequency of recessive lethal mutations in the species, noticed that when certain male lines were mated with a lab tester a genetic mutant was being recovered in excess of its Mendelian proportions after mating (Sandler *et al.* 1959). In fact, the trait was being passed on with such a distorted

proportionality (nearly 100%), that it quickly became clear that these males must have been carrying a meiotic drive element. Because of the skew in inheritance, the researchers named the meiotic drive element *Segregation distorter*.

Like *t*-haplotype, *SD* affects sperm maturation and, as such, only shows drive in males. In a SD$^+$/SD heterozygous male, spermatids carrying SD$^+$ fail to properly condense their chromatin following meiosis (Tokuyasu *et al.* 1977). This failure of DNA condensation causes these spermatids to not form correctly and so they die either in the testes or in the testicular duct (Peacock *et al.* 1972).

Both the killer element and target locus have been genetically identified in *SD*. The drive element, *Sd*, is a duplicated, mutant Ran GTPase activating protein (RanGAP) that has lost 234 amino acids from its C-terminus (Merrill *et al.* 1999), and the target locus, *Rsp*, is a 240 bp repeat of satellite DNA (Wu *et al.* 1988) (Figure I.8).

In eukaryotic cells, wild-type RanGAP is responsible for (among many other functions) efficient nuclear transport. It localizes to the cytosolic side of the nuclear pores and establishes a gradient of Ran-GTP and Ran-GDP between the cytosol and the nucleus so as to allow the transport of molecules into and out of the nucleus. Sd-RanGAP, however, is missing its nuclear localization signal and therefore mislocalizes to the cytosol and nucleus where it performs a yet unknown function (Kusano *et al.* 2001).

The role of *Rsp* in both wild-type and *SD* cells is still unclear. As mentioned, *Rsp* is a 240 bp repeat of satellite DNA. Flies containing anywhere from 100-2500 copies of
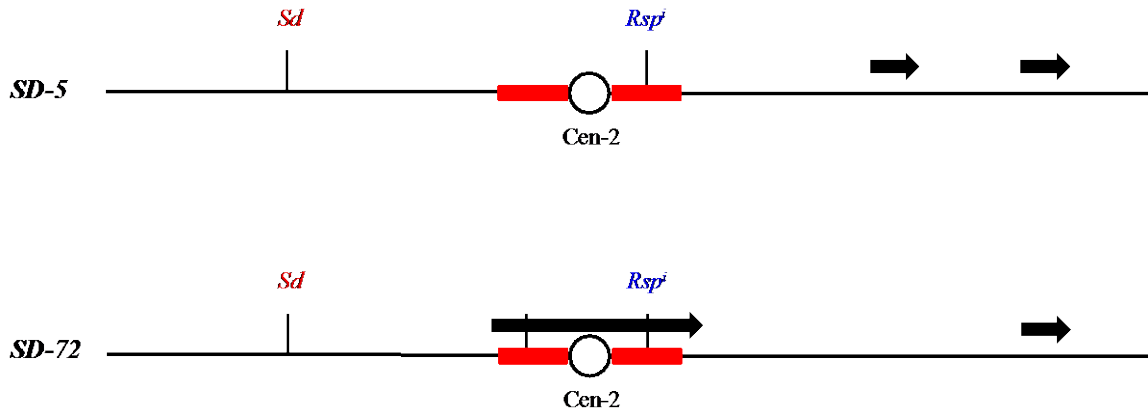
Figure I.8. Depictions of the two forms of SD

Segregation distorter exists in two forms in *Drosophila melanogaster*, *SD-5* and *SD-72*. The two forms differ only in the location of their chromosomal inversions. *SD-5* carries two paracentric inversions on 2R while *SD-72* carries the distal paracentric inversion of *SD-5* and a large pericentric inversion that encompasses *Rsp*. The red color denotes the centromeric heterochromatin. Modified from Larracuente 2012.

Rsp are sensitive to the driving effects of *Sd* while flies that have less than 20 copies of the satellite sequence are insensitive to *Sd* (*Rsp^i*) (Wu *et al.* 1988). A complete absence of *Rsp* also grants immunity to drive. Current theories suppose that the *Rsp* repeats might be generating rasiRNAs (repeat-associated small interfering RNAs) to direct chromatin remodeling following meiosis (Larracuente and Presgraves 2012). The molecular mechanism and the role of *Rsp* copy number have yet to be determined in this theory.

Although the two main elements of *Segregation distorter* have been genetically identified, the mechanism for how drive is created is still unclear. The mislocalization of Sd-RanGAP and the copy number of *Rsp* are certainly related to the phenomenon of segregation distortion, but how they interact to create meiotic distortion still remains a mystery.

For a comprehensive review of *Segregation distorter* in *Drosophila melanogaster*, please refer to Amanda Larracuente's 2012 review entitled "The Selfish *Segregation Distorter* Gene Complex of *Drosophila melanogaster*."

I.4. Spore killer

I.4.i. General features and cytology

Three similar, but unique meiotic drive elements have been discovered in the fungal genus *Neurospora*: *Spore killer-1* (*Sk-1*) was discovered in *N. sitophila*, and *Spore killer-2* (*Sk*-2) and *Spore killer-3* (*Sk*-3) were both discovered in *N. intermedia*. Each of the drive elements creates drive by eliminating any progeny that do not inherit the drive element following meiosis. The three drive elements are susceptible to killing by the

other in a heterozygous cross and resistant strains have also been identified in the wild for each of the Spore killers [denoted *r(Sk-#)*].

The Spore killer drive elements show first division segregation (i.e. when homologous chromosomes split during meiosis) when crossed to a sensitive strain. This results in half of the progeny containing the *Sk* chromosome and the other half containing the non-killer chromosome. The killing phenotype manifests post-meiotically during ascospore development. As individual ascospores in the ascus begin to delimit and form their cell walls, only those ascospores containing the *Sk* element mature into black, full-sized ascospores while those spores that do not contain the drive element cease developing and appear white and undersized (Raju 1979) (Figure I.9A).

When any of the Spore killers are crossed to their respective naturally resistant strains, first division segregation is still observed but in these crosses all ascospores develop into full-sized, black ascospores and all are viable. Crosses between identical *Sk* systems result in all full-sized, black ascospores as well (Figure I.9C).

I.4.ii. *Sk-2* and *Sk-3*

Barbara Turner and David Perkins first identified the *Sk-2* and *Sk-3* meiotic drive elements from a collection of worldwide *Neurospora intermedia* isolates in the 1970s (1979). The Spore killers were identified by a characteristic 4 black (*Sk*) to 4 white (*WT*) ascospore pattern (Figure I.9A). Both of the drive elements were introgressed into *N. crassa* because of the availability of known genetic markers.

Sk-2 and *Sk-3* are very rare in natural populations (*Sk-2* has only been found in a few populations while *Sk-3* has only been found once) (Turner 2001). Both drive
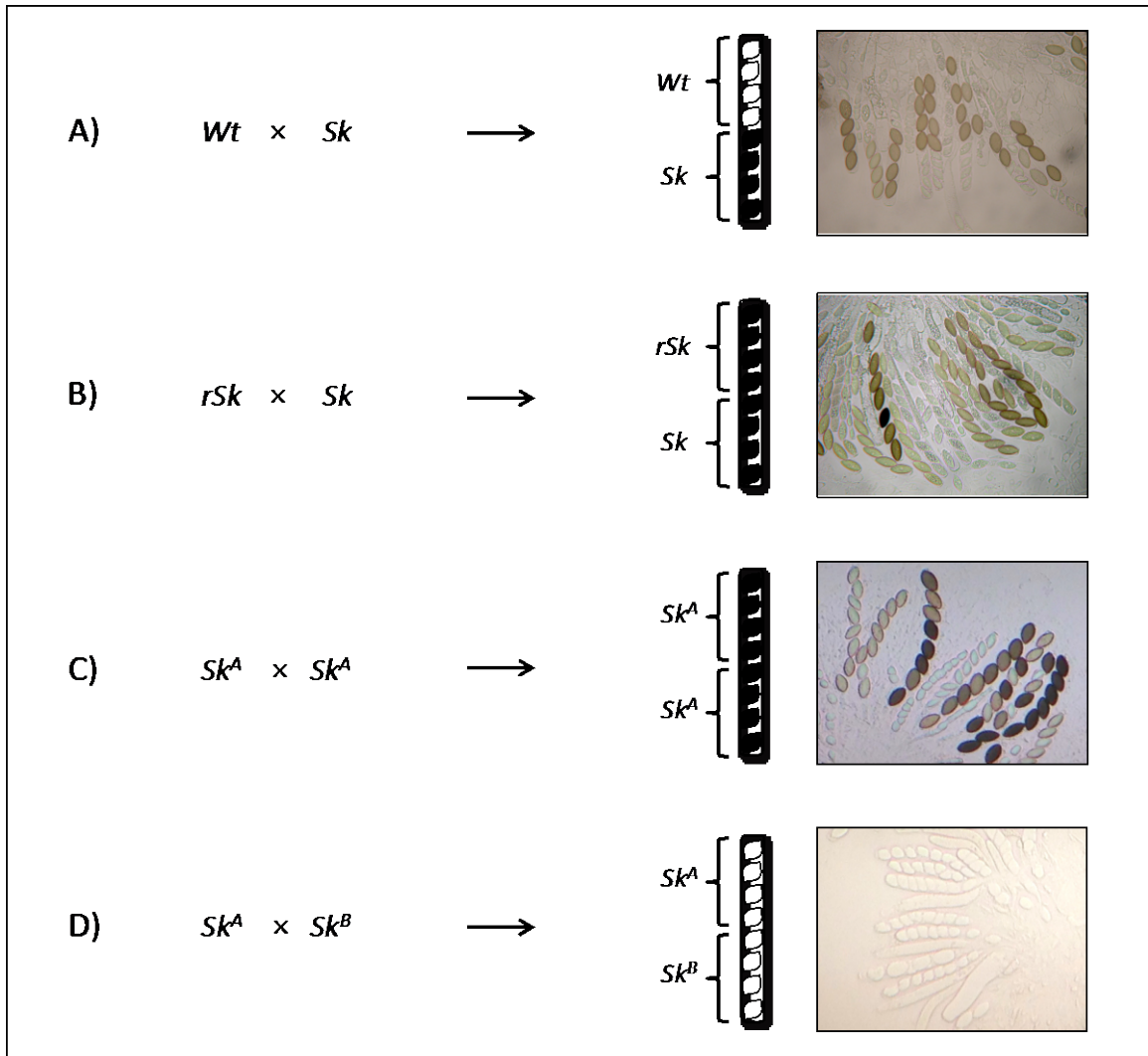
Figure I.9. The Spore killer phenotype

The three *Neurospora* Spore killer systems have similar phenotypic outcomes: (A) In *WT* × *Sk* crosses the *Sk* chromosome always shows first division segregation during meiosis which results in half of the progeny containing the *Sk* chromosome (black, viable) and the other half containing the *WT* chromosome (white, inviable), (B) *rSk* × *Sk* crosses also show first division segregation but result in all eight ascospores being black and viable (4*rSk*:4*Sk*), (C) *Sk* × *Sk* crosses of the same killer haplotype (e.g. *Sk-2* x *Sk-2*) result in all eight ascospores being viable (D) *Sk^A* × *Sk^B* (e.g. *Sk-2* x *Sk-3*) crosses result in all of the ascospores being killed off.
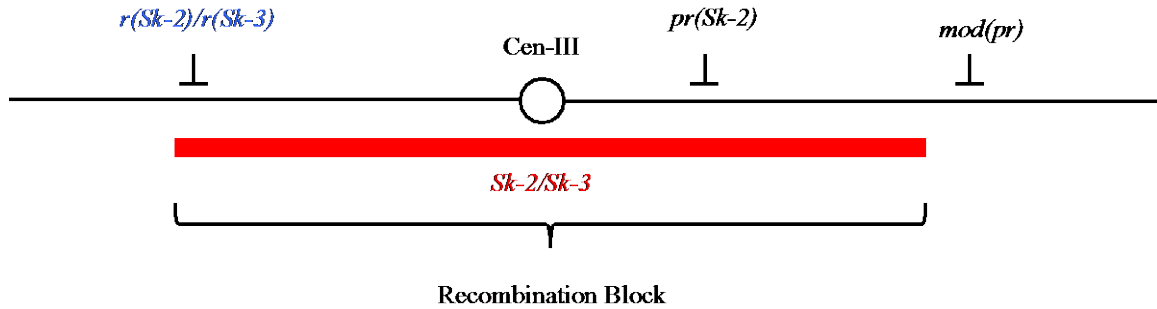
Figure I.10. The Spore killer chromosome in Neurospora
Located on chromosome III in *Neurospora crassa*, *Sk-2* and *Sk-3* are inherited as a 30
map unit pericentric block of DNA. *r(Sk-2)* and *r(Sk-3)* have been mapped to the left end
of the recombination block. Two additional loci have also been identified, with their
relative locations mapped (Turner 2003). *pr(Sk-2)*, found within the right arm of the
recombination block, is responsible for granting partial resistance to *Sk-2* in a cross.
mod(pr), found outside the right arm of the recombination block, does not grant
resistance to *Sk-2* by itself but, when paired with *pr(Sk-2)*, grants a full resistance
phenotype akin to *r(Sk-2)*.

elements were characterized, mapped, and cytologically studied as reported in two papers in 1979 by Turner and Raju. Naturally resistant strains were also discovered, both in *N. intermedia* [*r(Sk-2)* and *r(Sk-3)*] and *N. crassa* [*r(Sk-2)*]. Resistant strains are much more abundant than the killer haplotypes in natural populations (Turner 2001).

The *Sk-2* and *Sk-3* drive elements were both mapped to the same region on chromosome III in *N. crassa*. This region is a well-defined recombination block that spans the centromere and is ~30 map units in length (Campbell and Turner 1987). The resistant alleles, *r(Sk-2)* and *r(Sk-3)*, were both mapped to the left end of this recombination block.

Further work, by Barbara Turner, revealed two additional loci related to the *Sk-2* system, *pr(Sk-2)* and *mod(pr)* (2003). The *pr(Sk-2)* locus is found at the right end of the Spore killer recombination block and is not linked to *r(Sk-2)*. *pr(Sk-2)* does not give a full resistant phenotype when crossed to *Sk-2*, and most asci contain the characteristic 4B:4W in a *Sk* × *Wt* cross with the exception of a scattering of 8B:0W. *mod(pr)* confers no resistance to *Sk-2* when the two strains are crossed. However, when *pr(Sk-2)* and *mod(pr)* are both present in the same strain, they confer a full resistant phenotype when crossed to *Sk-2*.

I.4.iii. Sk-1

Sk-1 shares a near identical killer phenotype as *Sk-2* and *Sk-3* (Raju 1979). Unlike *Sk-2* and *Sk-3*, however, *Sk-1* is found in *Neurospora sitophila*. While *Sk-2* and *Sk-3* are very rare in natural populations, *Sk-1* is considered to be common and widely distributed

(Turner 2001). The opposite is true for the resistant strains of *Sk-1* - *r(Sk-1)* has only been discovered once in nature while *r(Sk-2)* and *r(Sk-3)* strains are abundant.

Little is known about *Sk-1* aside from the characteristic 4B:4W ascospore killing pattern and its geographic distribution. Interspecific crosses between *N. sitophila* and *N. crassa* yield almost no progeny and, since very little genetic work has been done in *N. sitophila*, researchers have only been able to determine that the *Sk-1* haplotype does not appear to be linked to mating type (Turner 1979).

II. General Methods

II.1. Molecular techniques

II.1.i. Long template PCR

All PCR was set up and run according to the protocol listed by the Roche Expand Long dNTP Pack (Roche Applied Science, Indianapolis, IN).

II.1.ii. Double-joint PCR

Double-joint PCR was conducted in the manner described in Yu *et al*. 2004.

II.1.iii. DNA gel electrophoresis

0.8% (w/v) agarose in 1× TAE with 0.5 μg/mL of ethidium bromide was utilized in both analytical and purification procedures. 50 mL gels were used for sample groups of ten or fewer, while samples greater than ten were run on a 200 mL gel. TAE buffer was made in accordance to Sambrook and Russell (2001), page A1.17. Gels were run at varying voltages depending on the size of the DNA bands in question.

II.1.iv. Plasmid miniprep

Plasmid minipreps were performed as described in Sambrook and Russell (2001), pages 1.32-1.34.

