Distinct Type of Transmission Barrier Revealed by Study of Multiple Prion Determinants of Rnq1

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Abstract

Prions are self-propagating protein conformations. Transmission of the prion state between non-identical proteins, e.g. between homologous proteins from different species, is frequently inefficient. Transmission barriers are attributed to sequence differences in prion proteins, but their underlying mechanisms are not clear. Here we use a yeast Rnq1/[PIN+]-based experimental system to explore the nature of transmission barriers. [PIN+], the prion form of Rnq1, is common in wild and laboratory yeast strains, where it facilitates the appearance of other prions. Rnq1’s prion domain carries four discrete QN-rich regions. We start by showing that Rnq1 encompasses multiple prion determinants that can independently drive amyloid formation in vitro and transmit the [PIN+] prion state in vivo. Subsequent analysis of [PIN+] transmission between Rnq1 fragments with different sets of prion determinants established that (i) one common QN-rich region is required and usually sufficient for the transmission; (ii) despite identical sequences of the common QNs, such transmissions are impeded by barriers of different strength. Existence of transmission barriers in the absence of amino acid mismatches in transmitting regions indicates that in complex prion domains multiple prion determinants act cooperatively to attain the final prion conformation, and reveals transmission barriers determined by this cooperative fold.

Introduction

An increasing number of proteins has been found to form β-sheet-rich aggregates called amyloid. In amyloid fibers, individual protein molecules are stacked on top of each other through the formation of inter-molecular β-strands perpendicular to the fiber axis. Existing fibers template the conformational conversion of the protein molecules with the same amino acid sequence, making amyloid an aggregate-based self-propagating protein conformation [1,2].

Intracellular aggregates or extracellular amyloid deposits are hallmarks of over 30 hereditary and sporadic disorders including Creutzfeldt-Jacob, Alzheimer’s, Parkinson’s and Huntington’s diseases and type II diabetes [2]. Several lines of evidence also suggest that the amyloid state has been functionally harnessed by organisms as diverse as bacteria, fungi and humans [3–5]. Discovery of infectivity of Creutzfeldt-Jacob disease and other spongiform encephalopathies, and linking the infectivity to the presence of the aggregated conformation of the PrP protein, PrPSc, single out these diseases as particularly hazardous, while PrPSc was termed “proteinaceous infectious agent”, or prion [6,7]. However, recent studies that revealed the inherent transmissibility of several mammalian amylloïds blurred the border between prions and other amyloïds [8,9].

In Saccharomyces cerevisiae, three epigenetic factors, [PSI+], [URE3] and [PIN+], manifest self-perpetuating amyloid conformations of Sup35, Ure2 and Rnq1, respectively [10]. The prion nature of these factors was established using genetic criteria proposed by Wickner [11], and the final proof of their protein-only transmission was obtained by infecting yeast cells with in vitro-made Sup35, Ure2 or Rnq1 amyloïds [12–15]. Another recently discovered prion, [SWT], satisfies the genetic criteria, but its amyloid nature has yet to be confirmed [16]. Yeast prions appear spontaneously or can be induced by transient overproduction of their respective prion-forming proteins [11,16–19]. Once established, they are efficiently transmitted to daughter cells in mitosis and segregate in a non-Mendelian fashion in meiosis, and the [PRIOV+] state is maintained in the population until it is spontaneously lost or selectively eliminated. Phenotypes caused by the presence of [PSI+], [URE3] and [SWT] are equivalent to loss-of-function mutations in, respectively, Sup35, Ure2 and Swi1, as these normally soluble proteins become sequestered into prion aggregates. For example, Sup35 is a translation termination factor, and [PSI+] increases the level of readthrough at stop codons [20], and can be detected as a suppressor of nonsense mutations [21]. Whether yeast prions are physiological epigenetic modifiers of cellular functions, egoistic elements or diseases is a subject of debate [10,22–30]. Nevertheless, yeast prions provide an excellent experimental model for addressing questions pertaining to self-propagating protein conformations.

The importance of direct templating in the transmission of the prion state is widely acknowledged, but the exact rules and determinants of this process remain unclear. Prion domains, which are terminally located in fungal prions, are essential and sufficient for prion formation and maintenance [18,31–34]. A recent study implicates short sequences within prion domains as nucleation/
Prions, self-propagating protein conformations and causative agents of lethal neurodegenerative diseases, present a serious public health threat: they can arise sporadically and then spread by transmission to the same, as well as other, species. The risk of infecting humans with prions originating in wild and domestic animals is determined by the so-called transmission barriers. These barriers are attributed to differences in prion proteins from different species, but their underlying mechanisms are not clear. Recent findings that the prion state is transmitted through the interaction between short transmitting regions within prion domains revealed one type of transmission barrier, where productive templating is impeded by non-matching amino acids within transmitting regions. Here we present studies of the prion domain of the [PIN⁺]−forming protein, Rnq1, and describe a distinct type of transmission barrier not involving individual amino acid mismatches in the transmitting regions. Rnq1’s prion domain is complex and encompasses four regions that can independently transmit the prion state. Our data suggest that multiple prion determinants of a complex prion domain act cooperatively to attain the prion conformation, and transmission barriers occur between protein variants that cannot form the same higher order structure, despite the identity of the region(s) driving the transmission.

Author Summary

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Figure 1. No one QN region of Rnq1 is essential for [PIN+] maintenance. (A) Schematic diagram of Rnq1 and deletion constructs. QN-rich regions are in red (patterned blocks indicate oligopeptide repeats), QG10 is in orange, hydrophobic patches are in blue. Numeric intervals indicate aa boundaries of respective QN regions. For deletion constructs, lines indicate regions present; nomenclature refers to deleted regions. (B) Deletion of any one QN region does not lead to the loss of ability to maintain [PIN+]. Indicated LEU2-marked constructs were transformed into [PIN+] [psi-] rmq1-D74-D694 carrying a URA3-marked RNQ1 maintainer and a HIS-marked pGAL-SUP35NM::YFP [PSI+] inducer. After selective elimination of the maintainer on FOA, expression of SUP35NM::YFP was transiently induced on SGal-Leu,His, and yeast were transferred to adeninelss media to score for [PSI+] (''from Gal''; shown is growth on SD-Ade after 10 days at 20°C) and to SD-Leu,His (''Growth Control''). In the control experiment yeast were grown on non-inducing SD-Leu,His instead of SGal-Leu,His (''from Glu''). (C) Rnq1 fragments introduced into the [PIN+] strain sustain the aggregated state after elimination of full-length Rnq1. Cultures carrying the indicated LEU2-marked plasmids were crossed to [pin-] 64-D697 carrying the URA3-marked pCUP-RNQ1::CFP. Diploids were selected on SD-Ura,Leu; reporter was induced by supplementing SD-Ura,Leu with 20μM CuSO4.

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constructs: (i) the non-QN-rich N-terminal domain preceding the A patch (aa 1–132) is retained; (ii) deletions start/stop at the borders of QG_{10} or QN-rich regions; (iii) internal deletions are “seamless”, i.e. no additional amino acids are inserted at the junctions; (iv) no tags are attached at the termini to avoid possible interference of tag sequences; (v) in yeast, the expression of deletion constructs is controlled by the native RNQ1 promoter, the native RNQ1 terminator sequence follows the ORFs, and the constructs are introduced on a single-copy CZ vector.

No one QN-rich region is essential for [PIN]\(^{+}\) maintenance

We first asked if any of the QN regions is essential for the maintenance of [PIN]\(^{+}\). Deletion constructs lacking one of the four QN regions, as well as an adjacent hypochromic sequence (Figure 1A), were transformed into a [PIN]\(^{+}\) strain expressing full-length RNQ1 controlled by the RNQ1 promoter. The plasmid-borne RNQ1 maintainer kept steady-state Rnq1 levels comparable to those in strains with endogenous RNQ1 and ensured stable maintenance of [PIN]\(^{+}\) (not shown); steady state levels of deletion constructs were similar to those of full-length Rnq1 (Figure S1A). Following transient co-expression of deletion constructs with full-length Rnq1 (to allow for transfer of the prion state), the maintainer plasmid was shuffled out, and the presence of prions composed of Rnq1 fragments, hereafter referred to as mini-[PIN]\(^{+}\), was scored using the [PSF] induction assay ([53,69]; see [PSF] Induction Assay in Materials and Methods).

The assay relies on the requirement of [PIN]\(^{+}\) for the de novo formation of [PSF] and requires that the strain carry a [PSF]-inducing construct and a reporter for the detection of [PSF]. Our strain carried the SUP25XM::YPF inducer, under the control of a tightly regulated GAL1 promoter, and the ade1-14 reporter, a premature stop in the chromosomal ADE1 gene allowing to score the appearing [PSF] colonies by their ability to grow on media lacking adenine. The Sup35NM::YPF fusion was used as an auxiliary reporter during [PSF] induction on galactose medium: its incorporation into newly forming [PSF] aggregates allowed their visualization by fluorescent microscopy ([70,71]).

Figure 1B demonstrates that, after elimination of full-length Rnq1, cultures expressing deletion constructs lacking any one QN region remained Pin\(^{+}\) (similar data for Δ2D and Δ3E not shown). The Pin\(^{+}\) phenotype was determined by mini-[PIN]\(^{+}\), as the propagation of the phenotype required the presence of Rnq1 fragments: the loss of deletion plasmids was always accompanied by the loss of ability to become Pin\(^{+}\). The Sup35NM::YPF fusion was used as an auxiliary reporter during [PSF] induction on galactose medium: its incorporation into newly forming [PSF] aggregates allowed their visualization by fluorescent microscopy ([70,71]).

