Sexual transmission of the [Het-s] prion leads to meiotic drive in *Podospora anserina*

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In the filamentous fungus *Podospora anserina*, two phenomena are associated with polymorphism at the *het-s* locus, vegetative incompatibility and ascospore abortion. Two *het-s* alleles occur naturally, *het-s* and *het-S*. The *het-s* encoded protein is a prion propagating as a self-perpetuating amyloid aggregate. When prion-infected [Het-s] hyphae fuse with [Het-S] hyphae, the resulting heterokaryotic cells necrotize. [Het-s] and [Het-S] strains are sexually compatible. When, however, a female [Het-s] crosses with [Het-S], a significant percentage of *het-S* spores abort, in a way similar to spore killing in *Neurospora* and *Podospora*. We report here that sexual transmission of the [Het-s] prion after nonisogamous mating in the reproductive cycle of *Podospora* is responsible for the killing of *het-S* spores. Progeny of crosses between isogenic strains with distinct wild-type or introduced, ectopic *het-s/S* alleles were cytologically and genetically analyzed. The effect of *het-s/S* overexpression, ectopic *het-s/S* expression, absence of *het-s* expression, loss of [Het-s] prion infection, and the distribution patterns of HET-s/S-GFP proteins were categorized during meiosis and ascospore formation. This study unveiled a *het-S* spore-killing system that is governed by dosage of and interaction between the [Het-s] prion and the HET-S protein. Due to this property of the [Het-s] prion, the *het-s* allele acts as a meiotic drive element favoring maintenance of the prion-forming allele in natural populations.

Meiotic drive is a process mediated by genetic elements, called segregation distorters, that actively bias Mendelian segregation in their favor. In metazoans, classic examples of meiotic drive include the *t* haplotype in mice and *Segregation Distorter* (SD) in *Drosophila* (1). In both these autosomal drive systems, heterozygous males produce gametes of the driver genotype in excess. The molecular mechanisms of meiotic drive remain largely elusive, except in the case of SD in *Drosophila*, where distortion involves a truncated form of a RanGAP protein and its mislocalization to the nucleus (2). The best studied examples of segregation distortion in fungi are the *spore killer* systems in the ascomycetes *Neurospora crassa* (3–6) and *Podospora anserina* (7). In these haploid organisms, the sexual progeny, the ascospores, are linearly arranged after meiosis. Ascomycetes therefore provide excellent opportunities to investigate the behavior of meiotic drive elements (8). Presence of a *killer* gene can readily be detected by directly analyzing the pattern of ascospore abortion. Spore killer genes exist as a *killer* and a *sensitive* allele or haplotype. In a *killer* × *sensitive* cross, the ascospores harboring only the sensitive genotype degenerate. Heterokaryotic spores, containing both a sensitive and a killer nucleus, escape abortion. Both in *Neurospora* and in *Podospora*, several spore killer loci have been genetically identified, but so far the mechanism of spore killing is unknown.

In 1965, Bernet (9, 10) described properties of the *het-s* gene in *Podospora*, now recognized to be analogous to a spore killer locus. The discovery that *het-s* encodes a prion protein (11, 12) places this observation into a new perspective. The *het-s* locus, together with at least eight other loci, determines vegetative incompatibility in *P. anserina* (13, 14). Two alleles are found at this locus, termed *het-s* and *het-S*. Strains of *het-s* genotype occur as two phenotypes, the active, prion-infected [Het-s] phenotype and the neutral [Het-s*]

phenotype (15). Fusion of [Het-s*] and *het-S* hyphae leads to viable heterokaryotic mycelium, whereas anastomosis between [Het-s] and *het-S* hyphae leads to an incompatibility reaction resulting in cell death of the heterokaryotic cells (15, 16). The prion form of HET-s appears *de novo* in [Het-s*] mycelium at low frequency. Cytoplasmic contact between [Het-s*] and prion-infected [Het-s] mycelium results in complete transformation of the [Het-s*] mycelium into [Het-s]. This transformation reflects the cytoplasmic infectious character of the prion form of HET-s (11). [Het-s] was proposed to propagate as a self-perpetuating amyloid aggregated form of the HET-s protein (17–19). HET-s forms amyloid fibers *in vitro* (18), and ballistic introduction of recombinant HET-s protein in its amyloid form transforms [Het-s*] mycelium into infected [Het-s] (19). The prion infection in *P. anserina* can be reversibly cured. First, crossing a maternal *het-S* strain with a [Het-s] strain yields its *het-s* progeny in the [Het-s*] form (9). Secondly, regeneration of protoplasts derived from [Het-s] mycelium yields a low number of [Het-s*] mycelia (20).

