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Over-expression of the molecular chaperone Hsp104 in *Saccharomyces cerevisiae* results in the malpartition of [*PSI*$^{+}$] propagons

Frederique Ness*, Brian S Cox, Jintana Wongwigkarn**,
Wesley R Naeimi*** and Mick F Tuite

Kent Fungal Group
School of Biosciences
University of Kent, Canterbury
Kent CT2 7NJ, UK

***Current Address:** LBMS-IBGC-UMR, 5095-CNRS, Université Victor Segalen - Bordeaux 2, 33076 Bordeaux, France;
****Current Address:** Department of Microbiology and Parasitology, Faculty of Medical Science, Naresuan University, Phitsanulok 65000, Thailand
*****Current Address:** University of Arizona Molecular & Cellular Biology, Life Sciences South, PO Box 210106, Tucson, Arizona 85721, USA.

Contact: Professor Mick F Tuite, School of Biosciences, University of Kent, Canterbury, Kent, CT2 7NJ, UK
Email: M.F.Tuite@kent.ac.uk
Phone: 01227 823699

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ABSTRACT

The ability of a yeast cell to propagate [*PSI*$^+$], the prion form of the Sup35 protein, is dependent on the molecular chaperone Hsp104. Inhibition of Hsp104 function in yeast cells leads to a failure to generate new propagons, the molecular entities necessary for [*PSI*$^+$] propagation in dividing cells and they get diluted out as cells multiply. Over-expression of Hsp104 also leads to [*PSI*$^+$] prion loss and this has been assumed to arise from the complete disaggregation of the Sup35 prion polymers. However, in conditions of Hsp104 over-expression in [*PSI*$^+$] cells we find no release of monomers from Sup35 polymers, no monomerisation of aggregated Sup35 which is not accounted for by the proportion of prion-free [*psi*$^-$] cells present, no change in the molecular weight of Sup35-containing SDS-resistant polymers and no significant decrease in average propagon numbers in the population as a whole. Furthermore, we show that over-expression of Hsp104 does not interfere with the incorporation of newly-synthesised Sup35 into polymers, nor with the multiplication of propagons following their depletion in numbers while growing in the presence of guanidine hydrochloride. Rather, we present evidence that over-expression of Hsp104 causes malpartition of [*PSI*$^+$] propagons between mother and daughter cells in a sub-population of cells during cell division thereby generating prion-free [*psi*$^-$] cells.

INTRODUCTION

[*PSI*$^+$] is an epigenetic phenomenon in the yeast *Saccharomyces cerevisiae* brought about by the prion properties of the translation termination release factor eRF3 (Sup35) (review; Tuite & Cox, 2006). Strains propagating the [*PSI*$^+$] prion show a nonsense suppression phenotype due to a deficiency in polypeptide chain termination most likely as a result of partial inactivation of the function of Sup35, while [*psi*$^-$] cells contain monomeric Sup35 and are proficient in termination (Cox, 1965, Tuite et al., 1983, Wickner, 1994, Patino et al., 1996, Paushkin et al., 1996). Both full-length and N-terminal fragments of Sup35 readily form amyloid fibres *in vitro* that can promote conversion of the normal soluble form of Sup35 to an aggregated amyloid state both *in vitro* (Glover et al., 1997, DePace et al., 1998) and *in vivo* (Tanaka et al., 2004, King & Diaz-Avalos, 2004).

Stable propagation of the prion form of Sup35 as with other native yeast prions, requires the ATP-driven molecular chaperone Hsp104 (Chernoff et al., 1995, Sondheimer & Lindquist, 2000, Moriyama et al., 2000, Ferreira et al., 2001, Jung and Masison, 2001, Du et al., 2008, Alberti et al., 2009). Hsp104 is a stress-inducible hexameric protein that is able to disaggregate both amorphous protein aggregates that form in stressed cells as well as, *in vitro,* the more ordered detergent-resistant, amyloid fibres formed by most yeast prions (Parsell et al., 1994, Glover & Lindquist, 1998, DeSantis et al., 2012). Amyloid disaggregation does not involve serial monomerisation from the ends of fibres, but rather occurs via fragmentation at internal sites as a consequence of the extraction of individual prion protein monomers from the fibrils (review: Winkler et al., 2012a). Hsp104 is recruited to these sites by Sis1, an Hsp40, and Ssa1/2, members of the Hsp70 family of chaperones (Tipton et al 2008, Winkler et al 2012b).

Yeast strains partially or completely devoid of Hsp104 function are unable to efficiently propagate [*PSI*$^{+}$] or any other native yeast prion (Chernoff et al., 1995, Hattendorf & Lindquist, 2002a, Hattendorf & Lindquist, 2002b Kurahashi & Nakamura, 2006, Kurahashi & Nakamura, 2007). For example, inhibition of the ATPase activity of Hsp104 by guanidine hydrochloride (GdnHCl) (Ferreira et al., 2001, Jung & Masison, 2001, Jung et al., 2002; Grimminger et al., 2004) provides a rapid and reversible means of studying the role of Hsp104 in prion propagation *in vivo* (Ness *et al.,* 2002; ; Byrne et al., 2007, 2009, Park et al., 2012). 3 – 5mM GdnHCl blocks the ability of Hsp104 to fragment the amyloid polymers and by so doing prevents the generation of the new [*PSI*$^{+}$] “seeds” necessary to multiply and transmit the prion from mother to daughter cells (Byrne et al., 2007, Eaglestone et al., 2000, Wegrzyn et al., 2001, Ness et al., 2002). We refer to these prion genetic determinants as propagons (Cox *et al.*, 2003) to emphasize their genetic properties and to avoid confusion with material having seeding properties in *in vitro* experiments, with *in vivo* aggregates of GFP fusion proteins or with SDS-resistant oligomers detected on agarose gels. Although it is commonly assumed that various sub-cellular objects, such as fluorescent punctate dots, ribbons or circles formed by Sup35:GFP protein fusions in [*PSI*$^{+}$] cells, or the SDS-resistant polymers of Sup35 commonly used to identify the [*PRION*$^{+}$] state (Kryndushkin et al., 2003) are the genetic determinants, there is no clear evidence that they have the properties required of genetic determinants, that is multiplication and transmission. The best that can be said of them is that they are associated with [*PSI*$^{+}$] cells or cell cultures and never with [*psi*$^{-}$] ones.

One of the paradoxes of the relationship between the [*PSI*$^{+}$] prion - and only the [*PSI*$^{+}$] prion - and Hsp104 is that elevating the cellular levels of Hsp104 in growing cells also de-stabilizes the [*PSI*$^{+}$] prion state leading to prion-free [*psi*$^{-}$] cells (Chernoff et al., 1995). Hsp104 breaks protein aggregates into lower molecular weight forms that can then be dealt with by the other components of the chaperone machinery (Grimminger-Marquardt & Lashuel, 2010, reviewed by Winkler et al., 2012a). The role of Hsp104 in [*PSI*$^{+}$] prion propagation therefore reflects a balance between the need to break up the amyloid fibres into transmissible propagons to keep pace with cell division, and some process that causes their loss from cells when it is over-expressed.

A popular assumption has been that the loss during over-expression is the consequence of over-rapid disaggregation returning the prions to the non-prion form (Kushnirov & Ter-Avanesyan, 1998, True, 2006, Helsen & Glover, 2012a,b, Park et al., 2014). The analysis of the activity of Hsp104 on Sup35 polymers and their disaggregation *in vitro* (Shorter & Lindquist, 2004, 2006, Krzewska & Melki, 2006, Krzewska et al., 2007,) largely supported this interpretation. However, it remains an anomaly that the [*PSI*$^{+}$] prion is unique among yeast prions in that it is the only one eliminated by Hsp104 over-expression, although all are dependent on Hsp104 ATPase activity for their propagation, presumably through its disaggregase activity.

Recent *in vivo* studies have suggested that the elimination of [*PSI*$^{+}$] by over-expression of Hsp104 may in fact be by a mechanism distinct from the supposed enhanced fragmentation process (Hung & Masison, 2006, Tipton et al., 2008 Moosavi et al., 2010, Reidy & Masison, 2010, Winkler et al., 2012b, Helsen and Glover, 2012a,b). The significant observations are: (1) that the N-terminal region of Hsp104 is dispensable for all prion propagation, but is necessary for curing of [*PSI*$^{+}$] by over-expression (Hung and Masison, 2006); (2) loss of the co-chaperones Sti1 and Cpr7 interferes neither with the propagation of [*PSI*$^{+}$] nor with the curing of [*PSI*$^{+}$] by growth in GdnHCl, but loss of either or both does almost abolish the curing by over-expression (Moosavi et al., 2010; Reidy and Masison, 2010); (3) an Hsp104 binding site in the M-region of Sup35 allows binding without the cooperation of Hsp70 or Sis1 (Helsen and Glover, 2012a,b, Winkler et al., 2012a, Frederick et al, 2014) and deletion of residues 131 – 140 in the M region eliminates curing by over-expression, but has no other effect on [*PSI*$^{+}$] propagation (Helsen and Glover, 2012a,b).

A new hypothesis that has emerged to explain why high levels of Hsp104 lead to [*PSI*$^+$] loss is that excess Hsp104 actually prevents or reduces prion polymer fragmentation (Helsen & Glover, 2012a, Winkler et al., 2012a). This hypothesis goes as follows. The normal productive interaction between Hsp104 and its prion polymer substrate that leads to fragmentation is achieved via Hsp70-mediated recruitment of Hsp104 to the prion polymers via its N-terminal domain (Tipton et al., 2008, Winkler et al., 2012b). However, Hsp104 is also able to bind non-productively to the prion polymer without the need for Hsp70 and does so via the Sup35M binding-site (Winkler et al., 2012b). This binding is Hsp70-independent in cells with elevated levels of Hsp104, and this "non-productive" interaction out-competes Hsp70 for the Hsp104 binding to the Sup35 polymer, which is otherwise Hsp70 and Hsp40-dependent. By so doing it affects Hsp104-mediated polymer fragmentation. Winkler et al. (2012b) proposed that the effect of the non-productivity is to permit growth of the aggregates such that their transmission to daughter cells is hindered and so [*psi*$^-$] daughter cells segregate. This may explain the paradox that although Hsp104 is essential for the propagation of most other native yeast prions, its over-expression eliminates only the [*PSI*$^+$] prion suggesting that the non-productive binding of Hsp104 to prion polymers is a unique feature of the Sup35 protein. The sequence in the M-region of Sup35 that is needed for this interaction is, in turn, also essential for elimination by over-expression of Hsp104 (vide (3) above) and also unique to Sup35. It is probably significant that the Hsp70-dependent binding of Hsp104 is dynamic and labile, whereas that in the M-region, which is Hsp70-independent, is relatively stable (Frederick et al., 2014) and this may explain some of the observations we report here.

Both hypotheses make predictions about molecular, genetic and kinetic effects in cells unique to each. Here we present results from molecular and genetic *in vivo* studies of full-length Sup35, tagged or not with a hexa-histidine (His_6), that test these predictions. The central issue is whether fragmentation by over-expressed Hsp104 leads to the recovery of Sup35 monomers or other low molecular weight forms of oligomeric Sup35 from any of the aggregates associated with the [*PSI*$^+$] state, as would be expected if the assertion by Park et al. (2014) were true. Secondly, it is not clear whether Winkler et al. (2012b) expect the "non-productivity" associated with Hsp104 over-expression to mimic the non-productivity caused by GdnHCl: if it were, presumably the kinetics of elimination of [*PSI*$^+$] would be similar for inhibition of fragmentation by over-expression and inhibition by growth in GdnHCl. Thirdly, if "non-productivity" caused malpartition, it would be expected to manifest in the inheritance of propagons.

