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Ness, F., Cox, B.S., Wongwigkarn, J., Naeimi, W.R. and **Tuite, M.F**. (2017) Overexpression of the molecular chaperone Hsp104 results in malpartition of [*PSI*$^+$] propagons. *Molecular Microbiology.* Jan 10.
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Over-expression of the molecular chaperone Hsp104 in *Saccharomyces cerevisiae* results in the malpartition of [*PSI*$^+$] propagons

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Key words: prion / molecular chaperone / Hsp104 / yeast / [*PSI*$^+$] prion / protein disaggregation / propagons/ malsegregation

37 **ABSTRACT**

38

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

56

57 **INTRODUCTION**

58

59 [*PSI*$^+$] is an epigenetic phenomenon in the yeast *Saccharomyces cerevisiae* brought
60 about by the prion properties of the translation termination release factor eRF3 (Sup35)
61 (review; Tuite & Cox, 2006). Strains propagating the [*PSI*$^+$] prion show a nonsense
62 suppression phenotype due to a deficiency in polypeptide chain termination most likely
63 as a result of partial inactivation of the function of Sup35, while [*psi*$^-$] cells contain
64 monomeric Sup35 and are proficient in termination (Cox, 1965, Tuite et al., 1983,
65 Wickner, 1994, Patino et al., 1996, Paushkin et al., 1996). Both full-length and N-terminal
66 fragments of Sup35 readily form amyloid fibres *in vitro* that can promote conversion of
67 the normal soluble form of Sup35 to an aggregated amyloid state both *in vitro* (Glover et
68 al., 1997, DePace et al., 1998) and *in vivo* (Tanaka et al., 2004, King & Diaz-Avalos,
69 2004).

70

71 Stable propagation of the prion form of Sup35 as with other native yeast prions, requires
72 the ATP-driven molecular chaperone Hsp104 (Chernoff et al., 1995, Sondheimer &
73 Lindquist, 2000, Moriyama et al., 2000, Ferreira et al., 2001, Jung and Masison, 2001, Du
74 et al., 2008, Alberti et al., 2009). Hsp104 is a stress-inducible hexameric protein that is
75 able to disaggregate both amorphous protein aggregates that form in stressed cells as
76 well as, *in vitro,* the more ordered detergent-resistant, amyloid fibres formed by most
77 yeast prions (Parsell et al., 1994, Glover & Lindquist, 1998, DeSantis et al., 2012).
78 Amyloid disaggregation does not involve serial monomerisation from the ends of fibres,
79 but rather occurs via fragmentation at internal sites as a consequence of the extraction of
80 individual prion protein monomers from the fibrils (review: Winkler et al., 2012a). Hsp104
81 is recruited to these sites by Sis1, an Hsp40, and Ssa1/2, members of the Hsp70 family
82 of chaperones (Tipton et al 2008, Winkler et al 2012b).
83
84 Yeast strains partially or completely devoid of Hsp104 function are unable to efficiently
85 propagate [*PSI*+] or any other native yeast prion (Chernoff et al., 1995, Hattendorf &
86 Lindquist, 2002a, Hattendorf & Lindquist, 2002b Kurahashi & Nakamura, 2006, Kurahashi
87 & Nakamura, 2007). For example, inhibition of the ATPase activity of Hsp104 by
88 guanidine hydrochloride (GdnHCl) (Ferreira et al., 2001, Jung & Masison, 2001, Jung et
89 al., 2002; Grimminger et al., 2004) provides a rapid and reversible means of studying the
90 role of Hsp104 in prion propagation *in vivo* (Ness *et al.,* 2002; ; Byrne et al., 2007, 2009,
91 Park et al., 2012). 3 – 5mM GdnHCl blocks the ability of Hsp104 to fragment the amyloid
92 polymers and by so doing prevents the generation of the new [*PSI*+] "seeds" necessary
93 to multiply and transmit the prion from mother to daughter cells (Byrne et al., 2007,
94 Eaglestone et al., 2000, Wegrzyn et al., 2001, Ness et al., 2002). We refer to these prion
95 genetic determinants as propagons (Cox *et al.*, 2003) to emphasize their genetic
96 properties and to avoid confusion with material having seeding properties in *in vitro*
97 experiments, with *in vivo* aggregates of GFP fusion proteins or with SDS-resistant
98 oligomers detected on agarose gels. Although it is commonly assumed that various sub-
99 cellular objects, such as fluorescent punctate dots, ribbons or circles formed by
100 Sup35:GFP protein fusions in [*PSI*+] cells, or the SDS-resistant polymers of Sup35
101 commonly used to identify the [*PRION*+] state (Kryndushkin et al., 2003) are the genetic
102 determinants, there is no clear evidence that they have the properties required of genetic
103 determinants, that is multiplication and transmission. The best that can be said of them is
104 that they are associated with [*PSI*+] cells or cell cultures and never with [*psi*-] ones.
105

106 One of the paradoxes of the relationship between the [*PSI*+] prion - and only the [*PSI*+]
107 prion - and Hsp104 is that elevating the cellular levels of Hsp104 in growing cells also de-
108 stabilizes the [*PSI*+] prion state leading to prion-free [*psi*-] cells (Chernoff et al., 1995).
109 Hsp104 breaks protein aggregates into lower molecular weight forms that can then be
110 dealt with by the other components of the chaperone machinery (Grimminger-Marquardt
111 & Lashuel, 2010, reviewed by Winkler et al., 2012a). The role of Hsp104 in [*PSI*+] prion
112 propagation therefore reflects a balance between the need to break up the amyloid fibres
113 into transmissible propagons to keep pace with cell division, and some process that
114 causes their loss from cells when it is over-expressed.
115
116 A popular assumption has been that the loss during over-expression is the consequence
117 of over-rapid disaggregation returning the prions to the non-prion form (Kushnirov & Ter-
118 Avanesyan, 1998, True, 2006, Helsen & Glover, 2012a,b, Park et al., 2014). The analysis
119 of the activity of Hsp104 on Sup35 polymers and their disaggregation *in vitro* (Shorter &
120 Lindquist, 2004, 2006, Krzewska & Melki, 2006, Krzewska et al., 2007,) largely supported
121 this interpretation. However, it remains an anomaly that the [*PSI*+] prion is unique among
122 yeast prions in that it is the only one eliminated by Hsp104 over-expression, although all
123 are dependent on Hsp104 ATPase activity for their propagation, presumably through its
124 disaggregase activity.
125
126 Recent *in vivo* studies have suggested that the elimination of [*PSI*+] by over-expression
127 of Hsp104 may in fact be by a mechanism distinct from the supposed enhanced
128 fragmentation process (Hung & Masison, 2006, Tipton et al., 2008 Moosavi et al., 2010,
129 Reidy & Masison, 2010, Winkler et al., 2012b, Helsen and Glover, 2012a,b). The
130 significant observations are: (1) that the N-terminal region of Hsp104 is dispensable for
131 all prion propagation, but is necessary for curing of [*PSI*+] by over-expression (Hung and
132 Masison, 2006); (2) loss of the co-chaperones Sti1 and Cpr7 interferes neither with the
133 propagation of [*PSI*+] nor with the curing of [*PSI*+] by growth in GdnHCl, but loss of either
134 or both does almost abolish the curing by over-expression (Moosavi et al., 2010; Reidy
135 and Masison, 2010); (3) an Hsp104 binding site in the M-region of Sup35 allows binding
136 without the cooperation of Hsp70 or Sis1 (Helsen and Glover, 2012a,b, Winkler et al.,
137 2012a, Frederick et al, 2014) and deletion of residues 131 – 140 in the M region eliminates
138 curing by over-expression, but has no other effect on [*PSI*+] propagation (Helsen and
139 Glover, 2012a,b).
140

141 A new hypothesis that has emerged to explain why high levels of Hsp104 lead to [*PSI*+]
142 loss is that excess Hsp104 actually prevents or reduces prion polymer fragmentation
143 (Helsen & Glover, 2012a, Winkler et al., 2012a). This hypothesis goes as follows. The
144 normal productive interaction between Hsp104 and its prion polymer substrate that leads
145 to fragmentation is achieved via Hsp70-mediated recruitment of Hsp104 to the prion
146 polymers via its N-terminal domain (Tipton et al., 2008, Winkler et al., 2012b). However,
147 Hsp104 is also able to bind non-productively to the prion polymer without the need for
148 Hsp70 and does so via the Sup35M binding-site (Winkler et al., 2012b). This binding is
149 Hsp70-independent in cells with elevated levels of Hsp104, and this "non-productive"
150 interaction out-competes Hsp70 for the Hsp104 binding to the Sup35 polymer, which is
151 otherwise Hsp70 and Hsp40-dependent. By so doing it affects Hsp104-mediated polymer
152 fragmentation. Winkler et al. (2012b) proposed that the effect of the non-productivity is to
153 permit growth of the aggregates such that their transmission to daughter cells is hindered
154 and so [*psi*-] daughter cells segregate. This may explain the paradox that although
155 Hsp104 is essential for the propagation of most other native yeast prions, its over-
156 expression eliminates only the [*PSI*+] prion suggesting that the non-productive binding of
157 Hsp104 to prion polymers is a unique feature of the Sup35 protein. The sequence in the
158 M-region of Sup35 that is needed for this interaction is, in turn, also essential for
159 elimination by over-expression of Hsp104 (vide (3) above) and also unique to Sup35. It
160 is probably significant that the Hsp70-dependent binding of Hsp104 is dynamic and labile,
161 whereas that in the M-region, which is Hsp70-independent, is relatively stable (Frederick
162 et al., 2014) and this may explain some of the observations we report here.
163
164 Both hypotheses make predictions about molecular, genetic and kinetic effects in cells
165 unique to each. Here we present results from molecular and genetic *in vivo* studies of full-
166 length Sup35, tagged or not with a hexa-histidine (His_6), that test these predictions. The
167 central issue is whether fragmentation by over-expressed Hsp104 leads to the recovery
168 of Sup35 monomers or other low molecular weight forms of oligomeric Sup35 from any
169 of the aggregates associated with the [*PSI*+] state, as would be expected if the assertion
170 by Park et al. (2014) were true. Secondly, it is not clear whether Winkler et al. (2012b)
171 expect the "non-productivity" associated with Hsp104 over-expression to mimic the non-
172 productivity caused by GdnHCl: if it were, presumably the kinetics of elimination of [*PSI*+]
173 would be similar for inhibition of fragmentation by over-expression and inhibition by
174 growth in GdnHCl. Thirdly, if "non-productivity" caused malpartition, it would be expected
175 to manifest in the inheritance of propagons.

