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# Rnq1: An Epigenetic Modifier of Protein Function in Yeast

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## Summary

**Two protein-based genetic elements (prions) have been identified in yeast. It is not clear whether other prions exist, nor is it understood how one might find them. We established criteria for searching protein databases for prion candidates and found several. The first examined, Rnq1, exists in distinct, heritable physical states, soluble and insoluble. The insoluble state is dominant and transmitted between cells through the cytoplasm. When the prion-like region of Rnq1 was substituted for the prion domain of Sup35, the protein determinant of the prion [*PSI*$^+$], the phenotypic and epigenetic behavior of [*PSI*$^+$] was fully recapitulated. These findings identify Rnq1 as a prion, demonstrate that prion domains are modular and transferable, and establish a paradigm for identifying and characterizing novel prions.**

## Introduction

A remarkable protein-only mechanism for the transfer of hereditary information has recently been discovered in yeast, by which changes in phenotype are propagated through proteins with altered, self-perpetuating conformations rather than through altered nucleic acids (Wickner, 1994; Lindquist, 1997; Wickner et al., 1999). This mechanism is similar to that hypothesized for the "protein-only" infectivity of the mammalian prion protein (PrP) in transmissible spongiform encephalopathies (Prusiner et al., 1998; Weissman, 1999), and these genetic elements have, therefore, been called yeast prions.

The two known yeast prions [*URE3*] and [*PSI*$^+$] were discovered accidentally—[*URE3*] in a screen for nitrogen uptake mutations (Lacroute, 1971), [*PSI*$^+$] in a screen for tRNA suppressors (Cox, 1965). These factors were suggested to be prions when it was recognized that their non-Mendelian patterns of inheritance could most easily be explained by the inheritance of altered, self-perpetuating conformations of the Ure2 transcriptional repressor and the Sup35 translation–termination factor, respectively (Wickner, 1994). These conformational changes result in unusual loss-of-function phenotypes that are dominant. This hypothesis is now supported by a wealth of genetic, cell biological, and biochemical data.

Both [*URE3*] and [*PSI*$^+$] are metastable elements and are spontaneously lost and gained at a low frequency (Lund and Cox, 1981; Wickner, 1994). Moreover, each element can be induced by a transient increase in the level of its protein determinant, Ure2 or Sup35 (Chernoff et al., 1993; Wickner, 1994). When the elements are induced, their protein determinants undergo characteristic changes in state, becoming protease resistant or insoluble (Masison and Wickner, 1995; Patino et al., 1996). Cells can be heritably cured of the elements by passage on medium containing a low concentration of the protein denaturant guanidine hydrochloride (GdnHCl) (Tuite et al., 1981; Wickner, 1994). [*PSI*$^+$] can also be cured by transient changes in the expression of Hsp104 (Chernoff et al., 1995), a protein whose only known function is to remodel the conformational states of other proteins (Parsell et al., 1994; Glover and Lindquist, 1998). In all cases, curing is accompanied by reversion of the protein determinant to its original physical state.

The self-perpetuating changes in the physical states of Ure2 and Sup35 in vivo have been modeled in vitro using recombinant proteins. Purified Ure2 and Sup35 convert from the soluble state to amyloid fibers after a characteristic lag phase (for review see Kushnirov, 1998). Small quantities of previously formed fibers seed the formation of amyloid, reducing the length of the lag phase (Glover et al., 1997; Thual et al., 1999). These findings suggest a hypothesis that explains both the conversion of cellular protein into a prion state and the unusual genetic behavior of prions. Prion-state protein is inactive and converts newly synthesized protein to the same inactive state. Because this protein is passed from mother cell to daughter and between partners during mating, the loss-of-function phenotype is heritable and exhibits dominant, non-Mendelian inheritance. It is not yet clear whether amyloid represents the in vivo prion state. However, in the case of [*PSI*$^+$], the pathway that leads to amyloid formation in vitro is closely linked to the pathway that leads to protein inactivation in vivo. Lysates from [*PSI*$^+$] cells enhance the conformational conversion of purified protein, whereas lysates of [*psi*$^-$] do not (Glover et al., 1997; Paushkin et al., 1997). Mutants that enhance or inhibit prion formation in vivo enhance or inhibit fiber formation in vitro in a complementary way (Glover et al., 1997; DePace et al., 1998; Liu and Lindquist, 1999).

Prions provide a remarkable opportunity for cells with identical genomes to display heritable variations in phenotype. Because they are metastable, they provide potential for phenotypic plasticity. A major question is just how common this novel mechanism for the inheritance of phenotypic states might be. How many proteins are capable of undergoing such transitions? [*PSI*$^+$] and [*URE3*] were found through serendipity. They were uncovered in screens that were intended to pick up conventional genetic loci and were studied in detail because they did not obey the conventional rules of Mendelian inheritance (Cox, 1965; Lacroute, 1971). Here we undertake a more deliberate strategy, using protein characteristics rather than phenotypes to identify prion candidates.

Our search uncovered several candidates including

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the open reading frame YCL028w, which we have analyzed in detail. The protein product of this gene, which we have named Rnq1 (for "rich in asparagine and glutamine"), shows dominant, heritable alterations of protein conformation that are "infectious"—that is, the conformation can be stably transmitted from one cell to another by cytoplasmic mixing. To determine if the prion-like domain of Rnq1 can produce an epigenetic modification of a heterologous functional protein, we fused it to the C-terminal domain of Sup35, which is known to be subject to prion-mediated inactivation. The "infectious" character of the Rnq1 protein was fully transferable, and the fusion protein recapitulated many of the characteristics of the [*PSI*$^+$] state.

## Results

### Identification of Prion Candidates

The characteristics of Sup35 and Ure2 suggested several criteria for identifying new prion candidates. Previous experiments have demonstrated that particular regions (residues 1–65 for Ure2 and residues 1–123 for Sup35) are critical for prion formation by these proteins (Ter-Avanesyan et al., 1994; Masison and Wickner, 1995; Patino et al., 1996). Overexpression of these regions is sufficient to induce the prion phenotype de novo. Deletion of these regions has no effect upon the normal cellular function of the proteins but prevents them from entering the prion state. These critical prion-determining domains have an unusually high concentration of the polar residues glutamine and asparagine and are predicted to have very little secondary structure. The domains are located at the ends of proteins that have an otherwise ordinary amino acid composition. We hypothesized that by searching for open reading frames with these characteristics we might find new prion proteins.

