Review

Structure and Function of the Molecular Chaperone Hsp104 from Yeast

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ABSTRACT:

The molecular chaperone Hsp104 plays a central role in the clearance of aggregates after heat shock and the propagation of yeast prions. Hsp104's disaggregation activity and prion propagation have been linked to its ability to resolubilize or remodel protein aggregates. However, Hsp104 has also the capacity to catalyze protein aggregation of some substrates at specific conditions. Hence, it is a molecular chaperone with two opposing activities with respect to protein aggregation. In yeast models of Huntington's disease, Hsp104 is required for the aggregation and toxicity of polyglutamine (polyQ), but the expression of Hsp104 in cellular and animal models of Huntington's and Parkinson's disease protects against polyQ and α-synuclein toxicity. Therefore, elucidating the molecular determinants and mechanisms underlying the ability of Hsp104 to switch between these two activities is of critical importance for understanding its function and could provide insight into novel strategies aimed at preventing or reversing the formation of toxic protein aggregation in systemic and neurodegenerative protein misfolding diseases. Here, we present an overview of the current molecular models and hypotheses that have been proposed to explain the role of Hsp104 in modulating protein aggregation and prion propagation. The experimental approaches and the evidences presented so far in relation to these models are examined. Our primary objective is to offer a critical review that will inspire the use of novel techniques and the design of new experiments to proceed towards a qualitative and quantitative understanding of the molecular mechanisms underlying the multifunctional properties of Hsp104 in vivo. © *2009 Wiley Periodicals, Inc. Biopolymers 93: 252–276, 2010.*

Keywords: Hsp104; ClpB; Hsp100; AAA$^+$-ATPase; disaggregation; molecular chaperones; neurodegenerative diseases; yeast prions; amyloid proteins; fibril formation; protein unfolding by chaperones

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INTRODUCTION

Several human diseases are caused by protein misfolding and the accumulation of toxic protein aggregates in the cytoplasm or extracellular space; these diseases include Alzheimer's and Parkinson's diseases, type II diabetes and the spongiform encephalopathies such as Creutzfeldt-Jakob disease. Therefore, understanding the molecular mechanisms involved in triggering and/or reversing protein aggregation in vivo is essential for elucidating the relationship between protein aggregation and disease and for developing effective therapeutic strategies to prevent, slowdown, or reverse the progression of these diseases. Several molecular chaperons have been shown to act individually or in concert with other chaperones to prevent protein misfold-

ing and aggregation, thereby blocking or reversing protein aggregation and toxicity in vitro and in vivo. However, the molecular mechanisms by which molecular chaperones modulate the aggregation, disaggregation, and clearance of protein aggregates in healthy and diseased states remain the subject of debate and intense investigation. Among the different classes of molecular chaperones, only the yeast heat shock protein Hsp104 has been described to both reverse and catalyze protein aggregation with different functional and cellular consequences for each activity.

Hsp104 is a member of the Hsp100/ClpB family of hexameric AAA^+-ATPases.[1–3] The family of Hsp100/ClpB proteins comprises bacterial, fungal, and plant Hsp100 ATPases. Hsp100/ClpB chaperones have the ability to bind and to remodel non-natively folded polypeptides. They are a member of the class of Clp ATPases which comprise prokaryotic hexameric protease subunits such as ClpA, ClpX, and ClpY.[4] However, while Hsp100/ClpB proteins are known to function as protein disaggregases, they do not possess a protease function. The molecular mechanisms underlying the disaggregase function are complex and not yet fully understood. Recent studies have demonstrated that Hsp100/ClpB unfolds proteins by extracting polypeptide chains from aggregates by processing them by asymmetrical cycles of ATP hydrolysis through the central channel of the Hsp100/ClpB hexamer.[5–8] The current understanding of their molecular and structural properties suggests that Hsp100/ClpB proteins, with the help of the Hsp70/40 chaperone system, break down large aggregates and recover proteins from a non-native insoluble or aggregated state. The spectrum of substrates for these chaperones includes unfolded polypeptides, amorphous aggregates of various proteins, and protease resistant amyloid structures (Table I). Protein disaggregation and clearance by Hsp100/ClpB proteins is crucial for thermotolerance of bacteria and low eukaryotes under stress conditions, e.g., heat shock, and protects against protein aggregation and toxicity in several cellular and animal models of neurodegenerative diseases.[29,32–34,41–43] The elucidation of the structure-function relationship of Hsp104 presents a unique opportunity for determining how protein aggregation is modulated and reversed in nature. Future work could utilize this knowledge to develop strategies to prevent or treat protein misfolding diseases by preventing or reversing protein aggregation in vivo.

STRUCTURE AND FUNCTION OF Hsp104

Hsp104 Function is Required Under Stress Conditions

Hsp104 was initially identified as the most potent factor contributing to induced stress tolerance of yeast.[41] Under normal growth conditions, Hsp104 is not required for yeast viability but rather plays a role in prion propagation,[44,45] and in the distribution and inheritance of oxidatively damaged proteins.[46,47] A genetic *HSP104* knockout yeast strain (Δ*hsp104*) does not show any defects at normal growth temperatures, but a severe viability defect becomes evident upon exposure to 15–20% ethanol or upon heat treatment at 37°C followed by a heat shock of 42–50°C, i.e., induced heat shock. Under these stress conditions Δ*hsp104* or mutant *HSP104* yeast strains show a 1000–10,000-fold reduced survival rate when compared to wild-type *HSP104* strains.[48,49] Thus, Hsp104 is essential for yeast survival under stress conditions. Electron micrographs of Δ*hsp104* and wild-type *HSP104* yeast cells reveal that the aggregation of proteins is reverted within 120 min after a 44°C heat shock in wild-type cells, while mutant cells are unable to clear aggregates and die.[49] Accordingly, Hsp104 was suggested to be involved in aggregate clearance, thereby promoting the thermotolerance of yeast. The protective function of Hsp104 under stress conditions cannot be attributed to a chaperone function in the "classical" sense as it is the case for GroEL. GroEL is a member of the Hsp60 family of ATP-dependent molecular chaperones, which are known for preventing the aggregation of proteins by binding to folding intermediates. The structure of GroEL provides a protected central cavity, the "Anfinsen's cage," in which the captured substrate can fold properly and be released after ATP hydrolysis.[50,51] Similar to GroEL, Hsp104 also shows ATP-dependent substrate affinity and has a large central channel that can accommodate unfolded polypeptides. However, in contrast to GroEL, Hsp104 does not assist in protein folding and has a significantly reduced capacity to suppress the aggregation of denatured substrates such as fire fly luciferase (FFL, see Table I), even at a chaperone to substrate ratio of 20:1, whereas GroEL reduces ~90% aggregation of FFL at a ratio of 1:1.[12] The disaggregation activity of Hsp104 also cannot be explained by a proteolytic degradation mechanism since Hsp104 has no intrinsic protease function[6,49] and no known protease subunit as the related Clp ATPases ClpX, ClpY, and ClpA have.[4] Nevertheless, the protective function of Hsp104 in thermotolerance and aggregate clearance has consistently been shown to depend on active Hsp104 with two functional nucleotide binding domains (NBDs). Notably, yeast strains carrying the *HSP104* ATPase point mutations K218T or K620T that render the NBDs dysfunctional, show almost the same reduced thermotolerance as *hsp104* deletion strains.[49,52]

Hsp104 Function Requires the Cooperation of Other Molecular Chaperones

The function of Hsp104 depends genetically on *HSP70*. A yeast strain with a deletion of both *HSP104* and *HSP70* shows

