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A Story Between s and S: [Het-s] Prion of the Fungus *Podospora anserina*

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ABSTRACT
In filamentous fungi, vegetative cell fusion occurs within and between individuals. These fusions of growing hyphae (anastomosis) from two individuals produce binucleated cells with mixed cytoplasm known as heterokaryons. The fate of heterokaryotic cells was genetically controlled with delicacy by specific loci named *het* (heterokaryon) or *vic* (vegetative incompatibility) as a part of self-/nonself-recognition system. When *het* loci of two individuals are incompatible, the resulting heterokaryotic cells underwent programmed cell death or showed severely impaired fungal growth. In *Podospora anserina*, *het-s* is one of at least nine alleles that control heterokaryon incompatibility and the altered protein conformation [Het-s] prion. The present study describes the [Het-s] prion in terms of (1) the historical discovery based on early genetic and physiological studies, (2) architecture built on its common and unique nature compared with other prions, and (3) functions related to meiotic drive and programmed cell death.

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1. More than a single protein: What is prion?

The term "prion" was coined by Stanley B. Prusiner in 1982 [1]. The letters denote "proteinaceous" (an entity composed solely of proteins with no associated nucleic acids), "infectious" (capable of being transmitted), and "~on" (a relatively small particle). Early knowledge of prions was based on studies of scrapie disease in sheep and goats [2,3]. Subsequently, these scrapie agents were shown to cause prion diseases or transmissible spongiform encephalopathies (TSEs) [4]. The causal agents of bovine spongiform encephalopathies (Mad Cow Disease), scrapie, and human TSE (i.e., Creutzfeldt–Jakob disease and its variants) are all based on the abnormal form of the normal cell surface prion protein (PrP) [5,6]. Most prions can propagate as biophysically very stable amyloid (β – sheet rich filamentous protein polymers) by a self-templating mechanism [7–9]. Many amyloid-based human neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, type II diabetes, and amyotrophic lateral sclerosis, share common aspects with PrP-based prion diseases [10].

These prion diseases were likely present within mammals, including humans, long before the term suggested in 1982. However, several prions in eukaryotic microorganisms were first discovered as non-chromosomal genetic elements [11–13]. Two non-Mendelian genetic elements, [PSI+] and [URE3] and the prion form of the normal proteins Sup35p/eRF3 and Ure2p, respectively, of *Saccharomyces cerevisiae* were reported to be prion analogs based on three genetic criteria [14]. The first criterion is reversible curability, in which prions can arise again spontaneously at a low frequency after curing. The second criterion is elevated frequency of prion generation by PrP overproduction. Prions are an altered form of normal PrP. This altered protein catalyzes and supports prion formation. Overproduction of normal PrP leads to an elevated frequency of prion generation, which is immediate evidence of being a prion. The third criterion is phenotypic relationships. Prion propagation depends on the presence of normal PrP. Once the protein is altered to form a prion, its phenotype becomes similar to that of a loss-of-function mutant of the normal PrP. This could also be direct evidence of a prion. Since the first report of two prions in yeast, at least 10 prions have been reported and characterized in yeast [15].

The older notion that prions are predominantly present in eukaryotes, such as fungi, and mammals, including humans, was negated by the first discovery of a bacterial prion in 2017 [16]. The global

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transcription terminator Rho of the bacteria *Clostridium botulinum* is converted into an alternative conformation (prion form: [RHO-*X*-C+]) [16]. The highly conserved prokaryotic transcription regulator Rho, with hexameric helicase activity, was demonstrated to behave as a prion, able to self-propagate and aggregate in another well-known bacterium, *Escherichia coli*, and in *S. cerevisiae*; the latter has become a model fungus for prion studies. The structural PrP expressed in *E. coli* was initially screened for its potential as a prion based on a previously hidden Markov model-based algorithm designed for a systematic survey to identify yeast proteins capable of converting the prion form [17]. This sets this prion apart from prions in other organisms that are typically discovered based on observable phenotypic traits. Additionally, the single-stranded DNA-binding protein of *Camplyobacter hominis* (*Ch* SSB) can transit into two distinguishable forms: prions and non-prions [18]. The domain involved in prion formation is an internal disordered linker between the N-terminal DNA-binding domain and C-terminal acidic tip of *Ch* SSB. This domain adopts an aggregated prion-like conformation. The maintenance of this conformation depends only transiently on the presence of caseinolytic peptidase B, the bacterial ortholog of the yeast disaggregase heat shock protein 104 [18]. Two bacterial prions (or prion conformations) have been identified using a heterologous expression-based prion detection system rather than their own endogenous system. These recent findings provide substantial evidence suggesting that the appearance of bacterial prions or prion-like phenomena may have occurred even before the evolutionary divergence of bacteria and eukaryotes [19].

