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Site-specific structural analysis of a yeast prion strain with species-specific seeding activity

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Prion proteins misfold and aggregate into multiple infectious strain variants that possess unique abilities to overcome prion species barriers, yet the structural basis for the species-specific infectivities of prion strains is poorly understood. Therefore, we have investigated the site-specific structural properties of a promiscuous chimeric form of the yeast prion Sup35 from *Saccharomyces cerevisiae* and *Candida albicans*. The Sup35 chimera forms two strain variants, each of which selectively infect one species but not the other. Importantly, the N-terminal and middle domains of the Sup35 chimera (collectively referred to as Sup35NM) contain two prion recognition elements (one from each species) that regulate the nucleation of each strain. Mutations in either prion recognition element significantly bias nucleation of one strain conformation relative to the other. Here we have investigated the folding of each prion recognition element for the serine-to-arginine mutant at residue 17 of the Sup35NM chimera known to promote nucleation of *C. albicans* strain conformation. Using cysteine-specific labeling analysis, we find that residues in the *C. albicans* prion recognition element are solvent-shielded, while those outside the recognition sequence (including most of those in the *S. cerevisiae* recognition element) are solvent-exposed. Moreover, we find that proline mutations in the *C. albicans* recognition sequence disrupt the prion templating activity of this strain conformation. Our structural findings reveal that differential folding of complementary and non-complementary prion recognition elements within the prion amyloid core of the Sup35NM chimera is the structural basis for its species-specific templating activity.

Introduction

Several neurodegenerative disorders (e.g., Alzheimer and prion diseases) are related by improper folding and aggregation of specific proteins into similar types of β-sheet rich, oligomeric structures.[1,2] However, Prion diseases are unique among conformational disorders since the mammalian prion protein PrP misfolds into infectious β-sheet rich, aggregated conformers that are linked to several fatal infectious diseases in mammals (transmissible spongiform encephalopathies).[2,3] Many prions in yeast and other fungi have also been identified,[4-16] the most-well studied of which is Sup35.[5-8] Sup35 is a yeast translation-termination factor whose switch to its aggregated prion conformation reduces its activity, leading to an increase in read-through of stop codons and multiple phenotypic changes (e.g., change in colony color on adenine-deficient media).[5-8,17-19] Sup35's N-terminal (N) domain is highly amyloidogenic, and essential for prion formation and propagation.[5-8] This domain is rich in glutamine and asparagine residues, and contains 5.5 imperfect oligopeptide repeats ([P/Q]QGGYQ[Q/S]YN) reminiscent of the mammalian PrP's oligopeptide repeats (P[H/Q]GGGWGQ).[20-23] The middle (M) domain is rich in charged residues and acts to solubilize the N domain.[5-8,10] Sup35's C-terminal domain encodes its translation termination function.[5-8,10] Together, the N and M domains (NM) can form infectious prion conformers with or without the C-terminal domain of Sup35.[24,25]

Both PrP and Sup35 display two of the most important and puzzling aspects of prion biology. First, both proteins adopt not just one aggregated prion conformation,[26] but rather several related yet structurally distinct strain conformations.[19,24,27-37] Each strain conformation self-perpetuates and confers a distinct biological phenotype.[24,27-31,33,38,39] Second, the transmission of the prion state between proteins of different species is limited by a species barrier that can be occasionally crossed.[40-55] Interestingly, the ability to establish and overcome species barriers in both yeast and mammals appears to be linked to the ability of prions to form distinct strains,[5,37,40,44,47,49,54-63] yet the structural mechanisms linking prion strains and species barriers are poorly understood. In yeast, transmission of Sup35 prions from *S. cerevisiae* (Sc) to *C. albicans* (Ca) and vice versa is limited by a species barrier.[43,46,56,60] However, a promiscuous chimeric Sup35 prion consisting of domains of Sc and Ca Sup35 (**Fig. S1**) can form two strain conformations with unique specificities to overcome this species barrier.[37,43,56,60]