II.1.v. Restriction endonuclease digestion

Restriction enzyme digests were conducted in accordance with the manufacturer's suggested parameters.

II.1.vi. Bacterial transformation

Transformation-competent cells (One-shot TOP10 chemically competent cells from Life Technologies) were thawed on ice for 15 minutes. Two μL of DNA were then added to the cells and gently mixed. Top10 cells/DNA mix was incubated on ice for another 30 minutes. Cell/DNA mix was heat-shocked for 30 seconds at 42°C before being placed back on ice for 2 minutes. Next, 250 μL of SOC liquid medium was added to the cell mix and the entire mixture was placed in a shaker for 1 hour at 37°C and 200 rpm. Finally, cells were plated on LB amp plates and left to incubate overnight at 37°C.

II.1.vii. Bacterial permanent stocks

Bacterial cultures containing constructs of interest were stored by mixing 1 mL of liquid LB culture with 1 mL of sterile 50% (v/v) glycerol and then placed in a freezer at -80°C.

II.2. Growth media

II.2.i. Vogel trace elements

Five g citric acid hydrate, 5 g zinc sulfate septahydrate, 1 g ferrous ammonium sulfate hexahydrate, 250 mg cupric sulfate pentahydrate, 50 mg manganese sulfate

hydrate, 50 mg boric acid, and 50 mg sodium molybdate dihydride were dissolved in 95 mL of nanopure water, then sterilized into 50 mL conical vials via a 0.45 μm pore filter (Vogel 1964). The solution was stored at room temperature.

II.2.ii. Vogel minimal media

25× Vogel's salts was made as follows: 100 g sodium citrate septahydrate, 166.5 g anhydrous monobasic potassium phosphate, 66.5 g ammonium nitrate, 6.65 g magnesium sulphate septahydrate, 3.34 g calcium chloride dehydrate, 1.67 mL of 100 ug/mL biotin solution, and 3.34 mL of Vogel's trace elements were mixed and brought to a total volume of 1 L with nanopure water. Five mL of choloroform was added as a preservative.

II.2.iii. Westergaard (WG) trace elements

To 1 L of water the following was added: 57 mg boric acid, 396 mg copper (II) sulfate pentahydrate, 72 mg manganese chloride hexahydrate, 4.2 g zinc chloride, 100 g sodium choloride, and 100 g calcium chloride dehydrate. The resulting solution was autoclaved for 30 minutes and stored at room temperature (Westergaard and Mitchell 1947).

II.2.iv. Westergaard (WG) media

20× WG salts were made as follows: 20 g potassium nitrate, 20 g potassium phosphate monobasic, 10 g magnesium sulfate septahydrate, 2 g sodium chloride, 2 g calcium chloride dihydrate were added to 1 L of nanopure water and autoclaved.

To make WG plates, an appropriate amount of 20× WG salts was diluted with water and 1.5% (w/v) sucrose. Next, 1.5% (w/v) Bacto Agar (BD), 1 mL WG Trace Elements per liter of media, and 5 ng/mL biotin were added. The media was autoclaved and 45 mL was added to $100 \times 100 \times 15$ mm square petri dishes (Westergaard and Mitchell 1947).

II.2.v. Brockman and de Serres (BDS) 20× solution

200 g sorbose, 10 g fructose, and 10 g glucose were added to 1 L of nanopure water, autoclaved for 30 minutes, and stored at room temperature.

II.2.vi. Brockman and de Serres (BDS) media

An appropriate amount of 25× Vogel's salts and 1.5% (w/v) Bacto Agar (BD) was added to water and autoclaved for 30 minutes. After sterilization, an appropriate amount of 20× BDS was added; the solution was mixed, and stored at room temperature until needed.

II.2.vii. 10× FIGS

100 g sorbose, 2.5 g fructose, 2.5 g glucose, and 1 g inositol were added to 500 mL nanopure water and autoclaved for 30 minutes. The solution was stored at room temperature.

II.2.viii. Top agar

Two g Bacto Agar (BD), 36.4 g sorbitol, 8 mL 25× Vogel's salts, and 172 mL

nanopure water were added to 500 mL bottle and autoclaved. After sterilization, 20 mL

of 10× FIGS was added. The agar was kept molten at 50°C until it was needed.

II.2.ix. Bottom agar

Twenty-four mL 25× Vogel's salts, 9 g Bacto Agar (BD), and 516 mL nanopure

water were added to a 1 L bottle and autoclaved for 30 minutes. After sterilization, 60 mL

10× FIGS was added to the molten mix. Twenty mL was poured into 100×15 mm round

petri dishes to solidify.

II.2.x. 20× BDS

Two hundred g sorbose, 10 g fructose, and 10 g glucose were added to 1 L of

nanopure water in a 2 L bottle, autoclaved for 30 minutes, and then stored at room

temperature.

II.2.xi. Lysogeny Broth (LB)-based media

LB powder mix (Sigma-Aldrich) was mixed into a concentration of 20 g/L for

liquid media. After autoclaving, glucose was added to a final concentration of 0.1% (w/v).

II.2.xii. Iodoacetic acid (IAA) tubes

Forty 18×150 mm tubes were placed in the appropriate tube rack, covered in

aluminum foil and autoclaved along with forty matching caps (caps were placed in a

beaker for autoclaving). In a 500 mL flask 7.5 mL of 4× WG, 1.5 g sucrose, 6 g agar, and

286 mL of water were added and then autoclaved for 30 minutes. During autoclaving, 124 mg of Na IAA (iodoacetic acid, sodium salt) was dissolved in 6 mL of sterile water (in a 50 mL tube) to make a concentration of 0.1M solution. The solution was then sterilized with a 0.45 μm Millipore filter. After the 500 mL solution was autoclaved, 3 mL of 0.1M Na IAA was added. 6 mL of media was then added to each of the forty tubes and the entire rack was placed on a slant to solidify.

II.3. *Neurospora* culture and handling

II.3.i. Genomic DNA isolation

Fifty mL of Vogel's liquid media was added to a 125 mL Erlenmeyer flask and then autoclaved for 30 minutes. Flasks were then inoculated with conidia from a strain of interest and placed in an incubator at 30°C. After 2-4 days, the resulting vegetative tissue was blotted dry with paper towels and then put in a vacuum chamber containing sodium hydroxide. After the tissue was dry, it was ground using a glass rod in a test tube and a vortexer. The resulting powder was then used with the DNeasy Plant Mini Kit from Qiagen.

II.3.ii. Fungal transformation

Recipient strains were inoculated to 100 mL of Vogel's agar (plus any additional necessary supplements) in a 500 mL flask. Flasks were then incubated at 30°C for 5-7 days to allow for robust conidial growth.

Conidia from the flasks was collected to 30 mL of 1M sorbitol, in 50 mL tubes, and then shaken. Next, conidia were passed through a 100 μm vacuum filter column into another 50 mL tube. One hundred μL of filtered conidia were diluted to 1000 μL by adding 900 μL of 1M sorbitol. Absorbance was measured at 420 nm on a spectrophotometer. The remaining filtered conidia were then spun down at 3200 rpm for 10 minutes. The supernatant was then gently removed and the conidial pellet re-suspended in 1M sorbitol at 1 unit/μL.

Transformation DNA (50 ng) was diluted to 10μL with water and 90 μL of the conidia/sorbitol mix was added to the DNA. The entire mix was then added to a 1 mm gap cell and placed on ice.

When all of the samples were ready, an electroporator was set to 1500 volts. Post-electrocution, 750 μL of 1M sorbitol was added to the gap cell before the entire mix was transferred to a new 50 mL tube and placed on ice. To aid in the recovery of the conidial cells, 4.2 mL of Vogel's liquid media was added to the cell mix and the entire solution was placed in a shaker for 3-4 hours at 30°C and 80 rpm.

Finally, 500 μL of the conidia mix was mixed with 20 mL of top agar and then poured onto a bottom agar plate and incubated at 37°C for ~3 days.

II.3.iii. Isolation of homokaryons using IAA

Heterokaryons were used to inoculate IAA slants, and then incubated at 30°C for 3 days at room temperature. 4 mL of water was added to the slant, vortexed for one minute, and then the water/conidia mix was filtered through a 100 μm vacuum-driven filter. The filtrate was transferred to a 50 mL conical vial lid, then drawn up into a 10 mL

syringe and filtered again with a syringe-driven 5 μm filter. Five hundred μL and the remaining filtrate were then spread on two separate BDS agar plates. The plates were incubated at 30°C for 2-4 days, and then colonies were picked to appropriate Vogel's media slants. The slants were allowed to conidiate.

II.3.iv. Permanent stocking of strains

Conidial strains were inoculated to agar/milk tubes (with appropriate nutrients) and incubated at 30°C for 2-4 days. After sufficient conidiation, tubes were vortexed to allow the sterile milk and conidia to mix. 1 mL of conidia mix was then pipetted into sterile silica stock tubes, vortexed and placed in the 4°C refrigerator. Anther 0.9 mL of the conidia mix was added to cryogenic vials containing 0.9 mL of 50% sterile glycerol, vortexed, and then placed in the -80°C freezer.

III. A neoclassical approach to fine-map

rsk

III.1. Introduction

In *Neurospora crassa,* the *Sk-2* and *Sk-3* meiotic drive elements are contained within a 30-map unit recombination block on chromosome III (Campbell and Turner 1987). The recombination block is believed to be comprised of several small chromosomal inversions, which might aid in preventing recombination when a Spore killer strain is crossed to a sensitive one. Without recombination, classical mapping techniques that analyze recombination frequencies between genes (to determine their order and distance) are impossible to conduct. As such, mapping the resistance and killer genes of *Sk-2* and *Sk-3* by this method would be a fruitless endeavor.

Naturally resistant non-killer strains, however, do not contain a recombination block. Therefore, locating the resistance gene using classical mapping techniques should be viable. Accordingly, the first step in elucidating the Spore killer drive elements began with the mapping of the *resistant to Sk-2* gene, *r(Sk-2)*, in these naturally resistant strains.

III.2. Materials and Methods

III.2.i. Strains

Strain name	Genotype
F1-16	*rid; fl; Sk-2 a*
F2-19	*rid; fl; Sk-2 A*
P8-11	*cum r(Sk-2) acr-7 A*
P8-38	*r(Sk-2) acr-7 a*
P10-25 (FGSC# 11742)	*NCU09520$^{\Delta}$::hph a*
P10-27 (FGSC# 13127)	*NCU09502$^{\Delta}$::hph a*
P11-03 (FGSC# 11743)	*NCU09520$^{\Delta}$::hph A*
P11-04 (FGSC# 11749	*NCU09175$^{\Delta}$::hph A*
P11-07 (FGSC# 13256)	*NCU09159$^{\Delta}$::hph A*
P16-07	*NCU09526$^{\Delta}$::hph a*
P16-08	*NCU09496$^{\Delta}$::hph a*
P16-09	*NCU09145$^{\Delta}$::hph a*
P16-10	*NCU09190$^{\Delta}$::hph A*

Table III.1. Strains for mapping *rsk*
Gene descriptions can be found in the *Neurospora crassa* e-Compendium
(http://www.fgsc.net/2000compendium/2000compend.html).

The *Neurospora crassa* genome knockout project, conducted by Park and others at Dartmouth, created a collection of *N. crassa* mutants in which each strain has exactly one gene deletion and the entire collection comprises a knockout of all known open reading frames (Park *et al.* 2011). The strains for this study were obtained from Dartmouth through the Fungal Genetics Stock Center (FGSC; McCluskey *et al.* 2010). Those not listed with an FGSC# are from our own collection. A complete list of strains used in this study is found in Table III.1.

III.2.ii Three-point crosses

In a three-point cross, three linked genes with discernable phenotypes are separated by spans of DNA called intervals (Figure III.1). To identify the gene order and the distance between genes, the frequency at which crossing over occurs in the intervals between the three genes must be calculated. The frequency of a single cross over is always greater than that of a double cross over. Utilizing this information, the order of the three genes can be established. And, by calculating the percentage of recombination in each of the two intervals, we can predict the map distance between each of the genes.

The three-point crosses in this study were conducted in accordance to the protocol described by David D. Perkins in the Neurospora Online Protocol Guide (http://www.fgs c.net/neurosporaprotocols/How%20to%20determine%20gene%20order%20using%203-point%20crosses%20final.pdf). A list of mapping crosses is shown in Table III.2.

35
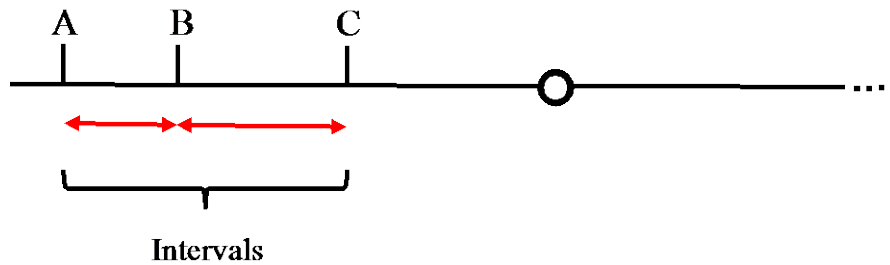
Figure III.1. Analyzing three-point testcrosses
In a three-point testcross, a strain containing three genes of unknown order is crossed to a recessive strain. The three genes (A, B, and C) are separated by two intervals; one between A and B, and the other between B and C. Assuming that a crossover occurring in one interval is more likely than a crossover happening in each of the two intervals simultaneously, the order of the three genes can be established by recording and analyzing the crossover frequency in these two intervals.

<u>III.2.iii *hph* and *acr-7* screening</u>

hph (hygromycin resistance) was screened by adding 200 μg/mL hygromycin to either Vogel's slants or plates. *acr-7* (acriflavine resistance) was screened by adding 10 μg/mL acriflavine to Vogel's plates. A toothpick-full of conidia from recombinant strains were added to 200 μL of water, from which 4 μL of conidia/water mix were then spotted to tubes or plates containing hygromycin, acriflavine, or both. Strains were then incubated for two days at 30°C before being screened for growth.

<u>III.2.iv. *r(Sk-2)* screening</u>

r(Sk-2) was screened for by spotting 4 μL of the conidial/water mixes from the *hph* and *acr-7* screens to *Sk-2* fluffy (highly fertile female) tester strains that had been grown on Westergaard plates for five days at room temperature. The fluffy *Sk-2* strains acted as the females and the conidial strains were the males. The strains were given time to undergo sexual reproduction and eject their ascospores (typically 12 – 18 days) before the presence or absence of *r(Sk-2)* was determined by examining the ratio of black to white ascospores shot to the surface of a glass plate placed directly above the perithecia (fungal fruiting bodies that house the asci).

III.3. Results

<u>III.3.i Analysis of three-point cross data</u>

Previous mapping of *r(Sk-2)*, conducted by Campbell and Turner (1987), placed the resistance allele between the two classical phenotypic markers *cumulus* (*cum*) and *acriflavine resistance-7* (*acr-7*). This region, on the left arm of chromosome III, is near

Gene deletion	Cross
NCU09145$^{\Delta}$	P16-09 × P8-11
NCU09159$^{\Delta}$	P11-07 × P8-38
NCU09175$^{\Delta}$	P11-04 × P8-38
NCU09190$^{\Delta}$	P16-10 × P8-11
NCU09496$^{\Delta}$	P16-08 × P8-11
NCU09502$^{\Delta}$	P10-27 × P8-11
NCU09520$^{\Delta}$	P10-25 × P8-11
NCU09526$^{\Delta}$	P16-07 × P8-11

Table III.2. Three-point crosses
Gene deletion strains were crossed to a *r(Sk-2) acr-7* strain (either P8-38 or P8-11 depending on the mating types available).
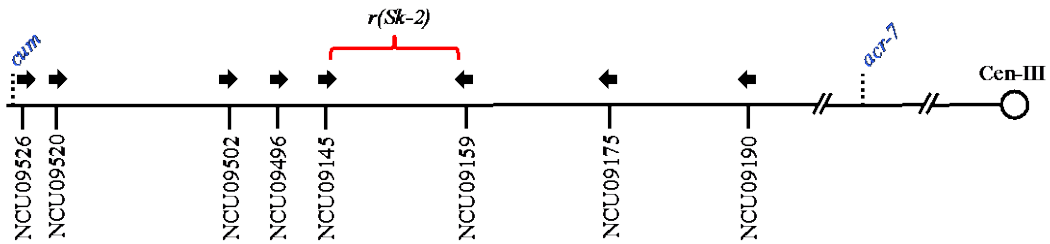
Figure III.2. Three-point cross analysis
Depicted above is the left arm of chromosome III in *N. crassa*. Previous mapping experiments placed the location of *r(Sk-2)* between the markers *cum* and *acr-7*. Gene deletions that were predicted to fall in between these two markers were chosen from the *N. crassa* knockout library for three-point cross analysis. The arrows above the gene knockouts indicate the direction *r(Sk-2)* lies relative to the knockout based on our three-point cross data. Figure is not to scale.

the left end of the *Sk-2* recombination block [*r(Sk-3)* also maps to this region]. The two

markers, although not physically mapped, recombine with *r(Sk-2)* strains (*cum* shows 1-

2% recombination and *acr-7* shows 7% recombination) to provide an estimate of the

chromosomal location of *r(Sk-2)*. To further refine the location of *r(Sk-2)* via three-point

crossing, new markers would need to be generated.

