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 specified 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 co-chaperones 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* moderates 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 (*MAT α* , *kar1-1*, *SUQ5*, *ade2-1*, *his3 Δ 202*, *leu2 Δ 1*, *trp1 Δ 63*, 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° 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* codons 124–685 driven by the *SUP35* promoter. Plasmid pAK64, carrying the *sis1H34Q/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.

Table 1 Plasmids used in this study

Plasmid	Description (protein designation)	Origin
pRS313	<i>CEN HIS3 Amp^R</i>	Sikorski and Hieter (1989)
pRS314	<i>CEN TRP1 Amp^R</i>	Sikorski and Hieter (1989)
pRS315	<i>CEN LEU2 Amp^R</i>	Sikorski and Hieter (1989)
pRS316	<i>CEN URA3 Amp^R</i>	Sikorski and Hieter (1989)
pRS424	<i>2μ TRP1 Amp^R</i>	Christianson <i>et al.</i> (1992)
pYW17	pRS316SIS1 (WT)	Yan and Craig (1999)
pYW62	pRS314sis1 Δ 122–352 (Δ GMCTD)	Yan and Craig (1999)
pYW65	pRS314SIS1 (WT)	Yan and Craig (1999)
pYW66	pRS314sis1 Δ 173–352 (Δ CTD)	Yan and Craig (1999)
pYW116	pRS314sis1 Δ 72–121 (Δ GF)	Yan and Craig (1999)
pYW118	pRS314sis1H34Q (H34Q)	Yan and Craig (1999)
pGCH1	pRS314sis1K199A (K199A)	This study
pAK1	pRS314sis1 Δ 339–352 (Δ DD)	This study
pJ528	pRS315SUP35MC	This study
pMR26L	pRS315P _{CUP1} ::Hsp104	Reidy and Masison (2010)
pAK17	pRS314sis1H34Q/ Δ 339–352 (H34Q/ Δ DD)	This study
pAK18	pRS314sis1K199A/ Δ 339–352 (K199A/ Δ DD)	This study
pAK21	pRS314sis1 Δ 122–172 (Δ GM)	This study
pAK29	pRS314sis1 Δ 122–352c-myc (Δ GMCTDc-myc)	This study
pAK30	pRS314sis1 Δ 173–352c-myc (Δ CTDc-myc)	This study
pAK31	pRS314sis1 Δ 339–352c-myc (Δ DDc-myc)	This study
pAK32	pRS314SIS1-c-myc (c-myc)	This study
pAK33	pRS315SUP45 (Sup45)	This study
pAK50	pRS314HDJ1 (Hdj1)	This study
pAK64	pRS314sis1H34Q/K199A (H34Q/K199A)	This study
pRS313RNQ–GFP	pRS313 (Rnq1–GFP)	This study

Protein preparation and Western analysis

Yeast were grown at 30° in 100 ml of SD maintaining selection for appropriate plasmids to an OD₆₀₀ 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°. Lysate was clarified by centrifugation at 15,000 rpm at 4° 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° 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 (Thermo Scientific, Rockford, IL; cat. no. 23227).

For Western analysis, proteins in 5 μ g 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 8 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

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 μ M 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 Rnq1–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 affects 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

Figure 1 Sis1p domain structure mutants. 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 [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 [PSI⁺] propagation in *SSA1-21* cells or prion curing by Hsp104, in the presence of wild-type Sis1p (2x Sis1p) or when the mutant protein is the only source of Sis1p (1x Sis1p); n/a, not applicable (mutant does not support growth).

coexpressed with Sis1p in wild-type cells, neither wild-type Sis1p nor any of the Sis1p mutants affected stability of [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 [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 [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