Table I Substrates of Hsp104

Substrate	Related Phenotype	Modifications	Type of Assay	References
NONAMYLOIDOGENIC MODEL SUBSTRATES				
α-Lactalbumin		Carboxymethylated, labeled	SEC co-elution, anisotropy	7, 9, 10, 11
α-Casein			HAP/ClpP degradation assay	6
β-Galactosidase		Urea denatured	SEC (oligomerization), enzymatic	12, 13
FFL lysate assay		Yeast expressed FFL	Enzymatic	13
FFL		Urea denatured, heat denatured	Enzymatic	12
FFL-fusions		Peptide fusion	Enzymatic	9
GFP		Thermally denatured	Fluorimetric	14
GFP-RepA		Contains RepA (1-70) tag	Fluorimetric	14
La-EYFP		YPF with unfolded lactalbumin tag	Fluorimetric	7
Polylysine, 15 kDa			Binding assays	9, 15, 16
RepA			DNA binding by RepA	14
NATURAL AMYLOIDOGENIC SUBSTRATES				
Rnq1, yeast	[*RNQ*$^+$] prion (= [*PIN*$^+$])	Δ*hsp104* is [*rnq* $^-$]	Yeast in vivo	17, 18
Sup35, yeast	[*PSI*$^+$] prion	↑Hsp104 cures, Δ*hsp104* is [*psi* $^-$]	Yeast in vivo, biophysical studies	44, 19, 20–22, 11
Ure2, yeast	[*URE3*] prion	Δ*hsp104* is [*ure-o*]	Yeast in vivo, biophysical studies	23 24 25
EXOGENOUS AMYLOIDOGENIC SUBSTRATES				
Amyloid beta, human	Alzheimer's disease	In vivo: no enhanced aggreg. by Hsp104	Yeast in vivo, biophysical study	26, 27, 11, 28
α-Synuclein, human	Parkinson's disease	↑Hsp104: neuro protection	Rat in vivo, biophysical study	29
HET-S, *Podospora anserina* (*Pa*)	[Het-S] prion	Δ*hsp104:* HET-S aggreg. reduced, Δ*Pahsp104:* no curing	Yeast and *Pa* in vivo	30, 31
Huntingtin, PolyQ, human	Huntington's disease	Δ*hsp104:* no Htt aggreg., ↑Hsp104: neuro protection	Yeast in vivo, *C. elegans* in vivo, rat, mouse in vivo	32–38
PrP, human	CJD, PrP^{Sc} prion	Hsp104 promotes conversion into PrP^{Sc}	Biophysical studies	11, 39
PrP, mouse	ME7 prion	No effect	Mouse in vivo	40

↑Hsp104 corresponds to the effect of Hsp104 overexpression. [*RNQ*$^+$], [*PSI*$^+$], and [*URE3*] are the prion phenotypes caused by the intracellular amyloid aggregates of the proteins Rnq1, Sup35, and Ure2, respectively; the corresponding soluble protein states and prion-free phenotypes are [*rnq*$^-$], [*psi*$^-$], and [*ure-o*], respectively.

even less thermotolerance than the corresponding Δ*hsp104* deletion strain. Second, when *HSP104* is deleted, the overexpression of Hsp70 only partially restores thermotolerance. Moreover, *HSP104*, which is not essential under normal growth conditions, turned out to be essential when endogenous Hsp70 levels are reduced, indicating that Hsp104 and Hsp70 are functionally linked.[53] In vivo experiments showing the reactivation of heat-shock-induced aggregates of recombinant FFL also suggested that Hsp104 has an Hsp70-dependent disaggregation activity.[49] Hsp104 together with Hsp70 were shown to recover proteins from heat-induced aggregates and refolds them into their native state. This recovery process depends on fully functional Hsp104 and on the cooperation of the Hsp70/40 chaperone system as shown by the in vitro reconstitution of the FFL disaggregation assay.[12] Similar observations were made for the corresponding bacterial homologues ClpB and DnaKJE (prokaryotic Hsp70/40 with the nucleotide exchange factor GrpE).[54–56] Accordingly, 1 μM of each chaperone, Hsp104, Hsp40, and Hsp70, was required for the refolding of 20 nM of urea-denatured FFL to yield ∼50% of the activity of the native FFL control within 30–60 min in the presence of ATP. This refolding activity required a cooperatively acting chaperone, since none of the assay components exhibited this extent of reactivation activity individually.[12] Recent studies have provided evidence that the disaggregation activity of Hsp104 is enhanced when Hsp70/40 acts upstream of Hsp104, see later. The physical interaction of Hsp104 with Hsp40 and/or

Hsp70 to form a multichaperone complex has been proposed to explain the high efficiency of refolding activity by Hsp104. Indeed, yeast Hsp40 (yeast Ydj1) was found to be associated with Hsp104, as revealed by pull-down experiments using yeast lysate and His-tagged Hsp104.[12] As Hsp104 exclusively cooperates with the corresponding eukaryotic Hsp70 but not with prokaryotic DnaK,[12,18,57,58] a specific interaction or a formation of a transient multichaperone complex might be postulated but has not yet been reported.

Small heat shock proteins (sHsps), such as yeast Hsp26, were also shown to contribute to aggregate clearance by Hsp104. sHsps generally bind to partially folded proteins and associate with them in high molecular weight complexes[59–61] or coaggregate with the substrates.[62] sHsps, such as Hsp26, were shown to facilitate the disaggregation by Hsp100/ClpB when they are preincubated with the substrates before aggregate formation.[62–64] Likewise, the molecular chaperone set of Hsp104/70/40 is more efficient in the reactivation of proteins when it acts on aggregates that are less tightly packed such as those formed in the presence of sHsps. Beyond the functional chaperone cooperation between Hsp104 and Hsp70/40, evidence is accumulating that the tetratricopeptide repeat (TPR) domain-containing Sti1 and Cpr7 interact physically with Hsp104. Sti1 and Cpr7 are cochaperones that use a TPR domain for protein interactions with the acidic C-termini of eukaryotic Hsp90 and/or Hsp70 (VEEVD or MEEVD). The binding of Sti1 and Cpr7 to Hsp104 is dependent on its acidic C-terminal extension (IDDDLD), but the functional consequences of the interaction are not yet clear. Sti1 and/or Cpr7 might be involved substrate sorting after release from Hsp104.[15,65]

Structural Properties of Hsp104

To gain insight into the molecular mechanisms underlying the functions of Hsp104 and to understand how it modulates, accelerates, or reverses protein aggregation, it is essential to review the molecular and structural determinants that govern the structural properties and functions of this molecular machine in vitro and in vivo. The members of the family of Hsp100/ClpB proteins are "motorized" molecular chaperones that translate the energy from ATP hydrolysis into mechanical work, i.e., the unfolding of polypeptides. Similar to other molecular machines (e.g., the proteasome, polymerases, or F0/F1 ATPase), Hsp100/ClpB proteins possess an elaborate structural organization that enables the enzymes to fulfill their disaggregation function, an energetically unfavorable process. Yeast Hsp104 and its bacterial homologue ClpB are hexameric AAA^+-ATPases with two ATPase sites per protomer, both of which are essential for the chaperone activity. The two ATPase sites, or AAA-modules, are referred to as nucleotide binding domain 1 and 2 (NBD1 and NBD2), respectively (see Figure 1A). NBD1 and NBD2 are structurally related and share a similar, conserved AAA-fold and sequence motifs (Figure 1B), but they are not identical in their structure and function. Structural studies have shown that each type of NBD of Hsp100/ClpB is found in a parallel arrangement in the hexamer structure, resulting in a stacked double ring consisting of NBD1 and NBD2,[8,66,67] see Figure 1C. The tight allosteric regulation of the ATPase sites of the Hsp100/ClpB protomer and the intersubunit cross-talk within the hexamer is thought to allow substrate binding and drive substrate unfolding while threading it through the central pore of the hexamer.

