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Prion aggregate structure in yeast cells is determined by the Hsp104-Hsp110 disaggregate machinery

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Prions consist of misfolded proteins that have adopted an infectious amyloid conformation. In vivo, prion biogenesis is intimately associated with the protein quality control machinery. Using electron tomography, we probed the effects of the heat shock protein Hsp70 chaperone system on the structure of a model yeast [PSI+] prion in situ. Individual Hsp70 deletions shift the balance between fibril assembly and disassembly, resulting in a variable shell of nonfibrillar, but still immobile, aggregates at the surface of the [PSI+] prion deposits. Both Hsp104 (an Hsp100 disaggregate) and Sse1 (the major yeast form of Hsp110) were localized to this surface shell of [PSI+] deposits in the deletion mutants. Elevation of Hsp104 expression promoted the appearance of this novel, nonfibrillar form of the prion aggregate. Moreover, Sse1 was found to regulate prion fibril length. Our studies reveal a key role for Sse1 (Hsp110), in cooperation with Hsp104, in regulating the length and assembly state of [PSI+] prion fibrils in vivo.

Introduction

Protein quality control is essential for cellular function and viability (Dobson, 2003; Sontag et al., 2014; Wolff et al., 2014). The capacity of the quality control systems can be exceeded under certain conditions, resulting in the accumulation of misfolded proteins (Balch et al., 2008; Hartl et al., 2011). Protein misfolding and/or mislocalization are implicated in human neurodegenerative disease (Soto and Estrada, 2008; Aguzzi and Rajendran, 2009). In the amyloid diseases, the misfolded proteins associate into amyloid assemblies, formed by a self-templating β sheet–rich conformation (Carrell and Lomas, 1997; Fändrich, 2007). Prions, which occur in fungi and mammals, constitute a transmissible form of amyloid.

Cells have evolved diverse mechanisms to prevent and reverse protein misfolding and aggregation (Sontag et al., 2014; Wolff et al., 2014). In yeast, nonamyloid amorphous aggregates accumulate in various quality control compartments (Kaganovich et al., 2008; Wang et al., 2008; Gong et al., 2012; Escusa-Toret et al., 2013; Miller et al., 2015b). Deposits found in the nucleus (intranuclear quality control, previously described as juxtanuclear; Kaganovich et al., 2008) contain soluble, highly mobile, and ubiquitinated proteins that are likely destined for proteasomal degradation (Miller et al., 2015a). In contrast, other aggregates, such as prions, which may be more resistant to disaggregation and proteolysis, are sequestered into relatively immobile compartments for terminally aggregated proteins such as the insoluble protein deposit (Kaganovich et al., 2008).

Many of the cellular factors that target the different aggregates to these functionally distinct compartments are known (Sontag et al., 2014).

Yeast prions provide a favorable model for assessing amyloid formation and its interactions with the cellular quality control network (Liebman and Chernoff, 2012). Yeast possess many prion proteins, although perhaps the best characterized is that of the prion form of Sup35, [PSI+] (Liebman and Derkatch, 1999; Serio et al., 1999; Tuite and Cox, 2006; Crow and Li, 2011). Sup35 consists of a C-terminal translation termination factor GTPase domain after the prion-determining N-terminal (N) and middle (M) domains. The N domain, essential for [PSI+] induction and propagation, is rich in glutamine and asparagine (Q/N) residues and constitutes the minimal prion/fibril-forming region (Glover et al., 1997; King et al., 1997; Paushkin et al., 1997; Baxa et al., 2011). The function of the charged M domain is unknown, although it may mediate an interaction with the heat shock protein Hsp104 disaggregate, a protein remodeling factor essential for yeast prion propagation (Helsen and Glover, 2012). Fibril assembly by the prion-forming domain sequesters soluble Sup35 into insoluble amyloid aggregates, impairing efficient cellular translation termination (Liebman and Chernoff, 2012).

The key molecular chaperones involved in processing and propagation of yeast prions are Hsp70 and its cofactors Hsp40, Hsp104, and Hsp110/Sse1 (Liebman and Chernoff, 2012; Tyedmers, 2012; Winkler et al., 2012a; Chernova et al., 2014; Sontag et al., 2014). Although the small heat shock proteins Hsp42 and Hsp26 can influence de novo [PSI+] prionogenesis (Duennwald...
Published October 5, 2015
Published on December 17, 2015 jcb.rupress.org Downloaded from

et al., 2012), it is the Hsp70/Hsp40 system that is believed to
target the prion fragmentation machinery in yeast. Growing evi-
dence suggests that Hsp70s such as Ssa1 or Ssa2, together with
Sis1 (Hsp40), initially bind prion aggregates and recruit Hsp104
for remodeling of the prion aggregates (Misselwitz et al., 1998;
Higurashi et al., 2008; Tipton et al., 2008; Kampina and Craig,
2010). Sse1 (Hsp110), a nucleotide exchange factor for Hsp70,
regulates de novo [PSI+] induction and functions in prion prop-
agation by modulating the nucleotide occupancy of Hsp70 (Fan
et al., 2007; Kryndushkin and Wickner, 2007; Sadlish et al.,
2008). In addition, Sse1 may promote prion nucleation as well
as interact directly with prion aggregates (Bagriantsev et al.,
2008; Sadlish et al., 2008).

Mammals lack an Hsp104 homologue, but they have a
disaggugase system composed of Hsp70, Hsp40, and Hsp110
(Shorter, 2011; Rampelt et al., 2012). Furthermore, mammalian
Hsp110 has been shown to reverse the toxicity associated with
mutant superoxide dismutase 1 aggregation in an axonal trans-
port model (Song et al., 2013).