During the sexual cycle, a single mating-type culture of *P. anserina* differentiates both male (microconidia) and female (protoperithecia) reproductive structures. The protoperithecium emits a specialized hypha called the trichogyne. Fertilization occurs when a microconidium fuses with a trichogyne of opposite mating type. After migration of the male nucleus down the trichogyne into the female ascogonium, individualized male and female nuclei divide synchronously in a syncytial structure. Because the male gamete contributes very little cytoplasm to this heterokaryon, the cytoplasm of the zygote is essentially of maternal origin. Nuclei of opposite mating type then pair up, and karyogamy takes place in specialized cells. Importantly, at this stage there is a transition from a syncytial to a cellular state (21). Meiotic progeny are linearly arranged as four binucleate spores per ascus. Ascospores are homokaryotic for all markers undergoing first-division segregation and heterokaryotic for markers showing second-division segregation (22). The *het-s* locus shows 10% second division segregation. Thus, in a *het-s* × *het-S* cross, 90% of the asci will contain two *het-s* and two *het-S* spores. Bernet reported that crossing [Het-s] as maternal parent with [Het-S] at 18°C yielded 5%–60% asci, with two normal and two aborted spores (9). This result occurred exclusively at 18°C when the maternal strain was of the [Het-s] phenotype and the paternal strain was *het-S*. Invariably, the surviving spores display the *het-s* genotype, indicating that the two aborted spores are of *het-S* genotype. In other words, in this cross in a variable proportion of the asci, *het-S* spores degenerate. Details

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Abbreviations: *het-S*, Het-(large)-S allele; *het-s*, Het-(small)-s allele; *het-s0*, Het-s0 knockout mutant; HET-S, Het-S protein; HET-s*, Het-s* protein (inactive); HET-s, Het-s prion protein (active); [Het-S], Het-S phenotype; [Het-s*], Het-s* phenotype (noninfected); [Het-s], Het-s phenotype (prion-infected); [Het-s0], Het-s0 knockout phenotype (no HET-s expression); *ect-het-S*, ectopically integrated *het-S* gene; *ect-het-s*, ectopically integrated *het-s* gene; *pGPD-het-S*, ectopically integrated *het-S* gene with a strong *GPD* promoter; *pGPD-het-s*, ectopically integrated *het-s* gene with a *GPD* promoter; -GFP, fused to GFP; DAPI, 4',6'-diamidino-2-phenylindole.

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Fig. 1. Genotypes and phenotypes of ascospores from a [Het-s] female $\times$ [Het-S] male, crossed at 18°C. All spores are homokaryotic at the *het-s* locus as a result of first-division segregation of the *het-s* alleles, which occurs in 90% of meioses. Aborted spores are indicated by a dagger. Percentages shown above are for early ripening asci; percentages below are for late-ripening asci. The latter are from perithecia allowed to grow older without releasing their spores.

about this system are elaborated in Fig. 1. Of particular relevance is the fact that *het-s* spores originating from asci in which *het-S* spore abortion occurred yield [Het-s] (prion-infected) mycelia whereas *het-s* spores originating from four-spored asci yield [Het-s*] (prion-free) mycelia.

The present study reexamines Bernet's observation in the light of the recent evidence that [Het-s] is prion-infected. We provide evidence that the HET-s protein in its prion form can be sexually transmitted and is responsible for specific *het-S*-spore abortion. As a consequence, the *het-s* allele encoding the HET-s prion protein acts as a meiotic drive element.

Materials and Methods

Strains and Culture Conditions. *P. anserina* strain S, isolated in 1937 in Normandy, France, was used as the genetic background strain in all experiments. *Het-s* and *het-S* heterokaryon incompatibility alleles were crossed into this strain S yielding the [Het-s*] and [Het-S] strains. The knockout mutant and several transformants were as described by Coustou *et al.* (11, 17, 23). The list of abbreviations and Tables 1 and 2 display an overview of all different *het-s/S* genotypes and phenotypes mentioned in this paper. Culture conditions and

genetic analysis methods are as described in ref. 22. Standard growth medium was Cornmeal agar. All crosses were performed on moistened copromes (24) placed on sterile filter paper on top of cornmeal agar by spermatization of monokaryotic strains with microconidia. The notation of all crosses mentioned in this paper is: $\text{♀} \times \text{♂}$ (the maternal strain printed first).

