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Prion Dynamics and the Quest for the Genetic Determinant in Protein-Only Inheritance

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Abstract

According to the prion hypothesis, proteins may act in atypical roles as genetic elements of infectivity and inheritance by undergoing self-replicating changes in physical state. While the preponderance of evidence strongly supports this concept particularly in fungi, the detailed mechanisms by which distinct protein forms specify unique phenotypes are emerging concepts. A particularly active area of investigation is the molecular nature of the heritable species, which has been probed through genetic, biochemical, and cell biological experimentation as well as by mathematical modeling. Here, we suggest that these studies are converging to implicate small aggregates composed of prion-state conformers as the transmissible genetic determinants of protein-based phenotypes.

Introduction

The phenotype of an organism is the visible manifestation of its genetic identity. Historically, the molecular composition of this genetic information has been associated with nucleic acid determinants, which specify unique traits in chemical sequences. However, mounting evidence in a number of systems indicates that some proteins, known as prions, may also specify heritable phenotypes by encoding genetic information in their physical structures [1,2].

Once considered heretical, the prion or protein-only hypothesis provides a robust explanation for the transmission of a broad range of enigmatic traits (Table 1). In mammals, the prion protein PrP is believed to be the causative agent in a group of neurodegenerative diseases in mammals that exist in familial, sporadic and infectious forms [3,4]. In fungi, prions were first linked to a limited group of phenotypic traits that are transmitted through a non-Mendelian route [5,6], but computational approaches and genetic screens have more recently suggested that the prion mechanism may be a widespread regulatory strategy in fungi [7–12]. A number of key regulators of basic cellular processes (i.e. translation, transcription, and nutrient catabolism) have been confirmed to be prion proteins (Table 1), and given the breadth of the downstream targets of these factors, it is, perhaps, not surprising that changes in prion protein biogenesis have profound and complex effects on fungal phenotypes [13–18].

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By what mechanism do prion proteins heritably alter cellular phenotypes? According to the prion hypothesis, when a cellularly encoded protein adopts an alternate physical state, its normal function is compromised, or it acquires a new activity [19,20]. Since the new physical state specifies the new phenotype, distinct protein-only traits can only stably persist if their corresponding physical forms, hereafter referred to as conformers, are propagated at the expense of the other possible states. Consistent with this prediction, prion proteins “self-replicate” both *in vitro* and *in vivo* by templating the refolding of other conformers of the same protein [21–23].

What is the nature of the self-replicating prion physical state? Both in mammals and in fungi, prion transmission is associated with the transfer of protein aggregates composed of the prion conformer to a susceptible host [1,24–28], but the exact identity of the active species remains elusive. To date, two confounding factors have complicated this assessment. First, the infectious material is biochemically complex. *In vitro*, prion proteins assemble into mature linear fibrils through a hierarchical pathway encompassing several intermediate forms [19, 21], and *in vivo*, prion aggregates are heterogeneous in size and composition [1,28–33]. Second, prion aggregates are highly dynamic *in vivo* [34]; thus, an externally delivered aggregate may function as a prion template directly or alternately may give rise to the active species once acted upon by cellular factors.

Prion phenotypes in fungi, particularly those of the budding yeast *Saccharomyces cerevisiae*, provide tractable experimental models with which the identity of the protein-only genetic determinant can be interrogated. Cellular regulation of the inheritance of these traits has been characterized through a variety of complementary biological and computational approaches. In this review, we re-visit this wealth of information to synthesize a model for the inheritance of prion phenotypes based on the selective transfer of small aggregates composed of prion-state conformers.

Propagation of Prion Phenotypes *In Vivo*

The most extensive mechanistic studies on protein-only inheritance in fungi to date have focused on two phenotypes in *S. cerevisiae*, [*URE3*] and [*PSI*⁺], which are specified by the prion forms of the Ure2 and Sup35 proteins, respectively [35]. As is the case for nucleic acid-based phenotypes, the transmission of protein-based traits depends upon two key events: replication of the genetic determinant and transfer of at least one of the copies to another cell or individual. For protein-based traits, molecular insight into these processes has been aided by the characterization of treatments and manipulations that lead to loss of the prion state in yeast cultures, an outcome known as curing [36].

