Identification of a genetic element required for spore killing in Neurospora

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Running title

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ABSTRACT

Meiotic drive elements like *Spore killer-2* (*Sk-2*) in Neurospora are transmitted through sexual reproduction to the next generation in a biased manner. *Sk-2* achieves this biased transmission through spore killing. Here, we identify *rfk-1* as a gene required for the spore killing mechanism. The *rfk-1* gene is associated with a 1,481 bp DNA interval (called *AH36*) near the right border of the 30 cM *Sk-2* element, and its deletion eliminates the ability of *Sk-2* to kill spores. The *rfk-1* gene also appears to be sufficient for spore killing because its insertion into a non-*Sk-2* isolate disrupts sexual reproduction after the initiation of meiosis. Although the complete *rfk-1* transcript has yet to be defined, our data indicate that *rfk-1* encodes a protein of at least 39 amino acids and that *rfk-1* has evolved from a partial duplication of gene *ncu07086*. We also present evidence that *rfk-1*'s location near the right border of *Sk-2* is critical for the success of spore killing. Increasing the distance of *rfk-1* from the right border of *Sk-2* causes it to be inactivated by a genome defense process called meiotic silencing by unpaired DNA (MSUD), adding to accumulating evidence that MSUD exists, at least in part, to protect genomes from meiotic drive.

INTRODUCTION

In eukaryotic organisms, genetic loci are typically transmitted through sexual reproduction to the next generation in a Mendelian manner. However, some loci possess the ability to improve their own transmission rate through meiosis at the expense of a competing locus. These "selfish" loci are often referred to as meiotic drive elements (Zimmering *et al.* 1970). The genomic conflict caused by meiotic drive elements may impact processes ranging from gametogenesis to speciation (Lindholm *et al*. 2016). Meiotic drive elements are found across the eukaryote tree of life (Burt and Trivers 2008; Bravo Núñez *et al*. 2018) and classic examples include *SD* in fruit flies (Larracuente and Presgraves 2012), the *t*-complex in mice (Lyon 2003; Sugimoto 2014), and *Ab10* in *Zea mays* (Rhoades 1952; Kanizay *et al.* 2013). In the fungal kingdom, the known meiotic drive elements achieve biased transmission through spore killing (Raju 1994) and a handful of spore killer systems have been studied in detail. While the prion-based spore killing mechanism of *het-s* in *Podospora anserina* is the best characterized (Dalstra *et al.* 2003; Saupe 2011), the mechanisms by which other fungal meiotic drive elements kill spores are mostly unknown (*e.g.*, see Grognet et al. 2014; Hu et al. 2017; Nuckolls et al. 2017).

Two fungal meiotic drive elements have been identified in the fungus *Neurospora intermedia* (Turner and Perkins, 1979). This species is closely related to the genetic model *Neurospora crassa* (Davis 2000), and the mating processes in both fungi are essentially identical. Mating begins with fertilization of an immature fruiting body called a protoperithecium by a mating partner of the opposite mating type. After fertilization, the protoperithecium develops into a mature fruiting body called a perithecium. The nuclei from each parent multiply within the developing perithecium, and a single nucleus from each parent is sequestered into a tube-like

meiotic cell (Raju 1980). Meiosis begins with fusion of the parental nuclei and ends with production of four recombinant daughter nuclei. Each recombinant nucleus proceeds through a single round of mitosis, resulting in a total of eight nuclei in the meiotic cell. A process known as ascosporogenesis then constructs cell walls and membranes around each nucleus to produce sexual spores called ascospores. Maturing ascospores accumulate a dark pigment and develop the shape of a spindle; thus, at the end of ascosporogenesis, the mature meiotic cells appear to contain eight miniature black American footballs (Figure 1A). The meiotic cells also serve as ascospore sacs (asci) and a single perithecium can produce hundreds of asci, each derived from a unique meiotic event.

During an effort in the 1970s to collect and characterize *Neurospora* isolates from around the world, Turner and Perkins discovered pairs of compatible mating partners that did not produce asci with eight viable ascospores (Perkins 1974; Turner and Perkins 1979). This outcome was more common when crosses were performed between isolates from widely separated populations, and in some cases the abnormal asci were attributed to heterozygosity of chromosome rearrangements between mating partners. However, for a few isolates of *N. intermedia*, asci with atypical phenotypes were due to chromosomal factors called *Spore killer-2* (*Sk-2*) and *Spore killer-3* (*Sk-3*). *Sk-2* and *Sk-3* are not single genes; rather, they are complexes of genes that span approximately 30 cM of chromosome III, and they are transmitted through meiosis as single units due to a recombination suppression mechanism thought to be enforced by inversions (Turner and Perkins 1979; Campbell and Turner 1987; Hammond *et al.* 2012; Harvey *et al.* 2014). Unlike standard genetic elements, which display a Mendelian transmission rate of 50% through sexual reproduction, *Sk-2* and *Sk-3* are transmitted at levels approaching 100%

(Turner and Perkins 1979). This biased transmission occurs because *Sk-2* and *Sk-3* kill ascospores that do not inherit resistance to spore killing (Raju 1979; Turner and Perkins 1979). For example, in *Sk-2* × *Spore killer*-sensitive (Sk^S) crosses, asci with four black ascospores and four clear ("white") ascospores are produced (Figure 1A). This phenotype can be symbolized as 4B:4W. The four black ascospores are typically viable and nearly always of the *Sk-2* genotype, while the four white ascospores are inviable and presumed to be of the Sk^S genotype. The same phenomenon occurs in *Sk-3* × Sk^S crosses, except the four black ascospores are of the *Sk-3* genotype.

Although spore killers have not yet been detected in wild isolates of *N. crassa*, *Sk-2* and *Sk-3* have been introgressed into this species for genetic analysis. Introgression of *Sk-2* and *Sk-3* has allowed the discovery of resistance to spore killing in natural populations of *N. crassa* (Turner and Perkins 1979; Turner 2001). One of the *Sk-2*-resistant isolates (FGSC 2222) carries a resistant version of a gene whose function is best described by its name: *resistant to Spore killer* (*rsk*). Crosses of rsk^{LA} × *Sk-2*, where rsk^{LA} is the Louisiana allele of *rsk* carried by FGSC 2222, produce asci with an 8B:0W phenotype because ascospores inherit either rsk^{LA} or *Sk-2*, and both are sufficient for resistance to *Sk-2*-based spore killing (Hammond *et al.* 2012). Discovery of rsk^{LA} made identifying other *rsk* alleles possible, some of which do not provide resistance to the known spore killers. For example, the Oak Ridge *rsk* allele (rsk^{OR}), typical of most laboratory strains, is resistant to neither *Sk-2* nor *Sk-3*. Additionally, some *rsk* alleles confer resistance to *Sk-3* but not *Sk-2*. An example is rsk^{PF5123}, which exists in an *N. intermedia* isolate from French Polynesia. *Sk-2* and *Sk-3* also carry resistant versions of *rsk*, referred to as $rsk^{Sk\text{-}2}$ and $rsk^{Sk\text{-}3}$, respectively. Crosses homozygous for *Sk-2* (*i.e.*, *Sk-2* × *Sk-2*) or *Sk-3* produce asci with an

8B:0W phenotype because each ascospore inherits a resistant *rsk* allele. Furthermore, heterozygous crosses between different spore killers (*e.g.*, *Sk-2* × *Sk-3*) produce asci with a 0B:8W phenotype (Turner and Perkins 1979) because each ascospore inherits either $rsk^{Sk\text{-}2}$ or $rsk^{Sk\text{-}3}$ but not both (and $rsk^{Sk\text{-}2}$ ascospores are killed by *Sk-3* while $rsk^{Sk\text{-}3}$ ascospores are killed by *Sk-2*).

The Killer-Neutralization (KN) model has been proposed to explain how *Sk-2* and *Sk-3* achieve biased transmission through sexual reproduction (Hammond *et al.* 2012). The KN model holds that *Sk-2* and *Sk-3* each use a resistance protein and a killer protein (or nucleic acid) and both proteins are active throughout meiosis and ascosporogenesis. During the early stages of meiosis, in an Sk^{S} × *Sk-2* (or *Sk-3*) cross, both the resistance protein and the killer protein are hypothesized to diffuse throughout the meiotic cell. This unrestricted movement allows the resistance protein to neutralize the killer protein wherever the latter protein may be found. However, once ascospores are separated from the cytoplasm, the resistance protein becomes restricted to those ascospores that produce it (*e.g.*, *Sk-2* ascospores), and ascospores that do not carry a resistant version of *rsk* (*e.g.*, Sk^{S} ascospores) are subsequently killed. This model requires the killer protein to move between ascospores after ascospore delimitation or to have a long half-life that allows it to remain functional in sensitive ascospores.

Evidence for the KN model is seen in the outcome of Sk^{S} × *Sk-2* $rsk^{\Delta Sk\text{-}2}$ crosses, where the latter strain has been deleted of its *rsk* allele. These crosses do not produce ascospores; instead, they produce asci that abort meiosis before ascospore production (Hammond *et al.* 2012). Meiotic cells of these crosses lack a resistant RSK, which likely causes the killing process

to begin early in meiosis (at the ascus level) rather than during ascosporogenesis (at the ascospore level). The KN model is also supported by the existence of different *rsk* alleles. Previous studies have demonstrated the sequence of RSK to be the most important factor towards determining which killer it neutralizes (Hammond *et al.* 2012), suggesting that RSK and the killer may interact by a "lock and key" mechanism. To test this hypothesis, the killer must first be identified.

As described above, $Sk^{S} \times$ *Sk-2* $rsk^{\Delta Sk\text{-}2}$ crosses produce abortive asci. We recently used this characteristic to screen for mutations that disrupt spore killing (Harvey *et al.* 2014). Specifically, we fertilized an Sk^{S} mating partner with mutagenized *Sk-2* $rsk^{\Delta Sk\text{-}2}$ conidia (asexual spores that also function as fertilizing propagules). We reasoned that only an *Sk-2* $rsk^{\Delta Sk\text{-}2}$ conidium mutated in a gene "*r*equired *f*or spore *k*illing" (*rfk*) would produce viable ascospores when crossed with Sk^{S}. The screen allowed us to isolate six *rfk* mutants (ISU-3211 through ISU-3216). Complementation analysis of each mutant strain suggested all to be mutated at the same locus, which was subsequently named *rfk-1* and mapped to a 45 kb region within *Sk-2* on chromosome III. Here, we report the identification of *rfk-1* as a gene encoding a protein of at least 39 amino acids. In addition to identifying *rfk-1*, we have found that the cellular process of meiotic silencing by unpaired DNA places limits on the location of *rfk-1* within *Sk-2*. The implications of this finding with respect to meiotic drive element evolution are discussed.

MATERIALS AND METHODS

Strains, media, and crossing conditions

The strains used in this study are listed along with genotype information in Table 1. Vogel's minimum medium (Vogel 1956), with supplements as required, was used to grow and maintain all strains. Hygromycin B and nourseothricin sulfate (Gold Biotechnology) were used at a working concentration of 200 μg / ml and 45 μg / ml, respectively. Synthetic crossing medium (pH 6.5) with 1.5% sucrose, as described by Westergaard and Mitchell (1947), was used for crosses. Crosses were unidirectional and performed on a laboratory benchtop at room temperature under ambient lighting (Samarajeewa *et al.* 2014). After fertilization, crosses were allowed to mature for 12-16 days before perithecial dissection in 25 or 50% glycerol and asci were examined with a standard compound light microscope and imaging system. Ascus phenotype designations were based on qualitative observations. More than 90% of the asci from a cross had to display the same phenotype to receive one of the following designations: 8B:0W, 4B:4W, or aborted.

Genetic modification of *N. crassa*, genotyping, and sequence confirmations

A technique called double-joint PCR was used to construct all deletion vectors (Yu *et al.* 2004; Hammond *et al.* 2011). Transgene-insertion vectors were designed to insert transgenes along with a hygromycin resistance cassette (*hph*) next to *his-3* on chromosome I. Construction details for deletion and insertion vectors are provided in Supporting Information (Tables S1–S4). Transformations of *N. crassa* were performed by electroporation of conidia (Margolin *et al.* 1997). Homokaryons were derived from heterokaryotic transformants with a microconidium isolation technique (Ebbole and Sachs, 1990) or by crossing the transformants to standard

laboratory strains (F2-23 or F2-26) to obtain homokaryotic ascospores. Site-directed mutagenesis was performed essentially as described for the QuikChange II Site-Directed Mutagenesis Kit (Revision E.01, Agilent Technologies) and details for its use are provided in Table S5. All genotypes were confirmed by polymerase chain reaction (PCR) assays on genomic DNA isolated from lyophilized (freeze-dried) mycelia with IBI Scientific's Mini Genomic DNA Kit (Plant/Fungi). Sanger sequencing was used to confirm sequences and/or identify mutations in PCR products and plasmids.

Data availability

All strains and plasmids generated during this study are available upon request. Supplemental files available at FigShare.

196 **RESULTS**

197 **Deletion of a DNA interval spanning most of *Sk-2*^{INS1} eliminates spore killing**

198 The annotated 45 kb *rfk-1* region contains 14 protein-coding genes, two pseudogenes (denoted
199 with an asterisk), an inverted sequence (*Sk-2*^{INV1}), an inversion breakpoint, and an 11 kb
200 insertion sequence (*Sk-2*^{INS1}; GenBank: KJ908288.1; Figure 1B). To refine the location of *rfk-1*
201 within this 45 kb region, intervals *v3*, *v4*, and *v5* (Figure 1B and Table 2) were deleted and
202 replaced with *hph* and the resulting deletion strains were crossed with an *Sk*^{S} mating partner. We
203 found that while deletion of interval *v3* or *v4* had no effect on spore killing (asci are 4B:4W;
204 Figure 1, C and D), deletion of *v5* eliminated it (asci are 8B:0W; Figure 1E).

205

206 **A DNA interval between *ncu07838** and *ncu06238* is required for spore killing**

207 Interval *v5* spans most of *Sk-2*^{INS1} (Figure 2, A and B). To further refine the position of *rfk-1*
208 within *Sk-2*^{INS1}, we constructed nine additional deletion strains and crossed each one with an *Sk*^{S}
209 mating partner (Figure 2B and Table 2). Surprisingly, deletion of the annotated genes and
210 pseudogenes within *Sk-2*^{INS1} did not interfere with spore killing (Figure 3, A–D). In contrast,
211 deletion of the intergenic region between *ncu07838** and *ncu06238* eliminated spore killing
212 (Figure 3, E–I).