In addition to addressing these three issues, we include assays to compare the effects of over-expressing wild type Hsp104 with over-expressed Hsp104 deficient in its ATPase function (Hsp104:K218T+K620T, abbreviated here to Hsp104-2KT) and assays to determine whether Hsp104 over-expression interferes with regeneration of depleted propagon numbers or affects the size of SDS-resistant polymers. We describe experiments which examine the consequences of Hsp104 over-expression on the genetic determinants of [*PSI*$^+$] (i.e. propagons) and on the behavior of the Sup35-containing molecular structures associated with the [*PSI*$^+$] phenotype, such as SDS-resistant polymers and the higher molecular weight objects that sediment under ultracentrifugation. In particular, we assay the release of Sup35 from the latter objects to determine whether there is significant degradation under these conditions. However, we make no assumptions about the role, if any, of these structures in propagation of the [*PSI*$^+$] phenotype. We use the term “propagon” to indicate entities that are assayed by their inheritance, i.e. phenotypic effects in dividing cells, the terms “polymers” or “SDS-resistant polymers” to designate material identified immunologically on SDD-AGE gels and the term “aggregates” for microscopically identified fluorescent foci or for material found in the pellets of cell-extracts subjected to ultracentrifugation (see Materials and Methods). We make no assumptions about their relationships except that they all contain Sup35 in some form.

Our data are in part consistent with the findings of Winkler et al (2012b) and show no evidence of enhanced polymer fragmentation in cells over-expressing Hsp104. A decrease in electrophoretic mobility was observed by Winkler et al (2012b) in Sup35:YFP [*PSI*$^+$] foci in cells that results from Hsp104 over-expression, and could in principle be the cause of a block in transmission. Here we show that Hsp104 over-expression does indeed affect transmission of propagons to daughter cells in a minority of cell divisions at any one time, but, contrary to the proposal of Winkler et al. (2012b) there is little effect on productivity, i.e. the multiplication of propagons.

RESULTS

The kinetics of [*PSI*$^+$] elimination by over-expression of Hsp104.

When wild-type Hsp104 was over-expressed in the 74D-694 [*PSI*$^+$] strain, cells that had lost all [*PSI*$^+$] propagons and thus generated pure red [*psi*$^-$] colonies (i.e. with no white

[*PSI*$^+$] sectors), were first observed approximately 1 - 1.5 generations post-induction. The number of pure [*psi*$^-$] colonies then continued to increase linearly over ten generations at approximately 10% per generation (**Figure 1A**). There are typically 200 - 400 propagons in a [*PSI*$^+$] cell (Cox et al., 2003) and, for the observed kinetics of propagon loss to be observed, the propagons would all have to be eliminated in a minority of cells (i.e. about 10%) in each generation, but with some left intact in the remaining cells. Continued over-expression of *HSP104* was necessary for prion elimination as approximately one generation after transfer of the cells to YEPD, a glucose-based rich medium which represses the *GAL1* promoter, no further prion loss was detectable (data not shown).

The addition of 3 mM GdnHCl, a concentration known to inhibit several ATPase-dependent biological activities of Hsp104 (Ferreira et al., 2001; Jung and Masison 2001, Grimminger et al 2004), resulted in a reduction in the rate of [*PSI*$^+$] loss to 4% per generation in cells over-expressing Hsp104 (**Figure 1A**). This finding suggests that the induced loss of [*PSI*$^+$] was only partially dependent on the ATPase activity of Hsp104. However, the failure of 3 mM GdnHCl to completely prevent over-expression prion curing and instead induce curing by dilution may be due in part due to this concentration of GdnHCl being insufficient to inhibit completely the ability of propagons to replicate *in vivo* in the defined medium used here; in complex YEPD medium full inhibition of Hsp104 activity is seen at 3 mM (Ness et al., 2002, Byrne et al., 2007, 2009,). Alternatively, it may simply reflect the 20 - 40-fold higher levels of Hsp104 in the over-expressed cells.

The kinetics of [*PSI*$^+$] loss by over-expression of Hsp104-2KT, an ATPase-negative mutant of Hsp104 (Chernoff et al., 1995), were essentially identical to those observed when [*PSI*$^+$] cells were either over-expressing *hsp104-2KT* in the presence of 3 mM GdnHCl (**Figure 1B,** filled symbols) or not (open symbols). The kinetics of curing is also identical to that observed when cells are grown without over-expression of *hsp104-2KT* in the presence of 3mM GdnHCl alone (data omitted but see caption and Cox et al., 2003; Cole et al, 2004; Byrne et al, 2007, 2009). This observation is consistent with the competitive inhibition, by an excess of the ATPase-negative mutant, of the wild-type Hsp104 present in these cells (Chernoff et al., 1995, Wegrzyn et al., 2001, DeSantis et al., 2012). Importantly, the kinetics observed differ dramatically from the kinetics of [*PSI*$^+$] loss seen here when wild-type Hsp104 was over-expressed, most notably with regards to the increased lag before the appearance of [*psi*$^-$] cells and the rate of loss at 50% per generation once prion-free cells arise in the culture (**Figure 1B;** see also **Figure 6B**

showing the effect on propagon numbers during such treatment: both these Figures demonstrate a halving of propagon numbers/cell with each cell generation). The presence of 3 mM GdnHCl made no difference to the rate of [*PSI*$^+$] loss induced by over-expression of the *hsp104-2KT* allele (**Figure 1B**, filled symbols).

In analysing [*PSI*$^+$] loss following *HSP104* or *hsp104-2KT* over-expression, only colonies that were wholly red were scored as [*psi*$^-$]. However, a significant proportion of colonies scored carried red sectors and the nature of these sectors differed depending on whether or not it was the wild-type *HSP104* or the *hsp104-2KT* allele that was over-expressed (**Figure 1C**). For the *hsp104-2KT* allele, the number and size of red sectors in otherwise white [*PSI*$^+$] colonies increased with time, with hair-line red sectors clearly observable in colonies approximately three generations after galactose induction. By 5 generations approximately 50% of the colonies contained large red sectors, but subsequently this number declined and whole red [*psi*$^-$] colonies begun to appear. The same trend was seen when 3 mM GdnHCl was present during over-expression (data not shown). Sectored colonies do not appear on colonies plated during curing with 3 mM GdnHCl alone: only when *hsp104-2KT* is being over-expressed. When the wild type *HSP104* allele was over-expressed, sectoring was restricted largely to half and quarter red sectors appearing concomitantly with the first appearance of wholly red [*psi*$^-$] colonies (**Figure 1C**). *HSP104* and *hsp104-2KT* over-expression thus led to clear differences in both the kinetics of [*PSI*$^+$] elimination and in the nature of the red/white colony sectoring, indicating that [*psi*$^-$] cells arise by different means under the two treatments.

A small red [*psi*$^-$] sector in an otherwise [*PSI*$^+$] colony – as typically seen with the over-expression of the *hsp104-2KT* allele – suggests that prion loss is delayed until late in the development of the colony. Plating the galactose-grown cells onto ¼YEPD to score the [*PSI*$^+$] phenotype would lead to an immediate repression of the expression of the plasmid-borne *GAL1*-regulated *hsp104-2KT* gene. As growth of the cells in galactose was continued, both the number and size of [*psi*$^-$] red sectors increased. This is coupled with a reduced number of propagons through dilution at cell division.

A colony of ~1.5 mm diameter typically represents ~26 generations (~10^8 cells) from the time of plating of individual cells to the cessation of colony growth. This suggests that during *hsp104-2KT* over-expression there is a progressive accumulation or maintenance in cells of factors which affect the ability to generate or segregate [*PSI*$^+$] propagons at cell

division and that this condition is maintained in some cells for many generations after over-expression ends as a result of plating.

Over-expression of Hsp104 does not release soluble Sup35 from its polymers in [*PSI*$^+$] cells

If elevating the levels of Hsp104 in the absence of other chaperones leads to the complete disaggregation of Sup35 fibrils in [*PSI*$^+$] cells, then this should be evident from an analysis of the proportion of non-sedimentable Sup35 under such conditions. In a strong [*PSI*$^+$] variant of the strain 74D-694, 98% or more of cellular Sup35 is present in high molecular weight aggregates which can be pelleted from cell extracts by centrifugation at 96,000 x g (**Figure 2**). After 5 generations growth in the presence of 3 mM GdnHCl, the proportion of cellular Sup35 material appearing in the supernatant increased to 50% because less of the newly-synthesised Sup35 becomes incorporated into fibrils as the number of propagons per cell (prion seeds) decreases, although because fragmentation stops, and the aggregates continue growing, the amount of sedimentable material does not decrease by so much. (Ness et al., 2002). However, 97% of the cells still contained one or more propagons and were able to form [*PSI*$^+$] colonies. After 5 generations of over-expression of the *hsp104-2KT* allele, which also inhibits the ATPase activity of Hsp104, again approaching 100% of the cells contained one (or more) propagons although significantly less of the Sup35 appeared in the supernatant fraction when compared to GdnHCl-treated cells (**Figure 2**). In contrast, when the level of wild-type Hsp104 was elevated in the same strain in the absence of GdnHCl, 35% of the Sup35 was present in the non-sedimentable fraction after 5 generations, but in this case 30% of the cells had no [*PSI*$^+$] propagons and hence generated only [*psi*$^-$] colonies (**Figure 2**).

These results demonstrate that over-expression of Hsp104 for 5 generations leads to an increase in non-sedimentable Sup35 in a [*PSI*$^+$] strain, but in contrast to over-expression of the *hsp104-2KT* allele where a similar level of non-sedimentable Sup35 was detected, 30% of the cells had no propagons. These results do not clarify whether the observed non-sedimentable Sup35 is derived from the total monomerisation of pre-existing Sup35 polymers in 30-35% of cells or comes from the presence of a sub-population of cells which are [*psi*$^-$] that have been generated by some other means. We therefore next established the fate of the Sup35 molecules that were present in the prion polymers prior to induction of Hsp104 over-expression.

The 74D-694 [*PSI*$^+$] strain was engineered to express a fully functional form of Sup35 carrying a C-terminal hexa-histidine tag (Sup35-His_6; Ness et al., 2002) under the control of the *GAL1* promoter. The Sup35-His_6 protein behaves exactly as wild-type Sup35 in terms of its distribution between supernatant and pellet fractions in [*PSI*$^+$] and [*psi*$^-$] cells and in terms of its function in translation termination and it forms aggregates in [*PSI*$^+$] cells, but not in [*psi*$^-$] cells and loses them when converted to [*psi*$^-$] by growth in guanidine (Ness et al., 2002). These same cells also carried the *HSP104* gene under the control of the copper-inducible *CUP1* promoter, and in addition had the wild-type *SUP35* gene intact in the genome. Cells of this strain, initially grown in galactose, were switched to a glucose-only medium containing 40 μM $CuSO_4$ to repress production of Sup35-His_6 while at the same time inducing over-expression of Hsp104. The levels of both the Sup35-His_6 and the total Sup35 were then determined for both the soluble and pellet fractions after four generations post repression/induction.