The most robust of the curing agents is guanidine hydrochloride (GdnHCl), which induces a quantitative [*PRION*⁺] → [*prion*[−]] switch over several generations [35,37–39]. During this time course, [*prion*[−]] cells arise after a lag phase and thereafter increase linearly in the population as a function of generation time. Based on these observations, Tuite, Cox, and colleagues proposed that GdnHCl treatment blocks replication of the heritable, protein-only determinant, known as a propagon and that subsequent cell division dilutes the pre-existing propagons to a point at which mother cells contain insufficient copies of the determinant to segregate a [*PRION*⁺] daughter [40,41]. Thus, if all of the progeny of the original cell are retained and analyzed separately [42], the number of [*PRION*⁺] colonies recovered following GdnHCl treatment should represent the number of propagons in the original cell, as each propagon will theoretically give rise to a single [*PRION*⁺] colony following complete distribution of these species [39].

How do propagons replicate? *In vitro*, fibrillar prion aggregates self-replicate their conformations by incorporating soluble protein onto the ends of these linear complexes [43–

46]. *In vivo*, a related, if not identical, templating process occurs, with existing propagons directing the conversion of non-prion state protein, whether it be newly made or existing protein introduced by mating, to the prion form on the timescale of minutes [47–49]. This refolding event alone, however, does not fully explain the process of propagon replication, as GdnHCl treatment does not inhibit the incorporation of non-prion state Sup35 into existing prion complexes [41,50] or the concomitant appearance of the $[PSI^+]$ phenotype [50]. Thus, propagon replication requires at least one additional, GdnHCl-sensitive biochemical event.

Insight into this missing step was aided by the realization that GdnHCl treatment inhibits the activity of the molecular chaperone Hsp104 [51–53]. A member of the AAA+ ATPase family, Hsp104 performs a unique function in stress tolerance: the rescue of misfolded proteins from aggregates [54,55]. Through the binding and hydrolysis of ATP, Hsp104 recognizes substrates and extracts them from aggregates by mechanically unfolding individual molecules and releasing them for subsequent refolding [56,57]. Given its role in the resolution of stress-induced aggregates, Ter-Avanesyan and colleagues proposed that Hsp104 contributes to propagon replication by fragmenting these species, presumed to be aggregates, into smaller oligomers [58]. Propagon replication *in vivo* would then require two interdependent biochemical events: conversion of non-prion state protein into the prion form by its incorporation into existing templates and fragmentation of these species to produce new surfaces for conversion (Figure 1A). Consistent with this idea, the efficiency of incorporation of prion protein into existing aggregates *in vitro* is strongly dependent upon the concentration of fibril ends [46], and treatment of $[PRION^+]$ cells with GdnHCl limits the capacity of these cells to convert an excess of non-prion state protein to the prion form, presumably by decreasing the number of templates available [41,50].

Is this two-step model sufficient to explain prion propagation *in vivo*? Given the complexity of the system, mathematical models have emerged as an invaluable tool to study the forces impacting the stability of prion phenotypes. The most extensive efforts in developing such models have centered on mammalian prion propagation [59–63], but a recent study indicates that a fundamentally identical model is also applicable to yeast prions [64]. Strikingly, prion propagation can only be accurately described mathematically by evoking an aggregate composed of prion conformers as a template [65,66] and by accounting for the individual biochemical events that govern its dynamics (Figure 1), including the rates of prion protein synthesis (α), conversion (β), fragmentation (γ), and removal (i.e. decay in mammals (d_x, d_p) or dilution by cell growth in yeast (R)) [59–64]. These models predict that the system will settle into one of two steady states: a $[prion^-]$ state, devoid of prion complexes, or a $[PRION^+]$ state, containing a stable distribution of prion complexes, regardless of the initial configuration of the system. Thus, the biochemical processes described above are sufficient to account for both the progressive nature of neurodegeneration associated with prion diseases in mammals as well as the efficiency of $[prion^-] \rightarrow [PRION^+]$ switches in yeast by directing the amplification of a newly introduced or spontaneously arising prion aggregate to a phenotypically detectable level.

Prion Aggregates and Propagons: A Comparison

While experimental and computational efforts have clearly converged on prion aggregates as the genetic determinants of protein-based phenotypes, the exact identity of the active complex remains a fundamental, open and elusive question. The framework that has emerged above, however, provides a gateway into the identification of the heritable species by allowing predictions to be made about the biochemical behavior of propagons in response to Hsp104 inhibition. Several approaches have been developed to analyze the physical state of prion proteins both in lysates and in live yeast cells. Using sucrose gradients and semi-denaturing agarose gels, the distribution of prion aggregates can be readily separated and analyzed under

different culture conditions (Figure 2A) [29,49], and these studies have been complemented with direct visualization of prion-fluorescent protein fusions, which remain diffuse in [*prion*⁻] strains and form foci in [*PRION*⁺] strains, reflecting the differences in oligomerization in the two states (Figure 2B) [47,67,68].

