



# Prion-specific Hsp40 function: The role of the auxilin homolog Swa2

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**ABSTRACT.** Yeast prions are protein-based genetic elements that propagate through cell populations via cytosolic transfer from mother to daughter cell. Molecular chaperone proteins including Hsp70, the Hsp40/J-protein Sis1, and Hsp104 are required for continued prion propagation, however the specific requirements of chaperone proteins differ for various prions. We recently reported that Swa2, the yeast homolog of the mammalian protein auxilin, is specifically required for the propagation of the prion [URE3].<sup>1</sup> [URE3] propagation requires both a functional J-domain and the tetratricopeptide repeat (TPR) domain of Swa2, but does not require Swa2 clathrin binding. We concluded that the TPR domain determines the specificity of the genetic interaction between Swa2 and [URE3], and that this domain likely interacts with one or more proteins with a C-terminal EEVD motif. Here we extend that analysis to incorporate additional data that supports this hypothesis. We also present new data eliminating Hsp104 as the relevant Swa2 binding partner and discuss our findings in the context of other recent work involving Hsp90. Based on these findings, we propose a new model for Swa2's involvement in [URE3] propagation in which Swa2 and Hsp90 mediate the formation of a multi-protein complex that increases the number of sites available for Hsp104 disaggregation.

**KEYWORDS.** amyloid, cell stress, heat-shock, neurodegenerative, protein misfolding, protein folding, Ssa, Sup35, ure2

Within the baker's yeast *Saccharomyces cerevisiae*, prions are non-chromosomal protein-based genetic elements, most of which are self-propagating amyloid protein aggregates.

Of the nearly one dozen currently identified amyloid-based yeast prions, the 4 best characterized include [PST<sup>+</sup>], [RNQ<sup>+</sup>] (also called [PIN<sup>+</sup>]), [SWI<sup>+</sup>], and [URE3] which are

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aggregated forms of the cytosolic proteins Sup35, Rnq1, Swi1, and Ure2, respectively.<sup>2-4</sup> Prion aggregates serve as a template to induce more of the same soluble protein to misfold into the prion conformation.<sup>5,6</sup> Aggregates are passed from mother to daughter through cytosolic transfer during cell division, inducing the prion phenotype in offspring.<sup>7</sup>

Cytosolic molecular chaperone proteins are critical for continued propagation of yeast prions.<sup>8</sup> The disaggregase Hsp104 works in tandem with Hsp70 and Hsp40, extracting a portion of the amyloid aggregate and unfolding it, which acts to disrupt and ultimately fragment amyloid fibers.<sup>9-12</sup> Specifically, the Hsp70 Ssa and the Hsp40 Sis1 are central players in the current model of yeast prion propagation. Sis1 and Ssa are known to bind directly to prion aggregates, allowing the productive recruitment of Hsp104.<sup>13-19</sup> Numerous other chaperone and co-chaperone proteins have also been implicated in prion propagation. Most relevant here are additional members of the Hsp40 co-chaperone family and the chaperone Hsp90, for which its own indirect involvement, and direct involvement of its co-chaperones, has been described in [*PSI*<sup>+</sup>] propagation, formation, and curing.<sup>20-24</sup>

Hsp40s, hereafter called J-proteins, stimulate the ATPase activity of Hsp70, stabilizing its interaction with client proteins.<sup>25</sup> Sis1 is an important cytosolic J-protein that is specifically required for the continued propagation of all 4 amyloid-based prions ([*PSI*<sup>+</sup>], [*RNQ*<sup>+</sup>], [*URE3*], and [*SWI*<sup>+</sup>]) for which data are available.<sup>15-17</sup> However, the specific portions of Sis1 required for the propagation of these prions differ, indicating that the exact roles and requirements of chaperone proteins are not universal in yeast prion propagation.<sup>6,26-30</sup> In further support of this idea, one of us (JKH) previously determined that an additional J-protein, Ydj1, is required for the propagation of [*SWI*<sup>+</sup>] specifically,<sup>17</sup> and we recently reported the finding that [*URE3*] specifically requires the action of a third J-protein, Swa2.<sup>1</sup> Swa2 is the homolog of the mammalian protein auxilin, which is responsible for uncoating clathrin-coated vesicles during clathrin-mediated endocytosis.<sup>31,32</sup> Analogous to auxilin, Swa2 recruits Hsp70 to

clathrin and stimulates the ATPase activity of Hsp70 through its J-domain, disassembling the clathrin lattice following vesicle formation.<sup>31,33</sup> Others have also recently reported additional co-chaperone requirements for the propagation of [*URE3*].<sup>20</sup> Here we will review our most recent findings regarding Swa2 and prion propagation,<sup>1</sup> present additional data, and discuss these new results in the context of a recently published complementary investigation from another laboratory.<sup>20</sup> Collectively, these investigations suggest that Hsp90 may be the critical binding partner of Swa2 in [*URE3*] propagation, and we propose a new model for plausible Swa2-Hsp90 cooperation in [*URE3*] prion propagation.

