Chaperone-dependent amyloid assembly protects cells from prion toxicity

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Protein conformational diseases are associated with the aberrant accumulation of amyloid protein aggregates, but whether amyloid formation is cytotoxic or protective is unclear. To address this issue, we investigated a normally benign amyloid formed by the yeast prion [RNQ+]. Surprisingly, modest overexpression of Rnq1 protein was deadly, but only when preexisting Rnq1 was in the [RNQ+] prion conformation. Molecular chaperones protect against protein aggregation diseases and are generally believed to do so by solubilizing their substrates. The Hsp40 chaperone, Sis1, suppressed Rnq1 toxic protein, but instead of blocking Rnq1 protein aggregation, it stimulated conversion of soluble Rnq1 to [RNQ+] amyloid. Furthermore, interference with Sis1-mediated [RNQ+] amyloid formation exacerbated Rnq1 toxicity. These and other data establish that even subtle changes in the folding homeostasis of an amyloidoigenic protein can create a severe proteotoxic gain-of-function phenotype and that chaperone-mediated amyloid assembly can be cytoprotective. The possible relevance of these findings to other phenomena, including prion-driven neurodegenerative diseases and heterokaryon incompatibility in fungi, is discussed.

Hsp40 | neurodegenerative disease | Sis1 | Rnq1 | yeast prion

Alzheimer’s disease, transmissible spongiform encephalopathies, and polyglutamine diseases are representatives of a large group of neurodegenerative disorders that are associated with the misfolding and conversion of particular proteins into amyloids (1). Amyloids form in response to many perturbations in protein homeostasis, namely mutations in the amino acid sequence of a disease-related protein, expansion of simple sequence elements in disease genes, elevated protein levels, and age-associated cell stress (2). Amyloid fibrils share a cross-β structural motif, in which β-strands run perpendicular to the long fiber axis and accumulate in intra- and extracellular inclusions (3, 4). Fibril formation requires that a misfolded protein expose a pleated β-surface that is capable of serving as a template and hydrogen-bonding partner with an extra β-strand (1). Biochemical parameters for the classification of protein aggregates as amyloids include resistance to solubilization by the detergent SDS and the ability to bind indicator dyes such as thioflavin-T (2).

Amyloid deposits in the brain are a hallmark of protein conformational disease, but often there is only a poor correlation between the detection of amyloid fibrils and other markers of neurodegeneration (5). Thus, there is still intense debate about whether amyloids are the causative toxic protein species in neurodegenerative diseases. In fact, recent, still controversial work suggests that amyloids might be benign or cytoprotective and that difficult-to-characterize soluble oligomeric conformers are the toxic species of disease-causing proteins (6–8).

Cells buffer proteotoxic events related to intracellular protein misfolding via chaperone-mediated partitioning of nonnative conformers between pathways for proper folding, inclusion body formation, and degradation (9). Molecular chaperones also play a critical role in the propagation of yeast prions (10), which are examples of intracellular amyloids that, in general, are not inherently toxic (11, 12). However, the conversion of active soluble Sup35 and Ure2 into their prion states [PSI+] and [URE3], respectively, inactivates these proteins (11, 12). Yeast prion formation occurs spontaneously at a low frequency, and the prion state is then perpetuated through the templating of newly synthesized prion proteins by preexisting amyloid-like prions (13). Templated prion proteins then undergo stable changes in structure and function to enter an amyloid-like state that is propagated and passed from mother cells to their daughters in a molecular chaperone-dependent manner (10). Yeast prions thereby constitute cytoplasmically transmitted, protein-based elements of inheritance that are dominant in genetic crosses (prions are denoted by brackets, italics, and capital letters to reflect these properties).

The yeast prion [RNQ+] is determined by the conformational state of the Rnq1 protein, which contains a C-terminal asparagine-glutamine-rich prion domain and an N-terminal non-prion-forming domain (14, 15). The native form of Rnq1 has no known normal biological function and is nonessential. Yet the [RNQ+] prion can have important effects on yeast cells because it influences certain other proteins to convert to amyloid-like states (16–18). For example, [RNQ+] prions are required for the initial conversion of native Sup35 to the [PSI+] state. Indeed, [RNQ+] constitutes the cytoplasmically inherited factor known as [PIN+] and is the only known yeast prion that is commonly found in wild strains (19, 20). [RNQ+] also causes the exon 1 fragment of huntingtin protein, containing glutamine repeats, to become toxic in yeast (16). Thus, [RNQ+] prions can interact with other amyloid-forming proteins and thereby help drive their conversion into benign or toxic amyloid-like species.

Results

Overexpression of Rnq1 Is Toxic in [RNQ+] Cells. We recently discovered that moderate (i.e., ~5- to 10-fold) overexpression of Rnq1 from the GAL1 promoter was severely toxic in cells that harbored the [RNQ+] prion (Fig. 1a (Upper) Growth of serially diluted liquid cultures on agar. (Lower) Protein levels detected by Western blotting). This finding was surprising because Rnq1 overexpression was not toxic when endogenous Rnq1 was in the [rnq−] non-prion conformation, nor was it toxic in cells carrying a deletion of the RNQ1 gene, Δrnq1 (Fig. 1a). Cell growth defects observed were more extreme than any we have observed with
cells. Fixed yeast were decorated with /H9251
tion state of Rnq1-YFP by fluorescence microscopy. (d)
with the amyloid indicator dye, thioflavin-T. (fluorescent secondary antibody. The same cells were simultaneously stained
extracts.