Do the prion aggregates detected with these assays correspond to propagons? According to the working model for propagon replication presented above, propagon fragmentation is blocked upon Hsp104 inhibition, and this change in propagon dynamics should lead to an increase in their size at steady-state. Strikingly, visible foci of a Sup35-GFP fusion, which are dynamically remodeled in the presence of active Hsp104, become static upon inhibition of Hsp104, reflecting the block to fragmentation [50]. Under the same conditions, the size of fluorescent foci increases [69], and the distribution of Sup35 aggregates shifts to a slower migrating range as assessed by semi-denaturing agarose gel electrophoresis [29]. Thus, the behavior of detectible prion aggregates mirrors that predicted for propagons under these conditions.

The similar behavior of the genetically defined heritable species (propagon) and the molecularly characterized predominant form of the prion protein (aggregates) suggests, at first glance, that the two entities are identical; however, additional observations have called this interpretation into question. First, the number of propagons detected for a given prion (i.e. ~ 100 for [*PSI*⁺][40] and ~ 20 for [*URE3*][70]) does not always correlate with the number of fluorescent foci observed per cell [71]. For example, green fluorescent protein (GFP) fusions to Sup35 and Ure2 form single fluorescent foci in cells, when expressed at elevated levels [47,70]. Second, prion proteins can be induced to aggregate in [*prion*⁻] cells by over-expression, but these aggregates do not always support inheritance of prion phenotypes. For example, a deletion mutant of Sup35 ($\Delta 22/69$) cannot support stable inheritance of the prion phenotype despite its ability to form aggregates *in vivo* [72]. In addition, wildtype Sup35 will aggregate in [*psi*⁻] (i.e. [*prion*⁻]) cells when over-expressed, but the associated [*PSI*⁺]-like phenotype is not heritable [73,74]. Likewise, the *S. paradoxus* homologue of Ure2 readily forms aggregates when heterologously expressed in *S. cerevisiae* at elevated levels but cannot support [*URE3*] propagation under these conditions [75]. Together, these observations led to the proposition that detectible aggregates of prion proteins are not the heritable species, but rather, represent dead-end, inactive products [70,76].

Despite these potential inconsistencies, the available genetic evidence indicates that non-heritable aggregates and propagons are biochemically interconvertible. Upon removal of GdnHCl from the media, [*PSI*⁺] loss is immediately halted [39], and this stabilization of prion propagation is accompanied by an amplification of propagons, which double every 20 minutes [41]. Strikingly, this propagon replication occurs in the absence of new protein synthesis, strongly suggesting that reactivation of Hsp104 converts existing non-heritable aggregates of Sup35 into propagons.

What, then, is the biochemical distinction that causes these species to differ in their ability to support inheritance of protein-based phenotypes? We suggest that a re-inspection of the available observations with an eye toward the dynamic nature of the system (Figure 1) allows the differences between non-heritable aggregates and propagons to be reconciled. As detailed above, changes in prion aggregate dynamics, induced by alterations in the rates of synthesis, conversion and fragmentation, have all been linked to prion loss, indicating that they impact some aspect of propagon inheritance [39,64,67,77,78]. Remarkably, these manipulations have the common effect of shifting the steady-state size distribution of prion aggregates to larger species [29,47,69,73,74], suggesting that this event can account for the inheritance defects associated with each of the manipulations.

How would changes in the size distribution of prion aggregates affect the inheritance of protein-based phenotypes? Intriguingly, the mobility of Sup35 aggregates in live [*PSI*⁺] yeast cells is greatly reduced upon inhibition of Hsp104 [50,79], suggesting that prion aggregates of different sizes could be transmitted to daughter cells at different rates (δ , Figure 1), thereby impacting stability of the associated phenotype. According to this idea, propagons would reflect only that portion of the distribution of prion aggregates, presumably the smaller end, that has the highest probability of transfer to daughter cells, rather than the entire population of aggregates [80]. Changes in biochemical rates that shift the size distribution to larger complexes would, therefore, lead to prion loss by decreasing the proportion of aggregates within the optimal size range for efficient transfer (Figure 3).

