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Heterologous Prion Interactions are Altered by Mutations in the Prion Protein Rnq1p

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Abstract

Prions in the yeast *Saccharomyces cerevisiae* show a surprising degree of interdependence. Specifically, the rate of appearance of the $[PSI^+]$ prion, which is thought to be an important mechanism to respond to changing environmental conditions, is greatly increased by another prion, $[RNQ^+]$. While the domains of the Rnq1 protein important for formation of the $[RNQ^+]$ prion have been defined, the specific residues required remain unknown. Furthermore, residues in Rnq1p that mediate the interaction between $[PSI^+]$ and $[RNQ^+]$ are unknown. To identify residues important for prion protein interactions, we created a mutant library of Rnq1p clones in the context of a chimera that serves as proxy for $[RNQ^+]$ aggregates. Several of the mutant Rnq1p proteins showed structural differences in the aggregates they formed, as revealed by SDD-AGE. Additionally, several of the mutants showed a striking defect in the ability to promote $[PSI^+]$ induction. These data indicate that the mutants formed strain variants of $[RNQ^+]$. By dissecting the mutations in the isolated clones, we found five single mutations that caused $[PSI^+]$ induction defects, S223P, F184S, Q239R, N297S, and Q298R. These are the first specific mutations characterized in Rnq1p that alter $[PSI^+]$ induction. Additionally, we have identified a region important for the propagation of certain strain variants of $[RNQ^+]$. Deletion of this region (amino acids 284–317) affected propagation of the high variant but not medium or low $[RNQ^+]$ strain variants. Furthermore, when the low $[RNQ^+]$ strain variant was propagated by $\Delta 284\text{--}317$, $[PSI^+]$ induction was greatly increased. These data suggest that this region is important in defining the structure of the $[RNQ^+]$ strain variants. These data are consistent with a model of $[PSI^+]$ induction caused by physical interactions between Rnq1p and Sup35p.

Introduction

Prions are caused by transmissible protein aggregates. In mammals, the PrP protein forms a prion that is the causative agent of the transmissible spongiform encephalopathies (TSEs) such as scrapie in sheep, bovine spongiform encephalopathy (mad-cow disease) and Kuru in humans.¹ Prions also exist in fungal species such as the budding yeast *Saccharomyces cerevisiae*. First discovered as a system of non-Mendelian phenotypic inheritance, prions in yeast do not cause cellular toxicity, but do share some characteristics with mammalian prions.^{2–4} Yeast prions provide an excellent model for studying both the intrinsic properties of the proteins that enable prion propagation and the cellular factors that facilitate prion formation and maintenance. In the non-prion state, these proteins are soluble and able to perform their molecular function in the cell. Transition into a prion state causes the proteins to aggregate, which generally causes a loss-of-function phenotype.³ The frequency of the transition to the

prion state can be increased by overexpression of the prion-forming protein. Prion protein aggregates are transmitted between cells through the cytoplasm in both mitosis and meiosis. Due to the self-propagating nature of the prion structure, the associated phenotypes are always dominant. Maintenance of the prion phenotype is dependent on the gene encoding the prion protein; deletion of that gene, or its prion forming domain (PFD) abrogates the cognate prion phenotype.

Two of the most well-studied prions in yeast are $[PSI^+]$ and $[RNQ^+]$.^{5,6} $[PSI^+]$ is caused by aggregation of the yeast eukaryotic release factor (eRF3), Sup35p.^{7,8} When soluble and functional in $[psi^-]$ cells, Sup35p facilitates the faithful release of polypeptide chains from the ribosome at stop codons. In its prion form, Sup35p aggregates and the recognition of stop codons becomes inefficient, thereby resulting in global nonsense suppression. $[PSI^+]$ can be monitored using alleles in biosynthetic pathways that contain premature stop codons that are only translated in the $[PSI^+]$ state. The $[PSI^+]$ state has been proposed to be beneficial due to the read-through of naturally occurring stop codons which enables translation of otherwise silent regions of the genome.^{9,10} As a result of nonsense suppression, isogenic $[PSI^+]$ and $[psi^-]$ strains show differences in their ability to survive in various growth conditions.^{9,11} Thus, the induction and removal (curing) of $[PSI^+]$ may be a regulatory mechanism. In addition, the ability to sample new protein products due to the conversion to the $[PSI^+]$ state may be adaptive and evolutionarily advantageous.⁹⁻¹² Interestingly, the *de novo* appearance of $[PSI^+]$ is more efficient in the presence of the $[RNQ^+]$ prion, which is caused by the aggregation of a protein of unknown function, Rnq1p.¹³⁻¹⁵ The reason for this interdependence is not fully understood but may relate to the ability of $[PSI^+]$ to serve as a mechanism to react to shifting environmental conditions. Several $[RNQ^+]$ yeast strains have been found in nature and these appear to be competent to form $[PSI^+]$,^{16,17} indicating that $[RNQ^+]$ may provide an extra level of control on the induction of $[PSI^+]$.

The mechanism by which $[RNQ^+]$ promotes $[PSI^+]$ induction is not well elucidated. The gain-of-function of Rnq1p aggregates in the $[RNQ^+]$ state is an attractive model since the deletion of *RNQ1* has no obvious phenotype, nor does it enhance the induction of $[PSI^+]$. Some data suggest that a direct interaction between prion aggregates of Rnq1p and soluble Sup35p facilitates $[PSI^+]$ induction. Fluorescently-labeled Sup35p colocalizes with Rnq1p aggregates early in $[PSI^+]$ formation but not once $[PSI^+]$ is fully established.¹⁸ Once formed, the aggregates appear to be largely separate.¹⁹ Purified recombinant Rnq1p forms fibers that are able to seed in vitro amyloid formation of Sup35NM, albeit inefficiently.^{18,20} These data point to a templating model where Rnq1p aggregates transiently interact with monomeric Sup35p to nucleate the formation of Sup35p aggregates and generate $[PSI^+]$.¹⁴ This is also consistent with in vivo data, as the initiation of $[PSI^+]$ is enhanced by the transient overexpression of Sup35p in $[RNQ^+]$ cells, but once $[PSI^+]$ is formed, *RNQ1* can be deleted and $[PSI^+]$ continues to propagate.¹⁴

The phenotypic effects associated with individual yeast prions show a wide range of severity, often due to a phenomenon referred to as prion strain variants. This is also true in mammalian prions, as strains of the infectious forms of PrP exist and are correlated with differences in age of onset, severity of disease, and pathology.²¹ Strain variants are structurally distinct conformations of the prion-forming protein, each of which is self-propagating.²²⁻²⁶ Strain variants of $[PSI^+]$ manifest as different strengths of nonsense suppression that typically correlate to the amount of soluble Sup35p.²⁷⁻³⁰ Strain variants of $[RNQ^+]$ result in differing propensities to induce $[PSI^+]$.^{13,31} Under semi-denaturing conditions, prion protein aggregates from different $[PSI^+]$ and $[RNQ^+]$ strain variants show a change in electrophoretic mobility.^{19,32} Additionally, recent biophysical studies have shown that the stable amyloid core of fibers of the prion-forming domain of Sup35p differs among strain variants produced in vitro.^{24-26,33,34}

The propagation of yeast prions is dependent on the chaperone machinery of the cell. Deletion of the molecular disaggregase Hsp104p eliminates all known yeast prions.^{15,35–37} Transient inactivation of Hsp104p by growth on media containing millimolar amounts of guanidine hydrochloride (GdHCl) also cures yeast prions.^{15,37–40} Interestingly, the overexpression of Hsp104p cures *[PSI+]*, but not other yeast prions.^{15,35,41,42} Prions are also sensitive to changes in the levels of various Hsp104p co-chaperones such as members of the Hsp70 and Hsp40 families.^{43,44} For example, Sis1p, an Hsp40 family member that interacts with the Hsp70 Ssa1p, is required for the maintenance of *[RNQ+]*.^{45–48}

Prion formation in yeast is driven by PFDs. These domains are typically modular and as such, can transfer the prion-forming ability to unrelated proteins when expressed as fusions.^{7,15,49} These fusion proteins form aggregates that retain prion character and can be used to study the properties of the original prion-forming proteins. The PFD of prion-forming proteins in *S. cerevisiae* generally have high glutamine (Q) and asparagine (N) content. These residues may be important for prion formation as a screen for mutant alleles of Sup35p that were unable to propagate *[PSI+]* found mutations in single Q or N residues that were generally mutated to a charged amino acid such as arginine.⁵⁰ In contrast, the PFDs of both the mammalian PrP and the Het-s prion protein from the fungal species *Podospora anserina* are not Q/N-rich. Interestingly, the Q/N-rich region of Sup35p can be replaced with either the PFD of Het-s or an expansion of a set of oligopeptide repeats from PrP.^{51,52} These fusion proteins have a greatly reduced Q/N character but continue to propagate as a prion in *S. cerevisiae*. This indicates that high Q/N content is not an absolute requirement for prion propagation in budding yeast.