Even after 4 generations of growth under induction/repression conditions, no significant increase in the trace levels of Sup35-His_6 in the supernatant fraction was observed even though 25% of the cells were [*psi*$^-$] after 4 generations (**Figure 3A**). However, total Sup35 levels in the supernatant fraction did increase significantly by 4 generations. These data are therefore consistent with the Sup35His_6 being already present in a polymerised form prior to Hsp104 over-expression and remaining in this form thereafter. The Sup35 appearing in the supernatant after the over-expression of Hsp104 must have been synthesised in the newly-generated [*psi*$^-$] cells following Cu^{2+}-induced over-expression of the *HSP104* gene. These data suggest that Sup35 molecules already present in polymers in the [*PSI*$^+$] strain are not released when Hsp104 levels are elevated. It was clear that Hsp104 over-expression had not caused enhanced fragmentation and disassembly of the pre-exiting Sup35His_6 prion polymers.

The effect of over-expression of Hsp104 on incorporation of monomeric Sup35 into aggregates

To establish whether elevated levels of Hsp104 prevented newly synthesised Sup35-His_6 from entering the [*PSI*$^+$] prion aggregates, the synthesis of Sup35-His_6 was now switched on concomitantly with *CUP1*-induced Hsp104 over-expression. Under these conditions all newly-synthesised Sup35-His_6 appeared in the material pelleted at either 2,500 x g

(P^1) or 96,000 x g (P^2) (**Figure 3B**). Although we expected to see a small amount (~5 to 10%) of the Sup35-His_6 from the [*psi*$^-$] cells present in the culture at the time of sampling, only trace amounts of the non-sedimentable Sup35-His_6 could be detected in the supernatant fraction generated at 2,500 x g (S^1) centrifugation (**Figure 3B**). Elevated levels of Hsp104 therefore do not prevent newly synthesised monomers of Sup35 from being incorporated into fibrils: i.e. there is not a polymerisation defect. The lower centrifuge speed assay was included in case newly-formed Sup35His_6 aggregates were significantly smaller or unstable as a result of over-expression.

Sup35 polymer size does not change in [*PSI*$^+$] cells over-expressing Hsp104

The average molecular mass of the Sup35 polymers that form in [*PSI*$^+$] cells reflects the relative balance reached between the rate of Sup35 polymerisation (i.e. monomer addition) and the rate of fragmentation of the polymers by Hsp104 (Tanaka et al., 2006). Inhibiting the disaggregase activity of Hsp104 by 3 mM GdnHCl would therefore be expected to lead to an increase in the size of SDS-resistant Sup35 polymers and has been shown to occur in [*PSI*$^+$] strains by SDD-AGE analysis (Kryndushkin et al., 2003). The converse would be expected if dissaggregase activity by Hsp104 were increased.

SDD-AGE analysis of Sup35 polymers in [*PSI*$^+$] cells over-expressing Hsp104 was carried out after 0 and 4 generations of growth post induction of Hsp104 synthesis (**Figure 4**). After 4 generations of over-expression of Hsp104, 24% of the cells were [*psi*$^-$], and a decrease in the relative amount, but not the size, of the SDS-resistant Sup35 polymers was observed. This experiment was repeated many times by different workers and in no case has the decrease in polymer size predicted by an increase in fragmentation activity of Hsp104 been observed, nor has an increase in size indicative of a block in the fragmentation activity of Hsp104 on over-expression, as proposed by Winkler et al. (2012b) and Helsen and Glover (2012a). However, in their [*PSI*$^+$] strain, 5V-H19, Kryndushkin et al. (2003) showed an approximately two-fold increase in size of SDS-resistant polymers in an over-expression experiment.

Importantly, neither in **Figure 2,** nor in **Figure 4** is a distinction made between newly-synthesised monomers and any released from pre-existing polymers, but this distinction is so made in **Figure 3.**

HSP104 over-expression does not inhibit propagon multiplication

To establish whether elevated levels of Hsp104 prevented the *de novo* generation of new [*PSI*$^+$] propagons, we estimated the number of propagons (n_P) in individual cells post induction. The method used involved an analysis of the kinetics of loss of [*PSI*$^+$] in YEPD growth medium containing 3 mM GdnHCl (Cole et al., 2004, Eaglestone et al., 2000). Using the 74D-694 [*PSI*$^+$] strain transformed either with pUKC1832 (*GAL1-HSP104*) or pRS316 (the backbone plasmid) first the cells were grown on glucose-based selective medium for 4 generations in 3 mM GdnHCl (**Figure 5A**). This resulted in a significant drop in the numbers of propagons (n_P) (**Figure 5B**, Glucose control: **a,** without GdnHCl versus **b,** with GdnHCl) although as previously reported, in **b**, >99% of the cells still generated [*PSI*$^+$] colonies on ¼ YEPD (Byrne et al., 2009, Eaglestone et al., 2000). The GdnHCl-exposed cells were then transferred to a galactose/raffinose-based medium either with (**d**) or without (**c**) 3 mM GdnHCl. The cells were then allowed to go through one generation at which point the number of propagons (n_p) was again counted. Over-expression of Hsp104 had no effect on the amount or timing of regeneration of new propagons when released from the GdnHCl-induced propagation block with complete recovery of normal numbers being observed within one generation of removal of the block whether or not the levels of Hsp104 were elevated in those cells (see **Figure 3B** for Hsp104 over-expression after 0.5 to 1 generation in galactose/raffinose medium). Loss of [*PSI*$^+$] when Hsp104 levels are elevated is therefore not due to an inability to generate new propagons.

Transmission of [*PSI*$^+$] propagons to daughter cells is disturbed in cells over-expressing Hsp104

Given that high levels of Hsp104 *per se* did not modify the number of propagons (n_P) generated in over expressing cells, nor destroy pre-existing propagons in 74D-694 [*PSI*$^+$] cells, we next investigated whether the loss of [*PSI*$^+$] from these cells could be due a failure to transmit propagons to daughter cells at cell division. During cell division, propagons are normally effectively distributed between mother and daughter cells with a small but significant bias towards retention by the mother cell. This segregation bias is shown by experimentally determining π, the fraction of propagons in a mother-daughter pair that are transmitted to the daughter. This is typically ~ 0.4 (Cox *et al*. 2003; Cole *et al*. 2004; Byrne *et al*., 2007, 2009). If partition were affected by Hsp104 levels in any dividing cell, then this value would decrease if propagons were retained in the mother cell

(or increase if they passed preferentially to the daughter). Either of these situations would increase the variance i.e. the range of the numbers of propagons found in a population of cells.

To establish whether elevated levels of Hsp104 affected π, n_P was determined in both mother and daughter cells over a number of generations of growth post-induction of Hsp104 synthesis, using the single-cell isolation method of Cox et al. (2003) to determine n_P (**Figure 6A**). The spread of propagon numbers in both mother and daughter cells remained similar over time in the control cultures but the range overall and separately, in both mothers and daughters, increased dramatically at each time point. The data also show that the overall means for the distributions of mother and daughter numbers in the later over-expressed cultures changed with time, the mothers' increasing and the daughters' decreasing over the last three time points. Nevertheless the average number of propagons per cell when mother and daughter numbers were pooled over this period of HSP104 overexpression remained constant, indicating propagon propagation continued at normal levels in the culture as a whole. **Figure 6B** shows, for comparison, the propagon numbers found in cells over-expressing the ATPase-deficient Hsp104-2KT (also cf. **Fig1B**). The regression in this experiment shows a decline in numbers by one-half in each generation, as has been shown elsewhere when Hsp104 activity is inhibited by GdnHCl (Cox et al., 2003). In **Figure 7** the numbers of propagons at successive sampling times for mother-daughter pairs plotted in **Figure 6A** were replotted on separate graphs, plotting mothers' numbers against their daughters, with an indication of how they matched or otherwise the limits of the mother-daughter partitions observed in the controls. In the [*PSI*$^+$] cells induced to over-express Hsp104, over the 5.7 generations monitored, there was a progressive increase in the number of cell divisions in which the mother:daughter partition numbers fell outside the t_0 limits with an increasing number of daughter cells receiving few or no propagons. Of the 57 pairs of mother and daughter cells sampled after 5.7 generations post induction of Hsp104 over-expression, eight daughters received no propagons ($\pi=0$), but there was no significant change in either the mean or the median of the distribution of n_P among the mother-daughter pairs counted. It is apparent from the plots in **Figure 7** that as time passed, the number of propagons in daughter cells tended to decrease, falling below the level of the control box, while numbers in mother cells tended to increase and fall to the right of the control box. This is an indicator of malpartition rather than selective destruction of propagons.

Nevertheless, the malpartition of propagons between mother and daughter only occurred in a minority of cells; for example, after 5.7 generations, 75% (43/57) of the mother:daughter pairs had values of π falling within the control and the t_0 limits. The increase in the range of data illustrated in **Figures 6 & 7** was subjected to a meta- analysis to show the trends in variance (**Figure 8)**. A progressive increase in the variance in the numbers of propagons in the over-expressing culture was observed when propagons in mothers and daughters were summed (**Figure 8A**). This effectively measures the variances of the numbers in the undivided parent cells. In **Figure 8B,** mothers and daughters are considered, each cohort separately or as a single cohort, with the numbers pooled. In the t_0 and in the [*PSI*$^+$] control cell populations carrying the backbone plasmid pRS316, the ratio of the standard deviation to the mean, a coefficient of variance that normalises between populations that have different means, was typically ≤ 0.45 (**Figure 8B)**. This value remained unchanged over time for eight generations in control populations whereas in cell populations in which Hsp104 was over-expressed, this value approached 1.2 after 5.7 generations (**Figure 8A, B**). Comparison of the variances of mothers alone, daughters alone and both sets of values amalgamated (i.e. not summed pairs) revealed no significant differences in the progressive increase in variance between the different categories of cells overexpressing Hsp104 (**Figure 8B**). This is in spite of the fact that there was a progressive decline in the means of propagon numbers in daughter cells and a concomitant increase in the means of propagon numbers in mother cells. Finally, **Figure 7E** shows the data displayed in **Figure 6A** replotted as values of π, the fraction of propagons transmitted to daughter cells at cell division and serves as a visual illustration of the increase in the range of π-values in over-expression conditions compared to controls.

The regressions (slopes) of covariance on time are significant and significantly different between control and over-expressed cultures with many mother cells in this population and earlier ones having fewer propagons than normal. These are expected as these are descendants of daughters which have suffered malsegregation of propagons in earlier generations. Few of them seem to inherit the malsegregation defect and no longer belong to the subset of cells which do.

