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Propagation of *Saccharomyces cerevisiae* [*PSI*$^+$] Prion Is Impaired by Factors That Regulate Hsp70 Substrate Binding

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The *Saccharomyces cerevisiae* [*PSI*$^+$] prion is believed to be a self-propagating cytoplasmic amyloid. Earlier characterization of *HSP70* (*SSA1*) mutations suggested that [*PSI*$^+$] propagation is impaired by alterations that enhance Ssa1p's substrate binding. This impairment is overcome by second-site mutations in Ssa1p's conserved C-terminal motif (GPTVEEVD), which mediates interactions with tetratricopeptide repeat (TPR) cochaperones. Sti1p, a TPR cochaperone homolog of mammalian Hop1 (Hsp70/90 organizing protein), activates Ssa1p ATPase, which promotes substrate binding by Ssa1p. Here we find that in *SSA1-21* cells depletion of Sti1p improved [*PSI*$^+$] propagation, while excess Sti1p weakened it. In contrast, depletion of Fes1p, a nucleotide exchange factor for Ssa1p that facilitates substrate release, weakened [*PSI*$^+$] propagation, while overproducing Fes1p improved it. Therefore, alterations of Hsp70 cochaperones that promote or prolong Hsp70 substrate binding impair [*PSI*$^+$] propagation. We also find that the GPTVEEVD motif is important for physical interaction with Hsp40 (Ydj1p), another Hsp70 cochaperone that promotes substrate binding but is dispensable for viability. We further find that depleting Cpr7p, an Hsp90 TPR cochaperone and CyP-40 cyclophilin homolog, improved [*PSI*$^+$] propagation in *SSA1* mutants. Although Cpr7p and Sti1p are Hsp90 cochaperones, we provide evidence that Hsp90 is not involved in [*PSI*$^+$] propagation, suggesting that Sti1p and Cpr7p functionally interact with Hsp70 independently of Hsp90.

Hsp70 is a universally conserved essential protein that acts in many cellular processes where proteins are incompletely folded, such as translation and membrane transport (7, 25). Additionally, Hsp70 expression is increased by stress, whereupon it protects cells by binding to partially unfolded proteins and preventing hydrophobic interactions that lead to aggregation. Proper functioning of Hsp70 in its diverse roles depends upon a finely tuned cycle of binding and release of the substrate that is regulated by ATP hydrolysis and nucleotide exchange.

Hsp70 has an amino-terminal ATPase domain that regulates the function of an adjacent substrate-binding domain (SBD). When ATP is bound, the SBD is "open" and rapidly binds and releases the substrate. Hydrolysis of ATP to ADP converts the SBD to a "closed" conformation, establishing a tight association with the substrate. Nucleotide exchange restores the ATP-bound state, returning the SBD to the open conformation and allowing release of the substrate.

Hsp70's ATPase activity is stimulated by substrate binding (41), indicating two-way communication between the domains, and is influenced by interactions with cochaperone proteins. Hsp40s, a conserved family of Hsp70 cochaperones that also bind partially folded proteins, stimulate Hsp70's ATPase activity. This stimulation is markedly elevated in the presence of the substrate, coupling ATP hydrolysis with substrate capture (23, 65). Return to the ATP-bound state is regulated by nucleotide exchange factors.

Function of eukaryotic Hsp70 is also regulated by cochaperones that contain tetratricopeptide repeat (TPR) motifs (52). Mammalian Hop1 (Hsp70/90-organizing protein) has two TPR regions that distinctly and simultaneously bind Hsp70 and Hsp90, forming a physical link between them (49). Hsp90 is another essential and stress-induced protein chaperone. Although its function during stress is unclear, Hsp90 has a well-defined role in assisting folding of steroid receptors and signaling kinases (48). Hsp70 acts in this process and has been shown to "accept" substrates from Hsp40 and "present" them to Hsp90 through an interaction facilitated by Hop1 (26). Other proteins involved in this pathway include CyP-40 cyclophilins, which are peptidyl-prolyl isomerases that have high affinity for immunosuppressants. Cyclophilins compete with Hop1 for binding to Hsp90 and displace Hop1 and Hsp70 from the complex.

The Hsp90 TPR cochaperone machinery is conserved in yeast and includes the Hop1 homolog Sti1p, which was shown recently to stimulate Ssa1p ATPase, and the CyP-40 cyclophilin homolog Cpr7p (8, 16, 45, 60). In addition to interacting with Hsp70 and Hsp90, these TPR proteins also interact with Hsp104, a chaperone that disaggregates protein aggregates in a reaction aided by Hsp70 and Hsp40 (1, 20). Hsp70 and Hsp90 have similar conserved amino acid residues at their extreme C termini that mediate interactions with TPR proteins and Hsp40 (18, 49). The C terminus of Hsp104 is similar but less conserved.

Yeast prions are proteins that misfold and form self-replicating aggregates, which are believed to propagate as amyloid (35, 63, 64). Ure2p and Sup35p, the protein determinants of

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the *Saccharomyces cerevisiae* prions [*URE3*] and [*PSI*$^+$], respectively, rapidly and spontaneously form amyloid when purified (19, 32, 57). Additionally, fibrous structures of Ure2p have been detected in [*URE3*] but not [*ure-o*] cells (55), and the protease digestion pattern of Ure2p from [*URE3*] cells is identical to that of amyloid formed from purified Ure2p (40, 57).

In a manner analogous to mammalian amyloidoses, the aggregates act as "seed" to recruit and convert the soluble form of the protein into the same misfolded form as it joins the polymer. Yeast prions replicate in the cytoplasm and are infectious in that they are transmitted between cells during cell division and cell fusion. Sup35p (eRF3) encodes a translation release factor (56, 66). When [*PSI*$^+$] is present, much Sup35 protein is aggregated and unavailable to function in translation termination, which causes a nonsense suppression phenotype. [*PSI*$^+$] thus provides a simple yet powerful system for studying amyloid propagation in vivo.

In line with yeast prions being protein folding problems, altered abundance or function of a variety of protein chaperones can affect their propagation (9, 28, 33, 34, 42, 54). Most effects are observed upon overexpression of the chaperones, and mechanisms underlying the effects remain speculative. Among the chaperones Hsp104 is special in that both its lack or its overproduction can cause loss of [*PSI*$^+$] (9). So an intermediate level of Hsp104's disaggregating activity appears to be critical for efficient [*PSI*$^+$] propagation. Hsp104 plays an important role in maintaining prion seed number, presumably by breaking prion aggregates into more numerous self-propagating particles (9, 43, 47). The elimination of [*PSI*$^+$] by overproduced Hsp104 is moderated somewhat by simultaneously overproducing Hsp70 Ssa1p (44). Our previous characterization of an Ssa1p mutant (Ssa1-21p) showed that it reduced generation of [*PSI*$^+$] seeds (28). Despite impairing [*PSI*$^+$] considerably, Ssa1-21p has little effect on cell growth under optimal or stressful conditions, suggesting that it interacts differently with amyloid than with other substrates.