For yeast prions, the balance between fibril self-assem-
bly, growth, and chaperone-mediated fragmentation/disassembly
determines the outcome of prion propagation in vivo. We
therefore sought to determine the spatial relationship between
Hsp70-related molecular chaperones and prion aggregates in a [PSI+] model. We used a well-characterized overexpres-
sion system consisting of a fusion of the Sup35 NM domain
to YFP (NM-YFP) to amplify the prion aggregate structure in
our strains (Tyedmers, 2012). This allowed us to use correla-
tive light and cryoelectron microscopy to probe the confor-
mation of NM-YFP prion aggregates in vivo. We used these
organized [PSI+] deposits (Kawai-Noma et al., 2010; Tyedmers
et al., 2010; Saibil et al., 2012) to investigate the cellular de-
terminants of prion assembly and processing in yeast. Using
individual chaperone deletions, we modulated the balance be-
tween assembly and disassembly of NM-YFP dot aggregates
and revealed a novel, nonfibrillar form of prion aggregate. This
work demonstrates a key role for Sse1 (Hsp110), in coopera-
tion with Hsp104, in regulating the length and assembly state of
[PSI+] prion fibrils in vivo.

Results

Correlative microscopy and electron
tomography of a [PSI+] prion model

We exploited the well-characterized yeast [PSI+] prion ex-
pression model, NM-YFP, to visualize aggregates of [PSI+] in
vivo. This was constitutively overexpressed in cells lacking
the endogenous NM region of SUP35 (Tyedmers et al., 2010;
Saibil et al., 2012), so that NM-YFP aggregates were propa-
gated while leaving the endogenous Sup35p C-terminal trans-
lation termination domain free from toxic recruitment into the
prion (Chernoff et al., 1992, 1993; Vishveshwara et al., 2009).
The NM-YFP aggregates in our model system assembled into
one large focus or “dot” structure (Fig. S1 A, top). To study the
3D structural organization of these dot aggregate species in
cells, we used a combination of high-pressure freezing, freeze
substitution, resin embedding, and ultramicrotomy to generate
well-preserved stained sections for electron tomography. In
parallel, we used cryoelectron tomography of unstained vitri-
fied cell sections to confirm that the observed structures reflect
the true assemblies in their native hydrated state (Al-Amoudi
et al., 2004a,b). In both instances, the YFP fluorescence was
maintained by the preparation procedures, enabling direct cor-
relation with EM of the aggregates (Materials and methods).
The NM-YFP dot aggregates formed ordered fibrillar arrays
(Fig. 1 A and Fig. S1, B–E), in agreement with previous studies
(Kawai-Noma et al., 2010; Tyedmers et al., 2010; Baxa et al.,
2011; Saibil et al., 2012).

Deletion of individual molecular chaperones
causes aggregate remodeling

Transient inactivation of Hsp70 chaperones can influence the
organization of [PSI+] assemblies (Saibil et al., 2012) and their
association with chaperones (Winkler et al., 2012b). To sys-
tematically probe the chaperone actions, we constructed single
Hsp70 (Δssa1 and Δssa2) or Hsp110/Sse1 (Δsse1) chaperone
knockouts of our [PSI+] prion model strain (Materials and
methods), and we assessed the resulting alterations to the ag-
gregate structures in these cells by correlative fluorescence and
electron tomography. There was no obvious alteration in the
fluorescence pattern of NM-YFP aggregates in any of the sin-
gle chaperone deletion strains compared with cells with a wild-
type chaperone complement (Materials and methods). We
systematically probed the chaperone actions, and we conserned single
Hsp70 (Δssa1 and Δssa2) or Hsp110/Sse1 (Δsse1) chaperone
knockouts of our [PSI+] prion model strain (Materials and
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ggregate structures in these cells by correlative fluorescence and
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Hsp70 (Δssa1 and Δssa2) or Hsp110/Sse1 (Δsse1) chaperone
knockouts of our [PSI+] prion model strain (Materials and
methods), and we assessed the resulting alterations to the ag-
ggregates (Fig. 1, A–D; and Fig. S2, A–E). The deletions introduced in both Δssa1 or Δssa2 strains resulted in the appearance of featureless perimeter zones sur-
rounding the fibrillar core of the aggregate (Fig. 1, B and C; and
Fig. S2, A and C). Occasionally, the dot aggregates entirely con-
sisted of this nonfibrillar zone (Fig. S2 B), although this could
be a result of the section plane not passing through the center
of the dot. Where present, the fibrils in the core region were
similar in length to NM-YFP fibrils formed with a wild-type chaperone complement (Fig. 1 E, wild type, Δssa1, and Δssa2)
and had a normal packing arrangement (Fig. 1, A–C). In addi-
tion to the nonfibrillar perimeter shells, clumps of dark-staining
amorphous material were often seen interspersed with the NM-
YFP fibril assemblies in these two Hsp70 deletion strains. All
of these features could be readily identified in cryotomograms of
vitreous sections of these cells, precluding any significant
artifact from our sample preparation method (Fig. S2, E and F).

The deletion of SSE1 slowed the growth of our model
strain, as reported elsewhere (Mukai et al., 1993; Trott et al.,
2005; Abrams et al., 2014). Here, we noted extensive areas of
dark amorphous material intercalated with large but otherwise
normal-looking dot fibril arrays (Fig. 1 D and Fig. S2 D). This
amorphous material formed a coarse meshwork when present
therein toward the center of the NM-YFP fibril assemblies (Fig. 1 D
and Fig. S2 D). Although packing within these fibril arrays was
similar to those in cells with a wild-type chaperone comple-
ment, the individual fibrils themselves were found to be approx-
imately 60% longer (Fig. 1 E, wild type and Δsse1).