Serendipitously, a genome-wide knockout library had recently been created by the

Genome Project Group and had been made available through the Fungal Genetics Stock

Center (Park 2011). These gene-knockout strains provided us with additional markers

that would allow us to further refine the location of *r(Sk-2)* through three-point mapping.

Three-point crosses allow researchers to determine the order of three genes based

on the frequencies of recombination between the genes in question. The closer two genes

are to each other the lower the frequency of recombination between them. With this

information we can determine the order of our three genes by conducting experimental

crosses and analyzing the resulting recombination frequencies (Figure III.2).

To be able to conduct a three-point cross, three markers with scorable phenotypes

must be used. *r(Sk-2)* has a very discernable phenotype as it grants resistance to *Sk-2* in a

cross. The two markers *r(Sk-2)* had previously been mapped between, *acr-7* and *cum*,

each have their own distinguishable phenotype as well (*acr-7* grants *N. crassa* strains

resistance to acriflavine and *cum* causes the fungus to grow in a tight, colonial pattern).

We utilized *r(Sk-2)* and *acr-7* as two of the three markers for the three-point test crosses.

The third marker we used for mapping was one of eight gene-knockouts from the

knockout library. These knockouts were spread across the area between *acr-7* and *cum*'s

predicted locations (Figure III.2). The genes were each knocked out with the dominant

Table III.3

	Parentals		Interval 1 crossover		Interval 2 crossover		Double crossover		
	hph / + / +	+ / *r(Sk-2)* / *acr-7*	+ / + / +	*hph* / *r(Sk-2)* / *acr-7*	+ / *r(Sk-2)* / +	*hph* / + / *acr-7*	*hph* / *r(Sk-2)* / +	+ / + / *acr-7*	**Total**
NCU09526$^\Delta$	75	107	1	6	7	12	0	0	208
NCU09520$^\Delta$	269	-	-	15	-	40	1	-	325
NCU09502$^\Delta$	91	-	-	8	-	31	0	-	130
NCU09496$^\Delta$	93	104	2	1	15	16	0	0	231
NCU09145$^\Delta$	82	118	1	2	11	17	0	0	231

Table III.3. Three-point cross analysis 1
Crossing data indicates that *r(Sk-2)* lies to the right of these five knockout markers.

Table III.4

	Parentals		Interval 1 crossover		Interval 2 crossover		Double crossover		
	+ / *hph* / +	*r(Sk-2)* / + / *acr-7*	*r(Sk-2)* / *hph* / +	+ / + / *acr-7*	*r(Sk-2)* / + / +	+ / *hph* / *acr-7*	+ / + / +	*r(Sk-2)* / *hph* / *acr-7*	**Total**
NCU09159$^\Delta$	75	107	1	6	7	12	0	0	208
NCU09175$^\Delta$	269	-	-	15	-	40	1	-	325
NCU09190$^\Delta$	91	-	-	8	-	31	0	-	130

Table III.4. Three point cross analysis 2
Crossing data indicates that *r(Sk-2)* lies to the left of these three knockout markers.

marker *hph*, which, when present in a *Neurospora crassa* strain, grants resistance to the fungicide hygromycin. We crossed our gene knockout (*hph*) strains, one at a time, to a *r(Sk-2) acr-7* strain, and then analyzed the resulting progeny to determine the map order of these three genes (Table III.2).

The genotypic frequencies resulting from our three-point testcrosses are provided in Table III.3 and Table III.4. The data from these crosses suggests that *r(Sk-2)* lies between gene-knockout markers *NCU09145* and *NCU09159* (Figure III.2.). This region contains 13 predicted genes, encompassing about 55kb of DNA.

III.4. Discussion

Prior to genome sequencing and gene knockout libraries, three-point cross mapping was an invaluable tool that allowed researchers to determine an approximate location of mutant genes within an organism's genome. By crossing a candidate mutant strain to a strain that contained known genetic markers, researchers were able to map the chromosomal location of genes through the analysis of segregation and recombination frequencies. The more mutant genes available to cross a candidate strain to, the more precise the mapping.

Prior to this study, the location of *r(Sk-2)* had previously been estimated using these exact techniques; using classical genetic mutant strains to conduct a series of crosses that localized *r(Sk-2)* relative to the location of known mutant markers. These early studies placed *r(Sk-2)* in between the two mutants *cum* and *acr-7* (Campbell and Perkins 1987).

Utilizing these results we chose gene knockout markers that are localized to this

region to help us further map the location of *r(Sk-2)*. Eight gene knockouts spanning the estimated locations of *cum* and *acr-7* were crossed to an *r(Sk-2)* strain and the resulting recombinants were screened and counted. Analysis of the recombinants narrowed the location of *r(Sk-2)* to a region between and containing the genes *NCU09145* and *NCU09159*.

In this study we show that the utility of three-point mapping is still quite relevant to the mapping of genes in *Neurospora crassa*. The recent creation of the genome knockout library for *Neurospora crassa* has provided a plethora of new markers that, when used in tandem with the already established library of mutant phenotypic markers, can accurately map a candidate gene to a precise location before more lengthy and expensive techniques are required.

III.5. Publication

This work has been published as "Fine-scale mapping in *Neurospora crassa* by using genome-wide knockout strains" in the journal Mycologia, Volume 104(I), pages 321-323, in 2012 by Hammond, T.M., Rehard, D.G., Harris, B.C., and Shiu, P.K.T. My co-authors have contributed to the design, analysis, and executions of the experiments.

IV. Discovery and characterization of *rsk*

IV.1. Introduction

Although the Spore killer meiotic drive elements in *Neurospora* were discovered some 35 years ago, the molecular basis for these elements has remained a mystery. The elucidation of these drive elements at the molecular level should provide new insights into the nature of meiotic drive elements as well as provide a rare, molecular view of a drive element in a haploid eukaryotic species.

This chapter describes the identification of the gene responsible for resistance to killing in the *Sk-2* and *Sk-3* meiotic drive systems. The DNA and protein sequences of these resistance alleles are analyzed and compared among the two drive systems as well as among global resistant/sensitive isolates. From the characterization of these resistance alleles we were able to construct a model for how the killer and resistance elements might interact during ascospore development in *Neurospora*.

IV.2. Materials and Methods

IV.2.i. Three-point cross mapping of *r(Sk-2)*

The creation of our own intergenic *hph* markers allowed us to further refine the location of *r(Sk-2)* down to a 55kb region of chromosome III (Chapter III). Here we

created two additional intergenic markers and placed them between the genes *NCU09149*

and *NCU09150*, and genes *NCU09155* and *NCU09156*.

Strain	Genotype
F1-16	*rid; fl; Sk-2 a*
F2-19	*rid; fl; Sk-2 A*
F2-23	*rid; fl A*
F2-26	*rid; fl a*
F3-14	*rid; fl; Sk-3 A*
F3-16	*rid; fl; Sk-3 a*
F5-18	*rid his-3; fl; Sk-2; sad-2$^\Delta$::hph a*
F5-28	*rid; fl; 09148$^\Delta$::pyr-4$^+$ rsk$^\Delta$::hph mus-52$^\Delta$::bar A*
F5-30	*rid his-3; fl; Sk-3; sad-2$^\Delta$::hph a*
P3-07	*A*
P6-07	*rid A*
P6-08	*rid a*
P8-11	*cum rsk$^{r(Sk-2)-LA2222}$ acr-7 A*
P8-38	*rsk$^{r(Sk-2)-LA2222}$ acr-7 a*
P12-41	*cum rsk$^{r(Sk-3)-PF5123}$ a*
P14-18	*rsk$^{r(Sk-2)-BR4706}$ a*
P14-19	*rsk$^{r(Sk-2)-HT4715}$ a*
P14-20	*rsk$^{r(Sk-2)-CI4831}$ a*
P15-52	*rsk$^{r(Sk-2)-LA2222}$ A*
P15-54	*rsk$^{\Delta r(Sk-2)-LA2222}$::hph acr-7; mus-51$^\Delta$::bar A*
P15-56	*rid; rsk$^{\Delta OR::LA2222}$-hph A*
P15-57	*rid; Sk-2 rsk$^{\Delta Sk-2}$::hph; mus-51$^\Delta$::bar A*
P15-58	*rid; IIIR::rsk$^{r(Sk-2)-LA2222}$-hph; mus-51$^\Delta$::bar A*
P17-01	*rsk$^{r(Sk-2)-LA2222}$ 09153$^\Delta$::hph acr-7; mus-51$^\Delta$::bar A*
P17-02	*rsk$^{r(Sk-2)-LA2222}$ 09154$^\Delta$::hph acr-7; mus-51$^\Delta$::bar A*
P17-03	*rid; Sk-3 rsk$^{\Delta Sk-3}$::hph; mus-51$^\Delta$::bar A*
P17-04	*rid his-3$^+$::rsk$^{r(Sk-2)-LA2222}$; sad-2$^\Delta$::hph A*
P17-05	*rid his-3$^+$::09152^{LA2222}; sad-2$^\Delta$::hph A*
P17-06	*rid his-3$^+$::rsk$^{r(Sk-2)-LA2222}$ a*
P17-08	*rid; 09148$^\Delta$::pyr-4$^+$ rsk$^\Delta$::hph a*
P17-12	*09155-09156::hph a*
P17-13	*09149-09150::hph a*
P17-15	*rid; rsk$^{\Delta OR::r(Sk-3)}$-hph mus-52$^\Delta$::bar A*
P17-16	*rid; rsk$^{\Delta OR::Sk-2}$-hph mus-52$^\Delta$::bar a*
P17-17	*rid; rsk$^{\Delta OR::Sk-3}$-hph a*
P17-25	*rsk$^{S-CI4832}$ a*
P17-26	*rsk$^{S-HT4714}$ A*
P17-27	*rsk$^{S-BR4705}$ A*

Table IV.1. *rsk* strains

Primer	Sequence (5' → 3')	Uses
rSK-2-92	TTCTCCGTCCAGCGATTTGTCC	IIIR L Flank
rSK-2-93	GTTTCAGGGGTTTCGTTCGCTGTATTGCTCGCTTTCCTCGGGTCT	IIIR L Flank
rSK-2-94	GCTCTCTCTCTCCCACAACGGTCACTGTCGCTGTCGCTGTCTCTCG	IIIR R Flank
rSK-2-95	CCTCCATCCCCTCCCCAATCTT	IIIR R Flank
rSK-2-96	GTCGGCATCGTGGTCTTCCCTA	IIIR Nested
rSK-2-97	GTAACCCTCAACCGCCCCAAAG	IIIR Nested
pMF272-4537R	ACAGCGAACGAAACCCCTGAAAC	*r(Sk-2)::hph*
rSK2-85	TGACCGTTGTGGGAGAGAGAGAGC	*r(Sk-2)::hph*
rSK-2-86	TCGGCTTGGTTCACTACGACTGG	*r(Sk-2)* L Flank
rSK-2-87	ACGGGTTTCAGGGGTTTCGTTCGCTGTCAAATGGTGACATGGACGGGAGA	*r(Sk-2)* L Flank
rSK-2-88	GTCTAGAGGTACTCGGCAAGTTTCAAAGTG	*r(Sk-2)* R Flank
rSK-2-89	CGGCAAGAAAGACGAGGAAGCAT	*r(Sk-2)* R Flank
rSK-2-90	GAGAAGCCCCAGTCCTTTCAGCA	*r(Sk-2)* Nested
rSK-2-91	CCAGCATTCCTCCGCTAATCTCG	*r(Sk-2)* Nested
rSK2-57	TCTATGCAACCAGGCAACTCAGCA	*09151* amplification
rSK2-58	TGACCGTTGTGGGAGAGAGAGAGC	*09151* amplification
rSk-2-11-DGR	AGAAAGTTGGAAGCGAGCAT	*09151* sequencing
rSk-2-10-DR	ATCCGTCTTGTCTTTGGCACGAC	*09151* sequencing
rSk-2-12-DR	GCGGCCGCTGGCACGGTCACTTCGAGACT	*09151* sequencing
rSk-2-13-DR	TTCCATTACCCCGTCCAC	*09151* sequencing
rSk-2-14	TTGCGGCCGCTGGCACGGTCACTTCGAGACT	*09151* sequencing
rSk-2 DGR1	TCTATACGACGAGGCAACTCAGCT	*09151* amplification
Sk-3-106	ATGCCAAAGGGTTTTCCACA	*09151* amplification

Table IV.2. *rsk* primers

Three-point crosses and subsequent screening of the recombinant progeny were conducted in the same manner listed in section III.2.ii. Recombinant analyses can be seen in Table IV.4 and IV.5.

IV.2.ii. Deletion of *NCU09151, NCU09153, NCU09154* from *r(Sk-2)*

$NCU09151$, $NCU09153$, and $NCU09154$ were deleted from P8-38 [*r(Sk-2)*] (we were unable to delete $NCU09152$). The deletion vectors were generated by amplifying the corresponding wild-type sensitive (S) gene knockout cassette from the knockout library created by the Neurospora Genome Project (e.g. $NCU09151^{\Delta S}::hph$ was used to knockout $NCU09151^{r(Sk-2)}$) (Park *et al.* 2011).

Transformation with the deletion vector was carried out via standard protocol and the P8-38 strain was the recipient of the transformation fragment.

IV.2.iii. Vectors for introducing $NCU09151^{r(Sk-2)}$ into sensitive strains

Two transformation vectors were created to insert $NCU09151^{r(Sk-2)}$ into a sensitive strain. The first vector allowed us to place $NCU09151^{r(Sk-2)}$ at the *his-3* locus on chromosome I. The creation of the transformation vector proceeded as follows. $NCU09151^{r(Sk-2)}$ was amplified from P8-38 using primers r(Sk-2)-57 and r(Sk-2)-58. These primers amplify not only the coding region of $NCU09151^{r(Sk-2)}$ but also the promoter and terminator sequences. The resulting PCR product was cloned into pCR-II

TOPO (Life Technologies, Grand Island, NY) to create the plasmid pTH95.1.

NCU09151^{r(Sk-2)} was then digested out of the plasmid and placed into pBM61 (Margolin *et al.* 1997) (using restriction enzyme sites *Eco*RI and *Not*I to create plasmid pTH97.2). This plasmid was used as a transformation vector for targeting *NCU09151^{r(Sk-2)}* to the *his-3* locus.

The second transformation vector allowed us to introduce *NCU09151^{r(Sk-2)}* to its native locus in a sensitive strain. An *hph* marker was added to pTH97.2, adjacent to *NCU09151^{r(Sk-2)}*. This new plasmid, pDGR3.1, was used as the template for the center fragment for the double-joint PCR construction of the final transformation vectors that would ultimately allow *NCU09151^{r(Sk-2)}::hph* to replace *NCU09151^S*.

IV.2.iv. Insertion of *NCU09151^{r(Sk-2)}* to the right border of *Sk* recombination block

NCU09151^{r(Sk-2)} was inserted into the right border of the recombination block on the right arm of chromosome III near the 5` end of *NCU06449.*

Insertion of *NCU09151^{r(Sk-2)}* to chromosome IIIR was performed according to the double-joint PCR protocol developed by Yu *et al.* (2004). The primers used to amplify the double-joint PCR transformation constructs are listed in Table IV.2. The left and right flanks were amplified from wild-type DNA (P3-07) and the center piece, *NCU09151^{r(Sk-2)}::hph*, was amplified from the plasmid pDGR3.1. The final PCR product was inserted into strain P8-42 (*rid his-3 a; mus-51^Δ::bar*).