The existence of an array of fatal disease-causing prions in mammals and pathogenic yeast prions [PSI+] and [URE3] indicates that these prions are not “favorable” elements for these hosts [20]. However, this does not mean that all prions are necessarily harmful elements. For example, the [Het-s] prion of the filamentous fungus *Podospora anserina* has a well-defined function as a mediator of heterokaryon (vegetative) incompatibility in self-/nonself-recognition systems [9].

2. Discovery of “Petit s”: where the story began

Filamentous fungi generally grow as a long, branched filamentous structure termed hyphae. Hyphae are comprised of one or more cells surrounded by thick tube-shaped cell walls. In most higher true fungi, these hyphae are divided by internal cross-sectional cell wall septa. Hyphae grow at their tips by extension of the cell wall. These growing hyphae can branch by hyphal fusion (anastomosis) of somatic cells between strains, resulting in the formation of vegetative heterokaryons. However, the formation of multinucleate cells containing genetically different nuclei (heterokaryons) is genetically controlled by a set of *het* genes that exist with at least two or more polymorphic allelic variants as a crucial part of the fungal self-/nonself-recognition system [21]. The viability or fitness of fungal heterokaryons with different nuclear types derived from fused hyphae is guaranteed when both hyphae (or strains) have compatible *het* gene constitutions. Genetic differences in at least nine *het* loci in *P. anserina* lead to incompatibility, indicating that heterokaryons cause cell death or severe growth defects. This heterokaryon incompatibility between incompatible strains provides a barrier to block the transmission of harmful cytoplasmic elements, such as RNA replicons, fungal viruses, and DNA plasmids, or allows preservation of genetic individuality. In *P. anserina*, the incompatibility reaction creates an abnormal contact line termed the “barrage phenotype” as an indicator of programmed cell death which can be easily detected by confrontation of two strains on a solid medium. This simple phenotype-related genetic method has been used to classify fungal strains into vegetative compatibility groups, with all strains being compatible in each group.

The [Het-s] prion of *P. anserina* was originally identified as a non-Mendelian genetic element in 1952 by Georges Rizet, a French geneticist and founder of the French Fungal Genetics school [11]. While studying the self-/nonself-recognition process by observing the “s” phenotype (later renamed as [Het-s], barrage reaction, which is incompatible with the S strain) between strains that were originally compatible, Rizet found an unconventional phenotype in “*het-s*” progeny comprising segregants derived from the sexual cross between the *het-s* and *het-S* strain. The resulting s progeny displayed an unexpected “s^S” phenotype (later renamed [Het-s*], which is compatible with s and S strains), rather than the “s” phenotype [11]. Rizet also discovered maternal inheritance of the progeny from a cross between S and s^S strains [11]. He concluded that the strain harbors a cytoplasmic heritable particle that is absent or exists in a modified form in the s^S strain, and which disappears upon mating with the *het-S* strain (Figure 1).

Janine Beisson, a pioneer of cytoplasmic heredity [22], examined the reversion of s^S to S in her PhD work in the Rizet lab. She found that cytoplasmic contact is required for the reversion process which is