213

214 **An ascus aborting element exists between *ncu07838** and *ncu06238***

215 The above results suggest that *rfk-1* is found within the intergenic region between *ncu07838** and
216 *ncu06238* and that *rfk-1* is required for spore killing. But, is *rfk-1* also sufficient for spore
217 killing? To answer this question, we genetically-modified eight *Sk*^{S} strains to carry different
218 intervals of *Sk-2*^{INS1} (Figure 2C and Table 2) and found that each strain produced normal asci

when crossed with an *Sk*^{S} mating partner (Figure S1). The reason for this finding can be traced to a silencing process called meiotic silencing by unpaired DNA (MSUD; Hammond 2017; Aramayo and Selker 2013). In a standard cross, where only one mating partner carries an ectopic transgene (*e.g.*, an interval of *Sk-2*^{INS1}), MSUD identifies the transgene as unpaired and silences it for the duration of meiosis. Therefore, to detect a phenotype that requires the expression of an unpaired transgene during meiosis, it is often necessary to suppress MSUD. MSUD suppression can be achieved by deleting a gene called *sad-2* from one mating partner of a cross (Shiu *et al.* 2006). With this technique, we found that some *Sk-2*^{INS1} intervals have no effect on ascus development, while others abort it. For example, normal asci are produced by strains carrying intervals *AH4*$^{Sk-2}$, *AH6*$^{Sk-2}$, *AH14*$^{Sk-2}$, or *AH32*$^{Sk-2}$ (Figure 4, A–C and H), while aborted asci are produced by strains carrying intervals *AH30*$^{Sk-2}$, *AH31*$^{Sk-2}$, *AH36*$^{Sk-2}$, or *AH37*$^{Sk-2}$ (Figure 4, D–G). The ascus abortion phenotype can be explained by the presence of *rfk-1* without the presence of a resistant version of *rsk*. Taken together, these findings suggest that intervals *AH30*$^{Sk-2}$, *AH31*$^{Sk-2}$, *AH36*$^{Sk-2}$, and *AH37*$^{Sk-2}$ contain *rfk-1* and that *rfk-1* is sufficient for spore killing.

The *AH36* interval from an *rfk-1* strain does not cause ascus abortion

The shortest abortion-inducing interval identified by the above experiments is *AH36*, located between positions 27,899 and 29,381 of the 45 kb *rfk-1* region (Figure 2C and Table 2). Because the research path that led us to *AH36* began with mapping the position of *rfk-1* in strain ISU-3211 (Harvey *et al.* 2014), *AH36* in ISU-3211 (referred to as *AH36*^{3211}) should harbor at least one mutation that disrupts *rfk-1* function. To test this hypothesis, we transferred *AH36*^{3211} to an *Sk*^{S} genetic background and crossed the resulting strain with an *Sk*^{S} *sad-2*$^{\Delta}$ mating partner. As expected, we found that *Sk*^{S} *sad-2*$^{\Delta}$ × *Sk*^{S} *AH36*^{3211} crosses produce normal asci (Figure 5).

242

243 **The G28326A mutation disrupts the ascus-aborting ability of *AH36^{Sk-2}***

244 The different phenotypes associated with *AH36^{Sk-2}* and *AH36*^{3211} suggest that they differ at the
245 sequence level. Indeed, sequencing of these two alleles allowed us to identify seven guanine to
246 adenine transition mutations in *AH36*^{3211} (Figure 6A; G27904A, G27945A, G27972A, G28052A,
247 G28104A, G28300A, and G28326A). To determine if one (or more) of these mutations is
248 responsible for the inability of *AH36*^{3211} to cause ascus abortion, we examined six of the seven
249 mutations by site-directed mutagenesis. For each mutation, this involved mutating the base in a
250 clone of interval *AH36^{Sk-2}*, placing the mutated interval (*e.g.*, *AH36^{Sk-2}*$^{[G27945A]}$) in an *Sk^{S}* strain, and
251 crossing the transgenic strain to an *Sk^{S} sad-2$^{\Delta}$* mating partner. Through this procedure, we found
252 that only one of the six mutations examined (*i.e.*, G28326A) eliminates the ascus-aborting ability
253 of *AH36^{Sk-2}* (Figure 7).

254

255 We also identified a 46–48 bp tandem repeat (7.17 repeats) between positions 28,384 and
256 28,722 (Figure 6, A and B). The sequences of *AH36^{Sk-2}* and *AH36*^{3211} are identical between these
257 positions and thus the biological significance of the tandem repeats with respect to spore killing
258 is currently unknown.

259

260 **A putative start codon for RFK-1 is located within *AH36***

261 The G28326A mutation is 62 bp to the right of a putative start codon at position 28,264 (Figure
262 6). To test if this "ATG" could serve as the start codon for RFK-1, we constructed two deletion
263 vectors: v199 and v200 (Figure 8A). Vector v199 deletes the interval between 28,131 and 28,264
264 and replaces it with *hph* and the promoter of the *N. crassa ccg-1* gene, thereby inserting

hph-ccg-1(P) directly upstream of the ATG at position 28,264 (Figure 8B). As a control, we used vector v200 to place *hph-ccg-1*(P) directly upstream of position 28,354, located 90 bases to the right of the proposed *rfk-1* start codon. When inserted directly upstream of 28,264, *hph-ccg-1*(P) has no effect on spore killing (Figure 8, C and D). In contrast, when inserted 90 bases to the right of this position, *hph-ccg-1*(P) disrupts spore killing (Figure 8E). These findings demonstrate that the ATG at position 28,264 could serve as the *rfk-1* start codon. Furthermore, they suggest that placement of *hph-ccg-1*(P) directly upstream of position 28,354 interrupts the *rfk-1* coding region.

The arrangement of *rfk-1* within *Sk-2* protects it from MSUD

The right border of *Sk-2* is found at position 29,151 (Figure 9A, dotted line; Table 2; Harvey *et al.* 2014). To the right of this position, the sequences of *Sk-2* and Sk^S strains are very similar. For example, a simple ClustalW alignment (Thompson *et al.* 1994; Hall 1999) finds that *Sk-2* positions 29,152 through 35,728 are 94.4% identical to the corresponding positions within Sk^S (GenBank: CM002238.1, positions 2,011,073 to 2,017,662). In contrast, the sequences to the left of the *Sk-2* border are unrelated between *Sk-2* and Sk^S strains (Figure 9A). Interestingly, most of *AH36* is found to the left of the *Sk-2* border, and thus most of *AH36*, including *rfk-1*, is unpaired during meiosis in $Sk^S \times$ *Sk-2* crosses. If so, how does *rfk-1* avoid inactivation by MSUD? While the molecular details of how MSUD detects unpaired DNA are unknown, we considered the possibility that the distance of *rfk-1* from a "paired" sequence allows it to avoid MSUD (*e.g.*, see the *ncu06238* genes in *Sk-2* and Sk^S, Figure 9A). To test this hypothesis, we inserted *hph* immediately to the right of *AH36* in a standard *Sk-2* strain (Figure 9A). We refer to this particular allele as $v140^{\Delta}$*::hph*. The $v140^{\Delta}$*::hph* allele increases the distance of *rfk-1* from paired sequences

by a length of 1391 bp (the length of *hph* minus the 21 bp that were deleted by v140). As predicted, we found that spore killing is absent in Sk^S × *Sk-2* $v140^{\Delta}$*::hph* crosses (Figure 9B). To confirm that the lack of spore killing is a result of the increased distance of *rfk-1* from paired DNA during meiosis, we inserted *hph* at the corresponding location in an Sk^S strain (Figure 9A). We refer to this allele as $v150^{\Delta}$*::hph*. When an Sk^S $v150^{\Delta}$*::hph* strain is crossed with an *Sk-2* $v140^{\Delta}$*::hph* strain, spore killing is normal (Figure 9C). Thus, the proximity of *rfk-1* to paired DNA helps it avoid inactivation by MSUD. As a final test of this hypothesis, we crossed Sk^S $sad\text{-}2^{\Delta}$ and *Sk-2* $v140^{\Delta}$*::hph* mating partners and found that spore killing is also normal in this cross (Figure 9D), most likely because $sad\text{-}2^{\Delta}$ suppresses MSUD, which makes the distance of *rfk-1* from paired sequences irrelevant to the expression of *rfk-1* during meiosis.

The *rfk-1* gene does not include *ncu06238*

To confirm that *ncu06238*, the gene to the right of *rfk-1* (as depicted in Figure 10A), is not required for spore killing, we deleted *ncu06238* from both Sk^S and *Sk-2* and analyzed ascus phenotypes in crosses involving *ncu06238* deletion strains. However, we found that Sk^S $ncu06238^{\Delta}$ × Sk^S crosses produce asci with varying numbers of fully developed ascospores (Figure 10A). Therefore, we could not use ascus phenotype to determine if spore killing is functional in Sk^S $ncu06238^{\Delta}$ × *Sk-2* $ncu06238^{\Delta}$ crosses (Figure 10B). Instead, we calculated the percentage of progeny with an *Sk-2* genotype produced by a cross between Sk^S $ncu06238^{\Delta}$ and *Sk-2* $ncu06238^{\Delta}$ mating partners. We found that 46 of 47 progeny had the *Sk-2* genotype (data not shown). Therefore, because meiotic drive functions without *ncu06238*, the *rfk-1* coding region does not overlap or include positions occupied by *ncu06238.*

Replacement of *AH36*^{3211} with *AH36*$^{Sk\text{-}2}$ restores spore killing to an *rfk-1* mutant

The *rfk-1* mutant strain ISU-3211 carries seven mutations within its *AH36* interval (Figure 6). To confirm that at least one of these mutations (presumably G28326A) is responsible for ISU-3211's inability to kill ascospores, we replaced *AH36*^{3211} in a descendant of ISU-3211 (strain ISU-3222) with *AH36*$^{Sk\text{-}2}$*::hph* (Figure 11A and Table S4). Because the presence of an *hph* marker to the right of *AH36* disrupts spore killing in an MSUD-dependent manner (Figure 9B), we performed our test crosses with both a standard *Sk*^{S} mating partner and an *Sk*^{S} *v150*$^{\Delta}$*::hph* mating partner. As expected, we found that replacing *AH36*^{3211} with *AH36*$^{Sk\text{-}2}$ restores spore killing to a spore killing-deficient strain (Figure 11, B–G). These results demonstrate that the *AH36*^{3211} interval is responsible for the loss of spore killing in ISU-3211 and its *rfk-1* descendants.

The RFK-1 protein contains (at least) 39 amino acids

Assuming that the start codon for RFK-1 begins at position 28,264, and that the pre-mRNA for *rfk-1* includes no introns (see discussion), we can propose the following hypothesis: RFK-1 is a 39 amino acid protein encoded by DNA located between positions 28,263 and 28,384 (Table 2 and Figure 12A). We found support for this hypothesis by sequencing the *AH36* intervals in strains ISU-3211 through ISU-3216 (Figure 12A), which are the six *Sk-2 rfk-1* isolates obtained by our initial screen for spore killing-deficient mutants (Harvey *et al.* 2014). Specifically, we found that *AH36*^{3211} contains the previously discussed G28326A mutation, which changes the 21st codon from a tryptophan codon to a stop codon; *AH36*^{3212} contains an extra thymine within a run of six thymines between positions 28,281 and 28,288, which causes a frameshift mutation in the 9th *rfk-1* codon; and *AH36*^{3213} contains a G28348A mutation, which changes the 29th codon

from an alanine codon to a threonine codon. In addition, we found that the sequences of *AH36*^{3214}, *AH36*^{3215}, and *AH36*^{3216}, are all identical to the sequence of *AH36*^{3211}, suggesting that ISU-3211, ISU-3214, ISU-3215, and ISU-3216 were all "fathered" by the same mutagenized conidium. In all, we identified at least one potential codon-altering mutation between positions 28,263 and 28,384 in each of the six known *rfk-1* mutants. This strongly suggests that the interval between positions 28,263 and 28,384 contains at least part, if not all, of the RFK-1 coding sequence.

RFK-1 is related to NCU07086

To investigate the origin of *rfk-1*, we downloaded a list of predicted *N. crassa* proteins from the National Center for Biotechnology Information (NCBI)'s Genome Database (Accession No. GCA_000182925.2) and performed a BLASTP search (Camacho *et al.* 2009) on the list with the hypothetical 39 aa RFK-1 sequence as query (Figure 12A). We found that the most significant match (Expect = 2e-7) to RFK-1 is a hypothetical 362-aa protein called NCU07086 (NCBI Protein Database: XP_960351.1). NCU07086 is encoded by the *ncu07086* gene on *N. crassa* chromosome VI and is predicted to contain four introns (Figure 12B, I1 through I4; NCBI Gene Database, 3876500). A search of NCBI's conserved domain database (CDD v3.16; Marchler-Bauer *et al.* 2015) with the predicted sequence of NCU07086 identified a region with a low-scoring match to the AtpF Superfamily (Expect=2.32e-3; Figure 12B). Interestingly, RFK-1 is highly similar to the first 39 amino acids of NCU07086 (Figure 12C), and it appears that the 46–48 bp repeat within *AH36* (Figure 6) expanded from a single 47 bp sequence within *ncu07086*'s first intron (Figure 12D). These findings suggest that *rfk-1* evolved from a partial duplication of the *ncu07086* gene.

DISCUSSION

The biological mechanism used by the Neurospora Spore killers to achieve biased transmission is believed to require the action of a resistance protein and a killer protein. In a previous work, we isolated six *rfk* mutants (ISU-3211 through ISU-3216) and provided evidence that each is mutated at the same locus, subsequently named *rfk-1* (Harvey *et al.* 2014). The *rfk-1* locus in ISU-3211 was mapped to a 45 kb region of *Sk-2*. We began this study with the goal of identifying *rfk-1*. At first, we intended to use three point crossing assays to further refine the position of *rfk-1* within the 45 kb *rfk-1* region. These assays were to be performed with *hph* markers inserted between genes *ncu06192* and *ncu06191* (with vector *v3*) and between genes *ncu06239* and *ncu06240* (with vector *v4*); therefore, deletion vectors *v3* and *v4* were designed to delete relatively small intervals from the *rfk-1* region (25 bp and 261 bp, respectively; Table 2) and they were not expected to influence spore killing. Accordingly, they had no effect on spore killing (Figure 1, C and D). In contrast, *v5* was designed to delete a 10,718 bp interval, spanning most of the *Sk-2*^{INS1} sequence, in hopes that *rfk-1* would be found somewhere within it (Table 2). Fortunately, deletion of interval *v5* (intervals are named after the deletion vectors designed to delete them) was successful and its removal from *Sk-2* eliminated *Sk-2*'s ability to kill ascospores (Figure 1E). We were thus able to focus our efforts on deleting subintervals of *v5*, which allowed us to track *rfk-1* to the intergenic region between *ncu07238** and *ncu06238* (Figures 2, 3, and 10).

