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Destabilization and recovery of a yeast prion after mild heat shock

Gary P. Newnam, **Jennifer L. Birchmore**[1], and **Yury O. Chernoff**[*]
School of Biology and Institute for Bioengineering and Bioscience, Georgia Institute of Technology, Atlanta, Georgia, USA

Abstract

Yeast prion [*PSI*$^+$] is a self-perpetuating amyloid of the translational termination factor Sup35. Although [*PSI*$^+$] propagation is modulated by heat shock proteins (Hsps), high temperature was previously reported to have little or no effect on [*PSI*$^+$]. Our results show that short-term exposure of exponentially growing yeast culture to mild heat shock, followed by immediate resumption of growth, leads to [*PSI*$^+$] destabilization, sometimes persisting for several cell divisions after heat shock. Prion loss occurring in the first division after heat shock is preferentially detected in a daughter cell, indicating the impairment of prion segregation that results in asymmetric prion distribution between a mother cell and a bud. Longer heat shock or prolonged incubation in the absence of nutrients after heat shock lead to [*PSI*$^+$] recovery. Both prion destabilization and recovery during heat shock depend on protein synthesis. Maximal prion destabilization coincides with maximal imbalance between Hsp104 and other Hsps such as Hsp70-Ssa. Deletions of individual *SSA* genes increase prion destabilization and/or counteract recovery. Dynamics of prion aggregation during destabilization and recovery is consistent with the notion that efficient prion fragmentation and segregation require a proper balance between Hsp104 and other (*e. g.* Hsp70-Ssa) chaperones. In contrast to heat shock, [*PSI*$^+$] destabilization by osmotic stressors does not always depend on cell proliferation and/or protein synthesis, indicating that different stresses may impact the prion via different mechanisms. Our data demonstrate that heat stress causes asymmetric prion distribution in a cell division, and confirm that effects of Hsps on prions are physiologically relevant.

Keywords

Hsp; prion segregation; *Saccharomyces cerevisiae*; stress response; Sup35

INTRODUCTION

The yeast prion [*PSI*$^+$] is a self-perpetuating fibrous cross-β aggregate (amyloid) of the translational termination (release) factor Sup35 (eRF3). [*PSI*$^+$] and other yeast prions, such as [*URE3*] and [*PIN*$^+$], are inherited via the cytoplasm, providing a molecular basis for the protein-based inheritance (for review, see refs.[1,2]). Yeast prions serve as useful models for

[*]Corresponding author: Yury O. Chernoff, School of Biology, Georgia Institute of Technology, M/C 0230, 310 Ferst Drive, Atlanta, Georgia 30332-0230, USA. Tel. 404-894-1157, fax 404-894-0519, yury.chernoff@biology.gatech.edu.
[1]Present affiliation: VWR International, Suwanee, Georgia, USA

the mammalian prion diseases (transmissible encephalopathies, or TSEs) and other amyloidoses and neural inclusion diseases in mammals and humans, such as Alzheimer disease, Huntington disease, Parkinson disease, *etc.* (for review, see ref.[3]). TSEs and many other mammalian amyloid diseases are fatal and incurable. Yeast model provides a unique opportunity for systematic studies of conditions curing cells of amyloids.

Propagation of [*PSI*$^+$] requires an intermediate level of the yeast chaperone protein Hsp104.[4] Inactivation of Hsp104 by deletion or mutation, or transient overproduction of Hsp104 under an inducible promoter causes loss of [*PSI*$^+$]. Most other yeast prions also require Hsp104 for their propagation but are not cured by Hsp104 overproduction[5–9] with the exception of [MCA$^+$].[10] Hsp104[11,12] and its bacterial homolog, ClpB[13] were shown to promote disaggregation of heat-damaged proteins. Considerable amount of experimental evidence supports the model suggesting that Hsp104 promotes prion propagation due to its ability to shear large polymers into smaller “seeds”, initiating new rounds of aggregation.[14–17] Conventional wisdom suggests that excess Hsp104 eliminates [*PSI*$^+$] by disaggregating prion polymers into monomers, however direct evidence for this mechanism is lacking, and recently reported involvement of Hsp104 in the apparatus controlling asymmetric distribution of protein aggregates in cell divisions[18,19] provides grounds for alternative explanations. It is also shown that [*PSI*$^+$] curing by excess Hsp104 requires the N-terminal region of Hsp104 that is dispensable for thermotolerance and prion propagation.[20]

Hsp104 mediated protein disaggregation is achieved via interactions with other chaperones of the Hsp70 and Hsp40 families.[12] Overproduction of any protein from the major stress inducible yeast Hsp70 family, Ssa (Ssa1, 2, 3 or 4), counteracts [*PSI*$^+$] elimination by high levels of Hsp104.[21,22] However in the absence of excess Hsp104, overproduction of some Ssa proteins could become detrimental for some variants of [*PSI*$^+$][22–25] and for other prions.[26] Mutational alterations of some Ssa proteins negatively impact [*PSI*$^+$] propagation.[27–29] Ssa proteins physically interact with Sup35[22] and are incorporated into the large structures assembled from Sup35 prion aggregates *in vivo*.[30] Another Hsp70 protein, Ssb (which is not stress-inducible) facilitates the [*PSI*$^+$]-curing effect of excess Hsp104.[31,32] Major yeast Hsp40 proteins, Sis1 and Ydj1, also influence prion propagation[32–34] and prion-promoted polyglutamine aggregation[35] in yeast. Sis1, together with Ssa, is shown to assist Hsp104 in interactions with prion polymers: current model suggest that Ssa and Sis1 recruit Hsp104 to aggregates.[36] Hsp role in prion phenomena is confirmed by the observation that alteration of the heat shock factor (HSF), regulating Hsp expression, drastically influence initial prion formation.[37]

Levels of Hsps are known to increase during environmental stresses, and [*PSI*$^+$] is “cured” with high efficiency by some environmental stresses such as osmotic stress (for review, see[38]), that are shown to induce Hsp104.[39,40] Surprisingly, growth at high temperature (37–39°C), when Hsp104 levels are also elevated,[41] does not cause any detectable loss of [*PSI*$^+$], while severe heat shock at 50°C or higher causes only infrequent loss (for review, see[14,38]). One possibility is that increased levels of the other chaperones interfere with the ability of excess Hsp104 to eliminate [*PSI*$^+$] during heat shock.

Here, we perform a detailed analysis of [*PSI*$^+$] maintenance during heat shock conditions and show that [*PSI*$^+$] is grossly destabilized by short-term mild heat shock but recovers after longer incubation at high temperature or in case if resumption of cell proliferation is significantly delayed by other means. Our data demonstrate that heat shock impairs prion segregation in the divisions following resumption of cell proliferation, and support the notion that alteration of the proper balance between Hsp104 and other stress-inducible Hsps

(specifically, members of the Hsp70-Ssa family), rather than excess Hsp104 per se, is responsible for the heat shock mediated prion destabilization.

RESULTS

[*PSI*$^+$] destabilization by short-term mild heat shock

Previous reports indicated that continuous growth at 37°C does not affect [*PSI*$^+$], while severe heat shock at 50–55°C causes slight loss of [*PSI*$^+$] (for review, see[38]). However, effects of severe heat shock were difficult to study, as it kills most of the yeast population, therefore prolonged incubation in these conditions becomes impossible, and data on the effect of [*PSI*$^+$] on the cytotoxic consequences of heat shock are contradictory.[42–45] In order to overcome these complications, we have studied the effect of mild heat shock (39°C) on [*PSI*$^+$]. While cell growth was inhibited in these conditions, no cell death was observed (Fig. 1A). Nor did the lack of the [*PSI*$^+$] prion show any effect on the yeast viability at 39°C (Supplemental Fig. S1A). This enabled us to study [*PSI*$^+$] maintenance at different time points during heat shock without a concern in regard to differential sensitivities of the [*PSI*$^+$] and [*psi*$^-$] cells, previously reported for higher temperatures in some genotypic backgrounds.[42] Further increase of temperature, *e. g.* up to 43°C, caused significant cell death after prolonged incubation (Supplemental Fig. S1B), indicating that conditions used by us are at the edge of permissive area.