To determine which QN regions could drive prion-like aggregation of Rnq1, bacterially expressed Rnq1 fragments lacking three out of four QN regions were tested for the propensity to form amyloid in vitro. Incubation of proteins encompassing only QN2 (ΔB1D3E4), QN3 (ΔB1C2E4) or QN4 (ΔB1C2D3) with Thiollavin T (ThiT) resulted in a shift of the ThiT excitation spectrum and increase of ThiT fluorescence at 403 nm, indicative of amyloid formation (Figure 2A). Sigmoidal fluorescence kinetics was consistent with the presence of a rate-limiting nucleation step followed by a fiber growth phase. The QN1-bearing protein (ΔC2D3E4) did not form amyloid even at very high concentrations.

Aggregation kinetics of QN2 and QN4 was similar except that at equal protein concentrations the lag phase was slightly shorter for QN4. The threshold protein concentration for fiber formation was \(15–20\ \mu M\), and the length of the lag phase was reproducible and concentration-dependent in the 30–80 \(\mu M\) range (Figure 2B and not shown). When reactions were seeded by preformed homologous fibers, the lag phase was completely eliminated (Figure 2C). In the cross-seeding reactions QN2 and QN4 could efficiently seed and be seeded by larger Rnq1 fragments, as long as they encompassed, respectively, QN2 and QN4 regions, implicating these regions in specific interactions during cross-seeding (Figure 2E and Figure S3A). For QN3, the threshold concentration was higher, \(\sim 80\mu M\), and the lag phase was considerably longer compared to QN2 and QN4 (Figure 2A), indicating that QN3 has a weaker aggregation propensity. The kinetics of seeded QN3 reactions was also distinct from QN2 and QN4: the QN3 fluorescence curve remained sigmoidal and a 10–20 hr lag phase was observed regardless of the amount of seed added (Figure 2D and data not shown). Such unusual kinetics was previously observed for the PrP_{90-231} fragment ([72]). Also, QN3 was only capable of self-seeding, but could not template or be templated by a fragment including both QN3 and QN4 (QN3,4; Figure 2E and Figure S3A). QN3,4 aggregation could only be self-seeded or seeded by QN4 and there was no lag phase in these reactions ([ibid.]). This suggests that the conformation of the QN3 region in the fibers made from a Rnq1 fragment in which QN3 is the only aggregation determinant is different from conformations QN3 can take when combined with other QN regions.

Electron microscopy revealed networks of \(>1\ \mu m\)-long fibers in QN2, QN3, and QN4 samples (Figure 2F–2H and 2J–2L). Fibers were \(18–25\ nm\) in diameter and unbranched, but frequently 2 or more fibers were associated laterally for part of their length producing thicker rope-like structures. In addition to fibers, we observed ring-like structures strikingly similar to oligomeric species previously seen during fiber formation by other amyloidogenic proteins ([73,74]); the oligomers were either lying separately or distributed irregularly along the fibers (Figure 2J and 2L inset). Similar fibers and oligomers were formed in the same conditions by full-length Rnq1 (Figure 2I and 2M).

As expected of amyloid, QN2 and QN4 fibers were protease-resistant. After hydrolysis with papain, they remained long, but became thinner (<10 nm in diameter), QN4 fibers became very smooth (Figure 2P), and QN2 fibers retained their twisted appearance (Figure 2N). Ring-like oligomers also remained and their structure became even more obvious (Figure 2N insets).
Figure 2. QN2 (∆B1C2E4), QN3 (∆B1C2E4), and QN4 (∆B1C2D3), but not QN1 (∆C2D3E4), can drive amyloid fiber formation in vitro.

(A–D) Kinetics of in vitro aggregation of recombinant proteins monitored by ThT fluorescence. (A) Unseeded reactions. Concentrations: QN1 - 100 μM, QN2 - 70 μM, QN3 - 80 μM, QN4 - 70 μM. Shown are averages and standard deviations of 2-hour time points based on 3–5 independent experiments. (B) Concentration dependence of the lag phase for QN4 aggregation. (C) Elimination of lag phase in seeded reactions for QN2 and QN4. Concentrations of soluble proteins were 70 μM. (D) Retention of lag phase in seeded reactions for QN3. Concentration of soluble protein was 80 μM.

(E) Summary of the analysis of cross-seeding between Rnq1 fragments carrying different QN regions (see Figure S3A). QN1,2 and QN3,4 correspond, respectively, to ∆D3E4 and ∆B1C2 in Figure 1A. (+) indicates disappearance or, in the case of QN3, reduction of the lag phase; (−) indicates no change in the kinetics of fiber formation upon the addition of seeds to soluble proteins. (F–P) Transmission electron micrographs of negatively stained fibers. In (N–P) fibers were treated with papain. Arrows show lateral association of fibers (L), ring-like oligomers (J, L inset, M, N insets) and twisted appearance of QN2 fibers (F,N).

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Unexpectedly, papain treatment eliminated all long QN3 fibers, although occasional clusters of atypical aggregates with few protruding thread-like structures could still be detected (Figure 2O).

Thus, the Rnq1 prion domain encompasses three distinct QN-rich determinants that can independently drive aggregation of Rnq1 in vitro: QN2, QN3 and QN4. Among them, QN3 is weaker, and QN3 aggregates are somewhat atypical.

Lack of aggregation of QN1 suggests that its 12 aa long QN-rich stretch is not sufficient to drive aggregation of Rnq1 independently and confirms the lack of strong aggregation determinants upstream of QN1. However, using ThT fluorescence analysis and TEM, we demonstrated that the 12 aa peptide corresponding to QN1 region alone readily formed typical amyloid fibers (Figure 3SB and data not shown), suggesting that this region may function as aggregation determinant in the presence of other QN regions.

The prion state can be transmitted to Rnq1 fragments carrying either QN2 or QN4

To test which QN regions could maintain prion state in vivo, RNQ1 fragments encoding any one QN region or all possible combinations of two QN regions were substituted for the full-length RNQ1 in the [PIN]+psi74-D694 strain. As seen from the [PSF] induction test, all fragments carrying two QN regions could be converted into mini-[PSF]s as long as QN2 or QN4 were retained (Figure 3A; ΔB1Δ3 cultures were also Pin– and in this test were similar to ΔB1C2 and ΔB1E4, not shown). Furthermore, low-level [PSF] induction was detected even when using the constructs encompassing only QN2 or QN4 (Figure 3B). This weak Pin– phenotype was confirmed using the Sup35NM::Yfp reporter: cells with bright foci indicative of [PSF] appearance were readily detected in cultures expressing either QN2 or QN4, but not in the empty vector control (not shown).

On the contrary, cultures expressing Rnq1 fragments encompassing only QN1 and QN3, alone or together, became Pin– after wild type RNQ1 was shuffled out (Figure 3A and 3B; also no aggregate-containing cells were detected when Sup35NM::Yfp was used to screen for rare [PSF]+s). We also found no evidence that QN1 and QN3-bearing fragments were taking on a prion state that was incapable of inducing [PSF]+; these fragments remained soluble in [PIN]+ cells before elimination of RNQ1 (Figure 3C), and after the shuffle the lack of mini-[PIN+] aggregates was confirmed by a diffuse distribution of the reporter in the Rnq1::Cfp aggregation test (not shown).

Ability of Rnq1 fragments carrying either QN2 or QN4 to form mini-[PIN+] is in agreement with in vitro data and further indicates that these regions represent independent prion determinants. Also, considering the specificity of cross-seeding of QN2- and QN4-encompassing fragments in vitro (Figure 2E), transmission of the prion state from [PIN]+ to fragments carrying either QN2 or QN4 suggest that in the wild type [PIN]+ prion both QN2 and QN4 regions are involved in prion formation and are available for templating. (To prove that the prion state was transmitted to Rnq1 fragments from the pre-existing [PIN]+, and that Rnq1 fragments expressed from single-copy plasmids did not induce mini-[PIN]+s de novo, RNQ1 deletion constructs were substituted for wild type RNQ1 in [pin–]psi74-D694, which resulted in Pin– cultures; see Text S1). The more robust Pin– phenotype observed when QN2 or QN4 were combined with either QN1 or QN3 compared to QN2 or QN4 alone (Figure 3A and 3B) suggests that QN1 and QN3 are also involved in prion formation. However, we found no indication that the prion state could be transmitted to fragments carrying only these QN regions. One possibility, consistent with the weak amyloid-forming propensity of QN1 and QN3 in vivo, is that in vivo their aggregation is contingent on the presence of QN2 or QN4.

![Figure 3. Transmission of the prion state to Rnq1 fragments with one or two QN regions.](https://example.com/figure3.png)

(A-B) The prion state can be transmitted from [PIN]+ to Rnq1 fragments encompassing either QN2 or QN4. [PSF]+ induction assay was performed as in Figure 1B. Shown is growth on SD-Ade after incubation at 20 °C for 11 (A) and 13 (B) days. (C) Rnq1 fragments carrying only QN1 or/and QN3 do not co-aggregate with [PIN]+. Sedimentation analysis of the lysates of [PIN]+ cells expressing both full-length Rnq1 and indicated deletion constructs; the WT Rnq1 and the QN1,QN3 panels show same lanes in the top and the bottom parts of the Western blot. See Figure S1B and S1C for steady state levels of Rnq1 fragments used in these experiments.