We also tested various subintervals of *v5* for the presence of *rfk-1* by transferring them to an *Sk*^{S} strain and performing test crosses with an *Sk*^{S} *sad-2*$^{\Delta}$ mating partner (Figure 4). For this assay to yield positive results, *rfk-1* must be sufficient for spore killing. Indeed, we found this to

be the case when we identified four intervals (*AH30*, *AH31*, *AH37*, and *AH36*) that trigger ascus abortion. These four intervals all have the 1481 bp of *AH36* in common, and the ascus abortion phenotype associated with each interval is likely due to the presence of *rfk-1* without a compatible resistance gene. For example, the KN model holds that the resistance protein (RSK) and the killer are both active during early stages of meiosis (Hammond *et al.* 2012). Lack of a resistant version of RSK, along with expression of the killer, may cause asci to abort meiosis before ascospore delimitation. This phenomenon explains the abortion phenotypes of *AH30*, *AH31*, and *AH37*. However, for succinctness, we also referred to the phenotype associated with *AH36* as ascus abortion, although it may be more accurate to refer to it as a "bubble" phenotype. The bubble phenotype was originally described by Raju *et al*. (1987), and it is thought to arise when asci and/or ascospores abort shortly after ascospore delimitation. Therefore, one explanation for the existence of the two phenotypic classes is that ascus development progresses a bit further with *AH36* than it does with *AH30*, *AH31*, and *AH37*. Asci could progress further with *AH36* if *rfk-1* expression is lower from *AH36* than it is from *AH30*, *AH31*, and *AH37*. In line with this reasoning, *AH36* is the shortest of the abortion-inducing intervals, and, as a result, it may lack some of the regulatory sequences needed for full expression of *rfk-1*. It should be possible to address this hypothesis once the complete transcriptional unit of *rfk-1* is identified.

Although we have yet to identify *rfk-1*'s transcriptional start (+1) site and termination site, or confirm the presence/absence of introns, we have provided strong evidence that the *rfk-1* coding region includes the DNA interval between positions 28,263 and 28,384 (Figure 6). For example, a putative nonsense mutation at position 28,326 disrupts the ascus-aborting ability of interval *AH36* (Figure 7); spore killing functions when a non-native promoter is attached to the

403 putative RFK-1 start codon at position 28,264 (Figure 8); all six of the known *rfk-1* mutants
404 carry putative codon-altering mutations between positions 28,263 and 28,384 (Figure 12A), and
405 insertion of a non-native promoter in the middle of this region disrupts spore killing (Figure 8).
406 However, while our data indicate that the positions between 28,263 and 28,384 are part of the
407 *rfk-1* coding region, they do not eliminate the possibility that the coding sequences for RFK-1
408 include additional positions upstream and/or downstream of 28,263 and 28,384, respectively.
409 Indeed, our preliminary analysis of RNAseq data from Sk^S × *Sk-2* crosses (unpublished data)
410 strongly suggests that an intron may exist between positions 28,379 and 28,775. The 5' splice site
411 of this hypothetical intron is related to the 5' splice site of the first intron of *ncu07086* (Figure
412 12D). If this intron does exist within the *rfk-1* pre-mRNA, the RFK-1 stop codon would shift
413 downstream and the length of RFK-1 would increase to 101 aa (assuming position 28,264 is the
414 start codon and no other introns influence the stop codon position). Future work will seek to fully
415 characterize the *rfk-1* coding region by identifying the transcriptional start site, termination site,
416 and any introns that may exist for the primary *rfk-1* transcript, as well as for any biologically
417 significant variants, if they were to exist.
418
419 While this work represents a significant step towards understanding the mechanism of
420 *Sk-2*-based spore killing, many questions remain unanswered. For example, although it appears
421 that RFK-1 evolved from NCU07086, does RFK-1 interfere with NCU07086 function as part of
422 the spore killing mechanism? NCU07086 contains a region with slight homology to the AtpF
423 Superfamily (Figure 12D). Interestingly, the *atpF* gene in *E. coli* (also known as *uncF*; NCBI
424 Gene ID 948247) encodes subunit b of the F-type ATP synthase complex (Walker *et al.* 1984;
425 Dunn 1992; McLachlin and Dunn 1997; Revington *et al.* 1999). This hints that RFK-1 could

mediate spore killing by targeting eukaryotic F-type ATP synthases, which are associated with mitochondrial membranes in eukaryotes (Stewart *et al.* 2014). However, NCU07086 in *N. crassa* has not been investigated and a much more likely candidate for the b subunit of *N. crassa*'s F-type ATP synthase is found in NCU00502 (KEGG oxidative phosphorylation pathway: ncr00190, release 87.0, Kanehisa and Goto 2000; Kanehisa *et al.* 2016). Thus, at this point in time, a role for RFK-1 in disrupting mitochondrial function as part of the spore killing process is purely speculative.

Although the primary goal of this work was to identify *rfk-1*, the identity of which has been of interest to meiotic drive researchers since the discovery of *Sk-2* nearly four decades ago, we unexpectedly discovered the strongest evidence to date that genomes in some, if not all, lineages of eukaryotic organisms possess elaborate defense processes to protect themselves from meiotic drive. With respect to Neurospora genomes, this defense process appears to be MSUD. The first hint that MSUD defends Neurospora genomes from meiotic drive appeared in 2007, when it was discovered that *Sk-2* and *Sk-3* are weak MSUD suppressors (Raju *et al.* 2007). Next, in 2012, it was found that the position of *rsk* within *Sk-2* allows it to pair with *rsk* in the Sk^S genome during *Sk-2* × Sk^S crosses. If *rsk* is not paired during these crosses (*e.g.*, if it is deleted from the Sk^S mating partner), it is silenced by MSUD and the entire ascus is killed by the killer protein, which we now know to be RFK-1. In the current work, we found that the position of *rfk-1* within *Sk-2* is also critical for the success of meiotic drive because it allows *rfk-1* to escape inactivation by MSUD. However, unlike *rsk*, *rfk-1* is only found in *Sk-2* strains and it cannot be paired in *Sk-2* × Sk^S crosses. Evolution appears to have found a way to circumvent this problem by positioning *rfk-1* close to sequences that are paired during meiosis (*i.e.* close to *ncu06238* in

449 Figure 10). Our data indicate that the proximity of *rfk-1* to paired sequences allows it to escape
450 inactivation by MSUD, which is critical for the success of spore killing. Overall, our findings
451 add to accumulating evidence that MSUD antagonizes the evolution of meiotic drive elements by
452 placing significant constraints on the arrangement of critical genes within the elements.
453 Furthermore, our findings suggest that eukaryotic genomes like those of Neurospora fungi have
454 evolved elaborate defense mechanisms to protect themselves from meiotic drive.
455
456

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LITERATURE CITED

Aramayo, R., and E. U. Selker, 2013 *Neurospora crassa*, a model system for epigenetics research. Cold Spring Harb. Perspect. Biol. 5: a017921.

Bravo Núñez, M. A., N. L. Nuckolls, and S. E. Zanders, 2018 Genetic villains: killer meiotic drivers. Trends Genet. 34: 424–433.

Burt, A., and R. Trivers, 2008 *Genes in Conflict: The Biology of Selfish Genetic Elements*. Belknap Press of Harvard University Press.

Campbell, J. L., and B. C. Turner, 1987 Recombination block in the Spore killer region of Neurospora. Genome Natl. Res. Counc. Can. 29: 129–135.

Camacho, C., G. Coulouris, V. Avagyan, N. Ma, J. Papadopoulos *et al*., 2009 BLAST+: architecture and applications. BMC Bioinformatics 10: 421.

Dalstra, H. J. P., K. Swart, A. J. M. Debets, S. J. Saupe, and R. F. Hoekstra, 2003 Sexual transmission of the [Het-S] prion leads to meiotic drive in *Podospora anserina*. Proc. Natl. Acad. Sci. U. S. A. 100: 6616–6621.

Davis, R. H., 2000 *Neurospora: Contributions of a Model Organism*. Oxford University Press, USA.

Dunn S. D., 1992 The polar domain of the b subunit of *Escherichia coli* F_1F_0-ATPase forms an elongated dimer that interacts with the F_1 sector. J. Biol. Chem. 267: 7630–7636.

Ebbole, D., and M. Sachs, 1990 A rapid and simple method for isolation of *Neurospora crassa* homokaryons using microconidia. Fungal Genet. Newsl. 37: 17–18.

Grognet, P., H. Lalucque, F. Malagnac, and P. Silar, 2014 Genes that bias mendelian segregation. PLoS Genet 10: e1004387.

494 Hall, T. A., 1999 BioEdit: a user-friendly biological sequence alignment editor and analysis
495 program for Windows 95/98/NT. Nucleic Acid Symp Ser 41: 95–98.
496 Hammond, T. M., 2017 Sixteen years of meiotic silencing by unpaired DNA. Adv. Genet. 97:1–
497 42.
498 Hammond, T. M., D. G. Rehard, H. Xiao, and P. K. T. Shiu, 2012 Molecular dissection of
499 Neurospora Spore killer meiotic drive elements. Proc. Natl. Acad. Sci. U. S. A. 109:
500 12093–12098.
501 Hammond, T. M., H. Xiao, D. G. Rehard, E. C. Boone, T. D. Perdue *et al.*, 2011 Fluorescent
502 and bimolecular-fluorescent protein tagging of genes at their native loci in *Neurospora*
503 *crassa* using specialized double-joint PCR plasmids. Fungal Genet. Biol. 48: 866–873.
504 Harvey, A. M., D. G. Rehard, K. M. Groskreutz, D. R. Kuntz, K. J. Sharp *et al.*, 2014 A critical
505 component of meiotic drive in Neurospora is located near a chromosome rearrangement.
506 Genetics 197: 1165–1174.
507 Hu, W., Z. D. Jiang, F. Suo, J. X. Zheng, W. Z. He, and L. L. Du, 2017 A large gene family in
508 fission yeast encodes spore killers that subvert Mendel's law. ELife Sciences 6, e26057.

509 Kanizay, L. B., T. Pyhäjärvi, E. G. Lowry, M. B. Hufford, D. G. Peterson et al., 2013 Diversity
510 and abundance of the abnormal chromosome 10 meiotic drive complex in *Zea mays*.
511 Heredity 110: 570–577.
512 Kanehisa M., Goto S., 2000 KEGG: kyoto encyclopedia of genes and genomes. Nucleic Acids
513 Res. 28: 27–30.
514 Kanehisa M., Sato Y., Kawashima M., Furumichi M., Tanabe M., 2016 KEGG as a reference
515 resource for gene and protein annotation. Nucleic Acids Res. 44: D457–462.

516 Larracuente, A. M., and D. C. Presgraves, 2012 The selfish *Segregation Distorter* gene
517 complex of *Drosophila melanogaster*. Genetics 192: 33–53.
518 Lindholm, A. K., K. A. Dyer, R. C. Firman, L. Fishman, W. Forstmeier *et al*., 2016 The ecology
519 and evolutionary dynamics of meiotic drive. Trends Ecol. Evol. 31: 315–326.
520 Lyon, M. F., 2003 Transmission ratio distortion in mice. Annu. Rev. Genet. 37: 393–408.
521 Marchler-Bauer, A., M. K. Derbyshire, N. R. Gonzales, S. Lu, F. Chitsaz *et al.*, 2015 CDD:
522 NCBI's conserved domain database. Nucleic Acids Res. 43: D222–226.
523 Margolin, B. S., M. Freitag, and E. U. Selker, 1997 Improved plasmids for gene targeting at the
524 *his-3* locus of *Neurospora crassa* by electroporation. Fungal Genet. Newsl. 44: 34–36.
525 McCluskey, K., A. Wiest, and M. Plamann, 2010 The Fungal Genetics Stock Center: a
526 repository for 50 years of fungal genetics research. J. Biosci. 35: 119–126.
527 McLachlin D. T., Dunn S. D., 1997 Dimerization interactions of the b subunit of the *Escherichia*
528 *coli* F_1F_0-ATPase. J. Biol. Chem. 272: 21233–21239.
529 Nuckolls, N.L., M. A. B. Núñez, M. T. Eickbush, J. M. Young, J. J. Lange *et al.*, 2017. *wtf* genes
530 are prolific dual poison-antidote meiotic drivers. ELife Sciences 6: e26033.
531 Perkins, D. D., 1974 The manifestation of chromosome rearrangements in unordered asci of
532 Neurospora. Genetics 77: 459–489.
533 Raju, N. B., 1979 Cytogenetic behavior of Spore killer genes in Neurospora. Genetics 93: 607–
534 623.
535 Raju, N. B., 1980 Meiosis and ascospore genesis in Neurospora. Eur. J. Cell Biol. 23: 208–223.
536 Raju, N. B., D. D. Perkins, and D. Newmeyer, 1987 Genetically determined nonselective
537 abortion of asci in *Neurospora crassa*. Can. J. Bot. 65:1539–1549.

538 Raju, N. B., 1994 Ascomycete Spore killers: Chromosomal elements that distort genetic ratios
539 among the products of meiosis. Mycologia 86: 461–473.
540 Raju, N. B., R. L. Metzenberg, and P. K. T. Shiu, 2007 Neurospora spore killers *Sk-2* and *Sk-3*
541 suppress meiotic silencing by unpaired DNA. Genetics 176: 43–52.
542 Revington M., McLachlin D. T., Shaw G. S., Dunn S. D., 1999 The dimerization domain of the b
543 subunit of the *Escherichia coli* F(1)F(0)-ATPase. J. Biol. Chem. 274: 31094–31101.
544 Rhoades, M., 1952 Preferential Segregation in Maize, pp. 66–80 in Heterosis: a record of
545 researches directed towards explaining and utilizing the vigor of hybrids, edited by J. W.
546 Gowen. Iowa State College Press, Ames Iowa USA.
547 Samarajeewa, D. A., P. A. Sauls, K. J. Sharp, Z. J. Smith, H. Xiao *et al.*, 2014 Efficient
548 detection of unpaired DNA requires a member of the Rad54-like family of homologous
549 recombination proteins. Genetics 198: 895–904.
550 Saupe, S. J., 2011 The [Het-s] prion of *Podospora anserina* and its role in heterokaryon
551 incompatibility. Semin. Cell Dev. Biol. 22: 460–468.
552 Shiu, P. K. T., D. Zickler, N. B. Raju, G. Ruprich-Robert, and R. L. Metzenberg, 2006 SAD-2
553 is required for meiotic silencing by unpaired DNA and perinuclear localization of SAD-1
554 RNA-directed RNA polymerase. Proc. Natl. Acad. Sci. U. S. A. 103: 2243–2248.
555 Stewart A. G., Laming E. M., Sobti M., Stock D., 2014 Rotary ATPases--dynamic molecular
556 machines. Curr. Opin. Struct. Biol. 25: 40–48.
557 Sugimoto, M., 2014 Developmental genetics of the mouse *t*-complex. Genes Genet. Syst. 89:
558 109–120.
559 Thompson, J. D., D. G. Higgins, and T. J. Gibson, 1994 CLUSTAL W: improving the
560 sensitivity of progressive multiple sequence alignment through sequence weighting,

position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22: 4673–4680.