In our strains, presence of [*PSI*$^+$] can be detected by readthrough (suppression) of the reporter allele *ade1-14* (UGA), resulting from decreased activity of a translation termination factor (for review, see[46]). The [*psi*$^-$] strains bearing the nonsense allele *ade1-14* (UGA) are not capable of growing on –Ade and exhibit red color on YPD medium due to the accumulation of a red pigment generated by polymerization of one of the intermediates of adenine biosynthetic pathway. The [*PSI*$^+$] strains grow on –Ade and exhibit light-pink (as opposed to red) color due to readthrough (suppression) of the *ade1-14* mutation in conditions when the Sup35 protein is partially inactivated by aggregation and is therefore incompletely functional in the process of termination. One and the same prion protein is capable of forming different variants, or "strains" of a prion. In case of [*PSI*$^+$] prion, weak variants are characterized by lower efficiency of suppression of the reporter allele *ade1-14* (UGA), lower mitotic stability, higher sensitivity to [*PSI*$^+$]-curing treatments, larger polymer size and decreased polymer fragmentation, in comparison to strong variants (reviewed in ref.[15]). The variant-specific patterns of [*PSI*$^+$] are faithfully reproduced in cell divisions.[47] We employed two isogenic yeast strains of the 74-D694 series (see below, Materials and methods), containing different variants of a prion, "weak" (OT55) and "strong" (OT56).[21]

When the yeast [*PSI*$^+$] culture, growing exponentially at 25°C or 30°C, was shifted for a short time to 39°C, followed by plating the cells onto YPD and incubating them at normal (25°C or 30°C) temperature, red ([*psi*$^-$]) or sectored pink/red (that is, mosaic [*PSI*$^+$]/[*psi*$^-$]) colonies appeared on YPD medium. Notably, heat shock induced destabilization depended on a prion variant. Heat shock effect was most profound in the weak [*PSI*$^+$] strain, where more than 30% colonies, produced after 30 min heat shock at 39°C, exhibited [*PSI*$^+$] loss or destabilization (Fig. 1BC). Destabilization of weak [*PSI*$^+$] was also detected after a short-term shift of exponential culture to either 37°C or 43°C, followed by a return to 25°C or 30°C (data not shown). However, these conditions were more difficult to employ for the detailed analysis of the mechanisms of curing, as cell proliferation continued at 37°C, and prolonged incubation at 43°C was cytotoxic (see above). Most colonies exhibiting prion loss after heat shock were pink/red mosaics (Fig. 1BC). This demonstrates that heat shock treatment results in destabilization of [*PSI*$^+$] propagation, leading to the loss of prion in some of the progeny during the subsequent cell divisions.

Although heat shock of the isogenic strong [*PSI*$^+$] strain OT56, growing in the exponential conditions, caused more than a 100-fold increase in [*PSI*$^+$] loss over untreated culture, only a few colonies (0.4% out of 2277) exhibited prion loss (data not shown), because overall stability of strong [*PSI*$^+$] is much higher than stability of weak [*PSI*$^+$]. Thus, our further experiments were performed with the weak [*PSI*$^+$] strain OT55, where [*PSI*$^+$] destabilization was easier to detect. We have also observed that [*PSI*$^+$] destabilization by heat shock strictly depended on the exponential growth phase of the yeast culture, as no significant loss of [*PSI*$^+$] was observed in the heat-shocked stationary culture of the weak [*PSI*$^+$] strain OT55 (Table 1). Similar to exponential culture, stationary culture did not exhibit any viability decrease at 39°C (Supplemental Fig. S1C).

Asymmetry of [*PSI*$^+$] segregation in the cell divisions following heat shock

Metastable conditions for [*PSI*$^+$] propagation could persist for longer than one cell division, as cultures incubated in YPD at 25°C for 2 hrs after heat shock continued to produce both red and mosaic colonies (Fig. 1C), even though by that time a significant fraction of the cells have already reentered the proliferation mode. Separation of heat shocked cells (mothers) from their first buds (daughters) by micromanipulation confirmed that prion loss may occur either in the first division after heat shock or in subsequent divisions (Fig. 1D). However, in cases when [*PSI*$^+$] loss occurred in the first cell division after heat shock, there was a statistically significant bias towards prion retention in the mother rather than the daughter cell. This indicates that heat shock treatment partly impairs prion segregation from mother to daughter cell in the subsequent cell division(s).

[*PSI*$^+$] recovery after prolonged heat shock or in the absence of nutrients

When yeast cultures were incubated for longer periods of time at 39°C before shifting back to normal temperature, proportion of cells producing red or mosaic colonies decreased, cumulating in almost complete recovery from the [*PSI*$^+$]-destabilizing effect by 4-hr time point (Fig. 1E). To check whether [*PSI*$^+$] recovery was due to continuous incubation at high temperature or simply due to inability to resume cell divisions, we have incubated the [*PSI*$^+$] culture in water at 25°C, following 30 min heat shock at 39°C. Aliquots were taken after certain periods of time and plated onto YPD medium. Proportion of the red and mosaic colonies was decreasing upon incubation in water, however at much slower kinetics, as compared to heat shock conditions. Detectable recovery was observed only after 1 day of incubation, while almost complete recovery required 4 days (Fig. 1F). Thus, continuation of heat shock treatment results in much faster prion recovery, compared to incubation in the conditions that simply prevent resumption of growth.

Role of heat shock protein synthesis in [*PSI*$^+$] destabilization and recovery

Next, we determined whether [*PSI*$^+$] destabilization and recovery result from the direct effect of high temperature on the prion or are mediated by cellular proteins synthesized during heat shock. For this purpose, we treated yeast cultures with cycloheximide, an inhibitor of protein synthesis. Cycloheximide did not have any effect on viability of yeast cells at 39°C (Supplemental Fig. S1D). However, addition of cycloheximide before heat shock completely blocked [*PSI*$^+$] destabilization, while addition of cycloheximide at 30 min after the shift to 39°C antagonized [*PSI*$^+$] recovery (Fig. 1E). These data confirm that both [*PSI*$^+$] destabilization by short-term heat shock and [*PSI*$^+$] recovery during prolonged heat shock depend on the proteins synthesized at high temperature. Notably, cycloheximide had no significant effect on kinetics of [*PSI*$^+$] recovery in water (Fig. 1F), suggesting that in this case, continuous protein synthesis is not crucial for recovery.

Accumulation of Hsps during heat shock

As heat shock proteins (Hsps) are known to be induced by high temperature, and some Hsps are known to affect [*PSI*$^+$] (for review, see[16]), we have checked the accumulation of various Hsps during mild heat shock treatment. Our data (Fig. 2AB) demonstrate that different Hsps exhibit different kinetics of accumulation at 39°C. For instance, Hsp104, which is present at a very low level in an exponentially growing yeast culture, is accumulated very quickly during heat shock, reaching the 8-fold excess over basal level by 30 min. Only a moderate further increase in Hsp104 levels was observed during the next several hours at high temperature. In contrast, Hsp70-Ssa, that is present at considerably higher background levels than Hsp104 in the exponential cells, is accumulated slower at mild heat shock conditions, reaching only 1.5-fold excess over basal level at 30 min. This can be explained by the fact that only 2 out of 4 members of the Hsp70-Ssa subfamily, Ssa3 and Ssa4, are strictly heat shock inducible, while Ssa2 is constitutively expressed, and Ssa1 is characterized by high basal level of expression, which is further increased by heat shock.[48] However, accumulation of Hsp70-Ssa continues during the next several hours at 39°C, reaching 4-fold excess by the 4-hr time point (Fig. 2AB). Other Hsps tested (Hsp40-Ydj1, Hsp26 and Hsp82) were in general closer to the “slow” kinetics of accumulation observed in case of Hsp70-Ssa rather than to the “rapid” one detected for Hsp104 (Fig. 2A). An intermediate case is Sis1 that was accumulated during heat shock slower than Hsp104 but faster than Hsp70-Ssa (Fig. 2AB). Thus, the point of maximal curing (30 min at 39°C) did coincide with the maximal imbalance between Hsp104 and the majority of other heat shock proteins, especially Hsp70-Ssa. This imbalance was specific to the heat shocked exponential culture, as no or only slight increase in Hsp levels was detected in the heat shocked stationary culture (Fig. 2C). Notably, the Hsp imbalance persisted for at least 2 hrs after resumption of cell proliferation, as levels of Hsp104 stayed high, while no significant further increase in levels of Hsp70-Ssa was detected (Fig. 2D). Likewise, both Hsp104 and Hsp70-Ssa stayed at high level for at least 2 hrs when cells resumed proliferation after 4-hr heat shock. This agrees with the previous observations that Hsp104 is stable, so that after induction, its levels could be decreased only due to dilution in cell divisions (*e. g.*, see[49]).

