Chapter 4

The Role of the Hsp40 Chaperone Sis1 in Yeast Prion Propagation

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Additional information is available at the end of the chapter

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Abstract

Yeast prions are self-templating amyloid aggregates composed of misfolded cellular proteins. In order to propagate, yeast prions must be broken into heritable seeds that are passed to subsequent generations. The replication step of the prion propagation cycle is accomplished by the actions of molecular chaperones, which bind to and serve the fibers through a process called disaggregation. Prions can be thought of as molecular diseases that have hijacked the chaperones for their continued existence. When viewed in this way, the study of yeast prions has been very informative about the interactions among of the molecular chaperones. This chapter focuses on the role of a single Hsp40 or J-protein, Sis1, in the propagation of yeast prions. While Sis1 seems to be required for the maintenance of many different prions, various prions depend on Sis1 in different ways, perhaps due to differences in underlying amyloid structure. New evidence is emerging that Sis1 is important for processes that may not involve prion replication activity, providing an intriguing alternative explanation for the observed differences in the prions' reliance on Sis1.

Keywords: Sis1, yeast prion, [*PSI*$^+$], [URE3], [*RNQ*$^+$], Hsp40, J-protein, prion propagation, amyloid

1. Introduction

In 1994, Reed Wickner solved a puzzle that had beguiled *Saccharomyces cerevisiae* (budding yeast) geneticists for decades [1–4]. He showed that certain genetic elements that did not follow the classical rules of DNA-based inheritance were due to alternative structures that an otherwise normal protein could adopt [5, 6]. These cytoplasmic genetic elements, some examples being [*PSI*$^+$] or [URE3] (the brackets denote the non-Mendelian character

of the phenotype), were dominant in crosses but were present in all meiotic progeny, a clear violation of Mendelian inheritance. Inspired by contemporary work in human health that suggested the agent of long-known livestock diseases such as scrapie and its human counterparts kuru and Creutzfeldt-Jakob disease (CJD) was a misfolded variant of a protein found in all mammals (and coining a new term "prion"—for proteinaceous infectious virion-like particle [7]), Wickner's group demonstrated that [*PSI*$^+$] and [URE3] propagated as misfolded versions of the yeast Sup35 or Ure2 protein, respectively [8, 9]. Soon, a new field emerged and several other yeast prions have been discovered and characterized (the story of the discovery of yeast prions is reviewed in Ref. [10]). **Table 1** lists the yeast prions known to date and the protein determinant that adopts the alternative fold. It was quickly discovered that the prions' continued propagation in the cytosol of yeast cells was highly dependent upon the molecular chaperones [11, 12], a strictly conserved suite of diverse proteins that evolved to protect the other proteins in the cell from the harshness of the environment.

The molecular chaperones were first identified in *Drosophila melanogaster* in the late 1970s as proteins that appeared or greatly increased in abundance after exposure to stress, such as heat [19]. These heat shock proteins (HSPs) are characterized by their apparent molecular weight in poly-acrylamide gels, for example, Hsp90, Hsp70, Hsp60 and Hsp40. Each class of HSP usually contains multiple isoforms that have overlapping and specific roles in various processes. **Table 2** lists the major chaperone families and their general activities. The molecular chaperones protect cells from stress and to varying extents are able to overcome the effects of stress. Importantly, molecular chaperones are also involved in myriad processes that are not related to stress, but are crucial to the overall proper function of the cell. Such processes include transport of proteins across membranes, assisting in the proper folding of newly syn-

Name	Determinant	Phenotype	Notes	Reference
[*PSI*$^+$]	Sup35	Nonsense suppression	Weak and strong variants	[1]
[URE3]	Ure2	Derepression of nitrogen utilization pathways		[3]
[*RNQ*$^+$]	Rnq1	Rnq1 aggregation; decrease in de novo [*PSI*$^+$] appearance	Also known as [*PIN*$^+$], for [*PSI*$^+$] inducibility, has several variants	[13]
[*ISP*$^+$]	Sfp1	Antisuppression (reverse of [*PSI*$^+$] phenotype)	Not dependent on Hsp104	[14]
[*SWI*$^+$]	Swi1	Poor growth on alternative carbon sources		[15]
[*OCT*$^+$]	Cyc8	Cyc8 deletion		[16]
[*MOT3*$^+$]	Mot3	Pseudohyphal growth and biofilm formation		[17]
[*MOD*$^+$]	Mod5	Fluconazole resistance	Prion-forming domain is not Q/N rich	[18]

Table 1. Amyloid-based yeast prions identified to date.

Class	Structure and function	Yeast	*E. coli*
Hsp100	Hexameric AAA+ ATPase disaggregase	Hsp104	ClpB
Hsp90	Dimeric posttranslational modifier of client activity	Hsc82, Hsp82	HtpG
Hsp70	Holdase; binds and releases unfolded polypeptides	Ssa1 through Ssa4; Ssb1, Ssb2	DnaK
Hsp60	Tetradecameric mitochondrial chaperonin	Hsp60	GroEL
Hsp40	Also called J-protein; dimeric stimulator of Hsp70 ATPase; substrate specificity	Sis1, Ydj1, Jjj1; 23 others (reviewed in Ref. [20])	DnaJ, CbpA, DjlA
Small HSPs	Crystallins, promoters of aggregation	Hsp42, Hsp26	

Table 2. The classes and names of cytosolic molecular chaperones and their general functions.

thesized proteins, protein turnover and cell division. Indeed, several entire classes of chaperones, such as Hsp70 and Hsp90, are essential to eukaryotic viability.