Turner, B. C., 2001 Geographic distribution of Neurospora Spore killer strains and strains resistant to killing. Fungal Genet. Biol. 32: 93–104.

Turner, B. C., and D. D. Perkins, 1979 Spore killer, a chromosomal factor in Neurospora that kills meiotic products not containing it. Genetics 93: 587–606.

Vogel, H. J., 1956 A convenient growth medium for Neurospora (Medium N). Microb. Genet. Bull. 13: 42–43.

Walker J. E., M. Saraste, and N. J. Gay, 1984 The unc operon. Nucleotide sequence, regulation and structure of ATP-synthase. Biochim. Biophys. Acta 768: 164–200.

Westergaard, M., and H. K. Mitchell, 1947 Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34: 573–577.

Yu, J. H., Z. Hamari, K. H. Han, J. A. Seo, Y. Reyes-Domínguez *et al.*, 2004 Double-joint PCR: a PCR-based molecular tool for gene manipulations in filamentous fungi. Fungal Genet. Biol. 41: 973–981.

Zimmering, S., L. Sandler, and B. Nicoletti, 1970 Mechanisms of meiotic drive. Annu. Rev. Genet. 4: 409–436.

Figure Legends

Figure 1 *Sk-2*^{INS1} harbors a genetic element required for spore killing. (A) The diagram illustrates phenotypic differences between spore killing and normal ascus development. Asci that have undergone spore killing contain four black and four white ascospores. Viable ascospores may appear tan, brown, or black, depending on their level of maturity. (B) Annotation of the *rfk-1* region as described by Harvey *et al.* (2014). Genes and pseudogenes are depicted as black rectangles. Gene names (*e.g.*, *6197*) are listed above the rectangles. Pseudogene names are appended with an asterisk. Labels *v3*, *v4*, and *v5* mark DNA intervals of the *rfk-1* region that were deleted and replaced with an *hph* selectable marker. (C–E) The images depict asci from crosses between *Sk*^{S} and *Sk-2* strains lacking different intervals of *Sk-2*^{INS1}. The predominant phenotype of the asci produced by each cross is listed above each image. Crosses are as follows: (C) F2-23 × ISU-3023, (D) F2-23 × ISU-3017, and (E) F2-23 × ISU-3029.

Figure 2 Deletion and insertion maps. (A) A diagram of *Sk-2*^{INS1}. (B) Ten intervals of *Sk-2*^{INS1} were deleted and replaced with *hph*. For convenience, each interval is named according to its deletion vector (*e.g.*, interval *v5* is named after deletion vector v5). Orange rectangles mark intervals that disrupt spore killing upon deletion. Green rectangles mark intervals that do not disrupt spore killing when deleted. (C) Eight intervals of *Sk-2*$^{INS-1}$were transferred to the *his-3* locus of an *Sk*^{S} strain. Intervals were named according to the name of the plasmid used for cloning of the interval (*e.g.*, interval *AH4* is named after plasmid pAH4). Red and blue open rectangles mark killer (*i.e.*, abortion-inducing) and non-killer intervals, respectively.

Figure 3 Deletion of a genetic element between pseudogene *7838** and the right border of *Sk-2*^{INS1} eliminates spore killing. (A–I) The images depict asci from crosses between an *Sk*^{S} strain and an *Sk-2* mating partner lacking a specific interval of *Sk-2*^{INS1}. Crosses are as follows: (A) F2-26 × ISU-3311, (B) F2-26 × ISU-3313, (C) F2-23 × ISU-3315, (D) F2-26 × ISU-3318, (E) F2-23 × ISU-3321, (F) F2-26 × ISU-3478, (G) F2-26 × ISU-3482, (H) F2-26 × ISU-3483, and (I) F2-26 × ISU-3485.

Figure 4 A genetic element within *Sk-2*^{INS1} causes ascus abortion upon its transfer to an *Sk*^{S} strain. (A–H) Images depict asci from crosses between an *Sk*^{S} *sad-2*$^{\Delta}$ strain and an *Sk*^{S} mating partner carrying a specific interval of *Sk-2*^{INS1}. Crosses are as follows: (A) ISU-3037 × ISU-3224, (B) ISU-3037 × ISU-3228, (C) ISU-3036 × ISU-3243, (D) ISU-3037 × ISU-3656, (E) ISU-3037 × ISU-3658, (F) ISU-3036 × ISU-4269, (G) ISU-3037 × ISU-4271, and (H) ISU-3037 × ISU-3660.

Figure 5 The *AH36* interval from an *rfk-1* mutant does not cause ascus abortion. Images depict asci from crosses between an *Sk*^{S} *sad-2*$^{\Delta}$ strain and an *Sk*^{S} strain carrying either the *AH36* interval from (A) F2-19 (*rfk-1*$^{+}$) or (B) ISU-3211 (an *rfk-1* mutant). Crosses are as follows: (A) ISU-3037 × ISU-4273 and (B) ISU-3037 × ISU-4275.

Figure 6 The *AH36* interval from an *rfk-1* mutant contains seven point mutations. (A) The 1481 bp sequence of *AH36*$^{Sk-2}$ is shown. A region containing 7.17 repeats of a 46–48 bp sequence is highlighted with red and blue fonts. The colors alternate with each iteration of the repeated sequence. The *AH36*^{3211} sequence contains seven G to A transition mutations. The position of

each mutation is marked by a white character on a black background with the non-mutated character shown. The mutations in *AH36*^{3211} are (from left to right): G27904A, G27945A, G27972A, G28052A, G28104A, G28300A, and G28326A. A non-native promoter was placed directly upstream of a putative RFK-1 start codon (white font, green background) with vector v199. A non-native promoter was also placed directly upstream of a TTT triplet (white font, blue background) with vector v200. (B) Alignment of the repetitive sequences highlighted in panel A.

Figure 7 A point mutation within *AH36* eliminates its ability to abort ascus development. Six of the seven point mutations in *AH36*^{3211} were examined for a potential role in ascus abortion. Each mutation was placed individually in *AH36*$^{Sk\text{-}2}$ by site-directed mutagenesis. (A–G) Images depict asci from crosses between an *Sk*^{S} *sad-2*$^{\Delta}$ strain and an *Sk*^{S} mating partner carrying *AH36*$^{Sk\text{-}2}$ or one of its mutated derivatives. Crosses are as follows: (A) ISU-3037 × ISU-4273, (B) ISU-3037 × ISU-4551, (C) ISU-3037 × ISU-4552, (D) ISU-3037 × ISU-4553, (E) ISU-3037 × ISU-4554, (F) ISU-3037 × ISU-4555, (G) ISU-3037 × ISU-4556.

Figure 8 A putative start codon for RFK-1 exists within *AH36*. (A) A DNA construct consisting of *hph* and the promoter for the *N. crassa ccg-1* gene was used to make two transformation vectors. (B) Vector 199 (v199) was designed to replace 133 bp of *AH36* while fusing *ccg-1*(P) to a putative ATG codon at position 28,264 (Figure 6), thereby creating the *v199*$^{\Delta}$*::hph-ccg-1*(P) allele. Similarly, vector 200 (v200) was designed to replace 223 bp of *AH36* while fusing *ccg-1*(P) to position 28,354 (Figure 6), thereby creating the *v200*$^{\Delta}$*::hph-ccg-1*(P) allele. (C–E) Crosses were performed to determine the effect of each allele on spore killing. Images depict asci

646 from the following crosses (C) F2-26 × P15-53, (D) F2-26 × ISU-4557, and (E) F2-26 ×
647 ISU-4558.
648
649 **Figure 9** The native arrangement of *rfk-1* protects it from MSUD. (A) Interval *AH36* spans the
650 right border of *Sk-2* (marked by a black dotted line). An *hph* selectable marker was placed
651 immediately to the right of *AH36* in an *Sk-2* strain (with vector v140) to create the $v140^{\Delta}$*::hph*
652 allele and at the corresponding location in an Sk^{S} strain (with vector v150) to create the
653 $v150^{\Delta}$*::hph* allele. (B–D) Crosses were performed to determine the effect of each allele on spore
654 killing. Images depict asci from the following crosses: (B) F2-23 × ISU-4344, (C) ISU-4348 ×
655 ISU-4344, and (D) ISU-3036 × ISU-4344.
656
657 **Figure 10** The *ncu06238* gene is not required for spore killing. (A) Asci from a cross between
658 two Sk^{S} strains (ISU-4559 × P8-43), where one of the strains has had its *ncu06238* coding
659 sequences deleted. While some normal asci are detected, many asci are abnormal and some
660 mimic the spore killer phenotype (red arrows). (B) Asci from a cross between an Sk^{S} strain and
661 an *Sk-2* strain (ISU-4559 × ISU-4561), where both strains have been deleted of their *ncu06238*
662 coding sequences. Nearly all viable progeny isolated from this cross have the *Sk-2* genotype (47
663 out of 48, data not shown).
664
665 **Figure 11** Replacement of $AH36^{3211}$ with $AH36^{Sk\text{-}2}$ restores spore killing to an *rfk-1* mutant. (A)
666 Strain ISU-4526 was constructed by replacing $AH36^{3211}$ in ISU-3222 (upper diagram, red box)
667 with *nat1* (using vector v160; ISU-3222 is a descendant of ISU-3211). Strain ISU-4563 was then
668 constructed by replacing $AH36^{\Delta}$*::nat1* in ISU-4562 with $AH36^{Sk\text{-}2}$*::hph* (lower diagram, red box

669 and green rectangle). The locations of the two recombination flanks used to replace *AH36*$^{\Delta}$*::nat1*
670 with *AH36*$^{Sk\text{-}2}$*::hph* are indicated with black crosses and red-dotted lines. (B–D) Asci are from
671 crosses between F2-23 and (B) ISU-3222, (C) ISU-4562, and (D) ISU-4563. (E–G) Asci are
672 from crosses between ISU-4348 and (E) ISU-3222, (F) ISU-4562, and (G) ISU-4563.
673
674 **Figure 12** RFK-1 is related to NCU07086. (A) All known *rfk-1* mutations alter the predicted
675 amino acid sequence of RFK-1. (B) A coding region of 2105 bp is predicted for gene *ncu07086*
676 (from start codon to stop codon, including introns). Predicted introns are labeled I1 through I4.
677 An AtpF Superfamily domain (NCBI CDD, Accession No. cl28522) can be identified within the
678 N-terminal end of the NCU07086 protein sequence. (C) The first 39 amino acids of RFK-1 and
679 NCU07086 are similar. (D) The repetitive sequence identified within *AH36* (see Figure 6)
680 appears to have originated from within the first intron of *ncu07086*. The first intron of ncu07086
681 is predicted to be 141 nucleotides long (NCBI Gene ID: 3876500), and only the first 84
682 nucleotides are shown. In contrast, the entire *AH36* repeat region is shown along with an
683 additional four nucleotides upstream of "repeat A". These four nucleotides include the stop
684 codon for the hypothetical 39 amino acid version of RFK-1.
685

Table 1 Strains used in this study

Name (alias)	Genotype[a]
F2-19	*rid; fl; Sk-2; A*
F2-23 (RTH1005.1)	*rid; fl A*
F2-26 (RTH1005.2)	*rid; fl a*
ISU-3017 (RKS2.1.2)	*rid$^{?}$; Sk-2 leu-1 v4$^{\Delta}$::hph; mus-51$^{?}$ a*
ISU-3023 (RKS1.1.6)	*rid$^{?}$; Sk-2 leu-1 v3$^{\Delta}$::hph; mus-51$^{?}$ a*
ISU-3029 (RKS3.2.5)	*rid; Sk-2 leu-1 v5$^{\Delta}$::hph; mus-51$^{\Delta}$:bar a*
ISU-3036 (RTH1623.1)	*rid; fl; sad-2$^{\Delta}$::hph A*
ISU-3037 (RTH1623.2)	*rid; fl; sad-2$^{\Delta}$::hph a*
ISU-3211 (RTH1158.8)	*rid; Sk-2 rsk$^{\Delta}$::hph rfk-1^{3211}; mus-51$^{\Delta}$::bar a*
ISU-3222 (RTH1249.14)	*rid; Sk-2 rfk-1^{3211}; mus-51$^{\Delta}$::bar a*
ISU-3223 (RTH1294.17)	*Sk-2 leu-1; mus-51$^{\Delta}$::bar A*
ISU-3224 (HAH8.1.3)	*rid his-3^{+}::AH4$^{Sk\text{-}2}$::hph; A*
ISU-3228 (HAH10.1.1)	*rid his-3^{+}::AH6$^{Sk\text{-}2}$::hph; A*
ISU-3243 (HAH16.1.1)	*rid his-3^{+}::AH14$^{Sk\text{-}2}$::hph A*
ISU-3311 (RDS1.1)	*Sk-2 leu-1 v31$^{\Delta}$::hph; mus-51$^{\Delta}$::bar A*
ISU-3313 (RDS2.3)	*Sk-2 leu-1 v32$^{\Delta}$::hph; mus-51$^{\Delta}$::bar A*
ISU-3315 (RDS3.9)	*Sk-2 leu-1 v33$^{\Delta}$::hph a*
ISU-3318 (RDS4.8)	*Sk-2 leu-1 v34$^{\Delta}$::hph A*
ISU-3321 (RDS5.9)	*rid; Sk-2 leu-1 v35$^{\Delta}$::hph; mus-51$^{\Delta}$::bar a*
ISU-3478 (RDS13.9.1)	*rid; Sk-2 v37$^{\Delta}$::hph; mus-51$^{\Delta}$::bar A*
ISU-3482 (RDS14.4.2)	*rid; Sk-2 v38$^{\Delta}$::hph A*
ISU-3483 (RDS15.1.1)	*rid; Sk-2 v39$^{\Delta}$::hph A*
ISU-3485 (RDS16.4.1)	*rid; Sk-2 v40$^{\Delta}$::hph A*
ISU-3656 (HAH42.1)	*rid his-3^{+}::AH30$^{Sk\text{-}2}$-hph A*
ISU-3658 (HAH43.1)	*rid his-3^{+}::AH31$^{Sk\text{-}2}$-hph A*
ISU-3660 (HAH44.1)	*rid his-3^{+}::AH32$^{Sk\text{-}2}$-hph A*
ISU-4269 (RAH64.1.1)	*rid his-3^{+}::AH37$^{Sk\text{-}2}$-hph; mus-52$^{\Delta}$::bar a*