Addition of cycloheximide before heat shock antagonized Hsp (including Hsp104) induction (Fig. 2E), while addition of cycloheximide at 30 min after shift to 39°C did not significantly influence levels of Hsp104 (that has already been accumulated by that time), but prevented further accumulation of Hsp70-Ssa (Fig. 2F). It is worth noting that levels of the Sup35 protein itself were affected by neither heat shock (Fig. 2AC) nor cycloheximide (data not shown).

Effects of *ssa* deletions on [*PSI*$^+$] destabilization and recovery

As artificial overproduction of Hsp70-Ssa is shown to antagonize [*PSI*$^+$] elimination by excess Hsp104,[21,22] we have checked the effects of *ssa* deletions on [*PSI*$^+$] destabilization and/or recovery during mild heat shock. A set of isogenic OT55 derivatives, each bearing a deletion of one of the *SSA* genes (*1*, *2*, *3* or *4*), has been constructed. None of the *ssa* deletions influenced yeast viability at 39°C (Supplemental Fig. S1E), however they exhibited profound effects on [*PSI*$^+$]. The most severe defect was observed for *ssa2Δ*. Even without heat shock, exponentially growing *ssa2Δ* culture bearing a weak [*PSI*$^+$] variant produced about 35% of red ([*psi*$^-$]) or mosaic ([*PSI*$^+$]/[*psi*$^-$]) colonies (Fig. 3AB). After heat shock, almost all colonies have become red or mosaic. No clear [*PSI*$^+$] recovery pattern was detected in the *ssa2Δ* strain after prolonged incubation at 39°C, however the ratio of mosaic (that is, still partly [*PSI*$^+$]) to completely red ([*psi*$^-$]) colonies somewhat increased by 4 hrs, compared to earlier time periods (Fig. 3B). Effects of other *ssa* deletions were not as dramatic as *ssa2Δ*, because none of them has a detectable impact on [*PSI*$^+$] stability in a non heat shocked culture. However, both *ssa3Δ* and *ssa4Δ* significantly increased [*PSI*$^+$]

destabilization after 30 min at 39°C, and partly impaired [*PSI*$^+$] recovery after prolonged incubation at 39°C (Fig. 3A). *Ssa1Δ* decreased rather than increased [*PSI*$^+$] destabilization after 30 min at 39°C, however destabilization continued to increase during longer incubation at high temperature, and no clear recovery pattern was observed (Fig. 3A). Notably, *ssa* deletions, with the exception of *ssa2Δ*, did not influence kinetics of [*PSI*$^+$] recovery after heat shock in water (Fig. 3C), that is in agreement with the lack of requirement of protein synthesis for the [*PSI*$^+$] recovery in these conditions (see above). However, actual frequencies of prion loss in the *ssa2Δ* and *ssa3Δ* strains, maintained in water, remained higher (Fig. 3C) than in the wild type culture at respective time points (Fig. 1F), as initial level of prion destabilization was higher.

The *ssa* deletions exhibited slight or no effect on background Hsp104 levels and did not influence Hsp104 induction during heat shock (Fig. 3D), indicating that effects of Hsp70-Ssa proteins on [*PSI*$^+$] destabilization by heat shock are not due to changes in Hsp104 abundance. None of the deletions influenced the levels of Sup35 in both exponentially growing and heat shocked cultures (data not shown). Background Ssa levels were somewhat decreased in the *ssa2Δ* strain, and levels of total Ssa induced during heat shock were decreased by 25–40%, relative to wild-type, by the 4-hr time point in all the *ssa* deletion strains, with the most dramatic effect observed in *ssa2Δ* (Fig. 3E). Although these effects might not look drastic to someone, it is worth noting that certain members of the Hsp70-Ssa family undergo compensatory induction in conditions when other member(s) is/are absent (for review, see[16]). On the other hand, even 1.5–2 fold variations in total Ssa levels are shown to have detectable biological consequences, including effects on [*PSI*$^+$].[21] Nevertheless, alteration of the levels of total Hsp70-Ssa is probably not the only factor explaining the phenotypes of *ssa* deletions. Effects of *ssa2Δ* on [*PSI*$^+$] (Fig. 3ABC) are certainly more severe than it could be expected from its impact on total levels of Hsp70-Ssa (Fig. 3E). For instance, *ssa1Δ* impairs total Hsp70-Ssa levels in a manner not clearly distinguishable from *ssa3Δ* or *ssa4Δ* (Fig. 3E), however kinetics of its effects on [*PSI*$^+$] is certainly different (Fig. 3A). This suggests that while different Ssa proteins act on [*PSI*$^+$] in the same direction (see also ref.[22]), they also exhibit certain specificities and are not completely interchangeable. Such a conclusion agrees with the previous reports on the differential effects of various members of the Hsp70-Ssa family on different yeast prions (*e.g.*, see refs.[26,50]). Thus, not only the ratio between Hsp104 and total Hsp70-Ssa, but also ratios between Hsp104 and each individual member of the Hsp70-Ssa family could be important for [*PSI*$^+$] maintenance during and after heat shock.

Patterns of Sup35 aggregation during and after mild heat shock

To check if phenotypically detected prion destabilization coincides with the detectable changes in the aggregation status of Sup35 protein, we compared the Sup35 aggregation patterns before, during and after heat shock. In the exponential [*PSI*$^+$] culture, essentially all detectable Sup35 protein was present in the form of the SDS-resistant polymers, incapable of entering the polyacrylamide gel without boiling (Fig. 4A). After 30 min of incubation at 39°C, detectable soluble (monomeric) fraction of Sup35 emerged, in addition to polymers. This soluble fraction became even more abundant after 2-hr incubation at 25°C following 30-min heat shock at 39°C. Accumulation of soluble Sup35 protein coincided with prion destabilization that was detected phenotypically under these conditions. However, proportion of soluble Sup35 continued to increase after prolonged (4 hrs) incubation at 39°C, despite the fact that the prion was phenotypically recovered in these conditions. In this case, no further increase in proportion of soluble Sup35 was detected after the shift back to 25°C. Thus, changes in the ratio between the soluble versus aggregated Sup35 protein accurately reflect patterns of prion maintenance in proliferating cultures, but fail to predict whether [*PSI*$^+$] is destabilized or recovered in the heat shocked cultures.

We also employed the semi-denaturing agarose gel electrophoresis[51,52] to characterize the size distribution of the SDS-resistant Sup35 prion polymers in the exponentially growing and heat shocked cultures (Fig. 4B). Emergence of the polymer species of increased size was detected after 30 min at 39°C. Subsequent 2-hr incubation at 25°C resulted in shifting all the remaining Sup35 polymers to higher molecular weight fraction. Such a behavior of Sup35 polymers reflects patterns observed after Hsp104 overproduction in the proliferating [*PSI*$^+$] cultures.[51] However, further increase in polymer size was also observed in the cells kept at 39°C for 4 hrs (Fig. 4B). In this case, polymer size was decreased after shift to proliferating conditions, consistent with resumption of prion propagation that is associated with polymer fragmentation.

Ssa2Δ greatly decreased proportion of the aggregated Sup35 protein incapable of entering the polyacrylamide gel, relative to wild type (Fig. 4AD), and the proportion of the polymeric fraction further decreased during heat shock, consistent with high loss of [*PSI*$^+$] (Fig. 4D). However, other *ssa* deletions exhibited no dramatic effect on the ratio of monomeric versus aggregated protein. Changes in aggregation status of Sup35 in the *ssa1*, *3* and *4* deletion strains during heat shock generally followed the same pattern as in the wild type strain (Fig. 4CEF).