Genetic Analysis of Sexual Spores. Microscopic slides were prepared of the contents of perithecia, and spore numbers per ascus were determined microscopically. Vegetative compatibility was determined by confronting strains on solid Cornmeal agar. Details are described in refs. 15 and 23.

Fluorescence Microscopy. The content of ripe perithecia was carefully transferred to a microscope slide and examined under a Leica (Deerfield, IL) DMRXA fluorescence microscope with a Micro-max CCD (Princeton Instruments, Trenton, NJ). For detection of HET-s/S-GFP fusion protein signal, an FITC filter was used. Nuclei inside ascospores were stained with 0.2 $\mu\text{g}/\text{ml}$ 4',6-diamidino-2-phenylindole (DAPI) (in 0.14 M NaCl, 4 mM Na_2HPO_4 , 2 mM KH_2PO_4 , solution at pH 7.2) after 30-min fixation of fresh asci in 50%/50% formamide/water.

Results

The Prion Form of HET-s Is Required for *het-S* Spore Killing. Bernet's observations suggest that the HET-s protein in its prion form may be responsible for the abortion of *het-S* spores. We have repeated and expanded Bernet's experiments to test whether spore abortion is correlated with the presence or absence of [Het-s] in the ascus. The results of these experiments are shown in Table 1. Asci from several different crosses were analyzed, determining the frequency of asci with either one, two, three, or four mature spores. As in Bernet's observation, a significant percentage, 27% on average, of two-spored asci emerged in the [Het-s] $\times$ [Het-S] cross (Fig. 2C). The reciprocal [Het-S] $\times$ [Het-s] cross, and the control crosses, [Het-S] $\times$ [Het-S], [Het-s] $\times$ [Het-s] were analyzed in the same way and did not yield significant numbers of two-spored asci (Table 1).

Table 1. Ascus frequencies in crosses between strains with various *het-s* alleles

Maternal parent		Paternal parent		Asci analyzed	% asci containing:			
Phenotype	<i>Het-s</i> locus [†]	Phenotype	<i>Het-s</i> locus [†]		1 spore	2 spores	3 spores	4 spores
[Het-S]	<i>het-S</i>	[Het-S]	<i>het-S</i>	1,031	2	1	1	96
[Het-S]	<i>het-S</i>	[Het-s]	<i>het-s</i>	1,664	3	2	1	94
[Het-S]	<i>het-S</i>	[Het-s*]	<i>het-s</i>	331	5	5	7	83
[Het-s]	<i>het-s</i>	[Het-s]	<i>het-s</i>	515	5	5	7	83
[Het-s]	<i>het-s</i>	[Het-S]	<i>het-S</i>	2,157	4	27 [‡]	3	66
[Het-s*]	<i>het-s</i>	[Het-S]	<i>het-S</i>	845	7	8	9	76
[Het-S]	<i>het-S</i>	[Het-s0]	<i>het-s0</i>	737	4	5	10	81
[Het-s]	<i>het-s</i>	[Het-s0]	<i>het-s0</i>	501	6	9	12	73
[Het-s0]	<i>het-s0</i>	[Het-s0]	<i>het-s0</i>	1,101	6	7	9	78
[Het-s0]	<i>het-s0</i>	[Het-S]	<i>het-S</i>	495	4	6	11	79
[Het-s0]	<i>het-s0</i>	[Het-s]	<i>het-s</i>	1,523	6	7	12	75

All crosses were performed at 18°C.

[†]The allele at the *het-s* locus.

[‡]At 27°C 4% two-spored asci and 89% four-spored asci were observed in this cross.