To summarise, three important findings emerge from these data: (i) the range of values for n_P, is much higher after Hsp104 over-expression (**Figure 6A** diamonds) than in the parallel control cultures (**Figure 6A,** squares**),** but shows little change in the means of the

492 distributions. This is true of the total set of values (expressed numerically as their co-
493 variances in **Figure 8A,B**) disregarding whether they were taken together from both
494 mother and daughter sets, treating mother and daughter sets of numbers separately or
495 summing each mother-daughter pair for the calculation of variance. (ii) The n_P values for
496 daughter cells occupy a lower range with a smaller mean than those for mothers, in spite
497 of the increase in variance (i.e. the range of values) (**Figure 6A**). (iii) Segregations where
498 n_P in the daughter decreases show concomitant increases in the number of propagons in
499 the mother. This is most apparent in the increases in the maximum values observed in
500 mother cells and the decreases in minimum numbers in daughter cells, while the overall
501 median values of both control and over-expressed cultures remained close to 200 per
502 cell, over nearly six generations of treatment (**Figure 6A**).
503
504 These experiments provide *prima facie* evidence that the partition of propagons between
505 mother and daughter cells at cell division is disturbed in many (but not all) of the [*PSI*$^+$]
506 cells over-expressing Hsp104 and that this can account for the steady accumulation of
507 [*psi*$^-$] cells in the population when this chaperone is over-expressed (**Figure 1A**). At the
508 same time the numbers show that overall, there is no shift in the balance between growth
509 and fragmentation of propagons, meaning that both the numbers of propagons per cell in
510 the population as a whole remain constant, as do their sizes (**Figure 4**).
511
512
513 **DISCUSSION**
514
515 We have tested the hypothesis that over-expression of Hsp104 causes disaggregation of
516 the Sup35-containing aggregates present in [*PSI*$^+$] cells by determining the release of
517 monomeric (or non-sedimentable oligomeric) Sup35-His$_6$ previously incorporated into
518 sedimentable material. We were unable to detect any sign of such release over four
519 generations of growth, during which 42% of the population had become [*psi*$^-$]. In
520 conditions of over-expression all Sup35-His$_6$ remained sedimentable. Of course,
521 considerable amounts of non-sedimentable Sup35 were observed in these conditions,
522 and could be accounted for by new synthesis of non-tagged Sup35 in [*psi*$^-$] cells cured of
523 [*PSI*$^+$] (**Figure 3A**). There is one caveat which must be applied to this experiment, namely
524 that Sup35, modified or not, may in addition to amyloid-based aggregates, form
525 aggregates impervious to Hsp104 degradation (Salnikova et al 2005). However, neither

non-tagged nor His_6-tagged Sup35 form precipitable material in [*psi*$^-$] strains and such material invariably disappears when [*PSI*$^+$] strains are cured by GdnHCl.

Our results also show that Hsp104 over-expression does not interfere with the incorporation of newly-synthesised Sup35-His_6 into sedimentable material (**Figure 3B**). Furthermore, we find that over-expression of Hsp104 did not in any way interfere with the regeneration of propagon numbers after their numbers had been depleted by blocking the ATP-driven disaggregase function of Hsp104 by GdnHCl (**Figure 5)**. This suggests that the Hsp70-independent binding of Hsp104 in conditions of over-expression, as demonstrated by Winkler et al. (2012b), does not render propagons “non-productive”, as suggested by these authors.

The continued propagation of [*PSI*$^+$] prions when Hsp104 was over-expressed was also evident in the experiments which followed the inheritance at cell division of the [*PSI*$^+$] phenotype (**Figures 6 & 7**). These showed that in a fraction of the population of [*PSI*$^+$] cells in each generation, there was malpartition of propagons. This took the form of retention of propagons in the mother cell in about 10% of the divisions. That this was malpartition and not due to any selective destruction of propagons in daughter cells was implicit in the concomitant appearance of mother cells with numbers above the upper limit of the range observed both in control and t = 0 samples (**Figures 6A** & **7A - D**) and by the decrease in the means of propagon numbers in daughter cells but without a simultaneous increase in the mean of mother cells’ numbers. We also calculated the variance of propagon numbers at successive times during over-expression and found that variance increased with time equally in mother cells, daughter cells, dividing cell pairs (that is in the mother and daughter cell of each pair, summed) and in all cells pooled. Variance is a measure of the spread of data values about the mean and malpartition increases the spread because it generates mother cells with higher numbers of propagons than usual and daughters with fewer. We have illustrated this effect in **Figure 7A – D** in which the propagons in mother-daughter pairs are plotted, comparing t = 0 values with those observed in samples taken at different times during over-expression. **Figures 8A** and **B** plot the variances in the four types of population (mothers alone, daughters, mother-daughter pairs summed and all values pooled); **Figure 6A** illustrates the spread of values at each time point, distinguishing the daughter cell values (shaded) from those of mothers and **Figure 7E** shows the difference in the spread of values of π between control and over-expressed cells. It should be noted that the calculations are of

the coefficient of variance, i.e. *Standard Deviation/Mean*, which normalizes for any differences between the means of any data sets.

These experiments show that malpartition occurs during over-expression and quantitatively accounts for the curing of [*PSI*$^+$] in our conditions. We have failed to detect any degradation of sedimentable aggregates containing His_6-tagged Sup35 and SDS-resistant polymers showed no change in size during over-expression (**Figure 4**), suggesting neither disaggregation leading to a decrease in size, nor decline of fragmentation rates leading to any increase in size of such aggregates. That SDS-resistant aggregates increase in size during the over-expression of Hsp104 in 57V-H19 (Kryndushkiin et al., 2003) may reflect a difference in the relationship between fragmentation rate and polymer growth in this strain, leading to fewer propagons and greater size (Cole et al., 2004; Tanaka et al., 2006) but information about numbers is lacking. We note that over-expression in the Kryndushkin et al. experiments was achieved using a multicopy plasmid without any medium shift. The discrepancy does not affect our argument.

The kinetics of [*PSI*$^+$] elimination

While we have not established a mechanism of malpartition, it is possible to discern some of its properties. Firstly, malpartition is wholly or partly dependent on the ATPase activity of Hsp104. Over-expression in the presence of GdnHCl reduces the rate of curing by one half to 5% per generation (**Figure 1A).** When the ATPase activity of Hsp104 was inhibited by over-expressing the *hsp104-2KT* mutant, the curing kinetics were identical to that seen in GdnHCl-mediated curing (**Figure 1B**). There is evident no over-expression curing characteristic of wild type Hsp104 over-expression, which is characterized by a very short lag before [*psi*$^-$] segregants are observed and a subsequent linear loss of [*PSI*$^+$] cells. It is clear that *hsp104-2KT* over-expression curing occurs by dilution following the competitive inhibition of Hsp104 and doing this in the presence of GdnHCl made no difference to the kinetics. The most graphic demonstration of the difference between over-expressing the wild-type and the ATPase negative mutant Hsp104 is shown in **Figure 6**, which records propagon counts in the two situations. When wild-type is over-expressed, average propagon numbers are maintained over nearly six generations while [*psi*$^-$] segregants start appearing within less than two generations (and cf. **Figure 1B**). By contrast, over-expression of *hsp104-2KT* results in an immediate halving of the average

propagon numbers in each generation (**Figure 6B),** but a lag before the appearance of [*psi*$^-$] cells, of six generations (cf. **Figure 1B**).

We have followed the inheritance of propagons during curing by over-expression of *hsp104-2KT* as shown in **Figure 6B,** and found no increase in variance at any time point up to 2.56 generations, nor malpartition at this time point.

It should be noted that except for the mutations in the ATPase sites of Hsp104, there is no difference between the two alleles being over-expressed in these experiments. The N-terminal domain necessary for [*PSI*$^+$] prion curing by over-expression in dividing cells is present in both experiments as is the Sup35 M-region required for Hsp70-independent binding and important for curing by over-expression and malpartition, is present in the Sup35 of both experiments.

Although the ATPase activity of Hsp104 is required for both fragmentation and malpartition, the difference lies in the nature of the binding of Hsp104 to Sup35 (Frederick et al., 2014): either the Hsp70-dependent binding which may well be sequence-independent or the specific Sup35 M-region to which the Hsp104 N-domain binds when *HSP104* is over-expressed. This binding does not require Hsp70 cooperation, but may need Sti1 and Cpr7 (Moosavi et al., 2010; Reidy & Masison 2010). Winkler et al. (2012b) reported that GdnHCl inhibits the binding of over-expressed Hsp104 to Rnq1 prion aggregates, and it may partly do so with the Sup35 prion. This would account for a reduction of the curing effect of over-expression seen in **Figure 1A.**

There is also a difference in the dynamics of the two modes of Hsp104 interaction with Sup35 (Frederick et al., 2014: see below) which is relevant to the observations we have made. They show that Hsp104-dependent binding at normal levels of expression is labile, with a high turnover, but that with over-expression, the binding becomes stable and is independent of a requirement for Hsp70. We therefore conclude that there was not necessarily any difference in the amount of “Hsp70-independent binding” to the M-region of Sup35 when either the wild-type Hsp104 or the Hsp104$^-$2KT mutant was over-expressed, but the consequences differed because of the difference in ATPase activity.

Evidence of high-stability M-domain binding of Hsp104-2KT

There is evidence that over-expressing the Hsp104-2KT mutant has a unique long-term effect, lasting even after over-expression is terminated. The [*PSI*$^+$]/[*psi*$^-$] phenotypic status of cells during the over-expression period is assayed by plating the cells on ¼ YEPD agar, which has the effect of blocking transcription of the *hsp104-2KT* gene from the *GAL1-10* promoter. Plating is followed by the appearance of red [*psi*$^-$] sectors on several of the [*PSI*$^+$] colonies that grow (**Figure 1B**). These sectors are few in number and small in size in the colonies arising from samples taken early in curing, but become more numerous and larger in later samples (**Figure 1C**). It should be remembered that during the curing, by *hsp104-2KT*, the number of propagons is decreasing (**Figure 6B**) (Cox et al., 2003, Byrne et al 2014) but that no wholly [*psi*$^-$] colonies appear for six generations (**Figures 1B)**. Such sectors are clearly a hangover of the *hsp104-2KT* over-expression. They do not appear when [*PSI*$^+$] is cured by GdnHCl inhibition of the Hsp104 ATPase. The delay in their appearance following inhibition of over expression when plated on glucose medium is evidence for a stable change resulting from the interaction of *hsp104-2KT* and Sup35 propagons. The [*psi*$^-$] sectors may reflect the presence of aggregates (or propagons) containing Sup35M-bound Hsp104-2KT which malpartition, segregating [*psi*$^-$] cells at earlier and earlier stages in colony development, correlating with the progressive reduction in propagon numbers as over-expression continues.

Frederick et al. (2014) described the *in vivo* binding of Hsp104 to strong and weak variants of the [*PSI*$^+$] prion and showed two classes of binding, one labile, Hsp70-dependent and showing free exchange of Hsp104 with the pool of monomers and a stable one in the M region of Sup35 that shows little such exchange. It is the weak [*PSI*$^+$] variant which has the larger proportion of stable binding. “Weakness” is recognized by a less pronounced [*PSI*$^+$] phenotype and by its instability, an instability that is most plausibly due to malpartition.