The proposed PFD of Rnq1p consists of the Q/N-rich C-terminus (amino acids 153–405) that is broken up into a several different motifs with repeating sequences.^{53,54} These include a set of QG repeats at the start of the prion-forming domain and several QN tracts separated by hydrophobic regions that are predicted to form alpha-helices. Several naturally-occurring Rnq1p alleles have been found with deletions in either the QG region or the QN tracts that may be responsible for a defect in the propagation of *[RNQ+]*.¹⁷ A Rnq1p-GFP fusion has been used to determine which domains are important for prion formation by deletion mapping.⁵³ Deletion of the C-terminal 64 amino acids reduces the ability of the fusion protein to propagate the *[RNQ+]* prion, while deletion of another 100 amino acids completely abrogated the ability of Rnq1p-GFP to maintain *[RNQ+]*. Additionally, the N-terminus of Rnq1p was also demonstrated to play a role in *[RNQ+]* propagation, as deletion of the first 133 amino acids reduces the ability of the Rnq1p-GFP fusion to transmit *[RNQ+]*.⁵³ Despite extensive deletion mapping of the Rnq1p PFD, very little is known about internal regions or individual residues that are important for the maintenance of *[RNQ+]* or the ability of *[RNQ+]* to promote *[PSI+]* induction.

Here, we describe mutations within Rnq1p that alter the ability of the *[RNQ+]* prion to propagate and to induce *[PSI+]*. Using a chimeric protein reporter, we identified mutants by phenotypic changes. These mutants have subtle effects on *[RNQ+]* propagation but several reduce *[RNQ+]*-mediated *[PSI+]* induction. Additionally, we have found a region of *[RNQ+]* that is responsible for the propagation of certain strain variants. Together, the data are consistent with a model that posits a direct physical interaction between Rnq1p aggregates and Sup35p that mediates the induction of *[PSI+]*.

Results

RRP nonsense suppression is dependent on the *[RNQ+]* status of the cell

In order to better understand the primary structure requirements for *[RNQ+]* prion maintenance, we set out to find mutants in Rnq1p that were unable to propagate the *[RNQ+]* prion. Since the

function of Rnq1p is unknown, there is no phenotype to use in a screen to identify alleles that do not propagate [*RNQ+*]. Therefore, we created a fusion protein, called RRP ([*RNQ+*] reporter protein), that enables the [*RNQ+*] status of a cell to be monitored phenotypically. RRP is composed of the proposed PFD of Rnq1p (amino acids 153–405) and the M and C domains of Sup35p (amino acids 124–685). The C-terminus of Sup35p is responsible for the translation termination function.^{55,56} Aggregation of Sup35p or its functional equivalent, RRP, in the prion form presumably reduces its ability to function and creates the nonsense suppression phenotype associated with [*PSI+*].^{7,8} In a strain carrying the *ade1-14* allele, which contains a premature stop codon, cells cannot complete the adenine biosynthesis pathway due to efficient translation termination. These cells are red due to the accumulation of a biosynthetic intermediate of the adenine biosynthesis pathway.¹⁵ Isogenic cells with aggregated Sup35p or RRP, however, are light pink due to the production of Ade1p by nonsense suppression.

We replaced the endogenous *SUP35* locus in a [*psi-*] [*RNQ+*] strain with *RRP*. The integrants expressing RRP were light pink (Figure 1A) and this nonsense suppression phenotype was curable by transient growth on GdHCl (data not shown). We performed genetic tests to determine whether the nonsense suppression phenotype was dependent on the presence of wild type Rnq1p and the [*RNQ+*] prion. First, we generated a diploid that was homozygous for *SUP35* replacement by RRP and heterozygous for a deletion of *RNQ1*. The diploid was light pink and the sporulation of this diploid yielded tetrads with a 2:2 segregation pattern of the light pink to red phenotypes (Figure 1A). Western blot analysis of the progeny showed that the light pink cells expressed wild type Rnq1p while the red cells did not (Figure 1A). The red cells do not maintain the [*RNQ+*] prion because of the *RNQ1* deletion and also did not maintain the RRP-dependent nonsense suppression. Second, deletion of *RNQ1* in a light pink haploid strain expressing RRP eliminated the nonsense suppression phenotype, turning the strain red (data not shown). These data suggest that RRP does not maintain the prion phenotype independently of [*RNQ+*]. Third, integration of RRP into a [*rnq-*] strain produced only red cells (data not shown). As such, we conclude that the function of RRP in translation termination is indicative of the status of the [*RNQ+*] prion in the cell, and presumably RRP co-aggregates with Rnq1p in the [*RNQ+*] state to produce the nonsense suppression phenotype (Figure 1B). A similar Rnq1p-Sup35p fusion construct, has previously been reported¹⁵ but it lacks a 6 amino acid sequence in the C-terminus of Rnq1p (NNGNQN) and forms a prion independent of the [*RNQ+*] status of the cells.

Screen for mutations affecting [*RNQ+*] propagation

A library of autonomously replicating plasmids containing mutations in the *RNQ1* PFD of RRP was created and transformed into a [*RNQ+*] strain carrying wild type RRP on the chromosome, which is light pink. Transformants were first plated on SD-ura medium to select for the mutant plasmids, then scraped and plated onto rich media (YPD) to assess the color phenotype. The rationale for this screen is as follows: mutants that are unable to efficiently join the existing Rnq1p aggregates remain in a soluble state and are therefore able to terminate translation efficiently (antisuppressors). Such RRP mutants would generate colonies containing red sectors (Figure 1B). Red sectors indicate candidate mutants that are defective in joining the Rnq1p aggregates. The light pink sectors are produced when the strain loses the plasmid but remains [*RNQ+*]. Approximately 20,000 mutant clones were screened and 25 mutants were found that had a reproducible red sectoring phenotype. The clones were sequenced and these mutants are listed in Table 1. Even though single mutants were present within the library, the vast majority of the library clones with antisuppression phenotypes contained multiple mutations. While this complicated the initial analysis, some striking features of the mutants were readily apparent.

Almost all of the RRP mutant clones contained at least one mutated glutamine or asparagine residue which was usually changed to a charged amino acid such as lysine or arginine. This is consistent with the results of a similar screen performed with Sup35p that was conducted in search of mutants unable to support *[PSI+]* propagation.⁵⁰ Two of the mutants containing single point mutations, N307D and Q317R, fit this pattern.

Several non-glutamine or asparagine residues were altered multiple times, for example, S182, F214, and S345 (Figure 1C). Additionally, the other identified single mutation that suppressed the nonsense suppression phenotype was S223P. Several clones had small deletions in the N-terminal QG repeats, likely due to primer slippage. Finally, seventeen of the 33 amino acids between residues 284 and 317 were mutated in at least one clone. This suggests the importance of this region in the formation or maintenance of the *[RNQ+]* prion.

Effect of Mutations on the Solubility of Rnq1p

All of the RRP mutations found were generated using a reporter of the *[RNQ+]* prion status of the cell. Therefore, we next wanted to test whether the identified mutations had an effect on the propagation of *[RNQ+]* when cloned into full length Rnq1p. All mutations found in a mutant RRP plasmid were cloned *en masse* into native *RNQ1*. During the cloning process, the deletions found in the QG section were removed, with the one exception being Mut31. Wild type *RNQ1* in a *[RNQ+]* strain was replaced by the mutant alleles using a plasmid-shuffle method. Western blot analysis indicated that the steady state levels of the Rnq1p mutants were similar to that of wild type Rnq1p expressed from either the chromosome or a control plasmid (data not shown). The solubility of mutant Rnq1p protein was then assayed by high speed ultracentrifugation to separate the soluble and insoluble material in the lysates. The distribution of the Rnq1p protein was then assessed by western blot. In lysates from *[RNQ+]* cells, Rnq1p fractionates to the insoluble fraction, but in *[rnq-]* cell lysates, Rnq1p is found in the soluble fraction. Surprisingly, the mutated Rnq1p fractionated exclusively to the insoluble fraction in all but one of the mutants (Figure 2A and data not shown). Despite a clear effect on the nonsense suppression of RRP, the majority of the mutants appear to be competent to maintain the *[RNQ+]* prion as the only copy of *RNQ1* by this biochemical assay. Only Mut72 showed the presence of soluble Rnq1p though the majority of it appeared to be in the insoluble fraction after the plasmid shuffle immediately followed by six generations of growth in liquid medium (Figure 2B). After 36 generations in liquid medium, almost all of Mut72 had shifted from the insoluble fraction to the soluble fraction, indicating that Mut72 loses its ability to maintain *[RNQ+]* over time. The insoluble protein from the mutants was not due to non-specific aggregation. After growth of strains expressing mutant Rnq1p on GdHCl, the protein was found to be shifted into the soluble fraction (data not shown). Furthermore, following a plasmid shuffle through a *[rnq-]* strain, the mutants proteins segregated into the soluble fraction (data not shown).