Table 2. Ascus frequencies in crosses of strains with various *het-s* alleles at the normal locus × strains with ectopically (over-expressed) *het-s* alleles

Maternal parent			Paternal parent			% asci containing:				
Phenotype	<i>Het-s</i> locus [†]	Ectopic site [‡]	Phenotype	<i>Het-s</i> locus [†]	Ectopic site [‡]	Asci analyzed	1 spore	2 spores	3 spores	4 spores
[Het-S]		—	[Ect-het-s]			641	5	4	7	84
[Ect-het-S]			[Het-s]		—	665	2	3	3	92
[Ect-het-s]			[Het-S]		—	515	5	19	8	68
[Het-s]		—	[Ect-het-S]			732	5	19	9	67
[Het-s0]		—	[Ect-het-S]			521	3	4	6	87
[Ect-het-s]			[Het-s0]		—	544	5	6	7	82
[Het-S]		—	[pGPD-het-s]			2,556	5	10	9	76
[pGPD-het-S]			[Het-s]		—	1,609	6	7	9	79
[pGPD-het-S]			[pGPD-het-s]			944	7	11	10	72
[pGPD-het-s]			[Het-S]		—	1,227	7	86	4	3
[Het-s]		—	[pGPD-het-S]			1,267	4	6	3	87
[pGPD-het-s]			[pGPD-het-S]			1,221	10	13	8	70
[pGPD-het-s]			[Het-s0]		—	509	4	7	13	76

All crosses were performed at 18°C.

[†]The allele at the *het-s* locus.

[‡]The ectopic locus where the *het-s/S* gene construct is integrated.

In these standard sexual crosses of *P. anserina*, usually 2–7% of the asci contain one, two, or three normal spores (Table 1). These abnormal asci reflect the normal level of developmental errors in ascospore formation in this fungus. Consequently, spore killing due to the action and segregation of meiotic drive elements can be recognized only if it significantly exceeds this background level of spore abnormalities. The [Het-S] × [Het-s*], [Het-s*] × [Het-S] crosses also did not yield significant numbers of two-spored asci (Table 1). Fig. 2 compares asci from a [Het-s] × [Het-S] cross with a rosette from a [Het-s*] × [Het-S] cross. [Het-s*] × [Het-S] and [Het-s] × [Het-S] are completely identical in nuclear genotype. However, in the maternal [Het-s*], the HET-s protein is in its neutral non-prion form, whereas in the [Het-s] female, the HET-s protein is in its active prion form. These observations clearly demonstrate the necessity of the HET-s protein to be in its prion form for *het-S* spore killing to proceed. It is noteworthy that, in a [Het-s] × [Het-S] cross, the two *het-s* ascospores from asci containing four spores are [Het-s*] (Fig. 1), whereas the *het-s* spores in asci in which *het-S* abortion occurs display the [Het-s] phenotype. Here, the loss or absence of the prion form of HET-s coincides with absence of *het-S* spore killing.

Both the *het-s* and *het-S* Genes Are Required in *het-S* Spore Killing. No significant spore killing was observed in a sexual cross between a female [Het-s0][¶] (*het-s* knockout) strain, and a [Het-S] strain (Table 1). Moreover, the cross [Het-s] × [Het-s0] exhibited no spore killing either (Table 1). Whereas the cross [Het-s] × [Het-S] yielded 27% two-spored asci, the absence of the intact *het-s* or *het-S* gene in otherwise identical crosses gave a decrease of two-spored asci to

normal levels. Apparently, both *het-s* and *het-S* must be intact for killing to occur. A *het-s0* × *het-s0* cross results in asci with normal numbers of maturing ascospores (Table 1). So it seems that neither *het-s* nor *het-S* are essential for ascospore maturation, as already shown by Turcq *et al.* (25). The relatively high percentage of three-spored asci (Table 1) was a recurring feature when analyzing asci from crosses with one or two *het-s0* parents. The fourth “aborted” spore collapsed before final maturation, different from the early abortion observed in *het-S* spore killing (Fig. 2 B and C).

Killing of *het-S* Spores Can Be Restored When a *het-s* or *het-S* Gene Is Ectopically Integrated in a *het-s0* Strain. [Het-s] × [Ect-het-S]^{||} and [Ect-het-s] × [Het-S] crosses showed 19% two-spored asci (Table 2), whereas control crosses (no ectopically integrated *het-s/S*) did not show significant killing (Table 1). Clearly the *het-s* or *het-S* genes do not have to be expressed from the *het-s* locus to accomplish *het-S* spore killing. The reciprocal crosses and the [Het-s0] × [Ect-het-S] and [Ect-het-s] × [Het-s0] crosses did not show any significant spore killing (Table 2), disproving that mere ectopic presence of a *het-s/S* gene itself results in the observed 19% spore killing.