### **[*URE3*] Exhibits a “Secondary” J-Protein Requirement for Swa2**

While the requirement for Sis1 for the propagation of [*URE3*] was previously established, the dispensability or requirement of the other 12 remaining cytosolic J-proteins for this prion was unclear.<sup>16</sup> To determine if any additional cytosolic J-proteins were required for [*URE3*] propagation, as is the case for at least one other prion ([*SWI*<sup>+</sup>]),<sup>17</sup> [*ure-o*] strains carrying individual deletions of each of the cytosolic J-proteins were crossed with a strain bearing [*URE3-1*], the strong variant of [*URE3*] originally isolated by LaCroute and the most widely studied variant of the prion in the literature (commonly and hereafter just called [*URE3*]).<sup>16,34-36</sup> We isolated F1 haploids maintaining [*URE3*] but lacking a single J-protein gene in 11 of the 12 crosses, demonstrating that 11 of the 13 cytosolic J-proteins of *S. cerevisiae* are dispensable for [*URE3*] propagation.<sup>1</sup> However despite numerous attempts, [*URE3*] was lost in all *swa2-Δ* haploids, indicating that Swa2 might be required, in addition to Sis1, for [*URE3*] propagation. To begin to confirm this, we first verified that the biochemically complex color phenotype of [*URE3*] was not affected by the loss of Swa2 by performing a backcross between these F1 haploids and a cured version of the [*URE3*] parental strain. All diploids and *SWA2* haploids isolated from this backcross remained

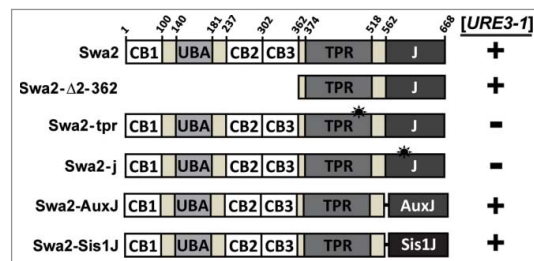
phenotypically [*ure-o*] (red colony color), confirming that [*URE3*] was indeed lost in the original cross.<sup>1</sup> To eliminate the possibility that [*URE3*] simply became unstable during meiosis when these parental strains were crossed, we mated a [*URE3*] strain and a strain bearing a genomic deletion of *SWA2* covered by a plasmid expressing *Swa2*. Following loss of the *Swa2*-expressing plasmid, cells again lost the [*URE3*] color phenotype, and exhibited the slow-growth phenotype characteristic of *swa2-Δ* cells.<sup>37,38</sup> Subsequent transformation by a plasmid expressing *Ure2*-GFP, followed by fluorescence microscopy revealed complete loss of *Ure2* protein aggregation, which again confirmed that the color phenotype accurately reported the prion status. Together these data demonstrated the rapid and total loss of [*URE3*] coincident with loss of *Swa2* expression.<sup>1</sup>

Because [*URE3*] is sensitive to altered levels of chaperone protein expression including *Ydj1*, *Sis1*, *Ssa1-4*, *Sse1* and *Hsp104* and others,<sup>16,39,40</sup> it was important to determine if loss of [*URE3*] was potentially due to an induced stress response resulting from this deletion, rather than directly from the loss of *Swa2*. We found no significant differences in protein expression of other molecular chaperones in the absence of *Swa2* that could explain prion loss, and no indication that *Swa2* deletion induces a general stress response.<sup>1</sup> Taken together, we concluded that our results strongly suggest the involvement of *Swa2* in [*URE3*] propagation.