A BLAST search of the NCBI database of nonredundant coding sequences using the prion-determining domains of Ure2 and Sup35 as the query sequence revealed approximately 20 open reading frames that had prion-like domains appended to polypeptides with an otherwise normal amino acid composition (Altschul et al., 1997). To restrict the number of likely candidates, we took advantage of recent global descriptions of mRNA expression patterns. In examining this data, we noted that Sup35 and Ure2 are expressed at nearly constant levels as cells transit from the log to the stationary phase of growth (DeRisi et al., 1997). Large fluctuations in expression would be inconsistent with the stability of both their heritable prion and nonprion states. The open reading frames from the BLAST search whose expression varies by less than 2-fold in the log-phase transition were selected for further analysis. They were fused to the coding sequence of green fluorescent protein (GFP) and expressed in the yeast strain 74D-694 (data not shown). Three of the proteins, *RNQ1*, YBR016w, and *HRP1*, showed coalescence of GFP, as previously described for Sup35 (Patino et al., 1996).

### Rnq1 Exists in Distinct States Controllable by Hsp104

We next asked if expression of the fusion protein in a strain that lacked the chaperone Hsp104 eliminated the coalescence of GFP, as it does for Sup35–GFP fusions (Patino et al., 1996). This is not a necessary criterion for prion proteins (an interaction with Hsp104 has not been demonstrated for [*URE3*]), but interaction with the chaperone provides a useful tool for further analysis. In wild-type yeast, fluorescence from the Rnq1–GFP fusion was found in one or more small, intense, cytoplasmic foci (Figures 1A and 1B). When the fusion protein was expressed in the isogenic Δ*hsp104* strain, fluorescence was diffuse (Figures 1C and 1D). The C-terminal end of Rnq1 (amino acids 153–405) contained the region rich in glutamine and asparagine residues. Fusion of this region alone to GFP gave an identical result to that seen with the full-length Rnq1–GFP fusion (data not shown). Since the effect of *HSP104* deletion upon the coalescence of the Rnq1 fusion was the most dramatic, it was chosen for further analysis.

Differential centrifugation was employed to determine if the coalescence observed with Rnq1–GFP fusion proteins reflected the behavior of the endogenous Rnq1 protein. Nondenatured total cellular lysates were fractionated by high-speed centrifugation, and supernatant and pellet fractions were reacted with an antibody against Rnq1 (Figure 1E). Rnq1 remained in the supernatant of a Δ*hsp104* 74D-694 strain but pelleted in the wild-type. Thus, the GFP coalescence is not an artifact of the fusion; the Rnq1 protein itself is sequestered into an insoluble aggregate in an Hsp104-dependent fashion. We also examined the solubility of Rnq1 in several

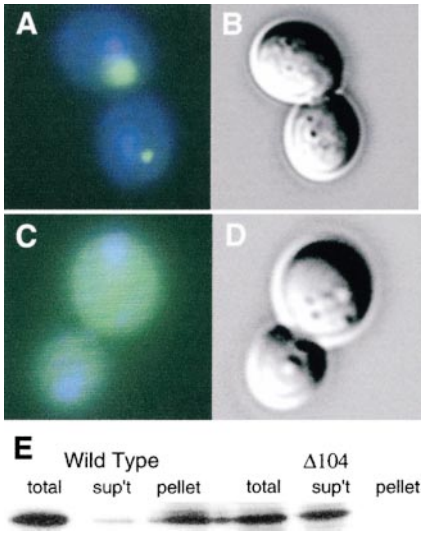


Figure 1. Rnq1 Aggregation and Hsp104 Effects

(A–D) Fluorescence microscopy of Rnq1–GFP fusion. Rnq1–GFP was expressed from the *CUP1* promoter in log-phase 74D-694 cells by the addition of 50 μM $CuSO_4$ for 4 hr. Cells were fixed with paraformaldehyde, DAPI stained, and immobilized on poly-lysine coated slides for viewing. Wild-type strains display cytoplasmic aggregates of Rnq1. Rnq1–GFP appears green, DAPI is blue (A), with Nomarski image (B) provided for comparison. (C and D) In Hsp104-deleted strains of 74D-694, Rnq1 is diffuse and spreads throughout the cell.

(E) Centrifugation analysis of Rnq1 protein in vivo. Native yeast lysates were produced from 74D-694 cells growing at $10^7$/mL. An aliquot was loaded prior to centrifugation (total), and the remainder was spun for 30 min to separate into supernatant and pellet fractions. The fractions were immunoblotted with α-Rnq1.

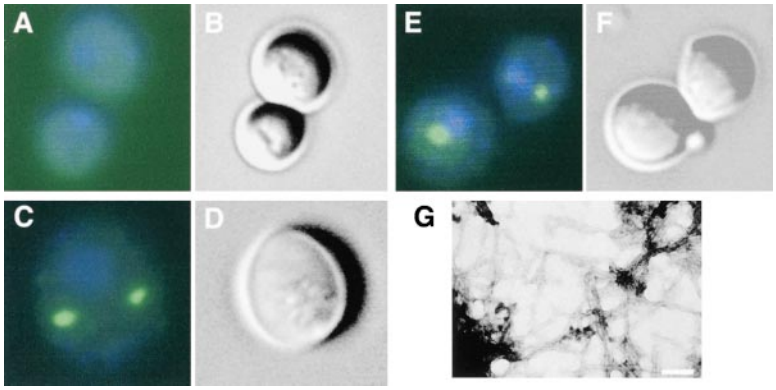


Figure 2. Prion Characteristics of Rnq1

(A–F) Rnq1–GFP was induced in 10B-H49 cells, which showed a soluble pattern of Rnq1 expression (A and B). This strain was cytoduced with W303, which has an insoluble pattern of fluorescence (C and D). Cytoductants were selected by growth on media lacking tryptophan that contained glycerol as its sole carbon source. This selected against both the W303 parent (which is *trp1–1*) and the noncytoduced 10B-H49 parent that lacks mitochondrial DNA. Selected cytoductants were induced to express the Rnq1–GFP fusion and showed an insoluble pattern of expression (E and F).

(G) Electron micrograph of Rnq1 fibers. Bacterially expressed, methanol-precipitated Rnq1 was dissolved in binding buffer to create a 10 μM solution. The protein was incubated on a roller drum spun at 60 rpm at room temperature for 28 hr. Scale bar, 100 nM.

unrelated yeast strains (data not shown). In four (S288c, YJM436, SK1, and W303), the protein fractionated in the pellet; in two (YJM128 and YJM309), it partitioned between the pellet and supernatant fractions; and in two others (33G and 10B-H49), the protein was chiefly recovered in the supernatant fraction. Thus, Rnq1 naturally exists in distinct physical states in different strains.