The first findings that molecular chaperones played a role in yeast prions were the discovery that overexpression of the AAA+ ATPase disaggregase Hsp104 "cured" cells of the [*PSI*$^+$] prion [11, 21]. While curing mediated by Hsp104 overexpression is a phenomenon unique to the [*PSI*$^+$] prion, (reviewed in Ref. [22]), the unexpected subsequent finding that deletion of the *HSP104* gene also resulted in inability to propagate [*PSI*$^+$] is a hallmark of most yeast prions, such as [URE3] and [*RNQ*$^+$] [11, 23, 24]. Yeast prions are highly ordered fibrous aggregates called amyloid, composed of a misfolded version of a normal yeast cytosolic protein. These amyloid fibers undergo a continual cycle of determinant protein addition and breakage that allows for sustained growth and transmission to new generations. Indeed, it is the action of the Hsp104 disaggregase, in concert with its Hsp70 and Hsp40 partners, that causes breakage and thus creation of new prion "seeds." These seeds are then passed to daughter cells where amyloid growth, breakage by chaperones and transmission to daughters continue unabated until the system is perturbed. Such perturbations include inhibition of Hsp104 activity by millimolar quantities of guanidine HCl or mutations in any of the chaperones involved. Thus, yeast prions are a valuable tool for studying molecular chaperones in vivo (reviewed in Ref. [25]).

This chapter focuses on the role of the Hsp40 Sis1 in yeast prion biology. **Figure 1** shows a cartoon of Sis1 domain structure. In addition to playing a central role in yeast prion propagation, Sis1 is essential for cell viability [26]. While the exact nature of Sis1's essential function remains unknown, it is clear that its regulation of Hsp70 function (via stimulation of Hsp70 ATPase, reviewed in Ref. [27]) is important, since the minimal Sis1 fragment required for growth is the Hsp70-interacting J-domain and the adjacent glycine/phenylalanine region [28]. Likewise, a single point mutation in any of the three universally conserved residues of the HPD (histidine, proline and aspartate) motif abolishes stimulation of Hsp70 ATPase in vitro [29] and is lethal in vivo [28]. Whether the essential function of Sis1 is also required for prion propagation is unclear, as will be discussed. The nonessential functions of Sis1 can easily

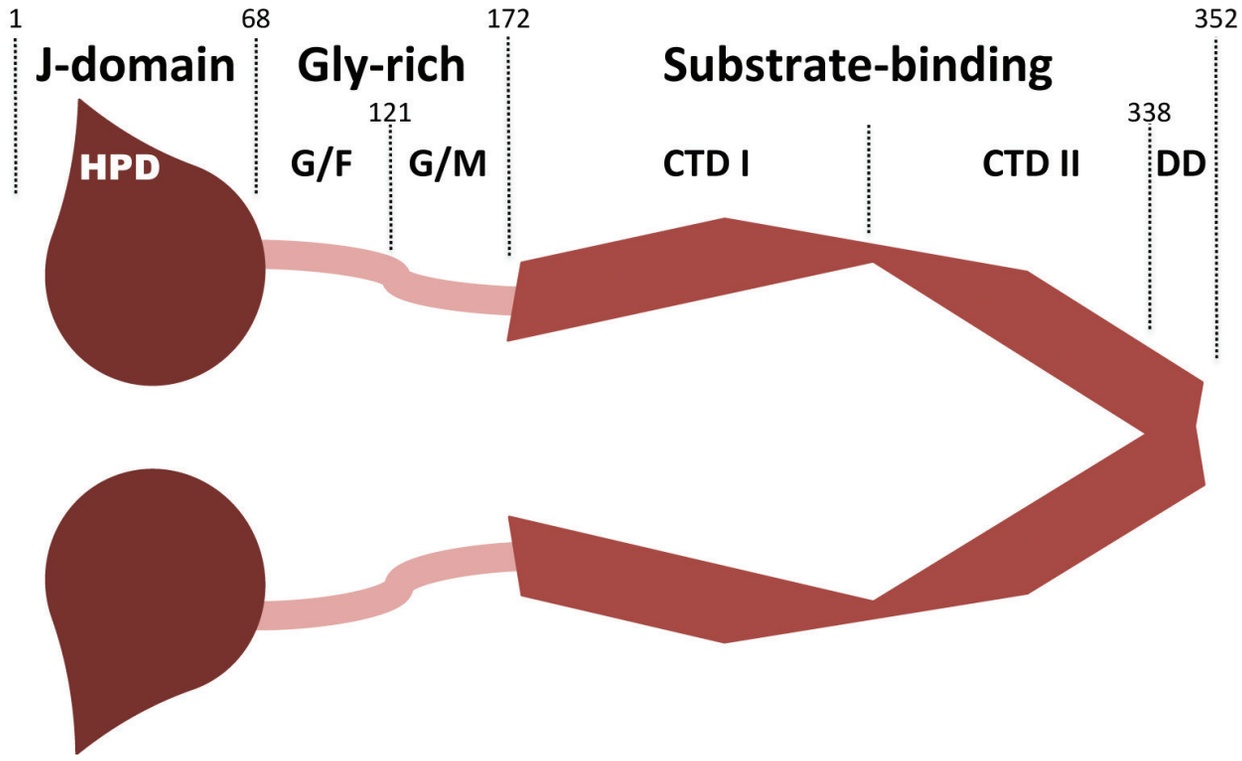

Figure 1. Cartoon of Sis1 domain structure. The J-domain is highly conserved and interacts with Hsp70 to stimulate ATPase and thus substrate transfer to Hsp70. The glycine-rich and substrate-binding domains are the areas of the most divergence between J-proteins and are thought to play a role in substrate selectivity. HPD: conserved Hsp70-interacting motif; Gly-rich: glycine-rich domain; G/F: glycine- and phenylalanine-rich region; G/M: glycine- and methionine-rich region; CTD I and CTD II are subdomains of the Sis1 substrate binding domain [34]; and DD: dimerization domain. The numbers above indicate amino acid position.

be studied using yeast prions, because the different yeast prions all seem to have differing requirements of Sis1 [30–33]. It is these nonessential Sis1 functions that are the focus of this chapter.