### *Swa2* Domain Requirements for [*URE3*] Propagation

*Swa2* is a multi-domain protein with 3 N-terminal clathrin-binding domains, a ubiquitin-associated (UBA) domain, a tetratricopeptide repeat (TPR) domain, and a J-domain (Fig. 1).<sup>32,41</sup> The clathrin-binding domains bind clathrin during uncoating of the clathrin lattice, however, only one domain is required for successful clathrin binding *in vivo*.<sup>32,37,42</sup> Although the exact biochemical role of the UBA domain is unknown, it presumably interacts with ubiquitinated cargo proteins during clathrin-mediated

FIGURE 1. Primary sequence diagrams of *Swa2* constructs and chimeras examined for [*URE3*] propagation. *Swa2* mutants were tested using plasmid-shuffling experiments.<sup>1</sup> Domains are denoted using the following notation: *CB* 1–3, clathrin-binding domains 1–3; *UBA*, ubiquitin associated domain; *TPR*, tetratricopeptide repeat domain; *J*, J domain;<sup>37</sup> *AuxJ*, J-domain of human auxilin; *Sis1J*, J domain of yeast *Sis1*. (\*) denotes single-point mutations. (+) or (-) denotes maintenance or loss, respectively, of [*URE3-1*], the specific strong variant of [*URE3*] used in these studies.



endocytosis.<sup>37,43</sup> In its C-terminal half, *Swa2* contains a TPR domain, comprised of 3 tetratricopeptide repeats. TPR domains typically facilitate protein-protein interactions but the physiological binding partner(s) of *Swa2*'s TPR domain are unknown.<sup>37</sup> The TPR domain is followed by the J-domain at the extreme C-terminus, which is an unusual placement as all other cytosolic J-proteins from *S. cerevisiae* contain N-terminal J-domains.<sup>31,32,44</sup> To further explore the potential role of *Swa2* in [*URE3*] propagation, we first determined the minimal regions of *Swa2* required for continued propagation of [*URE3*].<sup>1</sup> Because of *Swa2*'s role in clathrin-mediated endocytosis, we initially hypothesized that *Swa2*'s involvement in [*URE3*] propagation was mostly likely associated with an uncharacterized clathrin-mediated process. To address this idea, we conducted a "plasmid shuffling" experiment wherein we transformed our plasmid-shuffling strain with plasmids bearing individual, well-characterized deletions of *Swa2*'s N-terminal domains, and selected against cells expressing the plasmid encoding full-length *Swa2* (Fig. 1).<sup>1,37,45</sup> Surprisingly, [*URE3*] was maintained in all N-terminally truncated

constructs assayed, including a construct lacking all 3 clathrin-binding domains and the UBA domain. Based on these results, we concluded that it is highly unlikely that Swa2's role in [URE3] propagation involves clathrin.<sup>1</sup>

We next examined the requirement of Swa2's two C-terminal domains, focusing first on the TPR domain. A well-studied full-length mutant construct, previously referred to as Swa2-*tpr*,<sup>37</sup> consists of a single glycine-to-arginine mutation (Gly<sup>388</sup>→Arg) that is predicted to impede binding to client peptides due to disruption of a structurally conserved  $\beta$ -turn between 2 amphipathic helices within the TPR domain.<sup>31,37</sup> The Swa2-*tpr* mutation is unlikely to affect the overall fold of Swa2, as this mutant is expressed at normal levels and is still able to bind clathrin and stimulate the ATPase activity of Ssa1.<sup>31,37</sup> Expression of this construct as the sole copy of Swa2 led to loss of [URE3], indicating that Swa2's TPR domain must be functional to support [URE3] propagation.<sup>1</sup> Finally, to examine the importance of a functional J-domain, we expressed a full-length construct bearing a triple alanine substitution (HPD→AAA) in place of the conserved HPD motif that is responsible for stimulating the ATPase activity of Hsp70 (Fig. 1). This mutant, referred to as Swa2-*j*,<sup>37</sup> also expresses normally and, like Swa2-*tpr*, failed to support [URE3] propagation when expressed as the only copy of Swa2.<sup>1</sup> Taken together, these results indicate that the 2 Swa2 C-terminal domains are individually essential and together sufficient for [URE3] propagation.

### ***The Swa2 TPR Domain Is Responsible for [URE3]/Swa2 Specificity***

One of us (JKH) and coworkers previously showed that unlike [URE3], neither [RNQ<sup>+</sup>] nor a single strong variant of [PSI<sup>+</sup>] require Swa2 for propagation;<sup>16</sup> however the possibility that other [PSI<sup>+</sup>] variants might require Swa2 remained untested. To address this, we re-examined the potential for a Swa2 requirement by [PSI<sup>+</sup>] using four more previously untested variants (two strong and two weak). We found no effect of SWA2 deletion on the