We previously generated second-site mutations in Ssa1-21p that restored normal [*PSI*$^+$] propagation to understand better how Hsp70 affects yeast prion propagation (27). Among others, several mutations were located in the conserved C-terminal motif (GPTVEEVD) known to be important for interactions with TPR cochaperones. Here, we used a candidate gene approach to identify relevant interacting proteins and found that depletion of specific TPR cochaperones improved [*PSI*$^+$] propagation in *SSA1-21* cells. Although the Hsp90 cochaperones Sti1p and Cpr7p had significant effects on [*PSI*$^+$], we find evidence that Hsp90 is not involved in [*PSI*$^+$] propagation, uncovering a functional interaction between these cochaperones and Hsp70 that appears independent of Hsp90. Our characterization of Hsp70 interactions with cochaperones reveals the altered Hsp70 function that antagonizes amyloid propagation and provides an explanation for effects seen in some earlier overexpression studies.

MATERIALS AND METHODS

Strains, media, growth conditions, and plasmids. Yeast strains are listed in Table 1. Except for strains G658 through G663, which are transformants of G400-1C (*MAT***a** *kar1-1 SUQ5 his3 leu2 lys2 trp1 ura3 ssa1*::*KanMX ssa2*::*HIS3 ssa3*::*TRP1 ssa4*::*ura3-1f*/pRDW10 [27]), all are isogenic to 779-6A (*MAT*α *kar1-1 SUQ5 ade2-1 his3*Δ*202 leu2*Δ*1 trp1*Δ*63 ura3-52* [29]). Because Ssap function is essential, plasmids with *SSA* alleles can be maintained in G400-1C cells on nonselective media. Similarly, plasmids with *HSC82* alleles can be maintained in *hsc82*Δ *hsp82*Δ cells without selection. *SSA1* gene replacements were made as described previously (29) by transformation using alleles on BglII-SphI fragments from plasmids pJ126 and pJ127 (see below). These fragments contain the *SSA* coding region and 500 bp of 5′ and 3′ flanking DNA. The coding region of *SSA2* was precisely replaced with *HIS3* by transformation with DNA obtained by PCR-amplifying *HIS3* using primers with 5′ homology to *SSA2* untranslated DNA (3). Remaining genes were disrupted by transformation using *KanMX* cassettes (59). YPAD (excess adenine), 1/2YPD (limiting adenine), and synthetic media were as described previously (50, 51). Unless indicated otherwise, cells were grown at 30°C. Genetic methods were as described previously (22, 28). The presence or absence of [*PSI*$^+$] was confirmed by both guanidine curing (below) and cytoduction, which is transmission of cytoplasm between strains through abortive mating (10, 28).

The pRS series plasmids have been described (53). Plasmids pRDW10 (*URA3*) and pJ120 (*LEU2*) are single-copy vectors with *SSA1* (27, 28). Plasmid pJ121 is pJ120 with L483W (the *SSA1-21* mutation) in *SSA1* (27). Plasmids pJ126 and pJ127 are pJ120 and pJ121, respectively, with a *hisG*::*URA3*::*hisG* cassette (2) inserted at an AatII site engineered 200 bp 3′ to the *SSA1* termination codon. *SSA1*Δ*8* and *SSA1-21*Δ*8* are *SSA1* and *SSA1-21*, respectively, which lack the eight C-terminal codons (GPTVEEVD) and were made by site-directed mutagenesis of pJ120 and pJ121. The *URA3*-based plasmid pH*SSA1*, a gift from E. Craig (University of Wisconsin, Madison; named pRSETB-SSA1 by her), has an amino-terminal His_6-tagged *SSA1* under control of the *TEF2* promoter. Plasmids pH*SSA1-21*, pH*SSA1*Δ*8*, and pH*SSA1-21*Δ*8* are pH*SSA1* with the indicated allele in place of *SSA1*. Multicopy plasmids p423*STI1* and p423*FES1* are pRS423 with *STI1* and *FES1*, respectively. The single-copy plasmid pL116 is pRS314 with the intact *HSC82*(1-709) gene and 500 bp of 5′ and 3′ flanking DNA, PCR amplified from strain 779-6A, on a BamHI fragment. Plasmid pL118 contains the truncated *HSC82*(1-704) allele lacking the five C-terminal codons (MEEVD).

Mutagenesis. The *SSA1-21* allele of *SSA1* has a tryptophan in place of leucine at codon 483 (L483W). A screen for second-site suppressors of L483W was done as described previously (27). Briefly, plasmid pJ121 was randomly mutagenized with hydroxylamine and then used to transform strain G400-1C to leucine prototrophy. Transformant colonies were replicated onto plates lacking adenine (−ade) and containing 5-fluoro-orotic acid to simultaneously select for cells that propagated [*PSI*$^+$] and had lost pRDW10, which requires that the mutant proteins both restore [*PSI*$^+$] propagation and provide essential Ssa1p function. Site-directed mutagenesis on pJ126 and pJ127 using the QuikChange kit (Stratagene) and appropriate primers was done to create alleles for genomic gene replacements of *SSA1*.

Nonsense suppression (read-through) assays. A dual-luciferase assay system was used (24). The system consists of bicistronic mRNAs encoding a translational fusion of *Renilla* and firefly luciferase genes with UGG or UAA at the sixth codon of the firefly gene (J. W. Harger and J. D. Dinman, unpublished data). Because the enzymes are expressed from the same mRNA and the product is a fusion protein, abundance of mRNA and protein are controlled for internally. Cells with plasmids pYDL505 (UGG) and pYDL506 (UAA) were grown in plasmid selective medium to an optical density at 600 nm (OD_{600}) of 0.5 to 1. Cells in 1 ml of culture were centrifuged, washed, and then broken by agitation with glass beads in 0.3 ml of lysis buffer. Broken cells were centrifuged for 5 min at 8,000 rpm in a microcentrifuge (Sorvall; MC12) and luciferase activity of 5 ml of supernatant was assayed using the Promega dual-luciferase assay system in a Zylux FB15 luminometer.

Purification of His-tagged Ssa1p and Western blotting. His_6-tagged Ssa1p and Ssa1-21p were indistinguishable from the untagged proteins with respect to functions in growth and [*PSI*$^+$] propagation (data not shown). Strain 1012 [*psi*$^-$] transformants with the pH*SSA1* series plasmids were transferred from solid medium lacking uracil to YPAD liquid. Cultures (OD_{600} = 1.0) were washed, suspended in lysis buffer (0.5× phosphate-buffered saline [pH 7.4], 50 mM KCl, 5 mM MgCl2, EDTA-free protease inhibitor cocktail tablets [Roche]), and broken by agitation with glass beads. Proteins were cross-linked for 60 min at 4°C with 1 mM dithiobis(succinimidylpropionate) (Sigma). The reaction was stopped with 5 mM lysine for 30 min at 4°C. Lysates (3 mg of protein) were diluted with equal volumes of binding buffer (1× phosphate buffer [pH 7.4], 10 mM imidazole, HisTrap kit [Amersham]) and incubated with nickel-charged resin for 1 h at 4°C. After five washes with binding buffer and then five washes with washing buffer (1× phosphate buffer [pH 7.4], 40 mM imidazole, HisTrap kit [Amersham]), resin-bound proteins were eluted with 120 μl of elution buffer (1× phosphate buffer [pH 7.4], 500 mM imidazole, HisTrap kit [Amersham]). Samples were boiled in 30 μl of 5× sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer for 5 min, and 20-μl aliquots were subjected to