Maintenance of prions other than [*PSI*$^+$] during heat shock

Prions other than [*PSI*$^+$], such as [*PIN*$^+$] (prion form of Rnq1) and [URE3] (prion form of Ure2), depend on the Hsp104 chaperone but are not cured by excess Hsp104.[5,6] We have checked whether these prions are cured by short-term mild heat shock. Original OT55 contained both [*PSI*$^+$] and [*PIN*$^+$]. We have tested the [*psi*$^-$] colonies, that have lost [*PSI*$^+$] in result of 30 min incubation at 39°C, for the presence of [*PIN*$^+$] by mating them to the [*psi*$^-$ *pin*$^-$] strain bearing the multicopy Sup35 plasmid (for detailed description, see below, Materials and methods). If [*PIN*$^+$] is present in the parental haploid, resulting diploid can grow or extensively papillate on –Ade medium due to efficient induction of [*PSI*$^+$] by excess Sup35 in the presence of [*PIN*$^+$]. If both parents are [*psi*$^-$ *pin*$^-$, no growth on –Ade is detected, with the exception of rare spontaneous revertants. All heat-shock induced [*psi*$^-$] colonies that were tested retained [*PIN*$^+$], indicating that [*PIN*$^+$] is not lost after heat shock at the level comparable to [*PSI*$^+$] (Table 2). Likewise, no loss of [*PIN*$^+$] was detected when isogenic [*psi*$^-$ *PIN*$^+$] culture was treated by mild heat shock for 30 min. Presence or absence of [*PIN*$^+$] had no effect on [*PSI*$^+$] destabilization by mild heat shock, as heat shock treatment of the [*PSI*$^+$ *pin*$^-$] strain GT488 produced the same results as heat shock treatment of the isogenic [*PSI*$^+$ *PIN*$^+$] strain OT55 (data not shown).

Next, we checked the effect of mild heat shock on the [URE3] prion. Spontaneous loss of [URE3] in the strain YHE64 was detected at the frequency of about 8% and was not increased by heat shock (Table 2). Thus, mild heat shock does not destabilize [URE3] prion in this strain.

[*PSI*$^+$] curing by osmotic stressors

Some osmotic stressors are known to induce Hsp104[40,41] and were shown to cure [*PSI*$^+$].[38,53] We have compared the curing effect of osmotic stressors to that of heat shock. Several osmotic stressors were tested, of which some did not inhibit (glycerol) or only slightly inhibited (NaCl) cell proliferation, while others significantly inhibited (ethylene glycol, sodium glutamate) or completely blocked (KCl) cell proliferation (Fig. 5A). After 30 min treatment of the exponential OT55 culture, none of the osmotic stressors caused [*PSI*$^+$] destabilization at the level comparable to mild heat shock; most stressors had no observable effect at this time point, while glycerol and KCl had only a slight effect (Fig. 5B). Prolonged incubation in the presence of any osmotic stressor up to at least 24 hrs caused an increase in

[*PSI*$^+$] loss (Fig. 5B), indicating a lack of recovery pattern, such as one observed during heat shock. Addition of cycloheximide only partially antagonized [*PSI*$^+$] curing in case NaCl and KCl, had no effect on [*PSI*$^+$] curing by glycerol or sodium glutamate, and increased [*PSI*$^+$] curing by ethylene glycol. These data show that for most osmotic stressors, prion destabilization only partially depends, or does not depend at all on stress-induced protein synthesis. Notably, osmotic stressors, with the exception of KCl, preferentially produced red (complete [*psi*$^-$) rather than mosaic ([*PSI*$^+$]/[*psi*$^-$]) colonies, indicating that prion loss mostly occurred during stress rather than in the subsequent cell divisions. All these features clearly distinguish prion curing by osmotic stressors from prion destabilization by mild heat shock.

DISCUSSION

Role of Hsps in prion curing and recovery after heat shock

Our data show that [*PSI*$^+$] propagation is destabilized after short-term shift to high temperature followed by immediate resumption of normal cell proliferation. Heat shock effect is most severe in case of a weak prion variant. However, longer incubation at high temperature leads to prion recovery, that explains negative results of the previous studies. Both [*PSI*$^+$] destabilization and recovery depend on the protein synthesis during heat shock, as they are antagonized by the protein synthesis inhibitor cycloheximide (see Fig. 1E). This implicates proteins, synthesized during heat shock, as participants in the processes of [*PSI*$^+$] destabilization and recovery at high temperature. Maximal [*PSI*$^+$] destabilization coincides with the period of heat shock (about 30 min) when levels of the Hsp104 chaperone are already greatly elevated in comparison to the non heat-shocked exponential cells, while the levels of most other Hsps (such as Hsp70-Ssa) are increased only moderately (compare Figs. 1E and 2AB). These data, together with the anti-prion effects of *ssa* deletions in heat shock conditions (Fig. 3), strongly indicate that transient increase in the abundance of Hsp104 relative to the other chaperones is a cause of heat shock induced [*PSI*$^+$] destabilization.

Faster accumulation of Hsp104 during heat shock, compared to Hsp70-Ssa, may seem to be in contradiction with the previously reported data on rapid induction of the *HSP70-SSA* transcription during stress.[48] However, one should note that background levels of Hsp104 in exponential cells are very low, compared to relatively high background levels of Hsp70-Ssa. Thus, it is logical that even after immediate induction of transcription, it takes longer time to achieve the fold increase of Hsp70-Ssa protein over background levels that would be comparable to the fold increase of Hsp104.

Individual overproduction of Hsp104 from an inducible promoter is known to cause loss of [*PSI*$^+$].[4] Excess Hsp104 cures weak [*PSI*$^+$] variant more efficiently than strong [*PSI*$^+$] variant,[47] that agrees with heat shock destabilization results. It is possible that during heat shock, Hsp104 is partly aided by the Hsp40 chaperone Sis1, that shows an intermediate kinetics of accumulation at 39°C, compared to Hsp104 and Hsp70-Ssa (Fig. 2AB). Indeed, Sis1 was previously shown to play a role in the interaction of Hsp104 with prion aggregates *in vivo*,[36] and our preliminary results confirm that co-overexpression of Sis1 somewhat increases the [*PSI*$^+$]-curing effect of excess Hsp104 (N. Romanova and Y. Chernoff, unpublished data). However, Sis1 is not accumulated proportionally to Hsp104 at high temperature, and its exact role in the process of prion destabilization by excess Hsp104 and heat shock remains to be determined.

Notably, prions [*PIN*$^+$] and [URE3], that are not curable by excess Hsp104,[5,6] are also not curable by mild heat shock (Table 2), even though [*PIN*$^+$] was studied in the same genotypic background and in one version of the experiment, even in the same exact cell environment as [*PSI*$^+$]. Certainly interpretations of the negative results obtained for [*PIN*$^+$] and [URE3]

are not as straightforward as the positive result for [*PSI*$^+$]. In the absence of the color assay, we could not detect mosaics for [*PIN*$^+$] and [URE3] as reliably as for [*PSI*$^+$], where mosaics did constitute a majority of destabilized colonies. However, one should note that in case of [*PSI*$^+$], about 1/3 of all mosaics arise from prion loss in the first division after heat shock (Fig. 1D). Such mosaics would still be detectable after velveteen replica plating of the colonies to the selective medium that was used in detection assays for [*PIN*$^+$] and [URE3] (see below, Materials and methods). Another potential pitfall is that only one prion variant was tested for each [*PIN*$^+$] and [URE3], and we observed the differences in stringency of the prion-destabilizing effect of mild heat shock for two variants of [*PSI*$^+$] (see above). However, the [URE3] variant used in our studies was losing prion spontaneously at a detectable rate, thus resembling the weak variant of [*PSI*$^+$] (which was more efficiently destabilized by heat shock) rather than the strong variant. Overall coincidence between curability by overproduced Hsp104 and curability by heat shock for several different prions studied is in agreement with the causative role of Hsp104 induction in [*PSI*$^+$] destabilization by heat shock. These data also indicate that [*PSI*$^+$] loss is unlikely to be due to sequestration of Hsp104 by heat-damaged misfolded proteins, as in such a scenario, the loss of [*PIN*$^+$] and [URE3] prions, that also require Hsp104 for their propagation, would be expected.