propagation of these variants, confirming our previous findings regarding [PSI<sup>+</sup>] and further bolstering the argument that the requirement for Swa2 seems to be specific for [URE3].<sup>1</sup> This then raised the question: *what is the origin of the specificity between this single J-protein and this single prion?* To address this, we next asked if there was something specific, or possibly unique, about Swa2's J-domain that is required specifically for [URE3] propagation, or if Swa2's J-domain is functioning in a generic fashion as has often been observed for other J-domains of other J-proteins.<sup>16,17,38,46</sup> To determine if a specific structural characteristic within the J-domain is necessary for [URE3] propagation, we created a chimera replacing Swa2's own J-domain with the J-domain of its human homolog, auxilin (Swa2-AuxJ, Fig. 1). Both auxilin and Swa2 contain C-terminal J-domains that include an unusual extra helix and long loop insertion. These characteristics are unique among both yeast cytosolic J-proteins and human J-proteins.<sup>44,47</sup> As an additional test, we also constructed a second chimera (Swa2-Sis1J), using Sis1's J-domain (Fig. 1). The J-domain of Sis1 is frequently regarded as generic as it can successfully replace, or be replaced by, that of Ydj1 in multiple prion and non-prion assays.<sup>17,27,38,46,48-50</sup> Interestingly, both Swa2-AuxJ and Swa2-Sis1J were competent to support [URE3] propagation following plasmid shuffling, demonstrating that it is likely the function of a generic J-domain, rather than the unique characteristics of the Swa2 and Auxilin J-domains, that is critical for Swa2's role in prion propagation. Together with our results described above, these data indicate that Swa2's TPR domain expressed in *cis* with a functional J-domain, but not necessarily that of Swa2, is required for [URE3] propagation. Further, these observations collectively led us to the conclusion that, because only the 2 C-terminal domains of Swa2 are required, and the J-domain can be replaced by J-domains from other J-proteins (both orthologous and paralogous), the Swa2 TPR domain alone must determine the specificity of the presently unique genetic interaction between Swa2 and [URE3].<sup>1</sup>

### Analysis of the Swa2 TPR Domain

TPR motifs are common repeat sequences present in a wide range of proteins and organisms. The motif is defined by a varying set of tandem arrays containing a canonical sequence of 34 amino acids which is entirely absent of invariant residues.<sup>51,52</sup> Basic Local Alignment Search Tool (BLAST) comparisons reveal that the TPR domain of Swa2 bears significant resemblance to the TPR domains of human Hsp70-Hsp90 Organizing Protein (HOP): 27% identity ( $E = 6 \times 10^{-13}$ ) to TPR1 and 23% identity ( $E = 1 \times 10^{-5}$ ) to TPR2A. The TPR1 and TPR2A domains of HOP bind the C-terminal EEVD motifs of Hsc70 and Hsp90, respectively, with a high degree of specificity. We examined 2 individual crystal structures (PDB IDs: 1ELW and 1ELR) of these domains in complex with their respective heptapeptide (Hsc70) or pentapeptide (Hsp90) ligands<sup>53</sup> for critical intermolecular interactions responsible for docking stability. These residues were assessed for conservation in the Swa2 TPR domain using the protein sequencing tools CLUSTALW and LALIGN. Alignments against TPR1 reveal conservation of K<sup>8</sup>, N<sup>43</sup>, A<sup>46</sup>, K<sup>73</sup>, and R<sup>77</sup> as well as the potentially functional conservation of L<sup>15</sup>→F<sup>385</sup>. Alignments against TPR2A reveal conservation of K<sup>229</sup>, Y<sup>248</sup>, N<sup>264</sup>, A<sup>267</sup>, K<sup>301</sup> and R<sup>305</sup> as well as potentially functional conservation of Q<sup>298</sup>→K<sup>452</sup> and N<sup>308</sup>→R<sup>475</sup> (for details see Table 1). Of particular note is the nearly complete conservation of residues necessary to form the “dicarboxylate clamp”, the characteristic structure known to bind the dicarboxylate formed at the C-terminus of proteins ending in EEVD or EEVD-like motifs. This strongly supports the idea that Swa2 possesses sufficient structure to bind a C-terminal EEVD or EEVD-like acidic sequence at its TPR domain.

*Are there structural aspects of the Swa2 TPR domain that might indicate whether it would be competent to bind Hsp70 vs. Hsp90 in vivo?* It has been suggested that the ability of TPR regions to distinguish between client proteins is established by unique hydrophobic pockets that interact with residues upstream of the EEVD-