TABLE 1. Yeast strains and growth rates

Strain	Relevant genotype[a]				Generation time (min)[b]	
	SSA1	*SSA2*	*CPR7*	*STI1*	[*psi*$^-$]	[*PSI*$^+$]
1001	+	+	+	+	99	100
1002	+	*ssa2*Δ	+	+	100	109
1003	+	+	*cpr7*Δ	+	144	145
1004	+	+	+	*sti1*Δ	93	110
1005	+	*ssa2*Δ	*cpr7*Δ	+	117	122
1006	+	*ssa2*Δ	+	*sti1*Δ	108	111
1007	+	+	*cpr7*Δ	*sti1*Δ	175	151
1008	+	*ssa2*Δ	*cpr7*Δ	*sti1*Δ	192	167
1009	*SSA1*^{636S}	+	+	+	108	106
1010	*SSA1*^{636S}	*ssa2*Δ	+	+	108	111
1011	*ssa1*Δ	+	+	+	93	96
1012	*ssa1*Δ	*ssa2*Δ	+	+	141	173
1013	*SSA1-21*	+	+	+	84	84
1014	*SSA1-21*	*ssa2*Δ	+	+	102	NA
1015	*SSA1-21*	+	*cpr7*Δ	+	127	125
1016	*SSA1-21*	+	+	*sti1*Δ	90	108
1017	*SSA1-21*	*ssa2*Δ	*cpr7*Δ	+	133	NA
1018	*SSA1-21*	*ssa2*Δ	+	*sti1*Δ	111	NA
1019	*SSA1-21*	+	*cpr7*Δ	*sti1*Δ	170	153
1020	*SSA1-21*	*ssa2*Δ	*cpr7*Δ	*sti1*Δ	168	208
1021	*SSA1-21*^{636S}	+	+	+	94	103
1022	*SSA1-21*^{636S}	*ssa2*Δ	+	+	95	108
919	*fes1*Δ*SSA1*				145	126
921	*fes1*Δ*SSA1-21*				123	NA
G612	*hsc82*Δ*hsp82*Δ/p*HSC82*(1-709)				ND	ND
G616	*hsc82*Δ*hsp82*Δ/p*HSC82*(1-704)				ND	ND
G658	*ssa1,2,3,4*/p*SSA1*				90	ND
G659	*ssa1,2,3,4*/p*SSA1-21*				92	NA
G660	*ssa1,2,3,4*/p*SSA1*^{P636S}				105	ND
G661	*ssa1,2,3,4*/p*SSA1-21*^{P636S}				98	ND
G662	*ssa1,2,3,4*/p*SSA1*Δ*8*				136	165
G663	*ssa1,2,3,4*/p*SSA1-21*Δ*8*				265	NA

[a] All except G658 through G663 are isogenic to wild-type strain 779-6A (see Materials and Methods). Various allele combinations are indicated under relevant genotype. G658 through G663 are G400-1C (see Materials and Methods [27]) with the indicated plasmids in place of pRDW10.
[b] Growth rates were measured in liquid YPAD at 30°C. ND, not determined; NA, not applicable, since [*PSI*$^+$] is unable to propagate in these strains.

Western analysis. Mouse anti-Hsp70 and rabbit anti-Hsp104 antibodies (SPA-822 and SPA-1040) were from Stressgen, rabbit anti-Ydj1p polyclonal antibodies were a gift from J. Brodsky (University of Pittsburgh), and rabbit anti-Sti1p and anti-Cpr7p polyclonal antibodies were generated for this study.

Guanidine curing of [*PSI*$^+$]. [*PSI*$^+$] cells grown on −ade plates at 25°C were used to inoculate liquid YPAD cultures, which were grown overnight at 30°C. These cultures were diluted in YPAD containing 3 mM guanidine-hydrochloride and maintained at an OD_{600} of ≤0.8 by dilution into fresh guanidine-containing medium. Samples were removed at each cell number doubling, measured as doubling of OD_{600}, and spread onto three YPD plates at dilutions producing 300 to 500 colonies per plate. Entirely red colonies were scored as [*psi*$^-$]. When guanidine curing was used as a confirmation of the presence of [*PSI*$^+$], cells were grown to colonies on YPAD plates containing 3 mM guanidine and then assayed for the [*PSI*$^+$] phenotype on YPD.

RESULTS

Impairment of [*PSI*$^+$] prion propagation by Hsp70 mutation. [*PSI*$^+$] is a self-replicating aggregated form of Sup35p thought to be amyloid. Aggregation of Sup35p in [*PSI*$^+$] cells causes nonsense suppression because much Sup35 protein is unavailable to function in translation termination. Mutants with the *ade2-1* nonsense allele cannot grow without adenine and are red when grown on limiting amounts of adenine (e.g., on 1/2YPD) because of the accumulation of a pigmented substrate of Ade2p. Partial suppression of *ade2-1* by [*PSI*$^+$], which also requires the weakly UAA-suppressing tRNA *SUQ5*/*SUP16*, allows growth without adenine and eliminates the pigmentation (11). The relative strength of [*PSI*$^+$] can be estimated from the degree of pigmentation and rate of growth without adenine, which reflect the degree of nonsense suppression caused by [*PSI*$^+$] (13, 28). Another indicator of robustness of [*PSI*$^+$] propagation is its mitotic stability, which typically correlates with nonsense suppression. Spontaneous mitotic loss of [*PSI*$^+$] is seen as appearance of red, [*psi*$^-$] colonies.

Figure 1 shows the various [*PSI*$^+$] phenotypes of our strains, several of which were abnormal. To rule out the possibility that mutations were generating variants of [*PSI*$^+$] that propagated atypically, all strains with abnormal [*PSI*$^+$] phenotypes were used as cytoplasm donors in cytoduction crosses with wild-type [*psi*$^-$] cells (see Materials and Methods). In all crosses, the wild-type recipients had a normal [*PSI*$^+$] phenotype, indicating that the mutations weakened propagation of a normal form of [*PSI*$^+$]. This test also confirms absence of [*PSI*$^+$] in [*psi*$^-$] cells. Where appropriate, we confirm that colony color reflected levels of nonsense suppression by quantifying stop codon readthrough (Fig. 2).

Our wild-type [*PSI*$^+$] cells are white on YPD medium and grow well without adenine at both 25°C and the more stringent 30°C (Fig. 1, column 1, upper panels). [*PSI*$^+$] is very stable in these cells, and we do not observe spontaneous appearance of