Transformants were screened for resistance to hygromycin and to *Sk-2* via standard protocol. Strain DGR15.1-4 (*rid IIIR::r(Sk-2)-hph mus-51^Δ::bar a*) was recognized as a positive candidate for insertion of *NCU09151^{r(Sk-2)}* on the right end of the

Sk-2 recombination block. This strain is referred to as *IIIR* in the remainder of this thesis.

IV.2.v. Amplifying additional *NCU09151* alleles

NCU09151 alleles from *Sk-2*, *Sk-3*, *r(Sk-3)*, and *Sk*-resistant and *Sk*-susceptible strains from natural Neurospora populations were amplified and cloned in the following manner. The primers used for amplifying *NCU09151* from these strains are listed in Table IV.3. All of the alleles were initially cloned to pCRII-TOPO before being digested with *Eco*RI and *Not*I and inserted into the vector pTH152.2. pTH152.2 contains a multiple cloning site adjacent to an *hph* marker. The *NCU09151* alleles, along with *hph*, were then PCR amplified from these vectors and used as the center piece in a double-joint PCR transformation fragment when necessary. A list of the vectors can be seen in Table IV.3.

IV.2.vi. Sequencing of *rsk*

DNA sequencing was conducted at the MU DNA core. The primers used for sequencing are listed in Table IV.2.

IV.2.vii. Microscopy and photography

Rosettes of asci were dissected from 11-14 day old perithecia in 25% glycerol using a VanGuard 1231CM Trinocular Microscope. A Canon Power Shot S3 IS digital camera with a Canon conversion lens adapter was attached to this microscope and used to photograph the rosettes.

Group	Phenotype	Origin	Allele (Strain)	Primers	*Hph* vector
Alleles similar to Sk-susceptible *rsk^OR^*	*Sk^S^*	*N. crassa* (Oak Ridge)	OR2489 (P3-07)	rSk-2-57 & rSk-2-58	pDGR102.1
	*Sk^S^*	*N. crassa* (Haiti)	HT4714 (P17-26)	rSk-2-DGR1 & rSk-2-58	p4714.1*
	*Sk^S^*	*N. crassa* (Brazil)	BR4705 (P17-27)	rSk-2-DGR1 & rSk-2-58	p4705.1*
Alleles similar to *Sk-2*-resistant *rsk^LA^*	*r(Sk-2)*	*N. crassa* (Louisiana)	LA2222 (P8-38)	rSk-2-57 & rSk-2-58	
	r(Sk-2)	*N. crassa* (Brazil)	BR4706 (P14-18)	rSk-2-DGR1 & rSk-2-58	pDGR100.2
	r(Sk-2)	*N. crassa* (Haiti)	HT4715 (P14-19)	rSk-2-57 & rSk-2-58	pDGR101.1
	r(Sk-2)	*N. crassa* (Ivory Coast)	CI4831 (P14-20)	rSk-2-57 & rSk-2-58	pDGR105.2
	Sk-2	*N. intermedia* (Borneo)	BN7401 (F2-19)	rSk-2-57 & rSk-2-58	

Alleles similar to *Sk-3*-resistant *rsk^PF^*	*r(Sk-3)*	*N. intermedia* (French Polynesia)	PF5123 (P12-41)	rSk-2-DGR1 & rSk-2-58	pDGR107.1
	Sk-3	*N. intermedia* (Papua New Guinea)	PG3193 (F3-14)	Sk-3-106 & rSk-2-58	**
	*Sk^S^*	*N. crassa* (Ivory Coast)	CI4832 (P17-25)	rSk-2-DGR1 & rSk-2-58	p4832.1*

Table IV.3. Primer sets for amplifying *NCU09151* alleles and their corresponding vectors
*Alleles are in pCRII TOPO vectors instead of *hph* vectors
**Allele was not put into a vector

IV.3. Results

IV.3.i. Refinement of the *r(Sk-2)* locus

The classical mapping experiments, reported in the previous chapter, refined the location of *r(Sk-2)* to a region between (and including) genes *NCU09145* and *NCU09159*. This region, a ~55kb tract, contains 13 predicted open reading frames. To further refine the location of *r(Sk-2)*, we created two *hph* markers and placed one between *NCU09149* and *NCU09150* and the other between *NCU09155* and *NCU09156*.

These new markers allowed us to conduct additional three-point crosses (Figure IV.1). Analysis of these three-point crosses refined the location of *r(Sk-2)* to between the

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two *hph* markers. This narrowed our candidate field from thirteen genes to six (Table IV.1 & IV.2).

IV.3.ii. Deletion of *NCU09151*[r(Sk-2)] results in loss of resistance to *Sk-2*

Due to the close proximity of the remaining six genes, three-point testcrossing was no longer a viable mapping method. So, instead we decided to delete the remaining genes with the hope that one of the gene deletions might lead to a loss of resistance when crossed to *Sk-2*.

Deletion strains were created for *NCU09151*, *NCU09153*, and *NCU09154* (we were unable to delete *NCU09152*). We then crossed each deletion strain to *Sk-2* to see if resistance might be lost. While *NCU09153*[A] and *NCU09154*[A] resulted in no loss of resistance, deletion of *NCU09151* resulted in the production of completely aborted asci (Figure IV.2). All three of the deletion-strains gave normal asci when crossed to wild type.
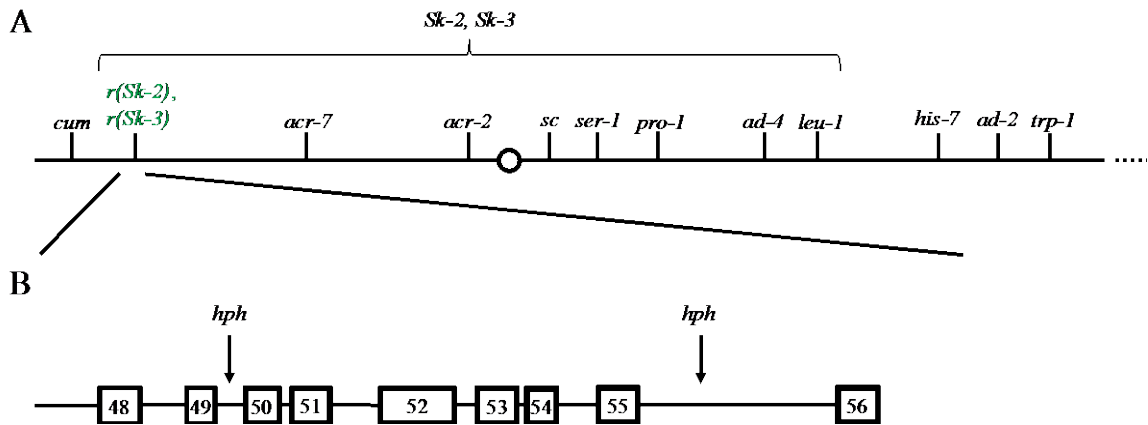
Figure IV.1. Refinement of the *r(Sk-2)* locus
A) Depicted is a segment of chromosome III in *N. crassa*. *Sk-2* and *Sk-3* are contained within a 30 map unit block of this chromosome (indicated by bracket). *r(Sk-2)* and *r(Sk-3)* have been mapped to the left border of this block. B) Our previous mapping experiments further localized r*(Sk-2)* to a region between, and including, the genes *NCU09145* and *NCU09159* (Hammond *et al.* 2012). To further refine the location of *r(Sk-2)*, two *hph* markers were created and placed in the regions indicated on the diagram. These new markers allowed us to conduct three-point mapping experiments to help determine the precise location of *r(Sk-2)*. Cross analysis revealed that *r(Sk-2)* lies between our two new *hph* markers. Map is not to scale.

Table IV.4. Three-point cross data suggests *r(Sk-2)* is to the right of the hygromycin marker *hph$^{49/50}$*

	Parentals		Interval 1 crossover		Interval 2 crossover		Double crossover		
	hph	+	+	*hph*	+	*hph*	*hph*	+	
	+	*r(Sk-2)*	+	*r(Sk-2)*	*r(Sk-2)*	+	*r(Sk-2)*	+	
	+	*acr-7*	+	*acr-7*	+	*acr-7*	+	*acr-7*	**Total**
hph$^{49/50}$	247	195	1	0	22	27	0	0	492

The hygromycin marker that we inserted between *NCU09149* and *NCU09150* (*hph$^{49/50}$*)

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was introduced into a wild-type *N. crassa* strain (P17-13) and then crossed to the strain *cum r(Sk-2) acr-7* (P8-11). Cross data suggest that the order of the three markers is $hph^{49/50}$ - *r(Sk-2)* - *acr-7*.

Table IV.5. Three-point cross data suggests *r(Sk-2)* is to the left of the hygromycin marker *$hph^{55/56}$*

	Parentals		Interval 1 crossover		Interval 2 crossover		Double crossover		
	+	*r(Sk-2)*	*r(Sk-2)*	+	*r(Sk-2)*	+	+	*r(Sk-2)*	
	hph	+	*hph*	+	+	*hph*	+	*hph*	
	+	*acr-7*	+	*acr-7*	+	*acr-7*	+	*acr-7*	**Total**
$hph^{55/56}$	233	222	2	2	28	21	0	0	508

The hygromycin marker that we inserted between *NCU09155* and *NCU09156* ($hph^{55/56}$) was introduced into a wild-type *N. crassa* strain (P17-12) and then crossed to the strain *cum r(Sk-2) acr-7* (P8-11). Cross data suggests that the order of the three markers is *r(Sk-2)* - $hph^{55/56}$ - *acr-7*.

To investigate the new aborted ascus phenotype associated with *NCU09151ᐟ* × *Sk-2*, we decided to re-examine this cross in a *sad-2ᐟ* background. We hypothesized that the aborted ascus phenotype might be due to the silencing of *Sk-2*'s own resistance allele. Deletion strains, like in this example, can create an unpairing event between the deletion and its homolog during meiosis and therefore cause silencing of the lone unpaired gene by the MSUD pathway (Shiu *et al.* 2001).

In our scenario, this would mean that no resistance protein would be expressed during meiosis in this cross and therefore all the ascospores, including those with the

killer haplotype, would be subject to killing by the killer element. However, when *sad-2^A*

is present in one of the parents, there is a complete knockdown of the unpaired-gene

silencing system (Shiu *et al.* 2006). This knockdown allows unpaired genes to be

expressed throughout meiosis. Indeed, when the deletion cross was conducted in a *sad-2^A*

background, restoration of ascospore production and the wild-type spore killing

phenotype (4B:4W) was observed (Figure IV.2). These results suggest that *NCU09151* is

the gene responsible for resistance in the *r(Sk-2)* strain. In addition, the results suggest

that the *Sk-2* resistance gene shares a similar sequence with *r(Sk-2)* because the MSUD

system only silences homologous alleles of the unpaired gene.

IV.3.iii. Expression of *NCU09151^r(Sk-2)* in a sensitive strain grants resistance to *Sk-2*

Since the deletion of *NCU09151^r(Sk-2)* corresponds to the loss of resistance to *Sk-2*,

we asked if the introduction of *NCU09151^r(Sk-2)* would confer resistance to *Sk-2* for a

sensitive strain. In order to perform this task, we cloned *NCU09151^r(Sk-2)* into two

different vectors. One vector would allow us to insert *NCU09151^r(Sk-2)* into *his-3*, a
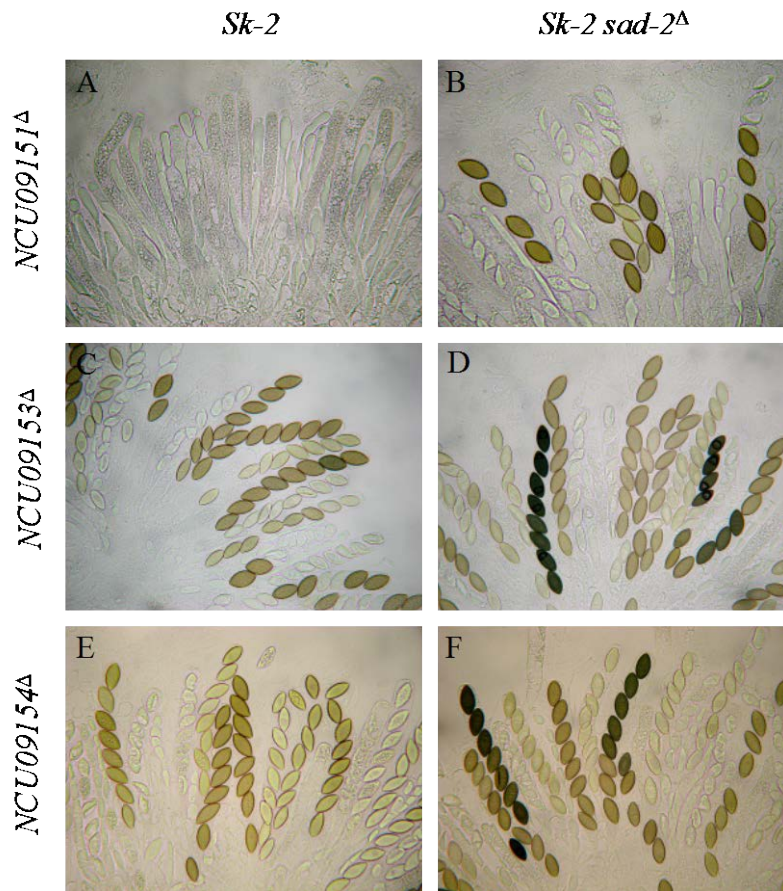
Figure IV.2. Candidate deletion strain crosses

Three $r(Sk-2)$ candidates were deleted and then crossed to $Sk-2$ and $Sk-2$ $sad-2^{\Delta}$ strains in order to determine if loss of resistance to $Sk-2$ would occur. (A) $Sk-2 \times NCU09151^{\Delta}$ (F1-16 × P15-54) crosses resulted in completely aborted asci, suggesting that the deletion of $NCU09151$ and resulting unpairing of the one $NCU09151$ copy in $Sk-2$ results in the loss of resistance in the entire ascus during early development. (B) When $sad-2^{\Delta}$ was added to the same cross (F5-18 × P15-54) ascospore production followed that of a typical WT × $Sk-2$ cross (4B:4W). (C-F) Deletions of $NCU09153$ (P17-01) and $NCU09154$ (P17-02) have no effect on $Sk-2$ resistance (all remained resistant to $Sk-2$, 8B:0W).

common transformation locus on chromosome I, and the other vector would allow us to

insert $NCU09151^{r(Sk-2)}$ into its native locus, replacing the sensitive allele.

Insertion of *NCU09151$^{r(Sk-2)}$* into the *his-3* locus was able to confer resistance to a sensitive strain (when it was crossed to *Sk-2*). The transformation was conducted in a *sad-2^Δ* background because ectopic insertion would unpair *NCU09151$^{r(Sk-2)}$* and possibly lead to another ascus abortion phenotype (later experiments revealed this to be the case). Because *his-3* is on chromosome I and *Sk-2* is on chromosome III, the two loci segregate independently and thereby give us asci with three different phenotypes: 8B:0W (four *NCU09151$^{r(Sk-2)}$::his-3$^+$* and four *Sk-2*, all viable), 4B:4W (four *Sk-2* survived, four *NCU09151^S* aborted), and 6B:2W (2 *NCU09151$^{r(Sk-2)}$::his-3$^+$*, two *Sk-2*, two *NCU09151$^{r(Sk-2)}$::his-3$^+$ Sk-2* survived, and 2 *NCU09151^S* aborted) (Figure IV.3).

Next we replaced the sensitive *NCU09151^S* allele in a wild type strain with *NCU09151$^{r(Sk-2)}$*. Introduction of *NCU09151$^{r(Sk-2)}$* into the native locus does indeed confer resistance in the wild type strain (when it is crossed to *Sk-2*) (Figure IV.4). It was not necessary to conduct this cross in a *sad-2^Δ* background because normal pairing at *NCU09151* occurs.