Functional Regulation and Substrate Processing by NBD1 and NBD2 of Hsp104

Both NBDs of Hsp104 are indispensable for the chaperone activity. The NBDs contain the highly conserved Walker A and Walker B sequence motifs for adenine nucleotide binding and hydrolysis, respectively,[68,69] see Figures 1A and 1B. The Walker A motif, GxxxxGKT (where x = any residue), is responsible for the binding of the nucleotide. The lysine residue coordinates the phosphate group of the nucleotide and corresponds to the residues K218 and K620 in the NBDs of Hsp104. A lysine to threonine mutation in the Walker A motif abolishes nucleotide binding in the affected NBD of related ATPases.[1,70–72] In Hsp104 and ClpB, this type of mutation was employed to inactivate one ATPase site, to determine its function, and to characterize the functional role of the remaining intact NBD.[73–78] A mutation of K218 in Hsp104 into threonine (K218T) results in a loss-of-function phenotype in vivo and a very strong reduction of the ATPase activity in vitro without altering the oligomerization state of the protein, see Table II. The corresponding mutation in NBD2 (K620T) also generates a loss-of-function phenotype, but in vitro assays have demonstrated that this mutant has an oligomerization defect. Under conditions that stabilize the hexamer, it regains up to 60% of the wild-type level of ATPase activity with a moderate chaperone activity.[49,75,76,82] Table II shows a summary of all Hsp104 mutations reported to date and their effect on in vivo function, on in vitro chaperone and ATPase activity, and on the oligomerization state. Together, these studies demonstrate that both NBDs are indispensable for the in vivo activity of Hsp104 but contribute to such activity via different mechanisms. The high k_{cat} of the ATPase of ~70 ATP/min per monomer of wild-type Hsp104[88] can be attributed to NBD1, whereas NBD2 mainly has a nucleotide-dependent

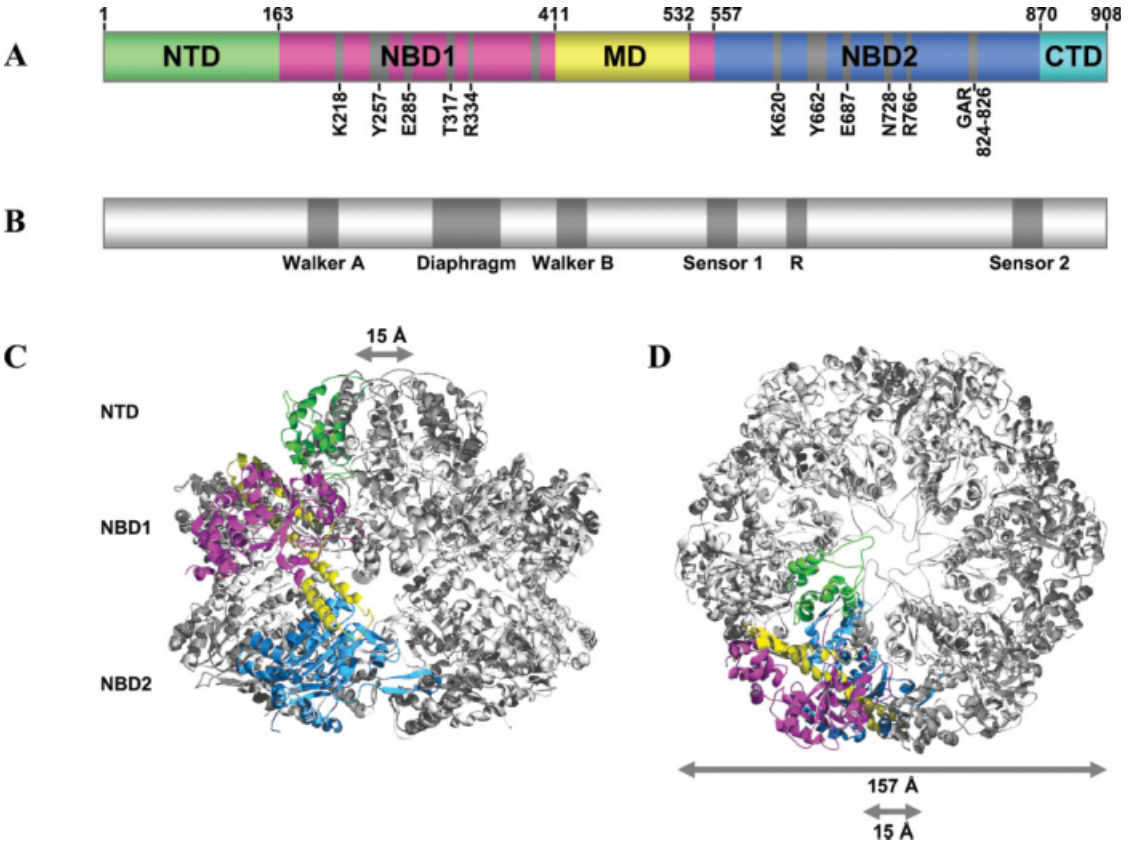

FIGURE 1 Structural organization of Hsp104. (A) Domain organization and key residues of Hsp104 as determined by sequence alignment analysis of Hsp100/ClpB proteins; NTD, N-terminal domain; molecular dynamics (MD), middle domain; CTD, C-terminal domain. (B) Main structural motifs of each nucleotide binding domain of Hsp104, NBD1 and NBD2. The indicated key resides of NBD1 and NBD2 in (A) correspond to the depicted motifs in (B). (C) Cryo-EM reconstruction of the $Hsp104^{N728A}$-ATPγS hexamer in a side view and (D) in a top view. The domains of one protomer within the hexamer are colored as follows: NTD in green, NBD1 in pink, MD in yellow, and NBD2 in blue. The CTD was not resolved in the structure and is missing from the structural model. The structure files of the Hsp104 reconstructions were kindly provided by P. Wendler and H. Saibil, London.[8]

oligomerization function.[75,82] These functional assignments are based on Walker A-type mutations, which prevent binding of nucleotide to the affected NBD. However, both mutants $Hsp104^{K218T}$ and $Hsp104^{K620T}$ show reduced nucleotide binding at one NBD, reflecting a nucleotide state that might not exist in the wild-type enzyme. Interestingly, other types of mutation that do not affect ATP binding but exclusively affect hydrolysis show a significantly different behavior, see below.[7,9,73] The glutamate residue of the Walker B motif (hhhhDE, where h = hydrophobic residue) is involved in the hydrolysis of ATP. A mutation of this glutamate inhibits ATP hydrolysis but does not affect ATP binding in related AAA^+-ATPases.[70,72,73,89] In Hsp104, the Walker B glutamate corresponds to the residues E285 and E687 in NBD1 and NBD2, respectively, see Figures 1A, 1B, and Table II. The corresponding E285Q/A mutants show an unexpected high ATP turnover of more than 300% of the wild-type activity that can be only accounted for the remaining intact NBD2,[7] suggesting that NBD2 contributes to the enzymatic function as well as to the oligomerization of the protein. Indeed, the ATPase activity of NBD2 was found to be strictly dependent on an ATP-bound state of NBD1.[7,90] ATP binding to NBD1 also induces a conformational change that promotes binding of polypeptide substrates.[10] Hence, both, activation of ATP hydrolysis at NBD2 and polypeptide substrate binding are controlled by the nucleotide state of NBD1 in order to allow substrate procession. ATP hydrolysis of either one of the two NBDs triggers polypeptide substrate release.[7] The tightly