Douglas
et al

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Fig. 1.

Overexpression of Rnq1 is toxic to [RNQ⁻] cells. (a) The effect of Rnq1 overexpression on yeast cell viability in the presence and absence of the [RNQ⁻] prion. (b) Thioflavin-T staining of Rnq1 in [RNQ⁻], [rnq⁻], and Δrnq1 cells. Fixed yeast were decorated with α-Rnq1 sera that was detected with a fluorescent secondary antibody. The same cells were simultaneously stained with the amyloid indicator dye, thioflavin-T. (c) Visualization of the aggregation state of Rnq1-YFP by fluorescence microscopy. (d) (Upper) Assembly status of Rnq1-YFP as determined by SDD-AGE. (Lower) Western blots of cell extracts.

other misfolded proteins in yeast (21, 22). At this modest level of Rnq1 overexpression, ~25% of [RNQ⁻] cells were dead within 4 h, as determined by the percentage of colony-forming units and dye exclusion (data not shown). Toxicity was accompanied by the accumulation of Rnq1 aggregates that stained with the common amyloid diagnostic dye thioflavin-T (Fig. 1b). Rnq1 overexpression was found to be toxic in [RNQ⁻] laboratory strains (W303, 74D-694, BY23, and BY4741), clinical strains (YJM269, YJM421, YJM436, and YJM653), a fermentation strain (Y12), and wine strains (I14, T73, and WE372) (M. Taipale and S.L., unpublished observations). Thus, Rnq1 toxicity is pervasive and not strain-specific.

Although not quite as deadly, a C-terminal Rnq1-YFP fusion protein behaved similarly to untagged Rnq1 and exhibited the same pattern of toxicity: toxic in [RNQ⁻] cells but not in [rnq⁻] or Δrnq1 cells. This result allowed us to correlate changes in toxicity with changes in protein distribution [Fig. 1c and supporting information (SI) Fig. S1]. While aggregated in [RNQ⁺] cells, Rnq1-YFP was distributed throughout the cytosol in [rnq⁻] or Δrnq1 cells. Using low- and high-copy plasmids that express Rnq1-YFP at different levels, we found that toxicity positively correlated with the degree of overexpression (Fig. S1a). Western blots of cell lysates separated by semidetergent-detergent-agarose gel electrophoresis (SDD-AGE) demonstrated that, in [RNQ⁺] but not [rnq⁻] cells, Rnq1-YFP assembled into a SDS-resistant high-molecular-weight species typical of amyloid assemblies of yeast prions (Fig. 1d and Fig. S1b) (23). Yet a pool of soluble Rnq1-YFP, which ran at the position of a monomer on SDD-AGE gels, also was present in [RNQ⁺] cells.

Growth of [RNQ⁺] cells overexpressing just the non-prion-forming domain or just the prion-forming domain of Rnq1, amino acids 1–183 and 154–405, respectively, was not hindered (Fig. S1c). When the prion-forming domain is expressed on its own, it assembles into an SDS-resistant species that runs as an amyloid on SDD-AGE gels (Fig. S1d). Therefore, the mechanism for Rnq1 toxicity does not appear related to the accumulation of large quantities of [RNQ⁺]-like amyloid per se.

The toxicity of Rnq1 overexpression in the presence of the [RNQ⁺] prion might seem similar to the toxicity of overexpressed Sup35 in the presence of its prion [PSI⁺]. Overexpression of Sup35 is toxic in [PSI⁺] cells because it drives too much of the essential Sup35 protein into an inactive amyloid conformation (24). In contrast, Rnq1 toxicity cannot be due to an inhibition of Rnq1 function because deletion of the gene-encoding Rnq1 has no detectable effect on yeast growth under hundreds of conditions tested (T.F. Outeiro and S.L., unpublished data). Furthermore, in contrast to Sup35, expression of Rnq1’s non-prion-forming domain does not rescue the toxicity caused by Rnq1 overexpression (data not shown).

The Hsp40 Sis1 Can Suppress Rnq1 Toxicity. Sis1, an essential Hsp40 chaperone, is required for the propagation of the [RNQ⁺] prion state (25). Sis1 specifies Hsp70 function and is required for protein synthesis, protein folding, and cell stress protection (26, 27). Overexpressing Sis1 by as little as 3-fold strongly suppresses Rnq1 toxicity (Fig. 2a). To examine whether other chaperones were capable of suppressing Rnq1 toxicity, an expression library of 4,954 yeast genes was screened (21). Sis1 was the only library includes, among many other chaperones, Ydj1, which is a member of the large Hsp40 family that is closely related to Sis1. It also includes Hsp70 Ssa1 (Hsp70) and Hsp104, which assist in shearing ones, Ydj1, which is a member of the large Hsp40 family that is closely related to Sis1. It also includes Hsp70 Ssa1 (Hsp70) and Hsp104, which assist in shearing

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required for interaction with the prion, the glycine- and phenylalanine-rich (G/F) region (29). A Sis1 G/F variant fails to promote the propagation of [RNQ\textsuperscript{+}], but can carry out Sis1’s essential functions. We overexpressed Sis1 G/F and found that, unlike Sis1, it could not suppress Rnq1 toxicity.

