Functions of Yeast Hsp40 Chaperone Sis1p
Dispensable for Prion Propagation but Important for
Prion Curing and Protection From Prion Toxicity

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ABSTRACT Replication of amyloid-based yeast prions [PSI⁺], [URE3], and [PIN⁺] depends on the protein disaggregation machinery that includes Hsp104, Hsp70, and Hsp40 molecular chaperones. Yet, overexpressing Hsp104 cures cells of [PSI⁺] prions. An Hsp70 mutant (Ssa1-21p) antagonizes propagation of [PSI⁺] in a manner resembling elevated Hsp104. The major cytosolic Hsp40 Sis1p is the only Hsp40 required for replication of these prions, but its role in [PSI⁺] curing is unknown. Here we find that all nonessential functional regions of Sis1p are dispensable for [PSI⁺] propagation, suggesting that other Hsp40’s might provide Hsp40 functions required for [PSI⁺] replication. Conversely, several Sis1p functions were important for promoting antiprion effects of both Ssa1-21p and Hsp104, which implies a link between the antiprion effects of these chaperones and suggests that Sis1p is a specific Hsp40 important for [PSI⁺] curing. These contrasting findings suggest that the functions of Hsp104 that are important for propagation and elimination of [PSI⁺] are either distinct or specific by different Hsp40’s. This work also uncovered a growth inhibition caused by [PSI⁺] when certain functions of Sis1p were absent, suggesting that Sis1p protects cells from cytotoxicity caused by [PSI⁺] prions.

PRIONS are cellular proteins that misfold into infectious self-templating amyloid conformations. Among numerous prions identified in Saccharomyces cerevisiae the most extensively investigated and best understood are [PSI⁺], [URE3], and [PIN⁺]/[RNQ⁺], which are formed by the Sup35p, Ure2p, and Rnq1p proteins, respectively (Cox 1965; Lacroute 1971; Wickner 1994; Derkatch et al. 1997; Sondheimer and Lindquist 2000). These prions propagate as amyloid that must grow, replicate, and be transmitted between dividing cells to be maintained in growing yeast populations. Interactions among components of the cellular chaperone machinery influence these aspects of prion propagation in complex ways and an understanding of the molecular basis underlying their effects on prions is limited.

Previously we identified a mutant of Ssa1p, one of the four cytosolic Hsp70 homologs of the essential Ssa subfamily that antagonizes [PSI⁺] propagation (Jung et al. 2000). This mutant, named Ssa1-21p, has the substitution L483W that alters intrinsic and co-chaperone regulated Hsp70 activities (Needham and Masison 2008). Ssa1-21p weakens phenotypic effects caused by [PSI⁺] and the prion's mitotic stability, causing [psi–] cells to arise in a growing population. Depleting Ssa1p does not inhibit [PSI⁺] this way, indicating that Ssa1-21p actively antagonizes prion propagation, which is consistent with its dominant effects (Jung et al. 2000). The weak unstable prion phenotype of SSA1-21 cells resembles that of cells modestly overexpressing the protein-disaggregating chaperone Hsp104 (Sharma and Masison 2008), which at normal levels acts with Hsp70 and Hsp40 to promote prion replication by severing prion polymers (Paushkin et al. 1996; Glover and Lindquist 1998; Eaglestone et al. 2000; Ferreira et al. 2001). Inhibiting this severing causes yeast to lose [PSI⁺], [URE3], and [PIN⁺] prions, showing dependency of yeast prion replication on Hsp104. However, transient high-level overexpression of Hsp104 “cures” cells of [PSI⁺] only (Chernoff et al. 1995; Derkatch et al. 1997; Moriyama et al. 2000). These and other data are consistent with a distinction between processes of prion propagation and curing and a difference in mechanism of curing by Hsp104 inhibition and overexpression. We and others...
recently showed that extragenic suppressors of the Ssa1-21p inhibition of [PSI+] also suppress the ability of overexpressed Hsp104 to cure cells of [PSI+] (Moosavi et al. 2010; Reidy and Masison 2010), which implies Ssa1-21p and elevated Hsp104 antagonize [PSI+] by a similar mechanism.

Alterations in function or abundance of Hsp70 cochaperones of the J-protein Hsp40 family, which activate Hsp70 ATPase activity and engage substrate proteins for interaction with Hsp70 (Cyr et al. 1992; Szabo et al. 1994; Walsh et al. 2004), strongly influence yeast prion propagation. Of these, the most widely studied both in general and in relation to prion biology are Sis1p, which is essential for viability, and Ydj1p. Sis1p modulates toxic effects caused by overexpressing Rnq1p in [PIN+] cells and is the only Hsp40 essential for propagation of [PSI+], [PIN+], and [URE3] (Sondheimer et al. 2001; Douglas et al. 2008; Higurashi et al. 2008). Upon depletion of Sis1p, [PIN+] and [URE3] prions are lost at a similar rapid rate, but loss of [PSI−] is delayed and more gradual (Higurashi et al. 2008). Additionally, partial analysis of Sis1p to identify regions important for prion propagation showed that a common Hsp40 glycine-phenylalanine–rich region of Sis1p is essential for propagation of [PIN+], but not [PSI+] (Sondheimer et al. 2001; Lopez et al. 2003; Aron et al. 2007; Higurashi et al. 2008). Here again [PSI+] interacts differently with the chaperone machinery than the other prions, but the activities of Sis1p that form the basis of this distinction, or that make Sis1p uniquely required among Hsp40s for [PSI+] propagation have not been investigated.

Our previous work showing that deleting Ydj1p enhances antiprion effects caused by Ssa1-21p suggested to us that Sis1p might be a particularly important Hsp40 involved in these effects (Jones and Masison 2003). Here, we characterized a series of engineered Sis1p mutants to test this prediction and to determine which activities of Sis1p are important for its role in [PSI+] propagation. We found alterations of Sis1p that affect the antiprion effects of both Ssa1-21p and overexpressed Hsp104, strengthening the link in how they inhibit prion propagation. None of the Sis1p mutants affected [PSI+] propagation in wild-type (WT) cells, however, suggesting that other Hsp40s can provide the Hsp40 functions required for [PSI+] propagation, and that the Hsp104 machinery requires different activities of Sis1p to promote propagation and elimination of [PSI+]. This work also uncovered a toxicity caused by [PSI+] when Sis1p functions are compromised, suggesting Sis1p protects cells from toxic effects of harboring [PSI+] prions.