Towards understanding how prion amyloid structure governs the selective infection of one species relative to another, we previously asked whether the nucleation of each strain conformation of the NM portion of the Sup35 chimera (herein referred

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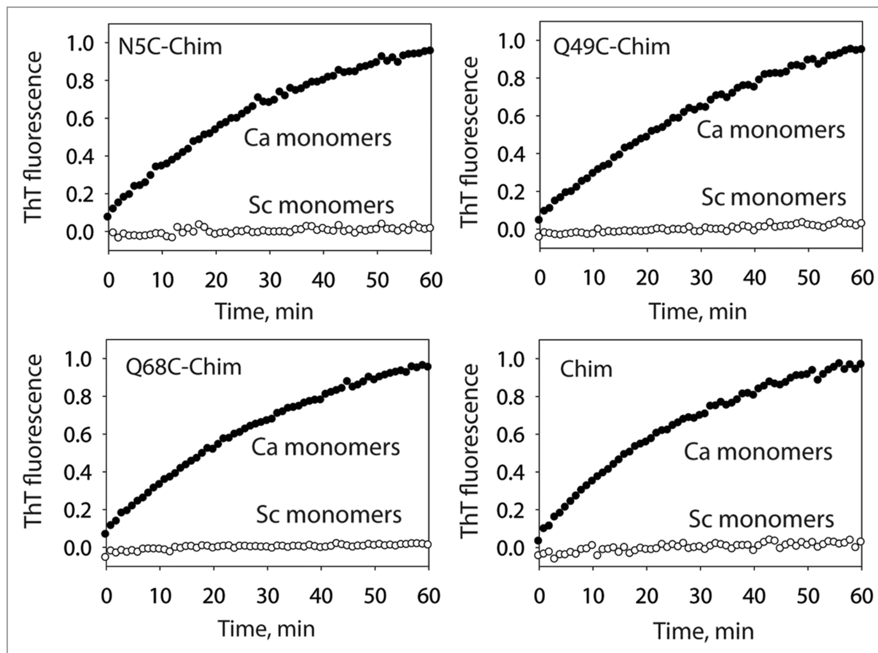

Figure 1. Templating specificity of the *C. albicans* prion strain conformation of the S17R Sup35NM chimera. The S17R Sup35NM chimera (Chim) and cysteine mutants thereof were assembled into prion amyloids and their templating specificity was analyzed using ThT fluorescence. The reported seeding activity of the chimeric Sup35NM fibrils is after subtraction of the contribution from the residual CaNM fibrils used to template the assembly of the Sup35NM chimera.

to as the Sup35NM chimera) could be localized to one or more small sequence segments within the chimeric prion protein.[64] Using peptide microarrays that displayed hundreds of overlapping 20mer peptides from Sc and Ca Sup35, we identified two prion recognition sequences that regulated the nucleation of each strain conformation. We found that a mutation (S17R) in the *S. cereviase* prion recognition element which favored nucleation of a prion strain conformation specific for infecting *C. albicans*[43,56] led to a highly specific interaction between the Sup35NM chimera and Ca peptides (residues 59–86).[64] In contrast, we found that mutations in the *C. albicans* recognition element (G70A, G71A, G80A, G81A) which promoted nucleation of a prion strain specific for infecting *S. cerevisiae*[43,56] led to specific association between the Sup35NM chimera and Sc peptides (residues 9–39).[64]