These large structural modifications to the NM-YFP dot
aggregates in each of the three chaperone deletions strains
(Δssa1, Δssa2, and Δsse1) did not affect the stability of prion
propagation. We monitored the presence of the fluorescent dot
aggregates in these strains in successive progeny (50 genera-
tions for Δssa1 and Δssa2, and 32 generations for Δsse1) and
found them to persist just as stably as dots in cells with a wild-
type chaperone complement (Fan et al., 2007; Kryndushkin
and Wickner, 2007; Sharma and Masison, 2008).

Similar to other insoluble protein deposit substrates (Ka-
ganovich et al., 2008; Kawai-Noma et al., 2009; Weisberg et al.,
2012; Yang et al., 2013), our overexpressed NM-YFP foci were immobile when analyzed by FRAP (Fig. S3). NM-YFP foci in each of the three individual chaperone deletion strains (Δssa1, Δssa2, and Δsse1) displayed similar recovery curves (Fig. S3), regardless of whether the bleached region of interest was located in the center or more toward the periphery of the dot (not depicted). This indicated that despite the structural differences in these NM-YFP assemblies, the cells were still sequestering both the fibrillar and nonfibrillar aggregates into discrete foci with very little cytosolic exchange.

**Chaperone deletion strains sustain a pool of small NM-YFP oligomers**

The NM-YFP expression level in each of the individual chaperone deletion strains (Δssa1, Δssa2, and Δsse1) was similar to that observed in cells with a wild-type complement of chaperones, whether NM-YFP was in the [PSI+] prion state or [psi−] soluble form (Fig. 2 A). Several lower molecular mass bands could also be seen in the blots. These bands were found to be specific to NM-YFP because an otherwise isogenic strain devoid of the NM-YFP cassette had no detectable signal on the Western blots (Fig. 2 A, control lane). These additional bands were likely attributable to proteolytic degradation; accordingly, soluble NM-YFP present in the [psi−] background appeared more susceptible to this phenomenon than the NM-YFP present in the amyloid form.

The oligomeric properties of the NM-YFP aggregates in lysates from each of these strains were assessed by ultracentrifugation (Fig. 2 B). As expected for aggregates formed with a wild-type chaperone complement, a substantial part of the full-length NM-YFP was in the pellet fraction (Fig. 2 B, [PSI+]). We analyzed this fraction by correlative cryofluorescence/cryoelectron tomography and found that the normal fibrillar array organization was partly preserved for many of the dot aggregates (Fig. 2 D). For lysates from each of the Δssa1, Δssa2, and Δsse1 strains, the full-length NM-YFP population was clearly observed in both the supernatant and pellet fractions after ultracentrifugation (Fig. 2 B). These supernatant pools of NM-YFP were composed of a smaller oligomeric species, but not monomeric NM-YFP, as assessed by semidenaturating detergent agarose gel electrophoresis (SDD-AGE; Fig. 2 C). The presence of these small oligomeric aggregates coincides with the appearance of the amorphous material in dot assemblies in these mutant backgrounds as described earlier (Fig. 1, B–D).

**Hsp104 is localized to the periphery of NM-YFP aggregates**

To relate the observed structures to changes in chaperone levels, we monitored global Hsp104, Hsp70 (SSA), and Hsp110 (Sse1) expression levels in each of our single chaperone deletion strains. Global Hsp70 and Sse1 expression was not significantly altered in the deletion strains relative to their levels in cells with a wild-type complement of chaperones (Fig. 3 A). A weak Hsp110 signal was detectable in our Δsse1 strain, which was likely attributable to Sse2 (which has 76% identity to Sse1), although Sse2 does not functionally substitute for
Sse1 in [PSI+] prion biology (Moran et al., 2013). In contrast, Hsp104 levels were significantly altered in the single chaperone deletions (Fig. 3, A and B). In the Hsp70 deletion backgrounds (Δssa1 and Δssa2), Hsp104 levels increased three- to fivefold, whereas no statistically significant increase was observed in the Δsse1 strain. Therefore, it appears that Hsp104 is increased to compensate for the loss of a particular Hsp70 (Ssa1 or Ssa2), as described elsewhere (Jung et al., 2000).

Molecular chaperones are abundant in yeast prion aggregates (Chernoff et al., 1995; Allen et al., 2005; Krzewska and Melki, 2006; Bagriantsev et al., 2008; Tipton et al., 2008; Tyedmers et al., 2010; Saibil et al., 2012; Yang et al., 2013). To assess the spatial distribution of molecular chaperones within the NM-YFP dot aggregates in our deletion strains, we created a series of mCherry fusions. C-terminally tagged Hsp104, Hsp70 (Ssa1 and Ssa2), and Hsp40 (Sis1) derivatives were constructed by integrating a copy of mCherry in frame with the endogenous coding regions. Confocal microscopy was then used to assess their respective cellular distributions. Hsp104, Hsp70 (Ssa1 and Ssa2), and Hsp40 (Sis1) derivatives were distributed evenly throughout the dot aggregates (Fig. 4, C). However, the strong Hsp104-mCherry fluorescence in the Hsp70 deletion strains (Δssa1 and Δssa2) clearly showed its peripheral distribution (Fig. 3 D and Fig. S4 B). In the Δsse1 strain, Hsp104 was almost exclusively confined to the perimeter of the dots (Fig. 3 E and Fig. S4 C), with the mCherry signal toward the center at background level. Hsp70 (Ssa1 and Ssa2) also showed a preferred localization toward the periphery of NM-YFP dots in the Δsse1 strain (Fig. 3 E).