Materials and Methods

Strains, media, and culture conditions

All strains are isogenic to 779-6A (MATα, kar1-1, SUQ5, ade2-1, his3Δ202, leu2Δ1, trp1Δ63, and ura3-52) (Jung et al. 2000). SIS1 was deleted by transforming strain 779-6A that carries pYW17 (pRS316-SIS1, see below) with a sis1::KanMX disruption allele that was PCR amplified from an S. cerevisiae gene knockout library strain (American Type Culture Collection).

Rich media used were YPAD (excess adenine) and 1/2YPD (limiting adenine). These and synthetic dextrose (SD) media, which contained only required nutrients and limiting adenine (8 mg/liter) for monitoring prions, were as described (Jung et al. 2000; Sherman 2002). SD solid agar medium used for the counterselection of strains containing the URA3 gene was supplemented with 1 g/liter of 5-fluoroorotic acid. Ingredients for growth media were purchased from Difco (Sparks, MD). All other chemicals and reagents were acquired from Sigma-Aldrich (St. Louis, MO) or Becton-Dickinson (Franklin Lakes, NJ).

Our starting strains were [PSI+][PIN+]. To cure cells of prions, they were first grown on 1/2YPD plates containing 3 mM guanidine-hydrochloride, which inactivates Hsp104 and causes loss of prions as cells divide (Ferreira et al. 2001; Jung and Masison 2001; Grimminger et al. 2004). [PSI+] and [PIN+] are not always cured simultaneously. Cells from the plates containing guanidine were then streaked onto 1/2YPD plates and red [psi−] colonies were isolated. The [PIN] status of [psi−] clones was assessed by fluorescence microscopy (see below) and we isolated [psi−] [PIN+] and [psi−][pin+] variants. White colonies arising on the 1/2YPD plates after the guanidine treatment were similarly assessed to identify [PSI+][pin+] variants.

To isolate cells with SIS1 alleles on TRP1 plasmids only, they were first grown in the presence of uracil to allow loss of the URA3 plasmid carrying the wild-type SIS1 allele and then transferred to FOA plates, which kills Ura+ cells (Boeke et al. 1987). Cultures were grown at 30°C except where indicated. Liquid cultures were shaken at 200 rpm.

Plasmids

Plasmids used, listed in Table 1, have a pRS shuttle vector base (Sikorski and Hieter 1989). Plasmid pJ528, carrying the SUP35MC allele, is single-copy pRS315 (LEU2) with SUP35 codonts 124–685 driven by the SUP35 promoter. Plasmid pAK64, carrying the his1H34Q/K199A double mutant allele, was constructed using the QuickChange site-directed mutagenesis kit (cat. no. 200523; Stratagene, La Jolla, CA) and pGCH1 as template. Epitope-tagged plasmids pAK29–pAK32 were constructed by subcloning SIS1 alleles from plasmids pYW62, pYW66, pAK1, and pYW65 (see Table 1) into the commercially available pESC-TRP vector (Stratagene, La Jolla, CA) followed by transposition of the c-myc–tagged SIS1 gene under control of its native promoter into single-copy plasmid pRS314 (TRP1).

Plasmid pAK50 was made by PCR amplification of the coding region of human type II Hsp40 gene HDJ1 (DNAJB1) from the commercial vector pcDNA5/FRT/TO-DNAJB1 (Addgene, Cambridge, MA; cat. no. 032373) and subsequent insertion into pRS314 with 500 bp of 5′ and 3′ flanking sequence from SIS1. Construction of all other plasmids in this study was done using alleles from existing constructs and conventional subcloning techniques.
Protein preparation and Western analysis

Yeast were grown at 30°C in 100 ml of SD maintaining selection for appropriate plasmids to an OD_{600} of ~1.0. Cells were collected by centrifugation, suspended in 2 ml of chilled lysis buffer (phosphate-buffered saline, pH 7.4, 0.01% Triton X-100, and 1 tablet/10 ml Complete EDTA-free protease inhibitor cocktail; Roche Diagnostics, Mannheim, Germany) and held on ice for 20 min. The cells were then lysed by vortexing with 0.5 mm silica beads (BioSpec Products, Bartlesville, OK) for three 20-sec pulses at 4°C. Lysate was clarified at 15,000 rpm for 15 min. Supernatant was transferred to a new tube on ice. Pellet fractions were solubilized in an equivalent volume of lysis buffer supplemented with 4% SDS and heated at 95°C for 10 min. The resulting soluble fraction was clarified once more as described above. Protein concentrations were determined using the bicinchoninic acid protein assay as directed by the supplier (Pierce, Rockford, IL; cat. no. 23227).

For Western analysis, proteins in 5 μg of lysate were separated by electrophoresis and then transferred to a PVDF membrane. Blotted membranes were blocked, probed, washed, and developed according to the manufacturer’s instructions using the SuperSignal West Pico Chemiluminescent Substrate kit (cat. no. 34080, Thermo Scientific). Blots were imaged by exposure to radiographic film and densitometric analysis was performed in triplicate using ImageJ image-processing software (http://rsb.info.nih.gov/ij/).

Antibodies were rabbit anti-c-myc (cat. no. ab9106; Abcam, Cambridge, MA), rabbit anti-Hdj1p (cat. no. SPA-400; Stressgen), rabbit anti-Sis1p (a kind gift from E. Craig, Madison, WI), and HRP-conjugated goat antirabbit (cat. no. 166-2408; BioRad, Hercules, CA).