176 In addition to addressing these three issues, we include assays to compare the effects of
177 over-expressing wild type Hsp104 with over-expressed Hsp104 deficient in its ATPase
178 function (Hsp104:K218T+K620T, abbreviated here to Hsp104-2KT) and assays to
179 determine whether Hsp104 over-expression interferes with regeneration of depleted
180 propagon numbers or affects the size of SDS-resistant polymers. We describe
181 experiments which examine the consequences of Hsp104 over-expression on the genetic
182 determinants of [*PSI*$^+$] (i.e. propagons) and on the behavior of the Sup35-containing
183 molecular structures associated with the [*PSI*$^+$] phenotype, such as SDS-resistant
184 polymers and the higher molecular weight objects that sediment under ultracentrifugation.
185 In particular, we assay the release of Sup35 from the latter objects to determine whether
186 there is significant degradation under these conditions. However, we make no
187 assumptions about the role, if any, of these structures in propagation of the [*PSI*$^+$]
188 phenotype. We use the term "propagon" to indicate entities that are assayed by their
189 inheritance, i.e. phenotypic effects in dividing cells, the terms "polymers" or "SDS-
190 resistant polymers" to designate material identified immunologically on SDD-AGE gels
191 and the term "aggregates" for microscopically identified fluorescent foci or for material
192 found in the pellets of cell-extracts subjected to ultracentrifugation (see Materials and
193 Methods). We make no assumptions about their relationships except that they all contain
194 Sup35 in some form.
195
196 Our data are in part consistent with the findings of Winkler et al (2012b) and show no
197 evidence of enhanced polymer fragmentation in cells over-expressing Hsp104. A
198 decrease in electrophoretic mobility was observed by Winkler et al (2012b) in Sup35:YFP
199 [*PSI*$^+$] foci in cells that results from Hsp104 over-expression, and could in principle be
200 the cause of a block in transmission. Here we show that Hsp104 over-expression does
201 indeed affect transmission of propagons to daughter cells in a minority of cell divisions at
202 any one time, but, contrary to the proposal of Winkler et al. (2012b) there is little effect on
203 productivity, i.e. the multiplication of propagons.
204
205 **RESULTS**
206
207 **The kinetics of [*PSI*$^+$] elimination by over-expression of Hsp104.**
208
209 When wild-type Hsp104 was over-expressed in the 74D-694 [*PSI*$^+$] strain, cells that had
210 lost all [*PSI*$^+$] propagons and thus generated pure red [*psi*$^-$] colonies (i.e. with no white

211 [*PSI*+] sectors), were first observed approximately 1 - 1.5 generations post-induction. The
212 number of pure [*psi*-] colonies then continued to increase linearly over ten generations at
213 approximately 10% per generation (**Figure 1A**). There are typically 200 - 400 propagons
214 in a [*PSI*+] cell (Cox et al., 2003) and, for the observed kinetics of propagon loss to be
215 observed, the propagons would all have to be eliminated in a minority of cells (i.e. about
216 10%) in each generation, but with some left intact in the remaining cells. Continued over-
217 expression of *HSP104* was necessary for prion elimination as approximately one
218 generation after transfer of the cells to YEPD, a glucose-based rich medium which
219 represses the *GAL1* promoter, no further prion loss was detectable (data not shown).
220
221 The addition of 3 mM GdnHCl, a concentration known to inhibit several ATPase-
222 dependent biological activities of Hsp104 (Ferreira et al., 2001; Jung and Masison 2001,
223 Grimminger et al 2004), resulted in a reduction in the rate of [*PSI*+] loss to 4% per
224 generation in cells over-expressing Hsp104 (**Figure 1A**). This finding suggests that the
225 induced loss of [*PSI*+] was only partially dependent on the ATPase activity of Hsp104.
226 However, the failure of 3 mM GdnHCl to completely prevent over-expression prion curing
227 and instead induce curing by dilution may be due in part due to this concentration of
228 GdnHCl being insufficient to inhibit completely the ability of propagons to replicate *in vivo*
229 in the defined medium used here; in complex YEPD medium full inhibition of Hsp104
230 activity is seen at 3 mM (Ness et al., 2002, Byrne et al., 2007, 2009,). Alternatively, it may
231 simply reflect the 20 - 40-fold higher levels of Hsp104 in the over-expressed cells.
232
233 The kinetics of [*PSI*+] loss by over-expression of Hsp104-2KT, an ATPase-negative
234 mutant of Hsp104 (Chernoff et al., 1995), were essentially identical to those observed
235 when [*PSI*+] cells were either over-expressing *hsp104-2KT* in the presence of 3 mM
236 GdnHCl (**Figure 1B,** filled symbols) or not (open symbols). The kinetics of curing is also
237 identical to that observed when cells are grown without over-expression of *hsp104-2KT*
238 in the presence of 3mM GdnHCl alone (data omitted but see caption and Cox et al., 2003;
239 Cole et al, 2004; Byrne et al, 2007, 2009). This observation is consistent with the
240 competitive inhibition, by an excess of the ATPase-negative mutant, of the wild-type
241 Hsp104 present in these cells (Chernoff et al., 1995, Wegrzyn et al., 2001, DeSantis et
242 al., 2012). Importantly, the kinetics observed differ dramatically from the kinetics of [*PSI*+]
243 loss seen here when wild-type Hsp104 was over-expressed, most notably with regards
244 to the increased lag before the appearance of [*psi*-] cells and the rate of loss at 50% per
245 generation once prion-free cells arise in the culture (**Figure 1B;** see also **Figure 6B**

246 showing the effect on propagon numbers during such treatment: both these Figures
247 demonstrate a halving of propagon numbers/cell with each cell generation). The presence
248 of 3 mM GdnHCl made no difference to the rate of [*PSI*+] loss induced by over-expression
249 of the *hsp104-2KT* allele (**Figure 1B**, filled symbols).
250
251 In analysing [*PSI*+] loss following *HSP104* or *hsp104-2KT* over-expression, only colonies
252 that were wholly red were scored as [*psi*-]. However, a significant proportion of colonies
253 scored carried red sectors and the nature of these sectors differed depending on whether
254 or not it was the wild-type *HSP104* or the *hsp104-2KT* allele that was over-expressed
255 (**Figure 1C**). For the *hsp104-2KT* allele, the number and size of red sectors in otherwise
256 white [*PSI*+] colonies increased with time, with hair-line red sectors clearly observable in
257 colonies approximately three generations after galactose induction. By 5 generations
258 approximately 50% of the colonies contained large red sectors, but subsequently this
259 number declined and whole red [*psi*-] colonies begun to appear. The same trend was
260 seen when 3 mM GdnHCl was present during over-expression (data not shown).
261 Sectored colonies do not appear on colonies plated during curing with 3 mM GdnHCl
262 alone: only when *hsp104-2KT* is being over-expressed. When the wild type *HSP104* allele
263 was over-expressed, sectoring was restricted largely to half and quarter red sectors
264 appearing concomitantly with the first appearance of wholly red [*psi*-] colonies (**Figure
265 1C**). *HSP104* and *hsp104-2KT* over-expression thus led to clear differences in both the
266 kinetics of [*PSI*+] elimination and in the nature of the red/white colony sectoring, indicating
267 that [*psi*-] cells arise by different means under the two treatments.
268
269 A small red [*psi*-] sector in an otherwise [*PSI*+] colony – as typically seen with the over-
270 expression of the *hsp104-2KT* allele – suggests that prion loss is delayed until late in the
271 development of the colony. Plating the galactose-grown cells onto ¼YEPD to score the
272 [*PSI*+] phenotype would lead to an immediate repression of the expression of the plasmid-
273 borne *GAL1*-regulated *hsp104-2KT* gene. As growth of the cells in galactose was
274 continued, both the number and size of [*psi*-] red sectors increased. This is coupled with
275 a reduced number of propagons through dilution at cell division.
276
277 A colony of ~1.5 mm diameter typically represents ~26 generations (~10^8 cells) from the
278 time of plating of individual cells to the cessation of colony growth. This suggests that
279 during *hsp104-2KT* over-expression there is a progressive accumulation or maintenance
280 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.

316
317 The 74D-694 [*PSI*$^+$] strain was engineered to express a fully functional form of Sup35
318 carrying a C-terminal hexa-histidine tag (Sup35-His$_6$; Ness et al., 2002) under the control
319 of the *GAL1* promoter. The Sup35-His$_6$ protein behaves exactly as wild-type Sup35 in
320 terms of its distribution between supernatant and pellet fractions in [*PSI*$^+$] and [*psi*$^-$] cells
321 and in terms of its function in translation termination and it forms aggregates in [*PSI*$^+$]
322 cells, but not in [*psi*$^-$] cells and loses them when converted to [*psi*$^-$] by growth in guanidine
323 (Ness et al., 2002). These same cells also carried the *HSP104* gene under the control of
324 the copper-inducible *CUP1* promoter, and in addition had the wild-type *SUP35* gene intact
325 in the genome. Cells of this strain, initially grown in galactose, were switched to a glucose-
326 only medium containing 40 μM $CuSO_4$ to repress production of Sup35-His$_6$ while at the
327 same time inducing over-expression of Hsp104. The levels of both the Sup35-His$_6$ and
328 the total Sup35 were then determined for both the soluble and pellet fractions after four
329 generations post repression/induction.
330
331 Even after 4 generations of growth under induction/repression conditions, no significant
332 increase in the trace levels of Sup35-His$_6$ in the supernatant fraction was observed even
333 though 25% of the cells were [*psi*$^-$] after 4 generations (**Figure 3A**). However, total Sup35
334 levels in the supernatant fraction did increase significantly by 4 generations. These data
335 are therefore consistent with the Sup35His$_6$ being already present in a polymerised form
336 prior to Hsp104 over-expression and remaining in this form thereafter. The Sup35
337 appearing in the supernatant after the over-expression of Hsp104 must have been
338 synthesised in the newly-generated [*psi*$^-$] cells following Cu^{2+}-induced over-expression of
339 the *HSP104* gene. These data suggest that Sup35 molecules already present in polymers
340 in the [*PSI*$^+$] strain are not released when Hsp104 levels are elevated. It was clear that
341 Hsp104 over-expression had not caused enhanced fragmentation and disassembly of the
342 pre-exiting Sup35His$_6$ prion polymers.
343
344 **The effect of over-expression of Hsp104 on incorporation of monomeric Sup35 into**
345 **aggregates**
346
347 To establish whether elevated levels of Hsp104 prevented newly synthesised Sup35-His$_6$
348 from entering the [*PSI*$^+$] prion aggregates, the synthesis of Sup35-His$_6$ was now switched
349 on concomitantly with *CUP1*-induced Hsp104 over-expression. Under these conditions
350 all newly-synthesised Sup35-His$_6$ appeared in the material pelleted at either 2,500 x g

351 (P^1) or 96,000 x g (P^2) (**Figure 3B**). Although we expected to see a small amount (~5 to
352 10%) of the Sup35-His$_6$ from the [*psi*⁻] cells present in the culture at the time of sampling,
353 only trace amounts of the non-sedimentable Sup35-His$_6$ could be detected in the
354 supernatant fraction generated at 2,500 x g (S^1) centrifugation (**Figure 3B**). Elevated
355 levels of Hsp104 therefore do not prevent newly synthesised monomers of Sup35 from
356 being incorporated into fibrils: i.e. there is not a polymerisation defect. The lower
357 centrifuge speed assay was included in case newly-formed Sup35His$_6$ aggregates were
358 significantly smaller or unstable as a result of over-expression.
359
360 **Sup35 polymer size does not change in [*PSI*⁺] cells over-expressing Hsp104**
361
362 The average molecular mass of the Sup35 polymers that form in [*PSI*⁺] cells reflects the
363 relative balance reached between the rate of Sup35 polymerisation (i.e. monomer
364 addition) and the rate of fragmentation of the polymers by Hsp104 (Tanaka et al., 2006).
365 Inhibiting the disaggregase activity of Hsp104 by 3 mM GdnHCl would therefore be
366 expected to lead to an increase in the size of SDS-resistant Sup35 polymers and has
367 been shown to occur in [*PSI*⁺] strains by SDD-AGE analysis (Kryndushkin et al., 2003).
368 The converse would be expected if dissaggregase activity by Hsp104 were increased.
369
370 SDD-AGE analysis of Sup35 polymers in [*PSI*⁺] cells over-expressing Hsp104 was carried
371 out after 0 and 4 generations of growth post induction of Hsp104 synthesis (**Figure 4**).
372 After 4 generations of over-expression of Hsp104, 24% of the cells were [*psi*⁻], and a
373 decrease in the relative amount, but not the size, of the SDS-resistant Sup35 polymers
374 was observed. This experiment was repeated many times by different workers and in no
375 case has the decrease in polymer size predicted by an increase in fragmentation activity
376 of Hsp104 been observed, nor has an increase in size indicative of a block in the
377 fragmentation activity of Hsp104 on over-expression, as proposed by Winkler et al.
378 (2012b) and Helsen and Glover (2012a). However, in their [*PSI*⁺] strain, 5V-H19,
379 Kryndushkin et al. (2003) showed an approximately two-fold increase in size of SDS-
380 resistant polymers in an over-expression experiment.
381
382 Importantly, neither in **Figure 2,** nor in **Figure 4** is a distinction made between newly-
383 synthesised monomers and any released from pre-existing polymers, but this distinction
384 is so made in **Figure 3.**
385