Table II Mutants of Hsp104 Identified in In Vivo Screens or Designed to Dissect the Function of Individual Domains or Motifs

Site	Mutant	In Vivo Phenotype	In Vitro Chaperone and ATPase Activity	Oligomerization	References
NTD	ΔN (Δaa1-147, aa1-152, or aa1-157)	[*PSI*$^+$], thermo tolerance +	100% FFL refolding, ΔNHsp104$_{TRAP}$ still binds to substrates	+	9, 67, 79
NTD	inv.110-121 (inverted DNA seq.)	Semi-dominant [*PSI*$^-$], thermotolerance loss			80
NTD	F130W	Thermotolerance +			16
NBD1	D184N	[*PSI*$^+$], insensitive to GdmCl			81
NBD1	C209Y	Semi-dominant [*PSI*$^-$], thermotolerance −			80
NBD1	G212D	Dominant [*PSI*$^-$], thermotolerance loss			80
NBD1, Walker A	G215V	Thermotolerance loss			49
NBD1, Walker A	G217V G217S	Thermotolerance loss	1–2% ATPase	+	76, 82, 83
NBD1 / MD	G217S/T499I	No growth at 25°C, conditional lethal			83
NBD1, Walker A	K218T [1]	Semi-dominant [*PSI*$^-$], thermotolerance loss	10–20% FFL refolding, no substrate binding in ATPγS-bound state, 1–2% ATPase	+	49, 12, 76, 82, 10, 84
NBD1 / NBD2, Walker A	K218T/K620T [1]	Dominant [*PSI*$^-$], thermotolerance loss	No nucleotide binding	Defect	44, 75, 84
NBD1	A220T	Semi-dominant [*PSI*$^-$], thermotolerance −			80
NBD1, Diaphragm	Y257A	Y257A: weak [*PSI*$^+$], thermotolerance −	<10% FFL refolding		6, 9, 16, 85
	Y257W [4]	Y257W: thermo tolerance +			
NBD1	G259D	Semi-dominant [*PSI*$^-$], thermotolerance −			80
NBD1, Walker B	E285Q/A [1]		Hsp70 independent unfolding activity, 300-500% ATPase with delayed kinetics		7, 9
NBD1 / NBD2, Walker B, TRAP or DWB mutant	E285Q/E687Q or E285A/E687A [2]		Tight binding to substrate in presence of ATP or ATPγS, ATP binding without hydrolysis	+	9, 10, 47
NBD1	K302N	Dominant [*PSI*$^-$], thermotolerance loss			37
NBD1, Sensor1	T317A [1]	Semi-dominant [*PSI*$^-$], thermotolerance −	10% ATPase	+	80, 86
NBD1, Arginine finger	R334M	Thermotolerance −	20–30% ATPase	−/+	67
NBD1	P389L	Semi-dominant [*PSI*$^-$], thermotolerance loss			80
MD	R419M	Thermotolerance −	20–30% ATPase	+	67
MD	R444M	Thermotolerance −	20–30% ATPase	+	67
MD	Y466W	Thermotolerance +			16
MD	L462R	Semi-dominant [*PSI*$^-$], thermotolerance +			80

Table II (*Continued from the previous page.*)

Site	Mutant	In Vivo Phenotype	In Vitro Chaperone and ATPase Activity	Oligomerization	References
MD	R495M	Thermotolerance −	300% ATPase	+	67
MD	T499I	Thermotolerance −	30% ATPase		83
MD	A503V	[*PIN*$^+$], but toxic to [*PSI*$^+$], conditional lethal, no growth at 37°C, but thermotolerance + +	140% ATPase		37, 83
MD	A503V/A509D	No growth at 37°C, conditional lethal			83
MD	Y507W	Thermotolerance +			16
MD	A509D	Semi-dominant [*PSI* $^-$], thermotolerance −	100% ATPase		37, 83
NBD1 extension	L553W	Thermotolerance +			16
NBD1 extension	P557L	Semi-dominant [*PSI* $^-$], but normal [*URE3*],[*RNQ*$^+$], thermotolerance +			80
NBD2, Walker A	G617V	Thermotolerance loss			52
NBD2, Walker A	S618T	Thermotolerance +			52
NBD2, Walker A	G619V		10–30% ATPase	Defect[a]	76, 82
NBD2, Walker A	K620T [1, 3]	Semi-dominant [*PSI* $^-$], thermotolerance loss (K620R: thermotolerance +)	30–50% FFL refolding, binds to substrate in ATPγS-bound state, 10–60% ATPase[a]	Defect[a]	10, 13, 49, 52, 75, 76, 82, 84
NBD2, Walker A	T621A		10% ATPase	+	76
NBD2	E645K	[*psi* $^-$], thermo tolerance loss	20% FFL refolding, 100% ATPase	+	85
NBD2	G661D	Dominant [*PSI* $^-$], thermotolerance loss			80
NBD2, Diaphragm	Y662F	Y662F: weak [*PSI*$^+$], thermotolerance −	Y662F: 100% FFL refolding, 107% ATPase	+	6, 8, 9, 85, 79
	Y662W [4]	Y662W:weak [*PSI*$^+$], thermotolerance −	Y662W: 85% FFL refolding, 160% ATPase		
	Y662A	Y662A: [*psi* $^-$], thermotolerance loss	Y662A: 0% FFL refolding, 100% ATPase		
	Y662K	Y662K: [*psi* $^-$], thermotolerance loss	Y662K: 0% FFL refolding, 105% ATPase		
NBD2, Walker B	E687Q/A [1]		Hsp70 independent unfolding activity in presence of ATP, 75-100% ATPase		7,9
NBD2	D704N	Semi-dominant [*PSI* $^-$], thermotolerance +			80
NBD2	I722T	Thermotolerance +	80% FFL refolding, 300% ATPase	+	13
NBD2, Sensor1	N728A [1]	Semi-dominant [*PSI* $^-$], thermotolerance − −	25% ATPase	+	8, 67, 86
NBD2, HAP mutant	G739I/ S740G/K741F [5]	[*PSI*$^+$], thermo tolerance +	100% FFL refolding		5, 6, 47
NBD2, Arginine finger	R765M [3]	Thermotolerance −	100% ATPase with delayed kinetics	Nucleotide independent oligomer	67

Table II (*Continued from the previous page.*)