like motifs.<sup>20</sup> In the case of HOP TPR1, A<sup>46</sup>, A<sup>49</sup>, F<sup>84</sup>, the  $\gamma$ -carbon of K<sup>50</sup>, and the  $\beta$ -carbon of E<sup>83</sup> form a hydrophobic pocket for I<sup>-5</sup> and P<sup>-7</sup> (numbered by convention from the C-terminal D<sup>0</sup> residue) of the Hsc70 C-terminus. Conservation of residues A<sup>46</sup>, K<sup>50</sup>, and E<sup>83</sup>, and nearly conserved residues A<sup>49</sup> → L<sup>423</sup> and F<sup>84</sup> → H<sup>479</sup> in Swa2 suggest that its TPR region is capable to accommodate Hsp70 through similar interactions. In the case of HOP TPR2A, Y<sup>236</sup> and the  $\gamma$ -carbon of E<sup>271</sup> form a small hydrophobic pocket around M<sup>-5</sup> of the Hsp90 C-terminus. Swa2 appears to also retain sufficient hydrophobic structure (Y<sup>236</sup> → F<sup>385</sup>) and residue chain length (E<sup>271</sup> → K<sup>424</sup>) to accommodate the Hsp90 M<sup>-5</sup> and thus might be competent for binding either Hsp90 or Hsp70 according to these sequence analyses alone.

These structure-based alignments also provided candidate residues for point mutation experiments to test TPR function *in vivo*. Using these sequence analyses, we identified targets for single point mutations which would eliminate critical salt bridges between the putative dicarboxylate clamp of Swa2 and a C-terminal EEVD-like acidic sequence in a theoretical binding partner. For this purpose, Lys<sup>468</sup> and Lys<sup>378</sup>, both of which are expected to mediate strong ionic (salt-bridge) interactions with the C-terminal residue of a putative binding partner,<sup>54</sup> were changed to Ala by site-directed mutagenesis in the context of either full-length Swa2 or Swa2 $\Delta$ 2–362.<sup>1</sup> These alterations produced a barely detectable color phenotype change in the context of full-length Swa2. However, in the context of Swa2 $\Delta$ 2–362, both mutant constructs caused a dramatic destabilization of [URE3] when expressed as the sole copy of Swa2 (Fig. 2A). The fact that 2 individual single amino acid substitutions each independently destabilize [URE3] makes it unlikely that the effect of these mutations are due to large-scale alterations of Swa2 or TPR domain structure. Likewise, the observation that these point-mutations destabilize the prion to such a great extent strongly supports the idea that the formation of a functional clamp interaction between the Swa2 TPR and the C-terminal EEVD-like motif of an as yet unknown

TABLE 1. Homology analysis of the Swa2 TPR domain relative to HOP TPR1 and TPR2A. Swa2 TPR residues aligned against HOP TPR1 and TPR2A residues that form critical intermolecular interactions with their respective EEVD ligands. Interactions were determined from crystal structure<sup>53</sup> analyses using the molecular graphics modeling system, PyMOL. Alignments were performed using protein sequence alignment tools LALIGN and CLUSTALW.

Hsp70	Interaction	HOP TPR1	SWA2 TPR	HOP TPR2A	Interaction	Hsp90
D <sup>0</sup>	salt bridge	K <sup>8</sup>	<b>K<sup>378</sup></b>	K <sup>229</sup>	salt bridge	D <sup>0</sup>
D <sup>0</sup>	ion-dipole	N <sup>43</sup>	<b>N<sup>417</sup></b>	N <sup>264</sup>	ion-dipole	D <sup>0</sup>
D <sup>0</sup>	ion-dipole	N <sup>12</sup>	→ <b>T<sup>382</sup></b>	N <sup>233</sup>	ion-dipole	D <sup>0</sup>
D <sup>0</sup>	salt bridge	K <sup>73</sup>	<b>K<sup>468</sup></b>	K <sup>301</sup>	salt bridge	D <sup>0</sup>
			→ <b>K<sup>452</sup></b>	Q <sup>298</sup>	ion dipole	D <sup>0</sup>
V <sup>-1</sup>	hydrophobic	L <sup>15</sup>	→ <b>F<sup>385</sup></b>			
V <sup>-1</sup>	hydrophobic	A <sup>46</sup>	<b>A<sup>420</sup></b>	A <sup>267</sup>	hydrophobic	V <sup>-1</sup>
			<b>Y<sup>397</sup></b>	Y <sup>248</sup>	hydrophobic	V <sup>-1</sup>
E <sup>-2</sup>	ion-dipole	K <sup>73</sup>	<b>K<sup>468</sup></b>			
E <sup>-2</sup>	ion-dipole	R <sup>77</sup>	<b>R<sup>472</sup></b>	R <sup>305</sup>	ion-dipole	E <sup>-2</sup>
			→ <b>R<sup>475</sup></b>	N <sup>308</sup>	ion-dipole	E <sup>-2</sup>
E <sup>-3</sup>	ion-dipole	R <sup>77</sup>	<b>R<sup>472</sup></b>	R <sup>305</sup>	ion-dipole	E <sup>-3</sup>