386
387 ***HSP104* over-expression does not inhibit propagon multiplication**
388
389 To establish whether elevated levels of Hsp104 prevented the *de novo* generation of new
390 [*PSI*+] propagons, we estimated the number of propagons (n_P) in individual cells post
391 induction. The method used involved an analysis of the kinetics of loss of [*PSI*+] in YEPD
392 growth medium containing 3 mM GdnHCl (Cole et al., 2004, Eaglestone et al., 2000).
393 Using the 74D-694 [*PSI*+] strain transformed either with pUKC1832 (*GAL1-HSP104*) or
394 pRS316 (the backbone plasmid) first the cells were grown on glucose-based selective
395 medium for 4 generations in 3 mM GdnHCl (**Figure 5A**). This resulted in a significant drop
396 in the numbers of propagons (n_P) (**Figure 5B**, Glucose control: **a,** without GdnHCl versus
397 **b,** with GdnHCl) although as previously reported, in **b**, >99% of the cells still generated
398 [*PSI*+] colonies on ¼ YEPD (Byrne et al., 2009, Eaglestone et al., 2000). The GdnHCl-
399 exposed cells were then transferred to a galactose/raffinose-based medium either with
400 (**d**) or without (**c**) 3 mM GdnHCl. The cells were then allowed to go through one generation
401 at which point the number of propagons (n_p) was again counted. Over-expression of
402 Hsp104 had no effect on the amount or timing of regeneration of new propagons when
403 released from the GdnHCl-induced propagation block with complete recovery of normal
404 numbers being observed within one generation of removal of the block whether or not the
405 levels of Hsp104 were elevated in those cells (see **Figure 3B** for Hsp104 over-expression
406 after 0.5 to 1 generation in galactose/raffinose medium). Loss of [*PSI*+] when Hsp104
407 levels are elevated is therefore not due to an inability to generate new propagons.
408
409 **Transmission of [*PSI*+] propagons to daughter cells is disturbed in cells over-**
410 **expressing Hsp104**
411
412 Given that high levels of Hsp104 *per se* did not modify the number of propagons (n_P)
413 generated in over expressing cells, nor destroy pre-existing propagons in 74D-694 [*PSI*+]
414 cells, we next investigated whether the loss of [*PSI*+] from these cells could be due a
415 failure to transmit propagons to daughter cells at cell division. During cell division,
416 propagons are normally effectively distributed between mother and daughter cells with a
417 small but significant bias towards retention by the mother cell. This segregation bias is
418 shown by experimentally determining π, the fraction of propagons in a mother-daughter
419 pair that are transmitted to the daughter. This is typically ~ 0.4 (Cox *et al.* 2003; Cole *et*
420 *al.* 2004; Byrne *et al.*, 2007, 2009). If partition were affected by Hsp104 levels in any
421 dividing cell, then this value would decrease if propagons were retained in the mother cell

422 (or increase if they passed preferentially to the daughter). Either of these situations would
423 increase the variance i.e. the range of the numbers of propagons found in a population of
424 cells.
425
426 To establish whether elevated levels of Hsp104 affected π, n_P was determined in both
427 mother and daughter cells over a number of generations of growth post-induction of
428 Hsp104 synthesis, using the single-cell isolation method of Cox et al. (2003) to determine
429 n_P (**Figure 6A**). The spread of propagon numbers in both mother and daughter cells
430 remained similar over time in the control cultures but the range overall and separately, in
431 both mothers and daughters, increased dramatically at each time point. The data also
432 show that the overall means for the distributions of mother and daughter numbers in the
433 later over-expressed cultures changed with time, the mothers' increasing and the
434 daughters' decreasing over the last three time points. Nevertheless the average number
435 of propagons per cell when mother and daughter numbers were pooled over this period
436 of HSP104 overexpression remained constant, indicating propagon propagation
437 continued at normal levels in the culture as a whole. **Figure 6B** shows, for comparison,
438 the propagon numbers found in cells over-expressing the ATPase-deficient Hsp104-2KT
439 (also cf. **Fig1B**). The regression in this experiment shows a decline in numbers by one-
440 half in each generation, as has been shown elsewhere when Hsp104 activity is inhibited
441 by GdnHCl (Cox et al., 2003). In **Figure 7** the numbers of propagons at successive
442 sampling times for mother-daughter pairs plotted in **Figure 6A** were replotted on separate
443 graphs, plotting mothers' numbers against their daughters, with an indication of how they
444 matched or otherwise the limits of the mother-daughter partitions observed in the controls.
445 In the [*PSI*$^+$] cells induced to over-express Hsp104, over the 5.7 generations monitored,
446 there was a progressive increase in the number of cell divisions in which the
447 mother:daughter partition numbers fell outside the t_0 limits with an increasing number of
448 daughter cells receiving few or no propagons. Of the 57 pairs of mother and daughter
449 cells sampled after 5.7 generations post induction of Hsp104 over-expression, eight
450 daughters received no propagons (π=0), but there was no significant change in either the
451 mean or the median of the distribution of n_P among the mother-daughter pairs counted. It
452 is apparent from the plots in **Figure 7** that as time passed, the number of propagons in
453 daughter cells tended to decrease, falling below the level of the control box, while
454 numbers in mother cells tended to increase and fall to the right of the control box. This is
455 an indicator of malpartition rather than selective destruction of propagons.
456

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

distributions. This is true of the total set of values (expressed numerically as their co-variances in **Figure 8A,B**) disregarding whether they were taken together from both mother and daughter sets, treating mother and daughter sets of numbers separately or summing each mother-daughter pair for the calculation of variance. (ii) The n_P values for daughter cells occupy a lower range with a smaller mean than those for mothers, in spite of the increase in variance (i.e. the range of values) (**Figure 6A**). (iii) Segregations where n_P in the daughter decreases show concomitant increases in the number of propagons in the mother. This is most apparent in the increases in the maximum values observed in mother cells and the decreases in minimum numbers in daughter cells, while the overall median values of both control and over-expressed cultures remained close to 200 per cell, over nearly six generations of treatment (**Figure 6A**).

These experiments provide *prima facie* evidence that the partition of propagons between mother and daughter cells at cell division is disturbed in many (but not all) of the [PSI^+] cells over-expressing Hsp104 and that this can account for the steady accumulation of [psi^-] cells in the population when this chaperone is over-expressed (**Figure 1A**). At the same time the numbers show that overall, there is no shift in the balance between growth and fragmentation of propagons, meaning that both the numbers of propagons per cell in the population as a whole remain constant, as do their sizes (**Figure 4**).

DISCUSSION

We have tested the hypothesis that over-expression of Hsp104 causes disaggregation of the Sup35-containing aggregates present in [PSI^+] cells by determining the release of monomeric (or non-sedimentable oligomeric) Sup35-His$_6$ previously incorporated into sedimentable material. We were unable to detect any sign of such release over four generations of growth, during which 42% of the population had become [psi^-]. In conditions of over-expression all Sup35-His$_6$ remained sedimentable. Of course, considerable amounts of non-sedimentable Sup35 were observed in these conditions, and could be accounted for by new synthesis of non-tagged Sup35 in [psi^-] cells cured of [PSI^+] (**Figure 3A**). There is one caveat which must be applied to this experiment, namely that Sup35, modified or not, may in addition to amyloid-based aggregates, form aggregates impervious to Hsp104 degradation (Salnikova et al 2005). However, neither

526 non-tagged nor His_6-tagged Sup35 form precipitable material in [*psi*⁻] strains and such
527 material invariably disappears when [*PSI*⁺] strains are cured by GdnHCl.
528

529 Our results also show that Hsp104 over-expression does not interfere with the
530 incorporation of newly-synthesised Sup35-His_6 into sedimentable material (**Figure 3B**).
531 Furthermore, we find that over-expression of Hsp104 did not in any way interfere with the
532 regeneration of propagon numbers after their numbers had been depleted by blocking
533 the ATP-driven disaggregase function of Hsp104 by GdnHCl (**Figure 5)**. This suggests
534 that the Hsp70-independent binding of Hsp104 in conditions of over-expression, as
535 demonstrated by Winkler et al. (2012b), does not render propagons "non-productive", as
536 suggested by these authors.
537

538 The continued propagation of [*PSI*⁺] prions when Hsp104 was over-expressed was also
539 evident in the experiments which followed the inheritance at cell division of the [*PSI*⁺]
540 phenotype (**Figures 6 & 7**). These showed that in a fraction of the population of [*PSI*⁺]
541 cells in each generation, there was malpartition of propagons. This took the form of
542 retention of propagons in the mother cell in about 10% of the divisions. That this was
543 malpartition and not due to any selective destruction of propagons in daughter cells was
544 implicit in the concomitant appearance of mother cells with numbers above the upper limit
545 of the range observed both in control and t = 0 samples (**Figures 6A** & **7A - D**) and by
546 the decrease in the means of propagon numbers in daughter cells but without a
547 simultaneous increase in the mean of mother cells' numbers. We also calculated the
548 variance of propagon numbers at successive times during over-expression and found that
549 variance increased with time equally in mother cells, daughter cells, dividing cell pairs
550 (that is in the mother and daughter cell of each pair, summed) and in all cells pooled.
551 Variance is a measure of the spread of data values about the mean and malpartition
552 increases the spread because it generates mother cells with higher numbers of
553 propagons than usual and daughters with fewer. We have illustrated this effect in **Figure**
554 **7A – D** in which the propagons in mother-daughter pairs are plotted, comparing t = 0
555 values with those observed in samples taken at different times during over-expression.
556 **Figures 8A** and **B** plot the variances in the four types of population (mothers alone,
557 daughters, mother-daughter pairs summed and all values pooled); **Figure 6A** illustrates
558 the spread of values at each time point, distinguishing the daughter cell values (shaded)
559 from those of mothers and **Figure 7E** shows the difference in the spread of values of π
560 between control and over-expressed cells. It should be noted that the calculations are of

561 the coefficient of variance, i.e. *Standard Deviation/Mean*, which normalizes for any
562 differences between the means of any data sets.
563
564 These experiments show that malpartition occurs during over-expression and
565 quantitatively accounts for the curing of [*PSI*+] in our conditions. We have failed to detect
566 any degradation of sedimentable aggregates containing His_6-tagged Sup35 and SDS-
567 resistant polymers showed no change in size during over-expression (**Figure 4**),
568 suggesting neither disaggregation leading to a decrease in size, nor decline of
569 fragmentation rates leading to any increase in size of such aggregates. That SDS-
570 resistant aggregates increase in size during the over-expression of Hsp104 in 57V-H19
571 (Kryndushkiin et al., 2003) may reflect a difference in the relationship between
572 fragmentation rate and polymer growth in this strain, leading to fewer propagons and
573 greater size (Cole et al., 2004; Tanaka et al., 2006) but information about numbers is
574 lacking. We note that over-expression in the Kryndushkin et al. experiments was achieved
575 using a multicopy plasmid without any medium shift. The discrepancy does not affect our
576 argument.
577
578
579 **The kinetics of [*PSI*+] elimination**
580
581 While we have not established a mechanism of malpartition, it is possible to discern some
582 of its properties. Firstly, malpartition is wholly or partly dependent on the ATPase activity
583 of Hsp104. Over-expression in the presence of GdnHCl reduces the rate of curing by one
584 half to 5% per generation (**Figure 1A).** When the ATPase activity of Hsp104 was inhibited
585 by over-expressing the *hsp104-2KT* mutant, the curing kinetics were identical to that seen
586 in GdnHCl-mediated curing (**Figure 1B**). There is evident no over-expression curing
587 characteristic of wild type Hsp104 over-expression, which is characterized by a very short
588 lag before [*psi*-] segregants are observed and a subsequent linear loss of [*PSI*+] cells. It
589 is clear that *hsp104-2KT* over-expression curing occurs by dilution following the
590 competitive inhibition of Hsp104 and doing this in the presence of GdnHCl made no
591 difference to the kinetics. The most graphic demonstration of the difference between over-
592 expressing the wild-type and the ATPase negative mutant Hsp104 is shown in **Figure 6**,
593 which records propagon counts in the two situations. When wild-type is over-expressed,
594 average propagon numbers are maintained over nearly six generations while [*psi*-]
595 segregants start appearing within less than two generations (and cf. **Figure 1B**). By
596 contrast, over-expression of *hsp104-2KT* results in an immediate halving of the average