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A barrier for the transmission of the prion state from [PIN+] to mini-[PIN+]s

As seen from Figure 3A and 3B, the Pin⁺ phenotype of cultures carrying shorter mini-[PIN+] s is notably reduced compared to the original [PIN⁺] strain. Furthermore, quantifying the frequency of [PSI⁺] induction using the Sup35::Yip reporter revealed that deletion of any QN region except QN1 resulted in a drop in the induction of [PSI⁺] (Figure S4). Reduced [PSI⁺] induction could reflect (i) the appearance of [pin⁺] cells due to inefficient transmission of the prion state from the wild type [PIN⁺], i.e., transmission barrier; (ii) accumulation of [pin⁺] cells because Rnq1 fragments were deficient in maintaining the prion state in the absence of full-length Rnq1; (iii) poor seeding of [PSI⁺] by mini-[PIN⁺]s.

To eliminate the last possibility as the only cause for weak Pin⁺ phenotypes in cultures expressing Rnq1 fragments, we analyzed the [PIN⁺] status of individual cells in these cultures. After shuffling out the full-length RNQ1, the cultures were colony purified, and individual colonies were screened via the [PSI⁺] induction assay (see Figure S5 for a scheme of experiments). If reduced [PSI⁺] formation were exclusively due to poor seeding of [PSI⁺] by mini-[PIN⁺] s, a weak Pin⁺ phenotype was expected for all colonies. However, most cultures yielded both Pin⁺ and Pin⁻ colonies (Figure 4A) indicating that substitution of deletion constructs for full-length RNQ1 lead to the loss of prion in some cells. Pin⁻ colonies were mitotically stable, and their [pin⁺] status was confirmed by the Rnq1::Gfp aggregation test (not shown) and, for several colonies, by sedimentation analysis (see e.g. Figure 4E).

Although the proportion of [pin⁺] cells varied widely in cultures expressing the same Rnq1 fragment, there was a clear dependence of distributions upon the number and identity of QN regions (see Figure S6 for distributions). In most cases, the proportion of [pin⁺] cells correlated with the degree of reduction of [PSI⁺] induction prior to colony purification (compare Figure 4A and Figure S4). In agreement with genetic data, soluble Rnq1 was detected in the lysates of the cultures expressing all Rnq1 deletion constructs except AB1, and the proportion of soluble Rnq1 correlated with the percentage of [pin⁺] cells (Figure 4D, Figure S7, and data not shown).

We next asked if Rnq1 fragments were unable to form efficiently propagating mini-[PIN⁺] s, and if this deficiency in prion propagation could alone account for the accumulation of [pin⁺] cells, or there indeed was a barrier for the transmission of the prion state from [PIN⁺] to mini-[PIN⁺] s. The Pin⁻ colonies obtained by the colony purification described in the previous paragraph represent single-cell derived mini-[PIN⁺] isolates. To analyze mitotic stability of these mini-[PIN⁺] s, we passed them once on SD-Leu,His, then colony purified again and determined what proportion of colonies remained Pin⁺ (Figure 4B; see also Figure S5 for a scheme of experiments). Even though [pin⁺] cells did accumulate in a considerable proportion of mini-[PIN⁺] isolates, the data clearly indicate the existence of transmission barriers. Specifically, for all single and double QN deletions, there were mini-[PIN⁺] s that transmitted to all mitotic progeny (Figure 4B).

Several such mini-[PIN⁺] s were selected for each construct and their high stability was confirmed by more extensive analysis (not shown). Furthermore, in these stable isolates, Rnq1 fragments were detected only in aggregated fractions (Figure 4E). This demonstrates that respective Rnq1 fragments are fully competent in maintaining the prion state. Also, for all deletion constructs, the average proportion of [PIN⁺] cells was much higher in mini-[PIN⁺] isolates than in original post-shuffle cultures, even though the number of cell divisions from a mini-[PIN⁺] cell to the second round of colony purification was no less than the number of divisions from the loss of full-length Rnq1 to the first round of colony purifications (compare Figure 4A and 4B and Figure S6). This indicates that the prion state was lost more frequently upon the elimination of full-length RNQ1 or soon after. Our finding of stable and unstable mini-[PIN⁺] isolates may reflect the process prion strain formation, which is another hallmark of transmission barriers.

In a different approach we analyzed the aggregation of Rnq1 fragments in [PIN⁺] cells prior to the elimination of RNQ1. The rationale was that in the case of a transmission barrier Rnq1 fragments should remain partially soluble even in the presence of [PIN⁺]. Indeed, even ΔC2, ΔD3 and ΔE4 single deletions, for which fairly weak transmission barriers were indicated by genetic analysis, were detected in soluble fractions, and only AB1, for which the weakest barrier was seen, appeared fully aggregated (Figure 4C and not shown). For double and triple deletions, only AB1C2 was mostly aggregated, whereas other Rnq1 fragments remained mostly soluble in the presence of [PIN⁺], consistent with strength of respective transmission barriers (not shown).

Thus, we demonstrated that there is a barrier for the transmission of the prion state from [PIN⁺] to mini-[PIN⁺]s. None of the QN regions can ensure a barrier-free transmission, and elimination of any QN region results in the transmission barrier (a barrier towards AB1 seen in Figure 4A is very weak but was confirmed in other experiments; MK and ID unpublished observations).

Contribution of oligopeptide repeats, hydrophobic patches, and QG10 to the transmission barrier

The complexity of Rnq1 prion domain goes beyond the presence of four QN-rich regions. Three QN regions, QN2, QN3 and QN4, encompass oligopeptide repeats (Figure 1A). To probe the contribution of these repeats to the transmission of the prion state, we tested if removing a part of a repeat-containing QN region is equivalent to its complete deletion. Experiments described in this section were performed with a set of C-terminal truncations gradually removing parts of regions QN3 and QN4 (Figure 5A and Figure S8). Comparison of Δ1/3E4 (terminates right after the first oligopeptide of the QN3 repeat) with ΔD3E4, and of Δ1/3A (the first oligopeptide of the QN4 repeat is preserved intact) with ΔE4, shows that retaining only the first oligopeptide of the repeat is enough to lower the transmission barrier compared to complete deletion of the respective QN region, but is not equivalent to retaining the whole QN region. This suggests that each oligopeptide contributes to prion formation, and that retaining the repeated structure is not essential for this contribution. The appearance of [pin⁺] cells even in the cultures expressing Δ1/3A/4 (retains the complete QN4 repeat but lacks the very C-terminal QN-rich stretch and the preceding non-QN-rich sequence) indicates that both the repeats and the unique part of QN4 contribute to the prion conformation. The gradual increase of the transmission barrier with progressing C-terminal truncations is consistent with observations of Vitrenko et al. [65] who noted an increase in the number of [pin⁺] cytoductants following the transmission of [PIN⁺] to a smaller set of Gfp-tagged C-terminally truncated Rnq1 fragments.

Another feature of Rnq1 prion domain is the alternating pattern of QN regions and hydrophobic patches (Figure 1A) with a QN-rich region at the C-terminus. Our analysis suggests the importance of the C-terminal location of the last QN region: compared to ΔE4 and ΔD3E4 ending with QN-rich regions, the proportion of Pin⁺ cells was sharply reduced in cultures expressing Δ4 and ΔE4 fragments carrying hydrophobic patches at their C-termini (Figure 5B). The importance of alternating QN regions
and hydrophobic patches is not as clear. For example, the proportion of mini-[PIN+] cells was lower in D2 cultures than in D2 and D2D, but there was no significant difference between D3, D3 and D3E (Figure 5C and Figure S9A and S9B). Finally, QG10, which does not lead to a transmission barrier when deleted alone (Figure S2C and S2D), may contribute to barriers if deleted with other regions. Compared to DQGB1C2, more of DQG1B1C2 remained soluble in [PIN+] cells, and after RNQ1 was eliminated and wild type [PIN+] was lost, a higher proportion of cells became [pin+] (Figure 5D, Figure S9C and S9D and data not shown). In this aspect QG10 is similar to QN1, for which effects on transmission barriers are seen mainly in the context of larger deletions.

Cooperative action of QN-determinants in prion conformation

As shown in the previous sections, prion domain of Rnq1 encompasses multiple QN-rich aggregation determinants. None of them is essential for the transmission of the prion state from [PIN+] to Rnq1 fragments, and analysis of transmission to Rnq1

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**Figure 4. Transmission barrier for the conversion of Rnq1 fragments into mini-[PIN+]s by wild-type [PIN+].** (A) Substitution of RNQ1 deletion constructs for full-length RNQ1 in a [PIN+] strain leads to the appearance of [pin+] cells. Plasmid shuffle was performed as in Figure 1B. After selecting for the loss of full-length RNQ1 on FOA, cultures now expressing only Rnq1 fragments were transferred to SD-Leu,His and then colony purified on this medium. 40 colonies from each culture were screened for the presence of mini-[PIN+]s via the [PSI+] induction test. Each data point represents the percentage of mini-[PIN+] colonies in one independent culture. The number of cultures analyzed for each deletion is shown below the graph. Bars indicate average for all independent cultures. (B) Analysis of mitotic stability of mini-[PIN+] isolates from the experiment described in 4A were passed once on SD-Leu,His and then colony purified again. Colonies from this second round of subcloning were analyzed as described in 4A. Several mini-[PIN+] isolates with high mitotic stability were also obtained for D1C2D3 in a separate experiment (MK and ID unpublished observations). (C–E) Sedimentation analyses of cell lysates. In [PIN+] cells still co-expressing wild-type Rnq1 (C) and after elimination of wild-type Rnq1 (D), ΔC2, ΔD3 and ΔE4 are detected in both soluble and aggregated fractions, whereas ΔB1 is aggregated. In (C) paired panels are from the same lanes at the top and the bottom of the Western blot. For (D), see Figure S7 for finer analysis of ΔD2 and ΔE4 aggregation. (E) Rnq1 fragments are fully aggregated in stable mini-[PIN+] isolates. A ΔD3 mini-[PIN+] and a [pin+] colony were isolated from a ΔD3-expressing culture in experiment described in Figure 4A; stability of the mini-[PIN+] was determined as in Figure 4B.