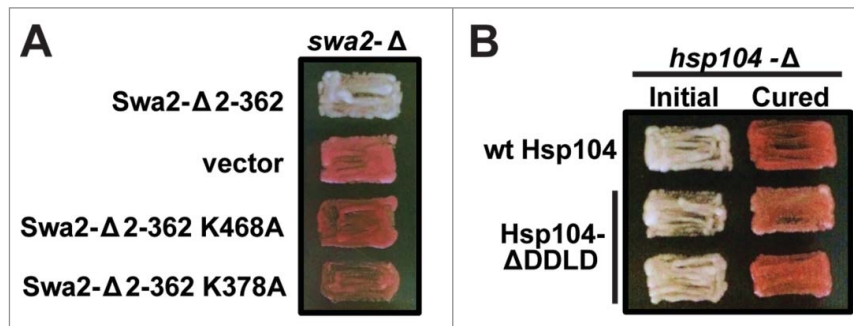
protein binding partner is critical for stable [URE3] propagation.

*What is the identity of the relevant Swa2 binding partner?* In a previous investigation we proposed two plausible models for the function of the Swa2 TPR domain.<sup>1</sup> The first was that the TPR domain binds the EEVD motif of the Hsp70 Ssa, forming a bipartite interaction that could enhance fiber fragmentation via repeated binding and release of the J-domain of Swa2 by Ssa's N-terminal ATPase domain. In this model, the association of the Swa2 TPR domain with the Hsp70 C-terminal EEVD effectively increases the local J-domain concentration in the immediate vicinity of the Hsp70 N-terminal domain, which would greatly accelerate the forward portion of the Hsp70 ATPase cycle. The second model asserted that the Swa2 TPR domain binds a C-terminal EEVD or EEVD-like acidic sequence of a different chaperone partner forming a ternary complex with Hsp70 binding at the J-domain. These two models are not mutually exclusive; that is, it is highly likely that Swa2 would engage in a bipartite interaction when either the J or TPR domain is engaged with Ssa, however an additional binding partner for the Swa2 TPR domain, in addition to Ssa, may also exist. Although there are many possible candidates, two became immediately apparent

to us: Hsp90 and Hsp104, which both contain C-terminal motifs that might participate in such an interaction. As described previously, the Hsp90 C-terminus ends with an EEVD motif and Swa2's TPR motif is highly similar to the Hsp90-binding TPR2A of the HOP protein. The Hsp104 C-terminus ends with a DDLDD sequence capable of binding TPR motifs,<sup>55,56</sup> and is therefore also a possible binding candidate. The hypothesis that Hsp104 is the relevant binding partner is highly intriguing because a plausible role for Swa2 in fiber fragmentation would then be immediately apparent: if Swa2 were able to dock both Hsp104 and Hsp70, it could promote prion fragmentation by directly recruiting additional Hsp104 to Hsp70 already engaged at [URE3] aggregates.

To test this hypothetical model, we replaced Hsp104 in [URE3] cells with a plasmid (pMR40 from Reidy and Masison, 2010) expressing Hsp104 lacking the C-terminal DDLDD motif which is known to bind to the TPR1 domains of Sti1 (the yeast homolog of mammalian HOP) and the Hsp90 co-chaperone Cpr7 in *S. cerevisiae*.<sup>22,55-57</sup> This construct, here called Hsp104ΔDDLDD, supports strong [PSI<sup>+</sup>] propagation similar to wild-type protein.<sup>22</sup> We were able to easily isolate strains (n = 7 of 8 examined) that stably maintained [URE3] with Hsp104ΔDDLDD as the sole copy

FIGURE 2. Residues of Swa2's TPR domain, but not Hsp104's C-terminal DDLD motif, are required for  $[URE3]$  propagation. (A) Haploid  $[URE3]$  cells of the W303 genetic background expressing His3 in place of genomic Swa2 and bearing the plasmid pRS416-SWA2 were transformed with plasmids (pRS315) expressing Swa2 variant constructs lacking the Swa2 N-terminal domains and containing a single amino acid substitution ( $K^{378} \rightarrow A$  or  $K^{468} \rightarrow A$ ). Swa2- $\Delta$ 2-362 and swa2- $\Delta$  were used as positive and negative controls for prion maintenance, respectively. Colonies exhibiting leucine prototrophy were placed on media containing 5-Fluoroorotic acid (5-FOA) to counter-select against the  $URA3$ -marked SWA2 plasmid. Red/white color phenotype assays are shown for representative transformants ( $n \geq 8$  for each variant); in this yeast genetic background red pigment accumulates upon  $[URE3]$  loss. (B) Haploid W303  $[URE3]$  cells possessing genomic HSP104 were crossed with one of 2 strains expressing Leu2 in place of genomic Hsp104, and expressing either wild-type Hsp104 (pRS313, row 1) or an Hsp104 construct lacking its 4 terminal residues: DDLD (pRS314, rows 2 and 3).<sup>22</sup> After passage on media selecting for adenine and leucine prototrophy, diploids were sporulated and subjected to tetrad dissection. Color phenotype assays are shown for representative haploids (right columns,  $n \geq 7$ ). Haploids were subsequently treated with GdnHCl to inhibit Hsp104 function (left columns,  $n \geq 7$  for each variant), to confirm that colony color still accurately reported prion status.