### The Insoluble State of Rnq1 Is Transmitted by Cytoduction

The heritability of the known yeast prions is based upon the ability of proteins in the prion state to influence other proteins of the same sequence to adopt the same state. Because the protein is passed from cell to cell through the cytoplasm, the conformational conversion is heritable, dominant in crosses, and segregates in a non-Mendelian manner. To determine if the insoluble state of Rnq1 is transmissible in this way, we used cytoduction, a well-established tool for the analysis of the [*PSI*$^+$] and [*URE3*] prion (Aigle and Lacroute, 1975; Fink and Conde, 1977). The karyogamy-deficient (*kar1–1*) strain 10B-H49 can undergo normal conjugation between Mat**a** and Matα cells but is unable to fuse its nucleus with its mating partner (Conde and Fink, 1976; Ter-Avanesyan et al., 1994). Cytoplasmic proteins and organelles are mixed in fused cells, but the haploid progeny that bud from them contain nuclear information from only one of the two parents.

10B-H49 shows diffuse expression of Rnq1–GFP and served as the recipient for the transfer of insoluble Rnq1 from W303, the donor (Figures 2A–2D). After cytoduction, colonies derived from haploid cells that contained the 10B-H49 nuclear genome but had undergone cytoplasmic mixing, as demonstrated by mitochondrial transfer, were selected. All showed single or multiple cytoplasmic aggregates of Rnq1–GFP—a pattern indistinguishable from that of the W303 parent (Figures 2E and 2F). Furthermore, density-based centrifugation of protein extracts indicated that cytoduction caused the endogenous Rnq1 protein of the 10B-H49 strain to shift from the soluble to the insoluble fraction (data not shown). Thus, exposure of 10B-H49 cells to the cytoplasm of W303 is sufficient to cause a heritable change in the physical state of Rnq1. Because *RNQ1* is a nuclear gene (not transmitted during cytoduction), the protein's insoluble state is not due to polymorphisms in its amino acid sequence, nor to any other trait carried by the W303 genome. Rather, like the Sup35 and Ure2 prions, its altered conformational state is "infectious," transmissible from one protein to another.

### Purified Rnq1 Forms Fibers by EM and Shows Seeded Polymerization

Both Sup35 and Ure2 have the capacity to form highly ordered amyloid fibers in vitro, as analyzed by the binding of amyloid specific dyes and by electron microscopy (Glover et al., 1997; King et al., 1997; Taylor et al., 1999). To examine conformational transitions of Rnq1 in vitro, the protein was expressed in *E. coli* and studied as a purified protein. Rnq1 was purified under denaturing conditions (8 M urea) because it had a tendency to form gels during purification in the absence of denaturant.

To determine if Rnq1 forms amyloids, we used Thioflavin T fluorescence. This dye exhibits an increase in fluorescence and a red shift in the $\lambda_{max}$ of emission upon binding to multimeric fibrillar β sheet structures characteristic of many amyloids, including transthyretin, insulin, β-2 microglobulin, and Sup35 (LeVine, 1995). The acquisition of Thioflavin T binding was sigmoidal (lag phase ≈ 6 hr; data not shown) suggesting a self-seeded process of protein assembly. The addition of 2% preformed fibers (see below) to fresh solutions of Rnq1 reduced the lag time—from 6.4 ± 0.2 hr to 4.3 ± 0.2 hr ($n = 4$; data not shown).

The formation of higher ordered structures was confirmed by transmission electron microscopy. The protein formed fibers with a diameter of 11.3 ± 1.4 nm (Figure 2G). This figure is comparable to the reported range for Ure2 (~20 nm) and Sup35 (~17 nm) fibers (Glover et al., 1997; Taylor et al., 1999). The fibers appeared to be branching, and the termini were unremarkable. The appearance of the fibers was coincident with the onset of rapid increases in Thioflavin T fluorescence.

### Rnq1 Disruption

[*URE3*] and [*PSI*$^+$] produce phenotypes that mimic loss-of-function mutations in their protein determinants (Wickner, 1994; Chernoff et al., 1995). To determine the

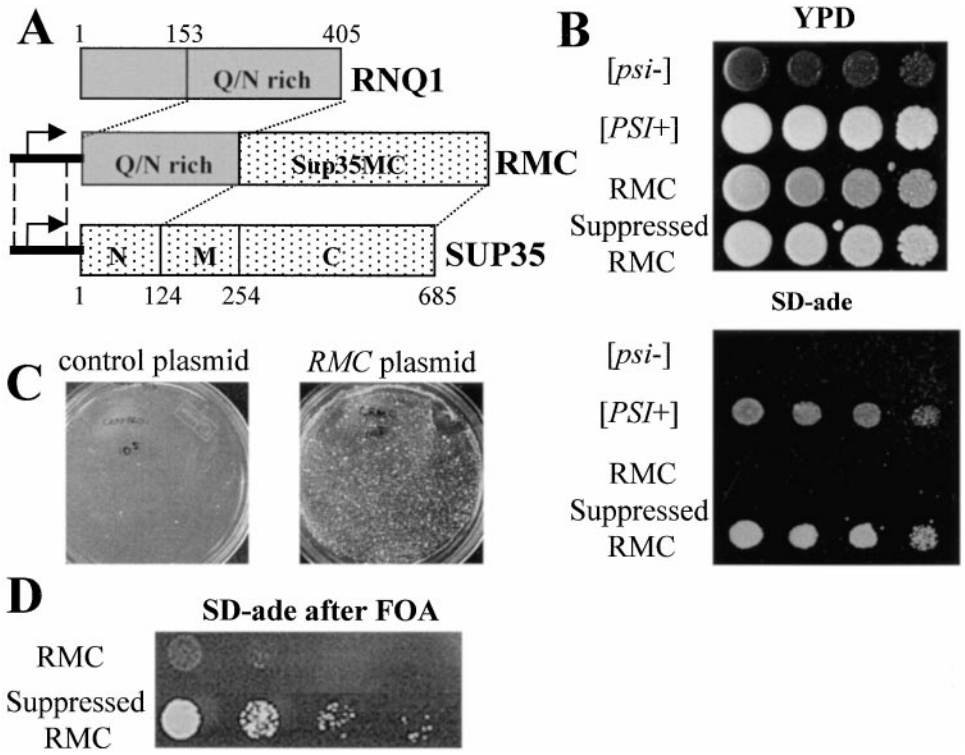


Figure 3. Creation of the Rnq1(153–405)–Sup35(124–685) Fusion and Resulting Phenotype

(A) Cloning scheme for the RMC fusion. The glutamine/asparagine-rich region of Rnq1 was inserted in place of the N-terminal domain (1–123) of Sup35. A pop in/pop out disruption was used that retained the endogenous *SUP35* promoter in the genome.