Figure 2 Prion and growth phenotypes of strains expressing wild-type (WT) and mutant Sis1 proteins. Wild-type (*SSA1*) strains are shown in A and *SSA1-21* strains are in B. Wild-type cells lacking prions are red (column 1) while those propagating [PSI⁺] are white (column 2). Weakened prion strength in *SSA1-21* cells is seen as partial accumulation of pigment (pink color) in [PSI⁺] cells (column 2). Reduced mitotic transmission of prions in *SSA1-21* cells results in appearance of red colonies (columns 5 and 10). Cells under the 2x Sis1p heading carry wild-type *SIS1* on a *URA3* plasmid and wild-type or mutant *SIS1*, as indicated on the left, on a *TRP1* plasmid. Patches of cells grown on plates lacking uracil and tryptophan were replica plated onto similar plates containing limiting adenine (column 2) or no adenine (columns 3 and 4) and incubated at 25° or 30° for 2 days. Solubility of Sup35p in [PSI⁺] cells increases as temperature increases, so [PSI⁺] phenotype is somewhat weaker at 30° (Jung *et al.* 2000). The plates with limiting adenine (columns 1 and 2) were subsequently replica plated onto medium containing uracil to allow loss of the *URA3* plasmid (not shown) and then onto similar plates containing FOA, which kills cells retaining the *URA3* plasmid. The FOA plates (not shown) were then replica plated onto plates with limiting (columns 6 and 7) or no adenine (columns 8 and 9). For clarity,

patches of cells grown on the same plates were rearranged as separate images to produce each column, and images have been omitted wherever growth is absent for an entire column of transformants (e.g., [psi⁻] cells on –Ade plates are not shown). Images of colonies (columns 5 and 10) are of cells taken from [PSI⁺] patches on limiting adenine plates (columns 2 and 7) and streaked onto similar medium. The frequency of red colonies is proportional to the degree of inhibition of prion transmission.

from the *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 *SIS1*. When the *Sis1*ΔGF and *Sis1*K199A mutants were expressed as the only source of *Sis1p* in *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° (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*ΔDD as the only source of *Sis1p*, however, had a strong and stable [*PSI*⁺] phenotype, more like that of wild-type cells (compare ΔDD 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.

Substrate binding is important for *Sis1*H34Q to counter *Ssa1-21p*

Since *Sis1*H34Q is defective in binding and regulating Hsp70, its ability to compete with wild-type *Sis1p* and strengthen [*PSI*⁺] in *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 ΔDD. The *Sis1*H34Q/K199A double mutant was as effective as wild-type *Sis1p* at weakening [*PSI*⁺] in *SSA1-21* cells (Figure 3), indicating that the ability of *Sis1*H34Q to interfere with wild-type *Sis1p* required normal substrate binding. Disrupting the ability of *Sis1*H34Q to dimerize, however, did not alter the dominant effects of the H34Q substitution. Together these results suggest the *Sis1*H34Q mutant interferes with antiprion effects of *Ssa1-21p* through nonproductive interactions with substrate.

We also combined K199A and ΔDD with each other to determine the relationship between these mutations. The double mutant *Sis1*K199A/ΔDD reduced prion inhibitory effects of *Ssa1-21p* like *Sis1*ΔDD (Figure 3), showing that effects of *Sis1*ΔDD are not dependent on substrate binding. Since *Sis1*ΔDD retains near normal ability to stimulate Hsp70 ATPase (Sha *et al.* 2000), these results suggest that both *Sis1*ΔDD and *Sis1*K199A/ΔDD 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).

Sis1p CTD protects cells from toxic effects of [*PSI*⁺]

We found that wild-type [*psi*⁻] cells expressing *Sis1*ΔCTD or *Sis1*ΔGMCTD as the only source of *Sis1p* grew somewhat

Figure 3 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, *Sis1*H34Q; K, *Sis1*K199A; and DD, *Sis1*ΔDD.

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 *et al.* 2001).

Although neither *Sis1*ΔCTD nor *Sis1*ΔGMCTD affected [*PSI*⁺] in the *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*ΔCTD than those expressing wild-type *Sis1p*, while cells expressing only *Sis1*ΔGMCTD 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*ΔDD 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*ΔGMCTD than in those expressing *Sis1*ΔCTD, 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

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° 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 Δ CTD 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 $[PSI^+]$.

Since comparison of growth differences on solid media is qualitative, we assessed the growth-inhibitory effects of $[PSI^+]$ quantitatively by measuring rates of growth in rich liquid medium at 30°. For the wild-type strain, both *Sis1ΔCTD* and *Sis1ΔGMCTD* supported growth of $[psi^-]$ cells like the wild-type Sis1p, indicating that optimal growth conditions eliminated any disadvantage to cells expressing these proteins as the only source of Sis1p that was exposed by growth on solid medium. The presence of $[PSI^+]$, however, considerably reduced the growth rate of cells expressing *Sis1ΔCTD*, and cells expressing *Sis1ΔGMCTD* grew even more slowly (Figure 4B). Growth rates of the *SSA1-21* strains mirrored those of the *SSA1* strain, with the $[PSI^+]$

cells displaying much slower growth than their $[psi^-]$ counterparts. These results demonstrate that $[PSI^+]$ prions can be very toxic and suggest that some function of the *Sis1p* CTD normally protects cells from this toxicity.