Elevated Levels of HET-s Prion Led to an Increase of *het-S* Spore Killing. In a *pGPD-het-s*^{||} strain, the HET-s protein is known to be at least ten times more abundant in vegetative mycelium, as compared with the wild-type *het-s* strain (11). Overexpressing *het-s* in such a *pGPD-het-s* strain strongly promotes the generation of the prion form of HET-s without impairing vegetative growth or the sexual cycle. The *het-S* locus shows ≈10% SDS, so in a *pGPD-het-s* × *het-S* cross ≈90% of all asci contain two spores with *het-S* nuclei next to

[¶]The *het-s0* knockout mutant was constructed by gene replacement (25) in *het-s*.

^{||}All ectopic *het-s* or *het-S* gene constructs were introduced in a *het-s0* strain (11, 23, 17).

Fig. 2. (A) [Het-s*] $\times$ [Het-S] rosette with normal asci. (B) [GPD-het-s] $\times$ [Het-S] rosette with all asci showing killing. (C) [Het-s] $\times$ [Het-S] asci, containing two transparent, aborted *het-S* spores and two black ripe spores.

two non-*het-S* spores. In a *pGPD-het-s* $\times$ *het-S* cross, the vast majority (86%) of the ripe asci contains just two mature spores (Table 2, Fig. 2B). Surviving ascospores, derived from 33 of these two-spored “killer” asci, all proved to be non-*het-S* spores. Clearly, the killed spores contained *het-S* nuclei. The ectopic *pGPD-het-s* is subject to over 50% SDS (H.J.P.D., unpublished data). Apparently, the high killing percentage in this cross reflects the high first division segregation (FDS) percentage of *het-S*. Neither in the reciprocal cross [Het-S] $\times$ [pGPD-het-s] nor in the cross [pGPD-het-s] $\times$ [Het-s0] was significant spore killing observed (Table 2). Thus, overexpression of *het-s* on its own does not lead to high spore-killing percentages.

The HET-s Prion and the HET-S Protein Are Present in Young Aborting Ascospores. Killed *het-S* spores are halted in their maturation some time after meiosis, when young developing ascospores are already clearly visible (Fig. 2C). Fluorescence microscopy studies of asci from crosses between a *het-s* and a *het-S-GFP* strain (17) revealed a GFP signal in either two or four of these young spores in the majority of the asci (Fig. 3D). The signal was mostly homogeneous and diffuse, indicating the presence of a soluble HET-S-GFP protein. In many spores, the signal was brighter in one or two spherical areas. These areas corresponded to the localization of DAPI-stained nuclei inside the spores (Fig. 3C). In a *pGPD-het-s-GFP* $\times$ *het-S* cross, HET-s-GFP protein was detected in developing

and ripe ascospores in its aggregated prion form (Fig. 3A and B). The HET-s-GFP fusion protein apparently retains its activity in *het-S* spore killing. However, as can be seen in Fig. 3A, the *het-S* spore abortion frequency was lower than the 86% observed when using non-tagged overexpressed HET-s. Asci showing two nearly mature ascospores and two aborted spores generally showed GFP signal in the two mature spores (Fig. 3A, I). However, some (nearly) killed spores still contain some fluorescent HET-s-GFP aggregates (Fig. 3A, II and B). The GFP signal could not be detected in every ascus (Fig. 3A, III). Interestingly, the first time after meiosis at which HET-S-GFP or HET-s-GFP signal could be detected in young ascospores coincided with the first time young *het-S* spores manifest abortion.

Results above show that both the HET-s and HET-S protein are present at the time and place of *het-S*-spore abortion. Furthermore, looking at the GFP signal in Fig. 3A and B, the condensed, intense, and particle-like nature of the signal indicates that the HET-s-GFP protein was in its prion form (17).

The Prion Form of HET-s Rarely Invades the Sexual Cycle When the Male Is the Sole Prion Donor. In a [Het-s0] $\times$ [Het-s] cross, penetration of the prion into the sexual cycle was investigated by analyzing the progeny. Using [Het-s0] as female excluded any possible prion propagating or neutralizing interference that a *het-s* or *het-S* female could assert. Among 280 ascospores from [Het-s0] $\times$ [Het-s], 8 (3%) were [Het-s]; the rest were either [Het-s*] or [Het-s0]. So, 3% of prion-infected [Het-s] males successfully introduced the HET-s prion into the sexual cycle. This finding explains the absence of significant *het-S* spore killing in all [(pGDP-) Het-S] $\times$ [(pGDP-) Het-s] type crosses (Tables 1 and 2).