Site	Mutant	In Vivo Phenotype	In Vitro Chaperone and ATPase Activity	Oligomerization	References
NBD2	F772S	[*psi* $^-$], thermo tolerance loss	65% FFL refolding, 150% ATPase	Defect	13
NBD2	K774E	Weak [*PSI*$^+$], thermotolerance loss	90% FFL refolding, 60% ATPase	Defect	13
NBD2	L814S	[*PSI*$^+$], thermo tolerance loss	75% FFL refolding, 80% ATPase	Mild defect	13
NBD2	Y819W [4]	Thermotolerance +	130% ATPase	+	85–87, 16
NBD2, Sensor 2	R826M	[*psi* $^-$], thermo tolerance −	45% ATPase	+	87
NBD2	L840Q	[*PSI*$^+$], thermo tolerance loss	110% FFL refolding, 90% ATPase	Mild defect	13
CTD	L892W	Thermotolerance +			16
CTD	Δ38 (Δaa871–908)	Weak [*PSI*$^+$], thermotolerance loss	<0.1% ATPase	Defect	15
CTD	Δ22 (Δaa887-908)	[*PSI*$^+$], thermo tolerance −/+	1.5% ATPase	Mild defect	15
CTD	Δ4 (Δaa905-908), putative TPR interaction site	[*PSI*$^+$], thermo tolerance +	80% ATPase	+	15

(+) refers to wild-type-like, (+ +) to enhanced, (−) to reduced, and (− −) to strongly reduced phenotype or trait. [*PSI*$^+$] refers to a normal, wild-type like Sup35 prion propagation, "weak [*PSI*$^+$]" to a mild suppression of the prion phenotype, [*psi* $^-$] to no prion propagation (such as Δ*hsp104* deletion phenotype), and [*PSI* $^-$] to an inhibition of prion propagation even if Hsp104^{WT} is present. Dominant or semi-dominant [*PSI* $^-$] applies to the effect of the mutant such as Hsp104$^{K218T/K620T}$ expressed in a wild-type background, the effect is most probably due to the formation of nonfunctional hetero-oligomers which depends for some mutants also on the type of yeast strain. This effect might be true for more mutants but it is hardly tested. The FFL refolding and ATPase values were roughly estimated based on published work and are given as percentage relative to the given wild-type activity. Several mutants are serving as specialized tools in functional studies: see [1], mutant serves to dissect NBD functions; [2], serves as trap for substrate binding assays; [3], for oligomerization dependency studies; [4], tryptophan fluorescence sensitive to nucleotide binding; and [5], provides new interaction site for ClpP protease and can serve as substrate trap when combined with inactive ClpP.

[a] The ATPase of this mutant dependents on oligomeric state but hexamer only present at low salt (20 m*M* salt), or at a protein concentration >100 μg/mL.

regulated interplay between NBD1 and NBD2 appears to provide the structural basis of the directed translocation of polypeptides through the central cavity form the N-terminal to the C-terminal exist site of the Hsp104 hexamer. In the case of the double Walker B mutant E285Q/E687Q (Hsp104^{TRAP}, see Table II), no ATP hydrolysis takes place at all and substrate release is very slow resulting in a highly stable Hsp104^{TRAP}-ATP-substrate complex.[7,10,91] This mutant has been used as a tool to generate tight binding in several polypeptide substrate interaction studies.[9,10,47,91–93]

Additional conserved structural motifs in the nucleotide binding domains, such as the Sensor 1 motif[94] and the Sensor 2 motif (see Figures 1A and 1B),[1] are in close proximity to the bound nucleotide. Their mutation in Hsp104 leads to an impaired nucleotide hydrolysis or nucleotide binding, respectively, see Hsp104^{T317A}, Hsp104^{N728A}, and Hsp104^{R826M} in Table II.[86,87] The Sensor 1 mutants T317A and N278A show nucleotide binding, but no ATP hydrolysis,[86] and have been used as tools to dissect the NBD functions. Unlike the Walker B mutants, the Sensor 1 mutants exhibit a reduced ATPase activity, suggesting a secondary functional defect, and they are not reported to from stable substrate complexes. Nevertheless, the Sensor 1 mutant Hsp104^{N728A} has been used in cryo-electron microscopy (Cryo-EM) studies to stabilize the ATP-bound state in NBD2,[8,67] see Figures 1C, 1D, and 2B.

Another important motif within each NBD is the Diaphragm loop, which is a highly flexible axial channel loop containing a tyrosine residue. Mutation of the tyrosine residue of the Diaphragm loop in NBD1 and NBD2 affects the protein disaggregation activity, see Hsp104^{Y257A} and Hsp104^{Y662A} in Table II.[9,85] This tyrosine is thought to extend into the central channel of the hexamer, where it may interact with other Diaphragm loops from the neighboring protomers, according to the structural model of the related ClpA ATPase,[95] and models of ClpB[96] and Hsp104.[8] It might restrict the size of the

central channel of Hsp100/ClpB and/or directly interact with polypeptide substrates. It has been suggested that the Diaphragm couples ATP hydrolysis to polypeptide procession though the channel of the hexamer.[5,85,92,97] Therefore, an ATP hydrolysis event in one subunit might be directly transmitted through movements of the Diaphragm, mediating the translocation of a polypeptide substrate, see model in Figure 3B. Together, these findings suggest that the structure of the NBDs provides a high level of molecular-mechanical regulation of Hsp104. The nucleotide is bound in a strategic position within each nucleotide binding domain and between adjacent subunits within the hexamer. Furthermore, a direct contact of core strands of the ATPase site to the polypeptide substrate in the central channel is very likely. This structural arrangement explains how the energy provided by ATP hydrolysis can be directly translated into conformational changes of the domains that drive the translocation of polypeptide substrates.

N-Terminal, Middle-, and C-Terminal Domain of Hsp104

The N-terminal domain, the middle domain, and the C-terminal domain, see Figures 1A and 1C, also play important roles in regulating the functional properties of Hsp104. These domains are involved in mediating substrate binding and/or specificity, in transmitting allosteric communication regarding ATP hydrolysis and substrate translocation, in the stabilization of the hexamer, or providing cofactor binding sites.

The N-terminal domain [amino acids (aa) 1–163] is thought to mediate the initial polypeptide substrate binding, as has been reported for ClpB from *E. coli*.[92,96,98] Although a deletion of the entire domain, see Hsp104$^{\Delta N}$ in Table II, does not have any effect on the in vitro disaggregation activity, in vivo thermotolerance, and prion propagation by Hsp104,[9,79] mutation of only a short stretch of amino acids in this domain (Hsp104$^{inv.\ 110–121}$) leads to a loss of prion propagation and thermotolerance,[80] see Table II. The function of the N-terminal domain is not well understood but it is conceivable that it serves to limit the access to the inner cavity in order to prevent nonspecific binding of polypeptides. Furthermore, it might be specifically required for the binding of prion protein substrates, since Hsp104$^{\Delta N}$ appears to be defective in the loss of the [*PSI*$^{+}$] prion phenotype upon overexpression.[79] In contrast, overexpression of wild-type Hsp104 leads a loss of the [*PSI*$^{+}$] prion state, a phenomenon called prion curing.[44] Accordingly, the deficiency in prion curing might arise via a partially reduced interaction of Hsp104$^{\Delta N}$ with prion proteins in the absence of the N-terminal domain.