**Sis1-Mediated Amyloid Formation Protects from Rnq1 Toxicity.** The toxicity produced by the overexpression of Rnq1 represents a dominant gain of function that requires endogenous Rnq1 protein to be in a [RNQ\textsuperscript{+}] prion conformation. It may be Sis1’s ability to facilitate [RNQ\textsuperscript{+}] prion propagation that ameliorates Rnq1 toxicity. Indeed, the suppression of Rnq1 toxicity by Sis1 overexpression was accompanied by a substantial increase in the formation of SDS-resistant [RNQ\textsuperscript{+}] amyloids (Fig. 2b). This seemed to be accompanied by a decrease in the pool of unassembled SDS-sensitive Rnq1. We have found, however, that although SDD-AGE is a reliable method for quantitatively detecting SDS-resistant species, it is not reliable for SDS-soluble species. To examine SDS-soluble species, we used gel-filtration chromatography. As shown in Fig. 2c, a large pool of unassembled Rnq1-YFP accumulated on Rnq1-YFP overexpression (Fig. 2c, compare Top and Middle). Suppression of Rnq1 toxicity by Sis1 correlated with a dramatic decrease in unassembled Rnq1-YFP pools and a corresponding increase in the pools of assembled forms (Fig. 2c, compare Middle and Bottom). These results suggest that cytotoxic Rnq1 conformers accumulate when levels of Rnq1 protein exceed the cell’s capacity to efficiently promote the template-driven formation of the SDS-resistant [RNQ\textsuperscript{+}] prion species. To test this hypothesis, we asked whether Rnq1 toxicity would be exacerbated when the efficiency of [RNQ\textsuperscript{+}] amyloid assembly was reduced.

**Identification of the Sis1-Binding Site in Rnq1.** First, we identified and mutated the chaperone-binding motif that Sis1 uses to interact with Rnq1. A peptide array was created that contained 25 residue N-acetylated peptides spanning the entire Rnq1 amino acid sequence. This array was incubated with purified Sis1 and washed, and Sis1-interacting peptides were identified by Western blot after transfer of bound chaperone to nitrocellulose (Fig. S2a). Tight binding of Sis1 was observed only with a few neighboring Rnq1 peptides, and these peptides were located in the non-prion-forming domain.

The amino acid sequence of this region is conserved in all known Rnq1 homologues and contains a classic, hydrophobic, chaperone-binding motif, LGKLALL (Fig. 3a). Thus, Sis1 forms a functional chaperone:substrate complex with peptides containing this chaperone-binding motif.

Next, to reduce the efficiency of Sis1’s interaction with [RNQ\textsuperscript{+}], we replaced hydrophobic leucine residues in the Rnq1 chaperone-binding motif with alanines (L91A, L94A, and L97A). As demonstrated by coimmunoprecipitation, the capacity of Rnq1-GFP to interact with Sis1 was strongly, but not completely, reduced by these mutations (Fig. 3c and data not shown).

**Mutations in the Sis1-Binding Site of Rnq1 Interfere with [RNQ\textsuperscript{+}] Amyloid Assembly.** To determine whether the Rnq1 chaperone-binding motif mutants were defective in the assembly of [RNQ\textsuperscript{+}] amyloid, we expressed them as Rnq1-GFP fusions from an extrachromosomal plasmid in cells expressing WT Rnq1 in its...
prion state. The L91A, L94A, and L97A mutations were expressed at levels similar to those of WT Rnq1 by using the CUP1 promoter, but they had a reduced capacity to form fluorescent foci (Fig. S2c). An L45A mutation, which also is located in the non-prion-forming domain, but lies outside of the chaperone-binding motif, had no detectable effect on the assembly of [RNQ⁺] prions (Fig. S2c). Further, a time-course analysis by SDD-AGE (Fig. 4a) and pulse–chase (Fig. S2d) revealed that the rate at which newly synthesized Rnq1-GFP protein was converted into SDS-resistant conformers in vivo was reduced several fold by the L94A mutation in comparison with the WT protein. In addition, in vitro-purified Rnq1 L94A could be templated by prion “seeds” present in [RNQ⁺] cell extracts to form SDS-resistant species (Fig. 4b). However, it was templated and converted to an SDS-resistant form with lower efficiency than the WT Rnq1 protein.

Finally, we asked whether the impairment of Rnq1 amyloid assembly increased the toxicity of Rnq1 overexpression. As we hypothesized, Rnq1 L94A was more toxic than WT Rnq1 when overexpressed in [RNQ⁺] cells (Fig. 4c). A triple mutant, Rnq1 L94A–L96A–L97A, was even more toxic than Rnq1 L94A (data not shown). As expected from the fact that the Rnq1 L94A mutation impaired, but did not eliminate, interaction with Sis1, the overexpression of Sis1 3-fold was still able to suppress the toxicity of the mutant protein (Fig. 4c).