(B) Conversion to a suppression phenotype. Logarithmically growing yeast cells were density matched and plated by 5-fold serial dilution to complete (YPD) media or media lacking adenine (SD-ade). SD-ade selects for strains suppressing the stop codon in the *ade1–14* allele. Suppression is detectable on YPD because of red pigment that accumulates in the adenine biosynthetic pathway in nonsuppressed strains. [*PSI*$^+$] and [*psi*$^-$] strains are presented as negative and positive controls. The suppressor RMC variant was isolated after plating the parental RMC strains to SD-ade at high density.

(C) Increased expression of Rmc enhances conversion. The RMC parent (nonsuppressing) was transformed with a *URA3* plasmid containing the *SUP35* promoter driving expression of the *RMC* gene. As a control, the RMC parent was transformed with the empty *URA3* backbone. Transformants ($10^5$) were plated to SD-ade and grown for 5 days.

(D) The suppression phenotype induced by Rmc overexpression is not dependent on continued presence of the *RMC* plasmid. Suppressing and nonsuppressing strains that have lost the *URA3* plasmid were selected by growth on media containing 5-FOA. Strains were plated by serial dilution onto SD-ade.

loss-of-function phenotype of *RNQ1*, the entire ORF was deleted by homologous recombination in a diploid 74D-694 strain using a kanamycin resistance gene. Upon sporulation, each tetrad produced four viable colonies, two of which contained the *RNQ1* disruption, confirmed by immunoblotting total cellular proteins with $\alpha$-Rnq1 antibody and PCR analysis of the genomic region (data not shown). The $\Delta$*rnq1* strain had a growth rate comparable to that of wild-type cells on a variety of carbon and nitrogen sources and was competent for mating and sporulation (data not shown). The strain grew similarly to the wild-type in media with high and low osmolarity and in assays testing sensitivity to various metals (cadmium, cobalt, copper) (data not shown).

### Fusion of Rnq1 (153–405) to Sup35 (124–685): Nonsense Suppression Phenotype

The lack of an obvious loss-of-function phenotype was not unexpected, as the two known yeast prions, [*URE3*] and [*PSI*$^+$], only exhibit phenotypes under unusual selective conditions. However, the absence of a phenotype presented difficulties in determining whether Rnq1 could direct the epigenetic inheritance of a trait. To determine if the prion-like domain of Rnq1 could produce an epigenetic loss-of-function phenotype, we asked if it could replace the prion-determining domain of Sup35. When the wild-type Sup35 translation termination factor enters the prion state, the loss-of-function phenotype it produces is nonsense suppression—the readthrough of stop codons (for review, see Serio and Lindquist, 1999). This phenotype can be conveniently assayed in the strain 74D-694 because it contains a UGA stop codon mutation in the *ADE1* gene. In [*psi*$^-$] 74D-694 cells, ribosomes efficiently terminate translation at this codon. Cells are therefore unable to grow on media lacking adenine (SD-ade), and colonies appear red on rich media due to the accumulation of a pigmented by-product. In [*PSI*$^+$] strains, sufficient readthrough occurs to support growth on SD-ade and prevent accumulation of the pigment on rich media.

The coding region for residues 153–405 of Rnq1 was substituted for 1–123 of Sup35 (Figure 3A), and the resulting fusion gene, *RMC*, was inserted into the genome in place of the endogenous *SUP35* gene. The resulting

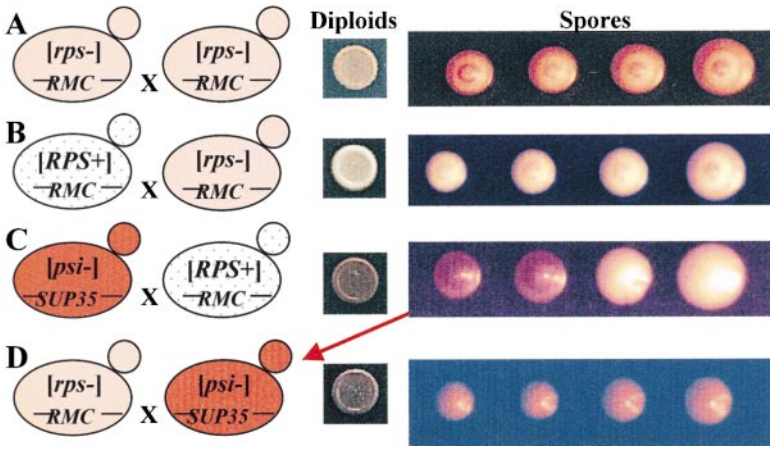


Figure 4. Genetics of RMC-Based Phenotypes

(A and B) [*RPS*$^+$] segregation is non-Mendelian. [*RPS*$^+$] strains were mated to an Mat**a** isolate of the parental RMC strain (which was [*rps*$^-$]). The diploid and spore progeny, showing the white color that indicates suppression, are shown on YPD (B). Diploids were sporulated and picked onto fresh YPD plates. Colonies represent growth of each of the four products of a single tetrad dissection. As a control, a mating between two [*rps*$^-$] strains is shown (A).

(C) [*RPS*$^+$] cannot be expressed in strains lacking *RMC*. An [*RPS*$^+$] strain was mated to 74D-694α, which contains the wild-type *SUP35* locus. The diploid of this cross was nonsuppressing (red), and sporulation was 2:2 reflecting the requirement of the underlying gene for expression of the phenotype.

(D) [*RPS*$^+$] cannot be maintained in a cryptic state in strains lacking *RMC*. A segregant from the cross in (C) that contained the *SUP35* gene (which had been exposed to the agent of [*RPS*$^+$]) was mated to an [*rps*$^-$] strain. Diploids of this cross were nonsuppressors. None of the spores from dissected tetrads were suppressors. This indicates that [*RPS*$^+$] could not be maintained in a cryptic state in a strain lacking the *RMC* gene.

strain, RMC, had a growth rate similar to that of wild-type cells on YPD, although the accumulation of red pigment was not as intense as seen in [*psi*$^-$] strains (Figure 3B). RMC strains showed no growth on SD-ade (Figure 3B) even after 2 weeks of incubation (data not shown). Thus, the protein encoded by the *RMC* gene (Rmc) fulfilled the essential translational termination function of Sup35.