We tested the ability of the mutants to transmit as a prion. Cells carrying the mutant protein in the insoluble form were mated to cells with wild type Rnq1p in the *[rnq-]* state. The resulting diploids were dissected and the *[RNQ+]* status of haploids carrying only wild type *RNQ1* was assessed by sedimentation assay. All of the mutants except Mut72 were able to convert wild type Rnq1p into the insoluble *[RNQ+]* state (data not shown). Mut72 likely failed to maintain a prion through the mating procedure and therefore failed to transmit. These results indicate that the other mutant proteins still propagate as a prion.

SDD-AGE reveals differences in mutant prion structure

We were surprised to find that all of the mutants but Mut72 failed to show any defect in *[RNQ+]* prion propagation by the biochemical solubility assay. Therefore, we turned to other methods to assess the structural integrity of *[RNQ+]* propagated by the mutants. To further examine the structure of the mutant *[RNQ+]* prion protein aggregates, we performed semi-

denaturing detergent agarose gel electrophoresis (SDD-AGE)³² on any mutant that shared a mutation with another mutant found in the screen. This technique resolves differences in high molecular weight protein aggregates and has been used to show differences in prion strain variants of both *[RNQ+]* and *[PSI+]*.^{19,32,57} Variants of *[RNQ+]* can be distinguished by the stability of protein aggregates following incubation in SDS at different temperatures. Most Rnq1 protein aggregates in *[RNQ+]* strain variants remain stable at moderate temperatures. However, the high *[RNQ+]* variant previously characterized³¹ breaks down into a presumably monomeric form upon exposure to increased temperature.¹⁹ Our *[RNQ+]* plasmid shuffle strains were made in strains that carry this thermolabile high *[RNQ+]* variant. Therefore, we asked if the aggregated mutants were still susceptible to thermal breakdown. Following incubation in sample buffer at either room temperature or 65°C, we performed the SDD-AGE analysis on the mutants (Figure 3).

Lysate from a *[rnq-]* strain showed little high molecular weight species, with nearly all of the monomeric Rnq1p located at the bottom of the blot. Lysate from a wild type *[RNQ+]* strain incubated at room temperature showed most of the protein migrating as a high molecular weight smear and little monomeric protein. Following treatment of the protein lysate at 65°C, however, the aggregates began to break down and a low molecular weight, monomeric Rnq1p band appeared. Many of the mutants tested harbor Rnq1p aggregates that show a pattern of SDS and temperature resistance similar to wild type. However, Rnq1p aggregates of Mut62, Mut83 and Mut84 did not break down upon heat treatment, indicating a more stable aggregate structure. Interestingly, two mutants, Mut27 and Mut72, showed large amounts of monomeric protein even without heat treatment. This is not surprising for Mut72, which is unable to stably maintain the *[RNQ+]* prion but likely indicates an alternate structure of Mut27 which maintains *[RNQ+]* mitotically. Other mutants, Mut33, Mut75, and Mut82, showed more subtle changes in the aggregate properties when subjected to heat treatment.

Next, we quantified the differences in temperature sensitivity of the aggregates seen in the SDD-AGE. For the thermosensitive mutant, Mut27, we subjected lysates to various temperatures and analyzed the amount of soluble protein by western blot. Rnq1 protein solubilized by heat treatment enters the gel while aggregated protein does not. Following SDS-PAGE, the protein was transferred to PVDF and probed with anti-Rnq1p antibody (Figure 3B, upper). Aggregates of Mut27 are sensitive to temperatures as low as 55° C while wild type Rnq1p aggregates shows no solubilization until 65° C. The quantification of three replicates is shown (Figure 3B, lower).

For the mutants that were more thermostable than wild type, we treated lysates at 65° C and then centrifuged them through a 20% sucrose solution. The aggregates entered the cushion while the solubilized protein did not. The amount of Rnq1 protein that did not enter the sucrose cushion was analyzed by western blot (Figure 3C). Both Mut83 and Mut84 showed significantly less soluble protein than wild type in this assay. These results, coupled with the SDD-AGE, establish that at least some of the mutants have different physical properties than wild type protein when in the prion state. These data suggest that the mutations are changing the structure of the aggregates, at least as long as the mutations themselves are present.

Effect of Mutant *[RNQ+]* on *[PSI+]* induction

As some of the mutants had apparently altered prion structures, we assessed whether those structures influenced the interaction between *[RNQ+]* and *[PSI+]*. The presence of the *[RNQ+]* prion has been shown to greatly enhance the appearance of the *[PSI+]* when Sup35p is overexpressed.^{13,14,41} Moreover, strain variants of *[RNQ+]*, which presumably propagate different prion structures, permit different efficiencies of *[PSI+]* induction.³¹ We wanted to determine if the *[RNQ+]* prions propagated by the mutant Rnq1 proteins were also capable of stimulating the induction of *[PSI+]*. Red *[psi-]* *[RNQ+]* cells expressing the Rnq1p mutants as

the only copy of Rnq1p were transformed with a plasmid that expresses Sup35p. Transformants were grown overnight in selective medium and plated on YPD. [PSI⁺] induction was scored by counting colonies with light pink sectors and dividing that by total number of colonies. Fifteen of the 25 mutant clones showed a significant decrease in [PSI⁺] induction as compared to wild type Rnq1p in [RNQ⁺] cells (Figure 4 and Table 1). These same mutants showed a defect in [PSI⁺] induction when expressed in a strain harboring the medium [RNQ⁺] variant as well, indicating the defect caused by the mutants was not specific to the high [RNQ⁺] variant (data not shown).

Single Mutants that Affect [PSI⁺] Induction

The presence of multiple mutations within most of the original [RNQ⁺] mutants made the interpretation of their individual effects challenging. Therefore, we cloned single mutations from the mutants into wild type Rnq1p. We chose to assess the individual mutations from Mut72 due to its inability to propagate the [RNQ⁺] prion. Further, we assessed all mutations that appeared in more than one RRP plasmid isolated from the screen by creating single mutants in *RNQ1* (Figure 1C and Table 1). When the same residue was mutated to multiple amino acids (as in the case of S345G and S345R), only one of the mutations was tested. Plasmids carrying *RNQ1* with each single mutation were then used to replace wild type *RNQ1* in a [RNQ⁺] strain by plasmid shuffle. We then tested the [PSI⁺] induction capability of the single mutants to identify which residues were responsible for the defects in [PSI⁺] induction. The majority of single mutations tested showed no significant defect in [PSI⁺] induction (data not shown). However, F184S, Q239R, N297S, and Q298R showed strong reductions in their ability to induce [PSI⁺], indicating that they may be primarily responsible for the defects seen in the originally isolated mutants (Figure 5A).

Additionally, we tested the solubility of the single mutant Rnq1 proteins. The single mutants that had defects in [PSI⁺] induction all partitioned into the insoluble fraction (Figure 5B). Additionally, none of the other single mutations altered the solubility of Rnq1p in the [RNQ⁺] strains as they separated into the pellet fraction (data not shown). None of the single mutants from Mut72 recapitulated the instability of the mutant prion as all remained in the insoluble fraction (data not shown). The insoluble Rnq1p single mutants were curable by GdHCl treatment and did not aggregate when shuffled through a [*rnq*[−]] strain (data not shown).

We also analyzed the structural properties of the four single mutants with [PSI⁺] induction defects by SDD-AGE. All of the aggregates were of similar size to wild type Rnq1p in the [RNQ⁺] state (Figure 5C). The single mutants also did not show a difference from wild type in SDS resistance at moderate temperatures as they were all partially broken down at 65°C but not at room temperature. These results indicate that the single mutants alone are not responsible for the alterations in thermal sensitivity seen when multiple mutations are present.

Next, we wanted to know if the single mutants that showed [PSI⁺] induction defects were able to pass those defects onto wild type Rnq1p. To answer this question, we mated the strains containing RRP mutants in the [RNQ⁺] state to a [*rnq*[−]] strain expressing wild type Rnq1p. The resulting diploids were sporulated and the haploid progeny showed a 2:2 inheritance of wild type *RNQ1* to the *RNQ1* deletion. Progeny with wild type *RNQ1* that did not maintain the mutant Rnq1p plasmid were assayed for [RNQ⁺] by Rnq1p solubility. In all cases the single mutants converted wild-type, soluble Rnq1p into its insoluble [RNQ⁺] form (data not shown), indicating that they maintain and transmit a prion. Additionally, for all mutants tested (all the multiple mutants, F184S, Q239R, and Q298R), [PSI⁺] induction from the converted wild type [RNQ⁺] was equivalent to the wild type parent (data not shown). This indicates that the mutants do not irrevocably alter the structure of the [RNQ⁺] prion nor create a new stably propagating strain variant. The high Q/N content of the Rnq1 protein and the large putative PFD, together

with our data that demonstrate that the majority of single mutants tested do not affect [RNQ+], suggest that the propagation of the [RNQ+] prion is difficult to disrupt.