Overexpression of *het-S* Influences the Process of *het-S* Spore Killing. The fact that *het-S* expression, whether at its original or at an ectopic locus, is required for a spore to be aborted suggests that *het-S* spore killing could result from interaction between the HET-s prion and the HET-S protein rather than between the prion and the *het-S* locus.

Raised levels of HET-S in the [Het-s] $\times$ [pGPD-Het-S] cross (Table 2) lowered the percentage of asci with *het-S* spore killing

Fig. 3. (A and B) Rosette and ascus from [pGPD-het-s-GFP] $\times$ [Het-S] cross. (A) Rosette displaying (I) asci with two prion-GFP aggregate infected mature spores next to two small killed spores, in which the prion-GFP aggregates already had degraded and (II) asci in which the two smaller spores that are being killed show prion-GFP aggregates. (III) Ascus with two mature and two killed spores, all without GFP signal. (B) Ascus with clear prion-aggregate-GFP signal in the two smaller, young spores that are being killed. (C and D) [Het-s] $\times$ [Het-S-GFP] asci. (C) DAPI-stained nuclei within young asci and ascogenous hyphae. (D) Green fluorescent GFP signal showing HET-S-GFP fusion protein expressed in young developing ascospores.

Fig. 4. Proposed model of the dynamics of HET-s prion inheritance and *het-S* spore killing. Depicted from left to right is the sequential formation of asci of a maternal [Het-s] × [Het-S] cross. Some time after fertilization, hook cells and ascogenous hyphae are formed; karyogamy is then followed by meiosis, after which ascospores develop and mature (21). Just before hook cell formation, HET-s(-GFP) and HET-S(-GFP) expression stops (unpublished microscopic data). Expression starts again after meiosis, when spores start maturing. So transmission of prion aggregates, synthesized before hook cell formation, determines which and how many asci will be infected after meiosis. The particle-like nature of these prion aggregates results in a limited number of prion infection seeds entering the first formed hook cells. The prion aggregates are then distributed in a stochastic way among the sequential hook cells that are formed from this initial hook cell. At some point, there are no aggregates left. Consequently, HET-s prion infection is restricted to the first formed ascogenous hyphae (type I). In later stages of development, these seeds of infection are enclosed in young, maturing spores. Enclosure of these prion seeds in *het-S* spores eventually yields infected [Het-s] spores. HET-S protein and the HET-s prion are known to interact when both are present in the same cytoplasm (11). Interaction between HET-s prion and HET-S is toxic. When prion aggregates are trapped in a young *het-S* spore, accumulated exposure to this toxic reaction ultimately leads to the abortion of the *het-S* spore. Depicted on the far right of the picture are the two types of asci that arise, the HET-s prion-infected type I, containing two aborted spores and two [Het-s] spores and the uninfected type II ascus, containing two [Het-s*] and two [Het-S] spores.

from 27%, observed in the [Het-s] × [Het-S] cross, to 6%. In the cross [pGPD-*het-s*] × [pGPD-*het-S*], spore killing was 13% (Table 2) as compared with 86% in [pGPD-*het-s*] × [Het-S]. Possibly, the HET-S protein itself is actively involved in the mechanism of *het-S* spore killing.

Do *het-s* + *het-S* Heterokaryons Survive? On average, some 70% of asci show no spore killing in the cross [Het-s] × [Het-S]. As *het-s* shows 10% SDS, only a small proportion of these four-spored asci are potentially *het-s* + *het-S* heterokaryons. However, in the *pGPD-het-s* × *het-S* cross, 3% (Table 2) of the asci contained four ripe spores, the normal number. The progeny of 16 such asci were analyzed. Heterokaryon incompatibility tests revealed the following ascospore genotypes: (*pGPD-het-s* + *het-S*), *het-S*, and *het-s0*. The mycelium germinating from a (*pGPD-het-s* + *het-S*) spore is [pGPD-*het-s** + *het-S*]. So, by neutralizing the prion into the non-prion HET-s*, these heterokaryotic spores survive. The possibility of [pGPD-*het-s** + *het-S*] viable mycelium was also observed by Coustou *et al.* (23). All viable [Het-S] spores from this cross exclusively arise next to [pGPD-*het-s** + *het-S*] spores. Again, absence of the prion form of HET-s results in HET-S spore survival.