Our discovery of two prion recognition elements within the Sup35NM chimera led us and others[37] to hypothesize that the structural basis of the species-specific infectivities of its two chimeric prion strains is selective inclusion of one recognition element within the amyloid core, while the other one is excluded from the core. Indeed, recent amide exchange and proteolysis experiments for the wild-type Sup35NM chimera revealed that the *S. cerevisiae* strain conformation contains the Sc recognition sequence within its amyloid core, while the Ca recognition element is excluded from the amyloid core.[37] However, the *C. albicans* strain conformation was found not only to contain the Ca recognition sequence within its amyloid core, but the Sc prion recognition element was also partially solvent protected. The partial protection of the Sc recognition element suggests that it may be aggregated within the *C. albicans* strain conformation of the Sup35NM chimera. Since we[64] and others[56,65] had previously found that the S17R mutation in the Sc recognition element inhibited nucleation of the *S. cerevisiae* strain conformation of the Sup35NM chimera, we hypothesized that the S17R mutation would prevent aggregation of the Sc recognition element in the *C. albicans* strain conformation. Herein, we report our site-specific structural analysis of each prion recognition element within the *C. albicans* strain conformation of the S17R Sup35NM chimera.

Results and Discussion

We first sought to define the solvent-shielded residues within the folded prion core relative to those excluded from the core. As a site-specific approach for identifying residues within the amyloid core, we synthesized a panel of single cysteine variants of the S17R Sup35NM chimera that could be used to report the extent of solvent accessibility via cysteine-specific labeling after folding each mutant into prion amyloids. We generated 24 single cysteine substitution mutants distributed throughout the prion (N) and nonprion (M) domains (**Fig. S1**). Each purified Sup35NM chimera mutant was templated specifically into prion amyloids via seeding with CaNM fibrils (37°C). All mutants formed SDS-resistant amyloids that were indistinguishable via SDS-PAGE analysis (**Fig. S2** and data not shown). We evaluated the templating activity of each cysteine mutant for specifically converting monomeric CaNM into prion fibrils relative to monomeric ScNM to evaluate if the cysteine mutations alter the biochemical activity of the chimeric prion amyloids. Importantly, all mutants display similar, significant seeding activity for CaNM monomers and negligible seeding activity for ScNM monomers (**Figs. 1 and S3**).

Once each cysteine mutant of the S17R Sup35NM chimera was assembled into the *C. albicans* strain conformation, we labeled the amyloids with a cysteine-specific dye (Lucifer yellow iodoacetamide) to identify solvent-shielded residues that are poorly reactive due to inclusion within the amyloid core.[39,66] We also labeled monomers of each mutant to evaluate the fibril-to-monomer labeling ratio as a measure of the extent of solvent accessibility of each residue. Importantly, we first confirmed that Lucifer yellow fails to label the original S17R Sup35NM chimera that lacks cysteine (<5%; data not shown). In **Figure 2A**, we report the extent of labeling of each cysteine mutant in the *C. albicans* strain conformation of the S17R Sup35NM chimera. We find that residues within and near to the Ca recognition element (residues 59–86) are the most protected (<50% solvent exposed except for Q68), while those residues that

flank this recognition element (including most of those in the Sc recognition element) are >50% solvent exposed (**Fig. 2A**). Notably, we find that residue Q38 in the Sc recognition element is protected (~20% solvent exposed), suggesting that the edge of the Sc recognition element folds within the amyloid core along with the Ca recognition element. Moreover, we find that other residues flanking the Ca recognition element (residues 88–142) also display a similar level of solvent exposure as the Sc recognition element. Finally, residues in the middle domain of the NM chimera (residues 168 and 213) are highly solvent accessible (>90% solvent exposed), consistent with the highly charged nature of this domain.[67,68]

We next evaluated the impact of the S17R mutation on the folding of the Sc prion recognition element within the *C. albicans* chimeric strain conformation by analyzing the wild-type Sup35NM chimera that lacks this mutation. We expected that the lack of the S17R mutation would decrease the solvent exposure of the Sc recognition element. Therefore, we repeated the labeling analysis for residues within the *S. cerevisiae* recognition element of the wild-type Sup35NM chimera in the *C. albicans* conformation. As expected, we find that the residues within the Sc recognition element are less solvent exposed for the wild-type Sup35NM chimera than for the S17R mutant (**Fig. 2B**).