Amorphous shells surrounding NM-YFP fibrils are enriched in Hsp110
We used immunogold EM on freeze-substituted sections to define the chaperone distributions in NM-YFP dots on the nanoscale. An anti–YFP antibody clearly localized NM-YFP fibrils to a well-preserved fibril array arrangement consistent with the example shown. Bar, 200 nm.
rimeter shells, and the dark amorphous clumps (Fig. 5 B, yellow, red, and blue outlines, respectively). For the Δsse1 strain, only a modest (approximately twofold) increase in Hsp70 labeling was observed on the dot aggregates compared with the cytosol (Fig. 5 D). Again, the mesh-like amorphous material in these strains was poorly labeled with the α-Hsp70 antibody (Fig. 5 C, blue outline).

Sse1 was not significantly concentrated in the dots in cells with a wild-type complement of chaperones (Fig. 6 A). In contrast, in Δssa1 or Δssa2 strains, there was a more than fourfold increase in the levels of Sse1 on the dot assemblies (Fig. 6, B and D). This redistribution of Sse1 was remarkable, given that Sse1 was not significantly up-regulated in these strains (Fig. 3 A). Furthermore, Sse1 was predominantly located in the nonfibrillar peripheral zones and the amorphous clumps with little fibril labeling (Fig. 6 B). As expected, barely detectable background labeling was observed with α-Sse1 labeling of sections from the Δsse1 strain, which was presumably attributable to Sse2, as described earlier (Fig. 6, C and D).

Consistent with the fluorescence observations (Fig. 3 B), our Western blots revealed a low abundance of Hsp104 in NM-YFP–expressing cells with a wild-type chaperone complement (Fig. 3 A), making it undetectable by immunogold EM. For cell sections of the Hsp70 deletions (Δssa1 and Δssa2), however, the elevated Hsp104 expression levels facilitated specific immunogold detection of Hsp104 (albeit with low labeling efficiency; Fig. 7, A–D), confirming the peripheral localization seen by confocal microscopy (Fig. 3 C).

The perimeter shells thus contained Hsp70 (Ssa1/Ssa2; Fig. 3 C and Fig. 5 B), Hsp40/Sis1 (Fig. 3 C), and Hsp104 (Fig. 3 C and Fig. 7, A–D) and were significantly enriched for Hsp110/Sse1 (i.e., all of the major components of the yeast disaggregase machinery; Fig. 6, B and D). Therefore, these nonfibrillar zones define a region of NM-YFP aggregates with an altered balance between aggregation and disaggregation.

Figure 3. NM-YFP assemblies have a nonuniform distribution of molecular chaperones. (A) Western blot of cell lysates showing Hsp104, Hsp70, and Sse1 expression levels in cells with a wild-type chaperone complement, which were either [PSI+] [NM-YFP aggregate dots] or [psi−] (soluble NM-YFP expressed but not converted to the prion state) and Δssa1, Δssa2, and Δsse1 cells. Glucose-6-phosphate dehydrogenase (G6PDH) was used as a loading control. (B) Plot of the quantitation of three independent immunoblots with the αHsp104 antibody, as indicated. The abundance of Hsp104 for each of the lysates is expressed relative to its level in the [PSI+] lysate and error bars correspond to the SD between the replicates. (C) Representative confocal Z-sections showing the distribution of mCherry-tagged Hsp104, Ssa1, Ssa2, and Sis1 on NM-YFP aggregates in cells with a wild-type complement of chaperones. The data are representative of at least 20 different NM-YFP dots for each labeled chaperone from two independent experiments. Imaging was optimized to visualize the fluorescence from the dot aggregates. A line plot illustrating the colocalization of Hsp104-mCherry with the NM-YFP dot in this strain is shown in Fig. 3B. Bars, 2 µm. (D) Representative confocal Z-sections showing the distribution of mCherry-tagged Hsp104, Ssa1, and Sis1 on NM-YFP aggregates in Δssa1 cells. Δssa2 cells displayed a similar chaperone distribution and thus have been omitted here. The data are representative of at least 20 different NM-YFP dots for each labeled chaperone from two independent experiments. Imaging was optimized to visualize the fluorescence from the dot aggregates. A line plot illustrating the colocalization of Hsp104-mCherry with the NM-YFP dot in this strain is shown in Fig. 3B. (E) Representative confocal Z-sections showing the distribution of mCherry-tagged Hsp104 and Hsp70 (Ssa1 and Ssa2) on NM-YFP aggregates in Δsse1 cells. We were unable to obtain a viable Sis1-tagged clone in this deletion strain. The data are representative of at least 20 different NM-YFP dots for each labeled chaperone from two independent experiments. Imaging was optimized to visualize the fluorescence from the dot aggregates. A line plot illustrating the colocalization of Hsp104-mCherry with the NM-YFP dots in this strain is shown in Fig. 3B.
Hsp104 and Sse1 can independently influence NM-YFP fibril assembly

Our immunogold labeling experiments suggested that NM-YFP fibrils are remodeled in regions enriched in Hsp104 and/or Sse1. To further test this hypothesis, we transiently overexpressed either Hsp104 or Sse1 individually (on plasmids pHsp104 or pSse1, respectively) in an otherwise wild-type chaperone background and examined the resulting NM-YFP dot assemblies by electron tomography.

In cells transiently overexpressing Hsp104, 80% of dot structures (40 dots examined) contained disorganized or amorphous regions (Fig. 8, A and B). The remaining dots contained large but normal NM-YFP fibrillar arrays (not shown). In the altered dot aggregates, large nonfibrillar zones were usually present, reminiscent of those seen in the Δssa1 and Δssa2 strains (Fig. 1, B and C; and Fig. 8 A). Both Hsp104 and Sse1 could be detected within the nonfibrillar zones in these dot aggregates (Fig. 8, C and D), consistent with their combined presence in these regions in the Δssa1 and Δssa2 strains. Other abnormal dot aggregates in this population comprised regions of highly fragmented and randomly arranged small fibrils that were often accompanied by dark-staining amorphous aggregates (Fig. 8 B).