597 propagon numbers in each generation (**Figure 6B),** but a lag before the appearance of
598 [*psi*⁻] cells, of six generations (cf. **Figure 1B**).
599
600 We have followed the inheritance of propagons during curing by over-expression of
601 *hsp104-2KT* as shown in **Figure 6B,** and found no increase in variance at any time point
602 up to 2.56 generations, nor malpartition at this time point.
603
604 It should be noted that except for the mutations in the ATPase sites of Hsp104, there is
605 no difference between the two alleles being over-expressed in these experiments. The
606 N-terminal domain necessary for [*PSI*⁺] prion curing by over-expression in dividing cells
607 is present in both experiments as is the Sup35 M-region required for Hsp70-independent
608 binding and important for curing by over-expression and malpartition, is present in the
609 Sup35 of both experiments.
610
611 Although the ATPase activity of Hsp104 is required for both fragmentation and
612 malpartition, the difference lies in the nature of the binding of Hsp104 to Sup35 (Frederick
613 et al., 2014): either the Hsp70-dependent binding which may well be sequence-
614 independent or the specific Sup35 M-region to which the Hsp104 N-domain binds when
615 *HSP104* is over-expressed. This binding does not require Hsp70 cooperation, but may
616 need Sti1 and Cpr7 (Moosavi et al., 2010; Reidy & Masison 2010). Winkler et al. (2012b)
617 reported that GdnHCl inhibits the binding of over-expressed Hsp104 to Rnq1 prion
618 aggregates, and it may partly do so with the Sup35 prion. This would account for a
619 reduction of the curing effect of over-expression seen in **Figure 1A.**
620
621 There is also a difference in the dynamics of the two modes of Hsp104 interaction with
622 Sup35 (Frederick et al., 2014: see below) which is relevant to the observations we have
623 made. They show that Hsp104-dependent binding at normal levels of expression is labile,
624 with a high turnover, but that with over-expression, the binding becomes stable and is
625 independent of a requirement for Hsp70. We therefore conclude that there was not
626 necessarily any difference in the amount of "Hsp70-independent binding" to the M-region
627 of Sup35 when either the wild-type Hsp104 or the Hsp104⁻2KT mutant was over-
628 expressed, but the consequences differed because of the difference in ATPase activity.
629

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

631

632 There is evidence that over-expressing the Hsp104-2KT mutant has a unique long-term
633 effect, lasting even after over-expression is terminated. The [*PSI*⁺]/[*psi*⁻] phenotypic status
634 of cells during the over-expression period is assayed by plating the cells on ¼ YEPD agar,
635 which has the effect of blocking transcription of the *hsp104-2KT* gene from the *GAL1-10*
636 promoter. Plating is followed by the appearance of red [*psi*⁻] sectors on several of the
637 [*PSI*⁺] colonies that grow (**Figure 1B**). These sectors are few in number and small in size
638 in the colonies arising from samples taken early in curing, but become more numerous
639 and larger in later samples (**Figure 1C**). It should be remembered that during the curing,
640 by *hsp104-2KT*, the number of propagons is decreasing (**Figure 6B**) (Cox et al., 2003,
641 Byrne et al 2014) but that no wholly [*psi*⁻] colonies appear for six generations (**Figures**
642 **1B)**. Such sectors are clearly a hangover of the *hsp104-2KT* over-expression. They do
643 not appear when [*PSI*⁺] is cured by GdnHCl inhibition of the Hsp104 ATPase. The delay
644 in their appearance following inhibition of over expression when plated on glucose
645 medium is evidence for a stable change resulting from the interaction of *hsp104-2KT* and
646 Sup35 propagons. The [*psi*⁻] sectors may reflect the presence of aggregates (or
647 propagons) containing Sup35M-bound Hsp104-2KT which malpartition, segregating [*psi*⁻
648] cells at earlier and earlier stages in colony development, correlating with the progressive
649 reduction in propagon numbers as over-expression continues.
650
651 Frederick et al. (2014) described the *in vivo* binding of Hsp104 to strong and weak variants
652 of the [*PSI*⁺] prion and showed two classes of binding, one labile, Hsp70-dependent and
653 showing free exchange of Hsp104 with the pool of monomers and a stable one in the M
654 region of Sup35 that shows little such exchange. It is the weak [*PSI*⁺] variant which has
655 the larger proportion of stable binding. “Weakness” is recognized by a less pronounced
656 [*PSI*⁺] phenotype and by its instability, an instability that is most plausibly due to
657 malpartition.
658
659 We propose the following. The Hsp70-independent binding of Hsp104 to the Sup35M
660 region, as described by Helsen and Glover (2012) and by Frederick et al. (2014), is very
661 stable. It is normally a relatively rare event and the principal interaction of Hsp104 and
662 the [*PSI*⁺] prion is Hsp70-dependent and transient, promoting the ATPase-driven
663 fragmentation of prion aggregates. In conditions of Hsp104 over-expression a second,
664 Hsp70-independent binding takes place at the M-region. This does not cause a cessation
665 of fragmentation, which proceeds normally, but has the effect of anchoring its substrate
666 to a sub-cellular structure, hydrolyzing an ATP in the process. Cytological assays of

667 mobility, such as FRAP or FLIP, as used by Winkler et al (2012b) might, as a result,
668 observe a loss of mobility and interpret it as due to a size change and ascribe that to "non-
669 productivity". Other interpretations of a decrease in mobility are possible. For example,
670 one might ask what the effect on the size of aggregates would follow stoichiometric stable
671 accretion of Hsp104 to the Sup35 protomers in the prion aggregates? When the Hsp104
672 involved lacks its intrinsic ATPase activity, Hsp70-independent binding takes place but
673 the anchoring not. Nevertheless, loss of the [*PSI*+] prion takes place as a result of the
674 competitive inhibition of the wild type activity, loss of fragmentation and dilution out of the
675 propagons with cell division. Towards the end of this process, aggregates probably
676 become so large as to fail, passively, to pass to daughter cells (Ness et al, 2002), but for
677 three generations at least of growth (potentially an eight-fold increase in size) partition
678 appears to be quite normal (**Figure 6B**).
679
680 Meanwhile, however, there are still propagons in every cell, and plating on glucose
681 medium will in due course restore wild type levels of Hsp104 activity. The appearance of
682 small red sectors on otherwise [*PSI*+] colonies could be a sign that the Hsp70-
683 independent binding of Hsp104 to [*PSI*+] aggregates is stable over several generations of
684 growth after plating and leads to occasional malsegregation, once wild-type Hsp104
685 becomes predominant and begins to exchange with the M-bound Hsp104-2KT. Were the
686 likelihood of malpartition to increase as propagons become larger and fewer during the
687 over-expression period, sectors would appear progressively earlier in colonies from cells
688 plated later (**Figure 1C**).
689
690 **Independent data supporting malpartition by overexpression of Hsp104.**
691
692 Park et al. (2014) explored the effects of over-expression of Hsp104 in considerable
693 detail, using different methods of assaying Sup35 aggregates and different experimental
694 procedures from ours. The major differences are that instead of the genetic method of
695 counting propagons, they used fluorescent microscopy to determine the presence and
696 numbers of aggregates of Sup35NM:GFP foci in individual cells and microcolonies and
697 FACS analysis for separating cells demographically. The observations they make largely
698 correlate with our findings, including the finding that [*PSI*+] curing by over-expression of
699 Hsp104 is hardly, if at all affected by the presence of GdnHCl. While we differ in our
700 interpretations, we can see nothing incompatible with our conclusions in the observations
701 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

738 overexpression; (5) no evidence of increase or decrease in fragmentation activity, as
739 measured by the average numbers of propagons, in the course of nearly six generations
740 of Hsp104 over-expression; and (6) no loss, during over-expression, of regeneration of
741 new propagons after depletion of their numbers by inhibition by GdnHCl of the Hsp104
742 fragmentation activity.
743
744 We present evidence that when fragmentation (referred to as 'productivity' by Winkler et
745 al. 2012b), is inhibited by competitive inhibition of Hsp104 through over-expression of the
746 mutant *hsp104-2KT*, neither fragmentation nor malpartition occur; that nevertheless there
747 is a long-term effect of overexpression of the mutant in the form of [*psi*⁻] sectors on
748 colonies growing on solid medium in which the over-expression was terminated. This
749 suggests that the underlying interaction of over-expressed Hsp104 is the same in both
750 mutant and wild-type Hsp104, but the consequences differ because of the lack of ATPase
751 activity in the cells over-expressing the mutant. We propose that the underlying events
752 brought about by over-expression and the modification of Hsp104 binding to Sup35 may
753 result from novel interactions with sub-cellular structures (anchoring) such that propagons
754 have decreased mobility.
755
756 We can now explain the observations in **Figure 1**, in which the effects of over-expressing
757 wild type *HSP104* or the ATPase-negative mutant *hsp104:2KT* allele for three
758 generations are compared and in which we can observe the role of the ATPase. When
759 the wild-type Hsp104 is over-expressed, whole [*psi*⁻] colonies are present from the first
760 generation onwards. Other colonies are either completely [*PSI*⁺] or sectored following a
761 division in which one cell inherits prions and the other none. In the ATPase negative
762 Hsp104 mutant however, after three generations 100% of the colonies are still wholly
763 [*PSI*⁺] or partly so, indicating that in every cell from which a colony grew there must have
764 been at least one [*PSI*⁺] prion propagon. Nevertheless, nearly every colony has red
765 sectors, in varying sizes and numbers. Wholly [*psi*⁻] red colonies did not appear in this
766 culture for two more generations (**Figure 1**). In this culture over-expressing the Hsp104-
767 2KT mutant apparently has the effect of complete competitive inhibition of the ATPase
768 activity of the resident wild-type Hsp104 and the curing is precisely that observed when
769 wild-type Hsp104 ATPase is inhibited by 3mM GdnHCl (Eaglestone et al. 2002, Cole et
770 al. 2004) and the curing occurs by dilution out of propagons during cell division (**Figure
771 6B** and Byrne et al., 2007). Nevertheless, in contrast, GdnHCl-induced ATPase inhibition
772 does not generate any red sectors in colonies in the early generations of curing by dilution.