At a low frequency, RMC variants appeared that were white on rich media and grew on SD-ade even more robustly than [*PSI*$^+$] cells did (Figure 3B). The frequency at which these variants appeared ($\sim 10^{-4}$; see Figure 3C) was far greater than expected for reversion of the UGA stop codon mutation in *ade1–14*, and subsequent analysis demonstrated that the allele had not reverted (see below). The suppressor phenotype of these variants was comparable in stability to that of [*PSI*$^+$]. Because Sup35 proteins that lack residues 1–123 are incapable of making such conversions (Ter-Avanesyan et al., 1994), these observations suggest that the Rnq1 prion-like domain can direct a prion conversion in the Rmc fusion protein.

Transient overexpression of Sup35 can produce new [*PSI*$^+$] elements, because higher protein concentrations make it more likely that a prion conformation will be achieved (Chernoff et al., 1993; Ter-Avanesyan et al., 1994). To test whether overexpression of Rmc can produce heritable suppressing variants, the original, nonsuppressing RMC strain was transformed with an expression plasmid for *RMC*. These transformants showed a greatly elevated frequency of conversion to the suppressor state compared to control strains carrying the plasmid alone (Figure 3C). Once a prion conformation is achieved, it should be self-perpetuating and normal expression should then be sufficient for maintenance. When the *RMC* expression plasmid was lost, all strains retained the suppressor phenotype (Figure 3D). Thus, transient overexpression of Rmc produced a heritable change in the fidelity of translation termination.

### Non-Mendelian Segregation of Rmc-Based Suppression Phenotype

To examine the genetic behavior of the suppressor phenotype in RMC strains, an isogenic Matα mating partner was created from a nonsuppressing Mat**a** RMC strain. When this strain was crossed to the original, nonsuppressing, RMC strain, neither the diploids nor their haploid meiotic progeny exhibited the suppressor phenotype (Figure 4A). However, when this strain was mated to RMC suppressor strains, the resulting diploids all displayed the suppressor phenotype, demonstrating that suppression is dominant (Figure 4B). In 14 tetrads dissected from two different diploids of this cross, all four haploid progeny showed inheritance of the suppression phenotype, instead of the 2:2 segregation expected for a phenotype encoded in the nuclear genome (Figure 4B). Following convention, we henceforth refer to the dominant, non-Mendelian suppressor phenotype as [*RPS*$^+$] (for Rnq1 [*PSI*$^+$]-like suppression) and the nonsuppressed phenotype as [*rps*$^-$].

To determine if the dominant, non-Mendelian [*RPS*$^+$] phenotype arises from the ability of Rmc protein to form a prion, we tested it for two additional unusual genetic behaviors that are not expected for other non-Mendelian genetic elements, such as viruses or mitochondrial genomes. First, it should become recessive and Mendelian in crosses to strains carrying a wild-type Sup35 allele. This is because Sup35 lacks the Rnq1 sequences that would allow it to be incorporated into an [*RPS*$^+$] prion. Wild-type Sup35, therefore, should cover the impaired translation-termination phenotype associated with the [*RPS*$^+$] prion. However, even when this phenotype has disappeared, Rmc protein in the prion state should still convert new Rmc protein to the same state. Therefore, in haploid meiotic progeny of this diploid, the phenotype will reappear in segregants carrying the *RMC* gene, but not in segregants carrying the *SUP35* gene (2:2 segregation).

Indeed, diploids of a cross between an [*RPS*$^+$] strain and an isogenic strain with a wild-type *SUP35* gene did not exhibit a suppressor phenotype. Upon sporulation, suppression reappeared in only two of the four progeny (Figure 4C). By PCR genotyping, these strains had the *RMC* gene at the *SUP35* locus (data not shown). Thus, the [*RPS*$^+$] factor had been preserved in the diploid, even though the phenotype had become cryptic.

Second, maintenance of [*RPS*$^+$] should depend upon

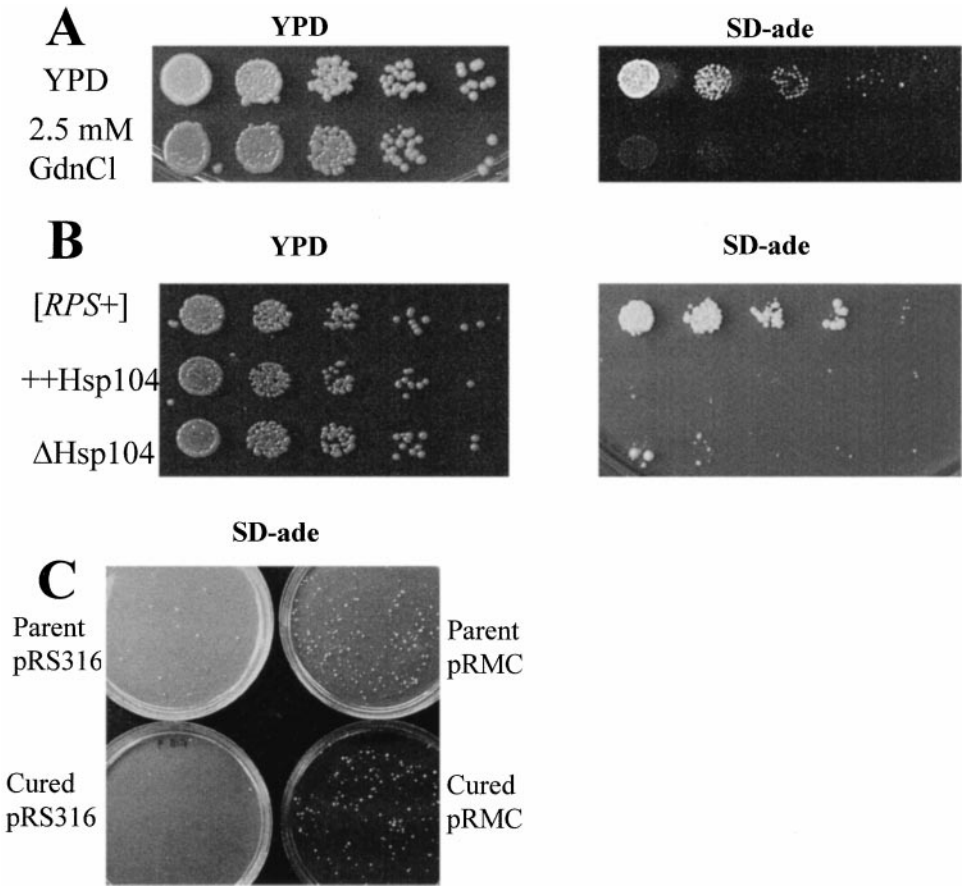


Figure 5. Curing the [*RPS*$^+$] Phenotype

(A) [*RPS*$^+$] is curable by plating on 2.5 mM GdnHCl. Strains were grown on solid YPD or YPD + 2.5 mM GdnHCl media for 3 days. Resulting colonies were plated by serial dilution onto YPD and SD-ade.