The middle domain (aa 412–532, Figure 1A) was originally assigned as a linker between NBD1 and NBD2[4]; however, based on sequence alignment analysis and structural studies, it is now considered an extension of the C-terminal end of NBD1.[66] As mutations within this domain result mostly in a loss of disaggregation activity for both ClpB and Hsp104, it was suggested to play a crucial role in the chaperone activity. The Cryo-EM reconstruction of the ClpB hexamer revealed that the middle domain forms a large coiled-coil stretch at the outer ring surface of the hexamer with similar appearance to that of propeller blades,[66] supporting a central function in the "crowbar model" of polypeptide disaggregation.[78,96,99–101] However, in the Cyro-EM reconstitution of Hsp104, this coiled-coil structure appears to be integrated between NBD1 and NBD2, as well as between the arrangement of NBDs from neighboring protomers,[8,67] see Figure 1C. It has been suggested that this domain offers potential binding sites for protein substrates in the wall of the inner cavity; there it could also provide interaction sites for residues involved in nucleotide coordination and hydrolysis.[67,102] Despite the apparent differences between Hsp104 and ClpB, their structures both suggest that the middle domain plays a role in translating the ATP hydrolysis within NBD1 into mechanical energy and movements of the central cavity and NBD2. The sequence of the middle domain is not highly conserved; however, mutagenesis studies support its relevance for the ATPase and chaperone activity of Hsp104. Conditional lethal mutants or gain-of-function mutants, such as G217S/T499I, A503V, or A503V/A509D, were isolated from in vivo screens. These mutations are located in the middle domain, see Table II. The mutants show an altered thermotolerance and/or prion propagation phenotype.[83] Furthermore, the Hsp104^{R495M} mutant shows an irregularly high ATPase activity.[67] Therefore, the middle domain appears to have a delicate function in the regulation of both the ATPase activity and the chaperone function of Hsp104.

The C-terminal domain (aa 871–908) flanking the NBD2 of Hsp104 has also been proposed to be involved in substrate binding.[16] However, related Clp ATPases that are associated with protease cofactors eject substrates at the C-terminal site in order to feed it to their protease subunit.[103,104] Recent work with the BAP mutant of ClpB and the HAP mutant of Hsp104, see Table II, in combination with the ClpP protease, indicated that Hsp100/ClpB proteins also use this domain as an "exit" site for the translocated substrates.[5,6] Furthermore, the C-terminal domain of Hsp104 plays a role in nucleotide-independent oligomerization. A deletion of more than 22 residues from the C-terminus of Hsp104 impairs its hexamerization.[15] In addition, it was shown that cofactor binding occurs at the extreme C-terminus of Hsp104 (aa 903–908),

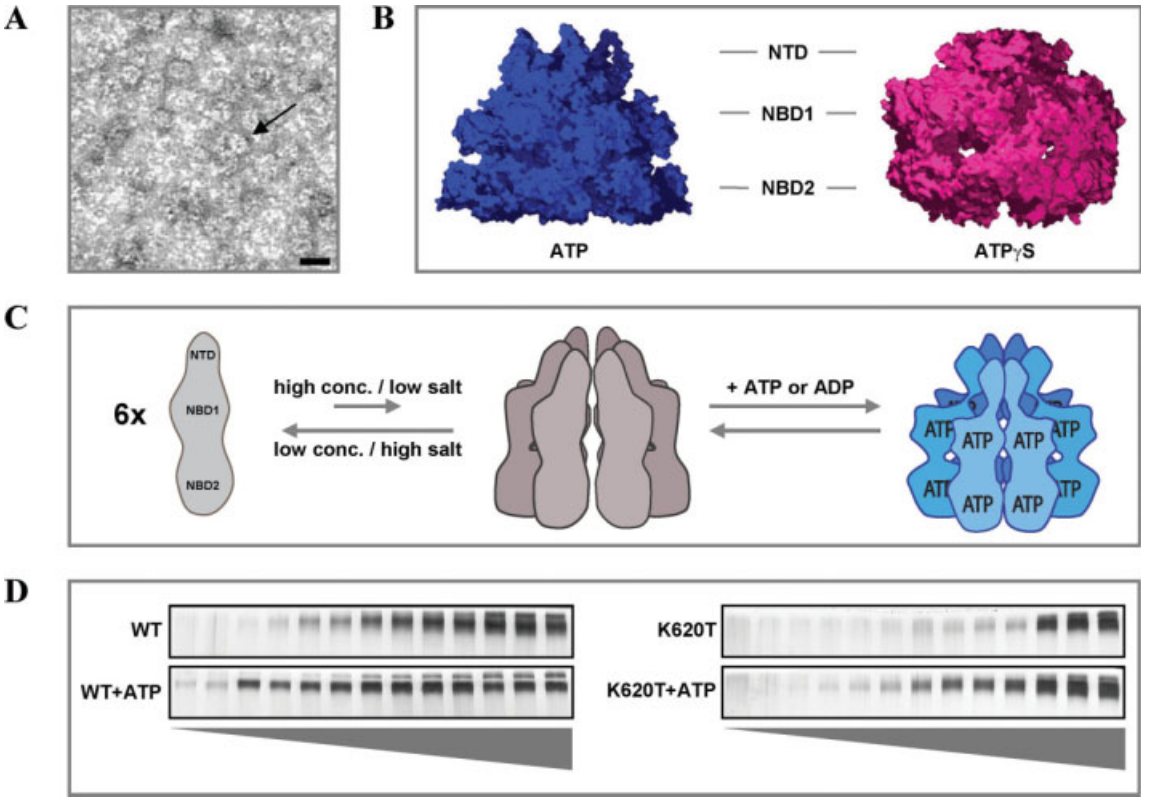

FIGURE 2 Oligomerization of Hsp104. (A) Current TEM image showing oligomeric Hsp104-ATP complexes at a concentration of 500 μg/mL; oligomer: see example at arrow; bar: 20 nm. (B) Side views of Cryo-EM reconstructions of $Hsp104^{N728A}$ showing different hexamer conformations depending on the type of bound nucleotide. The structure files of the Hsp104 reconstructions were kindly provided by P. Wendler and H. Saibil, London.[8] (C) The oligomerization equilibrium of Hsp104 is dependent on several factors, i.e., high protein concentration, binding of nucleotide, or low ionic strength stabilize the hexamer. The monomer is depicted in light grey, the apo-hexamer in grey, and the ATP-bound hexamer in blue. (D) Glutaraldehyde cross-linking of $Hsp104^{WT}$ and $Hsp104^{K620T}$ showing the hexamer formation dependent on the protein concentration and the presence of ATP, over an Hsp104 concentration range from 5.1 to 510 μg/mL (50–5000 n*M* monomer) after cross-linking. Samples were cross-linked in physiological buffer containing 150 m*M* KCl and 10 m*M* $MgCl_2$. A total of 80 ng protein was loaded in each lane of the SDS-PAGE gel and subjected to electrophoresis followed by silver staining; only the hexamer band is depicted.

an acidic C-terminal extension (IDDDLD) that is not present in ClpB and bears some similarity to the C-terminal cofactor binding motifs of eukaryotic Hsp90 and Hsp70 (VEEVD and MEEVD).[15,52,65,105] Hsp90 and Hsp70 interact with the TPR domain containing proteins via this stretch of residues.[106,107] It is conceivable that the C-terminus of Hsp104 contains a TPR protein binding motif, possibly linking Hsp104 to the Hsp70/90 chaperone network of TPR proteins, that might serve in substrate trafficking.