Deletion of Amino Acids 284–317 Alters Strain Variant Propagation

Seventeen of the 33 amino acids between positions 284 and 314, which we refer to as a mutational hotspot, were found to be mutated in one or more of the recovered RRP mutants (Figure 6A). We generated a deletion of this entire region to create an allele called *rnq1Δhot*. We then replaced the wild type copy of *RNQ1* in various [RNQ+] strain variants with the *rnq1Δhot* allele by plasmid shuffle. We next tested [PSI+] induction with the *rnq1Δhot* allele in the different [RNQ+] strain variant backgrounds. The deletion showed a markedly decreased ability to induce [PSI+] in the high [RNQ+] variant (Figure 6B). However, no such defect was observed in either the medium [RNQ+] or the low [RNQ+] variant (Figure 6B). Strikingly, the low variant seemed to have increased [PSI+] induction with the *rnq1Δhot* allele.

We also examined the solubility of the deletion proteins in lysates from the [RNQ+] strains. The Rnq1p encoded by the *rnq1Δhot* allele remained insoluble in the low and medium [RNQ+] strain variants (Figure 6C) and this property was reversible by GdHCl treatment (data not shown). Interestingly, a large amount of soluble protein was seen in the high [RNQ+] strain variant expressing *rnq1Δhot* (Figure 6C). This indicates that amino acids 284–317 are important for propagation of the high [RNQ+] variant and for the interaction of high and low [RNQ+] variants with Sup35p, but not for the propagation of the medium [RNQ+] strain variant.

Given the strain-specific alterations engendered by the *rnq1Δhot* allele, we wanted to ask if there were changes in the structure of the mutant prions by assessing the SDS and thermal sensitivity of the aggregates. We performed SDD-AGE on the [RNQ+] variants carrying this allele and compared them to wild type variant aggregates (Figure 6D). The aggregates in the strains carrying the *rnq1Δhot* allele showed a broader range of aggregate sizes than the wild type Rnq1p aggregates in the corresponding strain variants. Additionally, the monomeric form of the Rnq1Δhot protein migrates as a doublet, which may be a consequence of an interaction with another protein, an SDS-stable oligomer, or a degradation product. Finally, the Rnq1Δhot protein aggregates showed an increased sensitivity to temperature relative to the corresponding wild type protein. These data strongly point to a change in the structure of the prion protein aggregates propagated by the *rnq1Δhot* allele.

Discussion

We used a chimeric proxy for the [RNQ+] status of the cell to identify mutants with defects in [RNQ+] propagation. While the mutants clearly affected the nonsense suppression caused by the chimeric RRP, they failed to dramatically affect the solubility of Rnq1p when moved into the native protein. One possible reason for this seeming discrepancy is the addition of the N-terminus. It is clear that while the C-terminus of Rnq1p is sufficient for joining or forming a prion aggregate,¹⁵ the N-terminus contributes to propagation of [RNQ+]. Deletions within the N-terminus of Rnq1p cause [RNQ+] to be transmitted inefficiently.⁵³ Furthermore, expression of Rnq1p with a deletion of the first 100 amino acids is able to cure [PSI+] in a [RNQ+] background, indicating that the amino terminus may play a role in prion-prion interactions.⁵⁴ Finally, the amino terminus of Rnq1p has been shown to have a binding site for the chaperone Sis1p.⁵⁸ Sis1p is essential for the propagation of [RNQ+] and mutations in the Sis1p binding site of Rnq1p alter the ability of a Rnq1p-GFP fusion to join pre-existing aggregates.^{46,47,58} In an attempt to ask whether a reduction in Sis1p binding to Rnq1p would alter the strain variant or [PSI+] induction, we generated Rnq1-L94A that was recently shown to drastically reduce Sis1p-Rnq1p interaction.⁵⁸ Unfortunately, the Rnq1p-L94A mutant was unable to maintain the [RNQ+] prion state (unpublished data J.P.B. and H.T.). This suggests that drastic changes in Sis1p-Rnq1p interaction are not occurring in our Rnq1p mutants that

maintain the prion state. Finally, our data also suggest that the N-terminus is able to compensate for mutations in the C-terminus that reduce or inhibit interactions with prion aggregates.

Several of the Rnq1p mutants altered the efficiency of $[PSI^+]$ induction caused by the overexpression of Sup35p. A change in $[RNQ^+]$ -mediated $[PSI^+]$ induction efficiency is generally thought to be associated with changes in the structure of the $[RNQ^+]$ prion. We examined the aggregates biochemically by analyzing their sensitivity to temperature and SDS. Several of the mutants showed an increased resistance to heat treatment, while others showed increased sensitivity. The results of the SDD-AGE show that at least some of the mutants are forming $[RNQ^+]$ structures that are different than wild type. However, they are unable to pass any structural differences onto wild type Rnq1p during the conversion of soluble wild type Rnq1p into an insoluble prion state, as the wild type protein propagates the original strain variant (Figure 5D and data not shown). This indicates that either the structural information that specifies strain variants is not altered in the mutant conformations despite alterations in the overall structure, or that wild-type protein cannot adopt the structures specified by the mutant proteins.

Previously, the primary sequence domains of the Rnq1p PRD and their role in $[RNQ^+]$ prion maintenance have been defined using deletion analysis.^{15,53,54} However, these deletion analyses provided no clues as to the specific residues required for $[RNQ^+]$ propagation or for its ability to regulate $[PSI^+]$ induction. We have defined five specific mutations that decrease the ability of $[RNQ^+]$ to induce $[PSI^+]$ but do not appear to significantly affect $[RNQ^+]$ propagation. The substitution of proline for a serine residue (S223P) likely induces large structural changes within the protein.

The other four mutations may shed more light on the interaction between Sup35p and Rnq1p and the induction of $[PSI^+]$ by $[RNQ^+]$. Three of these mutations are in glutamine or asparagine residues (Q239R, Q298R, and N297S). Glutamine and asparagine have been shown to be important for the propagation of other yeast prions, as mutants in these residues of Sup35p can abrogate its ability to form $[PSI^+]$.⁵⁰ Additionally, the crystal structure of an amyloid-forming peptide of Sup35p shows the side chains of the glutamine and asparagine residues forming hydrogen bonds between adjacent members of individual beta sheets.⁵⁹ These hydrogen bonds are important for the positioning of residues within the sheets. The final mutation that affected $[PSI^+]$ propagation was in an aromatic residue, phenylalanine (F184S). In the same crystal structure, aromatic tyrosine residues showed stacking of their side chains.⁵⁹ Given that both glutamine and tyrosine are important as structural components of some amyloids, the fact that mutation of these residues alters $[PSI^+]$ induction implies that the heterologous interactions between prion proteins result from a similar set of amino acid interactions. This is consistent with direct templating model of $[RNQ^+]$ -mediated $[PSI^+]$ induction.

The deletion of amino acids 284–317 revealed a region of Rnq1p that is required for maintenance of the high and low $[RNQ^+]$ strain variants but not for the medium strain variant. Interestingly, replacement of a shorter stretch of amino acids within this region, 292–298, with alanines had no effect on $[RNQ^+]$ propagation (data not shown). Deletions within the hotspot region have previously been shown to exist as naturally occurring polymorphisms of Rnq1p.¹⁷ While strains carrying deletions within this region of the protein were shown to be $[rnq^-]$, their ability to support $[RNQ^+]$ upon mating to a $[RNQ^+]$ strain was not tested. Previously, both large domains and specific residues of Sup35p that are required for specific strain variants of $[PSI^+]$ have been defined.^{34,60–63} This represents the first such discovery for the $[RNQ^+]$ prion. Additionally, $[RNQ^+]$ propagated by the Rnq1 Δ hot protein showed similar levels of $[PSI^+]$ induction when derived from either medium or low $[RNQ^+]$, which may indicate that deletion of this region locks the mutant protein into a specific conformation when templated by those structures.

Here, we have defined residues in Rnq1p that are important for the induction of $[PSI^+]$. These data are consistent with a direct physical interaction between Sup35p and $[RNQ^+]$ prion aggregates. As the formation of $[PSI^+]$ is much less efficient than the templating of additional Rnq1p molecules onto existing $[RNQ^+]$ aggregates, the interaction between Rnq1p in the $[RNQ^+]$ state and monomeric Sup35p may be much more sensitive to the mutations. This could explain the relatively modest effects of the mutants on $[RNQ^+]$ propagation. Using the relative sensitivity of $[PSI^+]$ induction, it may now be possible to find compensatory mutations within Sup35p that overcome the $[PSI^+]$ induction defects of the mutant $[RNQ^+]$ prions, thereby providing stronger evidence for a direct physical interaction. Understanding which residues are important for this interaction is an important step in understanding how $[RNQ^+]$ controls the induction of $[PSI^+]$ and how this translates to the regulation of prion formation by other epigenetic elements.

Materials and Methods

Strain and Plasmid Construction

All *S. cerevisiae* strains were derived from 74-D694. Low, medium, and high $[RNQ^+]$ variants were kindly provided by Dr. Susan Liebman. Standard culturing and manipulation techniques were used for both yeast and *Escherichia coli*⁶⁴ YPD is a rich medium while SD is a synthetic medium usually lacking one or more amino acids to select for appropriate plasmids.