Impairment of prion segregation in cell divisions by heat shock

[*PSI*$^+$] loss in the first cell division following heat shock was biased towards a daughter cell (Fig. 1D), suggesting that heat shock induced physiological changes (specifically, induction of Hsp104) could impair prion segregation between the mother and daughter cells during the cell division. Crucial role of Hsp104 in the mother cell-specific accumulation of the stress-damaged aggregated proteins has been reported previously,[18] although its interactions with other chaperones during this process have not yet been specifically addressed. Our data indicate that excess Hsp104 impairs segregation of prion aggregates as well. In cases when prion loss occurs in the first division after heat shock, there is a clear and statistically significant bias towards prion retention in the mother cell (Fig. 1D). This shows that heat shock treatment impairs prion transmission from mothers to daughters.

It should be noted that impairment of prion transmission to the daughters is not absolute. Moreover, exceptional cases when prion was transmitted to daughter but lost in the mother were detected (Fig. 1D). However, it is possible that such cases represent occasional technical errors in mother versus daughter differentiation by experimentator. Another possibility could be that these exceptional pedigrees arise from old mother cells, that transmit prion to the first (separated by micromanipulation), but not to the second daughter, and die soon after thus eliminating prion from the resulting colony. As these exceptions are very rare and not predictable from the beginning of the experiment, it is technically difficult to determine the exact reason. On the other hand, it should be noted that even when prion is transmitted to a daughter, this does not necessarily mean that it is completely recovered from heat shock induced alterations. In some pedigrees, transmissibility defect persists for a number of generations, leading to prion loss after the first division and generation of mosaic colonies of complex nature (Fig. 1BD).

Notably, impairment of [*PSI*$^+$] proliferation in conditions when Hsp104 is inhibited by GuHCl also leads to the mother-cell specific accumulation of the remaining prion units (propagons).[54] This suggests that prion segregation is controlled by a proper balance between Hsp104 and other components of the prion-propagating chaperone complex, and can be altered by changing the balance of chaperone levels or activities in either direction.

Changes in the aggregation status of Sup35 during heat shock mediated [*PSI*$^+$] destabilization and recovery

As Hsp104 overproduction increases the proportion of the soluble fraction of Sup35 in the [*PSI*$^+$] cells, it has been suggested that Hsp104 "cures" yeast cells of [*PSI*$^+$] by solubilizing Sup35 aggregates.[55,56] Indeed, a slight increase in the proportion of monomeric Sup35 is detected after 30 min of heat shock (Fig. 4A). However, continuous increase in the proportion of monomeric Sup35 during prolonged (up to 4 hrs) incubation at 39°C is accompanied by recovery rather than further destabilization of [*PSI*$^+$]. This indicates that differences in aggregation patterns during stress *per se* can not be used as a reliable predictor of the effect on prion propagation. In contrast, dynamics of aggregation patterns in the first cell division following return to the normal growth temperature does coincide with prion maintenance, as proportion of the monomeric versus polymeric Sup35 is further increased after 2 hrs at 25°C following the 30-min heat shock, while no such increase is detected after 2 hrs at 25°C following the 4-hr heat shock (Fig. 4A). This agrees with the high percentage of mosaic colonies (Fig. 1BC) and confirms that actual loss of [*PSI*$^+$] preferentially occurs in the cell divisions following the stress treatment. Apparently, chaperone imbalance caused by the short-term stress and maintained in the first cell divisions after stress, rather than changes in the physical status of prion protein *per se*, becomes detrimental to [*PSI*$^+$] propagation once cell proliferation is resumed. Indeed, it is known that Hsp104 is a highly stable protein, so that restoration of its normal level after induction requires dilution in several subsequent cell divisions (for example, see[49]).

Measurements of the Sup35 polymer size during heat shock by using the SDD-AGE procedure (Fig. 4B) uncover accumulation of the larger polymers that become detectable at 30-min time point and predominant by the 4-hr time point. Thus, both increased polymer size and increased monomerization are detected when Hsp104 is present in an excess, compared to other Hsps (30-min time point and following 2 hrs at 25°C), while resumption of cell divisions in the conditions when chaperone balance is partly restored, albeit at higher absolute levels (that is, after 4 hrs of heat shock), results in the decrease of polymer size accompanied by phenotypic prion recovery.

Effects of heat shock on polymer size, detected in our experiments, agree with previous work employing artificial overproduction of Hsp104 in the growing yeast culture.[51] However, previous results could be explained by selection of the large polymers that are less sensitive to disaggregating effect of Hsp104. Such an explanation is unlikely in our case, as we detect appearance of the larger polymer fraction after 30-min heat shock, when total amount of the Sup35 polymers in the cell is not yet significantly decreased. Larger polymer fraction is not present at comparable level immediately prior to heat shock (compare lanes 1 and 2 from left on Fig. 4B), making it impossible to explain its emergence by the size-dependent selection. It can not be ruled out that disassembly of the smaller polymers may provide material contributing to the growth of the larger polymers, however direct evidence for such a mechanism is lacking. The most plausible explanation of our data is that the shift of the chaperone balance towards Hsp104 results in blockage of the polymer fragmentation and therefore leads to the appearance of the polymers of larger size. Possibly, this occurs when Hsp104 binds prion polymers in the absence of its disaggregating partners.

Recent data suggest that there might be a size threshold for transmission of the prion polymers to the daughter cell, that could be responsible for the decreased mitotic stability of the weak [*PSI*$^+$] variants, characterized by the larger polymer size.[57] This agrees with our observation that heat shock increases average size of Sup35 polymers (Fig. 3B) and decreases transmissibility of the weak [*PSI*$^+$] prion to the daughter cell in the first cell division following the high temperature treatment (Fig. 1D). Also, it explains why the transmissibility effect may persist for several generations after heat shock: even when a

larger polymer is occasionally acquired by a daughter cell, its ability to be efficiently transmitted in the next division would remain impaired (and may become impaired even further if the polymer continues to grow by immobilizing new Sup35 molecules). Thus, the defect might persist until normal chaperone ratios are restored and fragmentation into the smaller polymers occurs. Less dramatic effect of heat shock on strong [*PSI*$^+$], compared to weak [*PSI*$^+$], agrees with the fact that the initial average size of prion polymers in the "strong" prion variant is smaller than in the weak prion variant.[15,51] However, it remains to be determined which mechanism controls the preferential retention of the larger polymers by the mother cell.

Differential roles of Hsp104 in prion fragmentation and segregation

The most logical explanation of our results is that Hsp104 promotes prion polymer fragmentation when present in the balanced ratio relative to Hsp70-Ssa (and possibly some co-chaperones), however it blocks fragmentation and facilitates the mother cell-specific polymer accumulation when present in an excess, relative to its partners. From the biological point of view, mother cell-specific segregation impairment could be considered as an alternative mechanism of an anti-aggregate defense, that is triggered in the situation when fragmentation becomes inefficient. It is an intriguing possibility that N-terminus of Hsp104, implicated in overproduction-driven prion elimination,[20] is specifically involved in control of aggregate segregation.

Stringency of the chaperone ratios required for proper prion propagation could be different for different prions, possibly due to differences in the numbers of propagons and/or in accessibility of prion polymers by Hsps. Indeed, excess Hsp104 promotes rather than impairs propagation of the [*PSI*$^+$] derivatives that are characterized by the abnormally large aggregate size.[23,24] Such derivatives would likely be represented by only a few propagons per cell, so that their fragmentation would not be limited by the amount of Hsp70-Ssa. Instead, low accessibility of the large aggregates to Hsp104 could become a limiting factor, and this would be improved if levels of Hsp104 are increased. It is likely that differences in effects of excess Hsp104 and heat shock on various prions are controlled, at least in part, by the number of propagons per cell and/or by accessibility of the respective prions to Hsp104.