773 The ones observed here are plainly a result of the earlier over-expression of the Hsp104-
774 2KT mutant.
775
776 Over-expressing the Hsp104-2KT mutant therefore has two effects. One is inhibition of
777 aggregate fragmentation, causing prion loss by dilution following the characteristic
778 kinetics, and the other is an accumulation of aggregates modified by stable Hsp70-
779 independent binding of Hsp104. In the absence of ATPase activity, these segregate
780 normally, diluting out with cell division, but on being plated, the over-expression dilutes
781 out, ATPase activity is restored and anchoring or retention takes place so that [*psi*]
782 daughters are produced and red sectors appear in the mature colonies. It may be that the
783 balance of propagon-bound Hsp104 and Hsp104-2KT is altered by exchanges as the
784 relative amounts of the two forms change (Frederick et al., 2014). Our attempt to mimic
785 this effect by treating the Hsp104 over-expressing culture with GdnHCl (Fig. 1B, black
786 symbols) was not successful, since the characteristic curing by dilution was not observed
787 (cf Park et al., 2014): all that happened was that the rate of curing by malpartition was
788 reduced. We suppose this was because in the conditions of 20 – 30-fold excess of
789 Hsp104, the concentration of GdnHCl was inadequate for complete ATPase inhibition.
790 Higher concentrations of GdnHCl were lethal and could not be tested.
791
792
793
794 **EXPERIMENTAL PROCEDURES**
795
796 **Strains and plasmids.**
797
798 The strain of *S. cerevisiae* used in these studies was a strong [*PSI*$^{+}$] variant of 74-D694
799 (*MATα ade1-14 trp1-289 his3Δ-200 ura3-52 leu2-3,112* [*PIN*$^{+}$]) and was originally obtained
800 from Dr. Susan Liebman (University of Illinois at Chicago). For the expression of a
801 galactose-regulated hexa-histidine-tagged version of Sup35 (Sup35-His_6), we used
802 pUKC1809, a multicopy *LEU2* plasmid containing the expression cassette *GAL1-SUP35*-
803 His_6 (Ness et al., 2002). To analyse [*PSI*$^{+}$] curing by over-expression of the *HSP104* gene
804 either plasmid pUKC1832 carrying the wild-type allele of *HSP104* under the control of the
805 *GAL1* promoter (Ferreira et al., 2001; Ness et al., 2002) or plasmid pUKC1837 which has
806 *HSP104* under the control of the *CUP1* promoter (see below), were employed. To
807 examine [*PSI*$^{+}$] curing by over-expression of an ATPase-negative mutant of *HSP104*, the

808 *hsp104-2KT* allele (K218T, K620T; Chernoff et al., 1995) was used to construct plasmid
809 pUKC1831 with the *hsp104-2KT* allele under the control of the *GAL1* promoter (Ferreira
810 et al., 2001). All three constructs were based on the plasmid pRS316, using *URA3* as a
811 selectable marker.
812
813 Plasmid pUKC1837 was constructed by digesting pUKC1832 with *Cla*I and *Bam*HI and
814 the *GAL1* promoter replaced by the *CUP1* promoter. This promoter was generated by
815 PCR of total genomic yeast DNA using the following oligonucleotide primer pair:
816 *CUP1*-5' primer: 5'CCATCGATCCCATTACCGACATTTG3'
817 *CUP1*-3' primer: 5'CGA<u>GGATCC</u>GATTGATTGATTGATTGTACCAG3'.
818 The PCR product was cloned between the *Cla*I and *Bam*HI sites of pUKC1832.
819
820 **Growth media.**
821 The liquid complete medium used was YEPD (1% bacto-peptone, 1% yeast extract, 2%
822 glucose). Solid complete used was ¼ YEPD (1% bactopeptone, 0.25% yeast extract, 4%
823 glucose, 1.5% agar) since this allows development of the red colony colour characteristic
824 of *ade1-14* mutant strains and was used for distinguishing white, suppressed ([*PSI*$^+$]) from
825 red, unsuppressed ([*psi*$^-$]) strains (Cox, 1965). Synthetic complete (SC) medium was
826 0.67% Yeast Nitrogen Base (Difco), 2% glucose supplemented with required growth
827 supplement mixes of amino acids and bases (Formedium Ltd., Norwich) as required.
828 Strains carrying the desired plasmid were selected for on SC medium supplemented with
829 the appropriate single- or double-drop-out mix of supplements (Formedium Ltd).
830
831 To induce gene over-expression using the *GAL1* promoter, strains containing the plasmid
832 of choice were grown in appropriate SC-based selection medium substituting 2%
833 raffinose for glucose as the carbon source. Target gene expression was induced during
834 log phase growth by adding galactose to 2% or recovering the cells by centrifugation and
835 resuspending them in SC-based selection medium containing 2% raffinose and 2%
836 galactose. Induction of *HSP104* gene expression with the *CUP1* promoter was done by
837 adding copper sulphate to SC medium to 0.2 mM.
838
839 **Monitoring the [*PSI*$^+$] and [*psi*$^-$] phenotypes**
840 On ¼ YEPD, [*PSI*$^+$] *ade1-14* strains form white/pink colonies while [*psi*$^-$] colonies are dark
841 red. When counting the numbers of [*PSI*$^+$] and [*psi*$^-$] colonies on solid agar, white/red
842 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 ^oC). 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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REFERENCES

Alberti, S., R. Halfmann, O. King, A. Kapila & S. Lindquist, (2009) A systematic survey identifies prions and illuminates sequence features of prionogenic proteins. *Cell* **137**: 146-158.

Byrne, L. J., D. J. Cole, B. S. Cox, M. S. Ridout, B. J. Morgan & M. F. Tuite, (2007) The number and transmission of [*PSI*$^{+}$] prion seeds (Propagons) in the yeast *Saccharomyces cerevisiae*. *PLoS ONE* **4**: e4670.

Byrne, L. J., B. S. Cox, D. J. Cole, M. S. Ridout, B. J. Morgan & M. F. Tuite, (2009) Cell division is essential for elimination of the yeast [*PSI*$^{+}$] prion by guanidine hydrochloride. *Proc Natl Acad Sci USA* **104**: 11688-11693.

Chernoff, Y. O., S. L. Lindquist, B. Ono, S. G. Inge-Vechtomov & S. W. Liebman, (1995) Role of the chaperone protein Hsp104 in propagation of the yeast prion-like factor [psi+]. *Science* **268**: 880-884.

Cole, D. J., B. J. Morgan, M. S. Ridout, L. J. Byrne & M. F. Tuite, (2004) Estimating the number of prions in yeast cells. *Math Med Biol* **21**: 369-395.

Cox, B. S., (1965) [*PSI*], a cytoplasmic suppressor of super-suppressors in yeast. *Heredity* **20**: 505-521.

Cox, B. S., F. Ness & M. F. Tuite, (2003) Analysis of the generation and segregation of propagons: entities that propagate the [*PSI*$^{+}$] prion in yeast. *Genetics* **165**: 23-33.

DePace, A. H., A. Santoso, P. Hillner & J. S. Weissman, (1998) A critical role for amino-terminal glutamine/asparagine repeats in the formation and propagation of a yeast prion. *Cell* **93**: 1241-1252.

DeSantis, M. E., E. H. Leung, E. A. Sweeny, M. E. Jackrel, M. Cushman-Nick, A. Neuhaus-Follini, S. Vashist, M. A. Sochor, M. N. Knight & J. Shorter, (2012) Operational plasticity enables hsp104 to disaggregate diverse amyloid and nonamyloid clients. *Cell* **151**: 778-793.

Du, Z., K. W. Park, H. Yu, Q. Fan & L. Li, (2008) Newly identified prion linked to the chromatin-remodeling factor Swi1 in *Saccharomyces cerevisiae*. *Nat Genet* **40**: 460-465.

Eaglestone, S. S., L. W. Ruddock, B. S. Cox & M. F. Tuite, (2000) Guanidine hydrochloride blocks a critical step in the propagation of the prion-like determinant [*PSI*$^{+}$] of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci USA* **97**: 240-244.

Erjavec, N., L. Larsson, J. Grantham & T. Nystrom, (2007a) Accelerated aging and failure to segregate damaged proteins in Sir2 mutants can be suppressed by overproducing the protein aggregation-remodeling factor Hsp104. *Genes Dev* **21**: 2410-2421.

Erjavec, N. & T. Nystrom, (2007b) Sir2p-dependent protein segregation gives rise to a superior reactive oxygen species management in the progeny of *Saccharomyces cerevisiae*. *Proc Natl Acad Sci U S A* **104**: 10877-10881.

Ferreira, P. C., F. Ness, S. R. Edwards, B. S. Cox & M. F. Tuite, (2001) The elimination of the yeast [*PSI*$^{+}$] prion by guanidine hydrochloride is the result of Hsp104 inactivation. *Mol Microbiol* **40**: 1357-1369.

956 Frederick, KK, GT Debelouchina, C Kayatekin, Y Dominy, AC Jacavone, RG Griffin, & S
957 Lindquist, (2014) Distinct prion strains are defined by amyloid core structure and
958 chaperone binding site dynamics *Chem. Biol.* **21**:295-305.
959 Glover, J. R., A. S. Kowal, E. C. Schirmer, M. M. Patino, J. J. Liu & S. Lindquist, (1997)
960 Self-seeded fibers formed by Sup35, the protein determinant of *PSI*+, a heritable
961 prion-like factor of *S. cerevisiae*. *Cell* **89**: 811-819.
962 Glover, J. R. & S. Lindquist, (1998) Hsp104, Hsp70, and Hsp40: a novel chaperone
963 system that rescues previously aggregated proteins. *Cell* **94**: 73-82.
964 Grimminger, V., Richter, K., Imhof, A., Buchner, J. and S. Walter, (2004) The prion curing
965 agent guanidinium chloride specifically inhibits ATP hydrolysis by Hsp104. *J Biol*
966 *Chem.* 279:7378-7383.
967 Grimminger-Marquardt, V. & H. A. Lashuel, (2010) Structure and function of the molecular
968 chaperone Hsp104 from yeast. *Biopolymers* **93**: 252-276.
969 Hattendorf, D. A. & S. L. Lindquist, (2002a) Analysis of the AAA sensor-2 motif in the C-
970 terminal ATPase domain of Hsp104 with a site-specific fluorescent probe of
971 nucleotide binding. *Proc Natl Acad Sci U S A* **99**: 2732-2737.
972 Hattendorf, D. A. & S. L. Lindquist, (2002b) Cooperative kinetics of both Hsp104 ATPase
973 domains and interdomain communication revealed by AAA sensor-1 mutants.
974 *EMBO J* **21**: 12-21.
975 Helsen, C. W. & J. R. Glover, (2012a) Insight into molecular basis of curing of [*PSI*+] prion
976 by overexpression of 104-kDa heat shock protein (Hsp104). *J Biol Chem* **287**: 542-
977 556.
978 Helsen, C. W. & J. R. Glover, (2012b) A new perspective on Hsp104-mediated
979 propagation and curing of the yeast prion [*PSI*+]. *Prion* **6**: 234-239.
980 Hung, G. C. & D. C. Masison, (2006) N-terminal domain of yeast Hsp104 chaperone is
981 dispensable for thermotolerance and prion propagation but necessary for curing
982 prions by Hsp104 overexpression. *Genetics* **173**: 611-620.
983 Jung, G. M., G. Jones & D. C. Masison, (2002) Amino acid residue 184 of yeast Hsp104
984 chaperone is critical for prion-curing by guanidine, prion propagation, and
985 thermotolerance. *Proc Natl Acad Sci USA* **99**: 9936-9941.
986 Jung, G. M. & D. C. Masison, (2001) Guanidine hydrochloride inhibits Hsp104 activity *in*
987 *vivo:* A possible explanation for its effect in curing yeast prions. *Curr. Microbiol.* **43**:
988 7-10.
989 King, C. Y. & R. Diaz-Avalos, (2004) Protein-only transmission of three yeast prion
990 strains. *Nature* **428**: 319-323.
991 Kryndushkin, D. S., I. M. Alexandrov, M. D. Ter-Avanesyan & V. V. Kushnirov, (2003)
992 Yeast [*PSI*+] prion aggregates are formed by small Sup35 polymers fragmented by
993 Hsp104. *J Biol Chem* **278**: 49636-49643.
994 Krzewska, J. & R. Melki, (2006) Molecular chaperones and the assembly of the prion
995 Sup35, an *in vitro* study. *EMBO J* **25**: 822-833.
996 Krzewska, J., M. Tanaka, S. G. Burston & R. Melki, (2007) Biochemical and functional
997 analysis of the assembly of full-length Sup35p and its prion-forming domain. *J Biol*
998 *Chem* **282**: 1679-1686.
999 Kurahashi, H. & Y. Nakamura, (2006) Channel mutations in Hsp104 hexamer distinctively
1000 affect thermotolerance and prion-specific propagation. *Molecular Microbiology* **63**:
1001 1669-1683.