For cloning, the pRS series of vectors were used to express or integrate constructs into yeast.^{65,66} RRP as cloned into the yeast integrating vector pRS306 in three parts. The promoter of *SUP35* was amplified with the following oligonucleotides 5'-

CGGAATTCCTCGAGAAGATATCCATC-3' and 5'-

CCCGGATCCTGTTGCTAGTGGGCAGATATAG-3', digested with *EcoRI* and *BamHI* and ligated into an *EcoRI/BamHI* fragment of pRS306. The MC domains of *SUP35* (coding for amino acids 124–685) were amplified with the oligonucleotides 5'-

GGGCCGCGGATGTCTTTGAACGACTTTCAAAGC-3' and 5'-

GGGGAGCTCGTGATTGAAGGAGTTGAAACCTTGC-3', digested with *SacI* and *SacII* and ligated into the promoter construct cut with the same enzymes. Finally, the C-terminus of *RNQ1* (encoding the PFD domain- amino acids 153–405) was amplified with the following oligonucleotides 5'-CCCGGATCCATGCAAGGTCAGGGACAAGGTCAAGG-3' and 5'-

GGGCCGCGGGTAGCGGTTCTGGTTGCCGTTATTG-3', cut with *BamHI* and *SacII* and ligated into a *BamHI/SacII* fragment of the construct carrying the promoter and *SUP35* MC domain and promoter to generate pRS306-RRP.

A *ScaI* fragment containing the Cen/Ars4 sequence from pRS316 was cloned into plasmid pRS306-RRP to make a plasmid that ectopically expressed RMC. Libraries were made by 15 cycles of PCR using Taq polymerase with the oligos to amplify the PFD of *RNQ1* listed above. PCR reactions contained either 125 μ M or 40 μ M $MnCl_2$, but otherwise were done using the manufacturer's conditions. To maintain complexity, each library consisted of 3 pools made from two different PCR reactions. Each pool contained approximately 4,000 clones (12,000 clones per library).

A *XhoI/SpeI* fragment containing *RNQ1* from pRS315-RNQ1⁶⁷ was ligated into pRS313 or pRS316 to make pRS313-RNQ1 or pRS316-RNQ1 respectively. Mutants found in RRP were transferred *en masse* by the following procedure. The N-terminus of *RNQ1* was amplified using the oligonucleotides 5'-GTCACGACGTTGTAAAACG-3' and 5'-

GCCAAAGACGCCAAAGCAGTAAAAGA-3' with pRS313-RNQ1 as a template. The C-terminus of RNQ was amplified from the corresponding RRP mutant using the oligonucleotides 5'-TCTTTTACTGCTTTGGCGTCTTTGGC-3' and 5'-

CCCTACGTAAACAAAGGATAGAAGGCGAACTGAATCATCGTTTCAGTAGCGGT

TCTGGTTGCCGTTATTG-3'. The products of these reactions were used as a template for a third PCR reaction using the outer oligonucleotides. This product contained full length *RNQ1* with the desired mutations and was digested with *SpeI* and *SnaBI* and ligated into a *SpeI/SnaBI* fragment of pRS313-RNQ1. Due to a random PCR-generated error, all RRP and RNQ construct from the original screen encoded a Y277C mutant in Rnq1p. Rnq1p containing this mutation behaved identically to WT in all in vivo assays (data not shown).

Single mutants were also cloned with bridge PCR. The N-terminus of *RNQ1* was amplified with the oligonucleotide 5'-GGGGATATCATGGATACGGATAAGTTAATCTCAGAGG-3' and an oligonucleotide specific to the mutant. The reverse complement of the mutant specific oligonucleotide was then used along with the oligonucleotide 5'-CCCGTCGACTCAGTAGCGGTTCTGGTTGCCG-3' to amplify the C-terminus of *RNQ1*. The oligonucleotide specific to the mutant contained the desired mutation. The pieces were then used as templates for a PCR reaction with the non-mutant specific oligonucleotides used to generate each fragment. This produced full length *RNQ1* carrying the desired mutation which was digested with *EcoRV* and *SalI* and ligated into pRS313 that contained the *RNQ1* promoter on an *EcoRV/EcoRI* fragment and the *RNQ1* terminator on a *SalI/XhoI* fragment.

Strains carrying RRP integrated at the *SUP35* locus were generated by the pop-in pop-out method. Plasmid pRS306-RRP was linearized with *MluI* and transformed into 74-D694 strains carrying either the medium or high variant of [*RNQ+*]. Transformants were plated on SD-ura to select integrants. Integrants were then grown overnight in liquid YPD and loop-outs were selected by growth on plates containing 1 mg/ml 5-fluoroorotic acid (5-FOA). RRP expression was checked by western blotting with anti-Rnq1p antibody. Additionally, cells that carried the RRP allele turned pink on YPD. This process generated strains 74D-694-RRP(high) and 74D-694-RRP(med) respectively.

The *RNQ1* plasmid shuffle strains were created by deleting *RNQ1* using a kanMX4 cassette flanked by *RNQ1* sequence. The cassette was generated as described previously⁶⁸ using pFA6a-kanMX4 and 5'-GAACGTACATATAGCGATACAAACGTATAGCAAAGATCTGAAATGTCGTACGCTGCAGGTCGAC -3' and 5'-CAAATACGTAAACAAAGGATAGAAGGCGAACTGAATCATCGTTCAATCGATGAATTCGAGCTCG-3' as primers. The product was transformed into 74-D694 strain and deletions were selected on YPD with 200 µg/µl G418. Deletion of *RNQ1* was confirmed by western blot. To make this deletion strain [*RNQ+*], it was first transformed with pRS316-RNQ1. This strain was then mated to 74-D694 cells of the opposite mating type carrying either high, medium or low [*RNQ+*].³¹ The resulting diploid was then sporulated and G418 resistant, URA+ haploid colonies were selected. The [*RNQ+*] status of these strains was then checked by sedimentation assay.

Screen for RRP Mutants

Libraries with mutagenized RRP were transformed into strain 74D-694-RRP(high) and plated on SD-ura. The colonies were scraped off of the SD-ura plates, pooled and replated on YPD. Disruption of the nonsense suppression phenotype was assayed by red/white sectoring on YPD. Sectoring colonies were spotted onto SD-ura to confirm the presence of the plasmid. Spots on SD-ura were spotted back onto SD-ura, SD-ura-ade and 5-FOA. Plasmids were recovered from isolates that were unable to grow on SD-ura-ade and continued to sector on YPD. These plasmids were transformed back into 74D-694-RRP(high [*RNQ+*] variant) to confirm the sectoring phenotype was linked to the plasmid and then sequenced.

Rnq1p solubility assay

Yeast cells were lysed in buffer containing 100mM Tris-HCl pH 7, 200 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 5% glycerol, 0.5 mM dithiothreitol (DTT), 50 mM N-ethylmaleimide (NEM), 3 mM phenylmethanesulphonylfluoride (PMSF) and complete Mini protease inhibitor cocktail (Roche). Lysis was performed by vortexing with glass beads. Following lysis, an equal volume of RIPA buffer (50 mM Tris-HCl pH 7, 200 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS) was added to the lysate and cell debris were cleared by a brief centrifugation. This cleared lysate is the total protein. Insoluble protein was pelleted by centrifugation at 80,000 RPM in a Beckman TLA-100 rotor for 30 minutes. The supernatant containing the soluble protein was removed and the pellet was resuspended in a 1:1 mix of lysis buffer and RIPA buffer. Total, supernatant and pellet fractions were subjected to SDS-PAGE, transferred to PVDF membranes and probed with an anti-Rnq1p antibody.

SDD-AGE

SDD-AGE was performed as previously described⁴⁷. Briefly, lysates were prepared by vortexing with glass beads in 25 mM Tris pH 7.5, 100 mM NaCl, 10 mM MgCl₂, 1 mM EDTA, complete Mini protease inhibitor cocktail, 3 mM PMSF, 0.5 mM DTT, 5 µg/mL pepstatin 50 mM NEM. Lysates were cleared by brief centrifugation. Approximately 40 µg of protein was incubated at either room temperature or 65°C in sample buffer containing 60 mM Tris-HCl, 5% glycerol, 2% SDS for seven minutes and run on a 1.5% Tris-glycine agarose gel. Proteins were transferred to PVDF membranes and probed with an anti-Rnq1p antibody.

Thermal Stability

The sensitivity of prion aggregates to temperature was determined in two ways. Lysates were incubated in SDS-PAGE sample buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 100 mM DTT) at various temperatures for seven minutes. The lysate was subjected to SDS-PAGE. Solubilized protein entered the gel while aggregates were not able to enter the gel. The protein was separated by SDS-PAGE, transferred onto PVDF, and probed with an anti-Rnq1p antibody. Quantification of Rnq1p protein was performed using ImageJ and values were normalized to the amount of protein at the highest temperature (95° C). Alternatively, lysates were incubated at 65° C in SDD-AGE sample buffer and then layered onto a 20% (w/v) sucrose cushion. This was subjected to ultracentrifugation at 80,000 RPM in a Beckman TLA-100 rotor for 30 minutes. Soluble protein did not enter the sucrose cushion. The top layer, containing protein that did not enter the sucrose cushion was removed, subjected to SDS-PAGE, transferred to PVDF membranes and probed with either an anti-Rnq1p or anti-Pgk1p antibody. Rnq1p levels were normalized to Pgk1p levels and quantified using ImageJ.