ISU-4271 (RAH63.1.2)	*rid his-3^{+}::AH36$^{Sk\text{-}2}$-hph; mus-52$^{\Delta}$::bar A*
ISU-4273 (HNR12.6.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2}$-hph A*
ISU-4275 (HNR10.4.2)	*rid his-3^{+}::AH36^{3211}-hph A*
ISU-4344 (RAY1.13)	*rid; Sk-2 v140$^{\Delta}$::hph; mus-51$^{\Delta}$::bar A*
ISU-4348 (RAY6.5)	*rid; fl; v150$^{\Delta}$::hph a*
ISU-4551 (RNR29.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2[G27945A]}$-hph; mus-52$^{?}$ A*
ISU-4552 (RNR28.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2[G27972A]}$-hph; mus-52$^{?}$ A*
ISU-4553 (RNR27.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2[G28052A]}$-hph; mus-52$^{?}$ A*
ISU-4554 (RNR26.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2[G28104A]}$-hph; mus-52$^{?}$ A*
ISU-4555 (RNR25.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2[G28300A]}$-hph; mus-52$^{?}$ A*
ISU-4556 (RNR30.1)	*rid his-3^{+}::AH36$^{Sk\text{-}2[G28326A]}$-hph; mus-52$^{?}$ A*
ISU-4557 (RNR129.1.3)	*rid; Sk-2 v199$^{\Delta}$::hph-ccg-1*(P); *mus51$^{?}$ A*
ISU-4558 (RNR130.1.3)	*rid; Sk-2 v200$^{\Delta}$::hph-ccg-1*(P)*; mus51$^{?}$A*
ISU-4559 (RNR108.1.12)	*rid; fl; ncu06238$^{\Delta}$::hph mus-52$^{\Delta}$::bar a*
ISU-4561 (RNR109.3.2)	*rid; Sk-2 ncu06238$^{\Delta}$::hph; mus51$^{\Delta}$::bar A*
ISU-4562 (HNR92.1)	*rid; Sk-2 v160$^{\Delta}$::nat1; mus-51$^{\Delta}$::bar a*
ISU-4563 (HNR100.11.1)	*rid; Sk-2 v140$^{\Delta}$::hph; mus-51$^{\Delta}$::bar a*
P8-42	*rid; mus-51$^{\Delta}$::bar a*
P8-43	*rid; mus-52$^{\Delta}$::bar A*
P15-53 (RTH1122.22)	*rid; Sk-2; mus-51$^{\Delta}$::bar A*

[a]The *rid$^{?}$*, *mus-51$^{?}$* and *mus-52$^{?}$* designations are used if the genotype has not been determined for the indicated allele. Genotypes of all key alleles were determined by PCR or lineage analysis.

Table 2 Interval positions

name	start	stop
Miscellaneous intervals of the 45 kb *rfk-1* region		
Sk-2^{INS1}	18118	29151
rfk-1 coding[a]	28264	28383
repeats[b]	28384	28722
Intervals deleted from the 45 kb *rfk-1* region		
v3	15640	15664
v4	36166	36426
v5	18042	28759
v31	18042	21464
v32	18042	25268
v33	18042	26951
v34	18042	27667
v35	25837	28759
v37	27242	28759
v38	27602	28759
v39	28126	28759
v40	27602	28198
v140	29381	29401
v160	27740	29401
v175	29489	31883
v199	28131	28263
v200	28131	28353
Intervals transferred from the 45 kb *rfk-1* region to *Sk*^{S}		
AH4	16579	22209
AH6	19408	25648
AH14	25632	28324
AH30	27528	29702
AH31	27900	29702
AH32	28304	29702
AH36	27900	29380
AH37	27900	29512

The coordinates of each interval are as defined by GenBank sequence KJ908288.1. [a]hypothetical. [b]contains repeats of a 47 bp sequence.

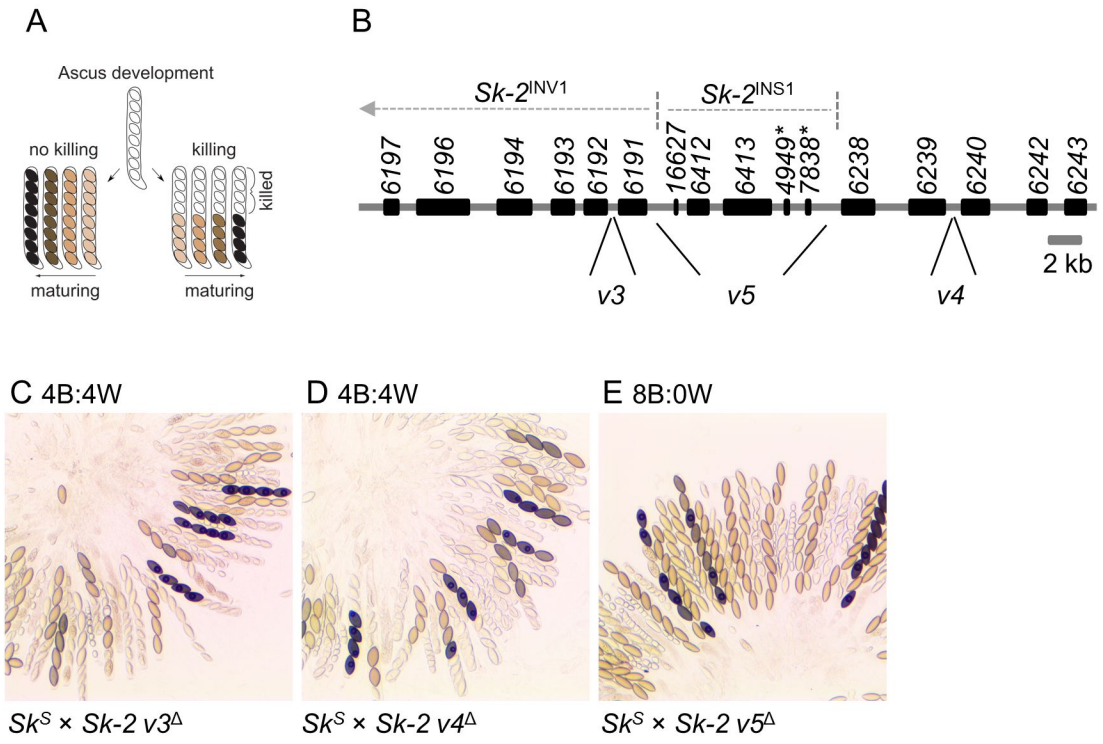
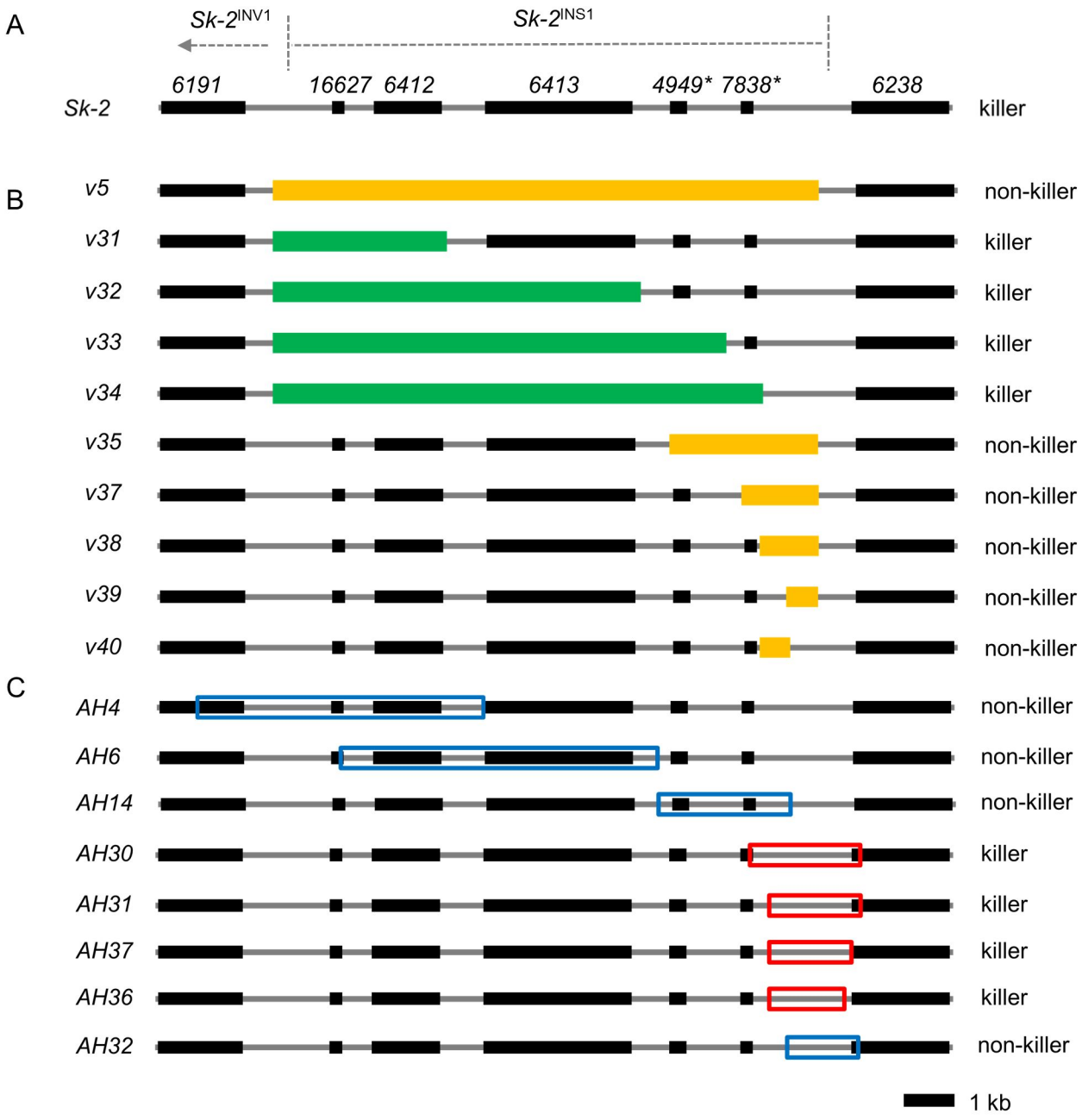
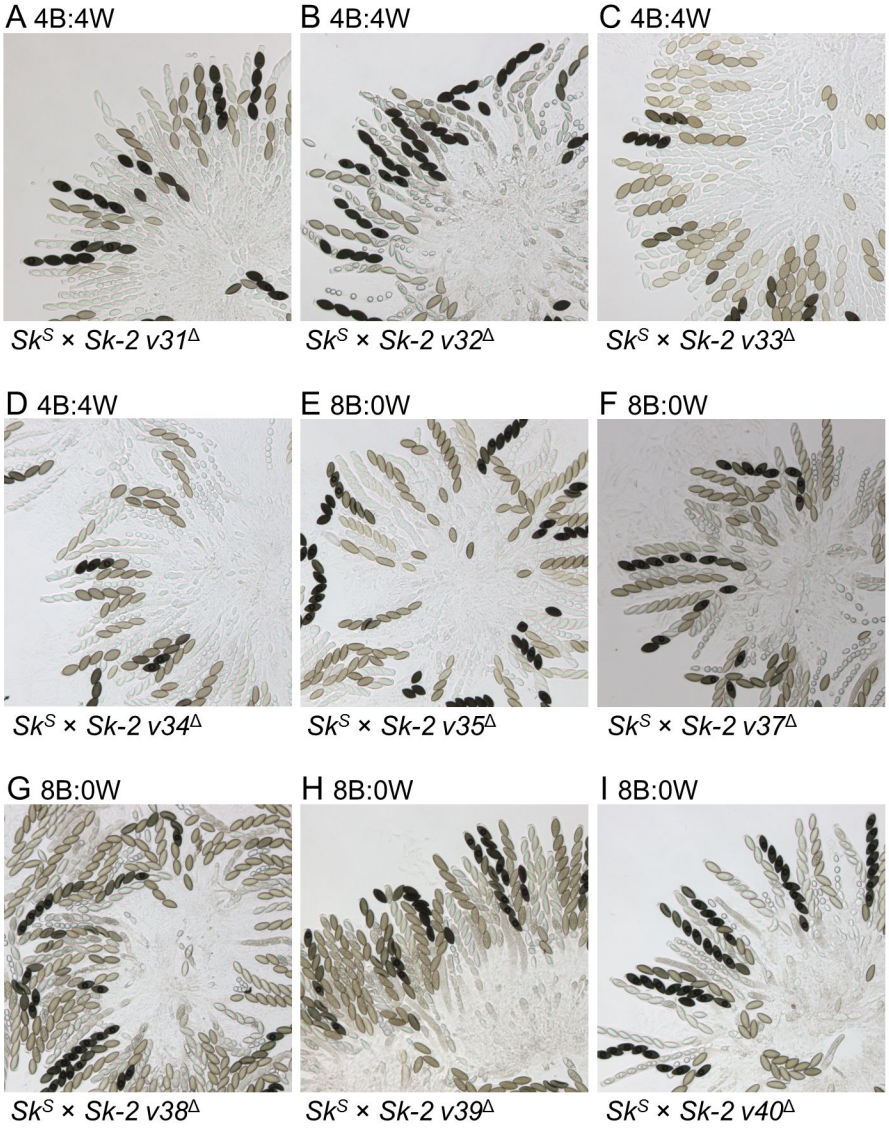