Thus far, we have shown that interfering with the assembly of Rnq1 into the [RNQ⁺] amyloid state is extremely toxic. Toxicity occurs when Rnq1 expression is higher than normal (Figs. 1 and 2) or when mutations in Rnq1 interfere with the efficiency of Sis1 interaction (Figs. 3 and 4). In addition, depletion of Sis1 from the cytosol reduces the efficiency of [RNQ⁺] prion assembly and exacerbates Rnq1 toxicity (Fig. S3). These collective data indicate that the efficient conversion of native Rnq1 into its SDS-resistant amyloid form prevents the accumulation of a toxic Rnq1 conformer.

**Suppression of Rnq1 Toxicity by Sis1 Requires [RNQ⁺] Prion Assembly.** Rnq1 L94A exhibits a higher propensity than WT Rnq1 to form SDS-soluble aggregates when [RNQ⁺] assembly is impeded via depletion of Sis1 (Fig. S3). The inability of cells to maintain Rnq1 L94A in a soluble state correlates with the enhanced toxicity of the L94A mutant. In this sense, Rnq1 L94A is similar to alleles of amyloidogenic proteins whose subtle defects in folding kinetics cause devastating protein conformational diseases (1). Thus, we wondered whether Rnq1 L94A would assume a toxic conformation in the absence of templating by [RNQ⁺] prion seeds. Indeed, the overexpression of Rnq1 L94A, but not WT Rnq1, was toxic in [rnq–] strains (Fig. 5a). Hence, a small amino acid substitution can cause Rnq1 to be toxic even in the absence of [RNQ⁺] amyloid formation.

Sis1-dependent [RNQ⁺] amyloid formation appears to protect cells from toxicity caused by the overexpression of Rnq1. If amyloid formation is a critical aspect of Sis1’s ability to suppress Rnq1 toxicity, then Sis1 overexpression should not protect [rnq–] cells from Rnq1 L94A-mediated death because these cells lack the [RNQ⁺] prion seeds required for amyloid assembly. Indeed, overexpression of Sis1, which binds Rnq1 L94A with reduced efficiency, protected [RNQ⁺], but not [rnq–], strains from Rnq1 L94A toxicity. This finding further confirms that Rnq1 toxicity is not caused by the sequestration of Sis1 into [RNQ⁺] prion complexes. Furthermore, the presence of the [RNQ⁺] prion assembly pathway and Sis1 overexpression are both required for the suppression of Rnq1 toxicity.

**Rnq1 L94A Does Not Form Prion Amyloids in [rnq–] Cells.** To rule out the possibility that Rnq1 L94A assembled into [RNQ⁺] prions spontaneously in [rnq–] cells, we compared its assembly status to that of WT Rnq1 in [rnq–] strains (Fig. 5b–e). In [rnq–] cells, Rnq1 L94A exhibited a higher propensity than WT Rnq1 to coalesce into foci (Fig. 5b). Gel-filtration chromatography showed that Rnq1 L94A formed high-molecular-weight aggregates in these cells (Fig. S4). Notably, these aggregates were not SDS-resistant and (Fig. 5c) were unable to bind the amyloid indicator thioflavin-T (Fig. 5d). Thus, Rnq1 toxicity is not related to the accumulation of excess pools of [RNQ⁺] amyloid and may be caused by a SDS-soluble Rnq1 species.

Because small prion seeds in the form of detergent-soluble prefibrillar species could have escaped detection by SDD-AGE and thioflavin-T staining, we applied another test for the existence of such forms of Rnq1 L94A in [rnq–] cell extracts
RNQ amyloids (Fig. 5) that contained toxic levels of Rnq1 L94A failed to seed assembly when coexpressed with the HET-S allele that cannot form toxic prion amyloid species. One of Sis1’s functions in [RNQ+ prion] propagation appears to be promoting the shearing of [RNQ+] amyloid fibers into smaller pieces, thereby creating new surfaces to more efficiently seed the assembly of the prion (28). This reaction also requires Hsp104 and Hsp70. Hence, binding of Sis1 to the non-prion-forming domain of full-length Rnq1 may help facilitate this shearing process. However, because overexpression of Hsp70 and Hsp104 does not suppress Rnq1 toxicity, Sis1 may have additional functions in [RNQ+] prion propagation that do not overlap with those of other chaperones. Sis1 stably associates with assembled [RNQ+] conformers in a 1:1 molar ratio (29). Therefore, Sis1’s binding to the non-prion-forming domain has the potential to stabilize [RNQ+] prions in a conformation that is optimal for efficient amyloid fibril growth.

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Although amyloid is not toxic, its interaction with soluble protein forms could give rise to pathogenic species via nonproductive templating. Case in point, when GPI-anchorless PrP was expressed together with WT PrP, it accelerated scrapie disease and resulted in increased deposits of both amyloid and nonamyloid proteinase K-resistant PrP (32). Similarly, in yeast, the toxicity of Huntington exon 1 depends on the [RNQ+] prion state (16, 22). This concept may even extend to the heterokaryon incompatibility mediated by the [Het-s] prion in Podospora anserina (34). HET-s in its prion form only leads to cell death when coexpressed with the HET-S allele that cannot form amyloid. Templating of the nonamyloidogenic HET-S protein by [Het-s] prion seeds could lead to the formation of a toxic species, whereas templating of HET-s protein would result in the non-toxic prion amyloid species.