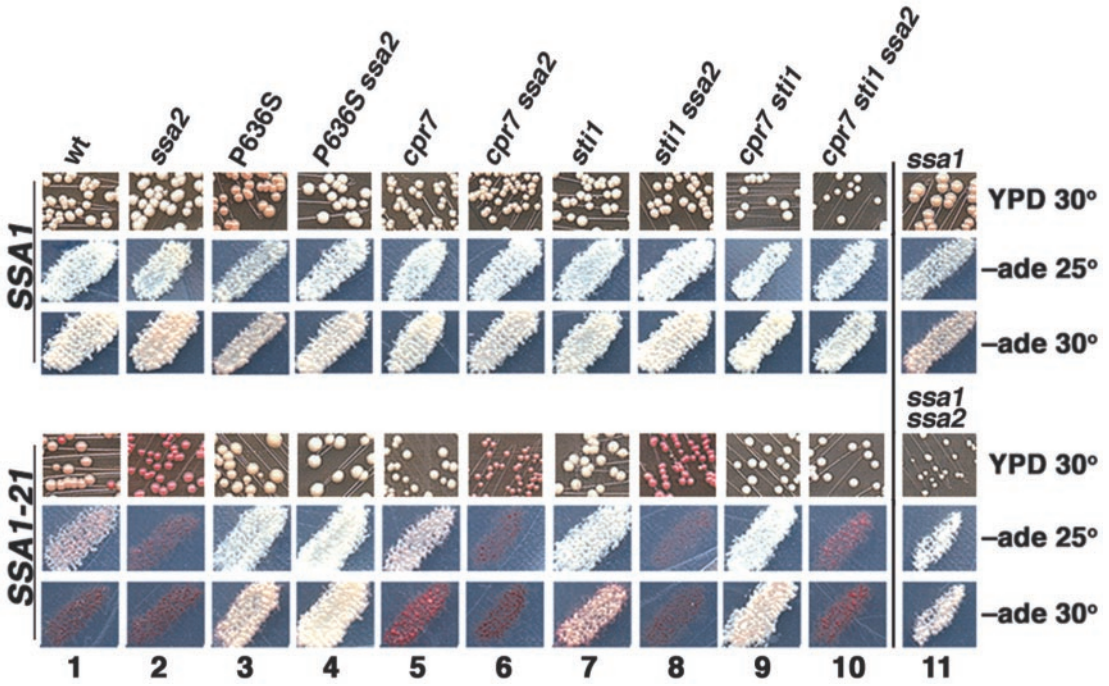

FIG. 1. Effects of *SSA1* and cochaperone mutations on [*PSI*$^+$] phenotype. Cells were grown as colonies on YPD for 2 days at 30°C followed by 3 days at 25°C or as patches of cells replica plated onto −ade plates, which were incubated for 5 days at 25°C (−ade 25°) or for 3 days at the more stringent 30°C (−ade 30°). Representative areas of growth on plates are shown. Except for those in column 11, strains in upper panels have wild-type *SSA1* and strains in lower panels have *SSA1-21*, as indicated. Strains in column 11 upper panels are *ssa1*Δ, and those in lower panels are *ssa1*Δ *ssa2*Δ. Relevant alleles of strains in each column are indicated; wt is wild type. Colors on YPD range from white to red, reflecting from most to least suppression of *ade2-1* (see the text). The extent of *ade2-1* suppression is also reflected as density of growth on −ade plates. Red cell color is enhanced on −ade plates compared with YPD plates, especially for nongrowing strains.

red [*psi*$^-$] colonies during routine handling. *SSA1-21*, an allele of the *HSP70* gene *SSA1* with the L483W mutation, causes frequent mitotic loss of [*PSI*$^+$] and reduces nonsense suppression (Fig. 1, column 1, lower panels; Fig. 2). Effects of Ssa1-21p on [*PSI*$^+$] are dominant over those of Ssa2p, which is another constitutively expressed cytosolic Hsp70 that is 97% identical to Ssa1p. When Ssa2p expression is abolished, Ssa1p abundance is elevated in a compensatory manner. While *ssa2*Δ cells have a normal [*PSI*$^+$] phenotype, [*PSI*$^+$] cannot propagate in *SSA1-21 ssa2*Δ cells (28) (Fig. 1, column 2).

C-terminal second-site suppressors of Ssa1-21p. To understand better how Ssa1-21p function was altered, we previously isolated second-site mutations in Ssa1-21p that restored [*PSI*$^+$] propagation in *SSA1-21 ssa2*Δ cells (27). Among others, three (P636S, E639K, and E640K) were in the extreme C terminus of Ssa1-21p. The conserved C-terminal octapeptide of Hsp70 (GPTVEEVD) mediates interactions with TPR-containing cochaperones and is necessary for optimal Hsp70 function (18, 49). Assuming that the three mutations similarly suppressed the impaired [*PSI*$^+$] phenotype by altering Ssa1-21p interaction with TPR cochaperones, we chose P636S for further study.

Isogenic strains that contained chromosomal *SSA1* and *SSA1-21* alleles with or without the P636S substitution (*SSA1-21*^{P636S} has both L483W and P636S) were constructed. An *ssa1*Δ strain and an isogenic *ssa2*Δ series were also tested. As originally isolated, *SSA1-21*^{P636S} *ssa2*Δ cells had a normal [*PSI*$^+$] phenotype (Fig. 1, compare column 4, lower panels, to column 1, upper panels). Therefore, the P636S substitution completely suppressed the [*PSI*$^+$]-inhibitory effects of the Ssa1p L483W substitution in *ssa2*Δ cells.

The presence of Ssa2p in *SSA1*^{P636S} or *SSA1-21*^{P636S} cells reduced ability of [*PSI*$^+$] to cause nonsense suppression (Fig. 1, compare columns 3 and 4; Fig. 2). The [*PSI*$^+$] phenotype was also slightly weakened in *ssa1*Δ cells but was normal *ssa2*Δ cells (Fig. 1, columns 11 and 2, upper panels; Fig. 2). These results show that Ssa2p weakened [*PSI*$^+$] in cells with compromised Ssa1p function and reveal a functional distinction between Ssa1p and Ssa2p with regard to [*PSI*$^+$] propagation.

Depletion of TPR cochaperones restores [*PSI*$^+$] propagation in *SSA1-21* cells. Since the extreme C terminus of Hsp70 is important for physical interaction with TPR motifs of cochaperones, we expected that the C-terminal suppressing mutations of Ssa1-21p were disrupting such interactions. Taking a candidate gene approach to identifying TPR cochaperones involved in the effects on [*PSI*$^+$], we deleted separately the nonessential cyclophilin homologs *CPR6* and *CPR7* and *HOP1* homologs *SGT2* and *STI1*. Deleting *CPR6* or *SGT2* had no effect on growth or [*PSI*$^+$] phenotype of wild-type or *SSA1-21* strains. Deleting *CPR7* or *STI1* in wild-type cells also had no obvious effect. In contrast, deleting either of them in *SSA1-21* cells reduced pigment accumulation and improved mitotic stability of [*PSI*$^+$] (Fig. 1, lower panels, compare columns 5 and 7 to column 1). Deleting *STI1* improved [*PSI*$^+$] propagation better than deleting *CPR7*. On medium lacking adenine at the more stringent 30°C, *SSA1-21* and *SSA1-21 cpr7*Δ cells were unable to form colonies but *SSA1-21 sti1*Δ cells grew well. As

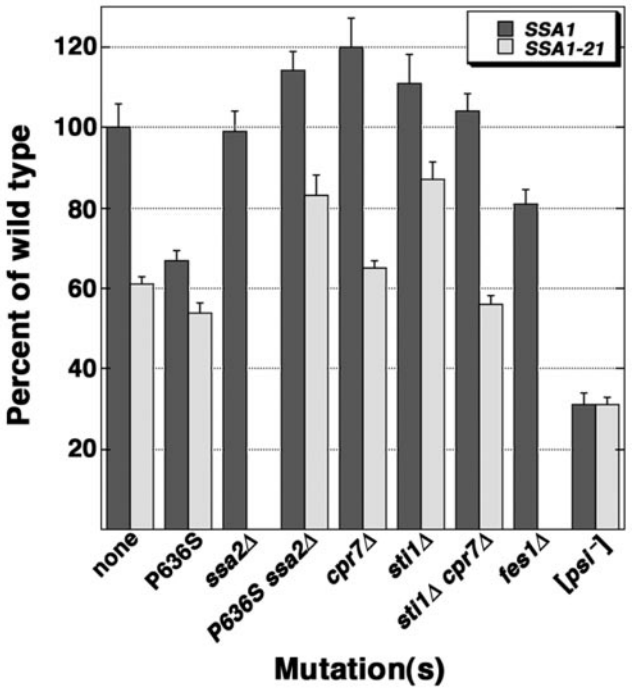

FIG. 2. Quantified levels of [*PSI*$^+$]-mediated nonsense suppression. Strains carry plasmids expressing a translational fusion of *Renilla* (upstream) and firefly (downstream) luciferases, with or without a termination codon (UAA) early in the firefly coding region. The ratio of activities of firefly to *Renilla* enzymes for the UAA construct versus the control construct [$(\text{firefly}/\textit{Renilla})_{\text{UAA}}/(\text{firefly}/\textit{Renilla})_{\text{control}}$] provides a measurement of UAA read-through. Read-through values for mutant strains are expressed as percentages relative to values for the wild-type strain. Assays were done in triplicate at least three times. *SSA1-21* derivatives of *ssa2*Δ and *fes1*Δ strains are not represented, since they are unable to propagate [*PSI*$^+$].