Physiological relevance of prion destabilization

Our results show that effects of Hsps on prion maintenance are not confined to artificial over- or underexpressor constructs, but rather reflect potential impacts of the physiologically relevant variations in relative abundances of these proteins, for instance during environmental stresses and recovery from stress conditions. Stresses other than heat shock, *e. g.* osmotic stress, also affect [*PSI*$^+$] propagation, however in this case, kinetics of prion destabilization was slower than during heat shock, and prion recovery after prolonged incubation in the stress conditions was not detected (Fig. 5B). This would be interesting to connect these parameters to the patterns of Hsp induction in the respective conditions. However, profound differences in the dependence of the stressor effect on protein synthesis and cell proliferation indicate that specific mechanisms of prion destabilization could be different for different stressors, therefore clarification of these mechanisms would require a thorough individual analysis of each stressor in the way similar to what has been done in case of heat shock.

Overall, our data confirm that some conditions frequently encountered by organisms in their natural environments, such as rapid temperature fluctuations, may have a dramatic effect on prion propagation. It appears that prion behavior during stresses depends on both general parameters of cellular response to accumulation of aggregated proteins, and individual properties of specific prions. Interestingly, environmental stresses, including some of those

studied in our work, were previously shown to increase *de novo* prion formation (*e. g.*[58–60]). Therefore, environmental conditions may influence inheritance of the protein-based traits in both directions, thus contributing to "epigenetic" heritable variability of the microbial population. Observation that environmental stresses are capable of counteracting self-perpetuating amyloids in yeast also raises a question of whether or not such an effect of stress is applicable to mammalian aggregation-related disorders.

MATERIALS AND METHODS

Yeast strains and growth conditions

Strains of *Saccharomyces cerevisiae*, used in this study, are listed in Table 3. [*PSI*$^+$] strains were derivatives of 74-D694,[4] bearing prion variants of different stringencies,[21,47] weak (OT55) and strong (OT56). We primarily used a weak variant for heat shock experiments, as heat shock induced prion destabilization was significantly more pronounced in the case of weak [*PSI*$^+$] (see above). OT60 is the isogenic [*psi*$^-$] strain.[61] All these strains also contained [*PIN*$^+$] (a prion form of Rnq1 protein). The strain GT488 was a [*PSI*$^+$ *pin*$^-$] derivative of OT55, that has been obtained by K. Gokhale via curing [*PIN*$^+$] with the overexpressed dominant negative allele of *HSP104* (*HSP104-KT*) as described previously.[49] GT234 was a [*psi*$^-$ *pin*$^-$] haploid strain of GT81 series.[62] This strain was transformed with the multicopy plasmid pSTR7, [63] bearing wild type *SUP35*. The plasmid-containing transformants were used in the genetic assay for the presence of [*PIN*$^+$] (see below). The [URE3] strain YHE64[64] was a gift of Dr. R. Wickner.

Strains containing disruptions of the *SSA1*, *SSA2*, *SSA3* or *SSA4* genes were constructed from OT55 by using the direct PCR-mediated transplacement with the *Schizosaccharomyces pombe HIS5* cassette, a counterpart of *S. cerevisiae HIS3* gene.[65] Disruptions were performed as described previously.[22]

Standard yeast media and cultivation conditions, as well as standard procedures for phenotype scoring, mating and transformation were used.[66] Cultures for heat shock experiments were usually grown at 25°C, in order to minimize the background levels of Hsps before heat shock. For other purposes, yeast cultures were grown at 30°C unless stated otherwise.

Prion detection

Presence of [*PSI*$^+$] prion was monitored by both color on the complete organic (YPD) medium and ability to grow on the synthetic medium lacking adenine (-Ade),[46] as described in more detail above (see Results). Presence of [*PIN*$^+$] prion in the [*psi*$^-$] colonies was monitored genetically by velveteen replica plating these colonies to a lawn of the [*psi*$^-$ *pin*$^-$ strain of the opposite mating type (GT234), that bears the multicopy plasmid pSTR7 with the *SUP35* gene. After mating, YPD plates were velveteen replica plated onto the medium selective for diploids, followed by replica plating to the –Ade medium. Multicopy *SUP35* causes suppression of *ade1-14* mutation due to *de novo* [*PSI*$^+$] induction that occurs efficiently only in the presence of [*PIN*$^+$].[5] Thus, diploids originated from [*PIN*$^+$] colonies exhibit growth or intense papillation on the –Ade medium, while diploids originated from [*pin*$^-$] colonies do not. It has been shown previously[67] and confirmed by us (not shown) that presence of [*PIN*$^+$] detected by such an assay in the strains of 74-D694 series correlates with the presence of aggregated Rnq1 protein. Presence of [URE3] prion was detected by velveteen replica plating yeast colonies to the medium with ureidosuccinic acid, where only [URE3] containing cells can grow.[2,46]

Heat shock experiments

In order to obtain exponential cultures, yeast cells were grown in liquid YPD medium for about 16 hrs with shaking at 250 rpm, and diluted down to OD 0.1, followed by incubation for an additional 2 hrs with shaking. (Typically, incubation temperature was 25°C, although heat shock induced prion destabilization was also detected in cultures grown at 30°C.) Then, heat-shocked cultures were moved to 39°C or 43°C and continued to be incubated with shaking. Aliquots were taken after specified periods of time. Serial dilutions were prepared, and cells were either plated (prion curability assays) or spotted (cell viability assays) onto YPD medium, and incubated at either 25°C or 30°C. Most experiments used 25°C, however we have not observed any significant effect of post-heat shock incubation temperature on the results. Once colonies were formed, the presence of prions was monitored as described above. Comparisons between different strains and/or conditions were always performed within one and the same experiment.

To obtain stationary cultures, yeast cells were grown in liquid YPD medium for 2 days, followed by heat shock as described above.

In the experiments that employed the protein synthesis inhibitor, cycloheximide (purchased from Sigma), it was typically added up to a final concentration of 100 μg/μl, either at 15 min before ("at start") or 30 min after ("at 30 min") the shift to the higher temperature. Experiments in which cycloheximide was added simultaneously with the shift to the higher temperature were also performed and produced the same results with those where an inhibitor was added at 15 min before heat shock.

Analysis of [*PSI*$^+$] segregation by micromanipulation

To determine the patterns of prion segregation between the mother and daughter cells in the first cell division after heat shock, individual cells from the OT55 culture heat shocked at 39°C for 30 min were placed onto the YPD plate, spaced apart by using the Singer micromanipulator MSM System Series 300 and allowed to form buds at 30°C. Once the first bud ("daughter") was separated from the original cell ("mother"), it was moved to a different location by micromanipulation, and plates were incubated at 25°C until each cell formed a colony. Presence or absence of [*PSI*$^+$] in each colony (or its portion) was monitored by color assay as described above.

Protein isolation and analysis

For protein level comparisons, cells were destroyed by vortexing with glass beads, and total proteins were isolated, boiled in the SDS-containing loading buffer and run on SDS-PAGE as described previously.[21] The resulting gels were blotted onto a nitrocellulose membrane, and reacted to the appropriate primary antibody, followed by reaction to the appropriate secondary antibody and chemiluminescent detection as described in the GE Healthcare protocols. Wherever necessary, relative protein levels were determined by densitometry using VisionWorks LS program from UVP

Ratio of aggregated (SDS-insoluble) versus monomeric (soluble) protein was determined by "boiled gel" assay.[52] This assay is based on the inability of prion polymers to enter the SDS-PAGE gel without boiling. Protein samples containing 2% SDS (but not boiled) were loaded on the SDS-PAGE gel and run for about 1 hr, so that soluble protein could enter the gel and run for sufficient distance. Then, electrophoresis was interrupted, and wells were filled with a new portion of acrylamide, in order to trap aggregated protein that remained in the wells. After polyacrylamide has solidified, the whole gel was incubated for 10–15 min in the boiling water bath and then cooled down, followed by resumption of electrophoresis. As polymers were destroyed by boiling, protein from aggregated fraction was now capable of

moving in the gel. After about 1 hr of electrophoresis, Western blotting was performed followed by reaction to antibodies. Upper band corresponded to the aggregated (polymer) fraction, while lower band corresponded to the monomeric fraction.