We propose the following. The Hsp70-independent binding of Hsp104 to the Sup35M region, as described by Helsen and Glover (2012) and by Frederick et al. (2014), is very stable. It is normally a relatively rare event and the principal interaction of Hsp104 and the [*PSI*$^+$] prion is Hsp70-dependent and transient, promoting the ATPase-driven fragmentation of prion aggregates. In conditions of Hsp104 over-expression a second, Hsp70-independent binding takes place at the M-region. This does not cause a cessation of fragmentation, which proceeds normally, but has the effect of anchoring its substrate to a sub-cellular structure, hydrolyzing an ATP in the process. Cytological assays of

mobility, such as FRAP or FLIP, as used by Winkler et al (2012b) might, as a result, observe a loss of mobility and interpret it as due to a size change and ascribe that to "non-productivity". Other interpretations of a decrease in mobility are possible. For example, one might ask what the effect on the size of aggregates would follow stoichiometric stable accretion of Hsp104 to the Sup35 protomers in the prion aggregates? When the Hsp104 involved lacks its intrinsic ATPase activity, Hsp70-independent binding takes place but the anchoring not. Nevertheless, loss of the [*PSI*$^+$] prion takes place as a result of the competitive inhibition of the wild type activity, loss of fragmentation and dilution out of the propagons with cell division. Towards the end of this process, aggregates probably become so large as to fail, passively, to pass to daughter cells (Ness et al, 2002), but for three generations at least of growth (potentially an eight-fold increase in size) partition appears to be quite normal (**Figure 6B**).

Meanwhile, however, there are still propagons in every cell, and plating on glucose medium will in due course restore wild type levels of Hsp104 activity. The appearance of small red sectors on otherwise [*PSI*$^+$] colonies could be a sign that the Hsp70-independent binding of Hsp104 to [*PSI*$^+$] aggregates is stable over several generations of growth after plating and leads to occasional malsegregation, once wild-type Hsp104 becomes predominant and begins to exchange with the M-bound Hsp104-2KT. Were the likelihood of malpartition to increase as propagons become larger and fewer during the over-expression period, sectors would appear progressively earlier in colonies from cells plated later (**Figure 1C**).

Independent data supporting malpartition by overexpression of Hsp104.

Park et al. (2014) explored the effects of over-expression of Hsp104 in considerable detail, using different methods of assaying Sup35 aggregates and different experimental procedures from ours. The major differences are that instead of the genetic method of counting propagons, they used fluorescent microscopy to determine the presence and numbers of aggregates of Sup35NM:GFP foci in individual cells and microcolonies and FACS analysis for separating cells demographically. The observations they make largely correlate with our findings, including the finding that [*PSI*$^+$] curing by over-expression of Hsp104 is hardly, if at all affected by the presence of GdnHCl. While we differ in our interpretations, we can see nothing incompatible with our conclusions in the observations reported by Park et al. (2014).

Relevance to other examples of prion instability

The sectoring observed in colonies growing from cells over-expressing the *hsp104-2KT* allele is reminiscent of many other examples of [*PSI*$^+$] instability: in weak variants and in various *PNM1* and *PNM2* mutants. A sector arises when a [*PSI*$^+$] cell segregates a [*psi*$^-$] one during the growth of the colony. It is an event associated with cell division and we would predict that it almost invariably is due to malpartition. Although this may often be a chance event due to low propagon counts, as is likely the case with weak [*PSI*$^+$] variants (Tanaka et al. 2006; see also Cox et al. 2003), the phenomenon in some variants or *PNM* mutants or in some stress conditions (Tuite et al. 1981, Newnam et al. 2011) cannot always be due to chance because of the numbers involved (e.g. **Figure 7D)**. The association of chaperone over-expression curing with malpartition opens up the possibility that sectoring of prion phenotypes may be an expression of normal metabolic activity involving the binding of Hsp104 to the M region of Sup35. We would not be surprised to find that this metabolism forms a component of the rejuvenation that occurs when aged cells divide (Erjavec, et al. 2007a,b; Spokioni et al., 2012; Zhou et al., 2014; Paoletti et al. (2016) or during sporulation (Ünal et al., 2014). What they have in common is retention and the participation of Hsp104.

CONCLUSIONS

We have reviewed the data accumulated over the last decade or so concerning the paradoxical situation that both inhibition and over-expression of Hsp104 cure the yeast [*PSI*$^+$] prion, uniquely among yeast prions. We show that data from our laboratory (this paper, Moosavi et al. 2010) and others (Hung and Masison, 2006, Reidy and Masison, 2010; Helsen and Glover, 2012a,b, Winkler et al., 2012a, Frederick et al, 2014) suggest clearly that two different mechanisms are involved, namely different patterns of Hsp104 binding which involve different binding sites, different chaperones and differing enzyme kinetics, all brought about by overexpression of Hsp104. In this paper we test predictions that arise from the two prevalent theories for the mechanism of curing by Hsp104 over-expression, namely hyperactive disaggregation of Sup35 prion aggregates or their malpartition. We demonstrate (1) the chronic occurrence, throughout the period of over-expression, of malpartition; (2) no release of non-sedimentable Sup35p::His_6 from pre-existing sedimentable forms of Sup35p::His_6; (3) no decrease in the amount of pre-existing sedimentable Sup35pHis_6 during over-expression; (4) no shift to either a higher or lower molecular weight of the prion-associated SDS-resistant aggregates during

overexpression; (5) no evidence of increase or decrease in fragmentation activity, as measured by the average numbers of propagons, in the course of nearly six generations of Hsp104 over-expression; and (6) no loss, during over-expression, of regeneration of new propagons after depletion of their numbers by inhibition by GdnHCl of the Hsp104 fragmentation activity.

We present evidence that when fragmentation (referred to as 'productivity' by Winkler et al. 2012b), is inhibited by competitive inhibition of Hsp104 through over-expression of the mutant *hsp104-2KT*, neither fragmentation nor malpartition occur; that nevertheless there is a long-term effect of overexpression of the mutant in the form of [*psi*$^-$] sectors on colonies growing on solid medium in which the over-expression was terminated. This suggests that the underlying interaction of over-expressed Hsp104 is the same in both mutant and wild-type Hsp104, but the consequences differ because of the lack of ATPase activity in the cells over-expressing the mutant. We propose that the underlying events brought about by over-expression and the modification of Hsp104 binding to Sup35 may result from novel interactions with sub-cellular structures (anchoring) such that propagons have decreased mobility.

We can now explain the observations in **Figure 1**, in which the effects of over-expressing wild type *HSP104* or the ATPase-negative mutant *hsp104:2KT* allele for three generations are compared and in which we can observe the role of the ATPase. When the wild-type Hsp104 is over-expressed, whole [*psi*$^-$] colonies are present from the first generation onwards. Other colonies are either completely [*PSI*$^+$] or sectored following a division in which one cell inherits prions and the other none. In the ATPase negative Hsp104 mutant however, after three generations 100% of the colonies are still wholly [*PSI*$^+$] or partly so, indicating that in every cell from which a colony grew there must have been at least one [*PSI*$^+$] prion propagon. Nevertheless, nearly every colony has red sectors, in varying sizes and numbers. Wholly [*psi*$^-$] red colonies did not appear in this culture for two more generations (**Figure 1**). In this culture over-expressing the Hsp104-2KT mutant apparently has the effect of complete competitive inhibition of the ATPase activity of the resident wild-type Hsp104 and the curing is precisely that observed when wild-type Hsp104 ATPase is inhibited by 3mM GdnHCl (Eaglestone et al. 2002, Cole et al. 2004) and the curing occurs by dilution out of propagons during cell division (**Figure 6B** and Byrne et al., 2007). Nevertheless, in contrast, GdnHCl-induced ATPase inhibition does not generate any red sectors in colonies in the early generations of curing by dilution.

The ones observed here are plainly a result of the earlier over-expression of the Hsp104-2KT mutant.

Over-expressing the Hsp104-2KT mutant therefore has two effects. One is inhibition of aggregate fragmentation, causing prion loss by dilution following the characteristic kinetics, and the other is an accumulation of aggregates modified by stable Hsp70-independent binding of Hsp104. In the absence of ATPase activity, these segregate normally, diluting out with cell division, but on being plated, the over-expression dilutes out, ATPase activity is restored and anchoring or retention takes place so that [*psi*$^-$] daughters are produced and red sectors appear in the mature colonies. It may be that the balance of propagon-bound Hsp104 and Hsp104-2KT is altered by exchanges as the relative amounts of the two forms change (Frederick et al., 2014). Our attempt to mimic this effect by treating the Hsp104 over-expressing culture with GdnHCl (Fig. 1B, black symbols) was not successful, since the characteristic curing by dilution was not observed (cf Park et al., 2014): all that happened was that the rate of curing by malpartition was reduced. We suppose this was because in the conditions of 20 – 30-fold excess of Hsp104, the concentration of GdnHCl was inadequate for complete ATPase inhibition. Higher concentrations of GdnHCl were lethal and could not be tested.

EXPERIMENTAL PROCEDURES

Strains and plasmids.

The strain of *S. cerevisiae* used in these studies was a strong [*PSI*$^+$] variant of 74-D694 (*MATα ade1-14 trp1-289 his3Δ-200 ura3-52 leu2-3,112* [*PIN*$^+$]) and was originally obtained from Dr. Susan Liebman (University of Illinois at Chicago). For the expression of a galactose-regulated hexa-histidine-tagged version of Sup35 (Sup35-His$_6$), we used pUKC1809, a multicopy *LEU2* plasmid containing the expression cassette *GAL1-SUP35*-His$_6$ (Ness et al., 2002). To analyse [*PSI*$^+$] curing by over-expression of the *HSP104* gene either plasmid pUKC1832 carrying the wild-type allele of *HSP104* under the control of the *GAL1* promoter (Ferreira et al., 2001; Ness et al., 2002) or plasmid pUKC1837 which has *HSP104* under the control of the *CUP1* promoter (see below), were employed. To examine [*PSI*$^+$] curing by over-expression of an ATPase-negative mutant of *HSP104*, the

hsp104-2KT allele (K218T, K620T; Chernoff et al., 1995) was used to construct plasmid pUKC1831 with the *hsp104-2KT* allele under the control of the *GAL1* promoter (Ferreira et al., 2001). All three constructs were based on the plasmid pRS316, using *URA3* as a selectable marker.

Plasmid pUKC1837 was constructed by digesting pUKC1832 with *Cla*I and *Bam*HI and the *GAL1* promoter replaced by the *CUP1* promoter. This promoter was generated by PCR of total genomic yeast DNA using the following oligonucleotide primer pair:
CUP1-5' primer: 5'CCATCGATCCCATTACCGACATTTG3'
CUP1-3' primer: 5'CGAGGATCCGATTGATTGATTGATTGTACCAG3'.
The PCR product was cloned between the *Cla*I and *Bam*HI sites of pUKC1832.

Growth media.

The liquid complete medium used was YEPD (1% bacto-peptone, 1% yeast extract, 2% glucose). Solid complete used was ¼ YEPD (1% bactopeptone, 0.25% yeast extract, 4% glucose, 1.5% agar) since this allows development of the red colony colour characteristic of *ade1-14* mutant strains and was used for distinguishing white, suppressed ([*PSI*$^+$]) from red, unsuppressed ([*psi*$^-$]) strains (Cox, 1965). Synthetic complete (SC) medium was 0.67% Yeast Nitrogen Base (Difco), 2% glucose supplemented with required growth supplement mixes of amino acids and bases (Formedium Ltd., Norwich) as required. Strains carrying the desired plasmid were selected for on SC medium supplemented with the appropriate single- or double-drop-out mix of supplements (Formedium Ltd).

To induce gene over-expression using the *GAL1* promoter, strains containing the plasmid of choice were grown in appropriate SC-based selection medium substituting 2% raffinose for glucose as the carbon source. Target gene expression was induced during log phase growth by adding galactose to 2% or recovering the cells by centrifugation and resuspending them in SC-based selection medium containing 2% raffinose and 2% galactose. Induction of *HSP104* gene expression with the *CUP1* promoter was done by adding copper sulphate to SC medium to 0.2 mM.