2. Yeast prion biology

Most yeast prions share a similar in-register parallel beta-sheet amyloid core composed of a prion-forming domain that is rich in asparagine and glutamine residues [35–37]. In the prion minus state, these domains are mostly unstructured and the protein is soluble and active. In the prion plus state, the prion-forming domains of newly synthesized prion protein molecules are recruited to the ends of an amyloid fiber. The in-register character of the beta-sheet core serves as a template for the incoming soluble molecule [38]. In addition to the different prions shown in **Table 1**, some prions such as [*PSI*$^+$] exhibit a range of distinct phenotypes called prion "variants" [39, 40]. In the case of [*PSI*$^+$], these variants roughly fall into two categories: strong and weak. It is known that phenotypically "strong" [*PSI*$^+$] variants are composed of thermodynamically unstable amyloid fibers, while "weak" [*PSI*$^+$] variants are composed of much sturdier amyloids [41]. Thus, the Sup35 protein can adopt multiple amyloid structures

that give rise to different phenotypes. It is likely that the locations of the turns between the beta-strands within the beta-sheet amyloid core govern amyloid thermostability and thus prion phenotype [42].

Most prion proteins also have globular domains that do not misfold to participate in the amyloid core [43, 44]. However, because the amyloid fiber as a whole is insoluble, these globular domains are essentially depleted from the cytosol. For two of the most studied prions, [*PSI*$^+$] and [URE3], depletion of the protein's function as a result of being in the prion plus state has been genetically coupled to the adenine biosynthetic pathway. Sup35, the determinant protein of the [*PSI*$^+$] prion, is the yeast eRF3 homolog, a GTPase that facilitates translation termination [45, 46]. When Sup35 is in the [*PSI*$^+$] state, there is read-through of stop codons [1, 2]. The presence of a nonsense mutation in certain genes of the adenine biosynthetic pathway, such as *ADE1* or *ADE2*, allows [*PSI*$^+$] to be monitored readily as growth on media-lacking adenine, due to suppression of the nonsense mutation. Likewise, [*psi*$^-$] cells are adenine auxotrophs. Using the adenine biosynthetic pathway to monitor prions has an added benefit. The substrates of the *ADE1* and *ADE2* gene products form pigments that are red in color and the accumulation of them within cells not expressing functional Ade1 or Ade2 proteins causes yeast colonies growing on limiting adenine media to appear red. Thus, [*PSI*$^+$] colonies are white and [*psi*$^-$] colonies are red. In the case of [*PSI*$^+$] prion variants, white colonies are categorized as phenotypically strong and weak variants appear pink on the same media. This variation in colony color is due to the relative amount of soluble, active Sup35 protein in the cell. The less soluble Sup35, the more read-through of the nonsense mutation and thus more Ade2 protein is made and less red pigment accumulates. The amount of soluble protein is directly related to the thermodynamic stability of the amyloid core of the prion. Strong phenotypes arise from thermodynamically unstable amyloids because they break more readily, exposing more ends and therefore effecting a more rapid depletion of the soluble prion protein pool. Conversely, weak variants have amyloids that are more thermostable, which break less and therefore have fewer sites of soluble protein recruitment, resulting in relatively more soluble, active protein [41]. This increase in Sup35 solubility leads to less read-through of the nonsense mutation and more accumulation of red pigment, resulting in pink colony color.

Ure2, the determinant of the [URE3] prion, is a regulator of yeast nitrogen catabolism [47, 48]. In the presence of certain nitrogen sources, soluble Ure2 binds to and sequesters transcription factors such as Gln3 in the cytoplasm [49]. When Ure2 is depleted, the transcription factors are free to move into the nucleus, where they activate genes such as *DAL5* that are essential for catabolism of poor nitrogen sources. By replacing the *ADE2* promoter with that from the *DAL5* gene, a similar red/white system for [URE3] was developed [50]. So, [URE3] cells are white and Ade$^+$ and cells lacking the prion, designated [ure-o], are red and ade$^-$. Rnq1, the determinant of the [*RNQ*$^+$] prion, has an unknown function [51]. Thus, it has not been linked to the adenine pathway without fusion to Sup35, which may complicate interpretations. The presence of the [*RNQ*$^+$] prion is most easily observed by visualization of prion aggregates in cells expressing green fluorescent protein (GFP)-tagged Rnq1 [52, 53].

Spontaneous breakage of amyloid fibers, even thermodynamically unstable ones, does not occur often enough to maintain the prion phenotype in an expanding yeast population. This

inefficient breakage is evident in the reliance of all amyloid-based yeast prions, except one (see **Table 1**) [54], on the activity of the Hsp104 molecular disaggregase. The absence of the *HSP104* gene is incompatible with amyloid-based prion propagation, as are inhibition of Hsp104 by millimolar amounts of guanidine HCl in the growth media [55, 56] or by expression of dominant negative alleles that poison the functional hexamer [57]. Hsp104 hexamers adopt an open barrel-like structure into which denatured and aggregated proteins are fed [58, 59]. Upon exit of the substrate through the central pore, it is then allowed to refold either spontaneously or assisted by other chaperones. While Hsp104 plays a pivotal role in yeast prion propagation, Hsp104 does not work alone. In fact, under normal conditions Hsp100 disaggregases, such as Hsp104 and its *Escherichia coli* ortholog ClpB, are inactive without the cooperation of Hsp70 and the obligate Hsp70 cochaperone J-protein (also known as Hsp40), both in vivo and in vitro [60, 61].