Toxicity in *Sis1p* truncation mutants is caused by $[PSI^+]$

To affirm 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

Figure 5 *[PSI⁺]* is responsible for the toxicity in cells expressing *Sis1p* mutants. All cells shown are of the wild-type (*SSA1*) strain. (A) *[PSI⁺]* and *[psi⁻]* cells expressing wild-type *Sis1p* from a *URA3* plasmid and *Sis1* proteins (indicated on left) from a *TRP1* plasmid were grown on medium selecting for both plasmids (not shown). These plates were then replica plated onto limiting adenine plates with uracil (columns 1 and 2), which allows loss of the plasmid encoding wild-type *SIS1*. These plates were then replica plated onto medium containing FOA (columns 3 and 4) and then the FOA plates were replica plated onto plates with limiting or no adenine as indicated (columns 5 through 8). (B) The same transformants were processed similarly except the initial limiting adenine plates (columns 1 and 2) contained 3 mM guanidine-hydrochloride to inhibit Hsp104-mediated prion replication.

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 *Sis1ΔGMCTD* 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 (Dagesamanskaya 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 promoter. 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 6, C and D). Wild-type cells, however, had more *Sis1p* in the insoluble fraction. This difference was not due to *[PIN⁺]*, which 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.

Figure 6 Toxicity of $[PSI^+]$ is not due to depletion of Sup35p or truncated Sis1 proteins. (A) Wild-type (SSA1) and SSA1-21 strains with and without $[PSI^+]$ (as indicated) and carrying a plasmid expressing Sup35MC were grown as patches of cells on limiting adenine plates with or without FOA. The reduced growth of $[PSI^+]$ cells expressing Sis1ΔGMCTD or Sis1ΔCTD as the only source of Sis1p (column 4) indicates that toxic effects of $[PSI^+]$ are not prevented by supplementing soluble Sup35p function in this way. (B) Wild-type (SSA1) $[PSI^+]$ and $[psi^-]$ cells carrying plasmids expressing wild-type Sis1p and c-myc-tagged truncation mutants were grown as patches on limiting adenine (columns 1 and 2) and then replica plated onto FOA and subsequently onto limiting adenine (as indicated by arrows). The c-myc-tagged proteins supported growth of $[psi^-]$ cells like their untagged counterparts (column 5, compare with Figure 4, column 6), and growth of cells expressing the tagged proteins was inhibited by $[PSI^+]$ (column 6). (C) Lysates of $[PSI^+]$ and $[psi^-]$ cells expressing indicated c-myc-tagged Sis1 proteins were separated by centrifugation into soluble (sol.) and insoluble (insol.) fractions and

then subjected to Western analysis probing with anti-c-myc antibody. Upper panels are discontinuous to align the different sized proteins for ease of comparison. Lower panels show a representative portion of amido-black stained membranes of the same blots as a loading and transfer control. (D) Signal intensity of bands on the blot in C was quantified and values are shown relative to the intensity of the insoluble band from wild-type $[psi^-]$ cells, set at 1.0.

$[PSI^+]$ 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 anti-prion 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