Monitoring [PSI+]

Our strains have the ade2-1 nonsense allele and are therefore auxotrophic for adenine. When grown on limiting adenine (i.e., 1/2YPD or SD containing 5 mg/liter adenine), ade2-1 cells are red due to accumulation of a metabolite of the induced adenine biosynthetic pathway. When [PSI+] is present, most of the Sup35p translation termination factor is depleted into the insoluble prion form, which suppresses ade2-1. This suppression, which also requires the weak SUQ5 ochre-suppressing tRNA, allows cells to grow without adenine and restores a normal white color. Weakened prion propagation increases the relative amount of soluble Sup35p, leading to intermediate accumulation of pigment (pink colony coloration), and reducing growth rate on media lacking adenine, in particular at elevated temperature (Jung et al. 2000). Weakened prion propagation can also cause loss of the prion as cells divide, which is detected as appearance of red colonies among a population of cells spread onto medium with limiting adenine. There is no discernible growth advantage or disadvantage associated with the ade2-1 detection system itself, provided that adenine is supplied as necessary.

Curing cells of [PSI+] by Hsp104 overexpression

Curing was done as described (Reidy and Masison 2010). Briefly, cells carrying pMR26L, which encodes copper-inducible

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**Table 1 Plasmids used in this study**

<table>
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<tr>
<th>Plasmid</th>
<th>Description (protein designation)</th>
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<td>pRS313</td>
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Hsp104, were grown on medium selecting for retention of SIS1 plasmids, pMR26L, and prions. Cells were then transferred to similar medium containing excess adenine and 100 μM CuSO4, to allow prion loss and to induce expression of Hsp104, respectively. Culture aliquots were removed periodically and spread onto 1/2YPD plates to determine the proportion of cells having lost [PSI+], as determined by color phenotype.

**Fluorescence microscopy for monitoring [PIN+]**

[PIN+]/[RNQ+] status was determined by observation of fluorescence of cells expressing an Rng1–GFP fusion protein encoded on plasmid pRS313RNQ–GFP. Fluorescence is diffuse in [pin+] cells, but punctate in [PIN+] cells. Fluorescent images were captured using an Olympus BX61 microscope with IPLab software and were processed using Adobe Photoshop software.

**Results**

*Functions of Sis1p dispensable for prion propagation but not for antiprion effects of Ssa1-21p*

Our [psi] ade2-1 mutants are red when grown on limiting adenine, but when [PSI+] is present the depletion of the Sup35p translation termination factor into prion aggregates suppresses ade2-1, which restores adenine prototrophy and a normal white colony color (see Materials and Methods). The Hsp70 mutant Ssa1-21p weakens both strength and stability of [PSI+] prions. SSA1-21 [PSI+] cells have less aggregated Sup35p, which reduces suppression of ade2-1 and leads to a pink colony phenotype and impaired growth on media lacking adenine (Jung et al. 2000). SSA1-21 cells also lose [PSI+] spontaneously as they divide, which gives rise to red [psi−] cells in growing populations.

Aiming to identify functions of Sis1p important for [PSI+] propagation and for Ssa1-21p inhibition of [PSI+], we initially characterized six Sis1p mutants (Figure 1) in wild-type (SSA1) and SSA1-21 strains that have a deletion of chromosomal SIS1 and carry a wild-type SIS1 gene on a single-copy URA3 plasmid to ensure viability. The strains were first transformed by single-copy TRP1 plasmids carrying the altered SIS1 alleles, and growth on media selecting for maintenance of both plasmids was used to monitor dominant effects of the mutations. To assess effects of the mutant Sis1 proteins as the only source of Sis1p, we then isolated cells carrying only the TRP1 plasmid on FOA medium (see Materials and Methods). Because Sis1p is essential for viability, cells with the empty vector depend on the wild-type SIS1 carried by the URA3 plasmid and were not viable on FOA. Cells expressing Sis1H34Q, which has a lethal mutation that disrupts ability of Hsp40 to interact functionally with Hsp70, also do not grow on FOA.

For both SSA1 and SSA1-21 strains, we recovered FOA-resistant cells expressing each of the remaining Sis1p mutants (Figure 2), indicating that all of the mutant Sis1 proteins tested except Sis1H34Q supported normal growth as the only source of Sis1p. For most of them, we saw no noticeable differences in growth rate, indicating the proteins were functionally expressed. Sis1p truncation mutants lacking the C-terminal domain (CTD), however, grew at reduced rates. Because effects on growth complicate interpretation of prion phenotypes, these mutants are discussed separately.

The [PSI+] phenotype of the wild-type (SSA1) strain was unaffected when wild-type Sis1p or any of the Sis1p mutants were coexpressed in cells carrying a plasmid with wild-type SIS1 (Figure 2A, columns 2–4). Thus, the presence of two SIS1 genes does not affect [PSI+] propagation in wild-type cells, and none of the mutant proteins had a dominant effect on [PSI+]. These results suggest that doubling SIS1 copy number does not disturb function of the chaperone machinery required for normal [PSI+] propagation.

In SSA1-21 cells, the presence of an extra copy of wild-type SIS1 enhanced the weakening of [PSI+] caused by Ssa1-21p (Figure 2B, compare wild type to empty vector, especially columns 3–5). Thus, as anticipated, some aspect of Sis1p function seems to help Ssa1-21p impair [PSI+] propagation, and doubling SIS1 copy number enhances this activity.

Coexpressing Sis1ΔGF or the substrate-binding defective Sis1K199A together with wild-type Sis1p also enhanced the prion-inhibitory effects of Ssa1-21p (Figure 2B), suggesting that intact glycine–phenylalanine (GF) and substrate binding activities are not necessary for this effect. Similarly, cells coexpressing Sis1ΔDD also had [PSI+] phenotypes much like those of cells with two copies of wild-type SIS1 (Figure 2B, columns 3 and 4), except that colonies of these cells appeared somewhat whiter, like those of cells carrying the empty vector (Figure 2B, column 5). Since intact Sis1p is dimeric and Sis1ΔDD is monomeric (Sha et al. 2000), this difference suggests dimerization-defective (i.e., monomeric) Sis1p can modestly interfere with the ability of Sis1p to assist Ssa1-21p inhibition of [PSI+]. Coexpressing Sis1H34Q interfered more effectively with prion inhibition by Ssa1-21p, restoring a white colony color and allowing cells to grow better without adenine than those expressing Sis1p from a single plasmid (Figure 2B). Thus, Sis1H34Q strongly antagonized the ability of wild-type Sis1p to promote Ssa1-21p antiprion activity.