Distribution of the Sup35 prion polymers by sizes was determined by using semi-denaturing electrophoresis in the agarose gel (SDD-AGE), as described previously.[22,51]

Polyclonal antibodies to Sup35C were a gift from Dr. D. Bedwell. Antibodies to Hsp104, Hsp26 and Hsp82 were a gift of Dr. S. Lindquist. Antibodies to Hsp70-Ssa were a gift of Dr. E. Craig. Antibodies to Ydj1 and Sis1 were a gift of Dr. D. Cyr. Antibodies to Ade2 were described previously.[22]

Statistical comparisons

Error bars shown on figures correspond to sample standard deviations (s), calculated according to the following formula: $s^2 = \Sigma(x_i - x)^2/(n - 1)$, where x_i refers to the value in each individual repeat, x to the average value (mean) of all repeats, and n to the number of repeats. Repeats of heat shock experiment for given strain and conditions always represented true biological replicates performed with independent cultures. The χ^2 statistics was used for evaluating the statistical significance of the asymmetry of [*PSI*$^+$] loss in the first division after heat shock (Fig. 1D). For more information, see ref.[68]

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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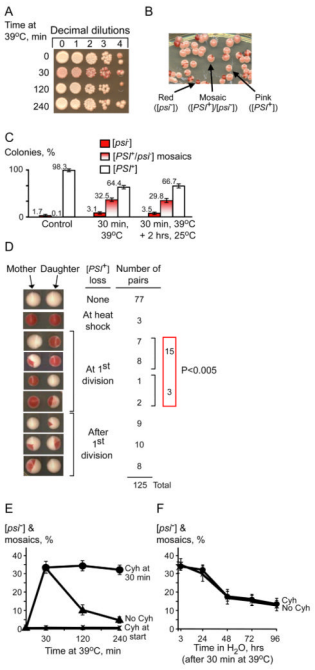

Fig. 1. [*PSI*$^+$] destabilization and recovery during mild heat shock

A - Cell viability during mild heat shock. Exponential yeast cultures were incubated at 39°C for the specified periods of time. Aliquots of each culture were taken before and after treatment, serial decimal dilutions were prepared, and 3 μl of each dilution were spotted onto YPD medium. Plates were photographed after 4 days at 30°C. B - Red ([*psi*$^-$]) and mosaic ([*PSI*$^+$]/[*pin*$^-$]) colonies, distinguishable from the predominant pink ([*PSI*$^+$]) colonies, are induced in by mild heat shock at 39°C. Heat shocked culture of the weak [*PSI*$^+$] strain OT55 was plated onto YPD medium, and [*PSI*$^+$] was detected by color as described in Materials and methods. C – Comparison of the proportions of pink, red and mosaic colonies in the OT55 culture plated immediately after 30-min heat shock at 39°C, and culture was allowed to grow in liquid YPD medium for additional 2 hrs at 25°C after heat shock and before plating. Averages of 6 experiments are shown, with error bars. D – Segregation of [*PSI*$^+$] in the first division after 30-min heat shock at 39°C. Mother and daughter cells were separated from each other by micromanipulation as described in Materials and methods. For each type of mother/daughter [*PSI*$^+$] distribution, numbers are shown. Probability of random deviation of the observed results from those expected in case of equal segregation of [*PSI*$^+$] between the mother and daughter cells is indicated. E - [*PSI*$^+$] destabilization and recovery during mild heat shock at 39°C, and effect of 100 μg/ml cycloheximide (Cyh) on these processes. F - [*PSI*$^+$] recovery from 30-min heat shock at 39°C during incubation in water at 25°C. Averages of at least 12 repeats are shown for each experiment, with error bars. In cases when error bars are not recognizable, they were less than the size of the symbol.

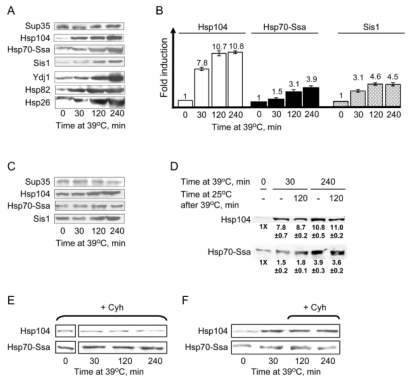

Fig. 2. Protein levels during and after mild heat shock

A - Levels of Sup35 and Hsps during heat shock. Exponential OT55 culture was heat shocked as described in Materials and methods and shown on Fig. 1. Aliquots were taken at each time point, total proteins isolated, run on the SDS-PAGE, transferred to the nitrocellulose membrane and reacted to the appropriate antibodies. Experiment has been repeated at least 2 times for each protein with similar results. B - Quantitative comparison of the Hsp104, Hsp70-Ssa and Sis1 levels in the exponential culture heat shocked at 39°C. Western blots, obtained with antibodies to Hsp104 and Hsp70-Ssa as described on panel A, were analyzed by densitometry, using Sup35 as a loading control. Fold induction relative to the level of respective Hsp in the non heat shocked culture, and standardized errors are shown. C – Comparison of the Hsp levels in the stationary culture, heat shocked at 39°C. Experiment has been performed at least 3 times for each protein with similar results. D - Comparison of Hsp104 and Hsp70-Ssa in heat-shocked cells and at 2 hrs after resumption of cell proliferation at 25°C. Protein ratios were normalized by using Ade2 protein as loading control. Experiment has been performed 3 times with similar results. D and E – Effects of 100 μl/ml cycloheximide (Cyh) added either before heat shock (C) or after 30-min heat shock (D) on Hsp104 and Hsp70-Ssa levels. Each experiment has been reproduced at least 5 times with similar results.

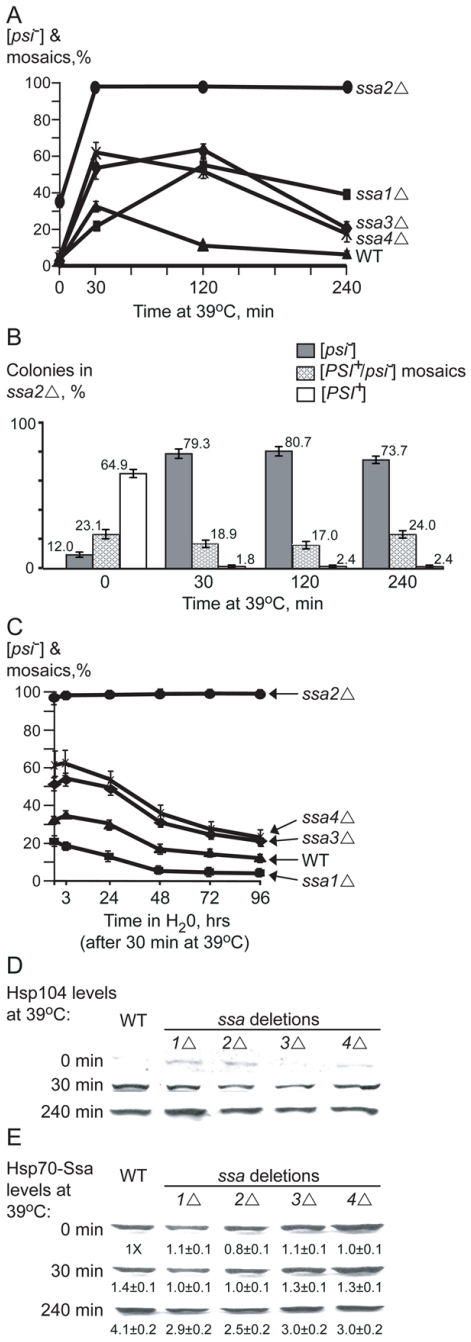

Fig. 3. Effects of *ssa* deletions during mild heat shock

A – [*PSI*$^+$] destabilization and recovery at 39°C in the *ssa* deletion strains. Wild type control (WT) is the same as on Fig. 1E ("No Cyh"). Averages of at least 7 experiments are shown for each *ssa* deletion strain, with error bars. B – Proportions of [*PSI*$^+$], mosaic [*PSI*$^+$]/[*psi*$^-$], and complete [*psi*$^-$] colonies in the *ssa2Δ* culture before and after heat shock. Averages of 7 experiments are shown, with error bars. C - [*PSI*$^+$] recovery from 30 min heat shock at 39°C during incubation in water at 25°C for *ssa* deletion strains. Averages of at least 4 experiments are shown with error bars. D - Hsp104 levels during mild heat shock are unaffected by *ssa* deletions. Densitometry results, using Ade2 as a loading control (not shown) confirm lack of statistically significant differences in Hsp104 levels between wild

type and *ssa* deletion strains for any time point. Each experiment has been repeated at least 3 times with similar results. E – Total Ssa levels in the wild type and *ssa* deletion strains before and during mild heat shock. Densitometry was performed by using Ade2 as a loading control. Numbers indicate levels of total Ssa relative to wild type exponential culture before heat shock. Each experiment has been repeated at least 5 times with similar results. On panels A and C, in cases when error bars are not recognizable, they were less than the size of the symbol.