Figure 7 Hdj1p cannot protect cells from *[PSI⁺]* toxicity and *[PIN⁺]* has minimal effects on growth. (A) Patches of *[PSI⁺]* and *[psi⁻]* cells of *SSA1* and *SSA1-21* strains carrying *SIS1* on a *URA3* plasmid and *HDJ1* on a *TRP1* plasmid were grown on medium selecting for both plasmids and containing limiting adenine (columns 1 and 2). These plates were transferred to similar medium containing uracil (not shown), then onto plates containing FOA (columns 3 and 4), and then onto medium lacking tryptophan and containing uracil and limiting adenine (columns 5 and 6). Hdj1p was unable to support growth in place of Sis1p in *SSA1* cells when *[PSI⁺]* is present so no FOA-resistant *[PSI⁺]* clones were isolated. Unstable prion propagation in the *SSA1-21* strain gives rise to *[psi⁻]* variants as cells divide, and all FOA-resistant clones isolated from this strain were *[psi⁻]*. (B) Fluorescence microscopy images of *[pin⁻]* (left) and *[PIN⁺]* (center) cells carrying pRS313RNQ-GFP are of *[psi⁻][pin⁻]* and *[psi⁻][PIN⁺]* FOA-resistant strains expressing Hdj1p (C). Proteins on the blot on the right are from *[psi⁻]* *[pin⁻]* cells expressing wild-type Sis1p or Hdj1p (as indicated) isolated from patches on the same FOA plates. The blot was probed with anti-Hdj1p antibody. (C) Hdj1p supports growth of cells propagating *[PIN⁺]*. Variants of *SSA1* cells propagating different combinations of *[PSI⁺]* and *[PIN⁺]* prions and carrying plasmids expressing Hdj1 or versions of Sis1p indicated on the left were treated as in A and compared side by side. *[PIN⁺]* does not prevent recovery of cells expressing only Hdj1p, confirming that Hdj1p supports *[PIN⁺]* propagation. All eleven clones from the *[PSI⁺][PIN⁺]* patch expressing Hdj1p that arose on the FOA plate were *[psi⁻]*.

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

Figure 8 *Sis1p* regions important for *Hsp104* curing of *[PSI⁺]*. Cells expressing *Sis1p* proteins indicated on left and carrying copper-inducible *Hsp104* were grown overnight in liquid medium selecting for maintenance of all plasmids and lacking adenine to select for cells with the prion. Cells were diluted into similar medium containing excess adenine and 100 μ M CuSO_4 and all cultures were grown for 4–5 generations. Loss of *[PSI⁺]* was monitored by spreading treated cultures on 1/2YPD and measuring proportion of red colonies. Rates of loss are presented as percentage of loss per generation.

SIS1 cured at a similar rate of ~18% per cell division. Curing of cells expressing *Sis1 Δ CTD* or *Sis1 Δ GMCTD* together with wild-type *Sis1p*, however, was reduced to ~60% of wild type. Although *[PSI⁺]* cells expressing *Sis1 Δ GMCTD* as the only source of *Sis1p* grew too poorly to be useful in this experiment, cells expressing only *Sis1 Δ CTD* did not cure at all. Cells expressing both *Sis1 Δ DD* and wild-type *Sis1p* cured normally, but those expressing only *Sis1 Δ DD* 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 *Sis1 Δ CTD* could be due mostly to deletion of the dimerization region.

Like *Sis1 Δ DD*, *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 *SSA1-21* cells, the *Sis1K199A/ Δ DD* 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 *Sis1*H34Q considerably reduced curing by *Hsp104* (Figure 8), and although cells expressing the *Sis1*H34Q/K199A or *Sis1*H34Q/ Δ DD double mutants cured approximately four- to fivefold better than those expressing *Sis1*H34Q, they did not cure as well as those expressing wild-type *Sis1p*. These data suggest that the inhibition of curing by H34Q depends only partially on *Sis1p* substrate binding and dimerization activities, which is consistent with our other data and indicate that *Sis1*H34Q and *Sis1* Δ DD can alter the curing machinery differently.

The only *Sis1p* mutant that affected the antiprion effects of *Ssa1*-21p and *Hsp104* differently was *Sis1* Δ GF. Although it promoted impairment of $[PSI^+]$ by *Ssa1*-21p, it was unable to support curing of $[PSI^+]$ by increased expression of *Hsp104*. These data suggest either a divergence in how *Ssa1*-21p and overexpressed *Hsp104* function to inhibit $[PSI^+]$ or that *Ssa1*-21p is capable of circumventing the function of the *Sis1p* GF domain in the process of restricting prion propagation.