Monitoring the [*PSI*$^+$] and [*psi*$^-$] phenotypes

On ¼ YEPD, [*PSI*$^+$] *ade1-14* strains form white/pink colonies while [*psi*$^-$] colonies are dark red. When counting the numbers of [*PSI*$^+$] and [*psi*$^-$] colonies on solid agar, white/red sectored colonies were counted as [*PSI*$^+$] since the cell from which the colony grew must

have contained at least one propagon at the time of sampling (Cox et al., 2003). With the 74D-694 strain, in log phase growth only about 5% of the colony-forming units are single cells, the remainder are one-, two- or three-budded. Consequently, what is seen on the plates after colonies have grown represents segregations from about half a generation earlier. No allowance was made for this in plotting the figures illustrating the kinetics of loss.

Analysis of Sup35 in cellular fractions.

Crude yeast extracts were prepared by lysis of yeast cells with glass beads in buffer P (10 mM Na phosphate, pH 7.5, 250 mM NaCl, 2 mM phenylmethanesulphonyl fluoride) and one tablet per 5 ml of a protease inhibitor cocktail (Boehringer). Cells were washed and resuspended in 150 µl of this buffer at approximately 3 x 10^8 cells ml^{-1}. Glass beads of mesh size 40 (0.4 mm) were added to the meniscus and the ice-cold mix vibrated for 3 x 1 min bursts. The lysate was recovered after adding 100 µl fresh ice-cold buffer P. To test the sensitivity of Sup35 to detergents, crude extracts were also prepared by lysis of yeast cells in buffer ST (components as for buffer P plus 1% SDS and 1% triton X100). All molecular experiment were repeated at least twice, usually more often: in each case only those from one of the repeats are shown (Figures 2, 3 and 4.).

Crude extracts were fractionated either by centrifugation at 50,000 rpm (~96,000 x g) or 8,000 rpm (~2,500 x g) for 15 min at 4 ^oC in a Beckman TLA100 rotor. The supernatant fraction was recovered and an equal volume of buffer P or ST was added to recover the pellet fraction. Aliquots of the supernatant, pellet and total fractions were kept for subsequent SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and analysed by western blotting as previously described (Ness et al., 2002).

Analysis of SDS-resistant aggregates of Sup35.

The method of Kryndushkin *et al.* (2003) was used to detect Sup35 polymers stable in conditions of mild SDS denaturation. The gels were 1.5% agarose (SeaKem LE agarose, Cambrex Bio Science, Rockland, ME) in 20mM Tris HCl pH6.8, 200mM glycine, 0.1% SDS. Cell lysates, prepared as described above, were mixed with 2x sample buffer (60mM Tris HCl pH 6.8, 10% glycerol, 2% SDS, bromophenol blue) and incubated at room temperature for 7 – 15 min before electrophoresis. High molecular weight protein markers used were obtained from rabbit myofibrils (Kryndushkin et al., 2003).

Transfer of proteins to Immobilon P membrane (Millipore) was achieved by semi-dry electroblotting. Sup35 was detected by a rabbit polyclonal antibody raised against full-length recombinant *S. cerevisiae* Sup35. In addition, hexa-histidine-tagged Sup35 (Sup35-His_6) was detected with an anti-penta-His polyclonal antibody (Qiagen), Hsp104 by a polyclonal anti-Hsp104 (StressGen).

Propagon counting

Two methods were used for estimating the numbers of propagons in [*PSI*$^+$] cells. The first was based on the rate of curing by GdnHCl of [*PSI*$^+$] cultures to [*psi*$^-$] measured over ten or more generations (Eaglestone et al., 2000, Cole et al., 2004). This method was also used to deplete cells of propagons in order to assay the effects of treatments on the recovery of propagon numbers (Ness et al., 2002).The second method used was that originally described by Cox *et al*. (Cox et al., 2003). Here, single cells from log-phase cultures were picked by micromanipulation to ¼ YEPD plates containing 3 mM GdnHCl and allowed to grow into colonies containing approximately 10^6 cells (i.e. after about 40 hr at 28 ^{o}C). The resulting colonies were then totally recovered and each spread on an agar plate of SC minus adenine supplemented with 2% (v/v) YEPD medium. During growth of the colonies on the ¼ YEPD + 3 mM GdnHCl medium, propagon replication is completely blocked and the remaining propagons segregate as the cells divide. Eventually, since propagons are not destroyed by incubation in ¼ YEPD + 3 mM GdnHCl medium (Byrne et al., 2007a), in each colony there are as many cells containing propagons as there were propagons in the original cell with each of these cells forming a colony of [*PSI*$^+$] cells on release from the GdnHCl block. [*PSI*$^+$] colonies were identified as Ade$^+$ colonies that were cured on 3 mM GdnHCl.

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FIGURE LEGENDS

Figure 1. Kinetics of elimination of [*PSI*$^+$] by over-expression of wild-type *HSP104* or the *hsp104-2KT* allele encoding an ATPase negative form of Hsp104. (A) The kinetics of [*PSI*$^+$] loss as a function of generation number when the wild-type *HSP104* gene was over-expressed using the galactose-inducible *GAL1* promoter. Cells were grown for up to 10 generations and the % of cells able to form [*PSI*$^+$] colonies when grown on ¼ YEPD was determined. ○, 74D-694 [*PSI*$^+$] grown in SC-GAL-Ura; ●, 74D-694 [*PSI*$^+$] grown in SC-GAL-Ura + 3 mM GdnHCl. SC-GAL-Ura medium was used to select for the *URA3*-based plasmid pUKC1832 and to induce over-expression of the *HSP104* gene it carries under the control of the *GAL1* promoter. Colonies from cells sampled after 3 generations post induction and plated on 1/4YEPD are shown. The data are pooled from three replicate experiments **(B)** The kinetics of [*PSI*$^+$] loss as a function of generation number when the ATPase-negative allele *hsp104-2KT* was over-expressed using the galactose-inducible *GAL1* promoter. Δ, 74D-694 [*PSI*$^+$] grown in SC-GAL-Ura; ▲, 74D-694 [*PSI*$^+$] grown in SC-GAL-Ura + 3 mM GdnHCl. SC-GAL-Ura medium was used in order to select for the *URA3*-based plasmid pUKC1831 (Ferreira et al., 2001) carrying the *hsp104-2KT* gene under the control of the *GAL1* promoter. The single trend curve was estimated from the two data sets as described by Cole et al. (2004), assuming complete inhibition of propagon replication, that a single remaining propagon was sufficient for the expression of the [*PSI*$^+$] phenotype and that partition between mother and daughter cells was in the ratio of 6:4, as determined by Byrne et al. (2007; see also **Figure 6B)**. Data from three replicate experiments have been pooled. Data from 74D-694 [*PSI*$^+$*]* with the *hsp104-2KT* plasmid growing in glucose medium +3mM GdnHCl followed the same trend line as the other two data sets, but has been omitted for clarity. Colonies from cells sampled after 3 and 5 generations post induction and plated on 1/4YEPD are shown.

Figure 2. Sub-cellular fractionation of Sup35 in cells over-expressing wild-type *HSP104* or the *hsp104-2KT* allele encoding an ATPase negative form of Hsp104. The 74-D694 [*PSI*$^+$] strain was grown for 4.8 generations either without treatment (control) or in the presence of 3 mM GdnHCl or with over-expression of the ATPase negative allele *hsp104-2KT* (plasmid pUKC1831) or with over-expression of the wild-type *HSP104* gene (plasmid pUKC1832). Total cell extracts were prepared at this point, split and fractionated by ultracentrifugation at 96,000 x g. Samples of the total extract (T), the pellet fraction (P)

and the supernatant fraction (S) were then separated by SDS-PAGE and the resulting blots probed with anti-Sup35 antibody. Total extracts were also probed with anti-Hsp104 antibody (right-hand panel). The % [*PSI*$^{+}$] cells in each culture at the time of sampling is shown as is the proportion of total Sup35p present in the pellet fraction (P). A sample was also prepared from a [*psi*$^{-}$] derivative of 74D-694 grown under the same conditions (lowest panel).

Figure 3. Over-expression of Hsp104 does not release non-sedimentable Sup35 from pre-existing aggregates nor does it prevent newly synthesised Sup35 from entering high molecular weight aggregates. The 74D-694 [*PSI*$^{+}$] strain was transformed with two plasmids: pUKC1837 expressing wild-type *HSP104* gene from the *CUP1* promoter, and pUKC1809 expressing a wild-type *SUP35* gene tagged at the C-terminus with hexa-histidine (*SUP35-His$_6$*) and under the control of the galactose-inducible *GAL1* promoter (Ness et al., 2002). **(A)** The strain was grown in SC-ura-leu medium for 16 hr on galactose/raffinose medium (Gal/Raf) to induce expression of the Sup35p-His$_6$ protein. The culture was then transferred to fresh medium with glucose as the sole carbon source and 0.2mM $CuSO_4$. This shuts down synthesis of the Sup35-His$_6$ and induces over-expression of Hsp104. **B.** A parallel culture was grown for 16 hr in SC-ura-leu glucose-based medium and cells were then transferred to fresh SC-ura-leu medium containing galactose and raffinose as sole carbon sources with 0.2 mM $CuSO_4$ to induce expression of the Sup35-His$_6$ encoded by the plasmid pUKC1809 and the over-expression of Hsp104. Samples were taken from '**A'** after ~2 (3 hr) and 4 generations (6 hr) growth and from '**B'** after 0.5 and 1.1 generations. Total cell extracts were prepared and centrifuged in aqueous buffer for 30 min at 96,000 xg. Total extract, pellet and supernatant (S) fractions were analysed by SDS-PAGE and western blot analysis using either an anti-hexahistidine antibody (Sup35-His$_6$: the top panels in **A** and **B**) or an antibody raised against full-length Sup35 to measure total Sup35 (**A,** lower panel) or an anti-Hsp104 antibody (**B** bottom panel). Total amounts were increased for the 2 and 4 generation time points in **A** to ensure that a sufficient signal was detectable to monitor any transfer of the Sup35-His$_6$ from the P (pellet) to the S (supernatant) fraction. At each of the time points the % of cells able to form [*PSI*$^{+}$] colonies was also determined. The % of Sup35 in the pellet fraction of each sample was determined by densitometry and is shown on the autoradiograph. In both **A** and **B,** two separate high molecular weight pellet fractions were generated by centrifugation at either 2,500 xg (P^1) or at 96,000 xg (P^2) for 30 min. Total extract, pellet and the respective supernatant fractions (S^1, S^2) were

analysed by SDS-PAGE and western blot analysis using the anti-hexa-histidine antibody. Only the data from **B** are shown: there was no detectable difference between the low *g* and high *g* samples in any culture.