These Sis1p alterations also dominantly affected the stability of [PSI+] in SSA1-21 cells in a manner that correlated roughly with their effects on strength of color phenotype (Figure 2B, column 5). When cells from plates selecting for both plasmids were streaked for isolated colonies, those carrying an extra copy of wild-type SIS1 had an increased frequency of red [psi−] colonies compared to those with empty vector. The Sis1K199A transformants also showed a slightly elevated frequency of prion loss and cells expressing Sis1ΔGF lost the prion more rapidly than all of the others. Conversely, Sis1H34Q reduced loss of [PSI+] from cells expressing Ssa1-21p, in line with the way it strengthened [PSI+] phenotype in SSA1-21 cells. In contrast, when
coexpressed with Sis1p in wild-type cells, neither wild-type Sis1p nor any of the Sis1p mutants affected stability of \([\text{PSI}^+]\), which agrees with their lack of effect on prion strength.

When the Sis1p mutants were expressed as the only source of Sis1p in the wild-type strain, none of them had any effect on either strength or stability of \([\text{PSI}^+]\) (Figure 2A, columns 7–10). Thus, although all of them supported growth like wild-type Sis1p, each of the functions affected by these mutations was dispensable for normal propagation of \([\text{PSI}^+]\) in otherwise wild-type cells.

As expected, SSA1-21 cells expressing wild-type Sis1p only from the single-copy TRP1 plasmid had a weak prion phenotype comparable to that of cells expressing Sis1p only from the single-copy URA3 plasmid (Figure 1). Sis1p is represented diagrammatically with domain abbreviations and amino acid residue positions that delimit the domains indicated at top. The N-terminal J domain (J) is important for physical interaction with Hsp70 and for stimulating Hsp70 ATPase activity (Wall et al. 1994; Tsai and Douglas 1996). The adjacent glycine–phenylalanine (GF)-rich region is important for specifying Sis1p function and for propagation of the \([\text{PIN}^+]\)/[RNQ+] prion (Yan and Craig 1999; Sondheimer et al. 2001), and the glycine–methionine (GM)-rich region contributes to specificity of substrate interaction (Fan et al. 2004). The C-terminal domain (CTDI and CTDII) and residue K199 are important for substrate binding (Lee et al. 2002) and the 14 C-terminal residues comprise a motif (DD) that promotes dimerization (Sha et al. 2000). Protein alterations are indicated on the left. Amino acid substitutions are shown as diamonds and domain deletions are indicated as gaps. The table on the right summarizes relative enhancing (upward arrows) or inhibiting (downward arrows) effects of the Sis1p mutations on \([\text{PSI}^+]\) propagation in SSA1-21 cells or prion curing by Hsp104, in the presence of wild-type Sis1p (2·Sis1p) or when the mutant protein is the only source of Sis1p (1·Sis1p); n/a, not applicable (mutant does not support growth).
from the \textit{URA3} plasmid (Figure 2B, 1x Sis1p panel). This weakened phenotype was due partially to a reduced number of [PSI\(^+]\) cells in the patches caused by the enhanced inhibition of [PSI\(^+]\) while the cells had two copies of \textit{SIS1}. When the \textit{Sis1\(\Delta\)GF} and \textit{Sis1K199A} mutants were expressed as the only source of Sis1p in \textit{SSA1-21} cells, both of them inhibited [PSI\(^+]\)-like wild-type Sis1p, causing pigment accumulation on media containing limiting adenine and the inability to grow without adenine at 30\(^\circ\) (Figure 2B, columns 7–10). These data suggest that the ability of Ssa1-21p to inhibit [PSI\(^+]\) does not depend on the GF or substrate binding functions of Sis1p. Cells expressing Sis1\(\Delta\)DD as the only source of Sis1p, however, had a strong and stable [PSI\(^+]\) phenotype, more like that of wild-type cells (compare \(\Delta\)DD images in Figure 2B with those in 2A). Thus, although the ability of Sis1p to dimerize is not necessary for [PSI\(^+]\) propagation, it is required for Sis1p to promote prion-inhibitory effects caused by Ssa1-21p. Together these results suggest that the requirements of Sis1p for [PSI\(^+]\) propagation are much less stringent than for [PSI\(^+]\) inhibition.

\textit{Substrate binding is important for Sis1H34Q to counter Ssa1-21p}

Since Sis1H34Q is defective in binding and regulating Hsp70, its ability to compete with wild-type Sis1p and strengthen [PSI\(^+]\) in \textit{SSA1-21} cells could be mediated through nonproductive interactions with either substrate or wild-type Sis1p, or both. To test these possibilities, we assessed the effects of combining H34Q with K199A or \(\Delta\)DD. The Sis1H34Q/K199A double mutant was as effective as wild-type Sis1p at weakening [PSI\(^+]\) in \textit{SSA1-21} cells (Figure 3), indicating that the ability of Sis1H34Q to interfere with wild-type Sis1p required normal substrate binding. Disrupting the ability of Sis1H34Q to dimerize, however, did not alter the dominant effects of the H34Q substitution. Together these results suggest the Sis1H34Q mutant interferes with antiprion effects of Ssa1-21p through nonproductive interactions with substrate.

We also combined K199A and \(\Delta\)DD with each other to determine the relationship between these mutations. The double mutant Sis1K199A/\(\Delta\)DD reduced prion inhibitory effects of Ssa1-21p like Sis1\(\Delta\)DD (Figure 3), showing that effects of Sis1\(\Delta\)DD are not dependent on substrate binding. Since Sis1\(\Delta\)DD retains near normal ability to stimulate Hsp70 ATPase (Sha \textit{et al.} 2000), these results suggest that both Sis1\(\Delta\)DD and Sis1K199A/\(\Delta\)DD interfere with Ssa1-21p by regulating Hsp70 in a nonproductive manner. Our results with the double mutants suggest that uncoupling of the Hsp70 ATPase and substrate trapping activities of Ssa1-21p can disrupt its ability to inhibit prions. Again, none of these Sis1p double mutants affected [PSI\(^+]\) phenotype in wild-type cells (Figure 3).