Discussion

Although *Sis1p* is proposed to be essential for propagation of $[PSI^+]$ prions, we show that all of the known nonessential Hsp40 activities of *Sis1p* are at least individually dispensable for the requirement of *Sis1p* for $[PSI^+]$ propagation. Moreover, *Sis1* Δ GMCTD, which contains only the J and GF regions, supports stable $[PSI^+]$ propagation. Since *Sis1p* lacking its GF region supports propagation of $[PSI^+]$ normally, and the only known functions of the J domain are to bind Hsp70 and regulate its activity, our findings suggest the most important function of *Sis1p* for $[PSI^+]$ propagation is its ability to regulate Hsp70. This function seems to be important and sufficient for prion propagation in general since *Sis1* Δ GMCTD also supports propagation of $[PIN^+]$ (Sondheimer *et al.* 2001), and specific binding of *Sis1p* to *Rnq1p* has been suggested to be dispensable for replication of $[PIN^+]$ prions (Aron *et al.* 2007).

Sis1p is believed to act in prion propagation as a specific Hsp40 component of the *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 *Sis1p* in prion replication, they are not consistent with the proposed role of *Sis1p* in delivering substrates to the disaggregation machinery (Tipton *et al.* 2008) being the essential activity in this process. Our finding that the J and GF regions of *Sis1p* are enough to provide Hsp40 function in prion replication suggests that functions of 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 $[PSI^+]$ prions. Alternatively, Hsp40s other than *Sis1p* must provide such activities. This latter explanation is consistent with a previous report showing that depletion of *Sis1p* causes only partial loss of fragmen-

tation of *Sup35p* prion polymers (Higurashi *et al.* 2008), which led the authors to suggest that other Hsp40s might contribute to the fragmentation process required for $[PSI^+]$ propagation.

Despite the similarities in the requirements $[PSI^+]$ and $[PIN^+]$ have for Hsp40 functions, GF function of *Sis1p* is essential for propagation of $[PIN^+]$ (Sondheimer *et al.* 2001), and depleting *Sis1p* by transcriptional repression causes $[PIN^+]$ and $[URE3]$ to be lost much more rapidly than $[PSI^+]$ (Higurashi *et al.* 2008). Our findings are in line with these observations, which indicate that the function of *Sis1p* in prion propagation is different or much less important for $[PSI^+]$ than for $[PIN^+]$ or $[URE3]$. Although cells lacking the *SIS1* gene divide about nine times, cells undergoing repression of *SIS1* transcription can grow for >100 generations, implying that a fraction of the normal amount of *Sis1p* is still present to allow continued cell division (Luke *et al.* 1991; Higurashi *et al.* 2008). Although such cells are “viable,” depletion of *Sis1p* this way certainly has pleiotropic effects on growth, raising the possibility that the reduced stability of $[PSI^+]$ when *Sis1p* is depleted is indirect. For example, depletion of *Sis1p* could impair cellular systems or alter expression patterns of stress response factors that produce conditions that interfere with $[PSI^+]$ propagation enough to cause eventual loss of $[PSI^+]$ (Newnam *et al.* 2011). Such effects would be expected to be specific to depletion of the essential *Sis1p*, since $[PSI^+]$ is stable in cells lacking any of the other Hsp40s, including *Ydj1p* whose depletion results in slow and temperature-sensitive growth (Caplan and Douglas 1991; Higurashi *et al.* 2008).

Although $[PSI^+]$ propagated normally in wild-type cells expressing any of the mutant *Sis1* proteins, specific functions of *Sis1p* were critical for both the antiprion effects of *Ssa1*-21p and the ability of overexpressed *Hsp104* to cure wild-type cells of $[PSI^+]$. These findings are consistent with our previous work, suggesting that *Ssa1*-21p antagonizes prions by a mechanism similar to that of overexpressing *Hsp104* (Hung and Masison 2006; Reidy and Masison 2010) and point to *Sis1p* as a specific Hsp40 involved in this mechanism. We also showed previously that depleting *Ydj1p*, the other major cytosolic Hsp40, enhances the anti- $[PSI^+]$ effects of *Ssa1*-21p (Jones and Masison 2003), which suggests that *Ydj1p* interferes with the prion impairment caused by *Ssa1*-21p. Taken together our data suggest that *Ydj1p* is not involved in curing of $[PSI^+]$ by *Hsp104*. Accordingly, the recessive character of *Sis1p* mutants that prevent curing indicates that the loss of curing is due to a loss of *Sis1p* function rather than to a defective *Sis1p* interfering with the action of another Hsp40. Therefore, we propose that *Sis1p* is a specific Hsp40 required for curing of $[PSI^+]$ by overexpressed *Hsp104*.