3. Generalized role of Sis1 in prion propagation

The first report that the essential yeast J-protein Sis1 was involved in prion propagation was in 2001 by a collaborative effort between Elizabeth Craig and Susan Lindquist [62]. Following up on earlier reports and communications that Sis1 co-immunoprecipitated with Rnq1 [26], the determinant of the [*RNQ*$^+$] prion [51], Sondheimer and colleagues first showed that Sis1 (and the Hsp70 Ssa1) interacted with Rnq1, but only in strains that were [*RNQ*$^+$]. Thus, Sis1 only interacted with Rnq1 when it was in its amyloid conformation. Furthermore, they identified the glycine/phenylalanine (G/F)-rich region of Sis1 as being crucial for the interaction with the amyloid form of Rnq1. This finding agreed with earlier findings from the Craig lab that the G/F region somehow functioned in substrate selectivity [28]. Using strains expressing GFP-labeled Rnq1, they then observed effects of Sis1 mutations on the size and distribution of Rnq1 aggregates (that result from being in the [*RNQ*$^+$] prion state), suggesting that certain Sis1 functions were important for prion maintenance. This idea was strengthened by the results of Sis1 mutational analysis on the propagation of a hybrid Rnq1-Sup35 prion (that allowed for assay of the prion state using the adenine-requiring system described above), which showed that Sis1 lacking any nonessential region was unable to support the hybrid prion. Thus, Sis1 was identified as playing a major role in the propagation of yeast prions. However, the nature of this role remained unknown, as important questions remained as to what step in the prion cycle Sis1 was involved.

A major step toward answering these questions came from the Craig lab in 2007 [63]. Aron and colleagues introduced into their strains a tetracycline-repressible system that shuts off transcription of target genes, which they called "TET-Off" [64], in this case *SIS1*. While the *SIS1* gene is essential, either the amount of expression is leaky enough or the turnover of the preexisting protein is slow enough, or some combination of these two factors that cells were able to grow up to 80 generations after initial treatment with the repressor. By expressing Rnq1-GFP in these cells, the authors showed that [*RNQ*$^+$] aggregates initially grew in size and then were accompanied by increase in diffuse fluorescence followed by appearance of cells that were completely diffuse and thus "cured" of the prion. They also demonstrated that the

diffuse fluorescence observed during the Sis1 depletion time course was from newly synthesized Rnq1, suggesting that the in the absence of Sis1 the amyloid had stopped recruiting new soluble molecules. This observation was consistent with the notion that amyloid growth is dependent upon continuous generation of new ends through fiber breakage. Importantly, the authors went on to show that Sis1 acted through Hsp70 in the propagation process, since mutations in the Hsp70-interacting HPD motif resulted in prion loss. Finally, a correlation of the effects of Hsp104 inactivation and Sis1 depletion on [*RNQ*$^+$] aggregates was demonstrated, suggesting that the two chaperones worked together in the same process. The authors concluded that Sis1 works together with Hsp104 and Hsp70 to break prion aggregates that are then transmitted to daughter cells. However, the exact role of Sis1 in the process was still unclear, for example, whether it worked upstream or downstream of Hsp104.

Having established that the [*RNQ*$^+$] prion relied on the activity of Sis1, the Craig lab subsequently showed the reliance on Sis1 of two other yeast prions, a strong variant of [*PSI*$^+$] and [URE3] [30]. In doing so, Higurashi and colleagues discovered that the three prions seemed to have differential dependence on Sis1. In this study, the authors first showed that any other nonessential yeast Hsp40 can be deleted and not affect either [*RNQ*$^+$] or [*PSI*$^+$] propagation, suggesting a unique role for Sis1. They then monitored loss of the prions after depleting Sis1 levels through the TET-Off system. While cells completely lost [*RNQ*$^+$] by the 20th generation after depletion of Sis1, [*PSI*$^+$] was not completely lost until the 80th generation. They next found that the [URE3] prion was most sensitive to Sis1 depletion, with all cells having become [ure-o] by the 10th generation of growth in doxycycline. These findings opened new paths of investigation that are discussed below. While this report was important for the discoveries therein, a robust understanding of the exact nature of Sis1's role in prion propagation was still lacking, but not for long.

In 2008 Jonathan Weissman's laboratory answered one of the most important remaining questions: What does Sis1 do in prion propagation [65]? By creating chimeric domain-swapped constructs between Hsp104 and the *E. coli* ortholog ClpB, which the authors showed is unable to rescue deletion of the *HSP104* gene when expressed exogenously in yeast, Tipton and colleagues determined that the "upper ring" of the Hsp104 barrel, composed of the N-terminal, first AAA ATPase domain and the coiled coil region, worked together to support prion propagation and thermotolerance. Thermotolerance is defined as survival to a lethal heat shock (~50°C) after a short, slightly elevated temperature pretreatment (37°C) and is a non-prion-related function of Hsp104 [66]. To maintain function, all three of these regions must be from the yeast version. However, the C-terminal AAA domain, which is thought to mainly play a role in hexamerization [67], could be swapped with the *E. coli* version without loss of Hsp104 activities. Next, the authors took advantage of the activity of this chimera and introduced a system previously used to monitor ClpB substrates in *E. coli*. A single point mutation in the C-terminal AAA ATPase domain of ClpB allows interaction with the chamber-like peptidase ClpP. By combining this mutant ClpB with a catalytically inactive version of ClpP called TRAP, which trapped substrates but did not destroy them, the identity of ClpB substrates were identified via co-immunoprecipitation with TRAP [68, 69]. Tipton and colleagues engineered their active Hsp104-ClpB chimera to feed substrates into co-expressed TRAP in yeast cells propagating various prions. They found Sup35 or Rnq1 co-immunoprecipitated with