IV.3.iv. Placement of *NCU09151$^{r(Sk-2)}$* at its native locus is crucial for resistance

From the previous experiments, two results were of special interest, i.e., the ascus abortion phenotype seen in (1) *NCU09151$^{Δ\,r(Sk-2)}$ × Sk-2*, and in (2) *NCU09151$^{r(Sk-2)}$::his-3$^+$ × Sk-2* (in a non-*sad-2^Δ* background). These results suggested that *NCU09151* must be paired during meiosis in order for any offspring to be viable in a cross between WT and *Sk-2*.
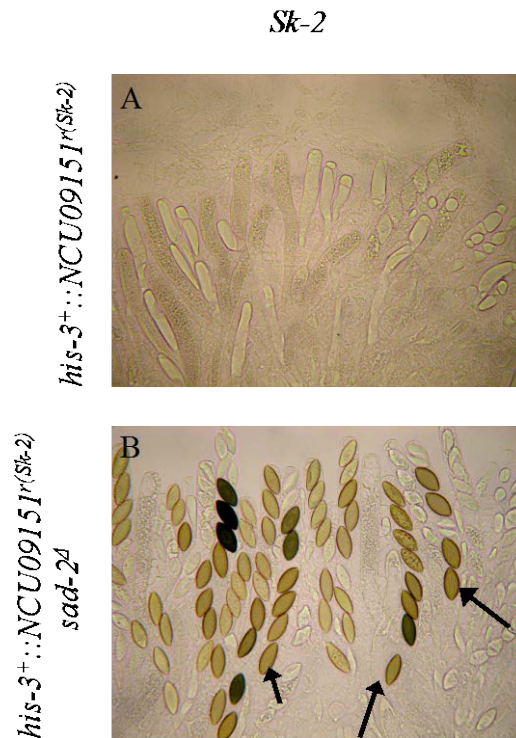
Figure IV.3. Ectopic insertion of *NCU09151*$^{r(Sk-2)}$ grants resistance to sensitive strains
A) Insertion of *NCU09151*$^{r(Sk-2)}$ at the *his-3* locus on chromosome I of a sensitive strain (P17-06) creates an aborted ascus phenotype when the transformant was crossed to an *Sk-2* strain (F2-19). B) However, when the same cross was done in a *sad-2*$^{\Delta}$ background (i.e. meiotic silencing deficient), resistance is conferred. Because chromosome I and III segregate independently, three different ascospore patterns are created: 4B:4W, 6B:2W, and 8B:0W (black arrows).
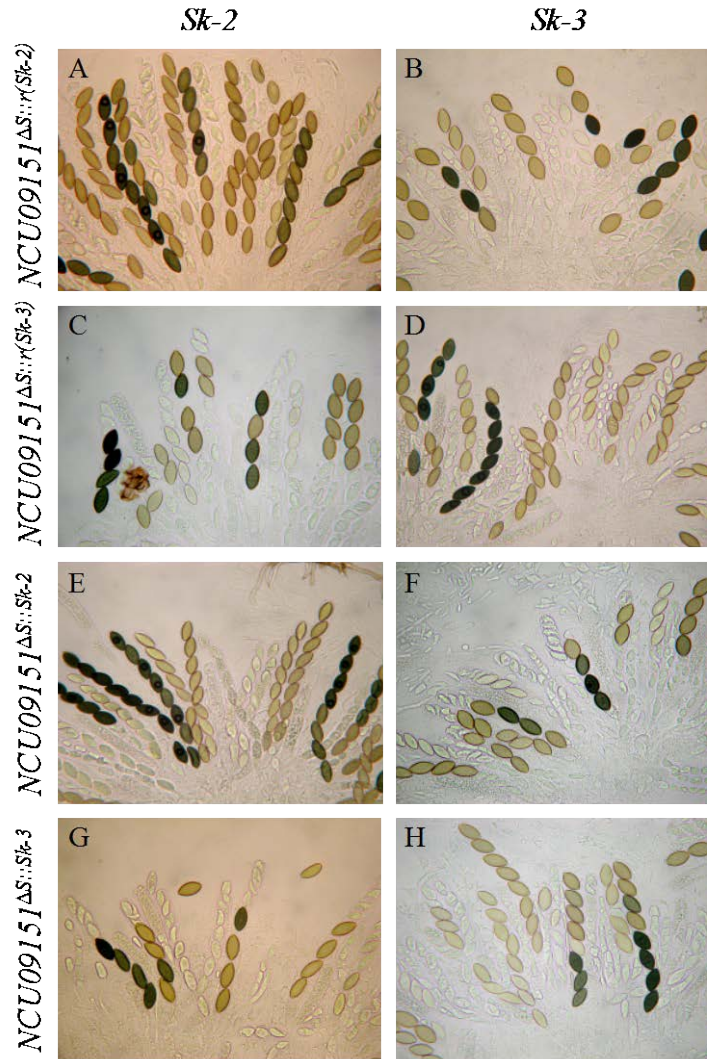
Figure IV.4. *NCU09151* from *r(Sk-2)*, *Sk-2*, *r(Sk-3)*, and *Sk-3* all grant resistance to a sensitive strain

NCU09151^S was deleted and replaced with either *NCU09151$^{r(Sk-2)}$*, $^{Sk-2}$, $^{r(Sk-3)}$, or $^{Sk-3}$ before being crossed back to *Sk-2* (F1-16 or F2-19) or *Sk-3* (F3-14 or F3-16). A,B) *NCU09151$^{r(Sk-2)}$* (P15-56) grants full resistance to *Sk-2* but is unable to grant resistance to *Sk-3*. C,D) *NCU09151$^{r(Sk-3)}$* (P17-15) is susceptible to killing by *Sk-2* but grants full resistance to *Sk-3*. E,F) *NCU09151^{Sk-2}* (P17-16) behaves exactly like *NCU09151$^{r(Sk-2)}$*. G,H) *NCU09151^{Sk-3}* (P17-17) behaves exactly like *NCU09151$^{r(Sk-3)}$*.

The observations from the above experiments make sense when we apply our knowledge of the MSUD system. The aborted ascus phenotype in these crosses is what we would expect for an unpaired gene in *Neurospora crassa*, namely that unpaired genes are silenced during meiosis. Since *NCU09151$^{r(Sk-2)}$* is unpaired, no resistance protein is being expressed in the asci and so all ascospores are subject to the effects of the killer molecule.

However, previous research by our group had clearly demonstrated that *Sk-2* and *Sk-3* have the ability to dominantly suppress meiotic silencing, thereby allowing unpaired genes to be expressed throughout meiosis (Raju *et al.* 2007). Why the MSUD suppression in *Sk-2* could alleviate the silencing of several different unpaired tester genes but was unable to suppress the silencing of its own resistance allele (the expression of which is absolutely crucial to the survival of the drive system), remains a mystery.

We hypothesized that perhaps the resistance allele might be immune from silencing if it is placed anywhere inside the recombination block (i.e. the recombination block might prevent unpaired genes from being silenced). To investigate this possibility, we placed *NCU09151$^{r(Sk-2)}$* on the other side of the centromere of chromosome III but still within the *Sk-2* recombination block (about 600kb within the right border of the block). When this new strain, denoted as *IIIR*, was crossed to *Sk-2*, it resulted in the same abortion phenotype as seen by *NCU09151$^{\Delta r(Sk-2)}$* and *his-3$^+$::NCU09151$^{r(Sk-2)}$*, suggesting that even though the resistance allele was on the same chromosome and within the *Sk* recombination block, its unpairing still led to the silencing of all copies of the *NCU09151* alleles present in the cross.

IIIR::NCU09151$^{r(Sk-2)}$

Figure positions (left margin labels, top to bottom): *Sk-2* (A), *Sk-2 sad-2$^\Delta$* (B), *Sk-3* (C), *Sk-3 sad-2$^\Delta$* (D)
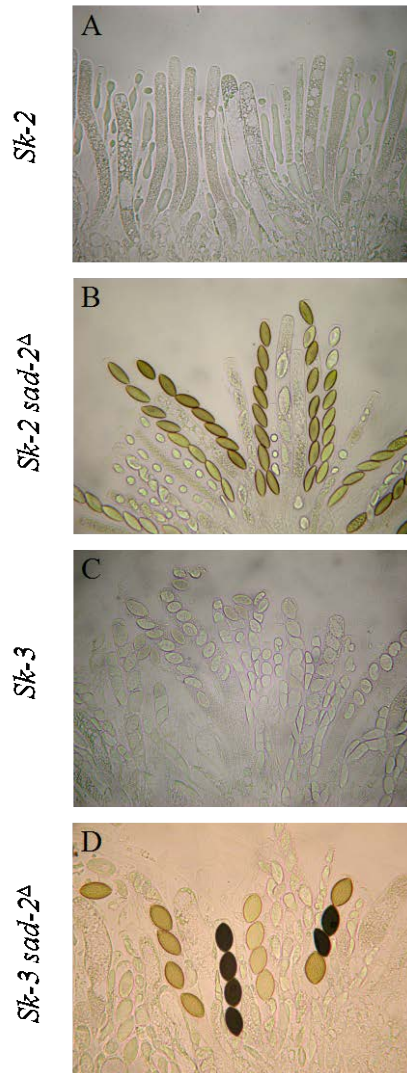
Figure IV.5. Ectopic insertion of *NCU09151$^{r(Sk-2)}$* into the *Sk* recombination block
NCU09151$^{r(Sk-2)}$ was transformed into a locus just inside the right border of the *Sk* recombination block on chromosome III (denoted *IIIR::NCU09151$^{r(Sk-2)}$* or simply *IIIR*; P15-58) of a sensitive strain. A) Since ectopic insertion of *NCU09151$^{r(Sk-2)}$* creates an unpairing event when crossed to *Sk-2* (F1-16), all copies of *NCU09151* are silenced during meiosis and one can see an ascus abortion phenotype similar to the ones observed in the *NCU09151$^{\Delta r(Sk-2)}$* crosses. This result suggests that the recombination block does not prevent an unpaired *NCU09151$^{r(Sk-2)}$* from being silenced by the MUSD machinery. B) When crossed to an *Sk-2* strain with a meiotic silencing deficient background (*sad-2$^\Delta$*; F5-18), resistance is restored. C) Crosses to *Sk-3* (F3-16) create another ascus abortion phenotype, which suggests that *Sk-3* contains a homolog of *NCU09151$^{r(Sk-2)}$*. D) Crosses to *Sk-3* in a meiotic silencing deficient background (*sad-2$^\Delta$*; F5-30) restore the typical 4B:4W pattern observed when different Spore killer haplotypes are crossed to each other. background, it resulted in the full restoration of the resistance phenotype. These results suggest that *r(Sk-2)* must be paired with a homolog in a cross involving a Spore killer and an active MSUD pathway.

IV.3.v. *Sk-2* uses *NCU09151* to confer resistance

After deducing that *NCU09151* was the locus responsible for granting resistance in *r(Sk-2)* strains, we then asked if the same locus was used by *Sk-2* strains. Since the deletion of *NCU09151* in our *r(Sk-2)* strain resulted in an ascus abortion phenotype when crossed to *Sk-2*, we believed there was a strong chance that *Sk-2* utilized the same gene for resistance.

To test whether *NCU09151* was indeed the resistance locus in *Sk-2*, we deleted this gene from an *Sk-2* strain. The deletion strain, when crossed to a sensitive strain, resulted in a similar self-killing ascus abortion observed in $NCU09151^{\Delta r(Sk-2)} \times Sk-2$ crosses. Next, we cloned *NCU09151* from *Sk-2* (denoted $NCU09151^{Sk-2}$) and introduced it to a sensitive strain, replacing the sensitive *NCU09151* to determine if resistance might again be conferred. Insertion of $NCU09151^{Sk-2}$ did in fact grant resistance to a sensitive strain when it is crossed to *Sk-2* (Figure IV.4). These results suggest that the resistance gene found in *r(Sk-2)* and *Sk-2* strains are allelic.

IV.3.vi. *Sk-3* also utilizes *NCU09151* for resistance

Since *Sk-2* and *Sk-3* are similar in their killing and resistance phenotypes, it was logical to ask whether *Sk-3* also utilizes *NCU09151* for resistance. Early evidence from the *r(Sk-2)* experiments had already hinted that *NCU09151* might be used by the *Sk-3* system. When $NCU09151^{\Delta r(Sk-2)}$ was crossed to *Sk-3*, an ascus abortion phenotype similar to that of *Sk-2* occurred (suggesting that an allele similar to $NCU09151^{r(Sk-2)}$ must be silenced in the cross) (Figure IV.5). Indeed, when $NCU09151^{\Delta r(Sk-2)}$ was crossed to *Sk-3* in a *sad-2$^\Delta$* (meiotic silencing deficient) background, the spore killing pattern of 4B:4W

occurred (4B *Sk-3* and 4W *NCU09151$^{\Delta r(Sk-2)}$* respectively).

Similar experiments to those done for *r(Sk-2)* and *Sk*-2 were then conducted for the *Sk-3* system. Deletion of *NCU09151* in *Sk-3* and *r(Sk-3)* strains resulted in an ascus abortion phenotypes when they are crossed to sensitive and *Sk-3* strains, respectively. Introduction of *NCU09151$^{r(Sk-3)}$* or *NCU09151^{Sk-3}* to a sensitive strain (at the native locus) confers resistance in a sensitive strain when it is crossed to *Sk-3* (Figure IV.4). These results suggest that *Sk-2* and *Sk-3* share a common resistance locus and that these two systems, although independent in their killing specificity, might share a common lineage.

IV.3.vii. New nomenclature for *NCU09151*

Since our naturally resistant strains, Spore killer strains, and sensitive strains all contain the locus *NCU09151*, we proposed to give them a more descriptive nomenclature. From now on *NCU09151* will be referred to as *rsk* (*resistant to Spore killer*). Alleles at this locus will be written as such: *rsk$^{r(Sk-2)}$* , *rsk^{Sk-2}*, *rsk$^{r(Sk-3)}$*, *rsk^{Sk-3}*, and *rsk^{S}* (where "S" stands for sensitive).

IV.3.viii. RSK sequence comparisons among sensitive and resistant alleles

The online *N. crassa* sequence website, administered by the Broad Institute, indicates that the sensitive version of *NCU09151*, hereafter referred to as *rsk^{S}*, encodes a 486-amino acid polypeptide with no currently discernable motifs. It also indicates that RSK is a fungal-specific protein found only in closely related fungi.

Sequencing and protein alignments were conducted on *rsk$^{r(Sk-2)}$* , *rsk^{Sk-2}*, *rsk$^{r(Sk-3)}$*, and *rsk^{Sk-3}* (Figure IV.6). Resistant alleles from naturally resistant strains and their

corresponding killer strains share high amino acid similarity: $RSK^{r(Sk-2)}$ and RSK^{Sk-2}
are ~94% identical while both share ~70% identity with RSK^S; $RSK^{r(Sk-3)}$ and RSK^{Sk-3} are
~97% identical and both share ~63% identity with RSK^S. $RSK^{r(Sk-3)}$ and RSK^{Sk-3} are only
~62% identical to $RSK^{r(Sk-2)}$ and RSK^{Sk-2}.

As seen in the depiction of the different alleles in Figure IV.7, the Spore killer
resistant alleles have unique insertion and deletion patterns and are both quite different
from the sensitive allele. Because the naturally resistant strains of each of the spore
killers share the same indel patterns as the killers themselves, one can hypothesize that
they are evolutionarily related.