Our findings that all nonessential activities of *Sis1p* are dispensable for *Hsp104* machinery function in replication of $[PSI^+]$ prions, yet specific functions of *Sis1p* are essential for curing cells of $[PSI^+]$ by overexpressed *Hsp104*, add to an accumulating body of evidence that points to a distinction

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 *Sis1 Δ DD* 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 *Sis1 Δ DD* 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 *ydj1 Δ* 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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Literature Cited

- Aron, R., T. Higurashi, C. Sahi, and E. A. Craig, 2007 J-protein co-chaperone Sis1 required for generation of $[RNQ^+]$ seeds necessary for prion propagation. *EMBO J.* 26: 3794–3803.
- Baxa, U., P. W. Keller, N. Cheng, J. S. Wall, and A. C. Steven, 2011 In Sup35p filaments (the $[PSI^+]$ prion), the globular C-terminal domains are widely offset from the amyloid fibril backbone. *Mol. Microbiol.* 79: 523–532.
- Boeke, J. D., J. Trueheart, G. Natsoulis, and G. R. Fink, 1987 5-Fluoroorotic acid as a selective agent in yeast molecular genetics. *Methods Enzymol.* 154: 164–175.
- Caplan, A. J., and M. G. Douglas, 1991 Characterization of YDJ1: a yeast homologue of the bacterial dnaJ protein. *J. Cell. Biol.* 114: 609–621.
- Chernoff, Y. O., S. L. Lindquist, B. Ono, S. G. Inge-Vechtomov, and S. W. Liebman, 1995 Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor $[psi^+]$. *Science* 268: 880–884.
- Chernoff, Y. O., G. P. Newnam, J. Kumar, K. Allen, and A. D. Zink, 1999 Evidence for a protein mutator in yeast: role of the Hsp70-related chaperone ssb in formation, stability, and toxicity of the $[PSI]$ prion. *Mol. Cell. Biol.* 19: 8103–8112.
- Christianson, T. W., R. S. Sikorski, M. Dante, J. H. Shero, and P. Hieter, 1992 Multifunctional yeast high-copy-number shuttle vectors. *Gene* 110: 119–122.
- Cox, B. S., 1965 “PSI” a cytoplasmic suppressor of super-suppressor in yeast. *Heredity* 20: 505–521.
- Cyr, D. M., X. Lu, and M. G. Douglas, 1992 Regulation of Hsp70 function by a eukaryotic DnaJ homolog. *J. Biol. Chem.* 267: 20927–20931.
- Dagkesamanskaya, A. R., and M. D. Ter-Avanesyan, 1991 Interaction of the yeast omnipotent suppressors SUP1(SUP45) and SUP2(SUP35) with non-mendelian factors. *Genetics* 128: 513–520.
- Derkatch, I. L., M. E. Bradley, P. Zhou, Y. O. Chernoff, and S. W. Liebman, 1997 Genetic and environmental factors affecting the de novo appearance of the $[PSI^+]$ prion in *Saccharomyces cerevisiae*. *Genetics* 147: 507–519.
- Derkatch, I. L., Y. O. Chernoff, V. V. Kushnirov, S. G. Inge-Vechtomov, and S. W. Liebman, 1996 Genesis and variability of $[PSI]$ prion factors in *Saccharomyces cerevisiae*. *Genetics* 144: 1375–1386.