The aggregation state and the toxicity of aggregation-prone proteins are strongly modulated by host factors such as Hsp70 and its associated cochaperones, but the mechanisms for chaperone-dependent conversion of soluble Rnq1 into SDS-resistant [RNQ+] amyloid is critical to prevent the formation of other toxic Rnq1 conformers. We demonstrate that toxic Rnq1 conformers accumulate in a nonamyloid form when [RNQ+] assembly is made inefficient by multiple means. We propose that nonproductive templating of Rnq1 monomers by [RNQ+] seeds predisposes Rnq1 to leave the amyloid pathway and accumulate as a toxic species, whose exact nature is not yet clear. Templating of native proteins to form amyloid is a basic feature of amyloidogenesis, and we suggest that inefficiencies in this process contribute to the proteotoxicity associated with certain protein conformational diseases. This templating model explains how amyloid formation can serve a protective function, whereas the [RNQ+] prion state is a prerequisite for toxicity of the WT Rnq1 protein.
eroine function in this process are just being defined (9). Molecular chaperones generally act to antagonize protein aggregation. Yet our observations that the chaperone-dependent assembly of amyloid conformers can be cytoprotective provide a different view of the effects of chaperones in conformational disease. Thus, molecular chaperones can antagonize protein toxicity in conformational disorders by two different mechanisms: They can solubilize misfolded proteins or aid in sequestering them into benign, amyloid-like species. The most central aspect of antagonizing toxicity of misfolded proteins appears to be preventing accumulation of the detergent-soluble misfolded species, rather than preventing the formation of amyloid conformers.

Methods

Strains. W303 and 74D-694 strains were used to take advantage of the different genetic markers or gene deletions. Unless noted, yeast harbored pRS315-\(\text{RNQ1-GFP}\) raffinose before 5-fold serial dilutions were spotted on either glucose or raffinose. Strains harboring pRS416-\(\text{RNQ1-GFP}\) were grown overnight in synthetic dropout media containing 2% galactose before dilutions were spotted on plates that contained 500 \(\mu\)M CuSO\(_4\). Plates were photographed after 3–5 days of incubation at 30°C.

Analysis of Rnq1 Cytotoxicity. Strains harboring pRS416-RNQ1 or pRS416-RNQ1-YFP were grown overnight in synthetic dropout media containing 2% raffinose. Core 5-fold serial dilutions were spotted on either glucose or galactose plates. Alternatively, strains that harbored pRS316-RNQ1-GFP or pRS315-RNQ1-GFP were cultured overnight in synthetic media containing glucose before dilutions were spotted on plates that contained 500 \(\mu\)M CuSO\(_4\). Plates were photographed after 3–5 days of incubation at 30°C.

Screening of an Rnq1 Peptide Array. A 25mer Rnq1 cellulose-bound peptide array was prepared by automated spot synthesis and screened essentially as described (11) (see also \textit{Si Methods}).

Pulse-Chase Analysis of [RNQ1]n Prion Formation. Pulse labeling of yeast to analyze the kinetics of Rnq1 assembly in 74D-694 cultures was performed essentially as described (30) (see also \textit{Si Methods}). Time points were taken after the addition of 50 \(\mu\)M CuSO\(_4\) to growing cells.

[\textit{RNQ1}]n Prion Formation by Fluorescence Microscopy. Assembly of Rnq1-GFP into fluorescent foci that represent prion amyloids was preformed essentially as described (26) (see also \textit{Si Methods}).

SDD-AGE. Rnq1 assembly into SDS-resistant [\textit{RNQ1}]\textsuperscript{n} prions was monitored by SDD-AGE as described (23) (see also \textit{Si Methods}).

Rnq1 Communoprecipitation with Sis1. Expression of Rnq1-GFP in log-phase cells harboring the indicated form of pRS316-Rnq1-GFP was induced by supplementation with 50 \(\mu\)M CuSO\(_4\). Native cell extracts were prepared 1 h later under the conditions described (26), and a-Sis1 was added to cell lysates. Rnq1-GFP was communoprecipitated with Sis1 and detected with a-\textit{GFP} by Western blot.

Seeded Polymerization of Purified Rnq1. Purified Rnq1-His was added to lysates of the indicated strains. Assembly was monitored by SDD-AGE (see also \textit{Si Methods}).

Size-Exclusion Chromatography. [\textit{RNQ1}]n or [\textit{RNQ1}]\textsuperscript{o} cells were grown overnight in synthetic media at 30°C. Rnq1-YFP expression was induced by the addition of 2% galactose for 4 h before the collection of 100 OD units of cells. Proteins in extracts created with a non-denaturing lysis buffer were resolved on a Superose 125 sizing column (Amersham Pharmacia). Indicated proteins in column fractions were detected by Western blot.