Transient overexpression of Sse1 also resulted in a majority of abnormal NM-YFP aggregate assemblies. Here, ~75% were disorganized, whereas the remaining assemblies were large but normal-looking dots. The disrupted dots had an extremely disordered fibrillar core surrounded by a web of darkly stained amorphous material (Fig. 9, A and B). This material did not contain NM-YFP (Fig. 9, C and D) and was reminiscent of the dark amorphous aggregates seen occasionally in fibril assemblies in the Δsse1 strain, as described earlier.

Discussion

We have explored the role of the Hsp70 chaperone system in the in situ organization of a [PSI+] prion model. We describe a novel nonfibrillar surface layer of amyloid-containing dot aggregates that appears to result from an altered balance between fibril assembly and disassembly. This material was indistinguishable from the “normal” fibrillar NM-YFP aggregates in our cells by fluorescence and could only be discerned by electron tomography.

We used a model system with a highly overexpressed but nontoxic form of the [PSI+] prion. This enabled us to visualize the 3D structure of the aggregates by correlative fluorescence and electron tomography and to probe the effects of modulating the major Hsp70-related chaperones. Although the fluorescent foci appear small and dispersed under native expression conditions (Satpute-Krishnan and Serio, 2005; Song et al., 2005; Park et al., 2014), it seems likely that similar molecular interactions would occur in the smaller aggregates.

Although yeast prion proteins can assemble into amyloid fibrils in vitro in the absence of additional cofactors, interactions with the protein quality control machinery are required for their assembly, deposition, and propagation in yeast cells (Tuite et al., 2011; Liebman and Chernoff, 2012; Tyedmers, 2012). Perturbing proteostasis through selective Hsp70 deletion revealed the nonfibrillar aggregates of NM-YFP, giving a
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nanoscale structural view of processes underlying previous observations of the cellular roles of Hsp70s on prion propagation (Sharma and Masison, 2008; Sharma et al., 2009). Our single Hsp70 deletions stably propagated dot aggregates of NM-YFP, but changes in expression level of the Hsp104 disaggregase appear to account for the observed dramatic structural changes. First, although the Δssa1 and Δssa2 strains maintained global Hsp70 and Sse1 expression levels, there was an up-regulation of Hsp104 (Fig. 3 A). Moreover, Hsp104 was primarily located around the periphery of NM-YFP dots (Fig. 3, C–E; and Fig. 7). In addition, there was a marked recruitment of Sse1 to this boundary region in Δssa1 and Δssa2 cells (Fig. 6 B). Our findings indicate that recruitment of Hsp104 and Hsp110/Sse1, rather than loss of a specific Hsp70 function, plays a key role in the structural changes, possibly by aggregate remodeling.

It is well established that Hsp104 is essential for [PSI+] propagation (Chernoff et al., 1995), most likely by fibril trimming/fragmentation in cooperation with Hsp70/Hsp40, to generate new seeds (Misselwitz et al., 1998; Ferreira et al., 2001; Wegezyn et al., 2001; Higurashi et al., 2008; Tessarz et al., 2008; Tipton et al., 2008; Kampinga and Craig, 2010; Moosavi et al., 2010; Winkler et al., 2012b). Although there is evidence that Hsp110/Sse1 is also involved in these processes, its precise role has been unclear. As a major nucleotide exchange factor for Hsp70, Sse1 can stimulate amyloid fragmentation by Hsp104 both in vivo and in vitro (Fan et al., 2007; Kryndushkin and Wickner, 2007; Sadlish et al., 2008; Shorter, 2011). Recent studies show that Sse1 (and Hsp110 in mammals), together with Hsp70/Hsp40, acts as a disaggregase (Shorter, 2011; Rampelt et al., 2012; Song et al., 2013; Torrente and Shorter, 2013), although the in vitro reaction with Sup35 NM fibrils was extremely slow (Duenwald et al., 2012; Torrente and Shorter, 2013).

What is the function of Sse1 in the nonfibrillar, peripheral regions of NM-YFP assemblies in our strains? This question was addressed by examining the morphology of dot aggregates generated in the absence of Sse1. Although deletion of SSE1 causes slow growth in yeast (Mukai et al., 1993; Trott et al., 2005; Abrams et al., 2014), our Δsse1 strain stably propagated NM-YFP dot aggregates. Interestingly, the fibrils within NM-YFP dots from Δsse1 cells were approximately 60% longer than those from Δssa1, Δssa2, and cells with a wild-type chaperone complement (Fig. 1 E). Unlike an Hsp104 knockout, which cannot propagate [PSI+] (Chernoff et al., 1995), it would appear that removal of Sse1 alters the balance toward [PSI+] fibril growth over cleavage, resulting in aggregates with longer fibrils. By contrast, the Hsp70 deletions (Δssa1 and Δssa2) did not affect the lengths of the remaining fibrils (Fig. 1 E), despite the elevated Hsp104 levels in these mutants. We hypothesize that in these strains, remodeling of the outer layer of the aggregates most likely results from elevated Hsp104 progressively disassembling fibrils in initially normal dots, starting from the outer surface.

The alterations in aggregate morphology suggest direct functional roles for both Hsp104 and Sse1 in fragmentation and disassembly of NM-YFP fibrils in vivo. But what are the individual contributions of these two molecular chaperones to this process? In our [PSI+] model, we found that transient overexpression of either Hsp104 or Sse1 separately had a significant effect on the resulting NM-YFP dot morphology. Hsp104 overexpression reproduced the nonfibrillar zones seen in aggregates in the Hsp70

Figure 5. Immunogold detection of Hsp70 on NM-YFP assemblies in Hsp70 and Hsp110 knockout cell sections. Same as for Fig. 4, but detection was with an α-Ssa antibody to label Hsp70. C, cytosol; D, dot aggregate; WT, wild type.
deletions (Fig. 8 A) with Sse1 recruited to these amorphous regions (Fig. 8 D). Overexpression of Sse1 produced more disorganized NM-YFP dot assemblies, often surrounded by mesh-like, amorphous, unidentified aggregates (Fig. 9, A and B). The Hsp104 overexpression result supports the notion that it simply disassembles NM-YFP fibrils. This conclusion was originally based on the reduction in aggregate size observed by SDD-AGE, a method that does not distinguish between individual polymers and clumps (Kryndushkin et al., 2003). Sse1, however, appears to regulate fibril length and organization in a more measured type of fragmentation.