- Douglas, P. M., S. Treusch, H. Y. Ren, R. Halfmann, M. L. Duennwald *et al.*, 2008 Chaperone-dependent amyloid assembly protects cells from prion toxicity. *Proc. Natl. Acad. Sci. USA* 105: 7206–7211.
- Eaglestone, S. S., L. W. Ruddock, B. S. Cox, and M. F. Tuite, 2000 Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [PSI⁺] of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* 97: 240–244.
- Fan, C. Y., S. Lee, H. Y. Ren, and D. M. Cyr, 2004 Exchangeable chaperone modules contribute to specification of type I and type II Hsp40 cellular function. *Mol. Biol. Cell* 15: 761–773.
- Ferreira, P. C., F. Ness, S. R. Edwards, B. S. Cox, and M. F. Tuite, 2001 The elimination of the yeast [PSI⁺] prion by guanidine hydrochloride is the result of Hsp104 inactivation. *Mol. Microbiol.* 40: 1357–1369.
- Glover, J. R., and S. Lindquist, 1998 Hsp104, Hsp70, and Hsp40: a novel chaperone system that rescues previously aggregated proteins. *Cell* 94: 73–82.
- Gokhale, K. C., G. P. Newnam, M. Y. Sherman, and Y. O. Chernoff, 2005 Modulation of prion-dependent polyglutamine aggregation and toxicity by chaperone proteins in the yeast model. *J. Biol. Chem.* 280: 22809–22818.
- Grimminger, V., K. Richter, A. Imhof, J. Buchner, and S. Walter, 2004 The prion curing agent guanidinium chloride specifically inhibits ATP hydrolysis by Hsp104. *J. Biol. Chem.* 279: 7378–7383.
- Higurashi, T., J. K. Hines, C. Sahi, R. Aron, and E. A. Craig, 2008 Specificity of the J-protein Sis1 in the propagation of 3 yeast prions. *Proc. Natl. Acad. Sci. USA* 105: 16596–16601.
- Hung, G. C., and D. C. Masison, 2006 N-terminal domain of yeast Hsp104 chaperone is dispensable for thermotolerance and prion propagation but necessary for curing prions by Hsp104 overexpression. *Genetics* 173: 611–620.
- Jones, G. W., and D. C. Masison, 2003 *Saccharomyces cerevisiae* Hsp70 mutations affect [PSI⁺] prion propagation and cell growth differently and implicate Hsp40 and tetratricopeptide repeat co-chaperones in impairment of [PSI⁺]. *Genetics* 163: 495–506.
- Jung, G., G. Jones, R. D. Wegrzyn, and D. C. Masison, 2000 A role for cytosolic hsp70 in yeast [PSI⁺] prion propagation and [PSI⁺] as a cellular stress. *Genetics* 156: 559–570.
- Jung, G., and D. C. Masison, 2001 Guanidine hydrochloride inhibits Hsp104 activity in vivo: a possible explanation for its effect in curing yeast prions. *Curr. Microbiol.* 43: 7–10.
- Kryndushkin, D. S., A. Engel, H. K. Edskes, and R. B. Wickner, 2011 Molecular chaperone Hsp104 can promote yeast prion generation. *Genetics* 10.1534/genetics.111.127779.
- Lacroute, F., 1971 Non-Mendelian mutation allowing ureidosuccinic acid uptake in yeast. *J. Bacteriol.* 106: 519–522.
- Lee, S., C. Y. Fan, J. M. Younger, H. Ren, and D. M. Cyr, 2002 Identification of essential residues in the type II Hsp40 Sis1 that function in polypeptide binding. *J. Biol. Chem.* 277: 21675–21682.
- Lopez, N., R. Aron, and E. A. Craig, 2003 Specificity of class II Hsp40 Sis1 in maintenance of yeast prion [RNQ⁺]. *Mol. Biol. Cell* 14: 1172–1181.
- Lu, Z., and D. M. Cyr, 1998 Protein folding activity of Hsp70 is modified differentially by the hsp40 co-chaperones Sis1 and Ydj1. *J. Biol. Chem.* 273: 27824–27830.
- Luke, M. M., A. Sutton, and K. T. Arndt, 1991 Characterization of SIS1, a *Saccharomyces cerevisiae* homologue of bacterial dnaJ proteins. *J. Cell Biol.* 114: 623–638.
- McGlinchey, R. P., D. Kryndushkin, and R. B. Wickner, 2011 Suicidal [PSI⁺] is a lethal yeast prion. *Proc. Natl. Acad. Sci. USA* 108: 5337–5341.
- Moosavi, B., J. Wongwigkarn, and M. F. Tuite, 2010 Hsp70/Hsp90 co-chaperones are required for efficient Hsp104-mediated elimination of the yeast [PSI⁺] prion but not for prion propagation. *Yeast* 27: 167–179.