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Transmission Barriers for Complex Prion Domains

Figure 5. Contribution of parts of regions QN3 and QN4 (A), hydrophobic patches (B, C), and GG10 (D) to the loss of prion state upon substitution of Rnq1 fragments for full-length Rnq1. Experiments were performed as in Figure 4A. Bars show average for 6 (A), 2 (B), 3 (C), and 3 (D) independent experiments; standard error of the mean (SEM) is shown for (A,C,D). See Figure S1D for steady state levels of Rnq1 fragments and Figure S8 and Figure S9 for map, data and the analysis of mini-[PIN+]s formed by these fragments.

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fragments with different sets of QN regions indicates that in the wild type [PIN+] prion several QN regions are aggregated and can be used for templating. Yet, transmission from [PIN+] to essentially any Rnq1 fragment involves crossing a barrier, which is similar to the barriers observed during interspecies transmission of other prions. These barriers are hard to explain if [PIN+] just included several relatively autonomous aggregated regions. Indeed, single aa mismatches, always present in the case of interspecies transmissions, could interfere with the transmission at particular short regions, but there are no single amino acid mismatches between [PIN+] and Rnq1 fragments; rather, whole aggregation-prone domains are removed. We hypothesized that transmission barriers are determined by an overall prion conformation and that the [PIN+] prion conformation is a result of co-operative action of multiple determinants that can transmit the prion state independently of each other.

Our model for conformational organization of [PIN+] and the mechanism of transmission barriers stipulates that transmission of the prion state between Rnq1 and its fragments involves templating by the exactly matching QN regions. Yet, having a common QN region capable of propagating the prion state is not sufficient for barrier-free transmission because all regions, including those not participating in templating, contribute to the overall conformation and, consequently, to conformational barriers. The conformation of the resulting mini-[PIN+] is also a product of co-operative action of all determinants present in the Rnq1 fragment and, consequently, is different from the original [PIN+], even though there is not a single amino acid mismatch in the templating region.

The following predictions can be made based on this model: (i) there should be a reciprocal barrier for the transmission of the prion state from mini-[PIN+] to mini-[PIN+] barriers may be different compared to the transmission from the original [PIN+], even if the same QN regions are involved in templating.

To test these predictions, we utilized stable mini-[PIN+] isolates obtained in experiments described in Figure 4B. LEU2-marked RNQ1 fragment constructs were substituted for the URA3-marked ones. Then wild type RNQ1 or various RNQ1 deletion constructs were introduced by transformation, and plasmid shuffle experiment was performed as previously described (see Figure 1B).

(i) Figure 6A shows the existence of transmission barriers from mini-[PIN+]s towards full-length RNQ1, confirming that mini-[PIN+]s indeed represent prion conformational variants distinct from the original [PIN+] supported by full length Rnq1. The strength of the reciprocal barrier correlated with the strength of the transmission barrier from [PIN+] towards the respective Rnq1 fragment for most constructs (e.g. D2 and AB1E4), but there were several notable exceptions to this rule (e.g. Δ3E).

(ii) Importance of the presence of a common QN region was confirmed by transmitting the prion state from mini-[PIN+]s to other Rnq1 fragments. Figure 6B illustrates the lack of transmission between the AB1C2 mini-[PIN+] and the Δ3E4 fragment. This result is in full agreement with for some level of transmission; (iv) relative strength of mini-[PIN+] to mini-[PIN+] barriers may be different compared to the transmission from the original [PIN+], even if the same QN regions are involved in templating.

Figure 5. Contribution of parts of regions QN3 and QN4 (A), hydrophobic patches (B, C), and GG10 (D) to the loss of prion state upon substitution of Rnq1 fragments for full-length Rnq1. Experiments were performed as in Figure 4A. Bars show average for 6 (A), 2 (B), 3 (C), and 3 (D) independent experiments; standard error of the mean (SEM) is shown for (A,C,D). See Figure S1D for steady state levels of Rnq1 fragments and Figure S8 and Figure S9 for map, data and the analysis of mini-[PIN+]s formed by these fragments.

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Figure 6. Cooperative action of QN regions determines transmission barriers for Rnq1-based prions. (A) Reverse transmission barriers for passing the prion state from mini-[PIN+]s to full-length Rnq1. Data for [PIN+] to mini-[PIN+] transmission are from Figure 4A. Reverse transmission from mini-[PIN+]s was analyzed the same way; shown are averages for 8–13 independent experiments and SEM. (B) Requirement of a common QN region for the transmission of prion state between Rnq1 fragments in vivo. Experiments were performed as in Figure 1B. Donors of prion state are indicated on top, recipients are listed on the left. Shown is growth on SD-Ade after 21 days at 20 C. Lack of transmission was confirmed by NM::Yfp and Rnq1::Cfp aggregation tests (not shown).

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in vitro data: ΔB1C2 (QN3,4) and ΔD3E4 (QN1,2) were unable to cross-seed each other while efficiently seeding QN4- and QN2-containing constructs, respectively (Figure 2E). Similarly, transmission was impossible from the ΔB1E4 mini-[PIN] to ΔD2E3 (not shown).

(iii) After establishing the specificity of QN-based templating, pairs of mini-[PIN]'s and Rnq1 fragments encompassing only one common QN region were used to demonstrate that each of the four QN regions could independently transmit the prion state. For QN2 and QN4, multiple examples of this ability were obtained, since the prion state could be transmitted to Rnq1 fragments encompassing only QN2 and QN4, respectively (Figure 7A; see also Figure 3B). Other examples include the transmission from ΔB1E4 mini-[PIN] to ΔD3 for QN2-driven transmission, and from ΔB1C2 to ΔD3 for QN4-driven transmission (Figure 7A; data shown in Figure 7D). The ability of QN1 to template matching sequences in Rnq1-based prions was deduced from the transmission from Δ2D mini-[PIN] to Δ3E4 (Figure 7A; data shown in 7E), despite no transmission from ΔB1C2 to ΔD3E4 (see Figure 6B). Finally, templating by QN3 was shown by transmission from ΔB1E4 mini-[PIN] to Δ2D (Figure 7A; data shown in 7C), despite no transmission from ΔB1E4 to Δ2D3E.

(iv) The strength of transmission barriers can be strikingly different when the same Rnq1 fragment is seeded by mini-[PIN]'s instead of [PIN] (Figure 7B-7E). Changes in transmission barriers emphasize the leading role of QN2 and QN4 regions in Rnq1-based prions as removal of one of these regions increases the importance of the other. For example, compared to [PIN], QN2-lacking mini-[PIN]'s Δ2D and ΔB1C2 are less efficient in converting ΔE4 (Figure 7B), and QN4-lacking ΔB1E4 mini-[PIN] has a dramatically reduced transmission to ΔC2 (Figure 7C). The contribution of QN3 to the transmission barrier is also further underscored. When a Δ3E mini-[PIN] is used for templating instead of [PIN], the transmission barrier is simultaneously increased towards Δ2D (Figure 7C) and reduced towards the QN4-lacking ΔE4 and ΔD3E4 fragments (Figure 7B and 7E). It appears that QN3 modulates the contribution of QN2 and QN4 to the prion conformation and/or transmission, and elimination of QN3 increases the involvement of QN2 and decreases the involvement of QN4. The more complex contribution of the QN3 region to the conformation of [PIN] is exemplified by the transmission to two similar constructs differing only in hydrophobic patch B: whereas [PIN] or ΔB1C2 could template Δ1C2D3 but not ΔB1C2D3, Δ3E mini-[PIN] transmitted prion state to both Δ1C2D3 and ΔB1C2D3 with similar efficiency (Figure 7A and data not shown).

Discussion

Multiple aggregation determinants of Rnq1

Together with [PSN'] and [URE3], [PIN] belongs to the class of prions with QN-rich prion domains. The unique feature of Rnq1 is that it carries four QN regions separated by hydrophobic sequences (Figure 1A). Using a set of constructs retaining the entire N-terminal part of Rnq1 but bearing single and multiple deletions of QN regions in the C-terminus, we demonstrated in vivo and in vitro that prion domain of Rnq1 encompasses multiple prion determinants that can independently drive aggregation and transmit the prion state. Specifically, all four QN regions are able to transmit the prion state in vivo. Such transmission was demonstrated in experiments where the aggregated template and the recipient fragment had only one common QN region (Figure 7A). That transmission indeed occurred through the common QN sequences (that in our experimental system match exactly) was strongly indicated by two lines of evidence: (i) in vivo transmission and in vitro cross-seeding were completely blocked in the absence of common QN regions (Figure 2E, Figure 6B); (ii) the presence of one common QN region was generally sufficient for the transmission of the prion state to Rnq1 fragments that were otherwise known to maintain it.