1002 Kushnirov, V. V. & M. D. Ter-Avanesyan, (1998) Structure and replication of yeast prions.
1003 *Cell* **94**: 13-16.
1004 Moosavi, B., J. Wongwigkarn & M. F. Tuite, (2010) Hsp70/Hsp90 co-chaperones are
1005 required for efficient Hsp104-mediated elimination of the yeast [*PSI*+] prion but not
1006 for prion propagation. *Yeast* **27**: 167-179.
1007 Moriyama, H., H. K. Edskes & R. B. Wickner, (2000) [*URE3*] prion propagation in
1008 *Saccharomyces cerevisiae*: requirement for chaperone Hsp104 and curing by
1009 overexpressed chaperone Ydj1p. *Mol Cell Biol* **20**: 8916-8922.
1010 Ness, F., P. Ferreira, B. S. Cox & M. F. Tuite, (2002) Guanidine hydrochloride inhibits the
1011 generation of prion "seeds" but not prion protein aggregation in yeast. *Mol Cell Biol*
1012 **22**: 5593-5605.
1013 Newnam, G.P., Birchmore, J.L. and Y.O.Chernoff, (2011) Destabilization and recovery of
1014 a yeast prion after mild heat shock. *J Mol Biol*. **408**:432-448.
1015 Paoletti, C, S. Quintin, A. Matifas & G. Charvin (2016) Kintetics of formation and
1016 assymetrical distribution of Hsp104-bound protein aggregates in yeast. *Biophys J.*
1017 **110**: 1605-1614
1018 Park, Y. N., D. Morales, E. H. Rubinson, D. Masison, E. Eisenberg & L. E. Greene (2012),
1019 Differences in the curing of [*PSI*+] prion by various methods of Hsp104 inactivation.
1020 *PLoS ONE* **7**: e37692.
1021 Park, Y.N., X Ziao, Y-I Yim, H Todor, R Ellerbrock, M Reidy, E Eisenberg, D.C. Masison,
1022 & L.E. Greene, (2014) Hsp104 overexpression cure *Saccharomycec cerevisiae*
1023 [*PSI*+] by causing dissolution of the prion seeds. *Euk Cell* **13**: 635-647.
1024 Parsell, D. A., A. S. Kowal, M. A. Singer & S. Lindquist, (1994) Protein disaggregation
1025 mediated by heat-shock protein Hsp104. *Nature* **372**: 475-478.
1026 Patino, M. M., J. J. Liu, J. R. Glover & S. Lindquist, (1996) Support for the prion hypothesis
1027 for inheritance of a phenotypic trait in yeast. *Science* **273**: 622-626.
1028 Paushkin, S. V., V. V. Kushnirov, V. N. Smirnov & M. D. Ter-Avanesyan, (1996)
1029 Propagation of the yeast prion-like [*psi*+] determinant is mediated by
1030 oligomerization of the *SUP35*-encoded polypeptide chain release factor. *EMBO J*
1031 **15**: 3127-3134.
1032 Reidy, M. & D. Masison, (2010) Sti1 regulation of Hsp70 and Hsp90 is critical for curing
1033 of *Saccharomyces cerevisiae* [*PSI*+] prions by Hsp104. *Mol Cell Biol* **30**: 3542-
1034 3552.
1035 Salnikova, A.B., Kryndushkin, D.S., Smirnov, V.N., Kushnirov, V.V. and M.D.Ter-
1036 Avanesyan, (2005) Nonsense suppression in yeast cells overproducing Sup35
1037 (eRF3) is caused by its non-heritable amyloids. *J Biol Chem.* **280**: 8808-12.
1038 Shorter, J. & S. Lindquist, (2004) Hsp104 catalyzes formation and elimination of self-
1039 replicating Sup35 prion conformers. *Science* **304**: 1793-1797.
1040 Shorter, J. & S. Lindquist, (2006) Destruction or potentiation of different prions catalyzed
1041 by similar Hsp104 remodeling activities. *Mol Cell* **23**: 425-438.
1042 Shorter, J. & S. Lindquist, (2008) Hsp104, Hsp70 and Hsp40 interplay regulates
1043 formation, growth and elimination of Sup35 prions. *EMBO J* **27**:2712-2724.
1044 Sondheimer, N. & S. Lindquist, (2000) Rnq1: an epigenetic modifier of protein function in
1045 yeast. *Mol Cell* **5**: 163-172.

Spokioni, R., O. Moldavski & D. Kaganovich (2012) Confinement to organelle associated inclusion structures mediates assymetric inheritance in budding yeast. *Cell Reports* **2:** 738-747

Tanaka, M., P. Chien, N. Naber, R. Cooke & J. S. Weissman, (2004) Conformational variations in an infectious protein determine prion strain differences. *Nature* **428**: 323-328.

Tanaka, M., S. R. Collins, B. H. Toyama & J. S. Weissman, (2006) The physical basis of how prion conformations determine strain phenotypes. *Nature* **442**: 585-589.

Tipton, K. A., K. J. Verges & J. S. Weissman, (2008) In vivo monitoring of the prion replication cycle reveals a critical role for Sis1 in delivering substrates to Hsp104. *Mol Cell* **32**: 584-591.

True, H. L., (2006) The battle of the fold: chaperones take on prions. *Trends Genet* **22**: 110-117.

Tuite, M.F., C.R. Mundy & B.S. Cox, (1981) Agents that cause a high frequency of genetic change from [*PSI*$^+$] to [*psi*$^-$] in *Saccharomyces cerevisiae*. Genetics **98:** 691-711

Tuite, M.F., B.S. Cox & C.S. McLaughlin (1983) In vitro nonsense suppression in [*PSI*$^+$] and [*psi*$^-$] cell-free lysates of *Saccharomyces cerevisiae*. Proc. Natl. Acad Sci USA **80**:2824-2828

Tuite, M. F. & B. S. Cox, (2006) The [*PSI*$^+$] prion of yeast: a problem of inheritance. *Methods* **39**: 9-22.

Ünal, E., Kinde, B., & A. Amon, (2014) Gametogenesis eliminates age-induced cellular damage and resets lifespan in yeast. *Science* **332**:1554-1557.

Versele, M. & J. Thorner, (2005) Some assembly required: yeast septins provide the instruction manual. *Trends Cell Biol* **15**: 414-424.

Wegrzyn, R. D., K. Bapat, G. P. Newnam, A. D. Zink & Y. O. Chernoff, (2001) Mechanism of prion loss after Hsp104 inactivation in yeast. *Mol Cell Biol* **21**: 4656-4669.

Wendler, P., J. Shorter, C. Plisson, A. G. Cashikar, S. Lindquist & H. R. Saibil, (2007) Atypical AAA+ subunit packing creates an expanded cavity for disaggregation by the protein-remodeling factor Hsp104. *Cell* **131**: 1366-1377.

Wickner, R. B., (1994) [*URE3*] as an altered URE2 protein: evidence for a prion analog in *Saccharomyces cerevisiae*. *Science* **264**: 566-569.

Winkler, J., J. Tyedmers, B. Bukau & A. Mogk, (2012a) Chaperone networks in protein disaggregation and prion propagation. *J Struct Biol* **179**: 152-160.

Winkler, J., J. Tyedmers, B. Bukau & A. Mogk, (2012b) Hsp70 targets Hsp100 chaperones to substrates for protein disaggregation and prion fragmentation. *J Cell Biol* **198**: 387-404.

Zhou, C., B.D. Slaughter & R. Li (2014) Organelle-base aggregation and retention of damaged proteins in asymmetrically dividing cells. *Cell* **159**: 530-542.

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)

1123 and the supernatant fraction (S) were then separated by SDS-PAGE and the resulting
1124 blots probed with anti-Sup35 antibody. Total extracts were also probed with anti-Hsp104
1125 antibody (right-hand panel). The % [*PSI*$^+$] cells in each culture at the time of sampling is
1126 shown as is the proportion of total Sup35p present in the pellet fraction (P). A sample was
1127 also prepared from a [*psi*$^-$] derivative of 74D-694 grown under the same conditions (lowest
1128 panel).
1129

1130 **Figure 3. Over-expression of Hsp104 does not release non-sedimentable Sup35**
1131 **from pre-existing aggregates nor does it prevent newly synthesised Sup35 from**
1132 **entering high molecular weight aggregates**. The 74D-694 [*PSI*$^+$] strain was
1133 transformed with two plasmids: pUKC1837 expressing wild-type *HSP104* gene from the
1134 *CUP1* promoter, and pUKC1809 expressing a wild-type *SUP35* gene tagged at the C-
1135 terminus with hexa-histidine (*SUP35-His$_6$*) and under the control of the galactose-
1136 inducible *GAL1* promoter (Ness et al., 2002). **(A)** The strain was grown in SC-ura-leu
1137 medium for 16 hr on galactose/raffinose medium (Gal/Raf) to induce expression of the
1138 Sup35p-His$_6$ protein. The culture was then transferred to fresh medium with glucose as
1139 the sole carbon source and 0.2mM $CuSO_4$. This shuts down synthesis of the Sup35-His$_6$
1140 and induces over-expression of Hsp104. **B.** A parallel culture was grown for 16 hr in SC-
1141 ura-leu glucose-based medium and cells were then transferred to fresh SC-ura-leu
1142 medium containing galactose and raffinose as sole carbon sources with 0.2 mM $CuSO_4$
1143 to induce expression of the Sup35-His$_6$ encoded by the plasmid pUKC1809 and the over-
1144 expression of Hsp104. Samples were taken from '**A**' after ~2 (3 hr) and 4 generations (6
1145 hr) growth and from '**B**' after 0.5 and 1.1 generations. Total cell extracts were prepared
1146 and centrifuged in aqueous buffer for 30 min at 96,000 xg. Total extract, pellet and
1147 supernatant (S) fractions were analysed by SDS-PAGE and western blot analysis using
1148 either an anti-hexahistidine antibody (Sup35-His$_6$: the top panels in **A** and **B**) or an
1149 antibody raised against full-length Sup35 to measure total Sup35 (**A,** lower panel) or an
1150 anti-Hsp104 antibody (**B** bottom panel). Total amounts were increased for the 2 and 4
1151 generation time points in **A** to ensure that a sufficient signal was detectable to monitor
1152 any transfer of the Sup35-His$_6$ from the P (pellet) to the S (supernatant) fraction. At each
1153 of the time points the % of cells able to form [*PSI*$^+$] colonies was also determined. The %
1154 of Sup35 in the pellet fraction of each sample was determined by densitometry and is
1155 shown on the autoradiograph. In both **A** and **B,** two separate high molecular weight pellet
1156 fractions were generated by centrifugation at either 2,500 xg (P^1) or at 96,000 xg (P^2) for
1157 30 min. Total extract, pellet and the respective supernatant fractions (S^1, S^2) were

1158 analysed by SDS-PAGE and western blot analysis using the anti-hexa-histidine antibody.
1159 Only the data from **B** are shown: there was no detectable difference between the low *g*
1160 and high *g* samples in any culture.

