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Insights into prion biology

Integrating a protein misfolding pathway with its cellular environment

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Protein misfolding and assembly into ordered, self-templating aggregates (amyloid) has emerged as a novel mechanism for regulating protein function. For a subclass of amyloidogenic proteins known as prions, this process induces transmissible changes in normal cellular physiology, ranging from neurodegenerative disease in animals and humans to new traits in fungi. The severity and stability of these altered phenotypic states can be attenuated by the conformation or amino-acid sequence of the prion, but in most of these cases, the protein retains the ability to form amyloid in vitro. Thus, our ability to link amyloid formation in vitro with its biological consequences in vivo remains a challenge. In two recent studies, we have begun to address this disconnect by assessing the effects of the cellular environment on traits associated with the misfolding of the yeast prion Sup35. Remarkably, the effects of quality control pathways and of limitations on protein transfer in vivo amplify the effects of even slight differences in the efficiency of Sup35 misfolding, leading to dramatic changes in the associated phenotype. Together, our studies suggest that the interplay between protein misfolding pathways and their cellular context is a crucial contributor to prion biology.

Key words: prion, protein misfolding, chaperones, amyloid, ordered aggregates, transmission, aggregate size, Sup35, Hsp104

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Prion Propagation In Vivo

According to the prion hypothesis, an alternative conformation of a normal, host-encoded protein, known as a prion, can function as an epigenetic determinant of transmissible phenotypic states in vivo. This idea was originally proposed as a disease mechanism for the Transmissible Spongiform Encephalopathies (TSEs), a group of severe mammalian neurodegenerative disorders associated with the misfolding of the prion PrP.[1,2] More recently, the prion hypothesis has provided a novel framework for understanding other enigmatic biological phenomena, including a group of metastable traits in fungi that are inherited through a non-Mendelian route.[3,4] Although once met with skepticism, this protein-only mechanism has now been proven in multiple studies, where new transmissible phenotypes were induced in experimental organisms simply by introducing particular conformational variants (conformers) of prions.[5-11]

Prion-associated phenotypes are thought to arise because conformational conversion alters the activity of the protein.[4] However, this link between discrete physical and functional states of a protein only provides a steady-state snapshot of the endpoints of a dynamic process. The appearance, spread and reversal of prion-associated phenotypes necessarily involve changes in protein physical state.[12] However, these transitions are only effective if the alternative conformer is preferentially amplified, at the expense of all others, to a level that can alter cellular physiology.

Much of our understanding of this conformational selection and amplification results from studies in vitro, where the assembly of prion conformers into amyloid aggregates directs the continued misfolding of the protein by providing a template for this conversion.[13-19] However, studies on the yeast prion Sup35 indicate that the process of conformational self-replication is more complex in vivo