Our data also suggest that, in Rnq1-based prions, multiple aggregation determinants simultaneously take on conformations allowing them to transmit the prion state. Indeed, in order to template a QN region in a soluble Rnq1 fragment, the corresponding QN region in a pre-existing [PIN] (or mini-[PIN]) has to be in a transmissible conformation, e.g. engage in a β-strand formation. And we found that [PIN] and mini-[PIN]'s were each able to convert Rnq1 fragments carrying different sets of QN regions. For example, the ability of ΔB1E4 to convert ΔD3 and Δ2D indicates that in ΔB1E4 both QN2 and QN3 were in a transmissible state, and conversion by ΔB1C2 of ΔD3 and ΔE4 implies the transmissible state of both QN3 and QN4 in the ΔB1C2 mini-[PIN] (Figure 7A). For the original [PIN], transmission to constructs carrying only QN2 or only QN4 proves simultaneous prionization of these QN regions, whereas aggregation of QN1 and QN3 is suggested by different strength of transmission barriers to constructs lacking or retaining these regions (Figure 3 and Figure 7).

Even though all four QN regions can transmit the prion state, the contribution of these determinants to the maintenance of [PIN] may not be equal. Indeed, in vivo mini-[PIN] formation was confirmed for all fragments encompassing either QN2 or QN4, but was not detected for fragments carrying only QN1 and/or QN3, suggesting that QN1 and QN3 cannot maintain the prion state in the absence of QN2 or QN4. We strongly favor this explanation for QN1, as QN1 construct retaining the upstream part of Rnq1 was shown to have very low aggregation propensity in vitro (only a short QN1 peptide free of N-terminal part of Rnq1 was able to form fibers). Furthermore, QN1’s inability to maintain the prion state is not unexpected, as the QN1 region corresponds to roughly a half of other QN regions in length, lacks oligopeptide repeats and has no Y residues that were hypothesized to facilitate the fragmentation of amyloid aggregates [75]. It is not so expected for the QN3 region, which is more similar to QN2 and QN4 in length and organization, and could independently drive Rnq1 aggregation in vitro, even though QN3 fibers formed after a notably longer lag phase and had atypical seeding kinetics and reduced protease resistance. So we also contemplated the possibility that the failure to obtain mini-[PIN]'s maintained by QN3 alone (or in combination with QN1) was due to extremely strong transmission barrier between [PIN] and ΔB1C2E4 (or ΔC2E4, respectively).

So far we were unable to bypass this presumptive barrier using mini-[PIN]'s as donors of the prion conformation (MK and ID, unpublished observations). For example, no transmission to ΔB1C2E4 or ΔC2E4 was detected from the ΔB1C2 mini-[PIN], for which transmissible state of QN3 was indicated by the efficient conversion of ΔE4 (Figure 7A). Our finding that ΔB1C2E4 in vitro aggregation could not be seeded by fibers formed by QN3-containing fragments (Figure 2E) is consistent with either of the above explanations, as it could merely reflect the inability of the ΔB1C2E4 fragment to form “mature” amyloid or indicate a conformational barrier. The remaining possibility that QN1 and
Figure 7. Transmission of the prion state between Rnq1 fragments. Experiments were performed as in Figures 1B and 4A. (A) Each of the QN regions can transmit the prion state. In the schematic diagrams of Rnq1 deletion fragments QN1, QN2, QN3 and QN4 are in red, yellow, green and orange, respectively. Arrows indicate possibility of transmission between the indicated constructs. The only common QN region responsible for templaring is indicated near each arrow. (B–E) Barrier strength for the transmission of the prion state to Rnq1 fragments depends upon what \([PIN]^{+}\) or mini-\([PIN]^{+}\) is templating the conversion. Data are grouped by the recipient Rnq1 fragments shown above the graphs, templating prions are listed under the graphs. Bars show averages of 3-12 independent experiments, SEM is shown for all datasets except the transmission from \(\Delta B1C2\) and \(\Delta B1E4\) to \(\Delta D3\), where only two experiments were performed. For strongest barriers averages are shown above the bars.

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Transmission Barriers for Complex Prion Domains

QN3-based mini-[PIN]s are unable to facilitate the induction of [PIN], thus making our most sensitive test for the transmission of the prion state non-informative, is a subject of our further explorations.

Our results provide a genetic framework for studies of the structure of [PIN]. Out of several possible arrangements where multiple QN regions participate in β-strand formation, our data are most consistent with the one where all QN regions form parallel in-register β-sheets. This is in full agreement with a parallel in-register structure recently proposed for in vitro-made Rnq1 amyloid by Wickner et al. [66], who analyzed fibers formed by the Rnq1_1,35-403 prion domain fragment by solid state NMR. Noteworthy, while Wickner et al. did not analyze the structure of different parts of the Rnq1 prion domain, their study utilized fibers with labeled Y residues, most of which are located in QN2, QN3 and QN4, indicating the applicability of the conclusion about parallel β-sheet structure to these QN regions. Another possible structure postulating aggregated state of several QNs is where QN regions of the same molecule interact with each other, e.g., pairwise in a pseudo-dimer [Het-s]-like arrangement [4,76]. Such structure appears unlikely for [PIN]; contrary to the expectation of such arrangements, we found no evidence that aggregation of any QN region in Rnq1-based prions depended on the presence of another QN region. For example, we detected transmission of the prion state relying on the QN4 aggregation from Δ2D, Δ3E or ΔB1C2; transmission relying on QN3 from either ΔB1C2 or ΔB1E4; and transmission relying on QN2 from either Δ3E or ΔB1E4 (Figure 7A). Finally, structures where duplicated oligopeptides within repeat-carrying QN regions engage in intramolecular self-interactions [49] are also not indicated by our data, since deleting one of the two oligopeptides in a repeat was not equivalent to complete elimination of the repeated region (Figure 5A), and such equivalency is expected if repeated oligopeptides interacted.

Do other prions encompass multiple aggregation determinants that maintain a certain degree of independence? Such possibility is feasible for prions with complex prion domains, such as [PSI'], PrPSc and [Het-s]. Indeed, recent evidence indicates the presence of two distinct self-interacting regions within the Sup35 prion domain, one within the first 40 aa, and the other at ~90–120 aa [34,49,50]. The second region appears to be expendable for the transmission of the prion state [48] and for fiber formation in vitro [77,78], whereas expendability of the first region has never been tested directly. Similarly, four self-interacting regions have been identified for PrP [79]. The first of them, although not needed for prion replication, affects the structure of amyloid fibers [80], and the last, while being expendable for fiber formation [46], was hypothesized to be implicated in prion conversion [81]. For [URE3], the existence of a secondary prion-inducing region was considered upon the discovery of sequences in the non-prion domain part of Ure2, which facilitated/inhibited the de novo prion induction [82], but there is no evidence of multiple aggregation domains.

On the other hand, prion domain of Rnq1 stands out as the most complex among known prion domains. The redundancy of its structure is the result of several duplication events, with most recent duplications that created oligopeptide repeats in three out of four QN regions still traceable at the DNA level ([26] and our unpublished data). Considering that so far no cellular function has been assigned to non-aggregated Rnq1, and that [PIN] was detected in industrial and pathogenic yeast isolates [24,26], and has been shown to interact with prions and aggregation-prone proteins affecting formation, stability and toxicity of various amyloids (see Introduction), functional performance of the Rnq1 protein may involve formation of oligomeric complexes, if not [PIN] itself. In this case redundant aggregation-prone sub-domains are likely to improve ability of Rnq1 to self-interact and broaden the repertoire of possible heterologous interactions.

Transmission barriers in the absence of amino acid mismatches in transmitting regions

Transmission barriers were discovered upon attempting to transmit scrapie infectious agent to goats and mice [83]. The presence and strength of transmission barriers determines the possibility and efficiency of prion transmission between non-identical proteins, e.g. the risk of infecting humans with prions originating in wild and domestic animals. In vitro and in vivo studies of mammalian and yeast prions relate these barriers to species-specific differences in primary sequences of prion proteins [37,46,84,85]. A search for the explanation of how differences in primary structure, and specifically single aa substitutions, can control the tightness of the transmission barriers led to the model based on the recognition element concept. According to this concept, transmission of the prion state requires the interaction between clearly defined critical regions, or recognition elements (see Introduction). Consequently, the model for transmission barriers postulates that non-matching aa residues within these regions may impose the barrier by disrupting the productive interaction of the recipient protein and the template [34,79,86,87]. Thus, the strength of such barriers depends on the degree of sequence dissimilarity within this short region, and on the conformation of the recognition element in the template (i.e. the prion strain), which determines whether, and to what extent, a particular aa mismatch will affect the barrier. An absolute barrier is expected if the interaction is impossible, e.g. in the absence of a common recognition element. In our experimental system transmission is driven by four QN regions. While we do not explore the effects on transmission of individual aa mismatches within the common QN regions, the lack of transmission between Rnq1 fragments with no common QN regions could exemplify the absolute barrier mentioned above.