Such a model is entirely consistent with available empirical observations. First, propagons can be regenerated from previously non-heritable aggregates [41], and mathematical models of the evolution of prion aggregates in a population indicate that the equilibrium distribution reflects the effects of conversion and fragmentation on the aggregates originally introduced into the system [62]. That is, the individual constituents of the population are all interconvertible. Second, altering the efficiencies of the conversion and fragmentation reactions allows aggregated forms of prion proteins, which are unable to support inheritance of prion phenotypes under normal conditions, to function in this capacity. For example, *S. paradoxus* Ure2 supports [*URE3*] propagation when expressed at lower levels in *S. cerevisiae* [78], and $\Delta 22/69$ Sup35 and over-expressed Sup35 support [*PSI*⁺] propagation when Hsp104 is over-expressed [72, 74]. In each case, the indicated manipulation has been proposed to alter the steady-state size of propagons. Third, such a model explains the relative stabilities of different yeast prions. For example, Sup35 can adopt multiple physical forms *in vivo* [81]. A prion variant with a size distribution shifted to smaller aggregates (strong) is three orders of magnitude more stable than another variant with a size distribution shifted toward larger aggregates (weak, Figure 2A) [29,82,83]. In addition, [*URE3*] is largely unstable in comparison with [*PSI*⁺] [35], and the steady-state distribution of Ure2 aggregates is shifted toward larger sizes relative to that of Sup35 aggregates [32]. Finally, smaller prion aggregates are the most infectious species in both mammalian and yeast models [28,31,84].

Resolving the Precise Nature of Protein-Only Determinants in Yeast

While the model proposed here suggests that a subpopulation of prion aggregates functions as propagons, a molecular definition of this species must still be generated to fully understand the regulation of prion inheritance and loss *in vivo*. By definition, the heritable species is that which is transmitted to daughter cells, and the goal moving forward must be a molecular characterization and quantification of this fraction. Such an assessment is, of course, complicated by the fact that prion inheritance in yeast occurs in a continually changing system in which cells grow and divide and prion aggregates interconvert on a rapid timescale. Thus, experimental interpretations must carefully weigh the impact of these dynamics on assay readouts. For example, prion aggregates increase in size in the presence of GdnHCl not only due to the block in fragmentation that this compound induces [29,41,50] but also through the continued incorporation of newly made prion protein into the existing complexes [41,50]. Thus, rather than assaying the number of heritable units present in a cell at the time of GdnHCl treatment, propagon counts likely underestimate this value, reflecting instead a transmissible fraction that is continually declining as propagons evolve into non-heritable aggregates.

Given the complexity of the system, definitive identification of the species, or more likely range of species, directing inheritance of protein-based traits in yeast will require continued parallel efforts through both *in vivo* and computational approaches. This goal, however, is within reach if population averages can be deconvoluted with high enough temporal resolution to directly monitor the transmission event, and minor adjustments to existing approaches can

greatly facilitate such advances. From the experimental side, existing biochemical approaches can be utilized to directly characterize the physical state of prion protein in daughter cells following simple fractionation of the population, and simultaneous inhibition of new protein synthesis and fragmentation should freeze aggregates at their initial sizes. In addition, quantitative microscopy-based assays can directly monitor the fraction of protein transmitted [79]. From the modeling side, sources of heterogeneity among cells in a growing population [85] strongly impact biological predictions, as has been recently demonstrated in probabilistic models developed to accurately measure propagon numbers from GdnHCl curing experiments [86–88]. Factors, such as the unequal distribution of prions between mother and daughter cells during division [40], the differences in cell cycle length between mothers and daughters [89], the effect of aggregate size on transmissibility [50], and the effect of changes in concentration of key players [67,77,78], are likely to alter prion aggregate dynamics and, therefore, should be incorporated into existing models of protein-only inheritance.

A detailed understanding of prion propagation *in vivo* requires a holistic view of the system, focusing on the interplay between the biochemical processes governing prion aggregate dynamics and the biological system in which they occur. The experimental and computational approaches that have already been implemented in this area are converging to a powerful path forward that will allow us to further appreciate, probe and predict the mechanisms by which self-replicating protein conformations specify transmissible traits.

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Figure 1.