- Moriyama, H., H. K. Edskes, and R. B. Wickner, 2000 [URE3] prion propagation in *Saccharomyces cerevisiae*: requirement for chaperone Hsp104 and curing by overexpressed chaperone Ydj1p. *Mol. Cell. Biol.* 20: 8916–8922.
- Nakayashiki, T., C. P. Kurtzman, H. K. Edskes, and R. B. Wickner, 2005 Yeast prions [URE3] and [PSI⁺] are diseases. *Proc. Natl. Acad. Sci. USA* 102: 10575–10580.
- Needham, P. G., and D. C. Masison, 2008 Prion-impairing mutations in Hsp70 chaperone Ssa1: effects on ATPase and chaperone activities. *Arch. Biochem. Biophys.* 478: 167–174.
- Newnam, G. P., J. L. Birchmore, and Y. O. Chernoff, 2011 Destabilization and recovery of a yeast prion after mild heat shock. *J. Mol. Biol.* 408: 432–448.
- Paushkin, S. V., V. V. Kushnirov, V. N. Smirnov, and M. D. Ter-Avanesyan, 1996 Propagation of the yeast prion-like [psi⁺] determinant is mediated by oligomerization of the SUP35-encoded polypeptide chain release factor. *EMBO J.* 15: 3127–3134.
- Reidy, M., and D. C. Masison, 2010 Sti1 regulation of Hsp70 and Hsp90 is critical for curing of *Saccharomyces cerevisiae* [PSI⁺] prions by Hsp104. *Mol. Cell. Biol.* 30: 3542–3552.
- Sha, B., S. Lee, and D. M. Cyr, 2000 The crystal structure of the peptide-binding fragment from the yeast Hsp40 protein Sis1. *Structure* 8: 799–807.
- Sharma, D., and D. C. Masison, 2008 Functionally redundant isoforms of a yeast Hsp70 chaperone subfamily have different antiprion effects. *Genetics* 179: 1301–1311.
- Sherman, F., 2002 Getting started with yeast. *Meth. Enzymol.* 350: 3–41.
- Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* 122: 19–27.
- Sondheimer, N., and S. Lindquist, 2000 Rnq1: an epigenetic modifier of protein function in yeast. *Mol. Cell* 5: 163–172.
- Sondheimer, N., N. Lopez, E. A. Craig, and S. Lindquist, 2001 The role of Sis1 in the maintenance of the [RNQ⁺] prion. *EMBO J.* 20: 2435–2442.
- Stansfield, I., K. M. Jones, V. V. Kushnirov, A. R. Dagkesamanskaya, A. I. Poznyakovski *et al.*, 1995 The products of the SUP45 (eRF1) and SUP35 genes interact to mediate translation termination in *Saccharomyces cerevisiae*. *EMBO J.* 14: 4365–4373.
- Szabo, A., T. Langer, H. Schröder, J. Flanagan, B. Bukau *et al.*, 1994 The ATP hydrolysis-dependent reaction cycle of the *Escherichia coli* Hsp70 system DnaK, DnaJ, and GrpE. *Proc. Natl. Acad. Sci. USA* 91: 10345–10349.
- Tipton, K. A., K. J. Verges, and J. S. Weissman, 2008 In vivo monitoring of the prion replication cycle reveals a critical role for Sis1 in delivering substrates to Hsp104. *Mol. Cell* 32: 584–591.
- Tsai, J., and M. G. Douglas, 1996 A conserved HPD sequence of the J-domain is necessary for YDJ1 stimulation of Hsp70 ATPase activity at a site distinct from substrate binding. *J. Biol. Chem.* 271: 9347–9354.
- Vishveshwara, N., M. E. Bradley, and S. W. Liebman, 2009 Sequestration of essential proteins causes prion associated toxicity in yeast. *Mol. Microbiol.* 73: 1101–1114.
- Wall, D., M. Zylicz, and C. Georgopoulos, 1994 The NH2-terminal 108 amino acids of the *Escherichia coli* DnaJ protein stimulate the ATPase activity of DnaK and are sufficient for lambda replication. *J. Biol. Chem.* 269: 5446–5451.
- Walsh, P., D. Bursac, Y. C. Law, D. Cyr, and T. Lithgow, 2004 The J-protein family: modulating protein assembly, disassembly and translocation. *EMBO Rep.* 5: 567–571.
- Wickner, R. B., 1994 Evidence for a prion analog in *S. cerevisiae*: the [URE3] non-Mendelian genetic element as an altered URE2 protein. *Science* 264: 566–569.
- Yan, W., and E. A. Craig, 1999 The glycine-phenylalanine-rich region determines the specificity of the yeast Hsp40 Sis1. *Mol. Cell. Biol.* 19: 7751–7758.

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