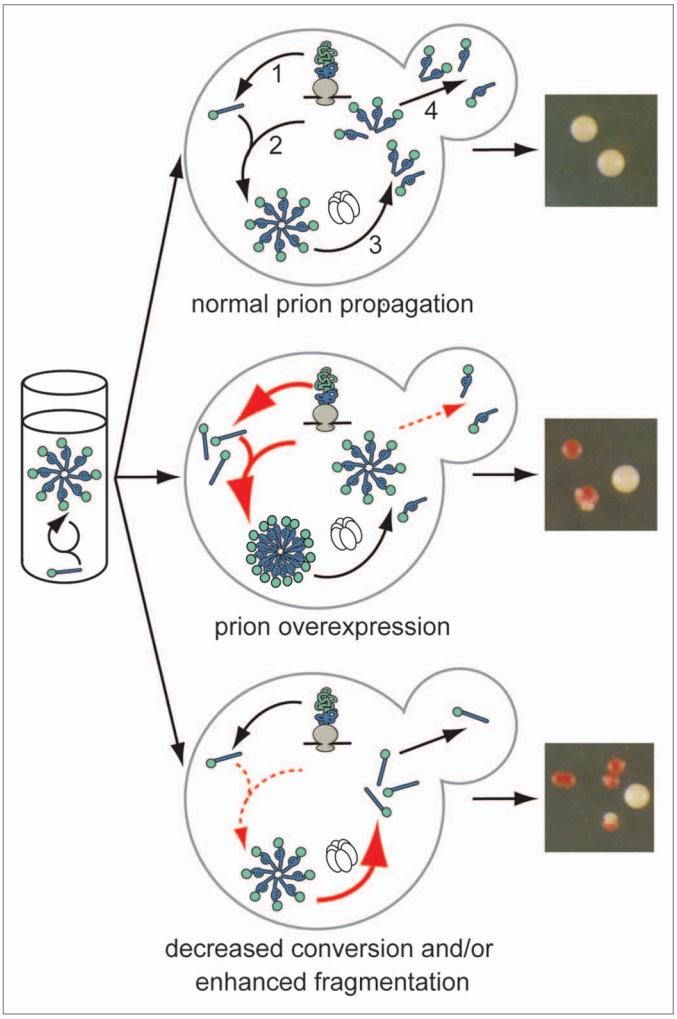

Figure 1. The cellular environment modulates prion misfolding pathways to create protein-based traits. (top) Self-replicating protein conformations create stable phenotypes (white colonies) in vivo when the processes of synthesis (1), conversion (2), fragmentation (3) by Hsp104 (hexamer) and transmission (4) are balanced to allow aggregates of prion proteins to persist in vivo. (middle) Overexpression of a prion protein promotes the conversion reaction (red arrows), leading to the accumulation of large aggregates that are inefficiently transmitted to daughter cells (dotted red arrow) and loss of the prion-associated phenotype (red colonies). (bottom) Dominant inhibition of prion propagation by mutants that decrease conversion efficiency (dotted red arrow) or enhance fragmentation efficiency (solid red arrow) promote aggregate disassembly (ball and stick) and induce prion loss (red colonies).

(**Fig. 1**, top), and a similar pathway has been proposed for prion propagation in mammals.[20] Based on this work, aggregates or their component prion-state protein must act in three roles to sustain the prion-associated phenotype in a population. As is the case in vitro, these aggregates template the conversion of newly made Sup35 to the prion conformer;[21,22] however, in vivo, simple growth of the aggregates will not allow the spread of prion-associated phenotypes in a dividing culture. Thus, these aggregates also must be continually fragmented to create new conversion surfaces[23] and transmitted to daughter cells (**Fig. 1**, top).[23,24]

Prion Variants

While the pathway of prion propagation in vivo explains how the appearance of a misfolded conformer can be amplified to induce and maintain a new phenotype, this scheme alone is not sufficient to explain the diversity of prion-associated phenotypes. Remarkably, each prion has the capacity to create a spectrum of phenotypic states, known as strains or variants, which retain their characteristics upon serial passage in the same host.[25-30] In mammals, variants of PrP are characterized by differences in host-specific incubation periods between initial infection and clinical disease,[31-34] the distribution of pathological changes in the brain,[25,33,35] clinical symptoms,[36] sensitivity to thermal inactivation[37-40] and the interspecies transmission of disease.[32,38,41-43] In contrast, variants of the fungal prions do not specify new phenotypes but rather alter the severity of the prototypical prion-associated phenotype to generate a continuum of similar traits.[26,27,29]

The existence of variants was once considered incompatible with a protein-only mechanism, but studies in both mammals and yeast have linked this phenotypic diversity to a parallel collection of unique conformers for each prion, which are characterized by changes in protease sensitivity, thermodynamic stability, and the extent of the core amyloid structure.[7,25,28,44-49] For both mammalian and fungal prions, these conformers are all self-replicated through the same pathway, but the physical differences between them affect the activities of aggregates and presumably their phenotypic consequences.[6,7,50] Aggregates composed of distinct prion conformers differ in their templating activity[49-51] and in their thermodynamic stabilities, which likely affect both their ability to be fragmented and to be cleared by quality control pathways in vivo.[49,52,53] What has emerged from these analyses is that unique combinations of conversion and fragmentation efficiencies define prion variants, but how these biochemical parameters created distinct biological outcomes remained a major unanswered question.