[PSI⁺] induction assays

Plasmid shuffled strains containing the Rnq1p mutants were transformed with pSUP2⁵⁶ and plated on selective media for both plasmids. Individual transformants were grown in selective media to OD₆₀₀ ~ 1.6 and plated on YPD. After five days of growth, [PSI⁺] cells were counted as any cell with a light pink sector. Representative colonies were checked for curing on plates containing 3 mM GdHCl. The vast majority (>95%) of colonies with light pink sectors were curable on 3 mM GdHCl (data not shown). Overexpression of Sup35p in a [RNQ⁺] strain has also been shown to create non-heritable amyloids of Sup35p that cause nonsense suppression similar to [PSI⁺].⁶⁹ Since this nonsense suppression is dependent on the overexpression of Sup35p, we selected cells with light pink sectors and spotted them onto medium containing 5-FOA. Cells that converted to [PSI⁺] remained light pink on 5-FOA medium, while cells with non-heritable amyloids reverted back to red. Experiments with both wild type Rnq1p and the 15 mutants revealed that on average, about 12% of the light pink sectors were the result of non-

heritable amyloids while the rest were *bona fide* [PSI+] (data not shown). This proportion is in line with the frequency of non-heritable amyloid previously reported.⁶⁹

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References

1. Prusiner SB. Prions. *Proc Natl Acad Sci U S A* 1998;95:13363–83. [PubMed: 9811807]
2. Wickner RB, Masison DC, Edskes HK. [PSI] and [URE3] as yeast prions. *Yeast* 1995;11:1671–85. [PubMed: 8720070]
3. Uptain SM, Lindquist S. Prions as protein-based genetic elements. *Annu Rev Microbiol* 2002;56:703–41. [PubMed: 12142498]
4. Bousset L, Melki R. Similar and divergent features in mammalian and yeast prions. *Microbes Infect* 2002;4:461–9. [PubMed: 11932197]
5. Liebman SW, Derkatch IL. The yeast [PSI+] prion: making sense of nonsense. *J Biol Chem* 1999;274:1181–4. [PubMed: 9880481]
6. Tuite MF, Cox BS. Propagation of yeast prions. *Nat Rev Mol Cell Biol* 2003;4:878–90. [PubMed: 14625537]
7. Patino MM, Liu JJ, Glover JR, Lindquist S. Support for the prion hypothesis for inheritance of a phenotypic trait in yeast. *Science* 1996;273:622–6. [PubMed: 8662547]
8. Paushkin SV, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. Propagation of the yeast prion-like [psi+] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor. *EMBO J* 1996;15:3127–34. [PubMed: 8670813]
9. True HL, Lindquist SL. A yeast prion provides a mechanism for genetic variation and phenotypic diversity. *Nature* 2000;407:477–83. [PubMed: 11028992]
10. Giacomelli MG, Hancock AS, Masel J. The conversion of 3' UTRs into coding regions. *Mol Biol Evol* 2007;24:457–64. [PubMed: 17099057]
11. True HL, Berlin I, Lindquist SL. Epigenetic regulation of translation reveals hidden genetic variation to produce complex traits. *Nature* 2004;431:184–7. [PubMed: 15311209] Epub 2004 Aug 15
12. Masel J, Bergman A. The evolution of the evolvability properties of the yeast prion [PSI+]. *Evolution* 2003;57:1498–1512. [PubMed: 12940355]
13. Derkatch IL, Bradley ME, Masse SV, Zadorsky SP, Polozkov GV, Inge-Vechtomov SG, Liebman SW. Dependence and independence of [PSI(+)] and [PIN(+)] : a two-prion system in yeast? *Embo J* 2000;19:1942–52. [PubMed: 10790361]
14. Derkatch IL, Bradley ME, Hong JY, Liebman SW. Prions affect the appearance of other prions: the story of [PIN(+)]. *Cell* 2001;106:171–82. [PubMed: 11511345]
15. Sondheimer N, Lindquist S. Rnq1: an epigenetic modifier of protein function in yeast. *Mol Cell* 2000;5:163–72. [PubMed: 10678178]
16. Nakayashiki T, Kurtzman CP, Edskes HK, Wickner RB. Yeast prions [URE3] and [PSI+] are diseases. *Proc Natl Acad Sci U S A* 2005;102:10575–80. [PubMed: 16024723] Epub 2005 Jul 15
17. Resende CG, Outeiro TF, Sands L, Lindquist S, Tuite MF. Prion protein gene polymorphisms in *Saccharomyces cerevisiae*. *Mol Microbiol* 2003;49:1005–17. [PubMed: 12890024]
18. Derkatch IL, Uptain SM, Outeiro TF, Krishnan R, Lindquist SL, Liebman SW. Effects of Q/N-rich, polyQ, and non-polyQ amyloids on the de novo formation of the [PSI+] prion in yeast and aggregation of Sup35 in vitro. *Proc Natl Acad Sci U S A* 2004;101:12934–9. [PubMed: 15326312]
19. Bagriantsev S, Liebman SW. Specificity of prion assembly in vivo. [PSI+] and [PIN+] form separate structures in yeast. *J Biol Chem* 2004;279:51042–8. [PubMed: 15465809]

20. Vitrenko YA, Gracheva EO, Richmond JE, Liebman SW. Visualization of aggregation of the Rnq1 prion domain and cross-seeding interactions with Sup35NM. *J Biol Chem* 2007;282:1779–87. [PubMed: 17121829]
21. Collinge J. Prion diseases of humans and animals: their causes and molecular basis. *Annu Rev Neurosci* 2001;24:519–50. [PubMed: 11283320]
22. Tanaka M, Chien P, Naber N, Cooke R, Weissman JS. Conformational variations in an infectious protein determine prion strain differences. *Nature* 2004;428:323–8. [PubMed: 15029196]
23. King CY, Diaz-Avalos R. Protein-only transmission of three yeast prion strains. *Nature* 2004;428:319–23. [PubMed: 15029195]
24. Tanaka M, Chien P, Yonekura K, Weissman JS. Mechanism of cross-species prion transmission: an infectious conformation compatible with two highly divergent yeast prion proteins. *Cell* 2005;121:49–62. [PubMed: 15820678]
25. Toyama BH, Kelly MJ, Gross JD, Weissman JS. The structural basis of yeast prion strain variants. *Nature* 2007;449:233–7. [PubMed: 17767153]
26. Krishnan R, Lindquist SL. Structural insights into a yeast prion illuminate nucleation and strain diversity. *Nature* 2005;435:765–72. [PubMed: 15944694]
27. Derkatch IL, Chernoff YO, Kushnirov VV, Inge-Vechtomov SG, Liebman SW. Genesis and variability of [PSI] prion factors in *Saccharomyces cerevisiae*. *Genetics* 1996;144:1375–86. [PubMed: 8978027]
28. Zhou P, Derkatch IL, Uptain SM, Patino MM, Lindquist S, Liebman SW. The yeast non-Mendelian factor [ETA+] is a variant of [PSI+], a prion-like form of release factor eRF3. *Embo J* 1999;18:1182–91. [PubMed: 10064585]
29. Uptain SM, Sawicki GJ, Caughey B, Lindquist S. Strains of [PSI(+)] are distinguished by their efficiencies of prion-mediated conformational conversion. *Embo J* 2001;20:6236–45. [PubMed: 11707395]
30. Kochneva-Pervukhova NV, Chechenova MB, Valouev IA, Kushnirov VV, Smirnov VN, Ter-Avanesyan MD. [Psi(+)] prion generation in yeast: characterization of the ‘strain’ difference. *Yeast* 2001;18:489–97. [PubMed: 11284005]
31. Bradley ME, Edskes HK, Hong JY, Wickner RB, Liebman SW. Interactions among prions and prion “strains” in yeast. *Proc Natl Acad Sci U S A* 2002;30:30.
32. Kryndushkin DS, Alexandrov IM, Ter-Avanesyan MD, Kushnirov VV. Yeast [PSI+] prion aggregates are formed by small Sup35 polymers fragmented by Hsp104. *J Biol Chem* 2003;278:49636–43. [PubMed: 14507919]
33. Tessier PM, Lindquist S. Prion recognition elements govern nucleation, strain specificity and species barriers. *Nature* 2007;447:556–61. [PubMed: 17495929]
34. Chang HY, Lin JY, Lee HC, Wang HL, King CY. Strain-specific sequences required for yeast [PSI+] prion propagation. *Proc Natl Acad Sci U S A* 2008;105:13345–50. [PubMed: 18757753]
35. Chernoff YO, Lindquist SL, Ono B, Inge-Vechtomov SG, Liebman SW. Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. *Science* 1995;268:880–4. [PubMed: 7754373]
36. Moriyama H, Edskes HK, Wickner RB. [URE3] prion propagation in *Saccharomyces cerevisiae*: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p. *Molecular & Cellular Biology* 2000;20:8916–22. [PubMed: 11073991]
37. Du Z, Park KW, Yu H, Fan Q, Li L. Newly identified prion linked to the chromatin-remodeling factor Swi1 in *Saccharomyces cerevisiae*. *Nat Genet* 2008;40:460–5. [PubMed: 18362884]
38. Wickner RB. [URE3] as an altered URE2 protein: evidence for a prion analog in *Saccharomyces cerevisiae*. *Science* 1994;264:566–9. [PubMed: 7909170]
39. Tuite MF, Mundy CR, Cox BS. Agents that cause a high frequency of genetic change from [psi+] to [psi–] in *Saccharomyces cerevisiae*. *Genetics* 1981;98:691–711. [PubMed: 7037537]
40. Eaglestone SS, Ruddock LW, Cox BS, Tuite MF. Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [PSI(+)] of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* 2000;97:240–4. [PubMed: 10618402]