Discussion and Conclusion

Bernet (9) reported that, in a [Het-s] × [Het-S] cross, a fraction of the *het-S* progeny degenerates. We now show that the *het-s* and *het-S* genes are both required for *het-S* spore killing and establish that HET-s has to be in its prion state for killing to occur. We demonstrate that killing can also occur when the *het-s* or *het-S* genes are ectopically integrated and that elevated concentrations of HET-s protein in its prion form dramatically increase killing of *het-S* spores, and we detected the presence of HET-s and HET-S proteins within young maturing or aborting ascospores. Considering the evidence presented above, it is plausible to conclude that the HET-s protein, in its prion form only, is essential for killing. Additionally, overexpression of HET-S causes a decline of spore killing to near background levels in crosses that otherwise exhibit significant spore killing. This finding indicates that the role of the HET-S protein is not merely a passive one. The HET-S protein also asserts a dosage-dependent effect, potentially neutralizing the active form of the HET-s prion.

This study provides clarification on a number of issues that go beyond Bernet's early description of the *het-S* spore abortion phenomena. Segregation distorters are often associated with ex-

tensive genomic rearrangements and are often inherited as haplotypes resulting from inhibition of recombination at the distorter locus. It is known that the *het-s* and *het-S* loci differ in genomic organization (25) and that all *het-s* strains contain a transposon long terminal repeat upstream and a 2-kb insertion, respectively, upstream and downstream of the *het-s* ORF. It was therefore conceivable that spore killing was not due to *het-s* but due to a closely linked genetic element. This possibility is now formally ruled out by the demonstration that inactivation of *het-s* by gene replacement abolishes *het-S* spore killing and that *het-S* spore killing can occur when *het-s* and *het-S* are expressed from ectopic loci.

What could be the mechanism of spore abortion? The *het-S* spore abortion mechanism bears much resemblance to the *het-s/het-S* vegetative incompatibility reaction. It is known that vegetative interaction between HET-s in its prion form and HET-S leads to cell death. This result is presumably also what happens during the sexual stage. One proposed model explaining this toxicity would be that HET-S interferes with HET-s amyloid aggregation, which leads to accumulation of toxic oligomeric aggregates (17).

Three major characteristics define a spore-killing system (26, 27): spores exclusively containing sensitive nuclei are aborted, spores containing a nucleus with the killer gene survive, and a killer nucleus protects a sensitive nucleus when contained within the same ascospore. The *het-S* spore-killing system is also subject to these rules. Homokaryotic spores containing exclusively *het-S* nuclei are aborted, and homokaryotic spores with *het-s* or *pGPD-het-s* nuclei survive as do heterokaryotic spores with both *pGPD-het-s* and *het-S* nuclei. As the *pGPD-het-s* gene is subject to >50% second-division segregation, one would expect more than the observed 3% asci in which four spores survive: two heterokaryotic spores with sensitive (*het-S*) + killer (*pGPD-het-s*) nuclei next to two spores with killer + knockout (*het-s0*) nuclei. Clearly, mere presence of a killer gene does not prevent spore abortion here. Interestingly, neutralization of the killer prion into its non-killer, non-prion form is what makes the 3% sensitive (*het-S*) + killer (*pGPD-het-s*) spores survive.

Two major differences distinguish *het-S* spore killing from the *Neurospora* spore-killing system. First, *het-S* spore killing is unidirectional. The female must be [Het-s] and the male [Het-S]. Second, *het-S* spore-killing frequencies do not reflect first-division segregation frequencies of *het-s*. At 28°C, *het-S*-spore killing is completely absent.

Involvement of HET-s in its prion form only leads to killing of *het-S*-spores. We observed some transmission of the prion by the

male parent. Transmission was so infrequent that the number of aborted spores was insignificant in [Het-S] × [Het-s] crosses. The spore killing in [Het-s] × [Het-S] crosses reflects the fraction of asci infected with the HET-s prion. Apparently wild-type *het-s* expression levels are not sufficient to infect all asci. By overexpressing the prion some 10-fold, practically all asci are infected, resulting in nearly all *het-S* spores being killed. Killing of *het-S* spores depends on dosage of the HET-s prion. Overexpression of HET-S or growth at higher temperatures prevent *het-S* spore killing, probably by preventing or neutralizing prion infection of asci. The mechanism behind this protection remains to be investigated, although it has been observed that growth at 18°C stabilizes the [Het-s] stage in *het-s/het-S* self-incompatible transformants (23). The presence of an ectopically inserted DNA sequence can lead to MSUD (meiotic silencing of unpaired DNA) in *Neurospora* (28). The observed expression of *het-S*-GFP some time after meiosis in young ascospores could be the result of an MSUD process acting in *Podospora*. It will be necessary to reinvestigate the potential of this process by using strains that are more suitable to address this question.