Connecting Prion Misfolding to Phenotype

In general, the thermodynamic stability of mouse, both natural and synthetic (i.e., in vitro generated), and yeast prion aggregates is inversely correlated with the severity of their associated phenotypes (**Fig. 2**), most typically scored as incubation time in mammals and extent of aggregation in fungi.[49,52,53] Based on these observations, it

was suggested that the efficiency of aggregate fragmentation in vivo, which should be a function of aggregate thermodynamic stability, has a profound effect on prion biology. If the system has reached steady-state, modest changes in fragmentation efficiency are predicted to inversely affect the size of aggregates and directly affect their accumulation, as prion synthesis is on-going,[54] and either (or both) of these variables could impact the creation of unique, conformation-based phenotypes. To distinguish between these size and abundance-based models of prion variants, we focused on the yeast protein Sup35, a component of the translation termination complex whose function is modulated by a prion cycle.[55-57] In the non-prion [(*psi*$^-$)] conformer, Sup35 is soluble and facilitates translation termination; however, in its prion [(*PSI*$^+$)] conformers, Sup35 aggregates to different extents, establishing a corresponding range of decreased translation termination efficiencies.[21,58,59]

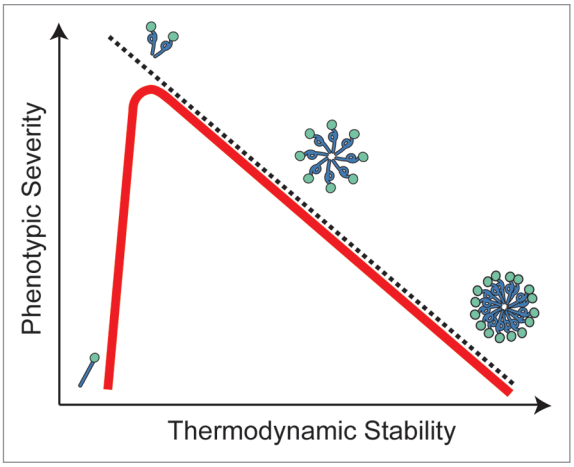

Figure 2. Relating prion phenotypic severity to aggregate thermodynamic stability. For many prion variants, there is a linear but inverse relationship between aggregate thermodynamic stability and phenotypic severity (black dotted line), but this trend cannot explain the phenotypes associated with all prion variants or the effects of dominant-negative prion mutants (see text for details). Our studies in vivo on the yeast prion [*PSI*$^+$] suggest that thermodynamic stability poses a limit on prion persistence at both extremes (red line) by impacting aggregate size and accumulation (shown schematically). The least thermodynamically stable aggregates are efficiently resolubilized, while the most thermodynamically stable aggregates are inefficiently transmitted.

Given the multiple roles of aggregates in prion propagation in vivo, we employed a computational approach to guide our experiments on the in vivo pathway through which prion variants are created. TSE propagation can be mathematically described in terms of PrP synthesis, conversion, fragmentation and decay rates,[20] and by substituting prion dilution during cell division for prion decay, the same formulation accurately captures the differences in soluble Sup35 levels found in two [*PSI*$^+$] variants, known as weak and strong.[27,49] However, this model cannot accurately predict other defining characteristics of yeast prion variants, including differences in the size distribution of aggregates,[60,61] the spontaneous frequency of prion loss,[27,62] and the elevated frequency of prion loss observed upon Sup35 overexpression,[63,64] a phenomenon that is also observed for another yeast prion.[65-67]