TRAP in prion plus, but not prion minus versions of their cells. This finding showed that Sup35 and Rnq1 are Hsp104 substrates only when they exist in their amyloid form, reinforcing the findings from the Sondheimer and Aron studies. This conclusion in turn implied that Sis1 or some other factor was involved in the selection of the amyloid form of Sup35 or Rnq1 over the soluble form. When Tipton and colleagues combined their TRAP system with the Sis1 TET-Off system borrowed from the Craig lab, they discovered that with reduced levels of Sis1, Sup35 and Rnq1 were no longer bound to TRAP. These results suggested that Sis1 worked upstream of Hsp104, delivering substrates to it, most likely in cooperation with Hsp70.

The investigations into the collaborative barrier that exists between prokaryotic and eukaryotic Hsp100s and Hsp70s ended up reinforcing the notion that Sis1 provides the disaggregation machinery's specificity for amyloid substrates. In 2010 and 2011, two studies revealed the underlying reason of why ClpB cannot function in yeast and Hsp104 cannot function in *E. coli*, even though the two proteins are 43% identical and share very similar structure [70, 71]. In one, Miot and coauthors created chimeras between Hsp104 and ClpB similar to those in the Tipton study, but using slightly different junction points and with more domain-swapped combinations. Through in vitro refolding reactions, where purified chaperones work together to reactivate denatured enzymes such as luciferase and in vivo thermotolerance experiments in yeast, the authors showed that the coiled-coil domain conferred a species-specific collaboration. Thus, a chimeric construct that contained only the coiled-coil domain of Hsp104, with the rest of the molecule being ClpB sequence, could rescue loss of Hsp104 activity in vivo or could effectively work with yeast Hsp70 and Hsp40 chaperones in vitro [71]. Both papers showed that it was the Hsp70 component of the machinery that interacted at the coiled-coil domain of Hsp104 or ClpB [70, 71]. Thus, Hsp104 could only work with yeast Hsp70 (Ssa1) and ClpB could only work with *E. coli* Hsp70 (DnaK). These findings implied that ClpB could not function properly in yeast because its preferred Hsp70 partner was absent, not because it was unable to disentangle yeast protein substrates. These findings were further substantiated in 2016 by the Bukau lab. Kummer and colleagues showed that ClpB and Hsp104 disaggregases use the same conserved mechanism [61]. The authors reported that Hsp70 interaction with the coiled-coil domain of the Hsp100, along with substrate binding, was an essential requirement for disaggregation activity in both the prokaryotic and eukaryotic systems. Furthermore, the authors went on to show that certain mutations in the Hsp104 coiled-coil domain, which were previously reported to activate Hsp104 in the absence of cofactors [72], also required Hsp70 to work properly [61]. This study highlighted the central role of Hsp70 and its essential cofactor Hsp40 plays in the disaggregation process.

In a follow-up to the Miot study, the Masison lab extended these findings to the prions [PSI^+], [URE3] and [RNQ^+] [60]. Reidy and colleagues tested the Hsp104-ClpB chimeras used in the Miot study for prion propagation and showed that the coiled-coil domain of Hsp104 was crucial for the maintenance of prions. Since a chimera that was mostly ClpB could propagate yeast prions as long as it contained the Hsp70-interacting domain from the yeast molecule, the authors reasoned that if other *E. coli* chaperones were co-expressed, perhaps wild-type ClpB could propagate prions and support thermotolerance in yeast. Thus, the authors used *hsp104*Δ yeast cells as living test tubes to find combinations of *E. coli* chaper-

ones that could rescue loss of Hsp104 functions. Remarkably, they found that in addition to the prokaryotic Hsp70 DnaK, which was expected, the Hsp70 nucleotide exchange factor GrpE was also required in order for ClpB to function properly in both thermotolerance and propagation of [*PSI*$^{+}$]. These findings conflicted with earlier reports that suggested that Hsp104 could work alone in luciferase reactivation and amyloid remodeling [73] but agreed with other reports that found Hsp104 required additional factors to process aggregates [74]. Furthermore, the results of the Reidy study showed that ClpB was able to work on amyloid substrates, as long as the cognate Hsp70 and nucleotide exchange factors were present, strong evidence against the notion that Hsp104 had coevolved with yeast prions [65, 73, 75, 76]. Regardless, the finding that the *E. coli* Hsp40, DnaJ, was not required in order for the prokaryotic system to function in yeast was intriguing and strongly suggested that a yeast J-protein was involved. This idea was not controversial since it was already known that the Hsp40s did not display the same species-specific barrier that the Hsp100s and Hsp70s exhibited. The collaboration of a yeast J-protein with the bacterial chaperones was demonstrated via a single point mutation in DnaK that was shown earlier to block interaction with Hsp40 [77]. Together with normal ClpB and GrpE, this mutant DnaK failed to function in yeast, indicating that a yeast J-protein was involved [60]. Finally, Reidy and colleagues took advantage of a known compensatory mutation in Hsp40's HPD motif that restored interaction with the defective DnaK [77]. They introduced this mutation into Sis1 or Ydj1, the two major yeast Hsp40s and after introduction into the system expressing ClpB, GrpE and the mutant DnaK, they discovered that, remarkably, Sis1 is the Hsp40 that the system collaborated with propagate prions, but the same system worked with Ydj1 in thermotolerance [60]. Thus, the Hsp40 component of the system specified the substrate type, with Sis1 required for amyloid substrates (prions) and Ydj1 required for heat-induced non-structured aggregates.