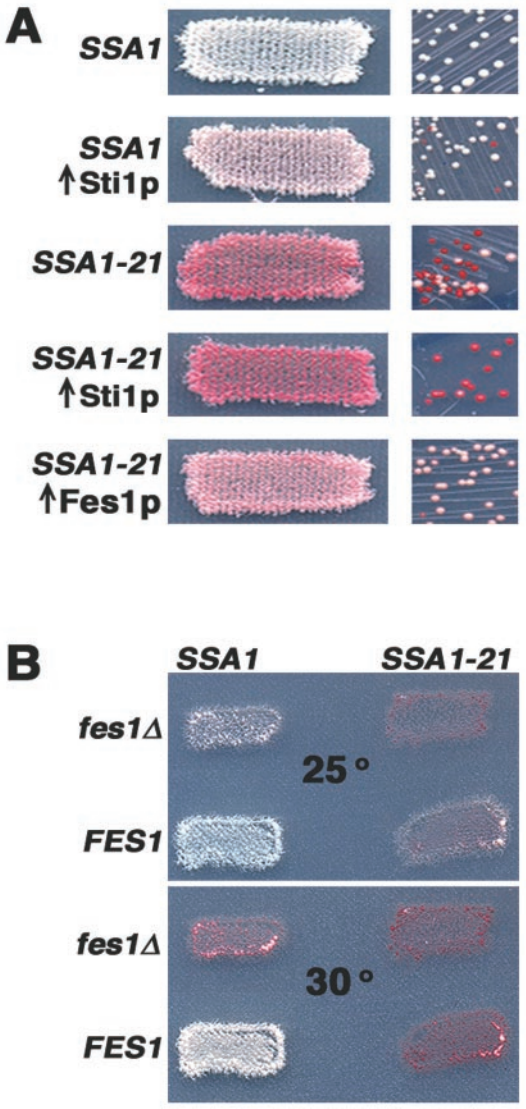

FIG. 3. Opposing effects of Sti1p and Fes1p on [*PSI*$^+$]. (A) Increased expression of Sti1p and Fes1p. Patches or colonies of [*PSI*$^+$] *SSA1* and *SSA1-21* transformants of a high-copy-number plasmid, with or without the indicated genes, were grown on selection plates with limiting adenine for 2 days at 30°C. While [*PSI*$^+$] can propagate in *SSA1-21* cells overproducing Sti1p, the enhanced weakening of [*PSI*$^+$] makes it difficult to distinguish its presence at the level of individual colonies. (B) Patches of *SSA1* and *SSA1-21* cells, having or lacking *FES1* as indicated, were replica plated onto plates lacking adenine and incubated at the indicated temperature (given in degrees Celsius) for 2 days.

observed previously (16), deleting *CPR7* reduced the growth rate (Table 1). These results show that Sti1p and Cpr7p were required for Ssa1-21p to have its full effects on impairing [*PSI*$^+$].

Unlike the P636S mutation, deletion of *STI1* or *CPR7* did not restore [*PSI*$^+$] propagation in *SSA1-21 ssa2*Δ cells. When both *STI1* and *CPR7* were deleted in *SSA1-21 ssa2*Δ cells, however, [*PSI*$^+$] propagated stably (Fig. 1, lower panels, column 10). These [*PSI*$^+$] cells did not grow without adenine, however, indicating that restoration of [*PSI*$^+$] propagation was incomplete. Compared with the complete restoration of [*PSI*$^+$] in *SSA1-21 ssa2*Δ cells by the P636S substitution in Ssa1-21p, this result suggests that other TPR cochaperones may affect [*PSI*$^+$] propagation. One candidate is the essential Cns1p, which is functionally redundant with Cpr7p (14, 39).

Overexpression of *STI1* weakens [*PSI*$^+$] and enhances *SSA1-21* effects. Since depleting Cpr7p and Sti1p improved [*PSI*$^+$] propagation, we anticipated that increasing their abundance would have the opposite effect. In fact, others have shown that overproduction of Sti1p can weaken propagation of weak or hybrid forms of [*PSI*$^+$] (33). We tested overproduction by transforming [*PSI*$^+$] cells with high-copy-number plasmids carrying *CPR7* and *STI1*. We also tested *CNS1* because excess Cns1p restores normal growth to *cpr7*Δ cells (39). In wild-type cells, [*PSI*$^+$] was not affected by excess Cpr7p or Cns1p, but overproduced Sti1p weakened [*PSI*$^+$] propagation, increasing pigmentation and reducing mitotic stability of [*PSI*$^+$] (Fig. 3A). Similarly, only excess Sti1p enhanced the impairment of [*PSI*$^+$] propagation in *SSA1-21* cells, increasing both pigment accumulation and frequency of mitotic loss of [*PSI*$^+$] (Fig. 3A). In *SSA1-21 cpr7*Δ cells, overproduced Cns1p restored both growth rate and impairment of [*PSI*$^+$] by Ssa1-21p (data not shown). Thus, in addition to being redundant with Cpr7p for

growth, Cns1p also overlaps functionally with Cpr7p with regard to [*PSI*$^+$] propagation through effects on Hsp70.

Deletion of *FES1* weakens [*PSI*$^+$] and enhances *SSA1-21* effects. While it is not known how the Hsp90 cochaperone Cpr7p affects Hsp70 function, Sti1p was recently shown to be an activator of Ssa1p ATPase (60) and thus promotes Ssa1p substrate binding. In contrast, the nucleotide exchange factor Fes1p accelerates release of ADP from Ssa1p (30), thereby facilitating Ssa1p's return to the open state and substrate release. Therefore, deleting Fes1p should prolong Ssa1p's closed state, which we predicted would adversely affect [*PSI*$^+$], while excess Fes1p was expected to have the opposite effects. Indeed, we found that [*PSI*$^+$] was unable to propagate in *SSA1-21* cells lacking *FES1* and propagated better in *SSA1-21* cells that overproduced Fes1p (Fig. 3A and B). Moreover, deleting *FES1* predictably weakened [*PSI*$^+$] in wild-type cells, reducing both nonsense suppression and mitotic stability of [*PSI*$^+$] (Figs. 2 and 3, data not shown).

TPR cochaperone deletion or P636S restores [*PSI*$^+$] seed number in *SSA1-21* cells. In order for [*PSI*$^+$] to be maintained in a population, the number of transmissible prion particles, or seeds, must double, on average, in the time it takes cells to divide. *SSA1-21* significantly reduces the average number of [*PSI*$^+$] seeds per cell (28), which explains why it causes frequent loss of [*PSI*$^+$]. We used guanidine curing of [*PSI*$^+$] to determine if P636S and the cochaperone deletions restored [*PSI*$^+$] seed generation in *SSA1-21* cells. At millimolar concentrations, guanidine in growth media arrests replication of [*PSI*$^+$] seeds, which then become diluted as cells divide, eventually giving rise to cells having lost [*PSI*$^+$] (17). After adding guanidine to growing cultures, the rate of appearance of [*psi*$^-$] cells provides an estimate of the average number of seeds per cell.