\textit{Sis1p CTD protects cells from toxic effects of [PSI\(^+]\]}

We found that wild-type \([psi\(^-\)]\) cells expressing Sis1\(\Delta\)CTD or Sis1\(\Delta\)GMCTD as the only source of Sis1p grew somewhat less densely than those expressing only wild-type Sis1p (Figure 4A, column 6). Thus, the Sis1p CTD region was important for normal growth on solid medium, an observation reported earlier (Sondheimer \textit{et al.} 2001).

Although neither Sis1\(\Delta\)CTD nor Sis1\(\Delta\)GMCTD affected [PSI\(^+]\) in the \textit{SSA1} strain carrying the plasmid encoding wild-type Sis1p (Figure 4A, columns 2–5), the recovery of [PSI\(^+]\) cells that lost this plasmid was considerably reduced (Figure 4A, lane 7). Notably, we recovered less than half as many cells expressing only Sis1\(\Delta\)CTD than those expressing wild-type Sis1p, while cells expressing only Sis1\(\Delta\)GMCTD appeared to be nonviable (Figure 4A, column 7), although we were able to isolate very slowly growing [PSI\(^+]\) versions of these cells after extended incubation on FOA lacking adenine. On medium lacking adenine, which allows only [PSI\(^+]\) cells to grow, the density of growth of the CTD mutants was similar (Figure 4A, columns 8 and 9). Thus, although [PSI\(^+]\] inhibited growth in these cells, it propagated stably in the absence of selection. Since [PSI\(^+]\] cells expressing only Sis1\(\Delta\)DD grew normally and had a wild-type prion phenotype with or without adenine (see Figure 2A, columns 7–10), the inability of the CTD mutants to protect cells from toxicity of [PSI\(^+]\] must be due to something more than loss of ability of Sis1p to dimerize.

Since [PSI\(^+]\] was more toxic in cells expressing Sis1\(\Delta\)GMCTD than in those expressing Sis1\(\Delta\)CTD, we tested whether the glycine–methionine (GM) region alone contributed to protection from prion toxicity. Regardless of prion status, cells expressing Sis1p lacking only its GM domain

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure3}
\caption{Growth and prion phenotypes of cells expressing Sis1 proteins with double mutations. SSA1 (wild-type) and SSA1-21 [PSI\(^+]\) strains expressing proteins indicated on right were processed as described in Figure 2. WT, wild type Sis1p; H, Sis1H34Q; K, Sis1K199A; and DD, Sis1\(\Delta\)DD.}
\end{figure}
Figure 4 [PSI+] is toxic to cells expressing Sis1p truncation mutants. (A) Patches of SSA1 and SSA1-21 cells expressing wild-type and Sis1p truncation mutant proteins were processed as described in Figure 2. (B) Growth of [psi+] (open symbols) and [PSI+] (solid symbols) variants of SSA1 (left panel) and SSA1-21 (right panel) strains expressing wild type (wt) or indicated Sis1p truncation mutants in liquid YPAD medium at 30°C was monitored as change in OD600 over time. As described earlier (Jung et al. 2000), [PSI+] is mitotically stable in SSA1-21 cells grown in liquid medium.

(residues 122–171), designated Sis1ΔGM, readily lost the plasmid with wild-type SIS1, and there was no effect on growth or [PSI+] phenotype when Sis1ΔGM was the only source of Sis1p in either the SSA1 or SSA1-21 strain (data not shown).

The [psi–] SSA1-21 strain expressing only Sis1ΔCTD or Sis1ΔGMCTD also grew slowly, and here again the prion reduced viability considerably (Figure 4A, columns 6–10). Again, despite its toxicity, [PSI+] was more stable in SSA1-21 cells that expressed these mutants than in those expressing wild-type Sis1p, as indicated by more confluent growth of cells on medium lacking adenine and increased prion stability (Figure 4A, note in column 10 the ΔCTD cells have more [PSI+] colonies than WT but most of them are very small). Together these results show that the CTD is dispensable for [PSI+] propagation, but important for protecting cells from toxicity caused by [PSI+].

To confirm that the slow growth was caused by the presence of [PSI+], we monitored the growth of wild-type cells expressing Sis1ΔCTD or Sis1ΔGMCTD after eliminating the prion. We first grew patches of cells on plates containing 3 mM guanidine hydrochloride, which inactivates Hsp104 (Glover and Lindquist 1998; Ferreira et al. 2001; Jung and Masison 2001; Grimminger et al. 2004). This treatment cures cells of [PSI+] as they divide, which produces a mixture of [PSI+] and [psi–] cells. After replica plating first onto FOA to eliminate cells expressing wild-type SIS1 and then from FOA onto plates with limiting adenine, both [PSI+] and [psi–] cells were isolated (Figure 5B, column 6). Viability of these [psi–] cells expressing only Sis1ΔCTD or Sis1ΔGMCTD was normal (Figure 5 and data not shown), which demonstrates that [PSI+] prions were responsible for the toxicity.

To confirm that the slow growth was caused by the presence of [PSI+], we monitored the growth of wild-type cells expressing Sis1ΔCTD or Sis1ΔGMCTD after eliminating the prion. We first grew patches of cells on plates containing 3 mM guanidine hydrochloride, which inactivates Hsp104 (Glover and Lindquist 1998; Ferreira et al. 2001; Jung and Masison 2001; Grimminger et al. 2004). This treatment cures cells of [PSI+] as they divide, which produces a mixture of [PSI+] and [psi–] cells. After replica plating first onto FOA to eliminate cells expressing wild-type SIS1 and then from FOA onto plates with limiting adenine, both [PSI+] and [psi–] cells were isolated (Figure 5B, column 6). Viability of these [psi–] cells expressing only Sis1ΔCTD or Sis1ΔGMCTD was normal (Figure 5 and data not shown), which demonstrates that [PSI+] prions were responsible for the toxicity.