Each of these characteristics is specific to prion propagation in vivo; therefore, we formulated a new model that incorporated aspects of the cellular environment that were known to impact prion propagation: the transmission of Sup35 protein to daughter cells[23,68] and the limitation on fragmentation efficiency imposed by the concentration of the molecular chaperone Hsp104, the catalyst for this reaction.[23,69,70] With this reformulation, we simulated [*PSI*$^+$] propagation via a transmission model that was either based on aggregate abundance or size and assessed their accuracy in computationally recapitulating all of the characteristics of the [*PSI*$^+$]^{weak} and [*PSI*$^+$]^{strong} variants described above. In contrast to in vivo observations, the prion state was completely stable under all conditions in the abundance-based model because aggregates, if they accumulated to any extent, were efficiently transferred to daughter cells, as had been previously predicted.[62] However, the severities, stabilities and aggregate size distributions of [*PSI*$^+$]^{weak} and [*PSI*$^+$]^{strong} variants were accurately recapitulated if a size threshold for aggregate transmission was imposed, but only if this limitation fell within the size range of SDS-resistant aggregates that distinguished the two variants.[60]

Our modeling suggested that the heritable prion species, known as a propagon,[71] represented a subset of aggregates that was distinguished simply by their size and that variants differed in their accumulation of these species. If true, the stability of variant-associated phenotypes should correlate with the proportion of Sup35 that was transmissible. To directly test this idea, we developed a fluorescence loss in photobleaching (FLIP) assay, in which daughter cells of yeast strains expressing Sup35-GFP are continually bleached prior to cytokinesis and fluorescence loss in the mother is monitored over time as a proxy for transmission (**Fig. 3**). Indeed, a [*PSI*$^+$]^{weak} variant transmitted 50% less Sup35-GFP and accumulated 50% fewer propagons than a [*PSI*$^+$]^{strong} variant, and these differences correlated with a three order of magnitude increase in prion loss during cell division (mitotic instability) for the [*PSI*$^+$]^{weak} variant.[27]

If aggregate size alone is responsible for these effects, shifting this distribution, in the absence of conformational changes, should also impact both the transmissibility of Sup35 and the stability of the associated phenotype. In vivo, the steady-state size of aggregates is a function of both the conversion and fragmentation reactions; therefore, we varied Sup35 expression level to modulate the conversion rate. The size of SDS-resistant aggregates was increased by mild overexpression of Sup35 (~5-fold) and dramatically decreased by repression of Sup35 synthesis for even one generation. By FLIP, conditions associated with the accumulation of larger aggregates resulted in a decrease in Sup35-GFP transmission (**Fig. 1**, middle), while those associated with smaller aggregates resulted in an increase in Sup35-GFP transmission.