In yeast, both the [PSI] and [URE3] prions are relatively unstable during meiosis (29). For [PSI], elevated levels of HSP104 during sporulation have been proposed to be a possible cause for this instability. In *Podospora*, maternal [Het-s] is lost in a fraction of the asci during its anisogamous sexual stage whereas [Het-s] is never lost during vegetative mitotic divisions. Occasional loss of maternal [Het-s] could be explained by taking into account that, before meiosis, there is a transition from a syncytial to a cellular state. In Fig. 4, we propose a mechanistic model that explains *het-S* spore killing as a consequence of vertical prion transmission. The cytoplasm of the ascogone is gradually compartmentalized into individual cells. Thus, in this case, when [Het-s] is lost in an individualized dikaryotic cell, there can be no reinfection by adjacent particles as occurs during the syncytial vegetative stage. This model might also explain why killing is more frequent among the first asci to ripen as compared with asci that develop later. In the model, the spore abortion or killing mechanism exclusively kills *het-S* spores in already prion-infected asci. Later, maturing asci correspond to cells that have undergone more divisions and will have had additional opportunities to lose the prion. From this point of view, it can readily be explained how overexpression of HET-s, in a [pGPD-Het-s] × [Het-S] cross, leads to far more ascogenous hyphae being infected and consequently to much higher *het-S* spore-killing frequencies. Contrary to the absence of spore killing in the [Het-s] × [Het-S] cross at 28°C, the [pGPD-Het-s] × [Het-S] displays high-frequency spore killing at this temperature (unpublished data). Together, these observations regarding temperature dependence, dosage effect, and variation in killing efficiency suggest that the fraction of asci that are infected by the HET-s prion, when spore maturation starts, ultimately determines the *het-S* spore-killing frequency.

Although segregation distortion has been studied in various eukaryotes, the molecular mechanisms of meiotic drive have remained largely elusive. The present study identifies the molecular components of a meiotic drive system in *Podospora*. The *het-s* allele is the driver element, by virtue of the prion protein it encodes, and the *het-S* allele is the trans-acting target. We propose that the mechanism of spore killing relies on the same molecular interactions that cause cell death by incompatibility during the vegetative stage. By killing *het-S* spores, the prion form of HET-s makes the *het-s* gene act as a meiotic drive element. The non-prion form of HET-s does not show this behavior, so it is truly the prion form that is responsible for this segregation distortion. The fact that 51% of the local *P. anserina* population (102 collected strains) around the town of Wageningen is [Het-s], 40% is [Het-S], and 9% is [Het-s*] (H.J.P.D., unpublished data) implies that *het-S* spore killing may occur in nature whenever outcrossing takes place. Prevention of amyloid formation has been proposed to represent a major constraint imposed on the evolution of protein sequences (30). This finding implies that variants leading to amyloid formation should be rapidly expurged from populations. Considering this result, it is striking that the amyloid-forming HET-s variant is so abundant in natural *Podospora* populations. It is not clear what determines the high frequency of the prion-encoding allele *het-s* in this population. Theoretically both phenotypes associated with the *het-s/het-S* polymorphism, heterokaryon incompatibility, and meiotic drive may affect the frequencies of the alleles. Assuming adaptive significance of heterokaryon incompatibility, balancing selection retaining the polymorphism over long time periods is plausible (31, 32). It is hard to see how the meiotic drive associated with spore killing may have adaptive significance for the fungus, as it effectively lowers fertility. It is therefore probably better regarded as a “Prion Disease,” following Couzin (33), who points out that prions in fungi do not kill an infected organism, as they do in mammals. Population genetic model analysis (34) suggests that spore killing may significantly influence the frequency of occurrence of the prion-encoding allele in fungal populations. It can thus be envisioned that the [Het-s] prion represents a selfish element promoting its own maintenance, not only at the protein level but also at the genetic level by actively destroying progeny bearing the non-prion variant (*het-S*).

In mammals, “vegetative” prion transmission by ingestion or infection has been observed to propagate prion diseases (35). In this study, we have demonstrated that, in the nonisogamously mating fungus *P. anserina*, prions of maternal origin are transmitted through a sexual pathway resulting in meiotic drive, with potential consequences for the level of prion infection in *Podospora* populations.

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