Yet, it would be an oversimplification to assume that all transmission barriers can be explained by primary structure dissimilarity in recognition elements [6,88,89]. Our study identifies and offers an explanation for transmission barriers not involving individual aa mismatches in recognition elements between the template and the recipient. We established that barriers exist for the transmission from full-length Rnq1 to Rnq1 fragments lacking any of the QN-rich aggregation determinants, and between all Rnq1 fragments encompassing non-identical sets of QN regions. At the same time, common QN regions, which drive the transmission across these non-absolute barriers, have fully matching sequences. Explaining these results in the framework of the abovementioned transmission barrier model is difficult, as it will involve postulating that deletion of any QN region changes the conformation of recognition elements in all other QN regions. However, our results are expected if overall conformation of [PIN] and other Rnq1-based prions were a product of cooperative action of several aggregation domains. In this case a transmission barrier will form as a result of the inability of a recipient lacking some (or having extra) QN regions to take on exactly the same higher-order fold as the template. This distinct type of barrier will reflect a requirement for a conformational change at a higher level of amyloid structure despite conservation of identical primary structure and possibility of transmission within the recognition element(s) in common QN regions.

Upon further analysis of across-the-barrier transmission we gained additional support for the existence of transmission barriers
determined by higher order conformational mismatches. (i) Since the recognition element is retained, such transmission barriers are likely to be non-absolute. Indeed, the presence of a common QN region was sufficient for the across-the-barrier transmission of the prion state to all constructs that were otherwise shown to be able to form mini-[PrP]s. (ii) Also, while the interaction at the templating interface in the absence of aa mismatches may determine the same arrangement of the templating region in the newly forming prion, higher order conformation of this prion will be different. So prions forming by overcoming such transmission barriers are expected to have reverse barriers toward the proteins they were templated with. Indeed, reverse barriers were detected when transmitting the prion state from mini-[PrP]s to full-length Rnq1; the strength of reverse barriers was not always reciprocal (Figure 3A). Importantly, inefficient reverse transmission is not predicted and was not observed for transmission barriers that are likely to be due to aa mismatches in recognition elements and where the transmission is presumed to occur through the selection of a recipient conformer compatible with the conformation of the template [6,46,47]. (iii) Recovery of both stable and unstable mini-[PrP]s that indicates formation of prion strains during across-the-barrier transmission (MK and ID unpublished observations) is also expected when new prion folds are forming.

Some naturally occurring transmission barriers may be very similar to the ones described in our work, being based exclusively on higher-order conformations. For example, deletions and expansions of oligopeptide repeats are common both in yeast and human prion proteins. Rnq1 variants lacking oligopeptide repeats in QN3 and QN4 of Rnq1 were uncovered in a significant proportion of natural isolates [26]. Our data predict that these differences will impose barriers for the transmission of [PrP] between yeast populations. The same study describes a Sup35 variant lacking two of the repeated oligopeptides. The Sup35 oligopeptide repeat region appears to be outside of recognition elements, but is involved in an ordered structure in the prion conformation [34,49–51]. In this case previously demonstrated inefficient transmission of [PSF] to Sup35 fragments with deletions in the oligopeptide repeat region can in part be due to transmission barriers. This possibility is consistent with a relatively high stability of “mini-[PSF]” isolates originating from cultures, which were predominantly [psf] after such transmissions [41]. Barriers determined by differences outside recognition elements may also play critical role in TSE epidemiology, specifically in limiting the spread of chronic wasting disease from cervidcs to other mammals. The 166–175 loop of PrP has been proposed to determine this transmission barrier, apparently without engaging in β-strand formation [90]. In summary, our work reveals the existence of transmission barriers in the absence of aa mismatches in transmitting regions and introduces the concept of distinct types of transmission barriers for complex prion domains. Forthcoming information on recognition elements for various prions should help determine the nature of established barriers and further elucidate their role in prion transmission.

**Materials and Methods**

**Plasmids**

Plasmids for expression of RNQ1 and its deletion alleles in yeast were constructed on the backbone of pRS416 (URA3) or pRS415 (LEU2) CEN vectors; the RNQ1 ORF and RNQ1 fragments are controlled by the RNQ1 promoter and followed by the RNQ1 terminator sequence. To obtain bacterial expression constructs RNQ1 fragments were amplified from corresponding yeast plasmids and cloned into pUC45 to yield N-terminally 10×His-tagged proteins. In CEN HIS3-marked pGAL-SUP35NM::YFP, the previously used Sup35NM::Yfp reporter [52] was placed under the control of the GAL1 promotor. In CEN URA3-marked pCUP-RNQ::CFP, a fusion of complete wild type RNQ1 ORF to CFP is controlled by the CUP1 promotor. Plasmid construction and primers are described in Protocol S1 and Tables S1, S2, S3.

**Strains**

Unless otherwise mentioned, all strains are derivatives of 74-D694 (MATa ade1-14 leu2-3,112 his3-A200 trp1-289 ura3-52; [91]). The [PrP][psf] derivative is Y1 [46]. The [psf] derivative is G4 [53], obtained from Y1 by GuHCl treatment [92]. The rnq1-Δ 74-D694 [PrP][psf] strain was constructed by seamlessly disrupting the complete RNQ1 ORF using the integration-excision approach [93]. The pRS406-based URA3 disrupting plasmid, pD130, contained the RNQ1 promotor inserted as the EcoRI - BamHI fragment (primers #19 and #3; see Table S3) and the sequence downstream of the RNQ1 gene inserted as the SacII - SacI fragment (primers #31 and #32). During the disruption, the [PIN] state was maintained by wild type RNQ1 expressed from the LEU2-marked maintainer (see Plasmids). To facilitate subsequent plasmid shuffles, the URA3-marked maintainer was substituted for the LEU2-marked maintainer after disruption. To monitor the presence of [PIN], the tightly regulated pGAL-SUP35NM::YFP CEN HIS3 plasmid was introduced prior to the disruption and was maintained in the rnq1-Δ 74-D694 [PrP][psf] strain during subsequent experiments. The [psf] version of rnq1-Δ 74-D694 was obtained from [PrP][psf] by transiently removing the RNQ1 maintainer. The [psf] strain 64-D697 MATa ade1-14 leu2-3,112 his3-A21 trp1-289 ura3-52 [53] was used in crosses to introduce pCUP-RNQ-CFP for the Rnq1::Cfp aggregation test.

**Yeast methods and cultivation procedures**

Standard yeast media and cultivation procedures were used [94,95]. Unless specifically mentioned, yeast were grown at 30°C on solid synthetic glucose media (SD) selective for plasmid maintenance. Cultures for transformation and protein isolation were grown in liquid organic complete YPD medium at 30°C with constant orbital agitation at 200 rpm. The GAL promotor was induced on synthetic media with 2% galactose as a single carbon source (SGal). The CUP promotor was induced on synthetic media supplemented with 20μM CuSO4. Media supplemented with 5-fluoroorotic acid was used for selective elimination of URA3-marked plasmids [96].

**[PSF] induction assay**

The [PSF] induction assay relies on the requirement of [PIN] for the de novo formation of [PSF] and requires that the strain carry a [PSF]-inducing construct and a reporter for the detection of [PSF] ([53]; reviewed in [69]). [PSF]-inducing constructs expressing the prion domain of the [PSF]-forming protein, Sup35, allow for the increase of [PIN]-dependent appearance of [PSF] to readily detectable levels. In our experiments, the strain carried the SUP35NM::YFP fusion under the control of a tightly regulated GALI promotor. The promoter remained repressed while yeast were growing on glucose media prior to shuffling out the wild type RNQ1 maintainer. After elimination of full-length RNQ1, expression of pGAL-SUP35NM::YFP was turned on by transferring yeast to galactose medium. To allow for the detection of [PSF], the strain carried the adel-14 reporter, a premature stop codon in the chromosomal ADE1 gene. The adel-14 mutation made the original [PIN][psf] rnq1-Δ 74-D694 strain unable to grow on media lacking adenine. However, in [PSF] cells that
appeared following \textit{SUP35NM::YFP} overexpression, translation
termination was compromised due to Sup35 aggregation, which
resulted in nonsense suppression, i.e. occasional readthrough of
the stop codon detectable as slow growth on -Ade.

**Indicated LEU2-marked constructs were transformed into**
[psi^-][PIN^+] \textit{rng1-} A 74-D694 carrying a \textit{URA3}-marked \textit{RNQ1}
maintainer and a HIS-marked pGAL-SUP35NM::YFP [PSF^+]-
deriv. Transformants were selected on SD-Leu,Ura,His and
then passed twice on SD-Leu,His to allow for the loss of the
inducer. Transformants were selected on SD-Ura,Leu,His
and supplemented SD-Ura,Leu with 20mM 3-AD
were selected on SD-Ura,Leu. The reporter was induced by
carrying the

**RNQ1::Cfp aggregation assay**

The assay [reviewed in \cite{69}] relies on the ability of fusions
of prion proteins with fluorescent reporters to join prion aggregates
and allow their visualization \cite{70}. The \textit{RNQ1::Cfp} reporter
was introduced by crossing \textit{rng1-}\textit{A} 74-D694 cultures carrying LEU2-
marked \textit{RNQ1} deletion constructs with the [psi^-] 64-D697 strain
carrying the \textit{URA3}-marked pCUP-RNQ1::Cfp plasmid. Diploids
were selected on SD-Ura,Leu. The reporter was induced by
supplementing SD-Ura,Leu with 20mM CuSO4. Presence of cells
with bright fluorescent foci was indicative of mini-[PIN^+]’s.

**Note:** moderate short-term (2–3 days) overexpression of
\textit{RNQ1::Cfp} does not include the \textit{de novo} appearance of
[PIN^+] in [psi^-] strains, which were used in our experiments \cite{19}. To
confirm lack of \textit{de novo} induction of [PIN^+] in the diploids, the \textit{rng1-}
\textit{A} 74-D694 carrying empty vector instead of \textit{RNQ1} deletion
con structs was included in all crosses.