Figure 4. Analysis of SDS-resistant Sup35-containing aggregates in [*PSI*$^+$] cells over-expressing wild-type *HSP104*. **(A)** Total extracts of strain 74D-694 [*PSI*$^+$], transformed with the plasmid pUKC2200 expressing the wild-type *HSP104* gene under the control of the *GAL1* promoter (+Hsp104). Cells were initially grown under repressed conditions (2% glucose; 0 gen) and then transferred to 2% galactose, 1% raffinose to induce Hsp104 synthesis. Samples taken at t = 0 and t = 4 generations were prepared for SDD-AGE analysis. The percentage of [*PSI*$^+$] colonies at each time point was scored. The locations of Sup35-containing SDS-resistant polymers and Sup35 monomers are indicated. (**B)** As above, but with the [*psi*$^-$] version of 74D-694. The MW of titin markers is given in kilodaltons and the largest, smallest and median sizes at t = 0 of the Sup35 polymers ("Sup35 units") which were detectable was calculated from the distances run relative to the markers, and are expressed as the numbers of Sup35 monomers in aggregates of that size. Representative example from eight repeats.

Figure 5. Over-expression of Hsp104 does not block the formation of new propagons in dividing [*PSI*$^+$] cells. Scheme and results of showing how the average number of propagons was determined under various growth conditions in the [*PSI*$^+$] 74D-694 strain carrying either the plasmid pRS316 (control) or pUKC1832 (Hsp104) that expresses the wild-type *HSP104* gene under the control of the *GAL1* promoter (Ferreira et al., 2001; Ness et al., 2002). For each culture (control or over-expressed Hsp104) the cells were initially grown for 4 generations in a glucose-based medium (Glu) in the presence of 3 mM GdnHCl. Cells from the guanidine-grown culture were then transferred to media containing either glucose (Glu) or galactose/raffinose (Gal/Raf) as the carbon source and growth continued for a further single generation. Samples for estimating propagon numbers were taken at the time of transfer to each new medium (t=0) and at the end of one generation of growth (t=2 hours, 1 generation)). For each of the cell samples, the average number of propagons in ten cells taken from each culture was estimated using the method of Cox *et al*. (2003). The doubling time for regeneration of propagons from very low numbers after removal from GdnHCl has previously been shown to be 20 min, so in these cultures they would have been expected to have undergone

approximately six doublings (64x) after release from the GdnHCl-induced block. **Fig. 3B** shows that Hsp104 is fully over-expressed in less than 0.5 generations, i.e. <1 hour.

Figure 6. The distribution of [*PSI*$^+$] propagons between mother and daughter cells following growth with over-expression of Hsp104 or Hsp104-2KT. (**A**) The [*PSI*$^+$] 74D-694 strain carrying either the plasmid pRS316 or pUKC1832 that expresses the wild-type *HSP104* gene under the control of the *GAL1* promoter (Ferreira et al., 2001; Ness et al., 2002) were grown in a glucose-based medium. At t = 0 the cells were transferred to a defined growth medium containing galactose as the carbon source in order to induce *HSP104* over-expression. Propagon numbers in both mother and daughter cells were estimated using the method of Cox *et al.* (2003). The numbers obtained were then plotted. pRS316-carrying cells (controls) are designated by open triangles (mothers) or filled triangles (daughters); pUKC1832-carrying cells over-expressing Hsp104 are designated by open circles (mothers) or filled circles (daughters). To help clarity, filled daughter symbols have been offset from the open mother symbols by six minutes on the x axis and the t=0 samples of each culture have also been offset from one another. Four repeats were recorded, data from two of them with matching t=0 propagon numbers are shown. (**B**) The same strain transformed with pUKC1831 with *hsp104-2KT* under the control of the *GAL1* promoter. At the first three time points, only un-budded single cells, i.e. those which had just completed a round of cell division were sampled. At the final time point, mother (open circles) and daughter cells (filled circles) were separated and sampled. Values of π were normal in the range from 0.28 to 0.5 in nine out of ten cell pairs; one cell pair was anomalous or misidentified, with π = .68

Figure 7. Analysis of the number of propagons in mother and daughter cells when Hps104 is over-expressed. Using the data from Hsp104 over-expressing cells (**Figure 6A**) mother-cell propagon numbers are plotted against the numbers of propagons in their daughters. Each panel shows the data from one time-point: in generations (g). **A**, t = 0; **B** =1.3 g; **C** = 3.6 g**; D** = 5.7 g. A dotted-line box drawn ('control box') on each plot shows the extremes of the values found in the t = 0 control and also two slopes (dotted lines) indicating the extreme values of π, the fraction of propagons in the dividing pair of cells found in the daughter at t = 0. In panel **D,** the set of divisions in which one or both of the cells involved were [*psi*$^-$] have also been boxed. The number of mother-daughter pairs assayed at each time point, n_g, is found by counting the number of points shown; for

example, the number at t = 5.7 is 59. **E**. A plot of the values of π for each division shown in Figure **6A.** An extra value for a population of 74D-694 after 8.1 generations of growth in galactose medium carrying the backbone plasmid and not over-expressing Hsp104 is included to extend the time range of the control samples. Dividing cells in the over-expressing culture are lozenges; those in the control culture and the t=0 sample are squares.

Figure 8. The coefficient of variance of the numbers of propagons in all mother and daughter pairs. (**A)** The coefficient of variance (standard deviation (SD) divided by the mean) of the numbers of propagons in all mother and daughter pairs, summed, in control compared with Hsp104 over-expressing cultures. (**B**) The coefficient of variance of propagon numbers in Hsp104 over-expressing cultures in mothers (◊), daughters (□) and in mothers and daughters pooled (Δ). NB: In panels **A** and **B**, y is the regression line equation in the format y = ax + b where a is the slope of the line and b is the intercept. R^2 is the square of the correlation coefficient and is a measure of the significance of the regression (i.e p =1 – R).