Next, we investigated the functional significance of the solvent-shielded residues within the *C. albicans* strain conformation of the S17R Sup35NM chimera using proline substitution mutagenesis. We sought to identify residues in the Sup35NM chimera involved in β-sheets (or other related folds) that are incompatible with proline residues.[38,69] Thus, we evaluated the templating activity of the *C. albicans* strain conformation for converting single proline mutants of the Sup35NM chimera from monomers into fibrils. We hypothesized that residues normally folded within the amyloid core will be inefficiently folded into the core when mutated to proline.[38,69,70] Therefore, we synthesized a panel of 22 single proline substitution mutations in the N and M domains of the prion chimera, and evaluated the rate at which each mutant is templated into the prion state by the *C. albicans* strain conformation of the S17R Sup35NM chimera (**Fig. 3**).

We find that proline mutations within or near the Ca recognition element (residues 38–99) are most disruptive to the seeding activity of the S17R Sup35NM chimera (>20% defect; **Fig. 3**). In contrast, proline mutations flanking the Ca recognition element (including most of those in the Sc recognition element) show little defect in templating activity, in agreement with the cysteine labeling experiments (**Fig. 2**). Mutating residue Q38 (at the edge of the Sc recognition element) to proline is also disruptive (~25% seeding defect), consistent with the fact that it is typically folded within the amyloid core of this strain conformation. Finally, we find that some proline mutations, especially residues in the Sc recognition element (residues 10, 12 and 33), accelerate the templating activity of fibrils of the S17R Sup35NM chimera. This finding suggests that the wild-type Sc recognition element participates in interactions that lower the templating activity of the *C. albicans* strain conformation of the Sup35NM chimera, and these interactions are attenuated via proline mutations in the Sc recognition sequence. Overall, the relative impact

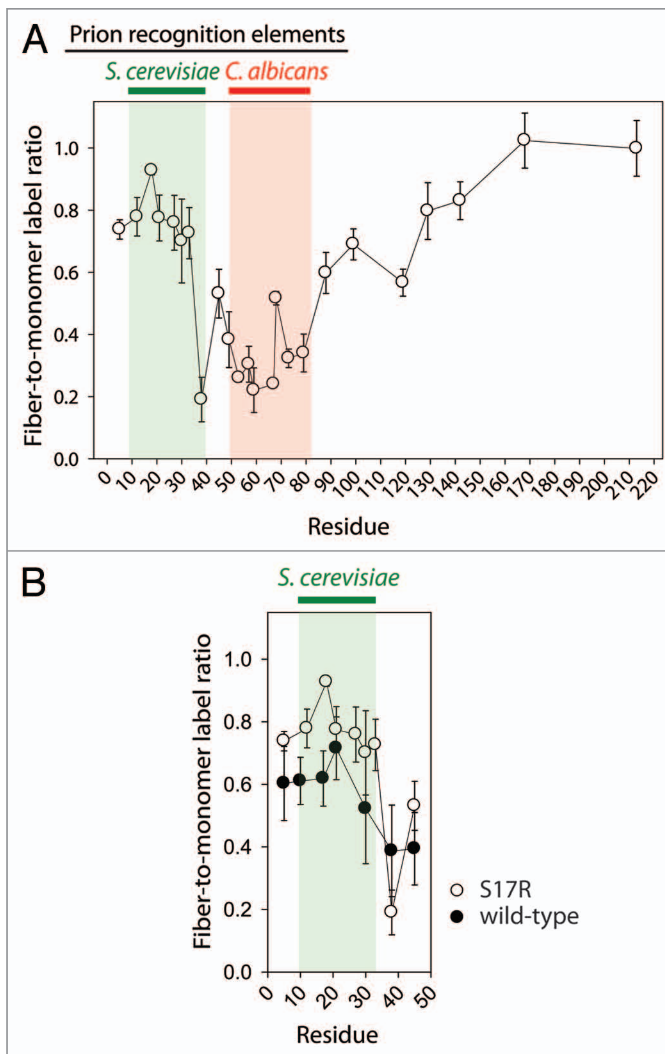

Figure 2. Solvent accessibility of cysteine residues in the *C. albicans* prion strain conformation of the Sup35NM chimera. (A) S17R Sup35NM chimera fibrils in the *C. albicans* strain conformation (as well as the corresponding monomers) were labeled using Lucifer yellow iodoacetamide. (B) Comparison of cysteine labeling of S17R and wild-type Sup35NM chimera fibrils in the *S. cerevisiae* domain. Each measurement is the average (±standard deviation) of at least three independent experiments.