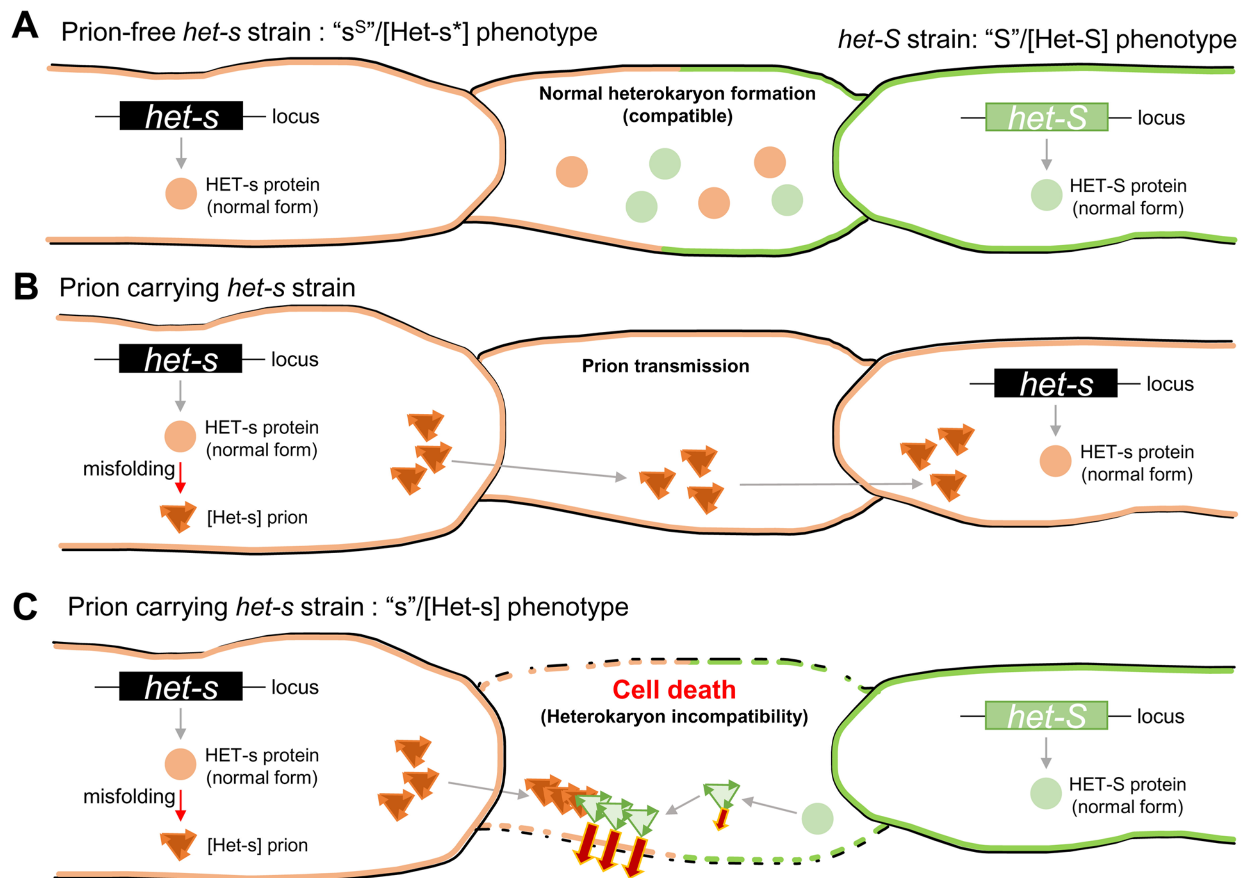

Figure 1. *het-s/S* alleles based heterokaryon incompatibility system in *Podospora anserina*. (A) When a prion-free *het-s* strain ([Het-s*] strain) fuses with a *het-S* strain ([Het-S] strain), results in viable heterokaryotic cells; heterokarton compatibility. (B) In the *het-s* strain, HETs can exist in at least two different forms: a soluble monomeric (normal) form and high-molecular-weight aggregated (or prion amyloid) form. This conformational change (transition to the [Het-s] prion form) of the HET-s protein occurs spontaneously and results in the [Het-s] prion being transmissible. (C) When a [Het-s] prion-carrying strain was fused with a *het-S* strain, the fused cells undergo cell death. Details are described in the section entitled "[Het-s] prion has functional amyloid."

10 times faster than the normal radial growth of hyphae [23]. Beisson proposed that a non-Mendelian genetic "s" element can control its own formation, explaining its cytoplasmic transmission and maternal inheritance. Although the fundamentally important findings by Rizet and Beisson revealed the genetics and physiology of the non-Mendelian genetic "s" element, many details of this element remain to be discovered.