In summary, our findings reveal the in situ roles of Hsp104 and Sse1 as principal determinants of the in situ amyloid structure. Our manipulations revealed an active zone in which these chaperones were enriched, likely remodeling the aggregate species found there. Deletion of SSE1 did not result in a nonfibrillar zone despite the clear localization of Hsp104 to the dot periphery (Fig. 3 E and Fig. S4 C). It therefore seems that both chaperone components are required to efficiently process fibrils of NM-YFP. Notably, these structural changes did not affect prion stability, still generating prion seeds that maintain epigenetic inheritance.

Materials and methods

Yeast strains media and plasmids

Yeast strains for the analysis of [PSI+] aggregates were derived from the 74D-694 background (MATa, ade1-14 ura3-52 leu2-3,112 trp1-289 his3-Δ200; Chernoff et al., 1995), which was manipulated to constitutively propagate an NM-YFP fusion in the prion state (Tyedmers et al., 2010). The NM domain–encoding region of the endogenous SUP35 gene was deleted in this parental strain and an NM-YFP fusion cassette, expressed from the constitutive GPD promoter, was integrated at the TRP locus. Strains were grown using standard culturing techniques in YPD media (1% yeast extract, 2% peptone, and 2% glucose) or synthetic dropout media (0.7% yeast nitrogen base without amino acids, 2% glucose) lacking the relevant nutrient for auxotrophic plasmid selection. Excess adenine, at 100 mg/l, was included in media when growing 74D-694 derivatives. Where appropriate, antibiotic selection was achieved using G418 (200 µg/ml) or hygromycin B (50 µg/ml).

Construction of the Δssa1, Δssa2, and Δsse1 deletion derivatives of our NM-YFP parental strain was achieved using a standard PCR-based deletion strategy (Baudin et al., 1993; Wach et al., 1994). In brief, the kanMX4 cassette from plasmid pDH6 (Yeast Resource Centre; Wach et al., 1997) was amplified by PCR, incorporating the appropriate flanking homology sequences for targeted integration and gene deletion by homologous recombination, using the oligonucleotide combinations listed in Table S1. Cells were transformed with these PCR products using a standard lithium acetate transformation protocol and were selected for resistance to G418. The integrity of the deletions was confirmed by colony PCR.

Fluorescent protein labeling of Hsp104, Ssa1, Ssa2, and Sis1 for colocalization studies with NM-YFP was conducted by a C-terminal
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fusion of the respective chromosomal coding sequence with mCherry (Shaner et al., 2004) using standard PCR targeting (Wach et al., 1997). The integration cassettes were amplified from plasmid pBS35 (Yeast Resource Centre) and contained the mCherry chromosomal coding sequence, the hygromycin B resistance cassette, and the appropriate flanking homology sequences for targeted integration. The oligonucleotide primer pairs used are listed in Table S1. Cells were transformed with these PCR products using a standard lithium acetate transformation protocol and were selected for resistance to hygromycin B. Correct integration was confirmed by PCR and the resulting fusions were verified by sequencing. Plasmids pHsp104 and pSse1 were constructed by PCR amplification of HSP104 and SSE1, respectively, and digestion with BamHI-SalI followed by ligation to p425Gal1 (Mumberg et al., 1995), which had been digested with the same restriction endonuclease pair. Oligonucleotide pairs used are listed in Table S1. Both plasmids were verified by sequencing. Galactose induction of the Gal1 promoter on p425Gal1 was conducted by growing cultures initially in SD media (lacking leucine) with 2% glucose to mid-late log phase. Cells were then pelleted by centrifugation (3400 rpm, 10 min), resuspended in media with 2% galactose, and grown for a further 6 h. Overexpression of Hsp104 and Sse1 was verified by Western blotting.

Fluorescence microscopy of yeast cells

For routine live-cell imaging, yeast cells were mounted on polysine slides (VWR International LLC) with coverslips (0.13- to 0.16-mm thick) in growth media. Conventional epifluorescence images were acquired from plasmid pBS35 (Yeast Resource Centre) and contained the mCherry chromosomal coding sequence, the hygromycin B resistance cassette, and the appropriate flanking homology sequences for targeted integration. The oligonucleotide primer pairs used are listed in Table S1. Cells were transformed with these PCR products using a standard lithium acetate transformation protocol and were selected for resistance to hygromycin B. Correct integration was confirmed by PCR and the resulting fusions were verified by sequencing.

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noise and total fluorescence in the NM-YFP dot were also monitored during the time course and used for normalization. The data were normalized using the easyFRAP program to account for differences in the starting intensities and for acquisition photobleaching during the time course (Rapsomaniki et al., 2012). The data presented for each strain were the mean of at least seven independent dot aggregates. Error bars in the graph correspond to the SD in the data.