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Supporting Information

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SI Methods

Strains and Plasmids. W303, MATa can1–100 ade2–1, his3–11,15 leu2–3,112 ura3–1 trpl–1; W303/rnq1, MATa can1–100 ade2–1 his3–11,15 leu2–3,112 ura3–1 trpl–1 rrnq1::KanMX4; 74D-694, MATa ade1–14 trpl–1–289 his3A–300 ura3–52 leu2–3,112; BY4741, MATa his3A leu2A met15A ura3A; W303/rnq1, MATa ade2–1 his3–11,15 leu2–3,112 ura3–1 trpl–1 sisd1–2 s1A::His3PRNQ1. All of these strains harbored Rnq1 in its [RNQ'] form, and the generation of isogenic [rnq−] strains was accomplished via sequential passage of cells on plates containing 3 mM guanidine-HCl (1). BY, W303 and 74D-694 strains were used to take advantage of the different markers or gene deletions. Identical results were obtained in studies carried out with both of these strains, so Rnq1 toxicity is not strain-specific.

Strains were transformed with plasmids and cultured in synthetic media as previously described (2). Plasmids that express the indicated protein under control of the GAL1 promoter include pRS416-RNQ1, pRS416-RNQ1-YFP, pRS426-RNQ1-YFP, and pYES2-SISI1, termed pGAL1-SISI1. All Rnq1 truncations were expressed as YFP fusions under control of the GAL1 promoter by using the pRS416 plasmid. Plasmids that express RNQ1 under control of the CUP1 promoter include pRS316-RNQ1-GFP and pRS315-RNQ1-GFP. The strains used were not sensitive to 50 μM CuSO4. The glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter controlled expression of SIS1 in pRS414-SISI1 and pRS414-sisI1αg/f. The QuikChange site-directed mutagenesis kit (Stratagene) was used to create the indicated point mutations in RNQ1. The pDEST17 vector was used to express His-Rnq1 WT and L94A in BL21AI cells.

Analysis of Rnq1 Cytotoxicity. W303 strains harboring pRS416-RNQ1 or pRS416-RNQ1-YFP were grown overnight in synthetic dropout media containing 2% raffinose before 5-fold serial dilutions were spotted on plates containing either 2% galactose or glucose. Alternatively, strains that harbored pRS316-RNQ1-GFP or pRS315-RNQ1-GFP were cultured overnight in synthetic media containing glucose before serial dilutions were spotted on agarose plates that contained 500 μM CuSO4. Plates were incubated for 3–5 days at 30°C and then photographed.

Screening of a Rnq1 Peptide Array. A 25-mer Rnq1 cellulose-bound peptide array was prepared by automated spot synthesis (Jerini Peptide Technologies). The array was screened according to the manufacturer’s instructions, with 100 nM Sis1 or Hsp70 Ssa1 in the presence or absence of 1 mM EDTA or Mg-ATP. Bound chromophores were transferred to a nitrocellulose membrane, and the peptide spots were identified by Western blot. Hsp70 Ssa1 bound a number of different peptides that contained clusters of hydrophobic amino acids (data not shown), yet peptides 27–30 were the only peptides that were Sis1 bound reproducibly with high affinity. In addition, peptide 28, which contains residues 82–106 of Rnq1, was the only peptide that was bound by Hsp70 Ssa1 in an Sis1- and ATP-dependent manner (Fig. 3b).

Pulse–Chase Analysis of [RNQ'] Prion Formation. 74D-694 cultures of Rnq1, was the only peptide that was bound by Hsp70 Ssa1 in the presence or absence of 1 mM EDTA or Mg-ATP. Bound chromophores were transferred to a nitrocellulose membrane, and the peptide spots were identified by Western blot. Hsp70 Ssa1 bound a number of different peptides that contained clusters of hydrophobic amino acids (data not shown), yet peptides 27–30 were the only peptides that were Sis1 bound reproducibly with high affinity. In addition, peptide 28, which contains residues 82–106 of Rnq1, was the only peptide that was bound by Hsp70 Ssa1 in an Sis1- and ATP-dependent manner (Fig. 3b).

Analysis of [RNQ'] Prion Formation by Fluorescence Microscopy. To examine the assembly of newly synthesized Rnq1 into [RNQ'] prions, Rnq1-GFP expression was induced from the CUP1 promoter by the addition of 50 μM CuSO4 to log-phase cultures, and live cells were photographed 1 h later. To examine the aggregation pattern of Rnq1, Rnq1-GFP, or Rnq1-YFP under conditions where growth defects are observed, protein expression under control of the CUP1 or GAL1 promoter was induced by the addition of either 500 μM CuSO4 or 2% galactose, respectively, and 4 h later live cells were photographed.