of proline mutations on prion templating activity (**Fig. 3**) is in excellent agreement with the cysteine labeling analysis (**Fig. 2**). These results suggest that residues in the Ca recognition element that are folded within the amyloid core directly mediate the species-specific templating activity of the *C. albicans* strain conformation.

A key outcome of our studies is site-specific structural details regarding the differential folding of the Sc and Ca prion recognition elements within one strain conformation of the promiscuous Sup35 prion chimera. There are several plausible structural models for how each recognition sequence could be differentially folded in the two strain conformations of the Sup35 chimera to yield unique capacities to overcome the *S. cerevisiae*/*C. albicans* species barrier. It is logical that the

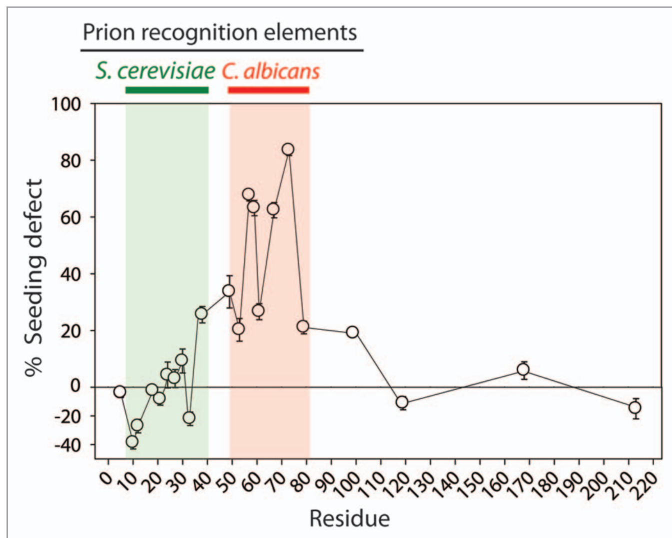

Figure 3. Impact of proline substitution mutants on the templating activity of the *C. albicans* strain conformation of the S17R Sup35NM chimera. S17R Sup35NM fibrils in the *C. albicans* strain conformation were evaluated for their activity to template proline substitution mutants of the Sup35NM chimera into prion fibrils. Each seeding rate (*k*) was calculated from the initial slope of the ThT measurements, and expressed as percent defect [100 x(1 - k_{mut}/k_{wt})], as described previously in reference 38.

Ca recognition sequence is folded within the amyloid core of the *C. albicans* strain conformation (as reported previously in ref. 37), yet it is less clear how the highly amyloidogenic *S. cerevisiae* recognition sequence is inactive in this strain conformation. Prior to performing these experiments, we envisioned at least four potential structural mechanisms for the inactivity of the Sc recognition sequence in the *C. albicans* strain conformation of the Sup35 chimera: (1) all residues within the Sc recognition element are unfolded; (2) a subset of the Sc residues are unfolded while the rest are folded within the amyloid core; (3) Sc residues are in an alternative non-β-sheet folded conformation in which they can participate in intermolecular contacts; and (4) Sc residues are folded in an alternative β-sheet conformation in which they do not participate in intermolecular contacts necessary for templating activity. Our combined cysteine labeling and proline seeding results are most consistent with the Sc recognition element being largely unstructured except for the edge of this region (residue Q38) being folded within the amyloid core (**Fig. 4**).