FIGURES

Figure 1
Ness

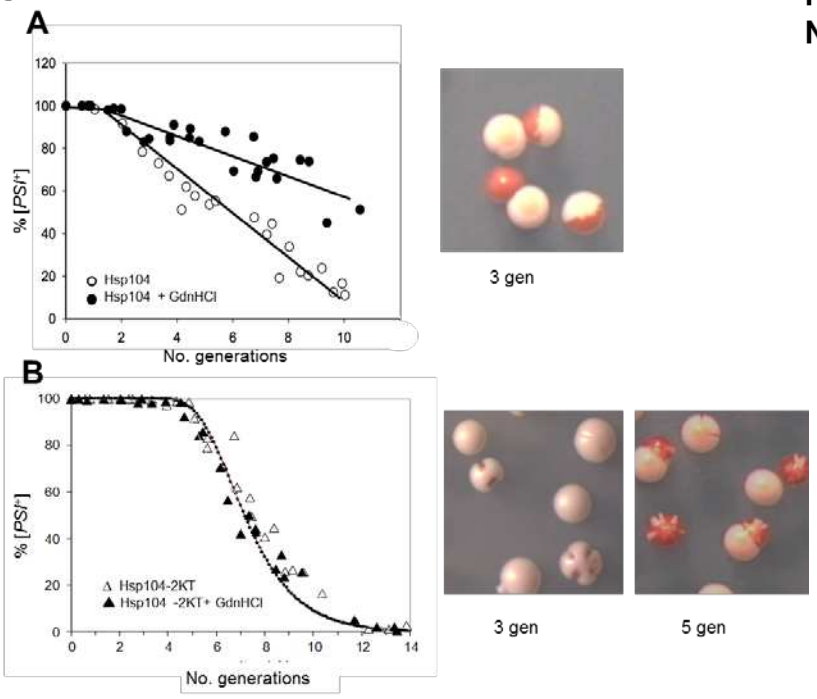

Figure 2
Ness

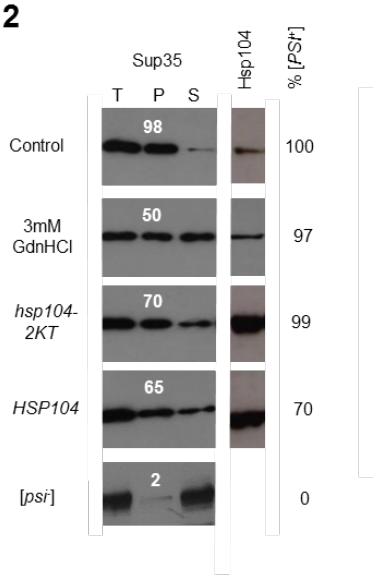

Figure 3
Ness

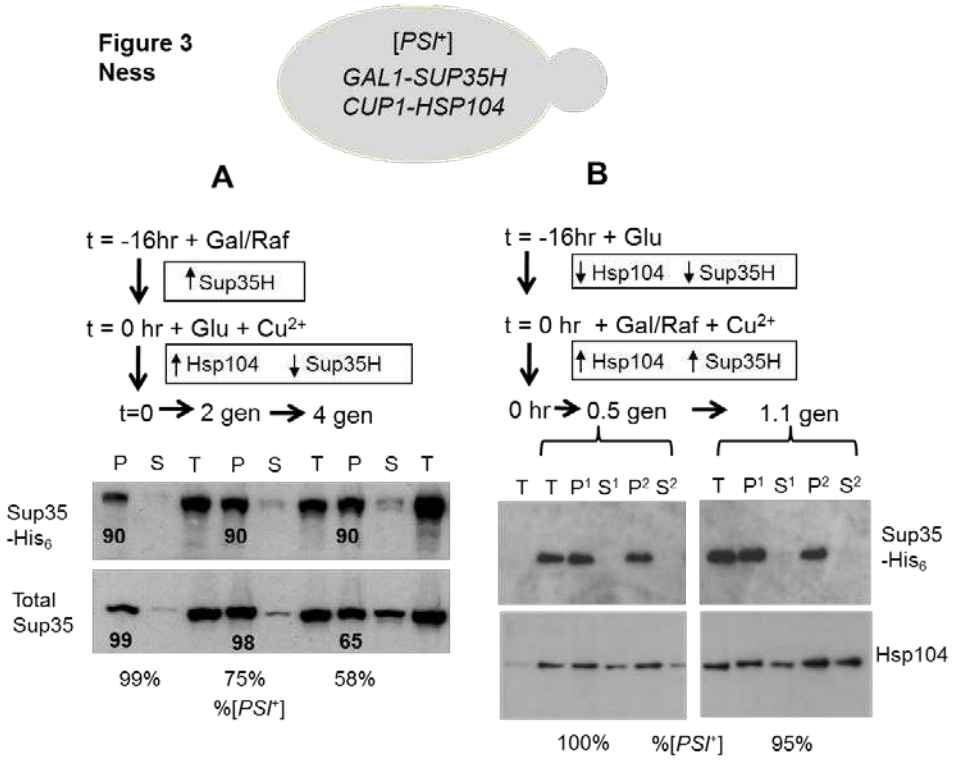

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Figure 4
Ness

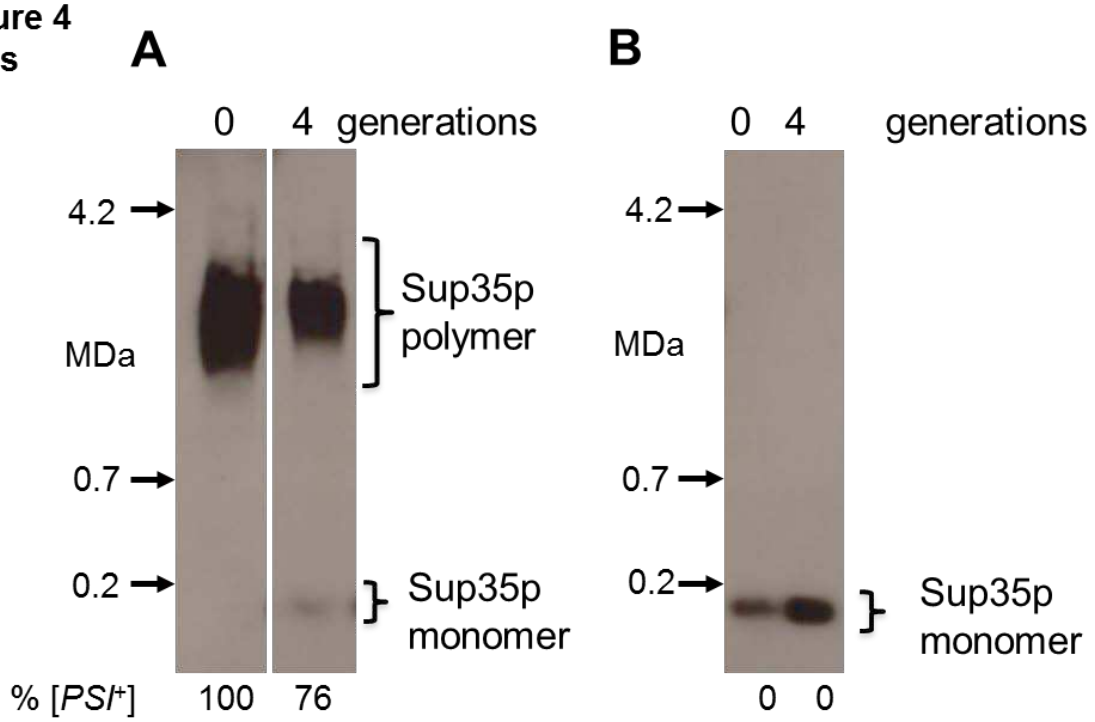

Figure 6
Ness

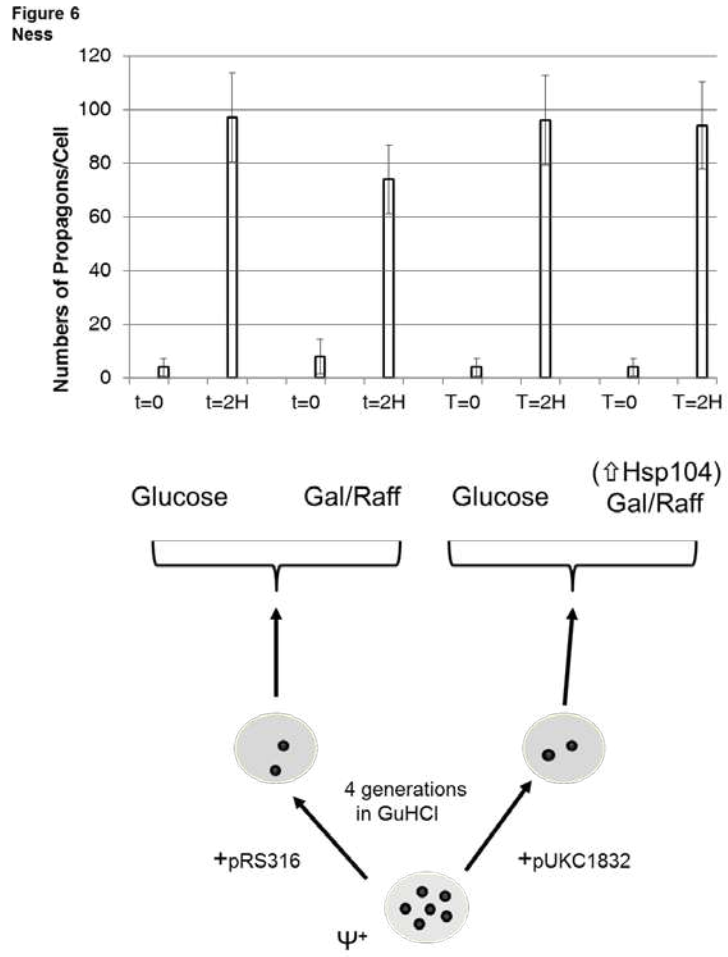

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Figure 6
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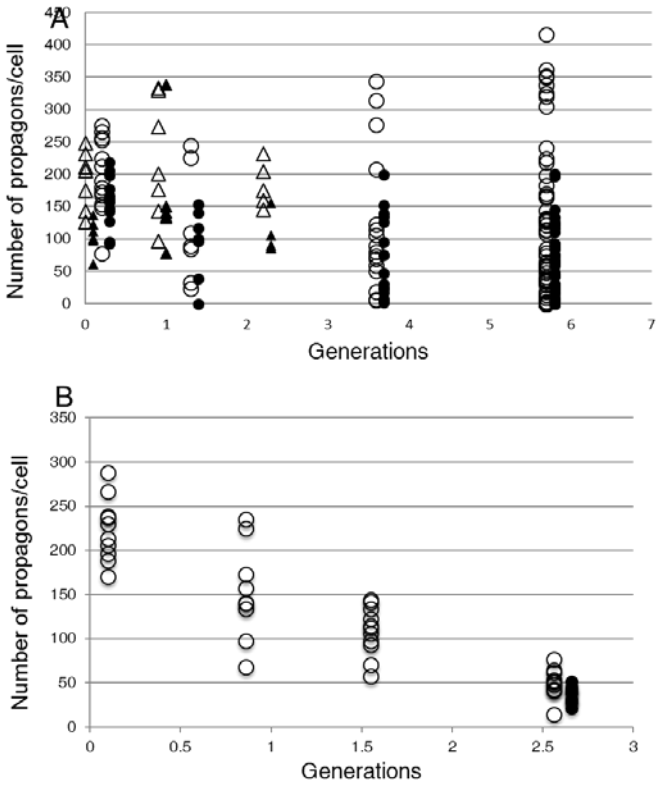

Figure 7
Ness

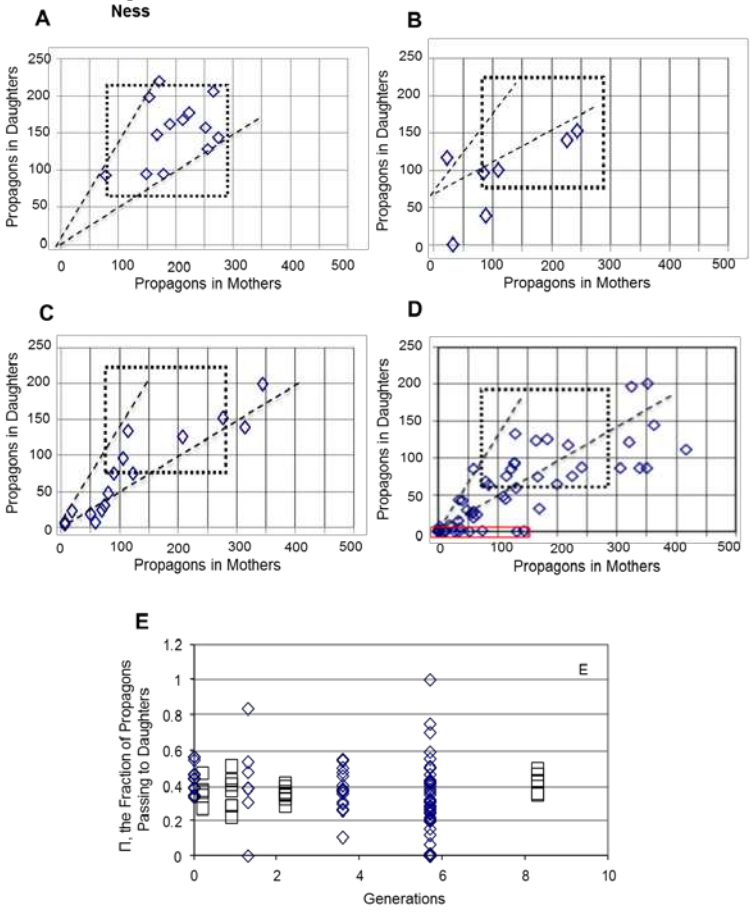

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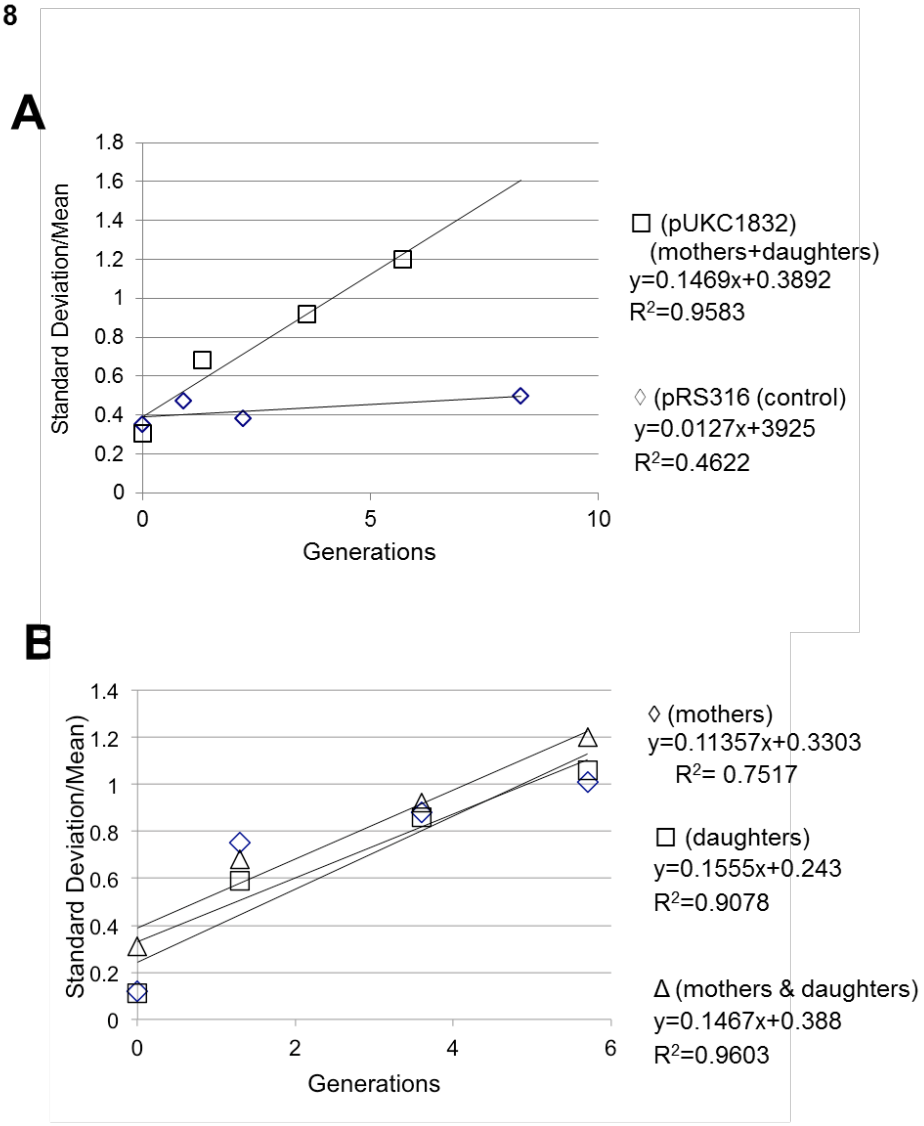
Figure 8
Ness



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