Kinetic regulation of prion propagation *in vivo*. We illustrate the underlying biochemical processes governing prion propagation *in vivo* and how these processes have been modeled with systems of differential equations both for mammals and for yeast. A. Prion dynamics in yeast results from the following processes: synthesis (light green and turquoise tangle on gray ribosome) of soluble protein (light green and turquoise ball and stick) at a rate α , conversion of soluble protein by incorporation into existing aggregates (green and blue ball and loop) at a rate β , fragmentation of aggregates into smaller complexes at a rate γ , and finally transmission of prion protein to daughter cells at a rate δ . B. These processes can be expressed as biochemical reaction equations governed by their respective rates, where we denote soluble protein as X

and a complex of i monomers by Y_i . Synthesis is the *de novo* ($\emptyset$) production of soluble protein (X). Conversion is the addition of a monomer, X , onto an existing aggregate of length i (Y_i), which increases the length of that aggregate by one subunit (Y_{i+1}). Fragmentation is the fracture of an aggregate of length i (Y_i) into two smaller complexes (Y_{i1} and Y_{i2}). In mammalian cells, the prion protein PrP is short-lived, and models must include decay ($\emptyset$) rates for both soluble protein (d_x) and prion complexes (d_p). In yeast cells, prions are transmitted from mother to daughter in a growing culture (boxed Y_i). C. Systems of differential equations were developed to study prion dynamics in mammals [63] and yeast [64]. As in B, x corresponds to monomers, but y represents the total number of prion aggregates, $y = \sum_i y_i$, and z is the total number of monomers in protein aggregates, $z = \sum_i i y_i$. Note that the mammalian equation for dy/dt represents the change in the number of aggregates per unit time while the yeast equation represents changes in the concentrations of aggregates per unit time, resulting in the difference in the term $z-y$. Despite the differences in dimensionality, the similarity between the two systems is evident. In each model, the system will reach one of two steady-state values (prion free, prion maintenance). The equations for the change in each term x , monomers, y , protein aggregates, and z , total aggregated monomers, are formed considering the relevant biochemical equations. While yeast prion proteins are long-lived *in vivo* [48,50], protein concentrations will be diluted by the growing cell culture, modeled to grow exponentially at rate R .

A **B** **Figure 2.**

Heterogeneity in prion protein physical states and its potential implications for inheritance. A. Semi-denaturing agarose gel electrophoresis followed by immunoblotting with antiserum directed against Sup35 reveals heterogeneity in the population of complexes adopted by the protein in the non-prion ($[psi^-]$) and prion ($[PSI^+]^{Strong}$ or $[PSI^+]^{Weak}$) forms. With this technique, prion complexes migrate as an extended smear toward the top of the gel, and Sup35 monomers are detected as a tighter band toward the bottom of the gel. Note the shifted distribution of prion complexes in $[PSI^+]^{Weak}$ vs. $[PSI^+]^{Strong}$, which corresponds to increased rate of prion loss in mitotically dividing cultures. B. Differences in the physical state of Sup35 in the same states is also apparent in strains expressing a Sup35-GFP fusion from the

endogenous Sup35 locus. Protein aggregates are detected a fluorescent foci in the prion states ($[PSI^+]^{\text{Strong}}$ or $[PSI^+]^{\text{Weak}}$, but the fluorescence pattern is diffuse in non-prion strains ($[psi^-]$), which are devoid of protein aggregates.

Figure 3.

A model for how the distribution of prion protein aggregates sizes could impact transmission efficiency and thereby mitotic stability of the prion phenotype. Conditions that impact the size distribution of complexes could impact the mitotic stability of prion phenotypes depending upon the location of a threshold for efficient transfer. Such shifts in distribution could convert a stably propagating prion (black) into an unstable, non-heritable aggregate (red) by decreasing the proportion of total protein that has the capacity to be passed to daughter cells (area under the curve that is to the left of the threshold).

Table 1

Prion Proteins and Their Phenotypes

Prion Protein	Prion Form	Phenotype	Organism	Reference
PrP	PrP ^{Sc}	neurodegeneration	Mammals	[1]
Ure2	[<i>URE3</i>]	nitrogen catabolism	<i>S. cerevisiae</i>	[35,90]
Sup35	[<i>PSI⁺</i>]	nonsense suppression	<i>S. cerevisiae</i>	[35,83]
Het-s	[<i>Het-s*</i>]	heterokaryon incompatibility	<i>P. anserina</i>	[91]
Rnq1	[<i>PIN⁺</i>]	prion inducibility	<i>S. cerevisiae</i>	[12,37,68,92]
Swi1	[<i>SWI⁺</i>]	transcriptional regulation	<i>S. cerevisiae</i>	[93]
Cyc8	[<i>OCT⁺</i>]	transcriptional regulation	<i>S. cerevisiae</i>	[94]
Mot3	[<i>MOT3⁺</i>]	transcriptional regulation	<i>S. cerevisiae</i>	[11]
Mca1	[<i>MCA</i>]	?	<i>S. cerevisiae</i>	[10]