Although guanidine cures cells of prions, the recovery of [PSI+] cells expressing Sis1ΔGMCTD was clearly increased when they were first grown on plates with guanidine (compare Figure 5B columns 7 and 8 to 5A columns 7 and 8). Since inactivation of Hsp104 by guanidine arrests prion replication, which causes the number of prions per cell to gradually diminish as cells divide (Eaglestone et al. 2000), this result could be explained if a reduction in the number of prions per cell was associated with reduced toxicity. A
prediction of this hypothesis is that upon outgrowth of the 
[PSI+] cells the eventual restoration of the number of prions per cell will again cause [PSI+] to become more toxic. Indeed, when cells from the Ade plates were streaked onto YPD plates, the [PSI+] cells formed very slow-growing colonies, indicating that the toxicity reappeared as prion propagation was restored to normal. These results support the conclusion that the toxicity is due to [PSI+] and suggest the degree of toxicity is related to the number of prions per cell. They are also consistent with the conclusion that Sis1[ΔCtD] supports stable [PSI+] propagation, despite the fact that [PSI+] is inhibitory to cell growth.

[PSI+]–dependent toxicity is not due to depletion of Sup35p, Sup45p, or Sis1p

Depletion of the essential translation termination factors Sup35p or Sup45p in [PSI+] cells is toxic under some conditions (Dagkesamanskaya and Ter-Avanesyan 1991; Stansfield et al. 1995; Derkatch et al. 1996; Gokhale et al. 2005; Vishveshwara et al. 2009). The Sis1p CTD mutants could cause [PSI+] to become toxic if they promoted increased rates of prion formation, thereby depleting too much Sup35p or its interacting partner Sup45p. To test this hypothesis, we first transformed cells expressing the truncated Sis1 proteins with a plasmid encoding Sup35MC, which contains the Sup35p middle and essential C-terminal regions, but lacks the N-terminal prion-forming domain. This protein is not depleted into prion aggregates and therefore restores a [psi–] phenotype (i.e., red and Ade–) even in cells propagating [PSI+]. Expression of Sup35MC did not restore viability of [PSI+] cells expressing either Sis1ΔCtD or Sis1ΔGMCTD (Figure 6A), indicating that depletion of Sup35p was not the cause of the toxicity. To test whether depletion of Sup45p was responsible for the toxicity, we expressed additional Sup45p from a plasmid under control of its native promotor. This approach also failed to improve the growth of either truncation mutant (data not shown). Thus, [PSI+] toxicity was not due to depletion of either Sup35p or Sup45p.

We next tested whether the toxicity was due to a difference in Sis1p abundance. Because the truncated proteins lack a substantial amount of potential Sis1p epitope, we used c-myc–tagged versions of the wild-type and mutant proteins to standardize the signal for Western analysis. The growth and prion toxicity phenotypes of cells expressing only the tagged proteins recapitulated that of cells expressing their untagged counterparts (Figure 6B), indicating the tags did not affect protein function. Whole cell lysates extracted from wild-type and SSA1–21 cells expressing the tagged proteins were separated into soluble and insoluble fractions by centrifugation and immunoblotted using a c-myc antibody probe. The mutant proteins were stably expressed and the relative amount of truncated Sis1p in the soluble fractions was at least as high as that in the wild-type cells (Figure 6C and D). Wild-type cells, however, had more Sis1p in the insoluble fraction. This difference was not due to [PIN+] or [PSI+] but to a [PIN+] cell that can titrate much Sis1p into the insoluble fraction, since all three strains lacked this prion. Importantly, for both CTD mutants the amount of Sis1p in both fractions was similar whether or not [PSI+] was present. Therefore, the toxicity cannot be explained simply by depletion of Sis1p in [PSI+] cells.
**[PSI] is lethal to cells expressing Hdj1p in place of Sis1p**

We tested whether the functions of Hsp40 important for cell growth in the presence of [PSI] were conserved by replacing Sis1p with the homologous human type II Hsp40, Hdj1p (DnaJB1). Hdj1p supports yeast cell growth and propagation of [PIN] prions (Lopez et al. 2003). Cells expressing both Sis1p and Hdj1p grew normally and Hdj1p had no dominant effect on [PSI] phenotype in either strain (Figure 7A). Thus, Hdj1p does not inhibit Sis1p function in supporting cell growth or [PSI] propagation or in promoting prion inhibition by Ssa1-21p.

We isolated FOA-resistant cells readily from [psi] variants of both SSA1 and SSA1-21 strains, confirming that Hdj1p provides the Hsp40 function of Sis1p that is essential for yeast growth. However, we did not recover FOA-resistant [PSI+] cells (Figure 7A, column 6). [PSI+] cells expressing Ssa1-21p lose the prion at a noticeable frequency as cells divide, so the corresponding patch of [PSI+] SSA1-21 cells expressing both Hdj1p and Sis1p contained a mixture of [PSI+] and [psi] cells. All of the FOA-resistant cells from this patch (Figure 7A, column 6) were [psi], indicating that Hdj1p would not support growth in place of Sis1p unless [PSI+] was lost. These data show that the toxicity was again due to [PSI+] prions, and that Hdj1p lacks a Sis1p function that is critical for yeast cell growth when [PSI+] is present.

Since cells expressing Hdj1p in place of Sis1p can support [PIN+] propagation (Lopez et al. 2003), and Sis1p can moderate a toxicity related to [PIN+] (Douglas et al. 2008), we tested whether our strains expressing Hdj1p supported [PIN+] and whether [PIN+] contributed to the toxicity we ascribed to [PSI+]. All of our [PSI+] starting strains expressing an Rnq1-GFP fusion protein displayed a punctate fluorescence, indicative of the presence of [PIN+] (Figure 7B). From these [PSI+][PIN+] strains we isolated [psi][PIN+], [PSI+] [pin+], and [psi][pin+] variants after growing cells on medium containing guanidine (see Materials and Methods).