**EM sample preparation**

Yeast cells grown to mid-late log phase were pelleted by centrifugation (3,400 rpm, 10 min) and resuspended in growth medium to achieve 500- to 1000-fold concentration. Aliquots were immediately transferred a Leica type A aluminum specimen carrier (0.2-mm indentation), covered with the flat side of a type B specimen carrier, and high-pressure frozen using the EM HPM100 system (Leica). Cryofixed samples were then freeze substituted and embedded with Lowicryl HM20 (Agar Scientific) using an EM AFS2 device (Leica) according to a protocol adapted from Hawes et al. (2007). In brief, cells were freeze substituted in dry acetone containing 0.1% uranyl acetate and 1% methanol at −90°C for 16 h. Samples were then warmed to −50°C at 20°C/h and washed twice in 100% ethanol for 30 min, before five exchanges with 1-h incubations into increasing concentrations of HM20 (25%, 50%, 75%, 100%, and 100%). A further exchange into 100% HM20 was then performed and incubation was continued at −50°C for 18 h. After a fresh HM20 exchange and a second 18-h incubation, the HM20 was polymerized under UV light at −50°C for 48 h. The samples were then warmed to 20°C at 10°C/h and polymerization was continued under UV illumination for an additional 48 h.

HM20-embedded sample blocks were then sectioned at RT on an EM UC7 Ultramicrotome (Leica) using an Ultra 45° diamond knife (Diatome). 65- to 100-nm-thick cryo-sections were cut at −140°C using an EM UC7 Ultramicrotome equipped with an EM FC7 cryochamber using a cryo 25°C diamond knife (Diatome). Cryosection ribbons were mounted on custom-made C-flat carbon-coated 200-mesh Gilder finder grids (Protochips Inc.) that had been covered with a 5-nm continuous carbon layer and coated with protein A gold. Cryosections were stored under liquid nitrogen for future use.

**Correlative cryo-fluorescence imaging**

HM20-embedded cell sections mounted on EM grids were visualized by wet mount as described elsewhere (Kukulski et al., 2011). 0.2-µm Blue Fluospheres (365/415; Life Technologies) were applied to grids as fluorescent fiducials for correlation with EM projection images. Fluorescence microscopy was performed using an AxioScope A1 microscope, equipped with an X-Cite Series 120Q lamp and an Orca R2
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CCD camera, and using a 63× Plan-Apochromat oil immersion objective lens (NA1.4). YFP signal was monitored using 546/512-nm and LP 590-nm excitation and emission filters, respectively. Blue FluoSpheres were imaged using G 365-nm and 445/450-nm excitation and emission filters, respectively. Correlation between fluorescence and low magnification EM images of the same sample was performed using the “cselect,” “cp2tform,” and “tformfwd” functions from the image processing toolbox in MATLAB (The MathWorks, Inc.). Affine transformations accounting for translation, rotation, and scaling differences between the two images were calculated using the coordinate positions of the blue FluoSpheres as control point pairs. The coordinate positions of NM-YFP dots on fluorescence images were then transformed onto the corresponding low-magnification EM projection images. This process was repeated for EM images of a magnification series up to that used for tilt-series acquisition.

Cryofluorescence imaging of vitreous cryosections was performed with the same microscope described above equipped with a cryostage (Linkam Scientific Instruments) operated at −195°C, using a 100× LD EC Epiplan-Neofluor objective lens (NA0.75). Correlation to NM-YFP dots in fluorescently mapped cryosections was performed in real time on the electron microscope using a software routine written in MATLAB and described elsewhere (van Driel et al., 2009). The four grid bar corners of a single EM grid square were used as landmarks for the registration of fluorescence images with a view of the same area on the electron microscope. The EM stage coordinates of these four known points were recorded by the software, which then calculated, by affine transformation, the stage coordinates corresponding to an NM-YFP dot location on the fluorescence image. The software was then used to set the EM stage to the location of the NM-YFP dot in the sample. This method had an expected accuracy of 0.5 µm (van Driel et al., 2009). NM-YFP dots typically occupied an area of 0.7–1.7 µm² in cell sections, so the EM field of view of 5.3 µm² used for tilt-series acquisition (see below) was chosen so as to always include the feature of interest. Precise correlation validation was subsequently performed using the MATLAB image processing toolbox, as outlined above for resin cell sections. The pattern of holes in the C-flat support film and autofluorescence from the cryosections (recorded using G 365-nm and 445/450-nm excitation and emission filters) facilitated accurate correlation.

Immunogold labeling of HM20-embedded cell sections

100-nm cell sections mounted on carbon-coated 300-mesh copper grids were used for on-section gold labeling. Grids were initially floated section-side down on 30-µl droplets of blocking buffer (1% BSA in PBS with 0.01% Tween 20) for 5 min. They were then transferred onto 30-µl droplets of the appropriate primary antibody diluted in blocking buffer and incubated at RT for 60 min. The grids were washed by six 2-min exchanges onto 60-µl droplets of blocking buffer before being transferred to 30-µl droplets of Protein-A gold, diluted 1:10 with blocking buffer. Incubation was continued at RT for 30 min. The grids were then washed with three changes of blocking buffer and three changes of PBS, for 2 min each. Labeling on the sections was then fixed by floating on 30-µl droplets of 1% buffered glutaraldehyde for 5 min, followed by four 1-min washes in milliQ water. NM-YFP was labeled with a mouse monoclonal α-YFP antibody (Takara Bio Inc.) at a working concentration of 20 µg/ml. A rabbit polyclonal α-Hsp104 antibody (Abcam) was used for Hsp104 labeling at a concentration of 50 µg/ml. Hsp70 labeling was with a rabbit polyclonal α-Ssa1 antibody diluted 1:50 (Winkler et al., 2012b). Sse1 was labeled with a rabbit polyclonal α-Sse1 antibody diluted 1:50.
EM data collection and processing

Grids mounted with HM20-embedded cell sections were placed into a Model 2040 dual-axis tomography holder (Fischione Instruments) and imaged using a Tecnai F20 microscope (FEI) operated at 200 kV with a 4k CCD camera (Ultrascan US4000; Gatan). Dual-axis tilt series were collected using SerialEM (Mastronarde, 2005) typically over a tilt range of ±60° with 2° increments at an unbinned pixel size of 0.585 nm. The nominal defocus was 0.5 µm. For reconstruction, tilt-series images were binned to a pixel size of 1.17 nm and dual-axis tomograms were generated using the IMOD package, version 4 (Kremer et al., 1996; Mastronarde, 1997). For plotting of individual fibril lengths in NM-YFP dots, fibrils were measured using the model tools of the 3dmod software package implemented in IMOD (version 4; Kremer et al., 1996). The data were analyzed using MATLAB.