Figure 2





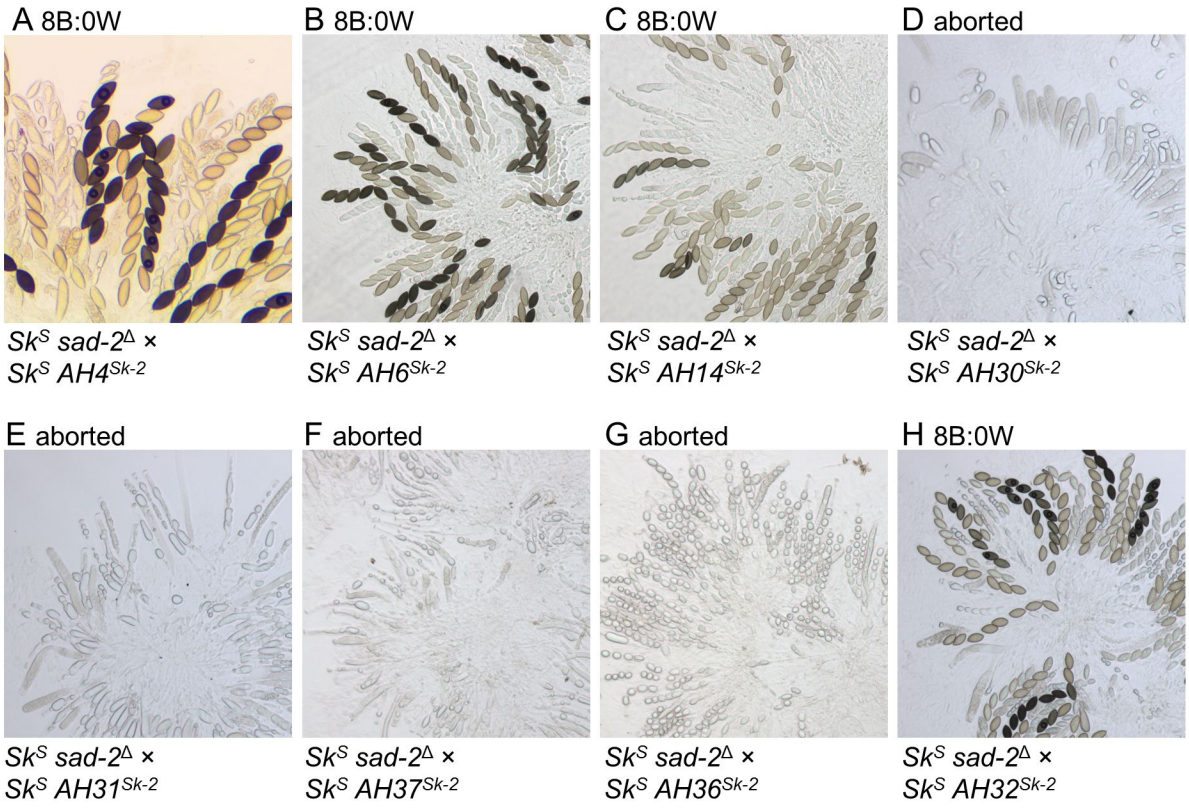
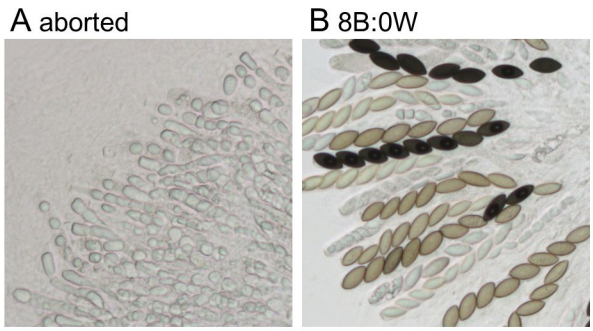


Figure 5



A

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GGGGCCCTGCCTTCCCTTAGTTCTCTCATGAAGCTGGAAGTTATATCCTAACCCACTACTGACAAAACAAAG
CAAGAGCTGCTGCCCCAACTATAGACAGCACGCTTTTCCACCTCAGTTGGGGCACCTAGAAAGCTATAAGAT
CCCTCTTCCCCCGGCCCAACCTCTCCTCAGAATTTCTTTTTTTCTCCAACATTGTTAAGAACTTTGTTTTTG
GAAAATGGCCTGCCCCACAGGGTTTTTTACCGCTCTTTTTGGCAAACTCCTCACCATCCCCATTTGGGTGTT
GGTGTTTGTATTCAATGCTCTGTTTGTCTTCCCCCGGTTTTGGGTCTGGTAAGTCTCCTTCATGTTCCAATT
CATTTTTGTTTTTTCCTTTCTCTTCTCGTCTCCTTCATGTTCCAATTCATTTTTGTTTTTTTCCTTTCTCTT
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CGTCTCCTTCATGTTCCAATTCATTTTTGTTTTTTTCCTTTCTCTTCTCGTCTCCTTCATGTTCCAATTCAT
TTTTTTTTGTCCTTTCTCTTCTCGTCTCCTTACAGTTTACCTTATCCTCTCGGTCCTCTCTGTCTTTCGCTA
ACCAGGAACAGGCGCTTACCACCACGGCTGCAACACGAGCAGCAGCAGCAGGACCGGAACGATGACGAATGG
CAGCGGCAGCAACAGGACAGGCGGGTTGTGGTTTGGCACCCACCGCCCCCTCCAGACGTGGAGATGGCCCTC
CAAGACAATCCCACTGCCGCCCCCGCAGAGCCGGCTGACCTCGACCACCCAGCGCCGTAGTGGCGGCGCTGG
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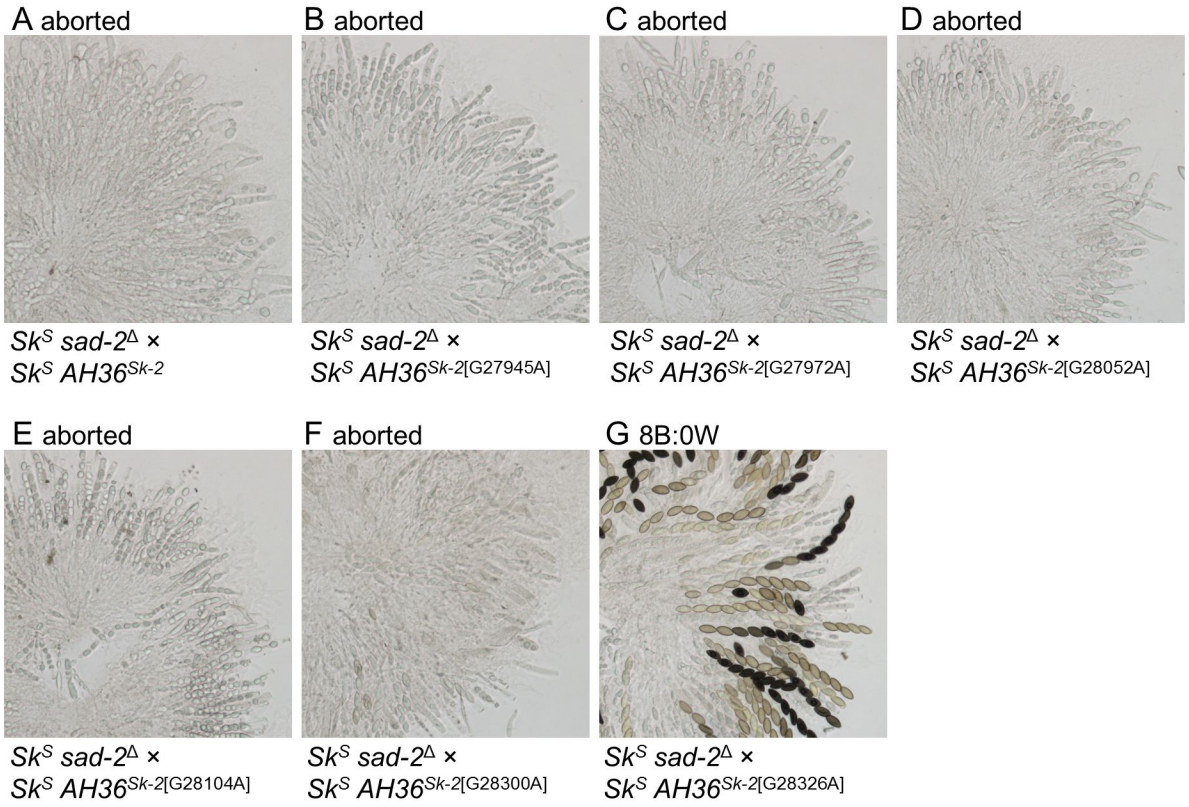
CCGAGAGGACACAGACAAGGCCCACGTCCGCCATCAGCATTCCCAGCTGGCCGACCGCACCGCCGCAACTCC
CACTTTACCTCAACACCAGAATACGGAATCGGTACATCGACAGCAGCATCATCATCATCAATATCACCACCT
CCACTTGGCGCGCACTTGCGGAAAACGTCCCGCTACACCGT
```

B

```
Repeat A  GTCTCCTTCATGTTCCAATTCATTTTTG-TTTTTTCCTTTCTCTTCTC 28430
Repeat B  GTCTCCTTCATGTTCCAATTCATTTTTGTTTTTTTCCTTTCTCTTCTC 28478
Repeat C  GTCTCCTTCATGTTCCAGTTCATTTTTG-TTTTTTCCTTTCTCTTCTC 28525
Repeat D  GTCTCCTTCATGTTCCAATTCATTTTTGTTTTTTTCCTTTCTCTTCTC 28573
Repeat E  GTCTCCTTCATGTTCCAGTTCATTTTTG-TTTTTTCCTTTCTCTTCTC 28260
Repeat F  GTCTCCTTCATGTTCCAATTCATTTTTGTTTTTTTCCTTTCTCTTCTC 28668
Repeat G  GTCTCCTTCATGTTCCAATTCATTTTT--TTTTTGTCCTTTCTCTTCTC 28714
Repeat H  GTCTCCTT------------------------------------------ 28722
```



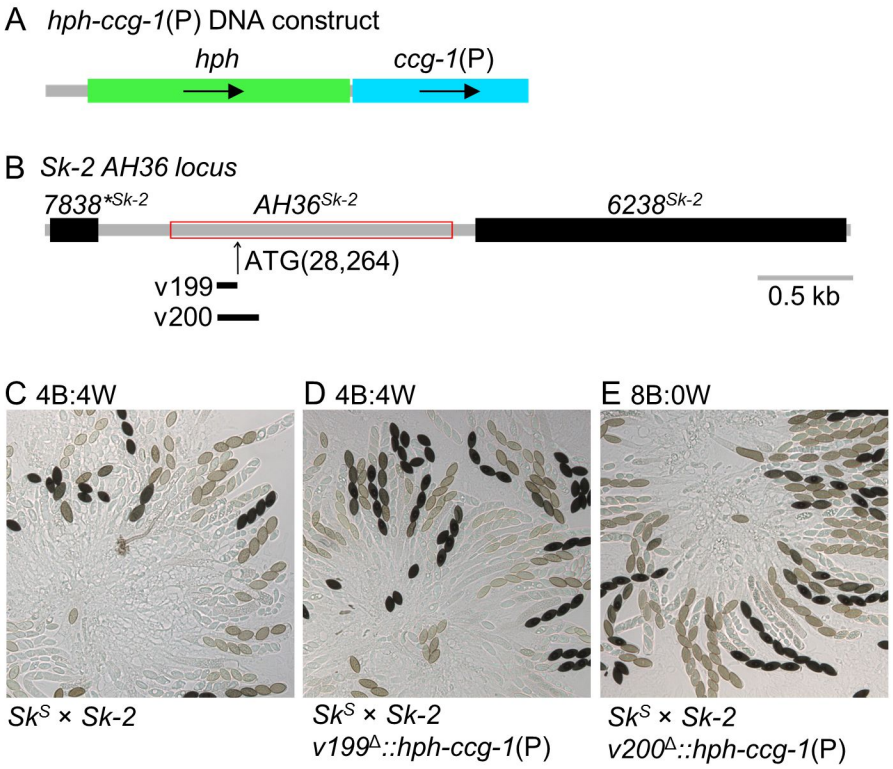


Figure 9

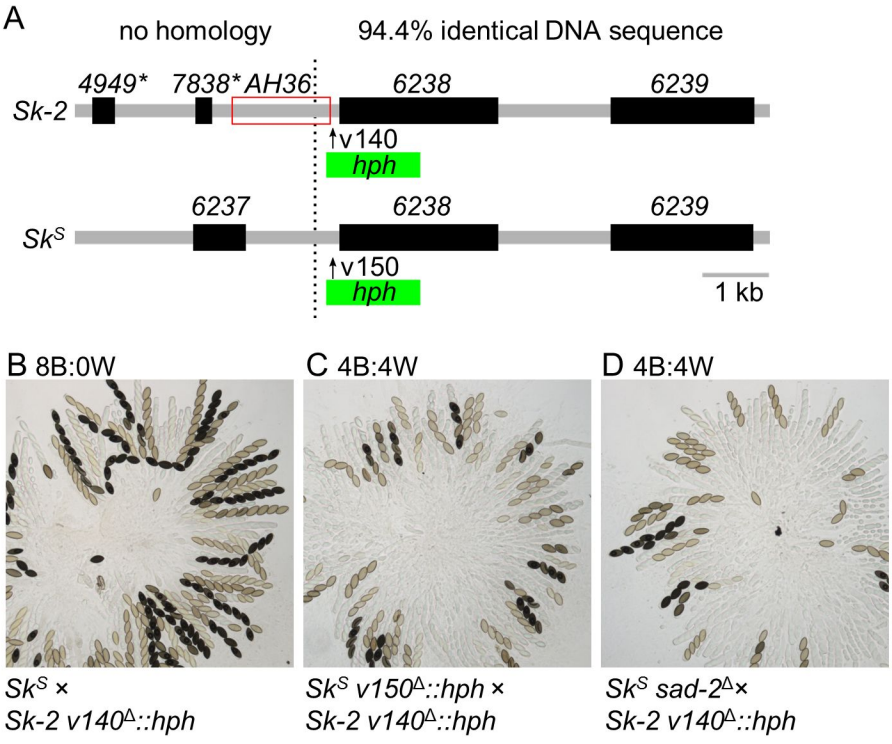
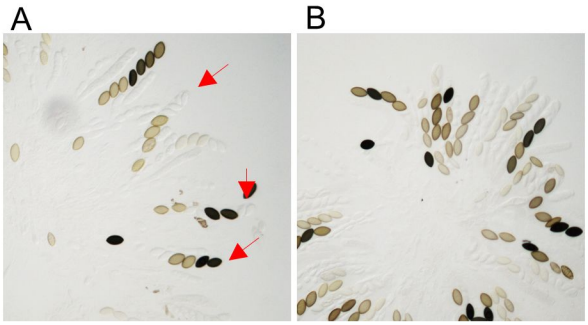
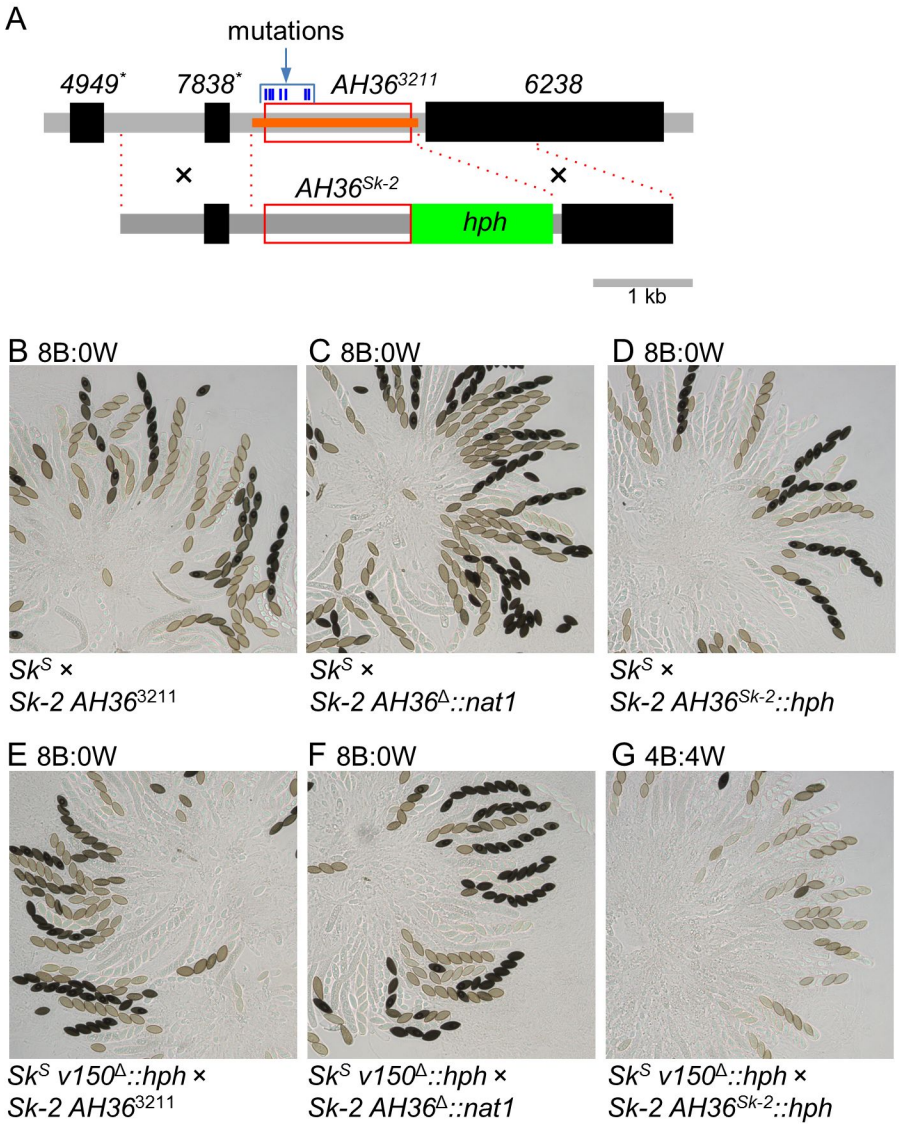


Figure 10

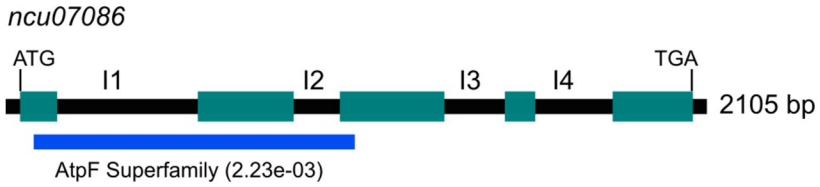




A