4. Specialized roles of Sis1 in propagation of the different prions

Amyloid fiber breakage is a process that is fundamental for the propagation of yeast prions. Left alone, intracellular amyloid fibers would be a fleeting phenomenon. This failure to propagate is due simply to the fact that without breakage, upon mitosis there are two cells but only one amyloid fiber. While it is easy to imagine an unstable amyloid fiber able to generate enough seeds for propagation spontaneously, prions that do not require Hsp104 have been rarely observed [54] and this does not necessarily mean that propagation occurs by spontaneous breakage. Rather, amyloid-based yeast prions require molecular chaperones and specifically they require Sis1, as we have seen. At the same time, not all amyloids are the same in terms of their thermodynamic and structural properties [41]. It makes sense that a thermodynamically sturdy amyloid fiber would need an increased capability to resolve complex structures compared to an amyloid composed of the same protein but in less stable conformation. The emerging view is that Sis1 plays a major role in meeting the different demands imposed by various amyloids. The discovery and characterization of distinct prion variants have proven to be a valuable tool in shaping the field's understanding of the redundancy and variability of chaperone functions [27].

As noted previously, Higurashi showed that while the three most-studied prions, strong [*PSI*$^+$], [*RNQ*$^+$] and [URE3], all required Sis1 for their existence, each of the three prions displayed different sensitivities to Sis1 depletion [30]. In 2011 the Masison laboratory showed that loss or impairment of any nonessential functions of Sis1, that is, mutations outside of the Hsp70-interacting J-domain, had no deleterious effect on the propagation of strong [*PSI*$^+$] [31]. Kirkland and coauthors reported that a minimal Sis1 construct consisting of the J-domain and the adjacent G/F region supported both growth and strong [*PSI*$^+$]. Mutations in any other region of Sis1 did not affect the prion. It remained unclear whether the Sis1 function required for strong [*PSI*$^+$] was the same function, in a different context, as the essential function of Sis1, or if Sis1 was not actually required by strong [*PSI*$^+$]. This latter possibility conflicted with reports from other groups, notably the Craig lab and the issue was further muddied by differences in prion variant and yeast genetic background. A report by the Masison lab discussed above that involved expression of bacterial chaperones in yeast showed unequivocally that Sis1 was required for the same strong [*PSI*$^+$] variant and genetic background as that used in the Kirkland study [31, 60]. The major findings of the Kirkland paper were not in doubt, however and were reinforced by a study from the laboratory of Justin Hines [32]. In complete agreement with Kirkland, Harris and colleagues observed multiple strong [*PSI*$^+$] variants were efficiently propagated by the minimal Sis1 construct. In contrast, Harris showed that these same constructs could not propagate any of three different weak [*PSI*$^+$] variants they tested. When Harris and coauthors compared the effects of various Sis1 domain truncations on the propagation of strong and weak [*PSI*$^+$] to [*RNQ*$^+$], they uncovered mutually exclusive requirements on the G/F region by weak [*PSI*$^+$] and [*RNQ*$^+$]. In other words, the G/F region of Sis1 was dispensable for weak [*PSI*$^+$] propagation, in contrast to [*RNQ*$^+$], for which the G/F is absolutely required [62]. Additionally, weak [*PSI*$^+$] required activities of the C-terminal domain of Sis1 that were not required by either strong [*PSI*$^+$] or [*RNQ*$^+$]. Thus, each prion tested had distinct requirements for Sis1. The underlying reason for these distinct Sis1 functional requirements is probably related to structure, since different amyloids composed of the identical polypeptide, for example, strong [*PSI*$^+$] and weak [*PSI*$^+$], had unique dependencies on Sis1.

Higurashi showed that [URE3] was lost much more rapidly than strong [*PSI*$^+$] and [*RNQ*$^+$] upon depletion of Sis1, suggesting that [URE3] is more dependent upon Sis1 [30]. These findings were confirmed and expanded upon in 2014 [33]. Reidy and coauthors first expanded upon their earlier findings that Sis1 was the Hsp40 working with the *E. coli* Hsp100, Hsp70 and nucleotide exchange factor (ClpB, DnaK and GrpE, respectively) in prion propagation and Ydj1 directed the same disaggregation machinery to heat stress-induced aggregates. By creating domain-swapped chimeras between Sis1 and Ydj1 and combining them with the prokaryotic chaperone compensatory system described above that forced the target Hsp40 to interact only with a mutant DnaK, the authors showed that the C-terminal domains of the Hsp40 molecule determined whether the chimera behaved like Sis1, able to propagate prions, or like Ydj1, able to provide thermotolerance. The authors then investigated the ability of their chimeras to complement Sis1 or Ydj1 function in normal yeast cells not expressing the prokaryotic chaperones. They found only the chimeras with the Sis1 C-terminal domain were able to support viability and propagation of strong [*PSI*$^+$] in strains carrying a deletion of the chromosomal *SIS1* gene. Likewise, only those chimeras that had the C-terminal domain

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of Ydj1 supported thermotolerance and Hsp90-related processes in cells lacking the *YDJ1* gene. Additionally, in vitro reactivation reactions using substrates that exclusively required either Sis1 or Ydj1 as the J-protein component of the disaggregation machinery in order to be resolved supported the in vivo results. The chimera that contained the Sis1 C-terminal domain could reactivate substrates that required Sis1 and the chimera that contained the Ydj1 C-terminal domain could reactivate substrates that required Ydj1.