SSA1-21^{P636S} cells had a near-wild-type curing profile, showing that the P636S substitution significantly restored seed number (Fig. 4). Deleting *STI1* or *CPR7* had no effect on [*PSI*$^+$] curing in wild-type cells. In *SSA1-21* cells, deleting *STI1* restored the curing profile, similar to that of *SSA1-21^{P636S}* cells, while deletion of *CPR7* restored seed number less well (Fig. 4). When both *CPR7* and *STI1* were deleted in *SSA1-21* cells, [*PSI*$^+$] seed number was restored completely. These results show that Cpr7p and Sti1p affect Hsp70 function with respect to [*PSI*$^+$] seed generation and are consistent with our other findings that altering Sti1p abundance had greater effects on [*PSI*$^+$].

Hsp70 C-terminal mutations variably affect interaction with cochaperones. To assess the effects of the C-terminal mutations on cochaperone interactions, we compared abundance of proteins that copurified with Ssa1p. Amino-terminal His_6-tagged versions of Ssa1p and Ssa1-21p were used to specifically isolate Ssa1p isoforms of Hsp70. Binding of Sti1p to Ssa1p and Ssa1-21p was reduced but not eliminated by both the P636S substitution and C-terminal deletion (Fig. 5). While these mutations modestly reduced the amount of Cpr7p associated with Ssa1p, they did not have a significant effect on the binding of Cpr7 to Ssa1-21p. Therefore, although the TPR interaction motif is important for binding of Sti1p and Cpr7p to Ssa1p, these cochaperones can bind Ssa1p at a different site. Nevertheless, since both point mutations and complete deletion of the C-terminal TPR interaction motif suppress Ssa1-21p impairment of [*PSI*$^+$], this secondary interaction must have incomplete or no functionality with regard to effects on [*PSI*$^+$]. Also, since more Cpr7p was associated with Ssa1-21p, the L483W substitution may enhance the secondary interaction with Cpr7.

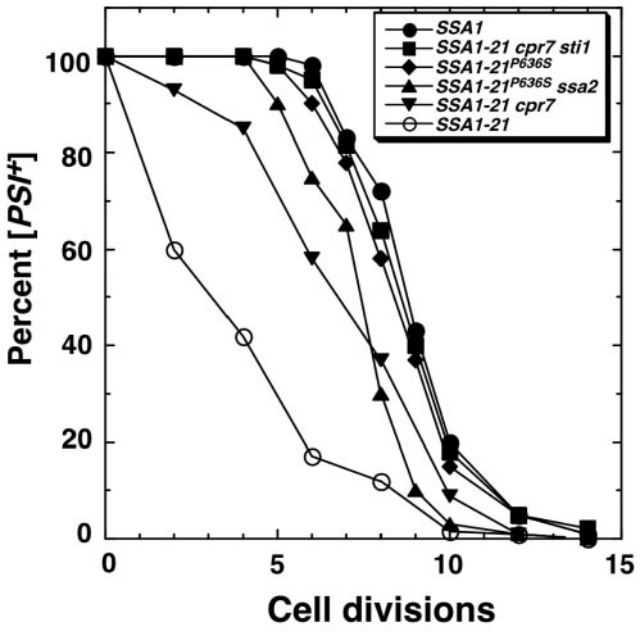

FIG. 4. Guanidine curing of [*PSI*$^+$]. The percentage of [*PSI*$^+$] cells remaining in log-phase cultures was monitored as a function of cell divisions after addition of guanidine-hydrochloride to a final concentration of 3 mM.

Deleting the C-terminal residues significantly reduced Hsp40 (Ydj1p) interaction, which agrees with earlier work showing that Hsp40 interaction with Hsp70 is reduced when Hsp70 lacks its four C-terminal residues (EEVD) (12). Hsp40s also interact with the ATPase domain of Hsp70 (31), and

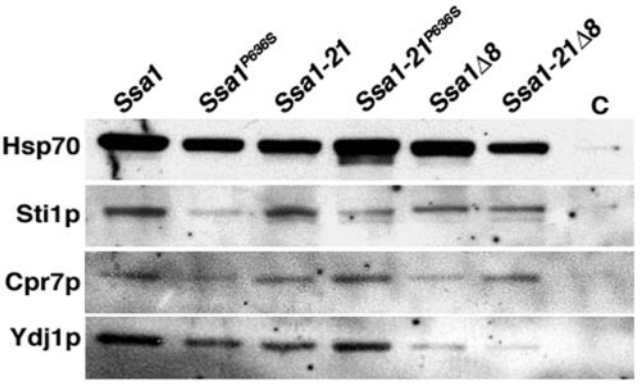

FIG. 5. Copurification of Hsp70-associated proteins. His_6-tagged versions of Ssa1p and Ssa1-21p were purified on nickel affinity resin and subjected to Western analysis. Purified His_6-tagged proteins are indicated at the top, and lane C is from a similar purification using lysates of cells expressing nontagged Ssa1p. The Hsp70 and Sti1p panels are from separate blots loaded with identical aliquots of the same samples. The Cpr7p and Ydj1p panels are from the Hsp70 and Sti1p blots, respectively, which were stripped and reprobed.

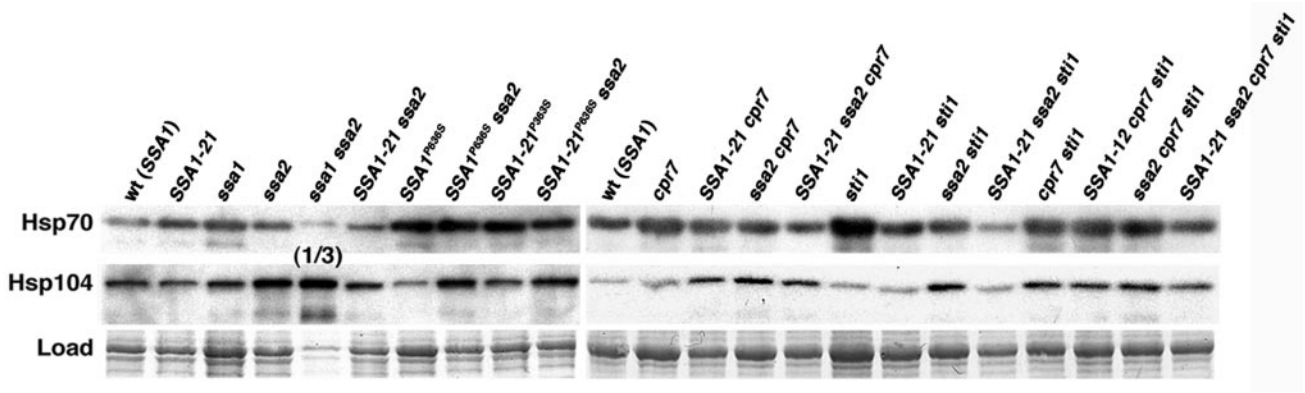

FIG. 6. Relative abundance of Hsp70 and Hsp104. Abundance of Hsp70 and Hsp104 was examined by Western analysis. Blots probed with anti-Hsp70 antibodies were stripped and reprobed with anti-Hsp104 antibodies. Representative portions of membranes, stained by amido black as a loading and transfer control, are shown (Load). Relevant alleles of strains are indicated at the top; wt is wild type. The *ssa1*Δ ssa2Δ sample was diluted 1:3, as indicated, because Hsp104 abundance in this strain is very high (compare amounts in the Load panel). The signal in the Hsp70 blot for this strain represents Ssa3p and Ssa4p, which are expressed when Ssa1p and Ssa2p both are absent.

residual binding of Hsp40 to the deletion mutants is likely mediated by this interaction. The P636S mutation had little affect on binding of Hsp40 to Ssa1p or Ssa1-21p, suggesting that suppression of L483W effects by P636S is not due to reduced ability of Hsp40 to interact with Ssa1p.