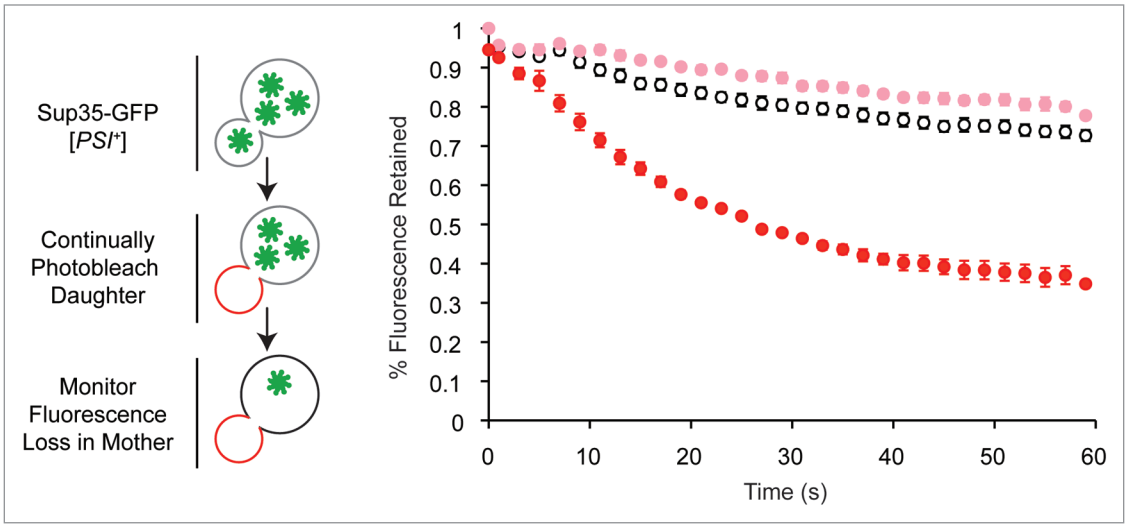

Figure 3. Transmission of Sup35 protein to daughter cells is conformation-dependent. (left) Schematic of fluorescence loss in photobleaching assay (FLIP) for Sup35 transmission to daughter cells. Bleached daughter (red) and monitored mother (black) are indicated. (right) Fluorescence retention in mother cells expressing Sup35-GFP in the [*PSI*$^{+}$]^{strong} (white), [*PSI*$^{+}$]^{weak} (pink) or [*psi*$^{-}$] (red) conformation.

As was the case for different variants, these differences in transmission correlated directly with the number of propagons and indirectly with the mitotic stability of the prion-associated phenotype. Thus, our studies suggest that there is no inherent functional difference between aggregates of the same conformer but rather that their behavior in vivo is modulated by differential interactions with the cellular environment.

Conformation-dependent differences in prion-associated phenotypes were previously thought to arise from distinct equilibria between soluble and aggregated protein (i.e., abundance-based model) in all cells in a population.[49] However, our size-based model suggested that prion-associated phenotypes arose through a population-based mechanism. Specifically, the size threshold for aggregate transmission is predicted to create heterogeneity in the population, with mother cells accumulating more and larger aggregates than their daughters. By assessing Sup35 physical state in either mother or daughter cells by FLIP or a gel-based assay following centrifugal elutriation, we provided experimental support for this prediction. These age-dependent differences and the fact that prion variants produce phenotypically homogeneous colonies suggested that the aggregate complement and associated phenotype must change over time. Our modeling indeed predicted that the fraction of Sup35 in aggregates, which determines translation termination activity,[72] and their size both increased with replicative age. Consistent with this prediction, we demonstrated that the number of propagons transmitted to daughters increases with the replicative age of the mother and that the fidelity of translation termination, as monitored by a fluorescent reporter, decreases with replicative age. Thus, prion-associated phenotypes arise through a dynamic pathway of prion biogenesis rather than a simple equilibrium between the prion and non-prion conformers.

The size-dependent transmission of aggregates provides a framework for understanding prion-associated phenotypes in a dividing yeast culture, but can differences in aggregate size also provide insight into prion biology in mammalian post-mitotic neurons? Mathematical models have suggested that aggregate size as well as abundance could be important contributors to prion propagation in mammals.[20] Indeed, biochemical analyses have linked small aggregates to high infectivity and short incubation times.[73,74] Mechanistically, aggregate size appears to impact converting activity,[73,75] but others have suggested an effect on transmission as well.[20,76] Consistent with this idea, more thermodynamically stable and presumably larger, aggregates are associated with localized pathology while less thermodynamically stable aggregates are associated with more widespread pathology.[77] Moreover, mammalian prion biology is altered by changes in expression of the prion protein, as is the case for yeast prion biology.[24,63-67] For example, the incubation time for clinical disease, but not for the generation of prion infectivity, is highly dependent on PrP expression level.[78,79] While other mechanisms are possible,[78,80,81] an intriguing model to explain these observations is that infectivity increases with aggregate abundance until reaching a plateau, which represents a limitation on the system. If that limitation is fragmentation activity, aggregates will increase in size but not abundance, and this process would proceed more rapidly at higher PrP expression levels. In this scenario, prion toxicity, corresponding to clinical disease, could result from the titration of other cellular factors through their association with aggregates, a mechanism that has been proven for the toxicity of overexpressed Sup35 in [*PSI*$^{+}$] yeast strains.[82] Thus, aggregate size may be an underappreciated contributor to the phenotypic outcomes of prion variants in mammals, as well.