```
RFK-1        MACPTGFFTALFGKLLTIPIWVLVFVFNALFVFPRFWVW* 40
RFK-1^3211   MACPTGFFTALFSKLLTIPI*VLVFVFNALFVFPRFWVW* 40
RFK-1^3212   MACPTGFFYRSFWQTPHHPHLGVGVCIQCSVCLPPVLGLV 40
RFK-1^3213   MACPTGFFTALFGKLLTIPIWVLVFVFNTLFVFPRFWVW* 40
```

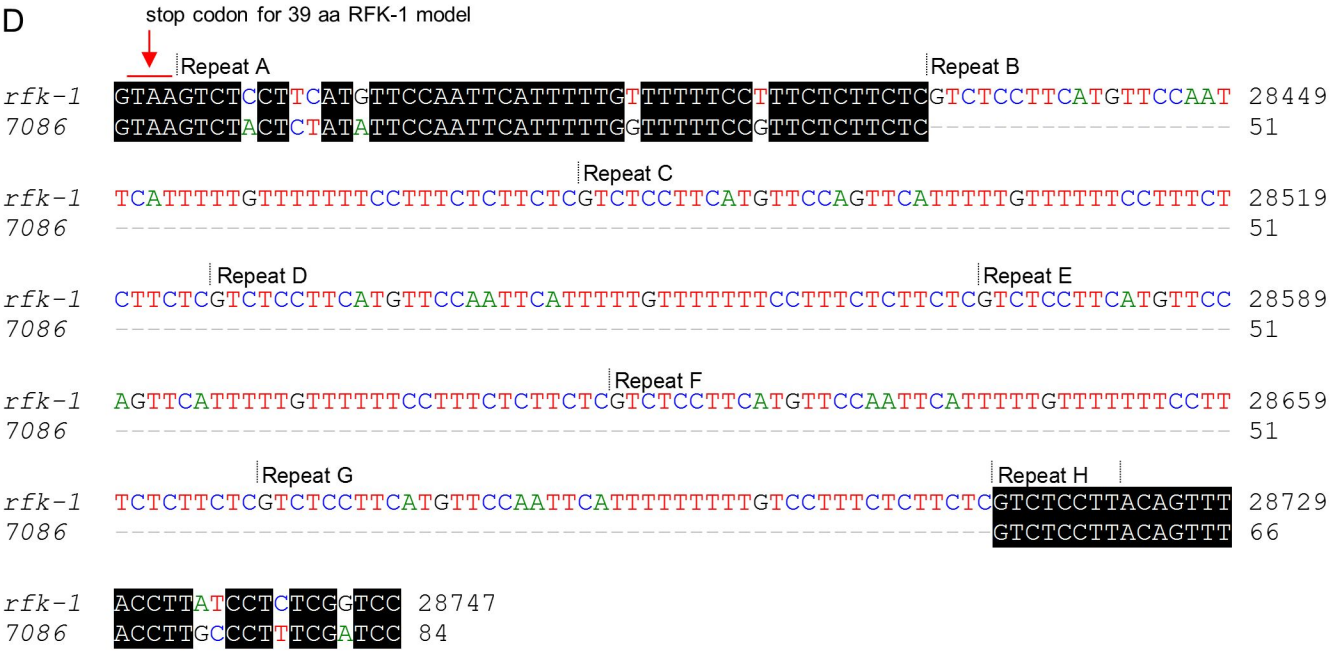
B



C