IV.3.ix. Sequence comparison among global *rsk*^{r(Sk-2)} isolates

Several globally isolated strains of *r(Sk-2)* have been identified from fungal
collections over the years (Perkins and Turner 1976; Perkins and Turner 1988; Turner
2001). Because these strains came from isolated populations, we were curious to see how
their *rsk* sequence compare to that of the classical resistance strain we characterized in
our study. Protein analysis revealed that the *r(Sk-2)* strains from the global collection
were >99% identical to our *r(Sk-2)* strain from Louisiana, with the exception of one strain
from the Ivory Coast which resembled a truncated *r(Sk-3)* protein (Table IV.3). These
identity comparisons indicate that the sequence of *r(Sk-2)* is highly conserved. They also
indicate that *Sk-2* has either spread across the globe (by natural or artificial means) or that
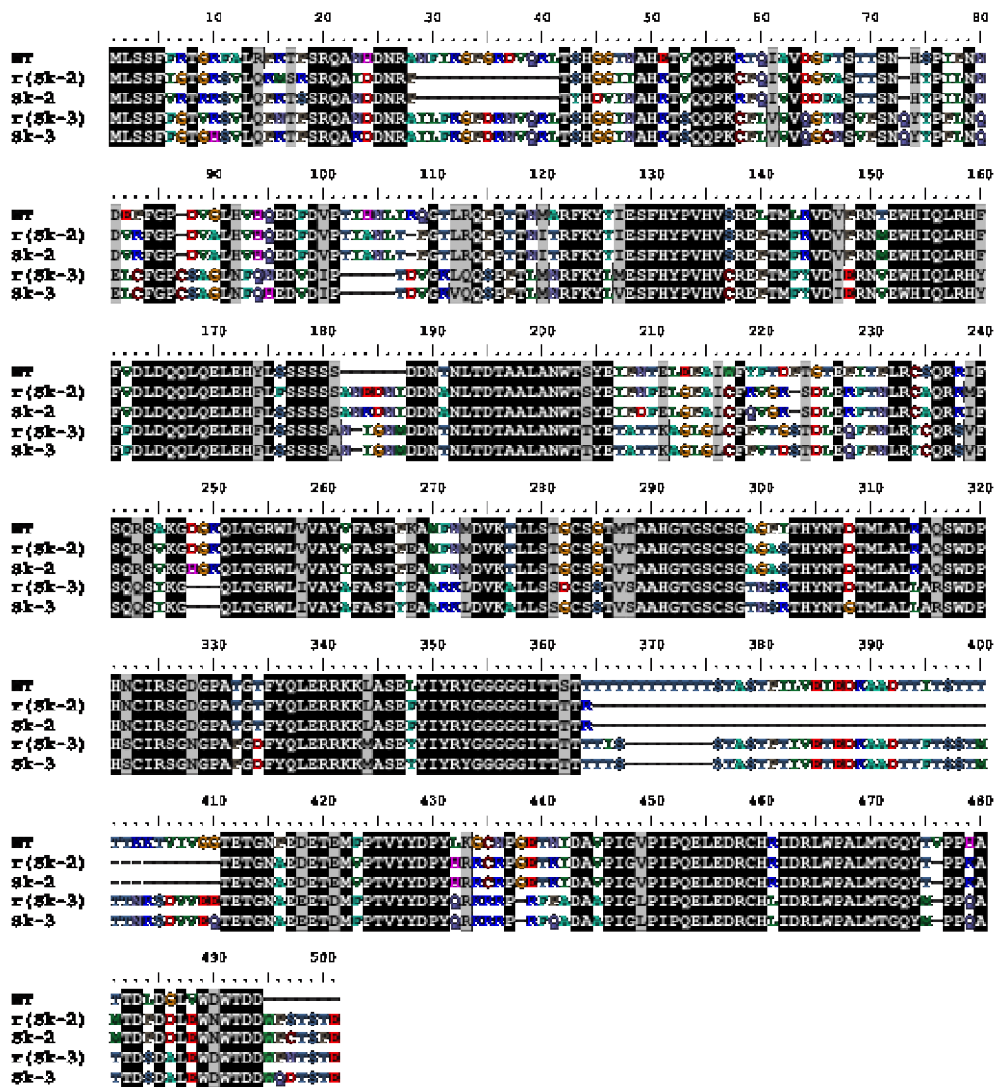
Figure IV.6. ClustalW alignment of RSK sequences
Protein sequences analysis was conducted on the following strains: WT (P3-07), *r(Sk-2)* (P8-38), *Sk-2* (F2-19), *r(Sk-3)* (P12-41), and *Sk-3* (F3-14).
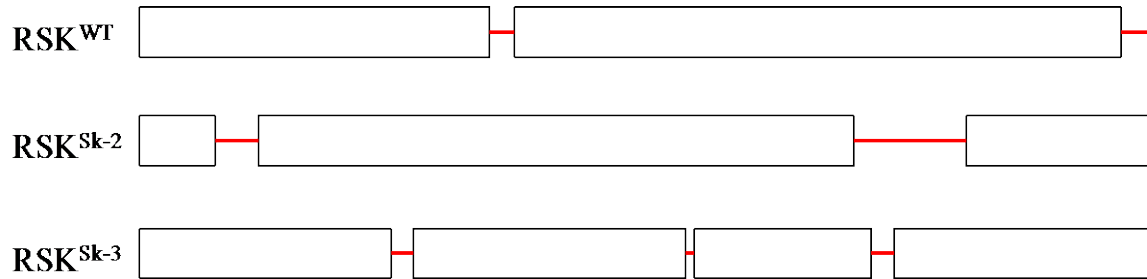
Figure IV.7. Insertion/deletion differences among RSK groups
RSK amino acids sequences can be categorized into three different groups; 1) WT
sensitive sequences, 2) Resistant to *Sk-2* sequences ($rsk^{r(Sk-2)}$ and rsk^{Sk-2}) and 3) Resistant
to *Sk-3* sequences [$rsk^{r(Sk-3)}$ and rsk^{Sk-3}].

Group	Phenotype	Origin	Allele (Strain)	Gene		Protein	
				Length	Identity	Length	Identity
Alleles similar to	Sk^S	*N. crassa* (Oak Ridge)	OR2489 (P3-07)	1461	100%	486	100%
Sk-susceptible	Sk^S	*N. crassa* (Haiti)	HT4714 (P17-26)	1470	96.9%	489	95.7%
rsk^{OR}	Sk^S	*N. crassa* (Brazil)	BR4705 (P17-27)	1470	96.4%	489	94.7%
Alleles similar to	*r(Sk-2)*	*N. crassa* (Louisiana)	LA2222 (P8-38)	1314	100%	437	100%
Sk-2-resistant	*r(Sk-2)*	*N. crassa* (Brazil)	BR4706 (P14-18)	1314	99.6%	437	99.5%
rsk^{LA}	*r(Sk-2)*	*N. crassa* (Haiti)	HT4715 (P14-19)	1314	99.8%	437	99.5%
	r(Sk-2)	*N. crassa* (Ivory Coast)	CI4831 (P14-20)	1314	99.7%	437	99.8%
	Sk-2	*N. intermedia* (Borneo)	BN7401 (F2-19)	1314	96.9%	437	94.3%
Alleles similar to	*r(Sk-3)*	*N. intermedia* (French Polynesia)	PF5123 (P12-41)	1449	100%	482	100%
Sk-3-resistant	*Sk-3*	*N. intermedia* (Papua New Guinea)	PG3193 (F3-14)	1449	98.5%	482	96.7%
rsk^{PF}	Sk^S	*N. crassa* (Ivory Coast)	CI4832 (P17-25)	1453	96.7%	103*	83.7%**

Table IV.6. *rsk* **sequence analysis of geographically distinct isolates.**

Figure IV.8. Phylogenetic relationships among various global *rsk* alleles
Numbers next to branches indicate the percentage of bootstrap support (based on 1000 replicates). Neighbor-joining tree constructed by MEGA5 (http://www.megasoftware.net/).

Sk-2 has arisen independently in these populations (Figure IV.8).
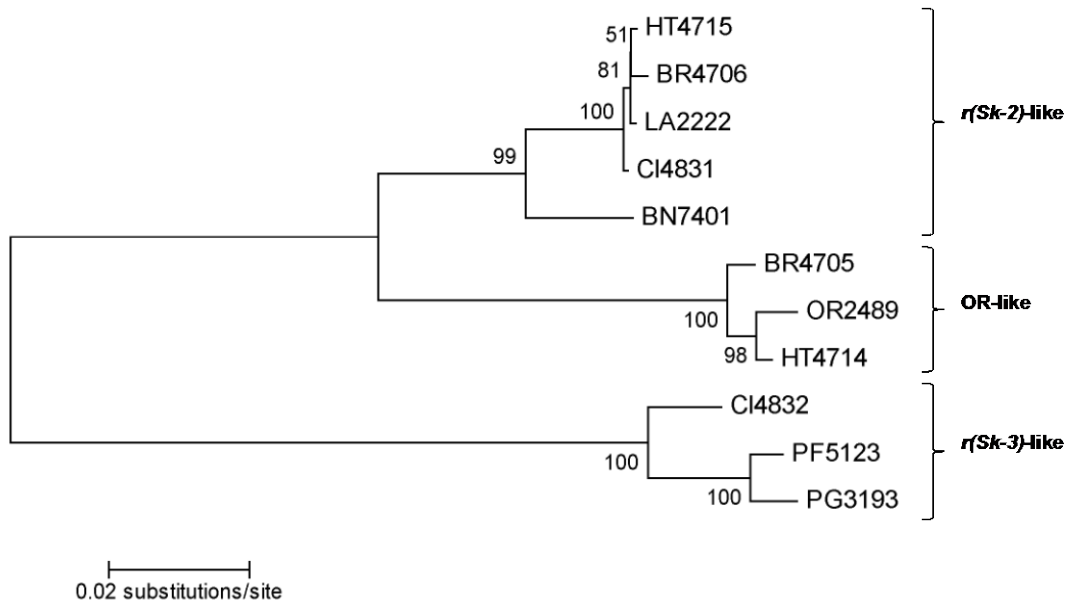
IV3.x. Model for killing and resistance in the Spore killer system

In the classic meiotic drive systems *t-haplotype* and *Segregation distorter*, the drive elements target sensitive loci and disrupt their function, which results in the improper formation of the gametes not containing the drive element. If the Spore killer meiotic drive systems fit this same model, we might predict that RSK^S is required for some aspect of ascospore development and that the killer targets the RSK^S protein for inactivation. This would mean that the killer element is unable to recognize RSK^R, thereby allowing development to proceed normally in the ascospores containing *rsk^R*. However, a key result contradicts this model. No readily observable dysfunction of ascospore development occurs in a cross that is homozygous for *rsk^S* deletion. This suggests that *rsk^S* is not required for development and therefore the killer element must act on some other molecule to create drive.

Based on our experimental results, we hypothesize that the killer and resistance loci work in a poison-antidote fashion; in a model we coined the RSK killer-neutralization (Figure IV.9). In this model, the killer element targets one or more molecules that are responsible for proper ascospore development. When RSK^R is absent from a cross (e.g. WT $\times$ *Sk rsk$^{\Delta}$*) the killer targets early ascospore development, which results in no ascospores being formed at all. If RSK^R is present in the cross, however, all ascospores are protected during early development from the effects of the killer element because RSK^R actively neutralizes the killer (Figure IV.9). Only when the ascospores delimit and become individual spores in later stages of development do the ascospores

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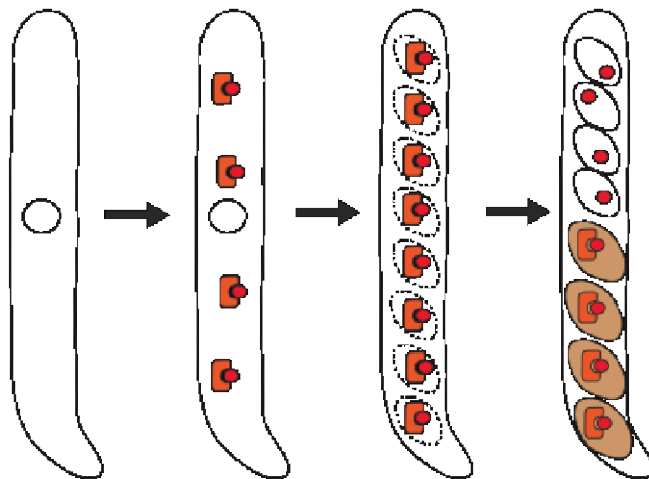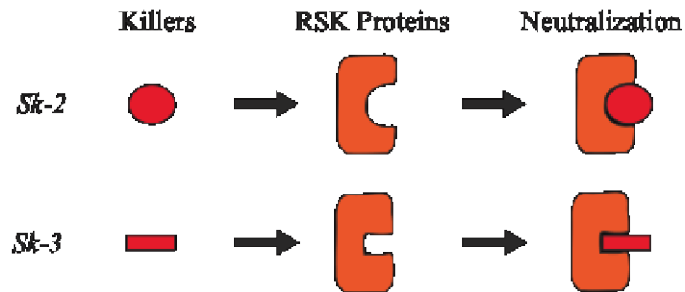
Figure IV.9. The RSK killer-neutralization model
A) The evidence garnered from our experiments supports a model in which different versions of RSK neutralize different killers [e.g. RSK$^{r(Sk-2)}$ neutralizes *Sk-2* and not *Sk-3*]. B) RSK^R and the killer are produced early in the ascus before individual ascospores begin to form. At this point RSK^R is actively neutralizing the effects of the killer. When the ascospores begin to delimit and form cell walls, only the ascospores containing *rsk^R* are able to continue to produce RSK^R and actively neutralize the killer. In the ascospores not containing *rsk^R* the killer element disrupts further development.

not containing the resistant protein begin to die off. The ascospores that contain rsk^R (50%) are able to keep producing RSK^R in their now separate cell plasmas while those that were previously protected by RSK^R before delimitation are now unable to produce the neutralizer and are therefore subject to the effects of the killer (Figure IV.9). This also suggests that the killer element must have a long half-life because it appears to still be active in the ascospores that do not carry the killer haplotype.

IV.4. Discussion

Meiotic drive elements are ubiquitous among the plant, animal, and fungal kingdoms. However, only a handful of these meiotic drive elements have been molecularly characterized and, of those, the mechanisms of drive are still far from understood. The *Neurospora* Spore killer meiotic drive elements fall into the category of classic drive elements whose mode of action, molecular characterization, and genetic identification are still unknown. As reported in the experiments above we genetically identified a critical component of the Spore killer drive systems, the *resistance to Spore killer* (*rsk*) gene.

rsk appears to be quite different from its counterparts found in other classical meiotic drive systems. In drive systems like *Segregation distorter* and *t*-haplotype, the drive element targets a specific locus in order to create drive during meiosis; alleles at this targeted locus can be either sensitive or neutral (resistant) to the effects of the drive locus. In the *Sk* system, however, *rsk* is not targeted by the drive element but instead creates a protein that targets the drive element itself. Because RSK^R prevents the drive function of the killer element, we coin their interaction the killer-neutralization model.

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Our killer-neutralization model can best be understood as a poison-antidote system. In the simplest iteration of a poison-antidote system the presence of the poison, within a cell or organism, dictates that the cell or organism will die unless otherwise supplied with the appropriate antidote. In the *Sk* system, the killer molecule is the poison and RSK^R is its antidote. Without the presence of RSK^R, the killer will eliminate all of the developing meiotic products, the ascospores, through some yet-to-be understood mode of action. In this model, the killer is absolutely dependent on the resistance allele to protect itself from self-killing.

The killer's dependency on RSK^R to protect itself from self-killing necessitates that the two elements be co-expressed during meiosis. Prior to our experiments, it was believed that *Sk* acted late in ascospore development to create drive, after ascospore delimitation, as the ascospore cell walls formed (Raju 1979). Our experiments, however, indicate that the killer functions throughout meiosis and ascospore development. When *rsk* is deleted from *Sk-2* and then crossed to wild type, the result is complete ascus abortion. This implies that the killer must be active early in ascospore development, before spore delimitation. It also implies that the RSK^R must be tandemly expressed alongside *Sk* in early development, otherwise the killer would eliminate all of the developing asci prior to RSK^R production.

Prior to our experiments it was not known whether *Sk-2* and *Sk-3* utilized the same locus for resistance. Here we report that *Sk-2*, *Sk-3*, *r(Sk-2)*, and *r(Sk-3)* strains all utilize the same gene (*rsk*) for resistance. However, while they share the same locus for resistance, *Sk-2* and *Sk-3* resistance alleles have different and specific indel patterns and either's resistant allele will not grant resistance to the opposite killer haplotype, even in

an MSUD deficient background. These differences in indel patterns might indicate that *Sk-2* and *Sk-3* evolved independently.

Although the function of RSK^R appears to be clear in both *Sk-2* and *Sk-3*, the role of RSK^S still remains a mystery. Homozygous deletion crosses of *rsk^S* produce no observable defects during the sexual phase and deletion strains appear to grow normally during the vegetative phase. Localization studies, utilizing a green fluorescent protein (GFP) tag, reveal that RSK^S is expressed throughout ascospore development in small foci within the ascus (our unpublished data). Further localization and protein interaction studies are needed to determine the role of RSK^S in the lifecycle of *N. crassa* and *N. intermedia*.

In 2007, our group discovered that *Sk-2* and *Sk-3* have the ability to dominantly suppress MSUD, a post-transcriptional gene silencing system (Raju *et al.* 2007). This means that when either of the two killers was present in the background of a cross they could allow for the expression of unpaired genes during meiosis (a feat not possible in a cross with a wild-type background). To date, this is the first example of a meiotic drive element, in any species, that has been shown to disrupt an RNAi system.