Balancing Aggregate Assembly and Disassembly Pathways

While fragmentation efficiency appears to be a limitation on prion propagation in vivo, there may also be an upper

boundary on the level of activity that is compatible with the prion state (**Fig. 2**). Several variants of hamster prion assemble into aggregates of lower thermodynamic stability than most other prion variants,[25,32,83,84] but their incubation periods are significantly longer.[28,49,52] One potential explanation for these disconnects is that the rate of aggregate disassembly might approach that of aggregate assembly for these variants, leading to clearance.[50,51,84,85] Consistent with this idea that aggregate assembly and disassembly pathways compete with one another in vivo, infectious particles delivered by direct inoculation, which presumably do not represent a load on the system, are largely cleared from experimental organisms,[78,86-90] and even established prion infections are reversed in vivo upon repression of new PrP synthesis.[91-95]

Other examples of the importance of competition between aggregate assembly and disassembly pathways in prion phenotypic outcomes may also be found in nature. Individuals with PrP polymorphisms are genetically less susceptible to prion disease.[96-105] These polymorphisms clearly restrict the range of conformations accessible to the prion and therefore its ability to replicate certain variants.[31,43,106-110] However, many of the same sequence changes also function as dominant inhibitors of prion propagation in vivo,[105,111-118] and a similar effect occurs upon co-expression of PrP homologues from different species.[79,119-124] Thus, these sequence variants must target crucial events in prion propagation by the wild-type protein, and elucidating their mechanisms of action could be instructive for developing therapeutic interventions for these diseases.[114-116]

Early models suggested that PrP dominant-negative mutants acted by titrating away a host-encoded cofactor (protein X) required for the conversion reaction;[122] however, these sequence variants also inhibit prion propagation in cell-free systems, suggesting that their effects are mediated directly through prion-prion interactions.[125-127] In this scenario, PrP sequence variants would interact with the templating surface on an aggregate and either slow, as has been proposed for a Q219K variant, or block, as has been proposed for a Q172R variant, conversion of wild-type PrP, depending on their affinities for one another.[125,126] Potentially supporting this affinity-based model, PrP dominant-negative mutants differ in their effective inhibitory ratios relative to wild-type protein,[111,125-129] but the same observation is also consistent with a model in which these mutants target different events in prion propagation in vivo. According to this latter idea, mutants that act at the templating surface would be effective at lower doses than those affecting fragmentation, which occurs along the length of the aggregate.[130]

Propagation of the yeast prion [*PSI*$^+$] is also disrupted by co-expression of Sup35 mutants, such as Q24R and G58D,[131-133] and we used this system to explore the mechanisms underlying prion dominant inhibition in vivo.[134] We found that Q24R, like PrP Q172R, acted at sub-stochiometric ratios while G58D, like Q219K, interfered only at higher ratios relative to wild-type protein.[113,125,135] Using a series of in vivo analyses, we linked these differences to defects in discrete events in the prion propagation pathway. While we detected no defect in prion transmission by FLIP, we determined that G58D, but not Q24R, could efficiently join wild-type complexes by monitoring conversion by a translation termination defect that appears upon Sup35 incorporation into aggregates,[22] as had been previously suggested.[64,131,136,137] In contrast, G58D, but not Q24R, incorporation into aggregates led to their destabilization, as measured by their sensitivity to disruption in SDS at increasing temperatures. Thus, Q24R expression decreases conversion efficiency while G58D expression likely increases fragmentation efficiency. In addition to revealing the molecular basis of the differences in effective inhibitory ratios, these effects on Sup35 biogenesis also explain the differential dominant interactions of Q24R and G58D with the [*PSI*$^+$]^{weak} variant, which is characterized by a reduced accumulation of aggregates due to inefficient fragmentation.[49] Q24R expression, which limits the conversion reaction and would further diminish aggregate accumulation, is completely incompatible with the [*PSI*$^+$]^{weak} variant (our unpublished observations), while G58D expression, which enhances fragmentation and would increase aggregate accumulation, promotes [*PSI*$^+$]^{weak} mitotic stability.[64]