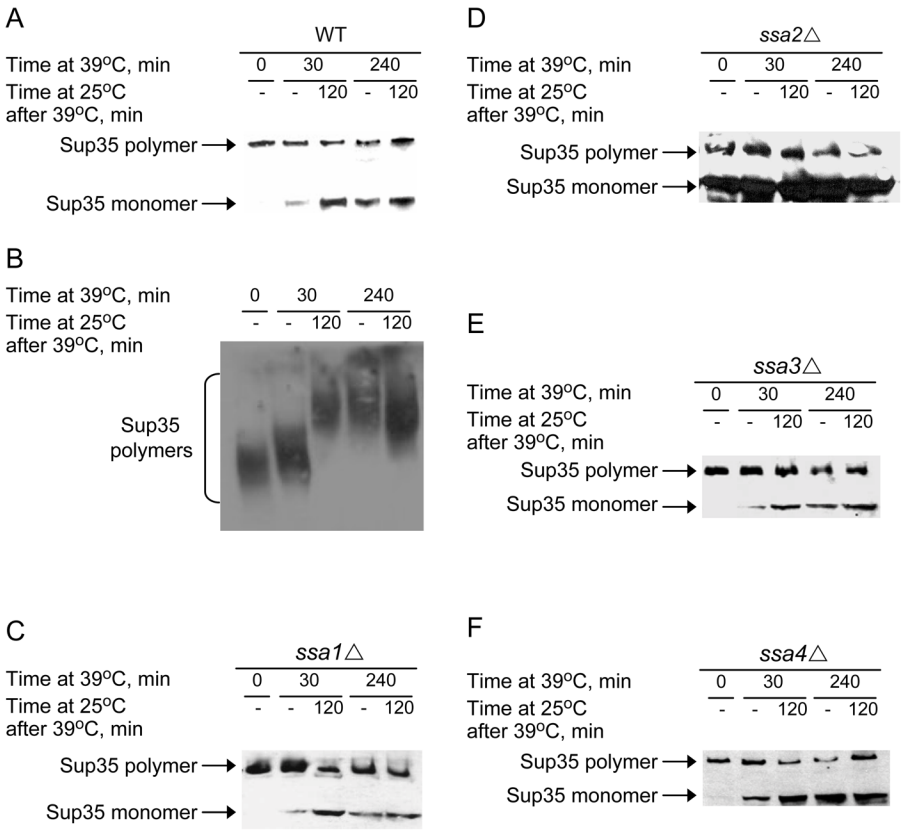

Fig. 4. Sup35 aggregation during and after mild heat shock

A – Proteins extracts the exponentially growing and heat shocked cultures of OT55 were fractionated by the "boiled gel" approach, as described in Materials and methods, and reacted to the Sup35C antibody. Experiment has been repeated 4 times with similar results. B – Distribution of the Sup35 polymers by size in the exponentially growing and heat shocked cultures of OT55, as visualized by semi-denaturing gel electrophoresis (SDD-AGE). Protein extracts were run on a 1.8% agarose gel at 4°C as described in Materials and methods, and reacted to the Sup35C antibody. Experiment has been repeated 5 times with similar results. C through F – Proportions of the polymeric versus monomeric Sup35 protein in the *ssa1Δ* (C), *ssa2Δ* (D), *ssa3Δ* (E), and *ssa4Δ* (F) cultures before and after heat shock, determined by "boiled gel" approach as on panel A. Each experiment has been repeated at least 3 times with similar results.

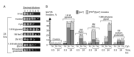

Fig. 5. Effects of osmotic stressors on yeast viability, proliferation and [*PSI*$^+$] maintenance

A – Effects of osmotic stressors on viability and proliferation of the yeast strain OT55 in liquid YPD medium. Yeast culture was grown to early exponential phase at 25°C, then diluted to 5×10^5 cells/ml in fresh YPD without (Control) or with specified chemical agent, and grown for additional 24 hrs, followed by washing cells three times with sterile water and spotting 4 μl of each serial decimal dilutions onto YPD plates, scanned after 4 days at 30°C. B - [*PSI*$^+$] curing by osmotic stressors, performed in the exponential cultures of the strain OT55, prepared as described for panel A and incubated in liquid YPD at 25°C, either in the absence (No) or in the presence (Yes) of the protein synthesis inhibitor cycloheximide (Cyh) at 100 μg/ml. Aliquots were taken after specified periods of time, cells washed three times with sterile water, resuspended in water and plated onto YPD. Presence of [*PSI*$^+$] was monitored by color assay as described in Materials and Methods, and in Fig. 1 legend. Proportions of complete [*psi*$^-$] and mosaic [*PSI*$^+$]/[*psi*$^-$] colonies are indicated by respective fillings. Each experiment has been performed 3 times with similar outcomes. Results of one typical experiment are shown in each case.

TABLE 1

Resistance of the stationary culture of weak [*PSI*$^+$] strain OT55 to the [*PSI*$^+$]-curing effect of heat shock

Time at 39°C, min	Colonies			Total number
	[*psi*$^-$]	Mosaic ([*PSI*$^+$]/[*psi*$^-$])	[*PSI*$^+$]	
0	0	4 (1.1%)	361	**365**
30	1 (0.4%)	1 (0.4%)	234	**236**
120	2 (0.8%)	2 (0.8%)	263	**267**
240	1 (0.3%)	4 (1.3%)	292	**297**

TABLE 2

Resistance of [*PIN*$^+$] and [URE3] prions to curing effect of heat shock

Time at 39°C, min	Prion composition of the yeast strain					
	[*PSI*$^+$ *PIN*$^+$]*		[*psi*$^-$*PIN*$^+$]		[*URE3*]	
	[*pin*$^-$]	[*PIN*$^+$]	[*pin*$^-$]	[*PIN*$^+$]	[*ure3-0*]	[*URE3*]
0		N/A	0	116	14 (8.2%)	157
30	0	56	0	292	12 (8.4%)	131
120	0	57	0	221	15 (8.5%)	161
240	N/T	N/T	0	162	16 (8.6%)	170

N/T- not tested, N/A - not applicable

*Presence of [*PIN*$^+$] was tested in [*psi*$^-$] colonies

TABLE 3

Yeast strains

Name	Genotypic background	Genotype	Prion composition	Reference
OT55	74-D694	*MAT***a** *ade1-14 his3 leu2 trp1 ura3*	[*PSI*$^{+}$ *PIN*$^{+}$]	21
OT56	74-D694	*MAT***a** *ade1-14 his3 leu2 trp1 ura3*	[*PSI*$^{+}$ *PIN*$^{+}$]	21
OT60	74-D694	*MAT***a** *ade1-14 his3 leu2 trp1 ura3*	[*psi*$^{-}$ *PIN*$^{+}$]	58
GT488	74-D694	*MAT***a** *ade1-14 his3 ura3 trp1 leu2*	[*PSI*$^{+}$ *pin*$^{-}$]	This study
GT234	GT81	*MATα ade1-14 his3 leu2 lys2 trp1 ura3*	[*psi*$^{-}$ *pin*$^{-}$]	47
YHE64	YHE64	*MATα leu2 trp1 ura2*	[*URE3*]	61