Growth of the four variants containing the different combinations of [PSI+] and [PIN+] is shown in Figure 7C. All of the [psi][PIN+] strains remained [PIN+] after they lost the plasmid encoding wild-type Sis1p, indicating that the Sis1p mutants and Hdj1 were capable of supporting propagation of [PIN+] prions, which is consistent with previously published data using strains with other backgrounds (Sondheimer et al. 2001; Lopez et al. 2003). Compared with [psi][pin+] cells, there was a slight, but noticeable reduction in growth of [psi][PIN+] cells expressing Sis1ΔGMCTD in place of Sis1p. However, viable FOA-resistant [PIN+] cells were recovered readily from all the [psi][PIN+] strains. In contrast, regardless of [PIN+] status, [PSI+] was toxic to cells expressing Sis1ΔCTD, Sis1ΔGMCTD, or Hdj1p, confirming that [PSI+] was the major contributor to the toxicity in these cells.

### Sis1p functions are important for Hsp104 curing of [PSI]

Although co-chaperones of Hsp70 are important for antiprion effects of both Ssa1-21p and overexpressed Hsp104, a specific requirement for Hsp40 in curing of prions by Hsp104 overexpression has not been established. Since the
antiprion effects of Ssa1-21p and overexpressed Hsp104 are connected, our data implied Sis1p was such an Hsp40. We therefore tested whether specific Sis1p functions were important for prion curing by overexpressed Hsp104 (Figure 8).

Although Sis1p was shown to enhance modestly the curing of [PSI+] by overexpressed Hsp104 (Kryndushkin et al. 2011), our cells expressing one or two copies of wild-type SIS1 cured at a similar rate of ~18% per cell division. Curing of cells expressing Sis1ΔCTD or Sis1ΔGMCTD together with wild-type Sis1p, however, was reduced to ~60% of wild type. Although [PSI+] cells expressing Sis1ΔGMCTD as the only source of Sis1p grew too poorly to be useful in this experiment, cells expressing only Sis1ΔCTD did not cure at all. Cells expressing both Sis1ΔDD and wild-type Sis1p cured normally, but those expressing only Sis1ΔDD were almost completely resistant to curing by overexpressed Hsp104. Together these results suggest that the extreme C terminus of Sis1p has an important role in promoting the Hsp104-mediated curing of [PSI+], and that the curing defect caused by Sis1ΔCTD could be due mostly to deletion of the dimerization region.

Like Sis1ΔDD, Sis1K199A did not have a dominant effect on curing, but when it was expressed as the only source of Sis1p, curing was reduced to ~40% of wild type. This result suggests substrate binding by Sis1p has a role in the curing. In line with its effects on [PSI+] propagation in SSS1-21 cells, the Sis1K199A/ΔDD double mutant also did not affect curing in trans, but when it was the only source of Sis1p Hsp104 was unable to cure cells of [PSI+]. These data point to a crucial role for dimerization of Sis1p in the curing of [PSI+] by overexpressed Hsp104.
Coexpressing \textit{Sis1H34Q} considerably reduced curing by \textit{Hsp104} (Figure 8), and although cells expressing the \textit{Sis1H34Q/K199A} or \textit{Sis1H34Q/ΔDD} double mutants cured approximately four- to fivefold better than those expressing \textit{Sis1H34Q}, they did not cure as well as those expressing wild-type \textit{Sis1}. These data suggest that the inhibition of curing by H34Q depends only partially on \textit{Sis1} substrate binding and dimerization activities, which is consistent with our other data and indicate that \textit{Sis1H34Q} and \textit{Sis1ΔDD} can alter the curing machinery differently.

The only \textit{Sis1} mutant that affected the antiprion effects of \textit{Ssa1-21p} and \textit{Hsp104} differently was \textit{Sis1ΔGF}. Although it promoted impairment of [\textit{PSI}] by \textit{Ssa1-21p}, it was unable to support curing of [\textit{PSI}] by increased expression of \textit{Hsp104}. These data suggest either a divergence in how \textit{Ssa1-21p} and overexpressed \textit{Hsp104} function to inhibit [\textit{PSI}] or that \textit{Ssa1-21p} is capable of circumventing the function of the \textit{Sis1} GF domain in the process of restricting prion propagation.

\section*{Discussion}

Although \textit{Sis1} is proposed to be essential for propagation of [\textit{PSI}] prions, we show that all of the known nonessential \textit{Hsp40} activities of \textit{Sis1} are at least individually dispensable for the requirement of \textit{Sis1} for [\textit{PSI}] propagation. Moreover, \textit{Sis1ΔGMCTD}, which contains only the \textit{J} and \textit{GF} regions, supports stable [\textit{PSI}] propagation. Since \textit{Sis1} lacking its \textit{GF} region supports propagation of [\textit{PSI}] normally, and the only known functions of the \textit{J} domain are to bind Hsp70 and regulate its activity, our findings suggest the most important function of \textit{Sis1} for [\textit{PSI}] propagation is its ability to regulate Hsp70. This function seems to be important and sufficient for prion propagation in general since \textit{Sis1ΔGMCTD} also supports propagation of [\textit{PIN}] (Sondheimer et al. 2001), and specific binding of \textit{Sis1} to \textit{Rnq1} has been suggested to be dispensable for replication of [\textit{PIN}] prions (Aron et al. 2007).

\textit{Sis1} is believed to act in prion propagation as a specific \textit{Hsp40} component of the \textit{Hsp104} disaggregation machinery that severs prion polymers, thereby generating new prions from existing ones (Aron et al. 2007; Tipton et al. 2008). While our data are consistent with a role of \textit{Sis1} in prion replication, they are not consistent with the proposed role of \textit{Sis1} in delivering substrates to the disaggregation machinery (Tipton et al. 2008) being the essential activity in this process. Our finding that the \textit{J} and \textit{GF} regions of \textit{Sis1} are enough to provide \textit{Hsp40} function in prion replication suggests that functions of \textit{Hsp40} other than direct regulation of Hsp70, such as simultaneous binding of Hsp70 and substrate to coordinate Hsp70 ATPase activity with substrate binding, are not necessary for the disaggregation machinery to promote replication of [\textit{PSI}] prions. Alternatively, \textit{Hsp40}s other than \textit{Sis1} must provide such activities. This latter explanation is consistent with a previous report showing that depletion of \textit{Sis1} causes only partial loss of fragmentation of \textit{Sup35p} prion polymers (Higurashi et al. 2008), which led the authors to suggest that other \textit{Hsp40}s might contribute to the fragmentation process required for [\textit{PSI}] propagation.