(B) [*RPS*$^+$] is curable by alteration of *HSP104* dosage. Strains were transformed with p2HG104 (expressing *HSP104* off a constitutive promoter), or a fragment containing a *LEU2* disruption of the *HSP104* open reading frame. Transformants were grown overnight and plated by serial dilution to YPD and SD-ade. *HSP104* expression levels were verified by immunoblotting (data not shown).

(C) [*RPS*$^+$] curing is reversible. Both the parental strain and a cured [*RPS*$^+$] strain were transformed with either control plasmid pRS316 or a plasmid containing a single copy of *RMC* driven from the *SUP35* promoter. Transformants were grown overnight, and $10^4$ cells were plated onto SD-ade and allowed to grow for 5 days.

continued expression of the Rmc protein. Although [*RPS*$^+$] is maintained in a cryptic state in diploids with a wild-type Sup35 gene, it should not be maintained in their haploid progeny whose only source of translational termination factor is wild-type Sup35. To determine if these progeny harbored the [*RPS*$^+$] element in a cryptic state, they were mated to an [*rps*$^-$] RMC strain whose protein would be converted if [*RPS*$^+$] were still present. When this diploid was sporulated, none of the progeny exhibited the suppressor phenotype (Figure 4D). Thus, the [*RPS*$^+$] element was not maintained in a cryptic state unless the Rmc protein was present.

**Curing of [*RPS*$^+$]**

One of the hallmarks of yeast prions is that cells can be readily and reversibly cured of them (Wickner, 1996). [*PSI*$^+$] is curable by several means, including growth on media containing low concentrations of the protein denaturant guanidine hydrochloride (Tuite et al., 1981) and transient overexpression or deletion of the protein remodeling factor *HSP104* (Chernoff et al., 1995).

Strains carrying [*RPS*$^+$] were passaged on medium containing 2.5 mM GdnHCl and then plated to YPD and to SD-ade to assay the suppressor phenotype. Cells passaged on GdnHCl no longer displayed the [*RPS*$^+$] phenotype, while cells not treated with GdnHCl retained it (Figure 5A). [*RPS*$^+$] was also lost when the *HSP104* gene was deleted by homologous recombination or when *HSP104* was overexpressed from a multicopy plasmid using the constitutive *GPD* promoter (Figure 5B). Cells that had been cured of [*RPS*$^+$] by overexpression of *HSP104* were passaged on YPD medium to isolate strains that had lost the overexpression plasmid. These strains remained [*rps*$^-$]. Thus, transient overexpression of *HSP104* is sufficient to heritably cure cells of [*RPS*$^+$] (data not shown).

Finally, we asked if Hsp104-mediated curing was reversible. Cells cured by overexpression of *HSP104* were retransformed with a plasmid bearing a single copy of *RMC*. Transformants were then plated onto SD-ade to assess the rate at which they converted to the [*RPS*$^+$] suppressor phenotype. [*RPS*$^+$] was regained at a rate

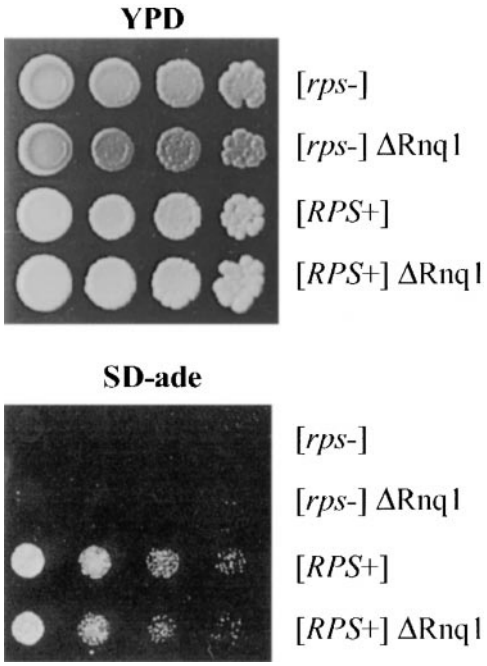


Figure 6. Effect of the Rnq1 Locus on [*RPS*] Phenotypes

[*RPS*$^+$] and [*rps*$^-$] yeast were disrupted at the Rnq1 locus by LFH-mediated disruption (see Experimental Procedures). Disrupted and parental strains were plated by serial dilution to SD-ade and YPD.

comparable to that seen in the parental RMC strain, indicating that the transient overexpression of *HSP104* caused no permanent alteration in susceptibility to [*RPS*$^+$] conversion (Figure 5C).

### The Effect of Endogenous Rnq1 on [*RPS*$^+$]

To determine if [*RPS*$^+$] can act as an independent genetic element, the gene encoding the endogenous Rnq1 protein was deleted in strains carrying the *RMC* replacement of *SUP35*. The deletion had no effect upon the maintenance of the [*RPS*$^+$] suppression phenotype. Growth on SD-ade was equally robust in [*RPS*$^+$] and [*RPS*$^+$] Δ*rnq1* strains (Figure 6). This indicates that Rmc can behave as an independent prion and is not dependent upon preexisting Rnq1 in an insoluble state.

### The Physical State of the Rmc Protein in [*RPS*$^+$] and [*rps*$^-$] Strains

Finally, we examined the localization of the Rmc fusion protein in the [*RPS*$^+$] and [*rps*$^-$] strains. Both strains were transformed with inducible plasmids that provided Rnq1(153–405)–GFP expression. Strains that lacked the endogenous *Rnq1* gene were used to prevent the GFP marker from localizing to the endogenous Rnq1 aggregate. Short-term expression of the GFP fusion protein prevented the formation of new [*RPS*$^+$] elements in the [*rps*$^-$] strain (data not shown).

Two distinct patterns of Rmc protein localization were revealed by this assay, and these correlated with the phenotypic differences between [*RPS*$^+$] and [*rps*$^-$] strains. In the nonsuppressing [*rps*$^-$] strains, the Rnq1(153–405)–GFP label was diffuse (Figure 7A). In the suppressing [*RPS*$^+$] strains, fluorescence was punctate and was

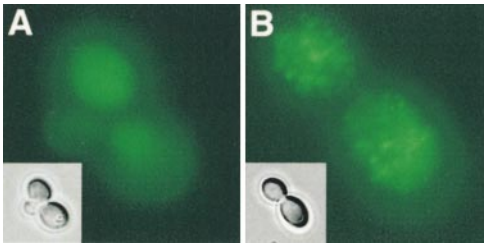


Figure 7. An Altered State of Rmc Is Present in [*RPS*$^+$] Strains

[*RPS*$^+$] and [*rps*$^-$] strains that were deleted for the Rnq1 gene were induced to express Rnq1(153–405)-GFP for 4 hr. The [*rps*$^-$] strain shows soluble fluorescence (A), whereas the [*RPS*$^+$] strain shows fine aggregations of the fusion protein (B). Inset figures are Nomarski images of the same yeast.

excluded from the nucleus (Figure 7B). This punctate pattern was different from that observed with the endogenous Rnq1 aggregates, as Rmc aggregates are numerous and very small (compare to Figure 1).