Deleting Hsp90's TPR interaction motif has no effect on [*PSI*$^+$]. Because Cpr7p and Sti1p are Hsp90 cochaperones, we anticipated that Hsp90 might be involved in the *SSA1-21* effects on [*PSI*$^+$]. If so, then disrupting the interaction between Hsp90 and TPR cochaperones would improve [*PSI*$^+$] propagation in *SSA1-21* cells. We tested this prediction by constructing strains that lacked both chromosomal *HSP90* genes (*HSC82* and *HSP82*) and expressed intact (Hsc82(1-709) or truncated (Hsc82(1-704) forms of Hsc82p. Expression of either Hsc82p or Hsp82, collectively referred to as Hsp90, is essential for viability. The Hsc82(1-704) protein lacks the C-terminal MEEVD amino acids, which mediate TPR interaction but are dispensable for viability (37).

The [*PSI*$^+$] phenotypes of both wild-type and *SSA1-21* cells were unchanged when the truncated form of Hsc82p was expressed in place of intact Hsc82p (data not shown). These results demonstrate that the Hsp90 TPR interaction motif is dispensable for [*PSI*$^+$] propagation and suggest that Hsp70 can functionally interact with TPR cochaperones independently of Hsp90.

Altered Hsp70 and Hsp104 abundance does not correlate with effects on [*PSI*$^+$]. Since altered abundance of Hsp70 and Hsp104 can have significant effects on [*PSI*$^+$] propagation (9, 34, 44), we addressed the possibility that the *SSA* and cochaperone effects on [*PSI*$^+$] were due to altered expression of Hsp70 and Hsp104 by comparing their abundance in our strains (Fig. 6). Although there was a complex pattern of Hsp70 and Hsp104 expression, there was no correlation between the levels of either of these proteins and [*PSI*$^+$] phenotype. Also, there was no correlation between Hsp70 or Hsp104 abundance and ability to propagate [*PSI*$^+$]. These results suggest that effects of *SSA* and cochaperone mutations on [*PSI*$^+$] propagation were due to altered activity of individual proteins or complexes rather than altered abundance of Hsp70 and Hsp104.

Deletion of *CPR7* correlated with elevated Hsp104 abundance, which is consistent with earlier data (15). Hsp104 was also elevated for most *ssa2*Δ strains but not for *ssa1*Δ strains, which reveals another functional distinction between Ssa1p and Ssa2p.

The Ssa1p C-terminal TPR interaction motif is dispensable for viability. The *SSA* subfamily of cytosolic Hsp70 genes, which consists of four members (Ssa3p and Ssa4p are expressed only under nonoptimal growth conditions), are the only *S. cerevisiae* Hsp70s with the GPTVEEVD motif. Expression of at least one *SSA* gene is essential for growth (62). To determine the importance of the GPTVEEVD motif for essential Ssap function, plasmids carrying *SSA1* and *SSA1-21* alleles without it (*SSA1Δ8* and *SSA1-21Δ8*, respectively) were expressed in a strain lacking all chromosomal *SSA* genes. Cells expressing intact *SSA1* or *SSA1-21* as the only *SSA* gene grew as well as the wild type (Table 1). Those expressing only *SSA1Δ8* grew more slowly (Table 1). Thus, the TPR interaction motif was important but dispensable for essential Ssap function. [*PSI*$^+$] propagated stably in this strain, but its presence slowed growth further. The *SSA1-21Δ8* allele also supported growth, but only very weakly and if cells were [*psi*$^-$] (Table 1). Because *SSA1-21Δ8* did not support growth of [*PSI*$^+$] cells, we could not test if the deletion overcame *SSA1-21* effects on [*PSI*$^+$] in the absence of other Ssap. However, as with the P636S substitution, the C-terminal deletion suppressed the effect of Ssa1-21p on [*PSI*$^+$] in cells expressing Ssa2p (data not shown).

DISCUSSION

We identify new proteins whose altered abundance affects [*PSI*$^+$] propagation and implies that amyloid propagation in vivo is impaired by alterations that promote conversion to, or stabilize, the substrate-bound state of Hsp70. Figure 7 depicts the Hsp70 reaction cycle and where these proteins act on it.

Our earlier work showed that second-site mutations in Ssa1-21p that overcome the [*PSI*$^+$]-impairing effect of the L483W substitution weaken substrate binding, which indicates that L483W enhances substrate binding of Ssa1p (27). Consistent

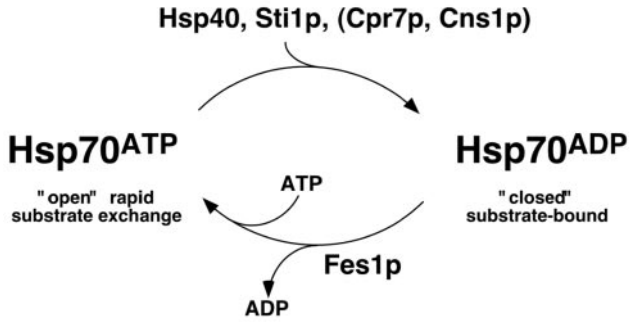

FIG. 7. Regulation of Hsp70 (Ssa1p) reaction cycle by cochaperones. Hsp70 function is finely tuned by ATP hydrolysis and nucleotide exchange, which regulate substrate binding. It is known that Hsp40 (Ydj1p, Sis1p) and Sti1p stimulate ATP hydrolysis, promoting substrate binding, and that Fes1p accelerates ADP release and nucleotide exchange, promoting substrate release. Our data suggest that enhancing conversion of Hsp70 to the ADP-bound state or stabilizing this state impairs [*PSI*$^+$] propagation. Our data also suggest that Cpr7p and Cns1p might promote or stabilize this step in the cycle.

with this interpretation, overproducing Sti1p, which promotes substrate binding by activating Ssa1p ATPase, also impaired [*PSI*$^+$] propagation. Conversely, deletion of Sti1p restored [*PSI*$^+$] propagation in *SSA1-21* cells. As predicted and in contrast to the case with Sti1p, overproduction of Fes1p, which facilitates substrate release (30), counteracted the impairment of [*PSI*$^+$] by Ssa1-21p, while depletion of Fes1p, which should prolong the substrate-bound state of Ssa1p, impaired [*PSI*$^+$] in both wild-type and *SSA1-21* cells. Thus, in addition to mutations in Hsp70 that alter its function directly, an imbalance of Hsp70 cochaperones can produce a similar [*PSI*$^+$]-impairing effect by disrupting regulation of the Hsp70 reaction cycle.