Despite the similarities in the requirements [\textit{PSI}] and [\textit{PIN}] have for \textit{Hsp40} functions, GF function of \textit{Sis1} is essential for propagation of [\textit{PIN}] (Sondheimer et al. 2001), and depleting \textit{Sis1} by transcriptional repression causes [\textit{PIN}] and [\textit{URE3}] to be lost much more rapidly than [\textit{PSI}] (Higurashi et al. 2008). Our findings are in line with these observations, which indicate that the function of \textit{Sis1} in prion propagation is different or much less important for [\textit{PSI}] than for [\textit{PIN}] or [\textit{URE3}]. Although cells lacking the \textit{SIS1} gene divide about nine times, cells undergoing repression of \textit{SIS1} transcription can grow for >100 generations, implying that a fraction of the normal amount of \textit{Sis1} is still present to allow continued cell division (Luke et al. 1991; Higurashi et al. 2008). Although such cells are “viable,” depleting \textit{Sis1} this way certainly has pleiotropic effects on growth, raising the possibility that the reduced stability of [\textit{PSI}] when \textit{Sis1} is depleted is indirect. For example, depletion of \textit{Sis1} could impair cellular systems or alter expression patterns of stress response factors that produce conditions that interfere with [\textit{PSI}] propagation enough to cause eventual loss of [\textit{PSI}] (Newnam et al. 2011). Such effects would be expected to be specific to depletion of the essential \textit{Sis1}, since [\textit{PSI}] is stable in cells lacking any of the other \textit{Hsp40}s, including Ydj1p whose depletion results in slow and temperature-sensitive growth (Caplan and Douglas 1991; Higurashi et al. 2008).

Although [\textit{PSI}] propagated normally in wild-type cells expressing any of the mutant \textit{Sis1} proteins, specific functions of \textit{Sis1} were critical for both the antiprion effects of \textit{Ssa1-21p} and the ability of overexpressed \textit{Hsp104} to cure wild-type cells of [\textit{PSI}]. These findings are consistent with our previous work, suggesting that \textit{Ssa1-21p} antagonizes prions by a mechanism similar to that of overexpressing \textit{Hsp104} (Hung and Masison 2006; Reidy and Masison 2011). Such effects would be expected to be specific to depletion of the essential \textit{Sis1}, since [\textit{PSI}] is stable in cells lacking any of the other \textit{Hsp40}s, including Ydj1p whose depletion results in slow and temperature-sensitive growth (Caplan and Douglas 1991; Higurashi et al. 2008).

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between the ways the Hsp104/70/40 machinery acts in fragmentation of prion polymers and elimination of [PSI+] prions. Although Sis1p does not functionally interact with Ssb Hsp70 in vitro (Lu and Cyr 1998), Ssb promotes curing of [PSI+] by overexpressed Hsp104 (Chernoff et al. 1999) and it might play a role in this distinction.

These data also imply that prion propagation requires only a specific regulation of Hsp70 by Hsp40, while curing of [PSI+] requires additional specific Sis1p functions. Since a single substitution (H34Q) in the J domain that disrupts the ability of Sis1p to regulate Hsp70 ATPase interfered strongly with prion curing by elevated Hsp104, regulation of Hsp70 is also important for Hsp40 function in the curing. The partial restoration of curing when Sis1K199A or Sis1ΔDD was combined with H34Q, however, points to a partial requirement of substrate binding and dimerization for the dominant effect of H34Q. Moreover, curing was nearly eliminated when Sis1ΔDD was the only source of Sis1p. Since J-domain function is enough for Sis1p to regulate Hsp70 ATPase, these results provide further evidence that coordination of Hsp70 ATPase and substrate binding activities contribute to the curing mechanism.

The severe inhibition of growth caused by [PSI+] in cells expressing the Sis1p CTD truncation mutants suggests that some aspect of Sis1p function conferred by the C-terminal region protects cells from toxicity caused by [PSI+] prions. However, [PSI+] propagated stably in these cells, indicating that activities of Sis1p required for tolerance of cells to [PSI+] prions are different than those required for [PSI+] replication. Thus, the way Sis1p protects cells from toxic effects of [PSI+] seems to be indirect and distinct from its role as a component of the Hsp104 disaggregation machinery. Possibilities for such a function include managing secondary effects of Sup35p fibril formation, such as preventing sequestration of essential cellular components by prion aggregates or easing a burden on one or more protein “quality control” processes. The lack of suppression of toxicity by Sup35MC argues against such a burden being caused by possible production of proteins with C-terminal extensions due to the read through of stop codons in [PSI+] cells. Recent data suggest that the globular C termini of the Sup35p monomers within amyloid fibers is accessible to interact with ribosomes, which might explain why the considerable depletion of soluble Sup35p in [PSI+] cells does not reduce growth (Baxa et al. 2011). It is possible that a function of the Sis1p CTD facilitates such an interaction. Regardless of the actual mechanism, this CTD function is specific to Sis1p, since [PSI+] propagates normally and does not cause toxicity in ydj1Δ cells (Jones and Masison 2003; Higurashi et al. 2008).

It was shown previously that Hdj1p can replace Sis1p to support both growth of yeast and propagation of [PIN+] (Lopez et al. 2003). Here, however, we find that it cannot replace Sis1p to support cell growth when [PSI+] is present. Thus the toxicity we observe is specific to [PSI+] prions and Hdj1p lacks an activity provided by Sis1p that confers tolerance to these toxic effects. These data also suggest that this function is distinct from Sis1p activity required to support growth. This lack of overlap of Sis1p activities required for cell growth and tolerance to [PSI+] is consistent with our other data that suggest toxicity caused by [PSI+] is not simply due to depletion of essential Sis1p function. On the whole the toxicity data are consistent with earlier evidence suggesting [PSI+] prions are a liability to cells rather than a benefit (Jung et al. 2000; Nakayashiki et al. 2005; McGlinchey et al. 2011).

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