## Discussion

The identification of new prion proteins is critical to understanding the importance of prions as a genetic mechanism. Here we demonstrate that a prion can be identified by a deliberate search rather than by the study of a preexisting phenotype. We suggest that the search method we have used will be applicable to the identification of prion proteins in many other organisms. Our demonstration that a new prion protein domain can substitute for that of another well-characterized prion, reproducing its phenotypic characteristics and epigenetic mode of inheritance, provides a crucial tool in the analysis of uncharacterized candidates.

We searched for prion candidates using three attributes of the known yeast prion proteins: unusual amino acid composition with a high concentration of the polar amino acid residues glutamine and asparagine, constant expression levels through log and stationary phase growth, and a capacity to switch between distinct stable physical states (in this case, insoluble and soluble forms). One of the candidates isolated in this search, Rnq1, has both the in vitro and in vivo characteristics of a prion.

For [*URE3*] and [*PSI*], two techniques have been used to analyze the transition between the prion and nonprion states in vivo—alterations in the physical properties of the protein determinant and phenotypic change. Heritable alteration in the physical state of Rnq1 was readily demonstrated. We have shown that Rnq1 exists in distinct physical states—soluble and insoluble—in unrelated yeast strains. The insoluble state can be transmitted through cytoduction and, once transmitted, is stably inherited.

Demonstrating phenotypic change was not as straightforward. Since our search was not based upon phenotype and since many yeast genes exhibit no obvious loss-of-function traits, it is not surprising that we have yet to detect a phenotypic difference between strains that can be ascribed to physical differences in the state of Rnq1. Nor, indeed, is there an obvious phenotype

associated with a complete disruption of *RNQ1*. We reasoned, however, that the prion-like domain of Rnq1 might be transferable to another protein to produce an epigenetic modification of function.

To test this possibility, we used the Sup35 protein, whose epigenetic behavior in the prion state has been well characterized. When the N-terminal prion-determining region of *SUP35* was replaced with the C-terminal domain of *RNQ1*, the hybrid Rmc protein provided translation termination activity, mimicking the phenotype of [*psi*$^-$] strains. At a low spontaneous frequency, the strain acquired a stable, heritable suppressor phenotype, [*RPS*$^+$], which mimicked the phenotype of [*PSI*$^+$] strains. Suppression was dominant and segregated to meiotic progeny in non-Mendelian ratios. The possibility that this phenotype is caused by an epigenetic factor unrelated to the fusion protein was ruled out by genetic crosses showing that the phenotype is not expressed and cannot be transmitted in strains that do not produce the fusion protein. The relationship of the suppression phenotype to protein conformation was further demonstrated by fluorescence localization of the hybrid protein in isogenic [*RPS*$^+$] and [*rps*$^-$] strains. In [*RPS*$^+$] strains, most of the protein is sequestered into small foci and is presumably inhibited in its function in translational termination.

Transient overexpression of Rmc greatly increased the frequency of conversion to [*RPS*$^+$]. It is highly unusual for overexpression of a protein to cause a loss-of-function phenotype. It is even more unusual for phenotypes produced by overexpression to be stable after overexpression has ceased. Yet these properties are shared by the two yeast prion determinants and, to our knowledge, have been uniquely shared by them until now (Chernoff et al., 1993; Wickner, 1994). They are believed to derive from stabilization of an otherwise unstable protein conformation by protein–protein interactions. Proteins in the altered form then have the capacity to recruit new proteins of the same type to the same form. The phenotype associated with this change is, therefore, stably inherited from generation to generation and transferred to mating partners in crosses.

The ability of Rnq1(153–405) to substitute for the N-terminal domain of Sup35 and recapitulate its prion behavior was by no means predictable. The C-terminal region of Rnq1 (residues 153–405) and the N-terminal region of Sup35 have no primary amino-acid sequence homology—only a similar enrichment in polar amino acids. Reconstituting the epigenetic behavior of a prion requires that the Rmc fusion protein achieve an unusual balance between solubility and aggregation. If the fusion protein is too likely to aggregate, the inactive state will be ubiquitous; if it is too likely to remain soluble, the inactive state will not be stable. To recapitulate the epigenetic behavior of [*PSI*$^+$], the fusion protein must be able to switch from one state to the other and maintain either the inactive or the active state in a manner that is self-perpetuating and highly stable from generation to generation. Even minor variations in the sequence of the N-terminal region of Sup35, including several single amino acid substitutions and small deletions, can prevent maintenance of the inactive state (Doel et al., 1994; DePace et al., 1998; Liu and Lindquist, 1999). And a small internal duplication destabilizes maintenance of the active state (Liu and Lindquist, 1999). Therefore, the ability of the Rnq1 domain to substitute for the prion domain of Sup35 and to fully recapitulate its epigenetic behavior provides a rigorous test for its capacity to act as a prion and suggests that it has been honed through evolution to serve this function.

The fusion of prion-determining regions with different functional proteins could be used to create a variety of recombinant proteins whose functions can be switched on or off in a heritable manner, both by nature and by experimental design. Another study in our laboratory has shown that when the N-terminal region of Sup35 is transferred to the glucocorticoid receptor, it can epigenetically modify the transcriptional activity of the protein. Although the physical states of the fusion protein have not been characterized, it can exist in two different functional states that are heritable and interconvertable by overexpression of the N region or Hsp104 (Li and Lindquist, 2000). This observation complements our finding that the C-terminal region of Rnq1 can substitute for the N-terminal domain of Sup35 to produce a new prion. The two regions that constitute a prion, a functional domain and an epigenetic modifier of function, are modular and transferable.

The modular nature of prion proteins has implications concerning their evolutionary origins. The sharp transitions between prion domains and functional domains apparent in the primary sequence are likely the result of gene rearrangements. We suggest that these juxtapositions are a mechanism for producing phenotypic plasticity. Once present, they allow adaptation through distinct heritable phenotypes that occur without mutation of nucleic acids. Which of these currently existing prions represent transitional evolutionary states and which have acquired selective advantages that have fixed them in the genome remains to be determined.