of Hsp104 (Fig. 2B), clearly eliminating Hsp104 as the essential binding partner for the Swa2 TPR domain in  $[URE3]$  propagation.

### *Hsp90 as a Plausible Binding Partner for Swa2 in $[URE3]$ Propagation*

Initially, we disfavored the idea of a ternary complex with Hsp90 for two reasons, the first of which was the greater similarity of the Swa2 TPR domain to HOP TPR1 (which binds Hsc70) rather than TPR2A (which binds Hsp90). The second was that, should such a complex exist, alterations of Hsp90 (specifically) or Hsp90 co-chaperones (plausibly) should greatly impact  $[URE3]$  similar to alterations in Swa2, yet no evidence indicating Hsp90 involvement in  $[URE3]$  propagation was present in the literature at the time of our original publication. We posited that the most

direct test of this hypothesis would be to ablate the EEVD motif of Hsp90 and examine the effect on  $[URE3]$  propagation as we did with Hsp104. However, a recent study by the Sharma laboratory (Kumar *et al.* 2015), coincident with our own, serendipitously did exactly that, providing considerable additional support for this alternative model. Most notably, the authors demonstrated novel genetic interactions between Hsp90 and one of its co-chaperones, and  $[URE3]$ .<sup>20</sup> Moreover, their results specifically implicated the Hsp90 EEVD motif, but not other functions of Hsp90. Normally Hsp90 exists as a homodimer with the dimer interface located in the C-terminal domains, and exhibits pincer-type conformational changes via cycling between bound and unbound states at its N-terminal nucleotide binding domains, mediated by ATP hydrolysis.<sup>58,59</sup> Kumar *et al.* demonstrated that neither inhibition of Hsp90

ATPase function nor removal of the charged linker region responsible for the pincer-type conformational change had any effect on [URE3] propagation. However, deletion of the C-terminal MEEVD pentapeptide significantly destabilized [URE3], suggesting a novel role for Hsp90 in [URE3] maintenance which is independent of ATPase activity and its normal chaperone functions. Furthermore, they demonstrated that the Hsp90 co-chaperone Cpr7 binds to Ure2 in pull-down assays and similarly reduces [URE3] stability when deleted. Finally, like our results with Swa2, they found no evidence of similar effects on weak or strong [PSI<sup>+</sup>] variants indicating that these effects are specific to [URE3].<sup>20</sup>

These new data from both our laboratories, which simultaneously implicate Hsp90 and exonerate Hsp104, lend significant support toward a model in which Swa2 aids in [URE3] propagation via simultaneous docking of Hsp70 at its J-domain and Hsp90 at its TPR domain. Interestingly, such a model must also explain the need for Hsp90 but without the need for Hsp90 conformational change or ATP hydrolysis. Here we propose a new model that accounts for all available data in which an Hsp90 dimer acts as a passive bridge bringing together multiple proteins via its 2 C-terminal domains to assemble a protein complex that could directly recruit Hsp104 to [URE3] fibers (Fig. 3). This model assumes that the ability of Cpr7 to bind soluble Ure2 extends to Ure2 in [URE3] amyloids; an assertion that seems likely given that Ure2 remains at least partially functional in [URE3] aggregates.<sup>60,61</sup> By virtue of its dual MEEVD motifs, Hsp90 could bind the Cpr7•[URE3] complex at one C-terminal MEEVD motif via the Cpr7 TPR domain while simultaneously docking the Swa2•Hsp70 complex at the second, nearby C-terminal MEEVD motif via the Swa2 TPR domain (Fig. 3). This multi-protein complex would bring together the [URE3] aggregate and Hsp70, allowing Hsp70 to remodel polypeptides from the aggregate and recruit Hsp104 for disaggregation. Thus the complex would effectively recruit additional Hsp104 directly to [URE3] fibrils in a productive fashion through Hsp70, enhancing aggregate remodeling by increasing the number of

sites available for fiber fragmentation. Deletion or mutation of any of these factors would be expected to result in prion instability, which is fully consistent with the current literature. It is also worth noting that because Cpr7 can also bind Hsp104 directly,<sup>56</sup> it may have a second function, independent of its action with Swa2 and Hsp90 in which it recruits Hsp104 directly to [URE3] aggregates by binding the DDLD motif of Hsp104 at its TPR domain. However, our experiment described above (Fig. 2B) clarifies that this second possible role for Cpr7 cannot be its major function in [URE3] maintenance since Cpr7 deletion results in prion loss but deletion of the Hsp104 DDLD motif does not, lending additional support for the model that Cpr7 acts primarily through Hsp90.