Advanced DNA transformation methods in the late 1980s enabled examination of the genetic properties of *het-s* and *het-S* genes. Joël Bégueret, a former PhD student of Rizet, constructed a *P. anserina* strain with an inactive s allele (*het-s*^{X}). Strains with this *s*^{X} allele were compatible with s and S strains same as "s^{S}" phenotype ([Het-s*]) [24,25]. Bégueret also demonstrated that *s*^{X} strains were unable to convert the "s" phenotype, indicating that "s" and "s^{S}" phenotypes are directly linked with *het-s* gene. The nomenclature of the heterokaryon incompatibility of *P. anserina* are presented in Table 1.

Table 1. Nomenclatures of [Het-s] prion related alleles, proteins, and phenotypes in *Podospora anserina*.

Notion	Description
het-s	*het-s* allele or locus
het-S	*het-S* allele
HET-s	A protein encoded from *het-s* allele, normal form state
HET-S	A protein encoded form *het-S* allele
[Het-s] "s"	Phenotype of [Het-s] prion carrying *het-s* strain, prion per se, incompatible with [Het-S]/"S"
[Het-s*] "s^{S}"	Phenotype of prion-free *het-s* strain, non-prion per se, compatible with both [Het-s]/"s" and [Het-S]/"S"
[Het-S] "S"	Phenotype of *het-S* strain, incompatible with [Het-s]/"s"

3. Proof of identity: [Het-s] is a prion form of HET-s protein

Even before the seminal discovery by microbial prions by Reed B. Wickner, the prion hypothesis for [Het-s] had been proposed by *het-s* researchers, including Carole Deleu [26]. In 1997, the HET-s protein was reported to behave as a prion based on simple and powerful approaches [27]. The demonstrations of the same expression levels of *het-s* and

the amount and apparent size of HET-s protein in [Het-s] and [Het-s*] strains (HET-s and HET-s*) indicate that the different reactivities to *het-S* strains are due to post-translational differences. The differences reflect different biochemical properties of the protein in each strain. For example, the HET- strain has proteinase K resistance and forms multimeric aggregates. This differential proteinase K sensitivity of HET-s and HET-s* is reminiscent of previous result of prions ([URE3] and [PSI+]) and PrP (Ure2p and Sup35p) in yeast [28,29].

A simple genetics-based experiment to test the transmission of [Het-s] prion in donor strain to recipient [Het-s*] strains or [Het-s°] strains (null mutant of *het-s* gene, same as s^X strains) was performed in the absence and presence of the translation inhibitor cycloheximide. The findings demonstrated that [Het-s] prion is capable of propagating in [Het-s*] strains, but not in [Het-s°] strains, indicating that the propagation depends on the transition of preexisting HET-s* into active HET-s form ([Het-s] prion) [27]. Our findings also supports the previous notion that *het-s* is required for the propagation of the [Het-s] prion [27], implying that *het-s* is a structural gene of the [Het-s] prion. Therefore, the increased frequency of the [Het-s] prion by the overproduced *het-s* gene, indicates that the [Het-s] element, whose identity had been unclear for 35 years, is evidently a prion in *P. anserina* [27].

4. Architecture portfolio of HET-s: From genetics to biophysics

Proteins are biochemical molecules that exist in different structural states, from polypeptides to properly folded or multimeric states, with biological activities for their particular functions. Even proteins with abnormal forms (typically misfolded) can exist in various forms, from monomers to multimers, such as amorphous aggregates and structurally organized amyloids. Most structurally characterized fungal prions are able to self-propagate in cells in the filamentous β-sheet-rich polymer form (amyloid) of normal PrP [29–32]. As a protein of the [Het-s] prion, HET-s can form a homodimeric complex (self-assembled form), as shown by the yeast two-hybrid system [27]. Moreover, HET-S, which is encoded by the incompatible *het-S* allele, can self-assemble, and HET-s and HET-S proteins can interact to produce heterodimers [27]. Protein-protein interaction analysis findings include the starter, a harbinger of the architectural discovery of the [Het-s] prion, and a catalyst foreshadowing excellent achievements that are not limited to the architecture of the [Het-s] prion.