Our results are complementary to those of Weissman and coworkers, who showed that the *S. cerevisiae* domain of the chimeric prion is partially solvent protected.[37] Importantly, this previous study demonstrated that the selective seeding of the *C. albicans* chimeric strain conformation does not require the *S. cerevisiae* domain, as it could be removed without altering seeding specificity or activity. Therefore, Weissman and coworkers conclude that the *S. cerevisiae* domain is in an aggregated, partially-protected conformation that is not competent for templating.[37] We also observe partial solvent protection of the Sc domain in the *C. albicans* strain conformation of the wild-type Sup35NM. Moreover, we find that the charged mutation S17R modestly increases the extent of solvent exposure of the Sc domain, especially within the Sc recognition element (**Fig. 2B**). This finding is consistent with other reports that the S17R mutation prevents aggregation of Sc Sup35NM[56,64] and Sup35,[65] and that this mutation strongly favors formation of the *C. albicans* strain conformation of the Sup35NM chimera.[56,64,65] Our results suggest that this charged mutation minimizes non-specific aggregation of the *S. cerevisiae* recognition element that otherwise occurs in the *C. albicans* strain conformation.[37] Our work reveals that prion amyloid structure is sensitive to single mutations within prion recognition elements.

Our findings also contribute to the broader understanding of how prion sequence, strain variation and species barriers are related. The amino acid sequence of a prion determines the spectrum of allowed strain conformations, and the ability of a donor prion to transmit its prion state to an acceptor prion is determined by whether their sequences encode compatible strain conformations.[5,37,41,49,51,60,70-74] Our work (combined with that of Weissman and coworkers[37]) provides a structural mechanism for defining the compatibility of prion strains formed by donor and acceptor prions. We find that inclusion of the Ca recognition element within the amyloid core of the Sup35NM chimera (donor) encodes its specific infectivity for CaNM (acceptor), and we posit that the Ca recognition element must be included within the CaNM amyloid core to form infectious *C. albicans* prions. Conversely, we find that partial exclusion of the Sc recognition sequence from the amyloid core of the prion chimera eliminated prion transmission to ScNM, which is consistent with previous findings that inclusion of the Sc recognition element within the amyloid core of ScNM is required for formation of infectious *S. cerevisiae* prions.[38,39] Thus, we posit that the spectrum of allowed strain conformations is determined by proper folding of prion recognition elements within the amyloid core of donor prion strains that are complementary to the prion recognition sequences of the acceptor prion. This hypothesis would predict mutations in highly localized peptide segments (but not outside these segments) that impact the folding of prion recognition elements in the donor prion will govern the ability of such prions to cross species barriers. Indeed, we find that the S17R mutation impacts the folding of the non-complementary prion recognition element within the *C. albicans* strain conformation of the Sup35NM chimera. Moreover, additional point mutations in Sup35,[56,65,69,75,76] as well as in PrP[54,58,77] have been identified that both prevent and promote interspecies prion transmission. We expect that future structural studies aimed at investigating how mutations impact allowed strain conformations for closely related prions (such as PrP[78,79] and Sup35[53,71] variants) will reveal further insights into the role of the folding of small sequence elements within the prion amyloid core on the efficiency of interspecies prion transmission.

Materials and Methods

Mutagenesis and protein purification. We used standard site-directed mutagenesis to generate single cysteine mutants of the

Sup35NM chimera (both wild-type and S17R), as well as single proline mutants of the wild-type Sup35NM chimera. Each protein was purified via its C-terminal 7xHis tag, as described in references 64 and 68. Purified NM proteins were buffer exchanged into guanidinium hydrochloride (4 M, pH 7.4, 100 mM phosphate, 0.5 mM EDTA, 2 mM DTT) and stored at -80°C.