## Experimental Procedures

### Yeast Strains, Culture, and Plasmids

Yeast strains were isogenic to 74D-694**a** [Mat**a**, *ade1–14, trp1–289, his3Δ–200, ura3–52, leu2–3, lys2*] except where noted. Yeast strains were grown in YPD or defined media lacking amino acids where necessary. Guanidine curing plates contained 2.5 mM guanidine hydrochloride (Fluka). Cytoduction studies were done using the strains W303 [Mat**a**, *his3–11,15, leu2–3,112, trp1–1, ura3–1, ade2–1*] and 10B-H49 [Mat$\alpha$, $\rho^\circ$ *ade2–1, lys1–1, his3–11,15, leu2–3,112, kar1–1, ura3::KANR*]. Cytoductants were selected after overnight mating on defined media lacking tryptophan that had glycerol as the sole carbon source. All other yeast manipulations were performed as previously described (Adams et al., 1997).

*RNQ1*, *SUP35*, and its promoter were cloned by amplification of 74D-694**a** genomic DNA. The *RNQ1* open reading frame was cloned using 5′-GGAGGATCCATGGATACGGATAAGTTAATCTCAG-3′ and (A) 5′-GGACCGCGGGTAGCGGTTCTGTTGAGAAAAGTTGCC-3′. *RNQ1* (153–405) was cloned using 5′-GAGGATCCATGCCTGATG ATGAGGAAGAAGACGAGG-3′ and (A). The *SUP35* promoter was cloned using 5′-CGGAATTCCTCGAGAAGATATCCATC-3′ and 5′-GGGATCCTGTTGCTAGTGGGCAGA-3′. *SUP35* (124–685) was cloned using 5-′GTACCGCGGATGTCTTTGAACGACTTTCAAA AGC-3′ and 5′-GTGGAGCTCTTACTCGGCAATTTTAACAATTTTAC-3′.

The *RMC* gene replacement was performed as described (Rothstein, 1991). To create the plasmid for pop in/pop out replacement in pRS306, the *SUP35* promoter was ligated into the EcoRI–BamHI site, *RNQ1* (153–405) was ligated into the BamHI–SacII site, and *SUP35* (124–685) was ligated into the SacII–SacI site. To create the disrupting fragment, this plasmid was linearized with MluI and

transformed. Pop outs were selected on 5-FOA (Diagnostic Chemicals, Ltd.) and verified by PCR. To create the single-copy *RMC* plasmid in pRS316, the ClaI–SacI fragment (including promoter and *RMC*) of the previous plasmid was ligated into the ClaI–SacI site. To create the plasmid used for GFP fluorescence studies, the Rnq1 fragment above was fused into plasmid pRSCUP1–3SGFP using the BamHI and SacII sites (Liu and Lindquist, 1999). *HSP104* overexpression plasmid p2HG104 and *HSP104* deletions were used as described (Chernoff et al., 1995).

**Rnq1 Deletion**

Strains deleted of the Rnq1 open reading frame were created using the long flanking homology PCR method (Wach, 1996). Primers 5′-GGTGTCTTGGCCAATTGCCC-3′ and 5′-GTCGACCTGCAGCGTACGCATTTCAGATCTTTGCTATAC-3′ or 5′-CGAGCTCGAATTCATCGATTGATTCAGTTCGCCTTCTATC-3′ and 5′-CTGTTTTGAAAGGGTCCACATG-3′ were used to amplify genomic DNA. These PCR products were used for a second round of PCR on plasmid pFA6a digested with NotI (gift of A. Wach). The product of the second PCR round was used to transform log-phase yeast cultures. Transformants were selected on YPD containing 200 mg/mL G418 (GIBCO-BRL) and verified by PCR and immunoblotting for Rnq1.

**Purification of Rnq1**

Rnq1 was cloned into pPROEX-HTb (GIBCO-BRL). Primers were 5′-GGAGGATCCATGGATACGGATAAGTTAATCTCAG-3′ and 5′-CCAAGCTTTCAGTAGCGGTTCTGTTGAGAAAAGTTG-3′, and the product was digested and ligated into the BamHI and HindIII sites. The plasmid was electroporated into BL21-DE3 lacIq cells.

Transformed bacterial cultures were induced at $OD_{600} = 1$ with 1 mM IPTG for 4 hr at 30°C. The cells were lysed in 8 M urea, 20 mM Tris-Cl (pH 8). Protein was purified over a Ni-NTA column (Qiagen) followed by Q-Sepharose (Pharmacia). The $His_6$ tag from the vector was cleaved under native conditions (150 mM NaCl, 5 mM KPi) using TEV protease followed by passage of the protease product over a Ni-NTA column to remove uncleaved protein. Protein was methanol-precipitated prior to use. The protein was used to create an antibody to Rnq1 (Cocalico).

**In Vitro Protein Studies**

Recombinant protein was resuspended in 4 M urea, 150 mM NaCl, 5 mM KPi (pH 7.4) at a concentration of 10 μM. Seeded samples were created by sonication of 1/50 volume of a 10 μM solution of preformed fibers verified by electron microscopy. The protein samples were incubated at room temperature on a wheel rotating at 60 rpm. Fluorimeter samples were prepared as 3.3 μM Rnq1, 50 μM Thioflavin T in buffer. Samples were analyzed on a Jasco FP750 with the following settings: $\lambda_{exc} = 409$ nm, $\lambda_{emi} = 484$ nm, bandwidth = 10 nm.

For electron microscopy analysis, 5 μl of a 10 μM protein solution was placed on a 400 mesh carbon-coated EM grid (Ted Pella), and allowed to adsorb for 1 min. The sample was negatively stained with 200 μl of 2% aqueous uranyl acetate and wicked dry (Spiess et al., 1987). Samples were observed in a Philips CM120 transmission electron microscope operating at 120kV in low dose mode. Micrographs were recorded at a magnification of 45,000 on Kodak SO-163 film.

**Centrifugation Analysis**

Log-phase yeast were lysed using a bead beater (Biospec) into 75 mM Tris-Cl (pH 7), 200 mM NaCl, 0.5 mM EDTA, 2.5% glycerol, 0.25 mM EDTA, 0.25% Na-deoxycholate, supplemented with protease inhibitors (Boehringer-Mannheim). Lysates were cleared of crude cellular debris by a 15 s 6000 rpm spin in a microcentrifuge (Eppendorf). Separation into supernatant and pellet fractions was performed in a TLA-100 rotor on an Optima TL ultracentrifuge (Beckman) at 280,000 × g (85,000 rpm) for 30 min. Protein fractions were resolved by 10% SDS-PAGE and immunoblotted with α-Rnq1.

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