**RNq1::Cfp aggregation assay**

Yeast cell lysates were prepared as described in \cite{69} except that
pre-clearing at 10,000 g was omitted. 60 mg of total protein
were centrifuged at 280,000 \textit{g} for 30 min at 4°C (Beckman Optima
TLX centrifuge, TLA 120.2 rotor). After removing the supernatant
(S), the pellet fraction (P) was resuspended in the protein
extraction buffer. The S and P fractions and 60 mg of total lysate
(T) were separated by SDS-PAGE. \textit{RNQ1} was detected by a
Western blot with polyclonal antibodies raised against full-length
\textit{RNQ1} (Type 2, a generous gift from S. Lindquist, Whitehead
Institute; Figure S2D) or the N-terminal \textit{RNQ1} fragment (\textit{RNq1A},
kindly provided by E. Craig, University of Wisconsin-Madison;
\cite{97}; Figure 3C, Figure 4C–4E, Figure S1, and Figure S2B).

**Supporting Information**

\textit{Escherichia coli} BL21-AI One Shot cells (Invitrogen) transformed
with \textit{p}[C43]-based expression constructs (see Plasmids and Table
\textit{S2}) were cultured in LB medium supplemented with 100 \textit{\mu}g/ml
ampicillin at 37°C. Protein expression was induced at mid-log
phase (OD\textsubscript{600}~0.4) by 1mM IPTG and 0.2% L-arabinose for
1.5 h. Cells were harvested by centrifugation (4°C, 1600 \textit{xg}) and
either processed immediately or frozen at -80°C. Due to limited
solubility of \textit{Rnq1} in aqueous solutions, purification was carried
out under denaturing conditions at room temperature. Cells were
lysed by gentle agitation in lysis buffer (100mM \textit{NaH}_{2}\textit{PO}_{4} \text{pH 7.4},
550mM NaCl, 8M urea) for 1h. Cell debris was removed by
centrifugation at 20,000 \textit{g} for 10 min. Supernatant was incubated
for 2 h with Ni-NTA Sepharose (Qiagen) equilibrated with lysis
buffer. The slurry was transferred to the column and washed
extensively with lysis buffer. Rnq1 was eluted with lysis buffer
containing 250mM imidazole. Protein enriched fractions were
determined by UV absorption at 280nm and concentrated on
Centricon (Millipore). Protein concentration was determined by
the BCA assay (Pierce). Purity of recombinant proteins was
estimated by SDS-PAGE as \textgreater95%.

**In vitro fiber formation**

The 200 \textmu\textit{l} reactions were set up in assembly buffer (1M urea,
final concentration; 100mM \textit{NaH}_{2}\textit{PO}_{4} \text{pH 7.4; 300mM NaCl}) in
the presence 5 \textmu\textit{M} ThT. Fiber formation was monitored by ThT
fluorescence \cite{99} in a Molecular Devices SpectraMax M-5 plate
reader (\textit{λem} 450 nm; \textit{λex} 483 nm; 24°C). Readings were taken
evry 15 min, samples were shaken for 5 sec prior to each reading.
For each protein, experiments were performed in duplicate and
repeated at least 3 times. To obtain fibers for seeded reactions,
suspensions of polymerized \textit{Rnq1} fragments were collected from
the wells and precipitated with 5 volumes of Met-\textit{OH}. Pellets were
washed 3 times with 70% Et-\textit{OH} to remove urea, and then dried
with anhydrous acetone. Seed powder was stored at 4°C and
dissolved in the assembly buffer right before adding to the samples.

Papain digestions were performed at 25°C for 30 min. Papain
(Sigma) was added directly to ThT reactions (enzyme:substrate
ratio 1:50).

**Fluorescence microscopy**

Cells were observed using an Axioplan2 Zeiss microscope. Images of representative fields were captured with a Zeiss
Axiocam digital camera and processed with Improvision OpenLab
software. Fluorescence and differential interference contrast
images (DIC) are shown for each field.

**Transmission electron microscopy (TEM)**

TEM was performed at the NYU School of Medicine Image
Core Facility. Fiber suspensions in the assembly buffer were
diluted 2.5-fold in water and 4 \textmu\textit{l} were applied onto the carbon-
coated 400 mesh Cu/Rh grids (Ted Pella Inc.). The grid was
dissolved 3 times with water to get rid of urea, and negatively
stained with 1% uranyl acetate (twice briefly and then for 5 min
at 25°C). Images were obtained using Philips CM12 transmission
electron microscope supplied with a Gatan 1kX1k digital camera
and processed using Gatan Digital Micrograph software.

**Figure S1** Expression of \textit{Rnq1} fragments in yeast. Western blot
analysis of the lysates of [PIN^+] [psi^-] \textit{rng1-} A 74-D694 cells co-
expressing \textit{RNQ1} and the indicated deletion constructs. Both full-
length \textit{RNQ1} and its fragments are controlled by the native \textit{RNQ1}
promoter (see Plasmids in Materials and Methods, and Protocol S1
for plasmid construction). Transformants were maintained on
the medium selective for both plasmids, but cultures for protein
isolation were grown in YPD. Yeast cell lysates were prepared as
described in Liebman et al. \cite{96} except that pre-clearing at
10,000×g was omitted. Rnq1 was detected with polyclonal antibodies raised against the N-terminal part of the protein ([Lopez et al., 2003]; kindly provided by E. Craig, University of Wisconsin-Madison). Panels show same lanes in the top and bottom parts of the Western blot of the same culture. All Rnq1 fragments ran in accordance with their expected size. At least 2 independent transformants were analyzed for each construct and experiments were repeated 2–3 times. The groups are: deletions of one (A), two (B) and three (C) QN regions with preceding hydrophilic patches used throughout the manuscript; and (D) other constructs used mostly in Figure 5, Figure S2, Figure S8, and Figure S9. (Liebman SW, Bagrantsiev SN, Derkatch IL (2006) Biochemical and genetic methods for characterization of [PIN+] prions in yeast. Methods 39: 23–34.) (Lopez N, Aron R, Craig EA (2003) The role of Sis1 on the maintenance of [RNQ+] prion. Mol Biol Cell 14: 1172–1181.)

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Figure S2 The QN-rich C-terminus is an essential part of the prion domain of Rnq1. N-terminal Rnq1 fragments were previously shown to be unable to join [PIN+] or to transmit the prion state [Vitrenko et al., 2007]. However, in those studies Rnq1 fragments were Gfp-tagged, and large tags sometimes interfere with prion properties [Dagkesamanskaia et al., 1997; Edskes et al., 1999]. We confirm that the QN-rich C-terminus is indispensable for mini-[PIN+]s in our experimental setup, and demonstrate that QG10 is not required for mini-[PIN+] establishment. (A,B) ΔB1C2D3E4; a Rnq1 fragment lacking all QN regions but retaining QG10, does not aggregate in [PIN+] cells and does not carry on the Pin− phenotype. (A) Cultures expressing AB1C2D3E4 become Pin− upon the loss of full-length Rnq1. Plasmid shuffle and [PSF+] induction test were performed as described in Figure 1B legend. The lack of [PSF+] formation was confirmed by fluorescent microscopy using the Sup35NM:Yfp reporter (not shown). (B) There is no evidence of AB1C2D3E4 aggregation even in the presence of [PIN+]. Sedimentation analysis of the lysate of [PIN+] cells co-expressing Rnq1 and AB1C2D3E4; panels show same lanes in the top and bottom parts of the Western blot of the same culture. Similar data for ΔI1C2D3E4 not shown. (C,D) QG10 is not essential for maintaining the prion state of Rnq1: ΔQGQ is aggregated, and cultures remain Pin− after elimination of Rnq1. (C) Plasmid shuffle and [PSF+] induction test were performed as described in Figure 1B legend. (D) Sedimentation analysis of cell lysates from cultures expressing indicated fragments after elimination of full-length Rnq1. Similar data for ΔAQQG not shown. (Vitrenko YA, Pavon ME, Stone SI, Liebman SW (2007) Propagation of the [PIN+] prion by fragments of Rnq1 fused to GFP. Curr Genet 51: 309–319.) (Dagkesamanskaia AR, Kushnirov VV, Paushkin SV, Ter-Avanesyan MD (1997) Fusion of glutathione S-transferase with the N-terminus of yeast Sup35 protein inhibits its prion-like properties. Genetika (Rus) 33: 610–615.) (Edskes HK, Gray VT, Wickner RB (1999) The [URE3] prion is an aggregated form of Ure2p that can be cured by overexpression of Ure2p fragments. Proc Natl Acad Sci USA 96: 1498–1503.)

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Figure S3 In vitro analysis of aggregation of Rnq1 protein fragments and QN1 peptide. (A) Cross-seeding between Rnq1 protein fragments lacking two or three QN regions. Kinetics of in vitro aggregation was monitored by ThT fluorescence. The soluble protein is indicated above the graphs, and seeds are listed to the right in brackets. “No seed” indicates unseeded polymerization. Proteins used: QN2 (ΔB1D3E4), QN3 (ΔB1C2E4), QN4 (ΔB1C2D3), QN1,2 (ΔD3E4), QN3,4 (ΔB1C2). Concentrations of soluble proteins were in the 60–80 μM range. (B) Transmission electron micrographs of negatively stained QN1 peptide fibers. The 12 aa long QN1 peptide (NSNNNNQQGQGNQ; GenScript; 98.5% purity) was pre-treated with 1,1,3,5,5-pentamethyl-2,4-hexadiol-2-isopropanol (Sigma) for 24 h at room temperature and lyophilized. The powder was re-suspended in water to a final concentration of 250 μg/ml. The 200 μl reactions were set up in the presence of 5 μM ThT. Samples incubated at 37°C for ~80 h were shaken for 5 sec every 10 min. Monitoring the aggregation kinetics by ThT fluorescence (see Materials and Methods) revealed a sigmoidal curve with a very short lag phase (not shown). TEM was performed at the NYU School of Medicine Image Core Facility as described in Materials and Methods. Long and very thin (<10 nm in diameter) fibers were frequently laterally associated and had either straight or twisted appearance.