Although genetics-based approaches have already revealed that HET-s could be present in two different states, with size exclusion chromatography and fractionation-based protein analysis providing direct evidence that the protein can exist in two biophysically different states: a soluble monomeric form in [Het-s*] strains and high-molecular-weight aggregated form in [Het-s] strains [33]. Visualization of highly expressed HET-s by green fluorescent protein tagging also confirmed the aggregation of the [Het-s] prion protein *in vivo* in the mycelia and protoplasts [33]. Moreover, *in vitro*, recombinant HET-s protein can reportedly aggregate by self-seeded polymerization. Electron microscopy examination has revealed that the HET-s protein aggregates to form unbranched amyloid-like structures 15–20 nm in width and several micrometers long [34]. When amyloid-like HET-s protein aggregates are introduced into [Het-s*] strains by biolistic transformation, the transition of the [Het-s] prion in the transformants is highly elevated, indicating that the amyloid aggregates of the HET-s protein are infectious, fulfilling the protein-only hypothesis for prions [35].

Infrared spectroscopy of the biophysical features of the aggregated form of HET-s protein has revealed a relatively high content of β-sheet with a proteinase K resistant core fragment of ~7 kDa [34]. This resistant-core was identified as the C-terminal part of the HET-s protein ranging from amino acid residues 218–289; this region retains the ability to form an infectious unbranched fibrillar amyloid structure ([Het-s] prion generation), similar to amyloids consisting of full-length HET-s protein [35,36]. The lack of the C-terminal part reportedly prevents *in vitro* aggregation of HET-s proteins and fails to support [Het-s] prion propagation *in vivo* [36]. Taken together with the failure of the barrage reaction using strains lacking the C-terminal region of HET-s, HET-$s^{218\text{-}289}$ serves a prion domain (PrD) required for prion formation and propagation, which are inevitably connected to the incompatibility function [36].

Solid-state nuclear magnetic resonance and mass-per-length measurements have revealed the structure of amyloid filaments in the PrD of HET-s [37]. This amyloid forming part (PrD, HET-$s^{218\text{-}289}$) adopts a left-handed β-solenoid structure comprising two repeated helically wound motifs. Each repeated motif consists of four β-strands; the first three form a triangular hydrophobic core and fourth sticks out from the core [37]. The molecular structure of HET-$s^{218\text{-}289}$ is unique and uniform. Most mammalian and yeast prions are heterogeneous in biology and pathology (strains/variants) [38–43]. This

heterogeneity is thought to be correlated with the variable structures of propagating amyloids derived from the same prion protein. However, the HET-s amyloid is quite uniform, suggesting that the [Het-s] prion has a distinctive feature, as discussed below, compared to other pathogenic prions in mammals and yeast [9].

5. [Het-s] prion is a functional amyloid

Amyloids have long been considered key features of many human diseases such as protein-based prion diseases, Alzheimer's disease, Parkinson's disease, amyotrophic lateral sclerosis, and type II diabetes [44]. Structural determination of infectious amyloids of yeast have revealed their folded, in-register, parallel β-sheet structure, which is similar to the amyloid structure of human disease related amyloid [45]. However, not all amyloids, including prions, have detrimental effects on host cells [46]. Thus, amyloids of the [Het-s] prion have been counted as functional prions that are beneficial to the host fungus based on clear and convincing evidence [46].

In 1952, Rizet noted that the [Het-s] phenotype was converted to [Het-s*] after sexual crossing with *het-S* strains. He described that the S factor can inactivate [Het-s] (transition to [Het-s*]/originally "s^{S}") indicating modification of "s" by "S" [11]. Jean Bernet, found an unusual phenotype of [Het-s] phenotype during the sexual cycle at low temperature (18 °C) [47]. Sexual crosses of maternal [Het-s] and parental [Het-S] reportedly provided two aborted spores, instead of four spores, in approximately 20% of the asci. In these two spores containing asci, the surviving spores were always of the *het-s* genotype and [Het-s] phenotype. However, in four fully living spores containing asci, the phenotype of *the het-s* strain was always [Het-s*] [47,48]. These observations clearly indicate that this genetic interaction between two antagonistic alleles (*het-s* and *het-S*) inhibits the [Het-s] prion, and triggers cell death or severe growth defects of heterokaryons formed between the two.