41. Derkatch IL, Bradley ME, Zhou P, Chernoff YO, Liebman SW. Genetic and environmental factors affecting the de novo appearance of the [PSI⁺] prion in *Saccharomyces cerevisiae*. *Genetics* 1997;147:507–19. [PubMed: 9335589]
42. Wegrzyn RD, Bapat K, Newnam GP, Zink AD, Chernoff YO. Mechanism of prion loss after Hsp104 inactivation in yeast. *Mol Cell Biol* 2001;21:4656–69. [PubMed: 11416143]
43. True HL. The battle of the fold: chaperones take on prions. *Trends Genet* 2006;22:110–7. [PubMed: 16378656]
44. Jones GW, Tuite MF. Chaperoning prions: the cellular machinery for propagating an infectious protein? *Bioessays* 2005;27:823–32. [PubMed: 16015602]
45. Qian X, Hou W, Zhengang L, Sha B. Direct interactions between molecular chaperones heat-shock protein (Hsp) 70 and Hsp40: yeast Hsp70 Ssa1 binds the extreme C-terminal region of yeast Hsp40 Sis1. *Biochem J* 2002;361:27–34. [PubMed: 11743879]
46. Sondheimer N, Lopez N, Craig EA, Lindquist S. The role of Sis1 in the maintenance of the [RNQ⁺] prion. *EMBO J* 2001;20:2435–42. [PubMed: 11350932]
47. Aron R, Higurashi T, Sahi C, Craig EA. J-protein co-chaperone Sis1 required for generation of [RNQ⁺] seeds necessary for prion propagation. *Embo J* 2007;26:3794–803. [PubMed: 17673909]
48. Aron R, Lopez N, Walter W, Craig EA, Johnson J. In vivo bipartite interaction between the Hsp40 Sis1 and Hsp70 in *Saccharomyces cerevisiae*. *Genetics* 2005;169:1873–82. [PubMed: 15687271]
49. Li L, Lindquist S. Creating a protein-based element of inheritance. *Science* 2000;287:661–4. [PubMed: 10650001]
50. DePace AH, Santoso A, Hillner P, Weissman JS. A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion. *Cell* 1998;93:1241–52. [PubMed: 9657156]
51. Tank EM, Harris DA, Desai AA, True HL. Prion protein repeat expansion results in increased aggregation and reveals phenotypic variability. *Mol Cell Biol* 2007;27:5445–55. [PubMed: 17548473]
52. Taneja V, Maddelein ML, Talarek N, Saupe SJ, Liebman SW. A non-Q/N-rich prion domain of a foreign prion, [Het-s], can propagate as a prion in yeast. *Mol Cell* 2007;27:67–77. [PubMed: 17612491]
53. Vitrenko YA, Pavon ME, Stone SI, Liebman SW. Propagation of the [PIN⁺] prion by fragments of Rnq1 fused to GFP. *Curr Genet* 2007;51:309–19. [PubMed: 17415568]
54. Kurahashi H, Ishiwata M, Shibata S, Nakamura Y. A regulatory role of the Rnq1 nonprion domain for prion propagation and polyglutamine aggregates. *Mol Cell Biol* 2008;28:3313–23. [PubMed: 18332119]
55. Eurwilaichitr L, Graves FM, Stansfield I, Tuite MF. The C-terminus of eRF1 defines a functionally important domain for translation termination in *Saccharomyces cerevisiae*. *Mol Microbiol* 1999;32:485–96. [PubMed: 10320572]
56. Ter-Avanesyan MD, Kushnirov VV, Dagkesamanskaya AR, Didichenko SA, Chernoff YO, Inge-Vechtormov SG, Smirnov VN. Deletion analysis of the SUP35 gene of the yeast *Saccharomyces cerevisiae* reveals two non-overlapping functional regions in the encoded protein. *Mol Microbiol* 1993;7:683–92. [PubMed: 8469113]
57. Bagriantsev SN, Kushnirov VV, Liebman SW. Analysis of amyloid aggregates using agarose gel electrophoresis. *Methods Enzymol* 2006;412:33–48. [PubMed: 17046650]
58. Douglas PM, Treusch S, Ren HY, Halfmann R, Duennwald ML, Lindquist S, Cyr DM. Chaperone-dependent amyloid assembly protects cells from prion toxicity. *Proc Natl Acad Sci U S A* 2008;105:7206–11. [PubMed: 18480252]
59. Nelson R, Sawaya MR, Balbirnie M, Madsen AO, Riekel C, Grothe R, Eisenberg D. Structure of the cross-beta spine of amyloid-like fibrils. *Nature* 2005;435:773–8. [PubMed: 15944695]
60. Bradley ME, Liebman SW. The Sup35 domains required for maintenance of weak, strong or undifferentiated yeast [PSI⁺] prions. *Mol Microbiol* 2004;51:1649–59. [PubMed: 15009892]
61. King CY. Supporting the structural basis of prion strains: induction and identification of [PSI] variants. *J Mol Biol* 2001;307:1247–60. [PubMed: 11292339]
62. Chien P, Weissman JS. Conformational diversity in a yeast prion dictates its seeding specificity. *Nature* 2001;410:223–7. [PubMed: 11242084]

63. Crist CG, Kurahashi H, Nakayashiki T, Nakamura Y. Conformation preserved in a weak-to-strong or strong-to-weak [PSI⁺] conversion during transmission to Sup35 prion variants. *Biochimie* 2006;88:485–96. [PubMed: 16364534]
64. Guthrie, C.; Fink, G. Guide to Yeast Genetics and Molecular Biology. In: Guthrie, CaFG., editor. *Methods in Enzymology*. Vol. 194. Academic Press, Inc.; San Diego: 1991.
65. Sikorski RS, Hieter P. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 1989;122:19–27. [PubMed: 2659436]
66. Mumberg D, Muller R, Funk M. Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene* 1995;156:119–22. [PubMed: 7737504]
67. Strawn LA, True HL. Deletion of RNQ1 gene reveals novel functional relationship between divergently transcribed Bik1p/CLIP-170 and Sfi1p in spindle pole body separation. *Curr Genet* 2006;50:347–66. [PubMed: 16972090]
68. Wach A, Brachat A, Pohlmann R, Philippsen P. New heterologous modules for classical or PCR-based gene disruptions in *Saccharomyces cerevisiae*. *Yeast* 1994;10:1793–808. [PubMed: 7747518]
69. Salnikova AB, Kryndushkin DS, Smirnov VN, Kushnirov VV, Ter-Avanesyan MD. Nonsense suppression in yeast cells overproducing Sup35 (eRF3) is caused by its non-heritable amyloids. *J Biol Chem* 2005;280:8808–12. [PubMed: 15618222]

Figure 1.

Description of screen for mutant alleles. (A) RRP nonsense suppression phenotype depends on the presence of Rnq1p. A diploid strain carrying RRP at both *SUP35* loci and heterozygous for *RNQ1* ($\Delta rnq1/RNQ1$) was sporulated and dissected. Top panels show western blots with anti-Rnq1p antibody of whole cell lysates from two tetrads. Bottom panel shows spottings of the same spores on YPD. (B) Schematic of screen for mutants in Rnq1p. Wild type RRP is able to be inactivated by coaggregation with wild type Rnq1p, in a $[RNQ+]$ background, causing nonsense suppression. Mutants that do not interact are able to terminate translation. Colonies carrying library plasmids on YPD are shown in photo on right. White sectors in the red colonies have lost the library plasmid. (C) Schematic representation of the Rnq1p prion forming domain (amino acids 153–405). Residues are indicated with their position in full-length Rnq1p. Residues marked with an asterisk are in Mut72. Other residues indicated appeared in more than one mutant or were of special interest.

Figure 2.