Consistent with these predictions, propagon accumulation in [*PSI*$^+$]^{strong} haploid strains decreased with Q24R expression but increased with G58D expression. However, these effects were modulated by the cellular environment: in [*PSI*$^+$] ^{strong} diploid strains, expression of either mutant decreased propagon accumulation. We hypothesized that these observations could be explained by the relative doses of Sup35 and Hsp104 under the two conditions (2:1 vs. 1:1, respectively). If true, modulating chaperone levels in diploids should affect the severity of the inhibition. Indeed, the dominant-negative effects of both Q24R and G58D were partially or completely reversed, respectively, by lowering Hsp104 levels and were enhanced by overexpression of Hsp104. Moreover, we found differences in the Sup35:Hsp104 ratio in yeast strains of different genetic backgrounds, providing a molecular explanation for known variations in G58D efficacy.[64,132]

The dependency of prion inhibition on chaperone level suggested that the disassembly pathway was competing more efficiently with the assembly pathway in the presence of the dominant-negative mutants. To determine if aggregates were actually being resolubilized, we monitored the fate of existing Sup35 after treating [*PSI*$^+$]^{strong} cultures with cycloheximide. In these experiments, Sup35 transitioned from an SDS-resistant to an SDS-sensitive form in yeast strains expressing both wild-type and dominant-negative mutants but not in those expressing wild-type protein alone. Mechanistically, we predict that Q24R and G58D induce this same outcome through different pathways: the conversion defect of Q24R would increase the ratio of enzyme (Hsp104) to substrate (aggregates) for the fragmentation reaction, while the efficiency of that reaction would increase in the presence of G58D (**Fig. 1**, bottom). Thus, in either case, expression of dominant-negative mutants skews the competition between aggregate assembly and disassembly pathways toward the latter, allowing the effective clearance of an established prion conformer in vivo

(**Fig. 2**). Given the heterogeneity in aggregate accumulation that we have observed in individual wild-type yeast cells, the balance between these opposing forces may also vary among cells in the population, contributing a currently unappreciated influence on the severity and stability of prion-associated phenotypes under normal conditions.

Strikingly, there are many parallels between Sup35 Q24R and PrP Q172R and between Sup35 G58D and PrP Q219K. In addition to displaying similar effective inhibitory ratios, PrP Q172R, like Sup35 Q24R, is conversion defective and is incompatible with prion variants of different thermodynamic stabilities.[28,117,125,135] In contrast, PrP Q219K, like, Sup35 G58D, converts to the prion conformer efficiently, destabilizes aggregates and is incompatible only with prion variants of reduced thermodynamic stability.[28,105,117,125,126] Thus, despite the distinct cellular environments in which mammalian and yeast prions propagate, the pathways of dominant inhibition that we have uncovered for Sup35 mutants may be applicable to PrP mutants and may provide mechanistic insight into the phenotypic outcomes of prion infections in that system.

Conclusions

Despite our detailed understanding of amyloidogenesis in vitro, our ability to mechanistically link this process to its biological consequences in vivo lags far behind.[138,139] Collectively, our studies on the yeast prion [*PSI*$^+$] have begun to bridge this gap by demonstrating that the cellular environment dramatically influences the physiological consequences of Sup35 misfolding. Notably, conformation-based phenotypes are created through the interplay among Sup35 folding, protein quality control and other aspects of yeast cell biology,[24] and imbalances in these forces lead to the dominant inhibition of prion propagation in vivo.[134] Given the many parallels between prion propagation in yeast and in mammals, the insight gained through these studies may provide a new framework for connecting protein misfolding pathways in vitro to disease mechanisms in vivo.

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Note Added in Proof

Recent studies by the Weissman group have determined that amyloid fibers composed of G58D alone, when present in a particular conformation, exhibit the same conversion and thermodynamic stability differences in comparison with wild-type fibers in vitro as we have identified for mixed G58D-wild-type prion aggregates in vivo.[140]

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