1161 **Figure 4. Analysis of SDS-resistant Sup35-containing aggregates in [*PSI*$^+$] cells**
1162 **over-expressing wild-type *HSP104*. (A)** Total extracts of strain 74D-694 [*PSI*$^+$],
1163 transformed with the plasmid pUKC2200 expressing the wild-type *HSP104* gene under
1164 the control of the *GAL1* promoter (+Hsp104). Cells were initially grown under repressed
1165 conditions (2% glucose; 0 gen) and then transferred to 2% galactose, 1% raffinose to
1166 induce Hsp104 synthesis. Samples taken at t = 0 and t = 4 generations were prepared
1167 for SDD-AGE analysis. The percentage of [*PSI*$^+$] colonies at each time point was scored.
1168 The locations of Sup35-containing SDS-resistant polymers and Sup35 monomers are
1169 indicated. (**B)** As above, but with the [*psi*$^-$] version of 74D-694. The MW of titin markers is
1170 given in kilodaltons and the largest, smallest and median sizes at t = 0 of the Sup35
1171 polymers ("Sup35 units") which were detectable was calculated from the distances run
1172 relative to the markers, and are expressed as the numbers of Sup35 monomers in
1173 aggregates of that size. Representative example from eight repeats.

1174

1175 **Figure 5. Over-expression of Hsp104 does not block the formation of new**
1176 **propagons in dividing [*PSI*$^+$] cells**. Scheme and results of showing how the average
1177 number of propagons was determined under various growth conditions in the [*PSI*$^+$] 74D-
1178 694 strain carrying either the plasmid pRS316 (control) or pUKC1832 (Hsp104) that
1179 expresses the wild-type *HSP104* gene under the control of the *GAL1* promoter (Ferreira
1180 et al., 2001; Ness et al., 2002). For each culture (control or over-expressed Hsp104) the
1181 cells were initially grown for 4 generations in a glucose-based medium (Glu) in the
1182 presence of 3 mM GdnHCl. Cells from the guanidine-grown culture were then transferred
1183 to media containing either glucose (Glu) or galactose/raffinose (Gal/Raf) as the carbon
1184 source and growth continued for a further single generation. Samples for estimating
1185 propagon numbers were taken at the time of transfer to each new medium (t=0) and at
1186 the end of one generation of growth (t=2 hours, 1 generation)). For each of the cell
1187 samples, the average number of propagons in ten cells taken from each culture was
1188 estimated using the method of Cox *et al.* (2003). The doubling time for regeneration of
1189 propagons from very low numbers after removal from GdnHCl has previously been shown
1190 to be 20 min, so in these cultures they would have been expected to have undergone

1191 approximately six doublings (64x) after release from the GdnHCl-induced block. **Fig. 3B**
1192 shows that Hsp104 is fully over-expressed in less than 0.5 generations, i.e. <1 hour.
1193
1194 **Figure 6. The distribution of [*PSI*+] propagons between mother and daughter cells**
1195 **following growth with over-expression of Hsp104 or Hsp104-2KT.** (**A**) The [*PSI*+]
1196 74D-694 strain carrying either the plasmid pRS316 or pUKC1832 that expresses the wild-
1197 type *HSP104* gene under the control of the *GAL1* promoter (Ferreira et al., 2001; Ness et
1198 al., 2002) were grown in a glucose-based medium. At t = 0 the cells were transferred to
1199 a defined growth medium containing galactose as the carbon source in order to induce
1200 *HSP104* over-expression. Propagon numbers in both mother and daughter cells were
1201 estimated using the method of Cox *et al.* (2003). The numbers obtained were then
1202 plotted. pRS316-carrying cells (controls) are designated by open triangles (mothers) or
1203 filled triangles (daughters); pUKC1832-carrying cells over-expressing Hsp104 are
1204 designated by open circles (mothers) or filled circles (daughters). To help clarity, filled
1205 daughter symbols have been offset from the open mother symbols by six minutes on the
1206 x axis and the t=0 samples of each culture have also been offset from one another. Four
1207 repeats were recorded, data from two of them with matching t=0 propagon numbers are
1208 shown. (**B**) The same strain transformed with pUKC1831 with *hsp104-2KT* under the
1209 control of the *GAL1* promoter. At the first three time points, only un-budded single cells,
1210 i.e. those which had just completed a round of cell division were sampled. At the final time
1211 point, mother (open circles) and daughter cells (filled circles) were separated and
1212 sampled. Values of π were normal in the range from 0.28 to 0.5 in nine out of ten cell
1213 pairs; one cell pair was anomalous or misidentified, with π = .68
1214
1215 **Figure 7. Analysis of the number of propagons in mother and daughter cells when**
1216 **Hps104 is over-expressed.** Using the data from Hsp104 over-expressing cells (**Figure**
1217 **6A**) mother-cell propagon numbers are plotted against the numbers of propagons in their
1218 daughters. Each panel shows the data from one time-point: in generations (g). **A**, t =
1219 0; **B** =1.3 g; **C** = 3.6 g**;** **D** = 5.7 g. A dotted-line box drawn ('control box') on each plot
1220 shows the extremes of the values found in the t = 0 control and also two slopes (dotted
1221 lines) indicating the extreme values of π, the fraction of propagons in the dividing pair of
1222 cells found in the daughter at t = 0. In panel **D,** the set of divisions in which one or both of
1223 the cells involved were [*psi*-] have also been boxed. The number of mother-daughter pairs
1224 assayed at each time point, n_g, is found by counting the number of points shown; for

1225 example, the number at t = 5.7 is 59. **E**. A plot of the values of π for each division shown
1226 in Figure **6A.** An extra value for a population of 74D-694 after 8.1 generations of growth
1227 in galactose medium carrying the backbone plasmid and not over-expressing Hsp104 is
1228 included to extend the time range of the control samples. Dividing cells in the over-
1229 expressing culture are lozenges; those in the control culture and the t=0 sample are
1230 squares.
1231
1232 **Figure 8. The coefficient of variance of the numbers of propagons in all mother and**
1233 **daughter pairs.** (**A)** The coefficient of variance (standard deviation (SD) divided by the
1234 mean) of the numbers of propagons in all mother and daughter pairs, summed, in control
1235 compared with Hsp104 over-expressing cultures. (**B**) The coefficient of variance of
1236 propagon numbers in Hsp104 over-expressing cultures in mothers (◊), daughters (□) and
1237 in mothers and daughters pooled (∆). NB: In panels **A** and **B**, y is the regression line
1238 equation in the format y = ax + b where a is the slope of the line and b is the intercept. R^2
1239 is the square of the correlation coefficient and is a measure of the significance of the
1240 regression (i.e p =1 – R).
1241

1242 **FIGURES**

1243

Figure 1
Ness

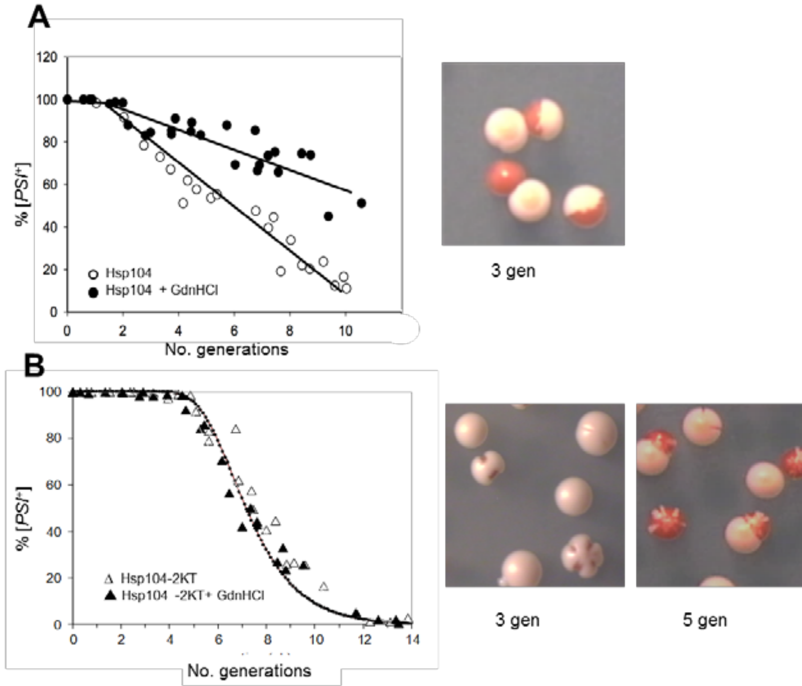
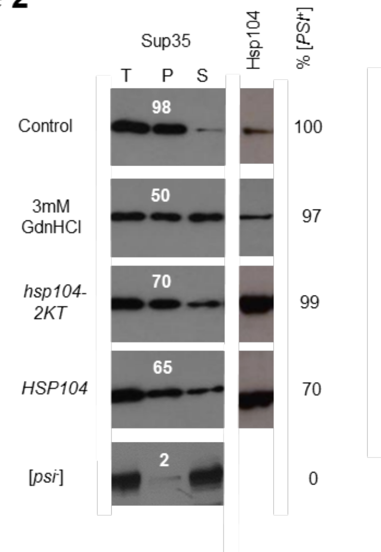


Figure 2
Ness



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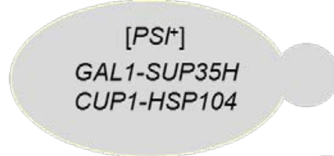
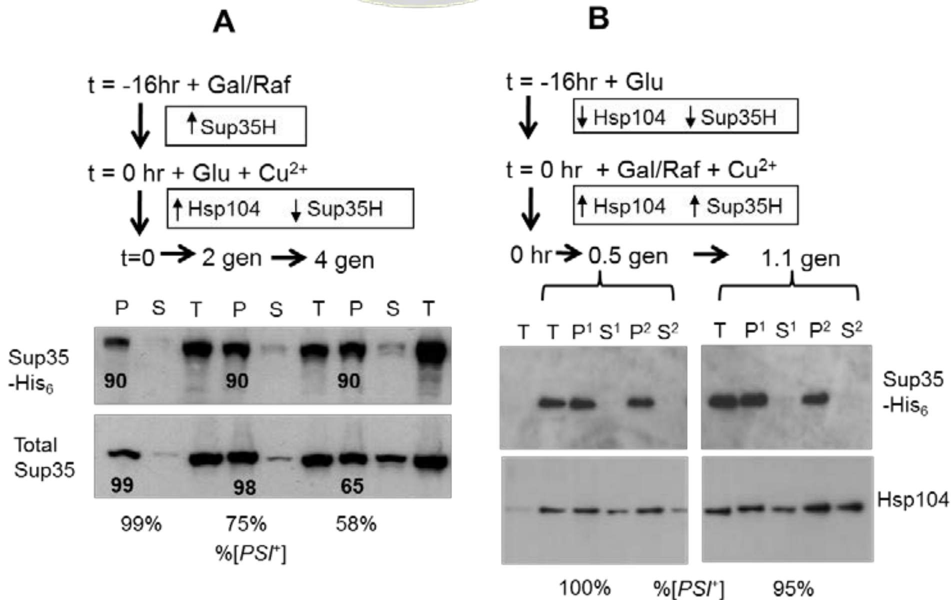