The Oligomerization State of Hsp104

The oligomeric state of Hsp104 has been investigated extensively using several biophysical and biochemical techniques including transmission electron microscopy (TEM), analytical ultracentrifugation, and glutaraldehyde cross-linking experiments.[10,75,76,88] These studies have consistently shown that Hsp104 exists predominantly as a hexamer. The first glimpse of the structure of the Hsp104 hexamer was provided by negative staining TEM studies of recombinant Hsp104 purified from yeast lysate that revealed ring-like particles with 6-fold symmetry in the presence of ATP,[75] see also current TEM image in Figure 2A. Recent structural studies employing Cryo-EM techniques combined with crystallographic methods confirmed these findings, showing a hexameric protein complex with a central channel.[8,66,67] The hexameric complex of Hsp104 has an outer dimension of around 160 Å, revealing a strong structural plasticity with nucleotide-induced conformational changes, and a large

central cavity whose inner diameter changes from 28 to 78 Å, depending on whether the protein is in an ADP, ATP-bound, or ATPγS-bound state (ATPγS is an ATP analogue, which can be slowly hydrolyzed by NBD1 but is not hydrolyzed by NBD2.[7]), respectively,[8,67] see also Figures 1C, 1D, and 2B. These emerging structures provide important insight into the structural basis of the disaggregation activity and the domain movements in the ATPase cycle of hexameric Hsp104.[8,66,67,96] The current structural model of Hsp104 also reveals significant domain movements upon ATP binding and hydrolysis. The movements of the N-terminal domain and NBD1, in particular, lead to the displacement of putative substrate binding sites, thus providing structural basis for substrate threading from the N- to the C-terminal site of the Hsp104 hexamer. Interestingly, the reconstitution of the hexameric structure in complex with ATPγS yields arrangements with a marked asymmetry. This suggests that the nucleotide-bound state of Hsp104 probably exists as a mixed ATP/ADP complex and/or that ATP hydrolysis by the hexamer takes place in a nonconcerted fashion,[8] possibly in order to allow a step-by-step translocation of polypeptide substrates through the central pore.

Hsp104 Oligomerization: Concentration, Nucleotide, and Ionic Strength Dependence

The oligomerization of Hsp104 exhibits concentration, ionic strength, and nucleotide dependence,[75,76,86] see Figure 2C. Cross-linking experiments showed that the wild-type protein is mostly hexameric at protein concentrations of $\geq$100 μg/mL ($\sim$1 μM monomer concentration). At lower protein concentrations, $<$100 μg/mL, Hsp104 exists in an equilibrium of hexamers and monomers, with the monomer being the predominant species at concentrations of $<$10 μg/mL, see Figure 2D. The oligomerization of Hsp104 is also driven by its binding to ATP, which stabilizes the hexamer. Hence, the majority of structure determination studies were carried out in the presence of ATP or its nonhydrolyzable or slowly hydrolyzable analogue ATPγS. In the absence of nucleotide, the hexamer is less stable, but can still form at a higher protein concentration of 500 μg/mL ($\sim$5 μM monomer concentration), see Figure 2D. The ATPase mutant Hsp104^{K620T} has a nucleotide binding defect in NBD2 and shows reduced hexamer formation even in the presence of nucleotide.[75] However, only the hexameric state of Hsp104 is enzymatically active. A direct relationship between hexamer formation and ATPase activity is supported by site-directed mutagenesis, concentration-dependent, and salt-dependent oligomerization studies.[76,82,86] Accordingly, several Hsp104 mutants, e.g., Hsp104^{K620T}, Hsp104^{G619V}, Hsp104^{F772S}, Hsp104^{K774E}, and C-terminal truncation mutants such as Hsp104$^{\Delta 38}$, exhibit a reduced hexamer formation and concomitantly a reduced ATPase and/or chaperone activity.[13,15,76] This reduction in activity can be recovered under conditions that promote hexamerization, i.e., high protein concentration ($\gg$100 μg/mL) or low salt conditions.[13,76] The salt dependency of the oligomerization was demonstrated by static light scattering experiments in the absence of ATP. At around 20 μg/mL, Hsp104^{WT} forms a $\sim$600 kDa complex in 20 mM NaCl, consistent with the hexamer as the predominant species. However, in the presence of 200–300 mM NaCl, the monomer becomes the predominant species in solution.[86] Accordingly, by carefully fine-tuning the ionic strength and the protein concentration, the hexamer can be stabilized in the absence of nucleotides or disassembled in the presence of nucleotides.[82,86,88] However, several Hsp104 mutants, e.g., Hsp104^{K218T}, Hsp104^{T317A}, Hsp104^{T621A}, and Hsp104^{N728A}, are ATPase deficient, despite their wild-type ability to form a hexamer.[10,75,76,82] Taken together, these findings demonstrate that the hexamer formation is necessary but not sufficient for Hsp104 ATPase and chaperone activity.

Hsp104 Oligomerization In Vivo

It is noteworthy that all structural and oligomerization studies described earlier exclusively employed in vitro techniques. Direct evidence of the existence of Hsp104 hexamers in vivo is still lacking. However, mutagenesis and functional studies consistently point to the hexamer as the primary functional species of Hsp100/ClpB in vivo. Mutants that are defective in oligomerization, such as Hsp104^{K620T}, Hsp104^{F772S}, Hsp104^{K774E}, and Hsp104$^{\Delta 38}$ (see Table II), are unable to fully complement *HSP104* deletions in yeast.[12,13,49,82] On the other hand, the oligomerization dynamics are required for Hsp104 function in vivo, since Hsp104^{R765M}, a hexamer stabilizing mutation, is deficient in yeast thermotolerance. In vitro experiments revealed that the R765M mutation stabilizes the Hsp104 hexamer and renders it insensitive towards nucleotide-induced oligomerization, although it retains up to 100% of wild-type ATPase activity,[67] see Table II. This mutation alters the putative Arginine Finger in NBD2 (see "R" in Figure 1B), a structural motif important for the allosteric regulation of related AAA$^+$-ATPases,[108] that might have an allosteric function beyond the stabilization of the hexamer. Indirect confirmation of the oligomerization of Hsp104 in vivo is derived from studies aimed at understanding the role of Hsp104 in prion propagation using Hsp104 point mutations. In these studies, the coexpression of wild-