When Reidy and colleagues investigated whether their Sis1-Ydj1 chimeras could propagate [URE3], they discovered that in addition to the C-terminal domain of Sis1, the glycine-rich domain (consisting of both the G/F region and the glycine/methionine (G/M) region) was also required. They then employed the suite of Sis1 domain truncations used in the Yan, Kirkland and Harris papers to determine the specific requirements for Sis1 on [URE3] propagation, information that was lacking. Remarkably, [URE3] was lost or greatly destabilized by any of the mutations in Sis1 that were tested [33]. In some cases, the prion could be selected for and maintained in the presence of a particular Sis1 truncation, such as deletion of the dimerization motif, by growth on media-lacking adenine. However, the prion was rapidly lost upon relief of the selection pressure. Such observations are noteworthy but do not necessarily constitute ability to efficiently support prion propagation. Thus, [URE3] exhibited the highest dependence on Sis1 function, confirming the initial finding by Higurashi.

Interestingly, in the 2008 Higurashi paper, the authors proposed that the strong reliance on Sis1 by [URE3] they observed may explain a phenomenon first reported in 2000 by Reed Wickner's group [23]. Moriyama and colleagues reported that in addition to requiring Hsp104 for propagation, the [URE3] prion was cured by overexpression of the Hsp40 Ydj1. [URE3] was unique among the yeast prions in being able to be cured by overexpression of Ydj1. Higurashi showed that a mutated Ydj1 that could no longer interact with Hsp70 failed to cure [URE3] when overexpressed and also showed that overexpression of just the J-domain of Ydj1 or a different J-protein called Jjj1 could cure [URE3] when overexpressed [30]. Higurashi concluded that the curing first observed by Moriyama was not due to Ydj1 itself, but rather the result of having too many J-domains in the cell that perhaps interfered with prion propagation through unproductive interactions with Hsp70. This theory was strengthened by a study published by the Masison group in early 2009 [78]. Sharma and colleagues determined that Ydj1 curing of [URE3] was mediated through Hsp70 by screening for random mutations in Ydj1 that failed to cure [URE3] when overexpressed. Similar to the finding in the Higurashi study that the J-domain of Jjj1 could also cure [URE3], Sharma reported that overexpression of the J-domain of Sis1 also resulted in [URE3] destabilization. Thus, the two studies complemented each other and supported the idea that overabundant J-domains destabilize [URE3], mediated somehow through Hsp70. These studies conflicted with a study published in 2006 [79]. Working with purified components, Lian and colleagues reported that Ydj1 interfered with the ability of Ure2 to form amyloid in vitro in a concentration-dependent manner. The authors extended these results to conclude that overexpressed Ydj1 cured [URE3] prions in vivo through direct inhibition of the Ure2 amyloid growth. However, the effect of Ydj1 on Ure2 amyloid formation was mostly limited to increasing the lag time of amyloid formation along with a decrease in overall yield. When Ydj1 was added to Ure2 amyloid reactions during logarithmic growth of amyloid fibers, conditions that arguably more closely resembled

the in vivo situation of overexpressing Ydj1 in a cell that contains actively growing amyloid, no effect on Ure2 amyloid formation kinetics was observed.

The Higurashi model of Ydj1-mediated curing of [URE3] was strengthened by findings in Sis1-Ydj1 chimera paper that further characterized [URE3]'s strong dependence on Sis1 [33]. When the chimeras that could not propagate [URE3] in place of normal Sis1 (those that did not have both the glycine-rich and C-terminal domains of Sis1) were overexpressed in wild-type cells, rapid loss of [URE3] was observed [33]. Removing the dimerization motif from the chimeras that contained the C-terminal domain of Sis1 resulted in amelioration of curing, suggesting that the chimeras destabilized [URE3] by dimerizing with normal Sis1 monomers that were thus unable to propagate [URE3]. The authors then reasoned that [URE3] loss was due to defects in Sis1's ability to propagate the prion and extended this idea to Ydj1-mediated curing. Perhaps, Ydj1 simply outcompeted Sis1 for interaction with the disaggregation machinery to such a level that was detrimental to [URE3]. Since [URE3] was much more sensitive to Sisi1 alteration than other prions, this may explain why overexpressed Ydj1 had no effect on them. The authors tested this hypothesis by co-overexpressing Sis1 at the same time as Ydj1. In line with their model, they observed that elevating Sis1 levels reduced Ydj1-mediated curing of [URE3] by tenfold, but had no effect on curing by expression of a dominant negative Hsp104 allele. Thus, the initial idea put forth by Higurashi regarding overexpressed Ydj1 curing of [URE3], that is, an imbalance of J-domains in the cell, was correct.