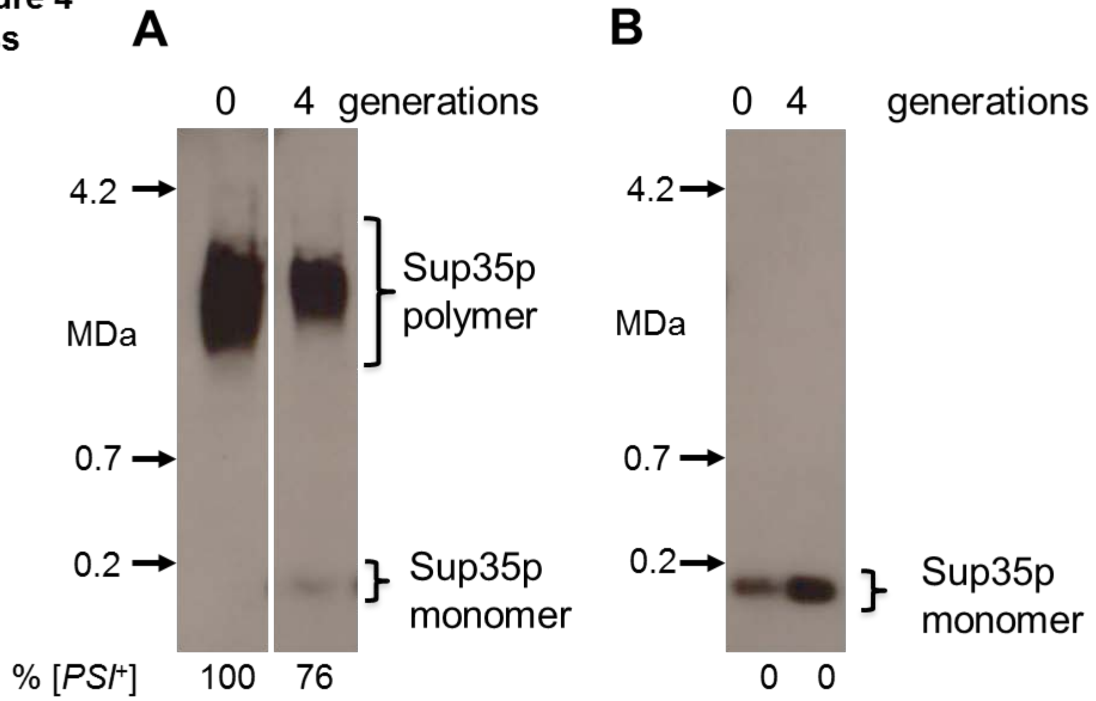


Figure 4
Ness
A
B
0
4
generations
4.2
MDa
0.7
0.2
Sup35p
polymer
Sup35p
monomer
% [PSI+]
100
76
0
0

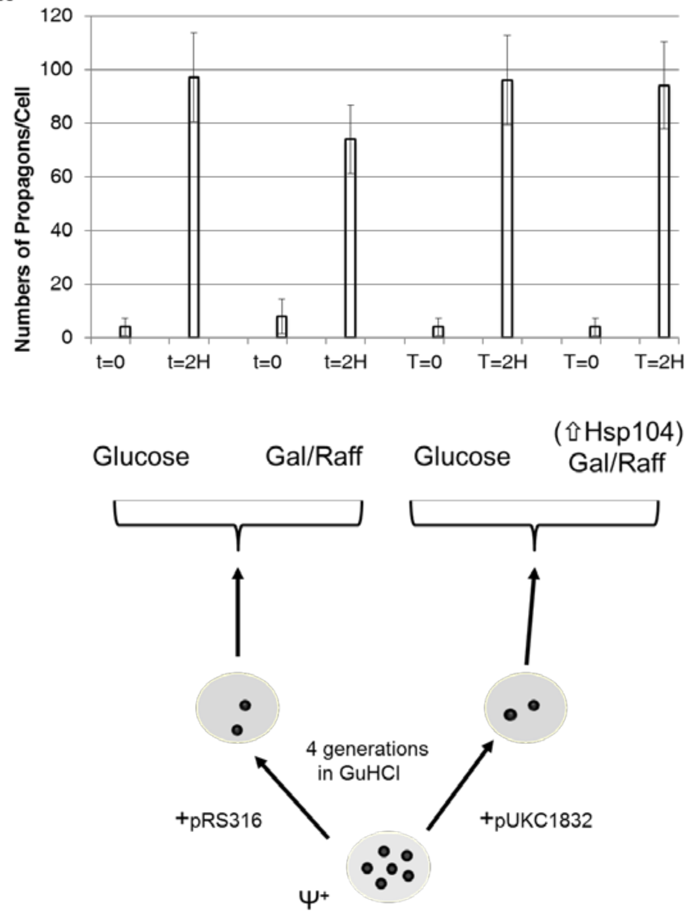
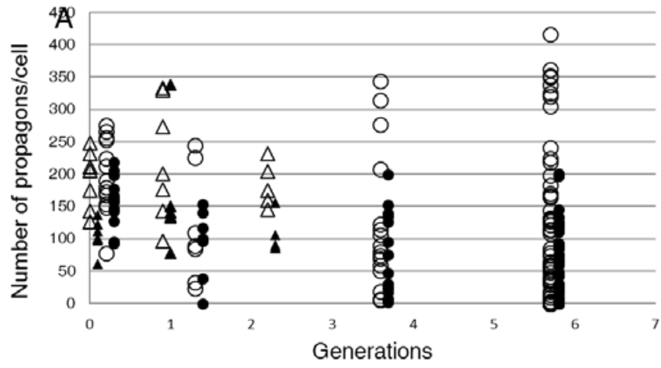
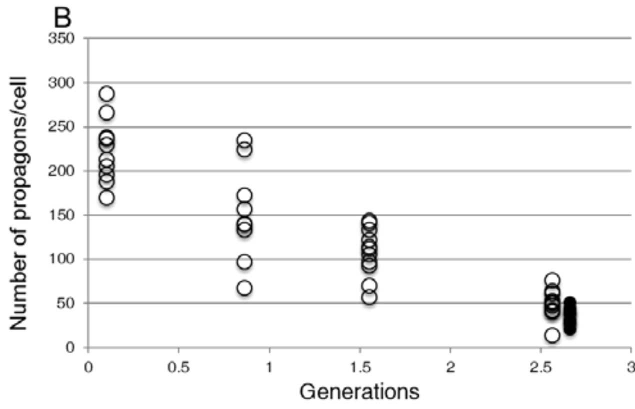


Figure 6
Ness
Numbers of Propagons/Cell
120
100
80
60
40
20
0
t=0
t=2H
T=0
T=2H
Glucose
Gal/Raff
(⇧Hsp104)
4 generations
in GuHCl
+pRS316
+pUKC1832
Ψ+

1245

Figure 6
Ness





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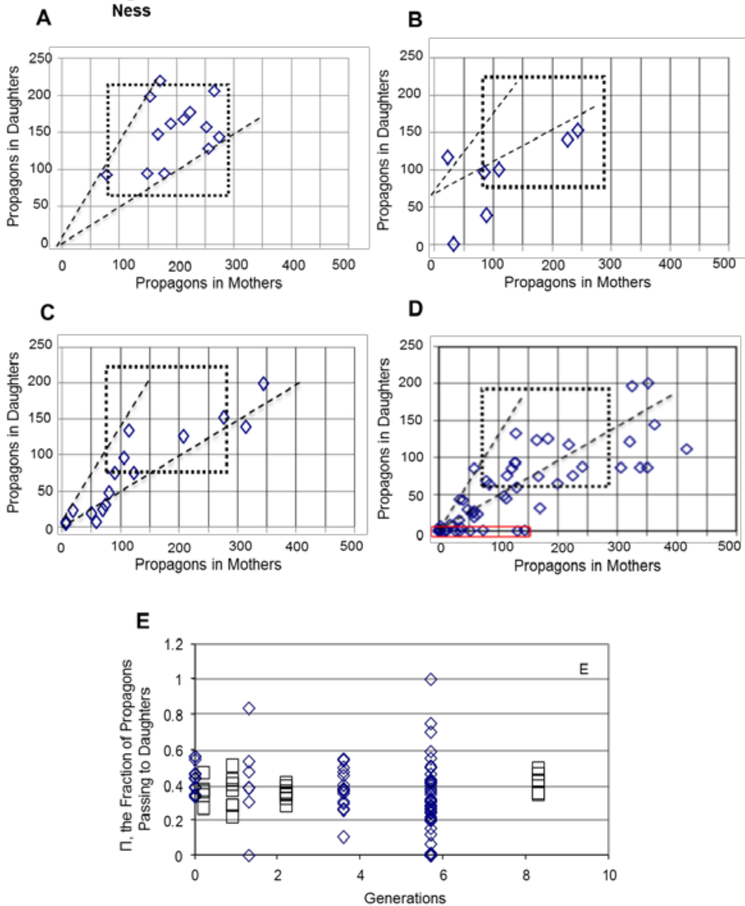
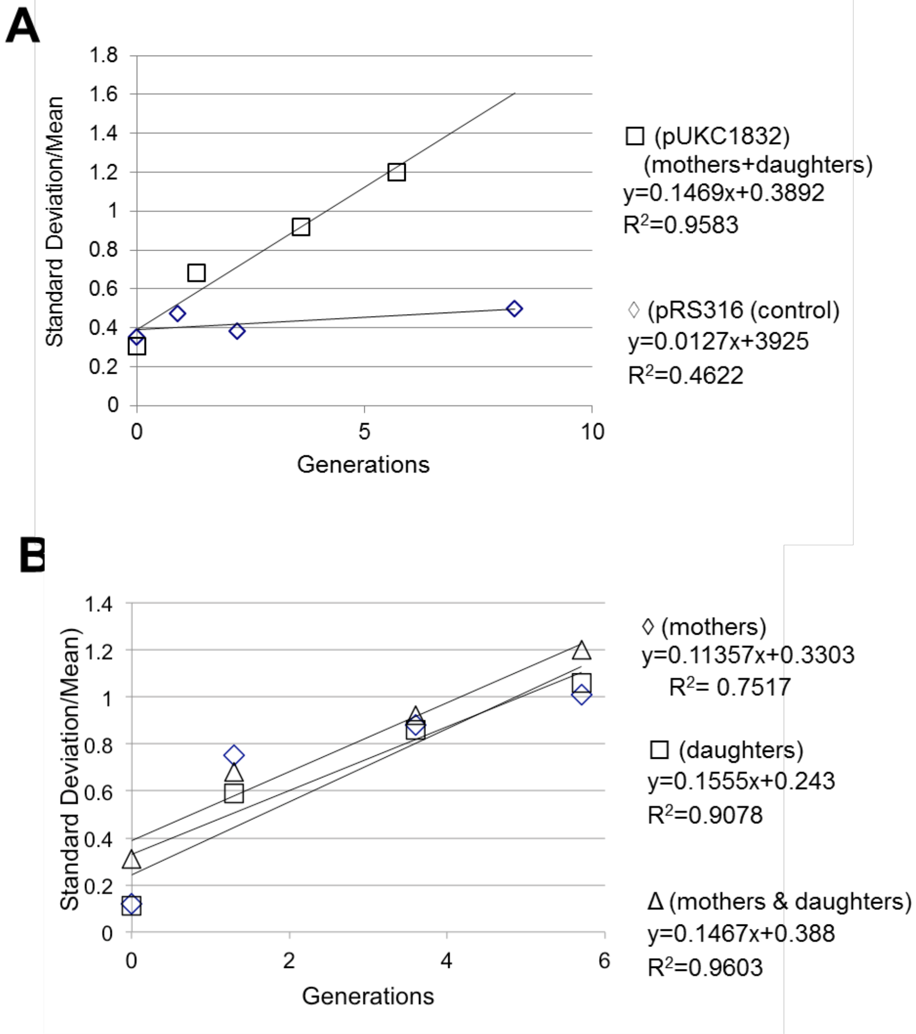


1246

Figure 8
Ness



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