```
RFK-1        MACPTGFFTALFGKLLTIPIWVLVFVFNALFVFPRFWVW* 40
NCU07086     MTCLNAFLTALFGKVLSAFIWLGVFAWNVYFVWPGWPLLN 40
```

D

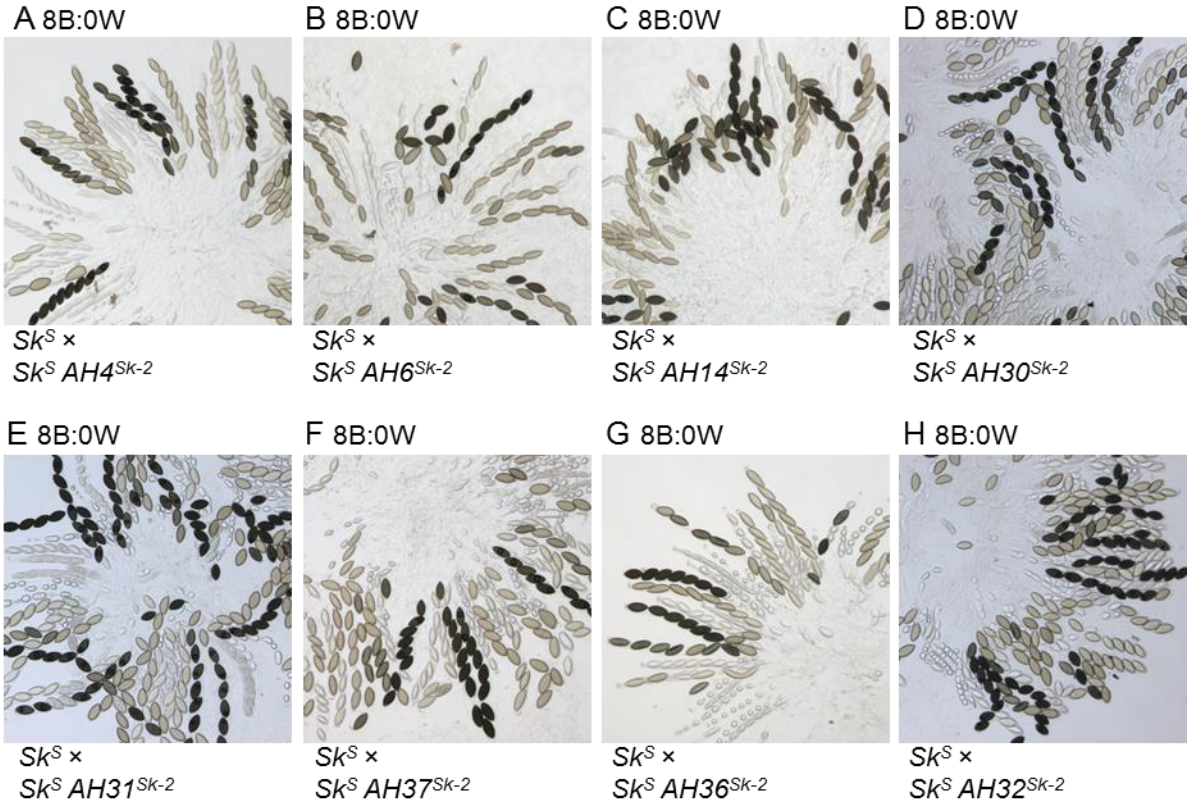


Supporting Information

Rhoades *et al*. "Identification of a genetic element required for spore killing in Neurospora".

Contents

Figure S1 Unpaired *Sk-2*^{INS1}-intervals do not kill ascospores in MSUD-proficient crosses.



Unpaired *Sk-2*^{INS1}-intervals do not kill ascospores in MSUD-proficient crosses. (A–H) The images depict asci from crosses between *Sk*^{S} strains, one of which carries an interval of the *Sk-2*^{INS1} locus (*e.g.*, *AH4*$^{Sk-2}$, *AH6*$^{Sk-2}$, etc.). All crosses produced asci with an 8B:0W phenotype. Crosses are as follows: (A) F2-26 × ISU-3224, (B) F2-26 × ISU-3228, (C) F2-23 × ISU-3243, (D) F2-26 × ISU-3656, (E) F2-26 × ISU-3658, (F) F2-23 × ISU-4269, (G) F2-26 × ISU-4271, and (H) F2-26 × ISU-3660.

Table S1 Primers for DJ-PCR-based construction of deletion vectors.

Nineteen deletion vectors were constructed by double joint (DJ)-PCR (Yu *et al.* 2004; Hammond *et al.* 2011). The table below lists the forward and reverse primer sequences (5′ to 3′) for the left recombination flank (L), the right recombination flank (R), and the nested amplification of each completed vector (N).

For each vector, the left and right DNA flanks were amplified from genomic DNA of the transformation host, which is also indicated in the table.

The center fragment for each vector is listed next to the name of each vector in the left-most column. Center fragments were either *hph*, *nat1*, or *hph-ccg-1*(P). See Table S2 for more information on the center fragments.

Vector (*center*) Primer numbers	*Name of transformation host* Primer sequences	Primer purpose
v3 (*hph*)	*Transformation host: ISU-3223*	
73	CAAGACCCAGAACAACGCCAACA	L
74	AAAAAATGCTCCTTCAATATCAGTTCCTCGCTCCTCTTCCGCAAATTA	L
75	GAGTAGATGCCGACCGGGAACCAGTTTGGTGGGATACTCGGTGCAGGTA	R
76	CGACACCTCGAATACGCCCTCTC	R
77	CCGGAAACGTCAGCAAACACGTA	N
78	GCGCCAGCTCCTCTACACTCTCC	N
v4 (*hph*)	*Transformation host: ISU-3223*	
79	CCAAGCCAAACTCAAGGGAATCG	L
80	AAAAAATGCTCCTTCAATATCAGTTAATGGCGGTGATCTTCGACTGCT	L
81	GAGTAGATGCCGACCGGGAACCAGTTGCCCAGACTCAGCTTGCATTGAC	R
82	TCACCTTGGCCCTGGAGTACCTG	R
83	CAAACGGGACGCAACCTCTATGA	N
84	CCAAGCGGGTTCCAGATAAGACG	N
v5 (*hph*)	*Transformation host: ISU-3223*	
85	CACCATGTAGTCGGAGCGGAAGA	L
86	AAAAAATGCTCCTTCAATATCAGTTTCATCTTGACGGGCAGAACTGAA	L
87	GAGTAGATGCCGACCGGGAACCAGTTGCTAACCAGGAACAGGCGCTTACC	R
88	CATCGAAAGGGAGAGGCACTTCG	R
89	GCCTTCCTTCTTCACACGGAGGT	N
90	ACAGGATCTGGTCATCCCGCTTC	N
v31 (*hph*)	*Transformation host: ISU-3223*	
85	CACCATGTAGTCGGAGCGGAAGA	L
86	AAAAAATGCTCCTTCAATATCAGTTTCATCTTGACGGGCAGAACTGAA	L
167	GAGTAGATGCCGACCGGGAACCAGTTATTGAGGTGAGGACAAGCGATGA	R
168	CATACGGCCCATGTTACCGCACT	R
89	GCCTTCCTTCTTCACACGGAGGT	N
170	CAACGAAGCAGGCTCCCATACAG	N
v32 (*hph*)	*Transformation host: ISU-3223*	
85	CACCATGTAGTCGGAGCGGAAGA	L
86	AAAAAATGCTCCTTCAATATCAGTTTCATCTTGACGGGCAGAACTGAA	L
173	GAGTAGATGCCGACCGGGAACCAGTTGTCGTCCGTGAATCGTGATCCTT	R
174	AATTCGCCGTGTACTTCGCTGTG	R
89	GCCTTCCTTCTTCACACGGAGGT	N
176	CGGTTGTATCTGCCGGTTTGAAGA	N
v33 (*hph*)	*Transformation host: ISU-3223*	
85	CACCATGTAGTCGGAGCGGAAGA	L
86	AAAAAATGCTCCTTCAATATCAGTTTCATCTTGACGGGCAGAACTGAA	L
3	GAGTAGATGCCGACCGGGAACCAGTTCATGGCAGTGAAGTGGACAAGCTG	R
4	GTGGTAAGCGCCTGTTCCTGGTTAG	R
89	GCCTTCCTTCTTCACACGGAGGT	N
6	TGCGGCCTGTTTACGAAATCCAA	N
v34 (*hph*)	*Transformation host: ISU-3223*	
85	CACCATGTAGTCGGAGCGGAAGA	L

86	AAAAAATGCTCCTTCAATATCAGTTTCATCTTGACGGGCAGAACTGAA	L
9	GAGTAGATGCCGACCGGGAACCAGTTCTCGATTGCCCGACACCTTCTGT	R
4	GTGGTAAGCGCCTGTTCCTGGTTAG	R
89	GCCTTCCTTCTTCACACGGAGGT	N
11	CGAAAGACAGAGAGGACCGAGAGGA	N
v35 (*hph*)	*Transformation host: ISU-3223*	
1	TCGGAAGGATTGCTGACTTGTGTGT	L
2	CCAAAAAATGCTCCTTCAATATCAGTTAGTTGGTAGCTGGCGCGGAAAG	L
87	GAGTAGATGCCGACCGGGAACCAGTTGCTAACCAGGAACAGGCGCTTACC	R
88	CATCGAAAGGGAGAGGCACTTCG	R
5	GCGCAGACGAACATCAAGGAGAA	N
90	ACAGGATCTGGTCATCCCGCTTC	N
v37 (*hph*)	*Transformation host: P15-53*	
7	GGCAGATACAACCGACGACCAAA	L
8	CCAAAAAATGCTCCTTCAATATCAGTTTCCGTTTCGCTTATGATGTTAATGATG	L
87	GAGTAGATGCCGACCGGGAACCAGTTGCTAACCAGGAACAGGCGCTTACC	R
88	CATCGAAAGGGAGAGGCACTTCG	R
10	CACGTAGGGAAGGAGGTTGAAGGT	N
90	ACAGGATCTGGTCATCCCGCTTC	N
v38 (*hph*)	*Transformation host: P15-53*	
309	ACGCCAAAAGGTGTAGGGGGATT	L
310	CCAAAAAATGCTCCTTCAATATCAGTTGACCGAACAACCGGAATGACCT	L
87	GAGTAGATGCCGACCGGGAACCAGTTGCTAACCAGGAACAGGCGCTTACC	R
88	CATCGAAAGGGAGAGGCACTTCG	R
311	AGGTCCGCAACTATTGTCCGTTT	N
90	ACAGGATCTGGTCATCCCGCTTC	N
v39 (*hph*)	*Transformation host: P15-53*	
309	ACGCCAAAAGGTGTAGGGGGATT	L
312	CCAAAAAATGCTCCTTCAATATCAGTTGCAGCTCTTGCTTTGTTTTGTCAGT	L
87	GAGTAGATGCCGACCGGGAACCAGTTGCTAACCAGGAACAGGCGCTTACC	R
88	CATCGAAAGGGAGAGGCACTTCG	R
311	AGGTCCGCAACTATTGTCCGTTT	N
90	ACAGGATCTGGTCATCCCGCTTC	N
v40 (*hph*)	*Transformation host: P15-53*	
309	ACGCCAAAAGGTGTAGGGGGATT	L
310	CCAAAAAATGCTCCTTCAATATCAGTTGACCGAACAACCGGAATGACCT	L
313	GAGTAGATGCCGACCGGGAACCAGTTCGGCCCAACCTCTCCTCAGAAT	R
88	CATCGAAAGGGAGAGGCACTTCG	R
311	AGGTCCGCAACTATTGTCCGTTT	N
90	ACAGGATCTGGTCATCCCGCTTC	N
v140 (*hph*)	*Transformation host: P15-53*	
1303	AACCAGGAACAGGCGCTTACCAC	L
1304	AAAAAATGCTCCTTCAATATCAGTTACGGTGTAGCGGGACGTTTTC	L
1305	GAGTAGATGCCGACCGGGAACCAGTTTCAACAAAGCGCGTGATCTTTCG	R
871	GAACTCGAACCACTCCACGCAAA	R
1306	ACAGGACAGGCGGGTTGTGGTTT	N
872	CATGTCGGTCTTGAGGTCGTTGC	N
v150 (*hph*)	*Transformation host: P8-43*	
1332	CCGCGAATGGTTAACTGCACGGC	L
1304	AAAAAATGCTCCTTCAATATCAGTTACGGTGTAGCGGGACGTTTTC	L
1305	GAGTAGATGCCGACCGGGAACCAGTTTCAACAAAGCGCGTGATCTTTCG	R
1333	GTCGGCATAGGCTGTGGTGGTCG	R
1334	ATGCGGCCTTGATGCACTGGCTG	N
1335	CGAGAGGGAGAGGCACTTCGCCA	N
v160 (*nat1*)	*Transformation host: ISU-3222*	
10	CACGTAGGGAAGGAGGTTGAAGGT	L
869	TGAATGCTAAAAGACACCATTTCCCACACTCCCTCAGCAAGTAAGCCGGTCACGATCC	L
870	GCTGGCTGCAATACAAGCGTTCCCACCTAACCAACTCAACAAAGCGCGTGATCTTTCG	R
871	GAACTCGAACCACTCCACGCAAA	R
311	AGGTCCGCAACTATTGTCCGTTT	N
872	CATGTCGGTCTTGAGGTCGTTGC	N

v175 (*hph*)	*Transformation host: ISU-3222*	
1433	GGAACAGGCGCTTACCACCA	L
1434	AAAAAATGCTCCTTCAATATCAGTTTGAAATGTTGATGCCTCCCTGGAT	L
1435	GAGTAGATGCCGACCGGGAACCAGTTGGGGTTTAGGGAGGGCTGCAT	R
1436	TTCCTTTCCCGCTCCGTTCG	R
1437	ACAGGACAGGCGGGTTGTGG	N
1438	CCGAATACCGACCCCCGATT	N
v176 (*hph*)	*Transformation host: P8-43*	
1439	TGGCAGGTCAAGGTCGATTGC	L
1440	AAAAAATGCTCCTTCAATATCAGTTTGAAATGTTGATGCCTCCCTAGAT	L
1441	GAGTAGATGCCGACCGGGAACCAGTTGGGGTTTAGGCAGGGCTGGAT	R
1442	TTCCTTTCCCGCTCCTTTCG	R
1443	CGGCCGCGAATGGTTAACTG	N
1438	CCGAATACCGACCCCCGATT	N
v199 [*hph-ccg-1*(P)]	*Transformation host: P15-53*	
1538	CGAAGGACAAGAGGAACGGGAAA	L
1539	GCAGCCTGAATGGCGAATGGACGCGCGGGCAGCAGCTCTTGCTTTGTTT	L
1544	TTCACAACCCCTCACATCAACCAAAATGGCCTGCCCCACAGGGTTT	R
1541	GTCACGGTGTAGCGGGACGTTTT	R
1542	GGGGCGGAGAGGAGAAGATGAGT	N
1543	GGAATTACAACCCCTGCGTGACC	N
v200 [*hph-ccg-1*(P)]	*Transformation host: P15-53*	
1538	CGAAGGACAAGAGGAACGGGAAA	L
1539	GCAGCCTGAATGGCGAATGGACGCGCGGGCAGCAGCTCTTGCTTTGTTT	L
1540	TTCACAACCCCTCACATCAACCAAATTTGTCTTCCCCCGGTTTTGG	R
1541	GTCACGGTGTAGCGGGACGTTTT	R
1542	GGGGCGGAGAGGAGAAGATGAGT	N
1543	GGAATTACAACCCCTGCGTGACC	N

Table S2 Primers for DJ-PCR center products.

The forward and reverse primers used to amplify the center fragments for construction of DJ-PCR deletion vectors are described below.

Center Primer number	*Name of template* Primer sequences
hph	*pTH1256.1(GenBank MH550659)*
12	AACTGATATTGAAGGAGCATTTTTTGG
13	AACTGGTTCCCGGTCGGCAT
nat1	*pNR28.12 (GenBank MH553564)*
297	GAGGGAGTGTGGGAAATGGTGTC
298	GTTGGTTAGGTGGGAACGCTTGT
hph-ccg-1(P)	*pTH1117.12 (GenBank JF749202)*
550	GCGCGTCCATTCGCCATTCA
1555	TTTGGTTGATGTGAGGGGTTGTGA

Table S3 Primers for cloning *Sk-2* intervals to pTH1256.1.

Eight intervals of *Sk-2*^{INS1} were cloned to the *Not*I site of pTH1256.1 (GenBank MH550659), using the primers listed below. These cloning procedures created plasmids pAH4, pAH6, pAH14, pAH30, pAH31, pAH32, pAH36, and pAH37. Each plasmid was then used to transform strain P8-43.

Plasmid name Primer number	*Name of transformation host* Primer sequences
pAH4	*Transformation host: P8-43*
248	AAAAGCGGCCGCAGGGTGGTGTGGGTGAGGATGT
249	TTTTGCGGCCGCGAGCGGAAGTGTTTGCTTGTGTGA
pAH6	*Transformation host: P8-43*
252	AAAAGCGGCCGCATCGCCAACGGGCATTCAAG
253	AAAAGCGGCCGCACCCGCCTACACATGCACCATC
pAH14	*Transformation host: P8-43*
302	AAAAGCGGCCGCTGCATGTGTAGGCGGGTATTGTG
314	AAAAGCGGCCGCGGGGCAGGGCAGCAAGTAAG
pAH30	*Transformation host: P8-43*
304	AAAAGCGGCCGCGAGGACCAGCTCGACGGTAGTAGG
251	AAAAGCGGCCGCGAGGAATAGGACGTGAGGGTGTGG
pAH31	*Transformation host: P8-43*
353	TTTTGCGGCCGCCATTGATACCGAGTCTTTCCGTTC
251	AAAAGCGGCCGCGAGGAATAGGACGTGAGGGTGTGG
pAH32	*Transformation host: P8-43*
351	AAAAGCGGCCGCAACTCCTCACCCATCCCCATTTG
251	AAAAGCGGCCGCGAGGAATAGGACGTGAGGGTGTGG
pAH36	*Transformation host: P8-43*
353	TTTTGCGGCCGCCATTGATACCGAGTCTTTCCGTTC
639	AAAAGCGGCCGCGACGGTGTAGCGGGACGTTTTCC
pAH37	*Transformation host: P8-43*
353	TTTTGCGGCCGCCATTGATACCGAGTCTTTCCGTTC
640	AAAAGCGGCCGCGTTCGCTGACTTTCCCGACCA

Table S4 Primers for amplification of *AH36^{Sk-2}::hph*.

The *AH36^{Sk-2}::hph* allele was amplified from ISU-4344 using the primers 10 and 871. These primers span the *v140$^{\Delta}$::hph* allele in ISU-4344 and produce a PCR product containing *AH36^{Sk-2}* and *hph* between recombination flanks suitable for replacing *AH36$^{\Delta}$::nat1* in ISU-4562 with *AH36^{Sk-2}::hph*.

Target Primer number	Template; *Name of transformation host* Sequence
AH36^{Sk-2}::hph	Amplify from ISU-4344; *Transformation host: ISU-4562*
10	CACGTAGGGAAGGAGGTTGAAGGT
871	GAACTCGAACCACTCCACGCAAA

Table S5. Primers for site directed mutagenesis of *AH36*$^{Sk\text{-}2}$.

Site directed mutagenesis was performed essentially as described for the QuikChange II Site-Directed Mutagenesis Kit (Revision E.01, Agilent Technologies). The *AH36* interval from *Sk-2* was cloned to the *Not*I site of a standard 3 kb bacterial cloning vector with primers 353 and 639 (Table S2). Site-specific mutations were introduced into the resulting plasmid (pNR9.1) by PCR with the primer sets described below. PCR products were digested with *Dpn*I and used to transform chemically-competent *E. coli* Ig^{TM} 5-alpha cells (Intact Genomics). Site directed mutations were confirmed by Sanger sequencing and mutated plasmids were used to transform P8-43.

Purpose of primers Primer number	Primer sequences
Change G to A at position 27945	
1138	GTTGGAGTGAGGATATAATCCGGCACGTCGAAG
1139	CTTCGACGTGCCGGATTATATCCTCACTCCAAC
Change G to A at position 27972	
1136	GATGATGACTGACTTATTTACTATAGTTCCTCCTTCGACGT
1137	ACGTCGAAGGAGGAACTATAGTAAATAAGTCAGTCATCATC
Change G to A at position 28052	
1134	CGACGGGGGCCCTACCTTCCCTTAGTT
1135	AACTAAGGGAAGGTAGGGCCCCCGTCG
Change G to A at position 28104	
1132	CTAACCCACTACTAACAAAACAAAGCAAGAGCTGCTGC
1133	GCAGCAGCTCTTGCTTTGTTTTGTTAGTAGTGGGTTAG
Change G to A at position 28300	
1130	GGGATGGTGAGGAGTTTGCTAAAAAGAGCGGTAAAAAAC
1131	GTTTTTTACCGCTCTTTTTAGCAAACTCCTCACCATCCC
Change G to A at position 28326	
1128	GAATACAAACACCAACACTCAAATGGGGATGGTGAG
1129	CTCACCATCCCCATTTGAGTGTTGGTGTTTGTATTC