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Figure S4 Reduction of the de novo formation of [PSF+] after substitution of deletion constructs for full-length Rnq1. Plasmid shuffle was performed in the [PIN+] [ψ+] rnl-A-74-D694 strain carrying the pGAL-SUP35NM:YFP [PSF+] inducer as described in Figure 1B legend. [PSF+] was induced by growth on SGal-LeuHis plates for 4 days. Relative levels of [PSF+] induction were calculated by determining the percentage of cells with Sup35NM-Yfp fluorescent aggregates in cultures expressing indicated constructs, and then normalizing it to the percentage of aggregate-containing cells in cultures carrying wild-type [PIN+] only. Each data point represents an independent experiment, in which the percentage of [PSF+] cells determined for three transformants expressing the deletion construct is normalized to the percentage of [PSF+] cells in three transformants expressing wild-type Rnq1 (a total of 500–1,000 cells were analyzed in each case; wild-type [PIN+] cultures carried 30–40% aggregate containing cells). Data for constructs ΔG2 and ΔA2D, and ΔD3 and ΔA3E were similar and are grouped. Among the constructs lacking only one of the four QN regions, elimination of QN4 had the biggest effect reducing [PSF+] induction ~5-fold, whereas deleting QN2 or QN3 reduced it ~2-fold. The effect of double deletions varied depending upon what QN regions were eliminated. Eliminating QN3 in conjunction with a lack of either QN2 or QN4 led to an almost 100-fold drop in [PSF+] induction, whereas deletion of QN1 only mildly increased the effect of eliminating QN3 and QN4 and, surprisingly, had a rescuing effect when deleted together with QN2. Finally, the level of [PSF+] induction was the lowest in cultures expressing the Rnq1 fragments with only one QN region.

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Figure S5 Scheme of the analysis of the transmission of the prion state from [PIN+] to Rnq1 fragments. Experiments are described in the text, Materials and Methods and Legends for Figure 1B and 1C. Figure 4A and 4B. Experiments described in Figure 6 and Figure 7 were performed similarly except the initial strain expressed a Rnq1 fragment and harbored a mini-[PIN+] and the prion state was transmitted to wild-type Rnq1 or to other Rnq1 fragments.

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Figure S6 Distributions of percentages of mini-[PIN+] cells in cultures expressing indicated Rnq1 fragments after the loss of wild-type [PIN+]. Analysis of data presented in Figure 4A (Round 1 colony purification) and in Figure 4B (Round 2 colony purification). Horizontal axes show percentage of [PIN+] cells in 10% increments. Vertical axes show frequency of cultures with corresponding percentages of mini-[PIN+] cells.

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**Figure S7** After elimination of full-length Rnq1, Δ2D, and ΔE4 were detected in both soluble and aggregated fractions. For finer analysis of aggregates formed by Rnq1 fragments, 0.5 ml (~1 mg) of total protein were loaded onto ~4.5 ml of 15%–40%–60% step sucrose gradient and centrifuged at 160,000×g for 60 min at 4°C (Beckman Optima L-90K centrifuge, SW55Ti rotor). The 0.5 ml fractions were collected from the bottom of the tube, resolved on SDS-PAGE and immunoblotted with anti-Rnq1A. The bottom fractions were collected from the bottom of the tube, resolved on sucrose gradient and centrifuged at 160,000×g for 60 min at 4°C.

**Figure S8** Transmission barrier for conversion of Rnq1 fragments lacking parts of regions QN3 and QN4 into mini-[PIN]+s. (A) Schematic diagram of Rnq1 and deletion constructs used here and in Figure 5A. QN-rich regions are in red; patterned blocks within QN regions indicate oligopeptide repeats; hydrophobic patches are in blue. Lines indicate regions present, and nomenclature refers to deleted regions. Δ1/2-A retains the oligopeptide repeat of QN4 but lacks the very C-terminus of Rnq1 that includes a non-QN-rich and a QN-rich stretch. In Δ2/3-A only the first oligopeptide of the QN4 region is preserved intact. Δ2/3-E4 terminates right after the first oligopeptide of the QN3 repeat. (B) Percentage of mini-[PIN]+ cells in cultures bearing indicated deletion constructs after wildtype [PIN] loss. See Figure 4A legend for full description of the experiment. (C) Analysis of mitotic stability of mini-[PIN]+s formed by the indicated fragments. Data points show percentage of mini-[PIN]+ isolates after ~20 generations of mitotic growth. See Figure 4B legend for full description of the experiment. The total number of independent cutures (B) or mini-[PIN]+ isolates (C) analyzed for each deletion construct is indicated on each graph. Bars indicate averages. In (B) note the gradual decrease in the proportion of mini-[PIN]+ cells in cultures with progressive truncations of each QN region. Significant differences between WT Rnq1 and Δ1/2-A; Δ1/2-A and Δ2/3-A; and Δ2/3-A and ΔE4 show that the C-terminal part of QN4, as well as each of the repeated peptides contribute to the transmission barrier (see SEM in Figure 4F). The significant difference between Δ1/2-E4 and Δ3D-E4 illustrates the contribution of the first of the repeated peptides in QN3. Data in (C) shows that stable mini-[PIN]+s can be obtained after transmission from [PIN]+ to any of the fragments.

**Figure S9** Analysis of the importance of non-QN-rich sequences within the C-terminal part of Rnq1. (A,B) Analysis of the importance of alternating QN regions with hydrophobic patches in the prion domain in Rnq1. (C,D) The QG10 deletion enhances within the C-terminal part of Rnq1. (A,B) Analysis of the importance of non-QN-rich sequences in the prion domain in Rnq1. (C) Analysis of mitotic stability of mini-[PIN]+s formed by the indicated Rnq1 fragments. See Figure 4A and 4B and Figure S8 legends for full description of the experiments. In (A,B) the total number of independent cutures or mini-[PIN]+s analyzed for each deletion construct is indicated on each graph. In (C,D) 3–5 independent cutures or mini-[PIN]+s were analyzed for each deletion construct. In (A), note the significant reduction of prion-containing cells in the cultures expressing the Δ2 Rnq1 fragment (alteration of QN regions and hydrophobic patches is disrupted and hydrophobic regions C and D are located next to each other), compared to ΔC2 and Δ2D (alternating pattern is preserved). Analysis of mitotic stability of mini-[PIN]+s formed by these fragments in (B) indicates that the alternating pattern is important for the establishment of stable mini-[PIN]+ strains. Yet, although not part of the experiment shown in (B), stable mini-[PIN]+ isolates were obtained for Δ2, confirming the ability of this fragment to faithfully maintain the prion state (MK and ID unpublished). For Δ3, the reduction in prion containing cells in post-transmission cultures (A), and the stability of mini-[PIN]+s isolates (B) is not significantly reduced compared to ΔD3 and Δ3E. In (C) note the significant reduction of prion-containing cells in the cultures expressing the ΔQGB1C2 compared to A1B1C2 (and lack of prion loss in cells expressing ΔQG). Ability of ΔQGB1C2 to form stable mini-[PIN]+ was confirmed in a separate experiment (MK and ID unpublished observations).

**Table S1** Constructs for expression of Rnq1 fragments in yeast. Found at: doi:10.1371/journal.pgen.1000824.s013 (0.03 MB DOC)

**Table S2** Constructs for bacterial expression. Found at: doi:10.1371/journal.pgen.1000824.s014 (0.04 MB DOC)

**Table S3** Primers used in this study. Found at: doi:10.1371/journal.pgen.1000824.s012 (0.07 MB DOC)

**Text S1** Mini-[PIN]+s result from the transmission of the prion state from the pre-existing [PIN]+ prion rather than from de novo prion formation. Found at: doi:10.1371/journal.pgen.1000824.s010 (0.05 MB DOC)

**Protocol S1** Plasmid construction. Found at: doi:10.1371/journal.pgen.1000824.s009 (4.02 MB TIF)

**Figure S5** After elimination of full-length Rnq1, Δ2D, and ΔE4 were detected in both soluble and aggregated fractions. For finer analysis of aggregates formed by Rnq1 fragments, 0.5 ml (~1 mg) of total protein were loaded onto ~4.5 ml of 15%–40%–60% step sucrose gradient and centrifuged at 160,000×g for 60 min at 4°C (Beckman Optima L-90K centrifuge, SW55Ti rotor). The 0.5 ml fractions were collected from the bottom of the tube, resolved on SDS-PAGE and immunoblotted with anti-Rnq1A. The bottom fraction is not shown. Predominately aggregated wild-type Rnq1 is not detected in the top (soluble) fraction, whereas partially soluble Δ2D and ΔE4 are detected in the top fraction, as well as in the same gradient fractions were WT Rnq1 is present. Consistent with Figure 4A and 4D, ΔE4 lysates have more soluble Rnq1 fragment than Δ2D lysates.

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