Imaging of immunogold-labeled cell sections was conducted on Tecnai T10 and T12 microscopes (FEI) at full voltage, and equipped with 1K Multiscan CCD cameras (Gatan). Images were collected at various magnifications within a calibrated pixel size range of between 1.5 and 4.3 nm. Quantification of labeling was performed using Fiji (Schindelin et al., 2012).

Vitrified cryosections were imaged using a Tecnai Polara microscope (FEI) operated at 300 kV with a 4k CCD camera (Ultrascan US4000; Gatan). Tilt series were collected using SerialEM (Mastronarde, 2005) over a tilt range of ±60° with 1.5° increments at nominal defoci of between 8.0 and 10.0 µm and an unbinned pixel size of 0.563 nm. Low-dose conditions were implemented to apply a total dose of 60 e/Å2 for each tilt series. Tomogram reconstructions were performed using IMOD (version 4; Kremer et al., 1996).

Yeast lysate preparation, Western blotting, and SDD-AGE

Yeast cells were grown in the appropriate growth media to mid-late log phase, pelleted (3400 rpm, 10 min), and resuspended in lysis buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 0.5 mM EDTA) with protease inhibitors (P8215; Sigma-Aldrich). Resuspended cells were lysed by explosive decompression with two passages through a French Pressure Cell (Amino; Thermo Fisher Scientific) operated at 10,000 pounds per square inch. The resulting crude lysates were centrifuged to remove unlysed cells and cellular debris (2,700 rpm, 10 min) and the clarified lysates were then stored at −80°C for future use.

SDS-PAGE and Western blots were performed using standard techniques. For Western analysis, 3 µg of each lysate was run on gels and equal loading was monitored with an α-glucose-6-phosphate dehydrogenase–HRP conjugate antibody (ab34601; Abcam). α-YFP (Takara Bio Inc.), α-Ssa1 (Winkler et al., 2012b), and α-Sse1 antibodies were used at a dilution of 1:10,000. α-Hsp104 (Abcam) was used at a dilution of 1:5,000. Band intensities were quantified using Fiji (Schindelin et al., 2012) and analyzed in MATLAB.

SDD-AGE analysis was performed as previously described (Kryndushkin et al., 2003; Halfmann and Lindquist, 2008). In brief, 40 µg of the lysates was incubated for 5 min at 37°C in 0.5 M Tris-acetate-EDTA, 2% SDS, 5% glycerol, and 0.05% bromophenol blue and subjected to electrophoresis in 1.5% agarose with 0.1% SDS, without heating the samples or the gel during running. Prion protein was detected by immunoblotting with α-YFP.

Sedimentation protifiling of yeast lysates was performed by centrifuging 1 ml of each lysate, adjusted to 0.5 mg/ml in lysis buffer, at 65,000 rpm in a TLA110 fixed angle rotor for 50 min using a Beckman Optima TLX Preparative Ultracentrifuge. The supernatant fractions were collected and the pellets were then resuspended in 1 ml of lysis buffer. Fractions were separated by SDS-PAGE or SDD-AGE and analyzed by Western blotting.

Database depositions

Representative tomograms have been deposited in the Electron Microscopy Databank with accession codes EMD-3118 (Fig. S2 E), EMD-3119 (Fig. S1 C), and EMD-3124 (Fig. 2 D).

Online supplemental material

Fig. S1 shows live-cell fluorescence/differential interference contrast microscopy and cryoelectron tomography of NM-YFP dot aggregates. The fluorescent dot aggregates are resolved as bundles of hexagonally packed fibril arrays by electron tomography of both freeze-substituted and vitreous cryosections. In Fig. S2, tomographic sections of dot aggregates in yeast strains Δssa1, Δssa2, and Δsse1 show marked structural changes. The fibril arrays are disrupted or absent in Δssa1 and Δssa2 strains, and dark, amorphous material is associated with the aggregates. These features are confirmed in tomograms of cryosections. In the Δsse1 strain, large aggregates with long fibrils are overlaid by a meshwork of amorphous material. Fig. S3 shows FRAP curves reporting on molecular mobility in NM-YFP dot aggregates. In all of the above strains, the prion protein is immobile, including the ones with nonfibrillar regions. Fig. S4 contains line traces of fluorescence images of dots in wild-type, Δssa2, and Δsse1 strains, showing that Hsp104 is located around the periphery of the dot aggregates. The oligonucleotides used in this study are listed in Table S1. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.201505104/DC1.

Acknowledgments

We thank David Houldershaw, Yanni Goudetsidis, and Richard Westlake for providing computing support, and Luchun Wang for EM technical assistance. We are very grateful to Bram Koster, Linda van Driel, and Frank Faas for installation of their correlative software package on our transmission EM and Richard Hayward for critical reading of the manuscript. We especially thank Jens Tyedmers for invaluable advice on yeast genetics, provision of background strains and cloning vectors, and critical reading of the manuscript. Finally, we thank members of the Birkbeck EM group, past and present, for helpful discussions.

This work was supported by Wellcome Trust Program and equipment grants 079605.086018, 089050, and 101488.

The authors declare no competing financial interests.

Submitted: 25 May 2015
Accepted: 8 September 2015

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