HET-S is 97% identical to HET-s, differing by 13 amino acids, with an identical domain structure with HET-s, comprising an N-terminal HeLo globular domain (residues 1–227) and C-terminal β-solenoid forming PrD (residues 218–289) [25,36]. These 13 different amino acids include a single amino acid substitution in HET-S (H33P) that alters the reactivity to the [Het-s] prion, and two amino acid substitutions in HET-s (D23A and P33H) that lead to the loss of spontaneous prion conversion [49]. While purified HET-s can form fibrils [34], recombinant HET-S does not form fibrils *in vitro* but inhibits HET-s fibril formation *in trans* and co-localizes with [Het-s] prion aggregates *in vivo* [50]. Taken together with results concerning the loss of HET-S activity by deletion [36] or mutation [50] of C-terminal PrD, the molecular interaction of HET-S and [Het-s] prion involves a structural adoption of HET-S PrD into the β-solenoid folds by the [Het-s] prion fibrils. This β-structure of HET-S leads to a conformational change of the HeLo domain [50]. The activated HeLo domain induces HET-S oligomerization [51]. In heterokaryon cells, HET-S oligomers/aggregates relocalize to the cell periphery and do not colocalize with [Het-s] prion aggregates [52], triggering cell death *via* cellular membrane leakage [51]. A short region (3–23) of the N-terminus of NWD2 (NATCH and WD repeat domain-containing protein) located immediately adjacent to *the het-S* locus reportedly has structural homology with the PrD of HET-s [53]. The NWD2 (3–23) peptide was able to form amyloid fibrils *in vitro* and adopted β-solenoid folds such as HET-s/S [54]. Moreover, green fluorescent protein-fused NWD2 (1–30) can induce [Het-s] prion formation and co-localizes with [Het-s] prion aggregates. NWD2 (1–30) also can lead to incompatibility by relocalizing HET-S to the membrane of heterokaryon cells [54]. In turn, the nucleotide-binding oligomerization domain-like receptor NWD2 for fungal signal transduction can activate the cell death effector HET-S *via* the same mechanism as the [Het-s] prion [55].

Population genetics-based analysis of [Het-s] prions in the wild also supports the notion that [Het-s] is a beneficial prion because of its clear role in preventing heterokaryon formation to secure the genetic stability of individuals. The frequency of this prion is produced from the equilibrium frequency between the spread by outcrossing or spontaneous gain and natural selection by the impaired survival of the host or spontaneous loss. To study [Het-s] prion frequency, a natural population of 112 *P. anserina* was genotyped for the allelic distribution of *the het-s/S* locus, showing 72 strains with *het-s* and 40 strains with *het-S* [56]. This biased distribution was derived from the meiotic drive of [Het-s] associated with the killing of *het-S* spores [47,48]. Among the *het-s* strains, 66 carried the [Het-s] prion (91.6%), showing a strong bias compared to infection involving the detrimental pAL2-1 (37/72), a mitochondrial senescence plasmid as a selfish genetic element [56]. The high natural prevalence of the [Het-s] prion in the wild indicates that [Het-s] prion has beneficial and detrimental effects owing to its role as a meiotic driver and a heterokaryotic cell death trigger [56].

6. Concluding remarks

Questions regarding prion-mediated heterokaryon incompatibility in *P. anserina* and other filamentous fungi remain unanswered. As described above, somatic cell fusion events are common in filamentous fungi and controlled by specific loci (*het* or *vic*). The comparative sequence alignment of HeLo/HET domain in PSI-BLAST analysis revealed 35 alleles with similar features to HET-s/S PrD (1–38 residues) and four alleles with identical *het-S-* and *nwd2*-like architecture, indicating the conservation of the HeLo domain in filamentous fungi as pore-forming toxins [51]. However, such a prion-related system, or fungal prions in other filamentous fungi, have not yet been reported.

Microbes, such as fungi and bacteria, contain prions [15]. Despite the distinct prion nature of [Het-s] compared to other pathological prions in yeast and humans [9,45], a series of studies on the [Het-s] prion and its mechanisms of action in cells have undoubtedly expanded our understanding of prion biology and fungal physiology. The important studies on filamentous fungi presented in this review may be beneficial for combating protein conformational disorders in humans.

Disclosure statement

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