There are many reasons why the Spore killers might dominantly suppress the MSUD system. Chromosomal inversions and translocations, although important for linking the killer to its resistance allele, almost assuredly unpair DNA in a heterozygous cross (*WT* × *Sk*). If any of the unpaired DNA contains meiotically important genes, they would be silenced and could therefore result in a failure of meiosis. Or, perhaps there might be an evolutionary advantage for the Spore killers to suppress MSUD. For example, it might allow the Spore killer strains to increase their mating potential by allowing them

to mate with strains that have altered genomic arrangements or to allow the strains to successfully mate with closely related cousins of *N. crassa* (Shiu *et al.* 2001).

Here we report on a limitation of Spore killer's ability to dominantly suppress MSUD. Although it has been demonstrated that *Sk-2* and *Sk-3* can dominantly suppress MSUD to allow for the expression of several meiotically expressed, unpaired genes [*act*, *asm-1*, *mei-3* (Raju *et al.* 2007)], our research indicates that the drive elements are unable to suppress MSUD enough to allow for the expression of an unpaired *rsk* allele. In fact, any deletion or insertion that leads to an unpairing of *rsk* in an *Sk* background results in the silencing of all *rsk* alleles present in the cross and thereby results in a self-killing phenotype where no meiotic products are viable.

These results suggest that *rsk* must remain paired in a cross containing *Sk* as a parent, despite *Sk*'s ability to dominantly suppress MSUD. It is unclear how *Sk* is able to suppress MSUD for some unpaired genes but not others. Perhaps certain unpaired genes, like *rsk*, are more prone to MSUD suppression no matter the presence of a suppressor of MSUD. Or, perhaps silencing is based upon the transcriptional activity of the gene (e.g. *rsk* may have a lower transcription rate and is therefore easier to silence). Whatever the case, how *Sk* is able to suppress MSUD, what advantages or disadvantages this suppression ability might impart on the evolutionary dynamics of *Sk*, and why *rsk* is unable to evade meiotic silencing still remain to be discovered.

We hope our discovery of *rsk* in the Spore killer meiotic drive system provides yet another stepping-stone along the path to understanding the molecular and evolutionary dynamics of these drive elements. Big questions remain, however. What is the killer element, how does it function, what does it target, how does it interact with the

resistant allele, and how does the drive element suppress meiotic silencing? The answers, we hope, are just around the corner.

IV.5. Publication

This work has been published as "Molecular dissection of *Neurospora* Spore killer meiotic drive elements" in the journal <u>PNAS</u>, Volume 109, pages 12093-12098, in July 2012 by *Hammond, T.M., *Rehard, D.G., Xiao, H., and Shiu, P.K.T. My co-authors have contributed to the design, analysis, and executions of the experiments. *Authors contributed equally to this work.

V. *sad(Sk)*

V.1. Introduction

The Spore killer meiotic drive element is located within a region of suppressed recombination (also known as a "recombination block") on chromosome III of *N. crassa*. In *Sk*-2, this recombination block encompasses ~3% (30 m.u.) of the *N. crassa* genome. The block, although beneficial in enforcing linkage between the *Sk-2* killer and resistant elements, might create gene-unpairing events during meiotic homologous pairing in a cross between *Sk-2* and a sensitive strain. A gene silencing system, Meiotic Silencing by Unpaired DNA (MSUD), recognizes these unpaired genes and silences their expression during meiosis (Shiu *et al*. 2001). If any of these genes within the recombination block are required for meiosis or spore development, the silencing of their expression could be detrimental to Sk-2's fitness unless *Sk-2* had a way to circumvent the silencing system.

In 2007, our group discovered that the *Sk-2* and *Sk-3* drive elements are capable of suppressing the function of MSUD and thereby allowing for the expression of unpaired genes during meiosis (Raju *et al*. 2007). The results demonstrate that when either of the Spore killers is present in a cross, the ability of the meiotic silencing machinery to suppress the expression of unpaired genes is substantially reduced. It was concluded that the Spore killers contain a dominant suppressor that interferes with the

meiotic silencing pathway. At the time, however, we were unable to determine what is within the *Sk* meiotic drive elements that allows it to knockdown meiotic silencing.

Since one of our MSUD gene candidates, *sad-p*, is located within recombination block, I set out to determine whether it is the gene responsible for MSUD suppression in the Spore killer system.

V.2. Materials and Methods

V.2.i. Sequencing of *sad-p* alleles

DNA sequencing was conducted at the University of Missouri DNA core. Primers used for sequencing are listed in Table V.2.

V.2.ii. Vector construction for *sad-p^{WT}* and *sad-p^{Sk-2}*

Both *sad-p^{WT}* and *sad-p^{Sk-2}* were PCR-amplified from laboratory strains and cloned into pCRII-TOPO. *sad-p^{WT}* was amplified from P3-07 (WT) using primers Sadp-Sk-05 and Sadp-Sk-06 and *Sad-p^{Sk-2}* was amplified from F2-19 (*Sk-2*) using primers Sadp-Sk-04 and Sadp-Sk-05. The resulting TOPO vectors were named pDGR227.1 and pDGR221.2, respectively.

Two additional vectors were created to tag the alleles with markers. The first vector, pTH256, was used to add a hygromycin (*hph*) marker to *sad-p^{WT}* and *sad-p^{Sk-2}* while the second vector, pBM61, contains a *his-3* tag to help target each allele to the *his-3* locus on chromosome I.

V.2.iii. Deletion vector creation for *sad-p^{Sk-2}* and *sad-p^{WT}*

Primer	Sequence (5` → 3`)	Uses
Sadp-Sk-01	GCGGCCGCGGCATTTCTCGGGCATTTCT	*sad-p* amplification
Sadp-Sk-02	GCGGCCGCGCACCTCAACCGCCGTCCTT	*sad-p* amplification
Sadp-Sk-03	GCGGCCGCAACCTAACCCCCGTCCATTTCC	*sad-p* amplification
Sadp-Sk-04	CCAGTTGCGGCCGCGGCATTTCTCGGGCATTTCT	*sad-p* amplification
Sadp-Sk-05	CCAGTTGCGGCCGCGCACCTCAACCGCCGTCCTT	*sad-p* amplification
Sadp-Sk-06	CCAGTTGCGGCCGCAACCTAACCCCCGTCCATTTCC	*sad-p* amplification
Sadp-Sk-07	TCATTGATCCAACACAGATTGC	*sad-p* amplification
Sadp-Sk-08	CGGCAATGCAGCTACGGATGC	*sad-p* amplification
Sadp-Sk-09	ATAGAGGGGTTATCGACTCTA	*sad-p* amplification
Sadp-Sk-10	CCGCTTTGCACAAGCTTCGCA	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-11	TTGGCATTTCTCGGGCATTTCT	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-12	ATGCTCCTTCAATATCAGTTGCGGTCATTAGTATCACACC	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-13	ATGCCGACCGGGAACCAGTTCTACACCTAGACGGAGATGC	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-14	GAATTGACCAACCAGTCAGC	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-15	GGCATTTCTCGGGCATTTCTAGG	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-16	GCACCTCAACCGCCGTCCTT	*sad-p* $^{Sk-2}$ deletion
Sadp-Sk-17	TGTGTGGAGCCCATACAAACT	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-18	TGCTTTGGGGGTTTATAATTATGCTGCCGCTTGATGAAAAGC	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-19	GCTTTTCATCAAGCGGCAGCATAATTATAAACCCCCAAAGCA	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-20	AATGACTCCGTACAGCGCCGTGGTTCCCGGTCGGCAT	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-21	AGTAGATGCCGACCGGGAACCACGGCGCTGTACGGAGTCATT	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-22	CCGGATCGTTCGAGGTTTGT	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-23	CAAACTGAGAGCCAAGACCA	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-24	CCATGACCCCAACCCAGAAC	*sad-p* $^{Sk-2}$ insertion
Sadp-Sk-25	CGCACATCACCGCAACTTCG	*sad-p* ^{WT} insertion
Sadp-Sk-26	GTTGGATCAATGACGGAGATATTTACTCCTTTGCTTTGGGGGTTGA	*sad-p* ^{WT} insertion
Sadp-Sk-27	TCAACCCCCAAAGCAAAGGAGTAAATATCTCCGTCATTGATCCAAC	*sad-p* ^{WT} insertion
Sadp-Sk-28	CGGGGTCCAGAATGCCTCCGTGGTTCCCGGTCGGCAT	*sad-p* ^{WT} insertion
Sadp-Sk-29	ATGCCGACCGGGAACCACGGAGGCATTCTGGACCCCG	*sad-p* ^{WT} insertion
Sadp-Sk-30	TTGTTCGACGGTCCCTGCGG	*sad-p* ^{WT} insertion
Sadp-Sk-31	AGATGCTCTCAACCCCGGCG	*sad-p* ^{WT} insertion
Sadp-Sk-32	AGTCCCGGATCGTTCGAGGT	*sad-p* ^{WT} insertion
Sadp-Sk-33	ATGTTCCTGGTCGTGGTGGGGGGGAGAGAGGGCGGGGATA	*sad-p* ^{WT} insertion
Sadp-Sk-34	TATCCCCGCCCTCTCTCCCCCCCACCACGACCAGGAACAT	*sad-p* ^{WT} insertion
Sadp-Sk-35	CCGCTTTCCCTCTGCCTCCGTGGTTCCCGGTCGGCAT	*sad-p* ^{WT} insertion
Sadp-Sk-36	ATGCCGACCGGGAACCACGGAGGCAGAGGGAAAGCGG	*sad-p* ^{WT} insertion
hph-cen-f	AACTGATATTGAAGGAGCATTTTTTGG	*hph* from pCB1004
hph-cen-r	AACTGGTTCCCGGTCGGCAT	*hph* from pCB1004
pBM61F	GCGAATTGGAGCTCCACCGC	pBM61 sequencing
pBM61R	ATATCGAATTCCTGCAGCCCGG	pBM61 sequencing

Table V.1. List of primers used in this study

Strain	Genotype
F1-16	*rid; fl; Sk-2 a*
F2-19	*rid; fl; Sk-2 A*
F2-23	*rid; fl A*
F2-26	*rid; fl a*
F2-35	*his-3::his-3$^+$ act$^+$; fl A*
F2-36	*his-3::his-3$^+$ bml^R; fl A*
F2-37	*his-3::his-3$^+$ act$^+$; fl a*
F2-38	*his-3::his-3$^+$ bml^R; fl a*
F3-14	*rid; fl Sk-3 A*
F3-24	*rid his-3$^+$::asm-1$^+$ fl asm-1$^\Delta$::hph a*
F5-17	*rid; fl; Sk-2; sad-2$^\Delta$::hph A*
F5-18	*rid his-3; fl; Sk-2; sad-2$^\Delta$::hph a*
HXP1	*sad-p$^\Delta$ A*
P3-07	*A*
P6-07	*rid A*
P6-08	*rid a*
P8-38	*rsk$^{r(Sk-2)-LA2222}$ acr-7 a*
P8-42	*rid his-3 a; mus-51Δ::bar*
P8-43	*rid his-3 A; mus-52Δ::bar*
P12-41	*cum rsk$^{r(Sk-3)-PF5123}$ a*

Table V.2. List of strains used for analyzing *sad-p*

The *sad-p^{ΔWT}* strain was obtained from the *Neurospora* knockout collection (Colot *et al.* 2006) via the Fungal Genetics Stock Center (McCluskey 2010). The strain was crossed to P8-43 in order to put the knockout in a *mus52^{Δ}::bar rid* background. The resulting strain was named RDGR301.5.

Double-joint PCR primers for deleting *sad-p^{Sk-2}* were created as similar to the ones used by the *Neurospora* Genome Project as possible (Table IV.2). The center fragment, an *hph* marker, was amplified from the plasmid pCB1004 using primers hph-cen-f and hph-cen-r (~1.4 kb fragment). The 5' flank was amplified from *Sk-2* (F2-19) using the primers Sadp-Sk-11 and Sadp-Sk-12 (~500bp). The 3' flank was amplified from *Sk-2* (F2-19) using the primers Sadp-Sk-13 and Sadp-Sk-14 (~1kb).

All three products were combined and run in a fusion PCR reaction with no primers. One μL of fusion product was used in a final PCR reaction with the nested primers Sadp-Sk-15 and Sadp-Sk-16 (~3kb).

The final PCR amplification product was used to transform P15-43 (*rid; Sk-2*; *mus51^{Δ}::bar A*). Two of the resulting transformant strains, RDGR310.1 and RDGR313.1, were stocked.

V.2.iv. Double-joint PCR construction for placing *sad-p^{Sk-2}* in a sensitive strain at its native locus

sad-p^{Sk-2}::hph was PCR-amplified from the plasmid pDGR224.1 using the primers Sadp-Sk-19 and Sadp-Sk-20 (~8.4kb). The two flanking regions were amplified

from *Sk-2* (F2-19). The 5' flank was amplified using Sadp-Sk-17 and Sadp-Sk-18 (~440bp). The 3' flank was amplified using Sadp-Sk-21 and Sadp-Sk-22 (~540bp).

All three bands were combined and run in a PCR fusion reaction with no primers. One μL of the fusion reaction product was used in the final PCR amplification reaction with the nested primers Sadp-Sk-23 and Sadp-Sk-24 (~9.2kb).

The *sad-p^{Sk-2}::hph* final double-joint PCR fragment was used to transform P8-42 (*rid his-3; mus51Δ::bar a*). Two *sad-p$^{ΔWT::Sk-2-hph}$* transformants, RDGR375.2 and RDGR376.1, were recovered. The introduction of the transformation fragment was confirmed by PCR amplification of the center segment with primers Sadp-Sk-19 and Sadp-Sk-20.

As a control, the *sad-p^{Sk-2}::hph* final double-joint PCR fragment was also inserted into an *Sk-2* strain (P15-43). The resulting strain was named RDGR380.1. Similar to the strains above, the insert was confirmed using PCR with the same primers listed.

V.2.v. Double-joint PCR construction for placing *sad-p^{WT}* in a *Sk-2* strain at its native locus

sad-p^{WT}::hph was PCR-amplified from the plasmid pDGR228.2 using primers Sadp-Sk-27 and Sadp-Sk-28 (~8.3kb). The flanking regions were amplified from a wild-type strain (P3-07). The 5' flank was amplified using Sadp-Sk-25 and Sadp-Sk-26 (~900bp). The 3' flank was amplified using Sadp-Sk-29 and Sadp-Sk-30 (~640bp). One μL of the fusion reaction was used in a final PCR reaction with the nested primers Sadp-Sk-31 and Sadp-Sk-32 (~9.7kb).

V.2.vi. Placing *sad-p^{Sk-2}* at the *his-3* locus

pDGR224.1 was inserted into RDGR301.5 to place *sad-p^{Sk-2}* at *his-3* on

chromosome I. Transformants were screened by selection on minimal media.

V.3. Results

V.3.i. Sequence comparison between *sad-p^{WT}* and *sad-p^{Sk-2}*

The nucleotide sequence of *sad-p^{WT}* is ~6 kilobases in length and encodes a

1,870-aa polypeptide (www.broadinstitute.org). A BLAST search of this sequence

predicts that the gene contains a SNF2-family helicase domain.

Illumina sequencing of the *Sk-2* haplotype has revealed the sequence for *sad-p^{Sk-2}*

(Figure V.1) (our unpublished data). A ClustalW protein alignment of the two sequences

revealed that the two proteins are 96% identical (98% similar). In addition, the

sequencing data revealed that a highly repetitive region of DNA flanks the 3' end of *sad-*

p^{Sk-2} (the repetitive region is absent in wild-type strains). The repetitive region prevents

us from constructing a full genetic map of this region but sequencing data indicates that

the gene may be near a possible chromosomal inversion within the Spore killer

recombination block (our unpublished data).