From these studies a correlation is evident that the demand on Sis1 increases with thermostability of the underlying amyloid. Based on the Kirkland, Harris and Reidy papers, one can rank the four prions by increasing dependence on Sis1 as strong [*PSI*$^+$] < weak [*PSI*$^+$] ≤ [*RNQ*$^+$] < [URE3]. A strong [*PSI*$^+$] variant is composed of a thermodynamically unstable amyloid; thus, it requires less Sis1 function than a weak variant of [*PSI*$^+$], which is composed of a more thermostable amyloid fiber. This model may be correct; however, there are parameters other than melting temperature that are more useful to understanding amyloid strength vis-à-vis a certain prion's reliance on Sis1. For example, amyloid formed from purified Ure2 had a melting temperature in the presence of SDS (T_M = 79 ± 4°C) very close to that of Sup35NM amyloid made at 37°C (T_M = 78 ± 7°C, called NM-37) [80]. In contrast Sup35NM amyloid prepared at 4°C (NM-4) had a melting temperature of 54 ± 2°C. Yet, [URE3] was more sensitive to Sis1 alteration than weak [*PSI*$^+$], which was derived from NM-37 amyloid. Since Ure2 amyloid and NM-37 have identical melting temperatures, there must be some other factor that governs dependence on Sis1. More biophysical data on these amyloids is needed to fully understand the relationship between amyloid structure and stability with dependence on Sis1. Interestingly, Ure2 does not exhibit temperature-mediated differences in thermostability the way Sup35 does, maybe because the prion-forming domain of Ure2 is less complex in terms of the types of amino acids present [80]. For example, the prion-forming domain of Ure2 contains no tyrosines, yet Sup35 has 20. Some of these tyrosines have been shown to play a role in governing formation of amyloids that give rise to strong [*PSI*$^+$] [81, 82] and aromatic residues like tyrosine have been shown to play a role in amyloid formation and stability of poly-glutamine and other amyloidogenic peptides [83, 84]. On the other hand, both [URE3] and weak [*PSI*$^+$], but not strong [*PSI*$^+$], were cured by expression of a human anti-amyloid protein called DNAJB6b [80]. This finding suggested that Ure2 and NM-37 amyloids shared a

common structural characteristic that is absent in NM-4 amyloid. What is needed is a robust, reliable and reproducible in vitro assay that could be employed to probe these unknowns.

5. The present and future of Sis1 research

Sis1 is a busy molecule, as we have seen. While the reliance on different functions of Sis1 is well documented, there remains a lack of understanding as to what these functions actually are. It is likely that these functions can be thought of as fine adjustments on the primary function of assisting Hsp70 in substrate delivery to Hsp104. However, this model does not satisfactorily explain why a minimal Sis1 molecule lacking the entire substrate-binding domain is able to propagate some prions such as strong [*PSI*$^{+}$] [31, 32]. One explanation is that certain amyloids simply do not require direct interaction with the C-terminal domain of Sis1 and Sis1's regulation of Hsp70 (but not that of other J-proteins) is sufficient to process these amyloids. Another explanation is that the G/F region that is part of the minimal construct is sufficient to interact with some amyloids but not others. Yet, the G/F region is dispensable for propagation of strong [*PSI*$^{+}$] when the C-terminal domain is present, so perhaps there is some overlap in G/F and C-terminal domain functions. Obviously, what Sis1 is actually doing in the cell is still very far from clear. Of course, all of these possible explanations assume that Sis1's only role in prion maintenance is in the disaggregation reaction with Hsp70, Hsp104 and nucleotide exchange factors.

One intriguing idea that has emerged is that Sis1 may be functioning in other pathways that are important for prion stability that are not well understood. In 2008 Reed Wickner's lab reported that overexpression of Btn2 or its homolog, a previously uncharacterized open reading frame that the authors named Cur1, cured cells of [URE3] [85]. Btn2 was shown previously to be involved with endosome trafficking [86]. Kryndushkin and colleagues also observed that deletion of both *BTN2* and *CUR1* genes stabilized [URE3], increased the de novo appearance of spontaneous [URE3]s and resulted in an increase of the average number of prion seeds per cell. The authors went on to show that Ure2-GFP and Btn2-RFP co-localized during the curing [85]. In 2014 the Wickner lab reported that normal, i.e., wild type, levels of Btn2 were sufficient to cure most [URE3] prions that arose either spontaneously or via induction [87]. The authors concluded that Btn2 and Cur1 comprise an "anti-prion system" that exists to protect yeast cells from prions by binding to them and preventing their transmission to daughter cells. These conclusions conflicted with a 2012 paper from the laboratory of Simon Alberti [88]. In that study, Malinovska and colleagues argued that Btn2 cured prions indirectly, by causing redistribution of chaperones and protein-sorting factors in response to stress-induced protein aggregation. The authors did not use [URE3] for their studies, but instead a hybrid prion composed of a fusion between the prion-forming domain of Nrp1 and the C-terminal functional domain of Sup35. According to their model, Btn2 overexpression cures prions not by directly binding to prion aggregates and sequestering them in the mother cell during mitosis, as proposed by the Wickner lab, but rather by depleting the cytosol of Sis1. However, this model did not explain the findings by the Wickner lab that Btn2 co-localized with Ure2, a finding that has been reported by another group [89]. Furthermore, while the Malinovska study did demonstrate interaction between Btn2 and Sis1,

their model does not explain how Btn2 and Cur1 are able to block de novo [URE3] prions at their wild-type levels, especially since there is much more Sis1 in the cell than either Btn2 or Cur1 [90]. A study by the Bukau group in 2015 added some understanding to the role of Sis1, Btn2 and Hsp42 (a small heat shock protein involved in aggregate formation and necessary for the curing of [URE3] by Btn2 overexpression [85]) in aggregate formation in various compartments that have been described [91]. These studies cursorily mentioned here merely illustrate that in addition to the disaggregation reaction, Sis1 also plays a role in protein aggregate sorting and processing that requires more work to understand. Since some of the described aggregate compartments have been shown to contain amyloidogenic proteins [92], it is not too great of a stretch to suggest that Sis1 exerts influence over prion maintenance through its role in these processes. If so, the idea that Sis1 plays a role in aggregate processing in addition to disaggregation may explain how all various prions rely on Sis1 to different degrees. Much work is still needed, such as comprehensive mutational analyses like those done for the prions, to determine the relationship between Sis1's various functions in aggregate formation and sorting. Unfortunately, the field has yet to resolve exactly what these aggregate compartments are and how they arise, since different groups all seem to have different names and markers for them (for a review of this subject, please see Ref. [93]). Perhaps, such deeper investigation into Sis1's role in these aggregates will provide the resolution or at least clarify our understanding a bit.

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