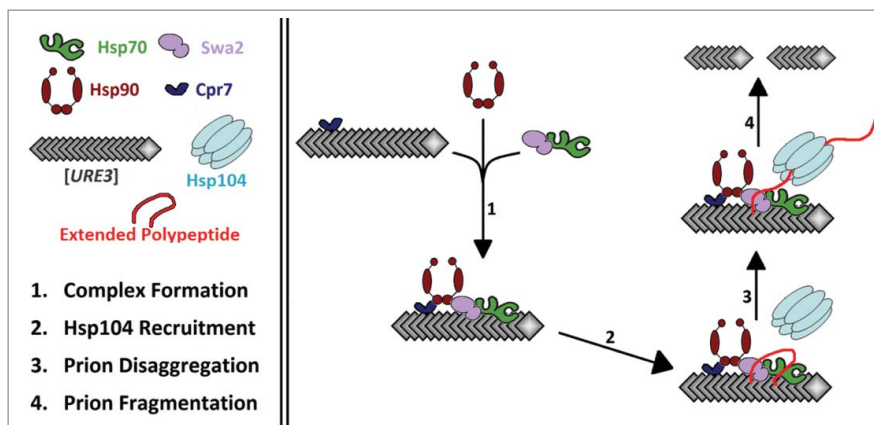
### ***Future Directions and Current Limitations***

It is reasonable to suggest that [URE3] might require additional chaperone complexes to increase sites of fiber fragmentation for continued propagation relative to other prions. [URE3] has been repeatedly characterized by relatively large intracellular aggregates and low propagon numbers, likely making it difficult for the prion to be fragmented and passed into progeny relative to other prions like [PSI<sup>+</sup>]. This idea is also consistent with numerous observations that [URE3] is highly sensitive to reductions in chaperone activity, particularly with regard to Sis1 and Hsp104.<sup>16,27,62,63</sup>

The model we propose herein makes many new testable predictions. First, the model asserts that Cpr7 binds to [URE3] aggregates in addition to monomeric Ure2. Second, this model would predict that alterations that block the proposed binding between Swa2 and Hsp90 would reduce Hsp104 recruitment to [URE3], a testable hypothesis, as is the hypothesis that Swa2 interacts directly with Hsp90. As already noted above, deletion or mutation of any of these chaperones or co-chaperones in a way that reduces their relevant functions would destabilize the prion, which is already consistent with the current literature. The expected



FIGURE 3. Proposed model of Cpr7-Hsp90-Swa2 cooperation and complex formation in [URE3] prion fragmentation. Hsp70 is recruited to [URE3] aggregates by formation of a chaperone bridge. Cpr7, bound to the aggregate, complexes with the EEVD of one Hsp90 monomer at its TPR domain while Swa2, bound to Hsp70 at its J-domain, complexes with the EEVD of the other Hsp90 monomer via the Swa2 TPR domain. This multi-chaperone protein complex allows Hsp70 to productively recruit Hsp104 and enhance prion fragmentation.



consequence of this reduction, however, would be an increase in aggregate size and decrease in propagon numbers before possible loss of the prion. Difficulties with testing this hypothesis are that [URE3] propagon numbers are already very low and aggregates very large relative to other prions, so it may be challenging to observe these expected changes without fully destabilizing the prion. One additional challenge is that methods to observe size changes for [URE3] aggregates are limited as [URE3] is known to become unstable in the presence of long-term ectopic Ure2 or Ure2-GFP expression,<sup>64</sup> and a reliable method to resolve [URE3] aggregates by using semi-denaturing detergent agarose gel electrophoresis (SDDAGE) has yet to be published. Ongoing efforts to establish alternative methods to follow [URE3] aggregates may resolve some of these issues and may ultimately lead to a more complete biochemical understanding of why some prions, but not others, require specific sets of chaperones for propagation.

### ABBREVIATIONS

ATP adenosine triphosphate  
SDDAGE semi-denaturing detergent agarose gel electrophoresis

### DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

The authors declare no competing interests.

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