Our results agree with previous data showing that excess Sti1p destabilizes some variants of [*PSI*$^+$] (33) and suggest that this destabilization is mediated through effects on Ssa1p activity rather than altered expression of other Hsps as previously suggested. Hsp40s also stimulate Hsp70 ATPase, and in particular Ydj1p and Sis1p stimulate Ssa1p ATPase (38). Consistent with our conclusion, overproduction of the Hsp40 homologs Ydj1p, Sis1p, and Apj1p impairs propagation of yeast prions (33, 34, 42), and a suppressing mutation in Ssa1-21p described earlier is in a residue expected to disrupt Hsp40 interaction (27). Additionally, weakening of [*PSI*$^+$] by Ydj1p overproduction is increased when Ssa1p is simultaneously elevated. Our data suggest that the previously described effects of Sti1p and Hsp40 overproduction on yeast prions can be attributed to stimulated Ssa1p ATPase.

Data presented here support our earlier interpretation that other mutations in Ssa1p that inhibit [*PSI*$^+$] propagation also increase ATPase activity of Ssa1p. Aside from L483W, all of eight previously described Ssa1p mutations that impair [*PSI*$^+$] are in the ATPase domain and cannot directly affect interaction with the substrate (27). The L483W substitution is within the substrate-binding domain but distant from the substrate-binding pocket, so its effects should also be indirect, perhaps altering interdomain communication. Together, effects of the Hsp70 and cochaperone mutations on [*PSI*$^+$] suggest that the increase in ATPase is reflected in the reduced generation of amyloid seeds from preexisting material. Since cells expressing Ssa1-21p as the only essential Ssap are unable to maintain [*PSI*$^+$] but grow as well as wild-type cells, an implication of our results is that it might be possible to therapeutically modulate ATPase activity of Hsp70 in other systems, directly or through cochaperones, in a way that would impair cytosolic amyloid propagation with minimal side effects.

How does promoting or prolonging substrate binding by Hsp70 impair [*PSI*$^+$] propagation? In addition to the continued growth of polymers, propagation of yeast prions (infectious amyloid) requires the generation of new self-replicating seeds from preexisting material and efficient distribution of seeds between mother and daughter cells. Generation of yeast prion seeds requires Hsp104 protein disaggregating function, which may break the polymers into smaller, more numerous pieces that continue to propagate the structure and are more readily transmitted to daughter cells (4, 43, 47). Since *SSA1-21* mutants have severalfold fewer seeds per cell, altered Hsp70 function can interfere with amyloid seeding. Because Ssa1-21p does not affect Hsp104's ability to provide thermotolerance (28) or to reactivate heat-denatured luciferase in vivo (G. Jung, unpublished data), its effect on [*PSI*$^+$] might be direct. The simplest way to explain our data is that Ssa1-21p reduces generation of [*PSI*$^+$] seeds by binding too avidly to Sup35p aggregates, which sterically restricts access to Hsp104 (27). If this explanation were true, then this altered function must affect disassembly or breakage of the presumed highly ordered amyloid fibers of yeast prions differently than disaggregation of amorphous, thermally denatured substrates.

A way to explain this difference is that amyloid is recognized differently as a substrate by Hsp70 and Hsp104. Since Hsp40 is thought to be able to present a substrate to Hsp70, and its substrate recognition overlaps that of Hsp70, Hsp40 also may contribute to the apparent substrate-specific effects on amyloid. In line with this idea, properties of a specific Hsp40 (Sis1p) have been suggested to enhance interaction of the yeast prion [*PIN*$^+$]/[*RNQ*$^+$] determinant Rnq1p with Hsp70 (36).

Another way that enhanced substrate binding by Hsp70 might impair [*PSI*$^+$] is that Hsp70's ability to assist Hsp104 in resolubilizing protein from aggregates is altered. Although the mechanism of protein disaggregation by Hsp104 is unknown, Hsp70 and Hsp40 cooperate in this process, and together these three chaperones can resolubilize large protein aggregates in vitro (20). The altered function of the mutant Hsp70 may affect an aspect of a transient physical interaction between Hsp70 and Hsp104 or of their ability to act sequentially or simultaneously on a substrate. In the homologous bichaperone system of *Escherichia coli*, the Hsp104 homolog ClpB binds the substrate first, increasing exposure of surfaces for subsequent Hsp70 interaction (21, 61). If the reaction is similar in yeast, a substrate produced from Hsp104 interaction with amyloid may interact with Hsp70 in a way that allows it to retain the ability to propagate the self-replicating structure. By binding too avidly with this substrate, Ssa1-21p may interfere with the ability of the self-replicating conformation to be maintained. Such a scenario may explain why Ssa1-21p has a detrimental effect on amyloid without greatly affecting other cellular processes that require Hsp70 function.

Our data suggest that Cpr7p, whose effect on Hsp70's enzymatic function is unknown, may also act to enhance substrate

binding by Ssa1p. Since more Cpr7p was associated with Ssa1-21p than with Ssa1p, and more Ssa1-21p is expected to be in the closed ADP-bound state, Cpr7p may preferentially bind the ADP-bound form of Hsp70. If so, then Cpr7p may be acting to stabilize the closed conformation rather than to induce conversion to this state. The additive effect of depleting both Cpr7p and Sti1p is consistent with this interpretation.

Deleting *CPR6* or *SGT2*, other yeast cyclophilin and Hop1 homologs, respectively, had no effect on [*PSI*$^+$] propagation in wild-type or *SSA1-21* cells, showing that the effect of TPR cochaperones on [*PSI*$^+$] was not a general one. Thus, with respect to [*PSI*$^+$] there are clear functional differences in TPR cochaperone interactions with Ssa1p, which are likely rooted in differences in specificity or affinity. Such differences also may underlie functional distinctions between Ssa1p and Ssa2p regarding [*PSI*$^+$] propagation (this study), the ability of excess Ssa1p but not Ssa2p to cure cells of the yeast [*URE3*] prion (50), or the specific requirement of Ssa2p for transport of certain proteins into prevacuolar (Vid) vesicles (6).

Our finding that deleting the TPR interaction motif (MEEVD) of Hsp90 had no effect on [*PSI*$^+$] suggests that Hsp90 is not involved in Sti1p and Cpr7p effects on [*PSI*$^+$] propagation. Despite wide interest in yeast prions and Hsp90 and the availability of many Hsp90 mutants, the only other experiments describing effects of Hsp90 on yeast prions were also negative, showing a lack of effect of overproduced Hsp90 on [*PSI*$^+$] (44). Whether Hsp90 is required for [*PSI*$^+$] propagation cannot be tested directly because it is essential for viability. However, we also found that [*PSI*$^+$] propagation was unaffected in both wild-type and *SSA1-21* cells by deletion of only *HSC82*, which reduces the overall abundance of Hsp90 to roughly 10% of wild-type levels (5), or when cells were treated with a wide range of concentrations of the Hsp90 inhibitors geldanamycin and radicicol (G. Jones, unpublished data). Together these observations suggest that Hsp90 function is not important for [*PSI*$^+$] propagation. Our data therefore provide evidence suggesting that Sti1p and Cpr7p can functionally interact with Ssa1p independently of Hsp90. Cpr7 and *CNS1* have been found together in complexes without Hsp90 (58), and our results may reveal effects of perturbation of such a complex.

Our findings illustrate the utility of yeast prions as a system for studying both amyloid propagation and protein chaperone function. In addition to advancing the understanding of how chaperones affect amyloid propagation in vivo, continued study with this unique system will provide new insights into the functions of the chaperone machinery in general.

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