Mutant Rnq1p continues to propagate as a prion. (A) Mutations that disrupt RRP-based nonsense suppression do not alter the solubility of Rnq1p. Plasmids containing the Rnq1p mutants were used to replace wild type Rnq1p by plasmid shuffle. Lysates from these strains were fractionated into total (T), soluble (S), and insoluble pellet (P) by high speed centrifugation, and then subjected to SDS-PAGE. A western blot was performed with an anti-Rnq1p antibody. Strain analyzed is indicated above the line. (B) Mut72 fails to maintain [RNQ+] over time. A strain carrying Mut72 was passaged continuously while maintaining plasmid selection. Samples were taken at the number of generations indicated above the line (6, 18 or 36) and used to generate lysates. Lysates were fractionated into total (T), soluble (S), and insoluble pellet (P) by high speed centrifugation, and then subjected to SDS-PAGE. A western blot was performed with an anti-Rnq1p antibody.

Figure 3.

The [RNQ+] forms of the Rnq1p mutants have structural differences from wild type (WT). (A) SDD-AGE of mutants. Lysates from strains carrying either wild type or mutant Rnq1p were incubated in SDD-AGE sample buffer at either room temperature or 65°C for 7 minutes and then run on an agarose gel containing SDS. Protein was transferred to PVDF membranes and blotted with an anti-Rnq1p antibody. The mutant tested is indicated at the top of the gel. Below the gel “-” and “+” symbols indicate the absence or presence of 65°C heat treatment prior to sample loading. (B) Mut27 protein aggregates are solubilized at a lower temperature than wild type Rnq1p. Lysates from strains carrying either wild type or mutant Rnq1p were incubated in SDS-PAGE sample buffer for 7 minutes at the temperature indicated above the western blot. These samples were then subjected to SDS-PAGE and solubilized protein entered the gel and was detected with an anti-Rnq1p antibody. Graph shows a quantification of the amount of soluble protein from three separate assays normalized to the amount of signal at 95° C. Error bars indicate standard error. The values at 55° and 65° C differ significantly between Mut27 and wild type as determined by Student’s t test ($p < 0.05$). (C) [RNQ+] Mut83 and Mut84 are not as sensitive to temperature as wild type [RNQ+]. Lysates from strains carrying either wild type or mutant Rnq1p were incubated in SDD-AGE sample buffer for 7 minutes at 65° C and then layered onto 20% sucrose. The insoluble fraction was separated from the soluble fraction by ultracentrifugation. The top fraction containing protein that did not enter the sucrose cushion was removed, subjected to SDS-PAGE, transferred to PVDF, and probed with an anti-Rnq1p or anti-phosphoglycerate kinase 1 (Pgk1p) antibody. Panels show three separate samples. Graph shows quantification of signal. Protein loading is normalized to Pgk1p and values shown

are normalized to wild type. Error bars indicate standard error. The means of each mutant differ significantly from wild type as determined by one way ANOVA and Bonferroni's post test ($p < 0.01$).

Figure 4.

Rnq1p mutants have a defect in *[PSI+]* induction. *[psi-]* strains carrying either wild type (WT) or mutant *[RNQ+]* were transformed with a plasmid that overexpresses Sup35p and plated on YPD. *[PSI+]* was scored by the appearance of light pink sectoring on the colonies. Controls and secondary tests were performed as described in materials and methods. Graph indicates the percentage of sectoring cells with wild type normalized to 100 %. The mutant tested is indicated on the X axis. Error bars indicate the standard error of at least three separate replicates. The means of each mutant differ significantly from wild type as determined by one way ANOVA and Bonferroni's post test ($p < 0.01$ or greater). Data is representative of multiple experiments.

Figure 5.

Analysis of single point mutants. (A) *[PSI+]* induction assay of single mutant *[RNQ+]* strains. *[psi-]* strains carrying either wild type (WT) or mutant *[RNQ+]* were transformed with a plasmid that overexpresses Sup35p and plated on YPD. *[PSI+]* was scored by the appearance of light pink sectoring on the colonies. Graph indicates the percentage of sectoring cells with wild type set to 100 %. The mutant tested is indicated on the X axis. Error bars indicate the standard error of at least six biological replicates. The means of each mutant differ significantly from wild type as determined by one way ANOVA and Bonferroni's post test ($p < 0.001$). (B) Rnq1p solubility assay of single mutants that have replaced wild type Rnq1p by plasmid shuffle. Lysates from these strains were fractionated into total (T), soluble (S), and insoluble pellet (P) by high speed centrifugation, and then subjected to SDS-PAGE. A western blot was performed with an anti-Rnq1p antibody. Strain analyzed is indicated above the line. (C) SDD-AGE of single mutants with *[PSI+]* induction defects. Lysates from strains carrying either wild type or mutant Rnq1p were incubated in sample buffer at either room temperature or 65°C for 7 minutes and then run on an agarose gel containing SDS. Protein was transferred to PVDF membranes and blotted with an anti-Rnq1p antibody. The mutant tested is indicated at the top of the gel. Below the gel “-” and “+” symbols indicate the absence or presence of 65°C heat treatment prior to sample loading.

Figure 6.

Deletion of amino acids 284–317 shows differential effects on [RNQ+] strain variants. (A) Sequence of Rnq1p amino acids 284–317. Residues found mutated in the screen are marked with an asterisk. (B) [PSI+] induction of strains carrying the *rnq1 Δ Hot* allele. [psi–] [RNQ+] strains carrying either wild type (WT) or mutant Rnq1p were transformed with a plasmid that overexpresses Sup35p and plated on YPD. [PSI+] was scored by the appearance of light pink sectoring on the colonies and is indicated without normalization. Graph indicates the percentage of sectoring cells. The [RNQ+] variant tested is indicated on the X axis. Error bars indicate the standard error of at least nine separate replicates. The means of the mutant in the low and high [RNQ+] background differ significantly from wild type as determined by one way ANOVA and Bonferroni's post test ($p < 0.001$). (C) Rnq1p solubility assay of *rnq1 Δ Hot* allele in various [RNQ+] strain variants. Lysates from these strains were fractionated into total (T), soluble (S), and insoluble pellet (P) by high speed centrifugation, and then subjected to SDS-PAGE. A western blot was performed with an anti-Rnq1p antibody. The [RNQ+] strain variant, either low, medium (Med), or high, is indicated above the line. (D) SDD-AGE of *rnq1 Δ Hot* allele in various [RNQ+] strain variants. Lysates from strains carrying either wild type or mutant Rnq1p were incubated in sample buffer at either room temperature or 65°C for 7 minutes and then run on an agarose gel containing SDS. Protein was transferred to PVDF and probed with an anti-Rnq1p antibody. The variant tested is indicated at the top of the gel. Below the gel “–” and “+” symbols indicate the absence or presence of 65°C heat treatment prior to sample loading.

Table 1

Summary of mutations found

<u>Clone</u>	<u>[PSI+] Defect</u>	<u>Mutations</u>
Mut25	N	deletion 169–172, N219K, N286Y, G337V, H384R
Mut26	N	L207P, <u>F214Y</u> , Q242R
Mut27	Y	<u>F214L</u> , S223P, Q284L
Mut29	N	N219I, <u>N222Y</u> , Y335R
Mut31	Y	deletion 157–166, N246K, Q247L, <u>G280V</u>
Mut32	N	<u>S182P</u> , <u>F214I</u> , Q287K
Mut33	Y	<u>S182T</u> , F184S , N296S, Q309R, S323N, <u>S345G</u>
Mut34	Y	S173P, N186K, Q248R
Mut35	Y	Q360L, N377T, <u>F393S</u>
Mut38	Y	F174S, <u>N291K</u> , Q293L, Q295E, <u>N296D</u>
Mut52	Y	F184S , <u>A266T</u> , N297S
Mut58	Y	Q292K, <u>Q298K</u> , <u>S345R</u> , <u>N361H</u>
Mut62	Y	L177M, <u>N222D</u> , <u>G280D</u> , G320C
Mut68	N	Q317R
Mut70	N	N190S, Q300R, Y308C
Mut71	Y	Q193R, <u>N291K</u> , Q312R
Mut72	Y	<u>N220Y</u> , Q239R , <u>S345G</u> , <u>N353I</u> , <u>F393L</u>
Mut73	N	S199P, F204L, Q240H, L336P, Y374D
Mut75	Y	Q298R , Q315R, <u>N361D</u>
Mut76	N	N189S, N253D, Q312L, E385A
Mut77	N	N307D
Mut78	N	<u>N222D</u> , Q304L, F325S
Mut82	Y	S223P
Mut83	Y	<u>F214S</u> , Q242R, <u>Q282L</u> , L328M
Mut84	Y	<u>N220D</u> , L279S, <u>Q282R</u> , N379I

Mutant name is indicated under “Clone” column “[PSI+] Defect” indicates defect in [PSI+] induction. Mutations found in each clone are listed. Underlined mutations were tested as single mutants. Mutants in the same position are also underlined. Mutants in bold showed an effect on [PSI+] induction as single mutations.