Indirect immunofluorescence of Rnq1 and thioflavin-T staining of Rnq1 aggregates was performed as follows. Log-phase cells harboring pRS416-RNQ1 that were cultured in synthetic raffinose media were supplemented with 2% galactose for 4 h to induce expression of either Rnq1 or Rnq1 L94A. Cells were fixed with 4% formaldehyde for 1 h and washed twice in buffer containing 1.2 M sorbitol before being converted to spheroplasts via 45-min incubations at 30°C in the presence of 5 mg/ml zymolyase-20T. Cells were then permeabilized in PBS containing 0.1% Triton X-100 for 5 min. Cells were washed and incubated with PBS that contained 0.001% thioflavin-T for 10 min. Next, thioflavin-T-stained cells were washed four times and incubated in PBS containing 1.0% BSA and 0.025% Triton X-100. Blocked cells were then incubated with polyclonal rabbit α-Rnq1 (1:50 dilution) for 1 h and then washed four times. Cells were then decorated with goat α-rabbit-conjugated Texas red (1:1,000) second antibody (Molecular Probes). Decorated cells were spotted on glass slides and photographed with a Nikon fluorescence microscope, and images were processed with Meta-morph and Adobe Photoshop Software.

SDD-AGE. Rnq1 assembly into SDS-resistant [RNQ'] prions was monitored by SDD-AGE as described (3) with the following exceptions. Cells were lysed in buffer containing 2% SDS, 5% glycerol, 75 mM Tris-HCl (pH 6.8), 2 mM EDTA, 8% 2-mercaptoethanol, 0.05% coomassie blue, 2 mM phenylmethylsulfonyl fluoride, and a protease inhibitor mixture (Roche). Proteins resolved by the 1.5% agarose gel were electrophoretically transferred to PVDF in a submerged transfer apparatus for 1.5 h at 24 V. PVDF membranes were then decorated with α-GFP, and bands were visualized with ECL reagent.

Rnq1 Coimmunoprecipitation. Expression of Rnq1-GFP in log-phase cells harboring the indicated form of pRS316-Rnq1-GFP was induced by supplementation with 50 μM CuSO4. Cell extracts were prepared 1 h later under the nondenaturing conditions described for the pulse-chase analysis. Cell debris was removed by centrifugation, and the resulting supernatant was then immunoprecipitated with α-GFP (Roche) in the presence of 0.1% SDS. 35S-labeled proteins in immunoprecipitates were resolved by SDS/PAGE and detected by autoradiography.
centrifugation and washed three times with lysis buffer. Immunoprecipitated proteins were resolved by SDS/PAGE and detected by Western blot.

**Rnq1 Toxicity and [RNQ⁺]** *Assembly in Sis1-Depleted Cells.* A strain in which Rnq1-GFP expression was controlled by the CUP1 promoter and Sis1 expression was controlled the GAL1 promoter was constructed by transforming W303Δsis1 with pRS315-Rnq1-GFP and pGAL1-SIS1. To examine [RNQ⁺] toxicity at different Sis1 levels, strains were generated by the plasmid shuffle technique (4) and then grown on synthetic dropout plates containing 2% galactose. A single colony was picked and diluted into sterile H2O, and 5-fold serial dilutions were spotted onto plates containing either galactose or glucose in the presence of 500 μM CuSO₄. To examine the effect that depletion of Sis1 had on [RNQ⁺] assembly, transformants grown on galactose plates were transferred to liquid media containing either glucose or galactose and cultured overnight at 30°C. Cells were then diluted and grown an additional 12 h. Rnq1-GFP expression was then induced by the addition of 500 μM CuSO₄, and the formation of SDS-resistant [RNQ⁺] particles was measured by SDD-AGE. To demonstrate that Sis1-depleted cells were still capable of [RNQ⁺] prion assembly, Sis1 levels were restored by spotting cells cultured in glucose media back onto synthetic galactose plates. After the restoration of Sis levels, [RNQ⁺] assembly was monitored as described by SDD-AGE.

**Seeded Polymerization of Purified Rnq1.** His-Rnq1 WT and L94A were expressed from pDEST17 vector in BL21AI cells for 4 h at 30°C, followed by lysis in a buffer that contained 6 M guanidium-HCl and 100 mM potassium phosphate buffer (pH 7.0). His-Rnq1 was purified by standard techniques on Ni-NTA agarose that was washed with 8 M urea and 100 mM potassium phosphate buffer (pH 7.0). His-Rnq1 was eluted with the same buffer at pH 3.0, concentrated by methanol precipitation, and stored at −80°C. Before use, His-Rnq1 was resuspended in 6 M guanidium-HCl and filtered through a Microcon YM-100 spin-column. Yeast extracts containing [RNQ⁺] seeds were prepared as follows. The indicated form of Rnq1 was expressed from the GAL1 promoter for 4 h. Cells were isolated by centrifugation, and extracts were created by bead disruption in buffer composed of 40 mM Hepes (pH 7.5), 150 mM KCl, 2 mM DTT, 5% glycerol, 8 mM PMSF, 10 mg/ml aprotinin, and 10 mg/ml leupeptin. An aliquot of purified Rnq1 was adjusted to a final concentration of 5 μM in cell lysate and incubated at 25°C for 30 min without agitation. Samples were then analyzed by SDD-AGE as described above.