V.3.ii. Deletion of *sad-p^{Sk-2}* does not eliminate *Sk-2*'s ability to suppress MSUD

Previously, we reported that *Sk-2* has the ability to suppress MSUD, thereby

allowing for the expression of unpaired genes during meiosis (Raju *et al.* 2007). To test

this ability, we developed strains that contained an extra copy of particular wild-type

genes. When one of these strains, which we designate as "unpaired testers", is crossed to

a wild-type strain, the MSUD machinery recognizes the unpaired gene (as well as any

homolog of that gene) and silences its expression during meiosis. If the expression of
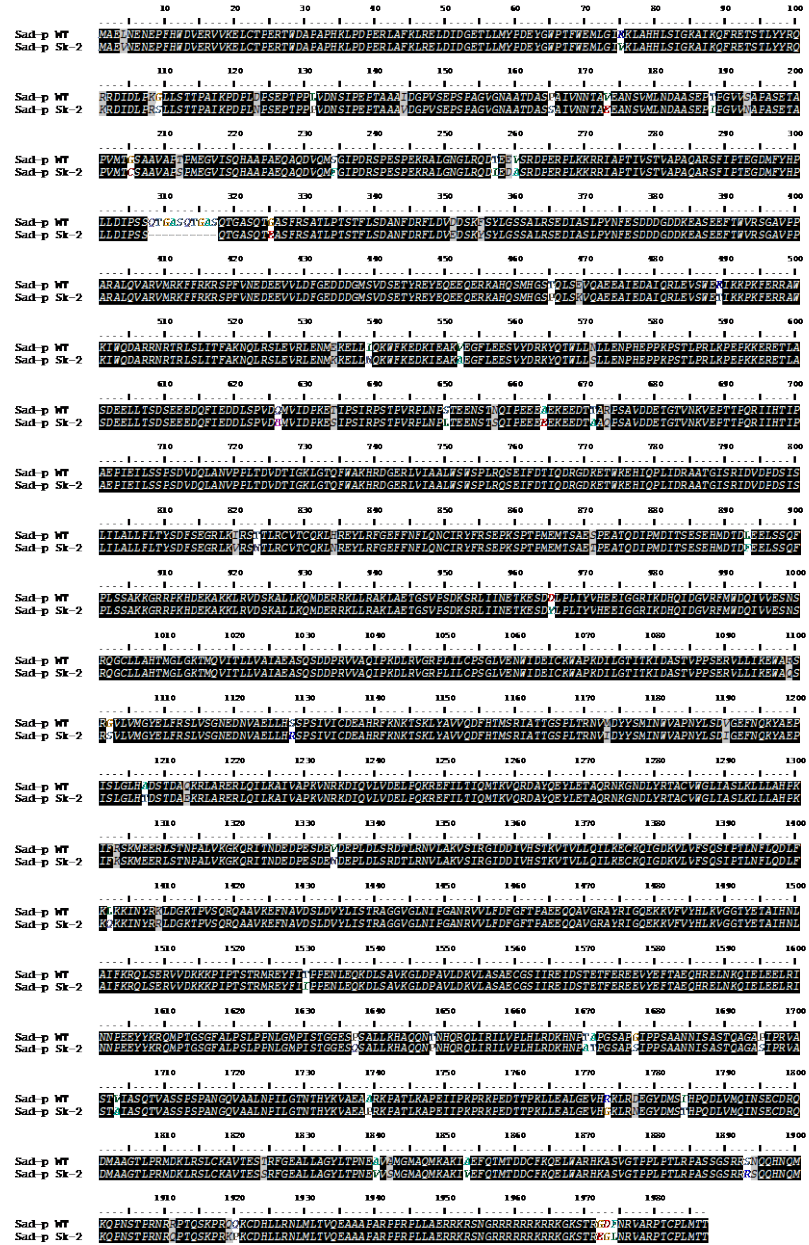

Figure V.1. ClustalW alignment of SAD-P^Sk-2 and SAD-P^WT
An amino acid alignment of SAD-P from *Sk-2* and WT reveals a 10-amino acid deletion

in *Sk-2* as well has a smattering of point mutations.

these unpaired genes is required for meiosis, then an observable phenotype is manifested. In this study, we utilized this approach to determine whether the deletion of *sad-p^{Sk-2}* has any effect on *Sk-2*'s ability to suppress MSUD.

We began by deleting *sad-p^{Sk-2}* from *Sk-2* (F2-19). The deletion strain was then crossed to the following unpaired testers: *::act* (F2-35 or F2-37) and *::bml^R* (F2-36 or F2-38). Qualitative analysis of these crosses indicate that *Sk-2* and *Sk-2 sad-p$^{Sk-2Δ}$::hph* are nearly identical in their ability to suppress MSUD (Figure V.2). This suggests that *sad-p^{Sk-2}* is not a dominant suppressor of the MSUD pathway.

V.3.iii. Replacement of *sad-p^{WT}* with *sad-p^{Sk-2}* in a wild-type strain

To further solidify that *sad-p^{Sk-2}* is not a dominant suppressor of the MSUD pathway, I inserted *sad-p^{Sk-2}* into a wild-type strain, replacing the wild-type allele.

As in the knockout experiment above, the replacement strain was crossed to the aforementioned unpaired testers. The result indicate that replacement of *sad-p^{WT}* with *sad-p^{Sk-2}* in a wild-type strain does not qualitatively affect its ability to silence unpaired genes (Figure V.3).

V.4. Discussion

For almost thirty years it was believed that the *Neurospora* Spore killers fit the classic model of a two-component meiotic drive system, consisting of a killer and

resistance element. However, in 2007, our group discovered a unique feature of *Sk-2* and

Sk-3, namely their ability to suppress MSUD (Raju *et al.* 2007). This discovery suggests

that *Sk* has a third, yet unknown, locus capable of disrupting the MSUD pathway. The
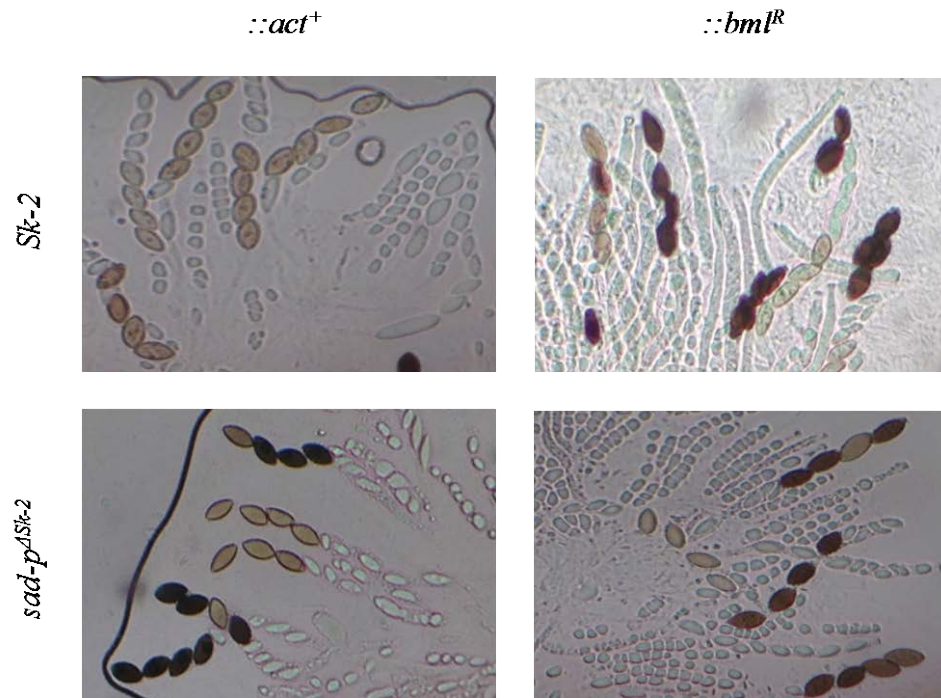
Figure V.2. Deletion of *sad-p^{Sk-2}* does not correspond to the loss of MSUD suppression in *Sk-2*

Deletion vectors were generated to knockout *sad-p* in *Sk-2*. The resulting deletion strains were then crossed to unpaired testers (*::act* and *::bml^R*) to determine whether suppression of MSUD still occurred. Dissection of the rosettes from these crosses indicate that *Sk-2* and *Sk-2 sad-p $^{\Delta Sk-2}$* are identical in their ability to suppress MSUD, suggesting that *sad-p^{Sk-2}* is not a dominant suppressor of MSUD.
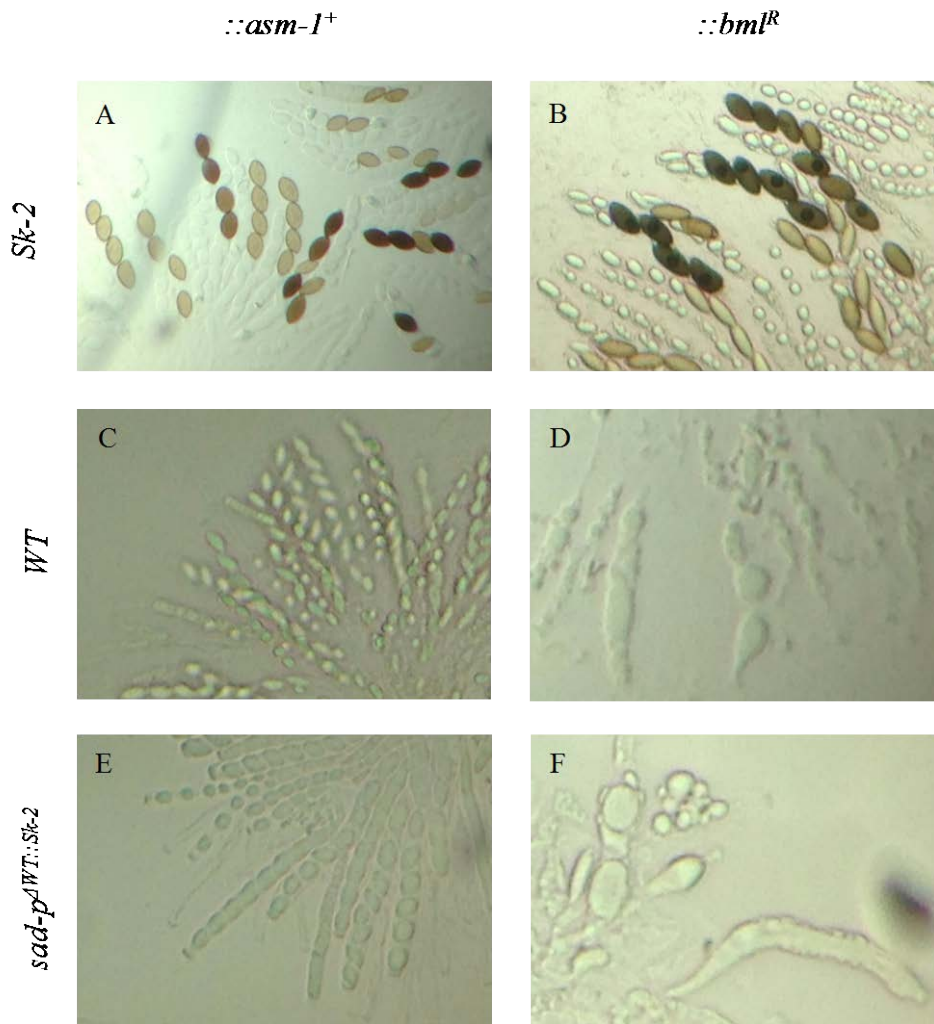
Figure V.3. *Sad-p^{Sk-2}* does not confer MSUD suppression on WT strains
sad-p^{Sk-2} was inserted into a WT strain in order to determine whether it could confer the ability to suppress MSUD in a WT strain. Two unpaired gene markers, *::asm-1⁺* and *::bml^R*, were utilized to test this. (A, B) Spore killer strains have the ability to dominantly suppress MSUD and thereby allow for the expression of unpaired genes during meiosis. However, in WT strains, the MSUD machinery is fully active and

silences the expression of the unpaired gene markers (C, D). Replacement of *sad-p^WT* with *sad-p^{Sk-2}* does not result in the dominant suppression of MSUD and therefor the unpaired gene markers are silenced throughout meiosis (E, F).

purpose of this study was to better understand what this third locus may be.

Two projects in our laboratory have allowed us to make a prediction about how Spore killer may evade meiotic silencing. The first project is a reverse genetics screen of knockout mutants defective in MSUD. The Neurospora Genome Project group has been working to knockout all 10,082 ORFs in the *Neurospora crassa* genome (Colot *et al.* 2006). Our laboratory has been testing these knockout strains for their involvement in the MSUD pathway by assessing their ability to silence unpaired genes during meiosis (Hammond *et al.* 2011).

When a gene required for MSUD is deleted, it results in the knockdown/inability of the MSUD system to silence unpaired genes during meiosis. Of the many MSUD candidate genes identified from the knockout screen thus far, only one of them (*sad-p*) is found within the *Sk* recombination block.

sad-p contains one recognizable motif, a Snf2-like helicase domain. Proteins that carry this domain are reportedly required for homologous recombination (Flaus, 2006). The role of *sad-p* in MSUD is still far from understood. Homozygous deletion of the gene leads to a reduction of MSUD efficiency but not the complete loss of MSUD function (our unpublished results).

The second project that could reveal how *Sk* may suppress meiotic silencing is the characterization of *rsk* (Hammond *et al.* 2012). In this project, we sequenced the *Sk-2* haplotype. Sequencing analysis revealed, among other things, a mutated sequence of *sad-*

p (our unpublished data). Not only is *sad-p^{Sk-2}* mutated, its 3' end also appears to be close to a highly repetitive region (a region not found in wild type). This suggests that *sad-p^{Sk-2}* may have reduced transcription efficiency, as its promoter is adjacent to a region of heterochromatic DNA.

To be able to utilize information from these two projects, we have to make some predictions on how *Sk* might exploit *sad-p* to suppress MSUD. There are many different ways *sad-p* might be used by the Spore killers to suppress MSUD: 1) *sad-p^{Sk-2}* could be unpaired, thereby creating a silencing-the-silencer situation which results in a reduction in MSUD function, 2) *sad-p^{Sk-2}* might be a dominant mutant that directly suppresses or disrupts the function of the MSUD pathway, or 3) the expression of *sad-p^{Sk-2}* could be down-regulated or suppressed, thereby allowing unpaired genes to be expressed to some extent.

Raju *et al*. tested the first prediction in 2007. If a deletion or an extra copy of a gene required for MSUD was present in the *Sk* haplotype, it should become unpaired in a cross to a wild-type strain, thereby leading to its own silencing ("silencing the silencer") and therefore a reduction in the efficiency of MSUD. In a homozygous cross between like Spore killers, however, the deletion of the MSUD gene should be completely paired and therefore a reduction in MSUD silencing efficiency should not be observed.

To test this hypothesis, a homozygous cross between two *Sk-2* strains was conducted in which one of the killer strains contains an unpaired marker gene. If *Sk*'s ability to suppress MSUD was granted through a "silencing the silencer" effect, it should no longer occur and therefore the MSUD machinery should silence the unpaired tester gene during meiosis. However, the results of the cross indicate that the expression of the

unpaired gene marker was not silenced during meiosis, which suggests that the suppression of MSUD in the Spore killers is not gained through an unpairing of genes required for MSUD.

A couple of possibilities remain as to how the *Sk-2* and *Sk-3* suppress MSUD (although other explanations are still possible), either they contain a dominant suppressor that directly disrupts the MSUD pathway, or the expression of a gene required for MSUD is reduced or completely turned off. In the experiments above, we attempted to determine if MSUD suppression is granted through the remaining two possible scenarios.

To test whether *sad-p^{Sk-2}* contains a dominant mutant disrupter (hypermorph) of the MSUD pathway, we replaced *sad-p^{WT}* with *sad-p^{Sk-2}* in a wild-type strain. If *sad-p^{Sk-2}* were a dominant mutant, we should observe a reduction in MSUD silencing efficiency on par with that of an *Sk-2* strain. Our data, however, indicate that there is no reduction in MSUD efficiency. In fact, wild-type strains carrying *sad-p^{Sk-2}* are qualitatively indistinguishable from wild-type strains in their MSUD efficiency. Although further experiments that directly quantify the efficiency of MSUD in these transformant strains are necessary, it is relatively safe to assume that *Sk*'s ability to suppress MSUD is not gained through a hypermorphic version of *sad-p*.

Although other complicated explanations could still justify the involvement of *sad-p* in *Sk*'s anti-MSUD ability, the data we have gathered so far are consistent with the notion that it is not the case. At the very least, the work presented here has allowed us to eliminate one (obvious) candidate from our search. Future *sad* candidates identified within the recombination block should allow us to paint a clearer picture on how *Sk* combats MSUD and how it may benefit from it.

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