Fibril assembly, solubility and templating analysis. We assembled fibrils of the Sup35NM chimera into the conformation specific for *C. albicans* by mixing monomeric wild-type or mutant chimeric protein of *C. albicans* NM (CaNM) fibrils overnight (20% w/v seed, PBS, 37°C, 1,200 rpm). Afterward, the chimeric fibrils were sedimented (110,000x g for 30 min at 4°C) and reconstituted in PBS. The SDS-resistance of fibrils was evaluated using a denaturing gel (NuPAGE 10% Bis-Tris gel) for prion samples before and after heating (100°C, 30 min). We evaluated the prion templating activity by following the rate at which chimeric prion fibrils (10% w/v) convert ScNM and CaNM monomers (2.5 μM) into Thioflavin T (ThT) positive conformers. We evaluated the ThT fluorescence (40 μM ThT) in 96-well plates using a Safire^2 plate reader (Tecan; λex = 450 nm, λem = 482 nm) to obtain initial seeding rates (~10 min).

Cysteine accessibility measurements. Fibrils of each cysteine chimera mutant (5 μM) were reduced with tris(2-carboxyethyl) phosphine (TCEP, 25 μM) and labeled with Lucifer yellow iodoacetamide (0.1 mM, PBS, pH 7.4) for 12 h at 25°C. Afterward, the labeled fibrils were centrifuged (110,000x g for 30 min at 4°C), dissociated (4 M GuHCl, 100°C, 5 min) and excess dye was removed (Zeba 96-well spin-desalting plates, Pierce Thermo Scientific). As a control, fibrils were also dissociated into monomers (4 M GuHCl, 100°C, 5 min) prior to labeling, and then monomers were labeled in the same manner as fibrils. The extent of labeling for both fibrils and monomers was evaluated using fluorescence (λex = 426 nm, λem = 531 nm) and protein (micro BCA assay kit, Pierce Thermo Scientific) assays.

Proline mutant seeding defect analysis. Fibrils of the S17R Sup35NM chimera (10% w/v) were mixed with monomeric proline mutants (2.5 μM, PBS, pH 7.4), and their ThT fluorescence was monitored (12 h). The initial seeding rates of each proline mutant were compared to the wild-type Sup35NM chimera and reported as % defect as described previously in reference 38.

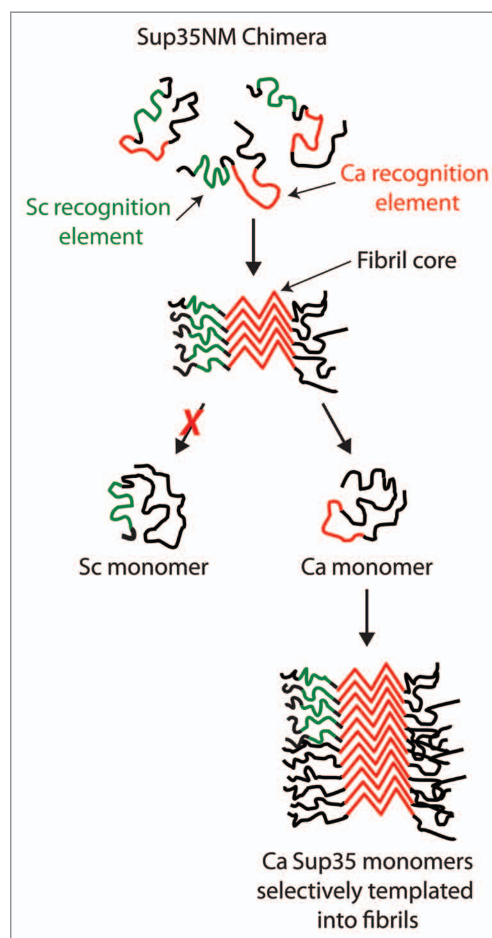

Figure 4. Selective folding of prion recognition elements within the Sup35NM chimera enciphers species-specific infectivity. The Sup35NM chimera contains two prion recognition sequences (green for the *S. cerevisiae* recognition element and red for *C. albicans* recognition element) that selectively nucleate each prion strain conformation. For the *C. albicans* strain conformation, we find that the Ca recognition sequence is folded within the prion amyloid core, while most of the Sc recognition sequence is excluded from the core. The resulting prion strain conformation is specific for infecting *C. albicans* Sup35 since its cognate recognition element is folded within the amyloid core and active for templating.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Note

Supplemental material can be found at:
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