Factors influencing Rnq1 toxicity include the expression level and presence of a carboxy-terminal tag. (a) Comparison of the toxicity of untagged Rnq1 to Rnq1-YFP that is expressed at different levels. (b) (Upper) SDD-AGE analysis of the assembly status of Rnq1-YFP expressed from low- and high-copy expression vectors. (Lower) Western blots depicting Rnq1-YFP levels in the indicated extracts. (c) (Upper) The effect on growth of $[RNQ^+]$ yeast caused by overexpression of full-length and truncated Rnq1-YFP fusion. Rnq1 1–153 corresponds to the Rnq1 N-terminal non-prion-forming domain. Rnq1 153–405 corresponds to the Rnq1 C-terminal prion-forming domain. Spots that contain 5-fold serial dilutions of indicated yeast strains were positioned horizontally across the plate. (Lower) Western blot of cell extracts detecting the indicated YFP fusion with GFP antisera. (d) SDS resistance of Rnq1-GFP or PrD-GFP fusions expressed under control of the CUP1 promoter as analyzed by SDD-AGE.
Fig. S2. Mutation of the chaperone-binding motif slows the rate of Rnq1 assembly into \( \text{RNQ}^+ \) prions. (a) Peptide spots in a 25-residue Rnq1 peptide array that were bound by purified Sis1. Shown is a schematic of the Rnq1 peptide array in which circles represent the peptide spots starting at amino acid 1 of Rnq1 and sequentially shift in register by 3 amino acids until the terminal peptide. Filled circles represent peptide spots where Sis1 binding could be detected at significant levels above background. The darkness of the spots correlates with the intensity of Sis1-binding signal. (b) Comparison of the Sis1-binding site in Rnq1 with similar regions in the two known Rnq1 homologs from \textit{Candida glabrata} and \textit{Eremothecium gossypii}. The hydrophobic core within the peptide identified in \( b \) is conserved between species in both sequence and proximity to the carboxy-terminal, Q/N-rich, prion-forming domain. aa, amino acid. (c) Fluorescence microscopy of live \( \text{RNQ}^+ \) cells after the indicated form of Rnq1-GFP was expressed for 1 h. Rnq1-GFP is under the control of the CUP1 promoter. Expression was induced by using 50 \( \mu \text{M CuSO}_4 \). (d) Pulse–chase analysis of the assembly of nascent Rnq1 into pelletable \( \text{RNQ}^+ \) prions. \( \text{RNQ}^+ \) cells were labeled with \( ^{35}\text{S}-\text{translabel} \). At the indicated times, cells were lysed and fractionated by centrifugation with an airfuge. Rnq1-GFP present in supernatant and pellet fractions of cell extracts was then isolated by immunoprecipitation with \( \alpha\)-GFP sera.
Depletion of Sis1 hinders assembly of nascent Rnq1-GFP into SDS-resistant [RNQ⁺] aggregates. (a) The influence of Rnq1-GFP and Rnq1-GFP L94A overexpression on cell viability when Sis1 levels are maintained or depleted. A [RNQ⁺] Δsis1 strain that harbored pGAL1-SIS1 was grown on plates that contained galactose or glucose as the carbon source to maintain Sis1 at close to normal or to deplete Sis1 levels. The same approach was previously used to characterize the effect of Sis1 depletion on cell growth (5). Rnq1-GFP and Rnq1-GFP L94A were expressed from the CUP1 promoter in the presence of 500 μM CuSO₄. Rnq1-GFP expression using the CUP1 promoter produces a lower concentration of protein than using GAL1 promoter (6). Thus, the growth defects observed when Rnq1-GFP is expressed from the CUP1 are not as severe as when Rnq1-GFP is overexpressed from the GAL1 promoter (compare Fig. S1a to Fig. S3a). Sis1 was depleted from a Δsis1 strain, which was maintained by a pGAL1-SIS1 plasmid, by shifting the strain from galactose to glucose medium. Consistent with a previous article (5), the near complete depletion of Sis1 did not cause yeast to exhibit growth defects on its own, but it did exacerbate Rnq1-GFP cytotoxicity. Further, this effect was far more severe for the L94A mutant than for WT Rnq1. (b) Coalescence of Rnq1-GFP and Rnq1-GFP L94A into foci in Sis1-depleted cells. (c) (Upper) SDD-AGE analysis of Rnq1-GFP and Rnq1-GFP L94A assembly into SDS-resistant conformers at normal and low Sis1 levels. (Lower) Levels of the indicated proteins as determined by Western blot. These data demonstrate that the foci formed by Rnq1 L94A under toxic conditions are sensitive to SDS treatment and thus do not appear to represent [RNQ⁺] prions. (d) (Upper) Restoration of [RNQ⁺] prion formation in Sis1-depleted cells upon reintroduction of normal Sis1 expression. (Lower) Sis1 and PGK levels in the indicated extracts as determined by Western blot. Data in d indicate that defects in the assembly of Rnq1 and Rnq1 L94A into SDS-resistant species observed upon depletion of Sis1 are not resultant from curing cells of [RNQ⁺] prion seeds.
Fig. S4. Analysis of the mobility of Rnq1 L94A in extracts from [RNQ⁺] and [rnq⁻] strains by gel-filtration chromatography. Rnq1-YFP L94A was expressed from the GAL1 promoter for 4 h in the indicated strain. Extracts were prepared under native buffer conditions and loaded onto a Superose 12S column. The mobility of Rnq1-YFP L94A was determined by Western blot of column fractions with anti-GFP sera.