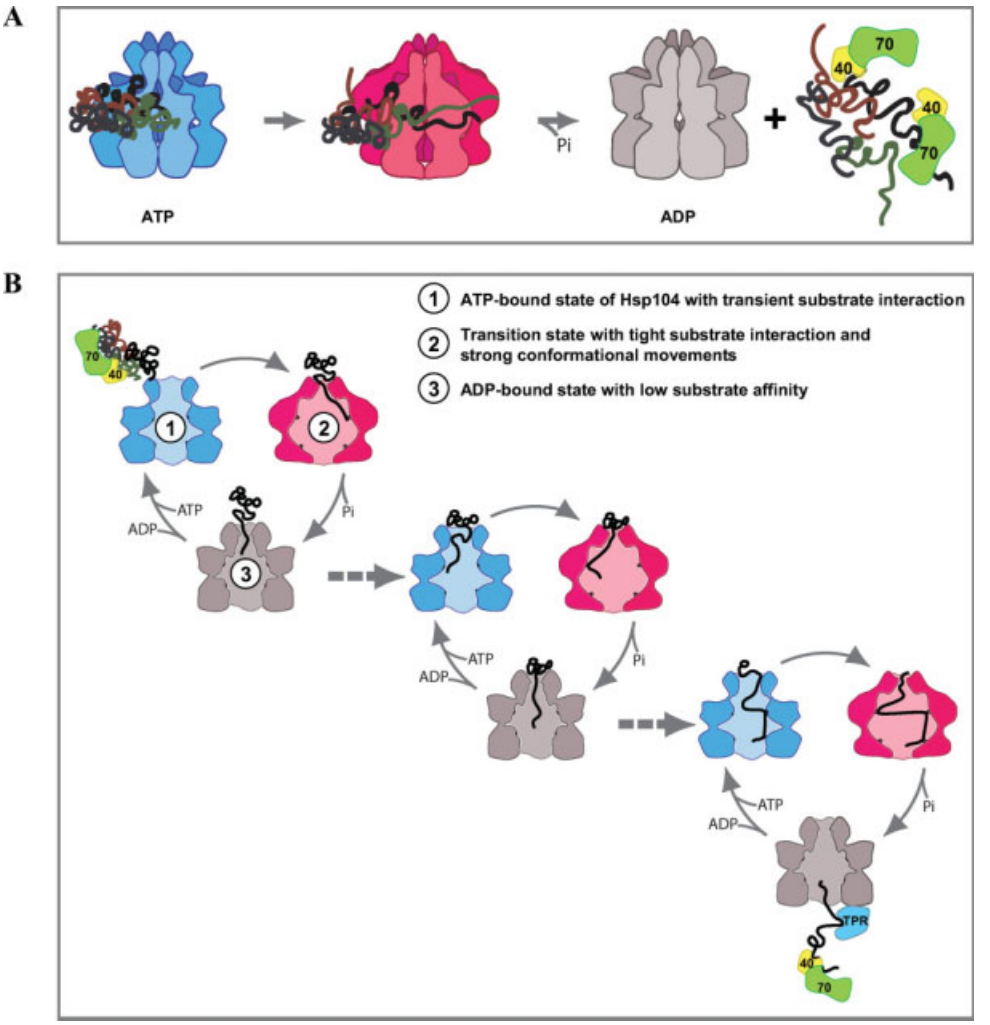

FIGURE 3 Models of the disaggregation activity of Hsp104. (A) Crowbar mechanism of Hsp104. The Hsp104 hexamer is presented in a side view. The aggregate is bound at several sites on the outer surface of Hsp104 and, by cycles of ATP hydrolysis along with significant conformational movements, Hsp104 induces partial unfolding or loosening of the bound aggregate. This mechanism was proposed to mediate mechanical fragmentation of large aggregates, e.g., aggregate loosening. (B) Substrate threading mechanism of Hsp104. Cross-sections of Hsp104 are depicted in three ATPase cycles. ATP binding and hydrolysis by Hsp104 drives conformational movements and the substrate binding cycle. First, substrates transiently interact with $Hsp104^{WT}$ in the ATP-bound state. This early substrate interaction might be supported by Hsp70/40 targeting the substrates for disaggregation. During the ATPase cycle, possibly in the ATP transition state, a high affinity state tightly binds the substrate to the Diaphragm loops in the central cavity of Hsp104, and Hsp104 undergoes strong conformational changes that cause a structural distortion of the bound substrate. The following ADP state has low substrate affinity and allows substrate release or it might allow transient relaxation of the substrate within the central channel. However, the ATP, ADP, and apo-states might be mixed within one hexamer and the role of each subunit upon translocation still needs to be investigated. Based on the available biophysical data it can be speculated that, by several sequential cycles of ATP hydrolysis, the substrate is dragged into the cavity and becomes translocated. The number of ATPase cycles may depend on the folding stability of the substrate or on the degree of aggregation. After release at the C-terminal site of Hsp104 the substrate is initially unfolded and might be subjected to the chaperone activity by Hsp70/40 to reach the native state and/or be handed over to TPR proteins for further substrate targeting.

type Hsp104 with the Walker A double mutant $Hsp104^{K218T/K620T}$ results in an elimination of the prion phenotype,[44,84] and a loss of thermotolerance and protein disaggregation activity.[109,110] This mutant has a dominant negative effect on the Hsp104-related functions when expressed in a wild-type background. Thus, the mutant generates the phenotype of an *HSP104* deletion even if wild-type Hsp104 is present in the cells.[44,84,109–112] Based on these in vivo observations, it was concluded that the inactivation of wild-type Hsp104 was due to the formation of mixed hetero-oligomers with the mutant. A close examination of the literature reveals that several further mutations can be classified as semi-dominant, i.e., they partly suppress wild-type Hsp104 activity. For example, the coexpression of the single point mutants $Hsp104^{K218T}$ and $Hsp104^{K620T}$ leads to a reduced thermotolerance and suppresses the [PSI^+] prion phenotype but does not cure it, as the double mutant $Hsp104^{K218T/K620T}$ does.[44,76,84] The effect of these semi-dominant mutants on [PSI^+] prion propagation by $Hsp104^{WT}$ also depends on the genetic background, i.e., the yeast strain,[44] and the dosage of mutant protein.[76,109] In vitro data support the hypothesis that hetero-oligomerization causes a loss of function, i.e., that one or two defective subunits within the hexamer are sufficient to "poison" the activity of the hexamer.[76,93] Accordingly, the incorporation of a substoichiometric amount of an inactive mutant into wild-type Hsp104 hexamers results in a significant reduction of the ATP turnover and chaperone activity in vitro, as well as impaired thermotolerance and prion propagation in vivo.

Taken together, the formation of putative mixed oligomers in vivo as well as the negatively stained images of hexameric Hsp104 protein purified from yeast[75] provide strong circumstantial evidence for the formation of the Hsp104 hexamer, but very little is known about the dynamics and cellular determinants of Hsp104 oligomerization in vivo. Hsp104 has a basal expression under physiological growth conditions and is upregulated under stress conditions. Its physiological level under nonstress conditions was estimated to be about 33,000 molecules per haploid yeast cell grown at 30°C.[113] This number of molecules corresponds to a minimal cellular concentration of 0.8 μM monomeric Hsp104 (82 μg/mL), assuming a cell volume of about 7 picoliters.[114] However, Hsp104 is a protein that is found in the cytosol and in the nucleus[115] and that occupies only a fraction of the total cell volume. Accordingly, it can be assumed that the physiological Hsp104 concentration is higher than 100 μg/mL, which would be sufficient to stabilize the hexamer, see Figure 2D. Interestingly, the addition of molecular crowding agents has been shown to restore the ATPase activity of oligomerization deficient mutants in vitro.[15] However, in vitro data on the oligomerization might not be applicable to in vivo conditions because unknown factors including protein–protein or protein–small molecule interactions could also influence the oligomerization of Hsp104 in vivo. Beyond the physiological conditions, yeast exhibits the highest stress resistance when cells are preconditioned by mild nonlethal stress that triggers increased expression of Hsp104 6–8-fold.[116] Thus, endogenous Hsp104 levels are strongly increased resulting in enhanced concentration-dependent oligomer formation under conditions where the survival of the yeast cell is dependent on a fully functional Hsp104. Importantly, the promoter of Hsp104 contains several stress-inducible *cis* acting elements including 5 HSE, 6 STRE elements and other transcription factor binding sites that play a role in drug and metal resistance. Therefore, the overexpression of Hsp104 is likely to be induced not only after heat shock induction but also by a wide range of other stress factors.[117–120]

Hsp104-MEDIATED DISAGGREGATION

The Crowbar Disaggregation Model

Several models have been proposed for Hsp104-mediated disassembly and clearance of protein aggregates. Initially, it was suggested that Hsp100/ClpB functions as a molecular crowbar on stable aggregates,[12,56,66,121,122] see Figure 3A. In the crowbar model, Hsp104 is thought to function by disrupting large aggregates into smaller particles, whereby the misfolded proteins become more accessible for the refolding performed by the Hsp70/40 chaperone system. This model has recently been supported by the Cryo-EM structure of ClpB; the middle domain of ClpB is found in the outer equatorial region of the hexamer structure.[66,99] The movement of this domain is thought to act as a propeller blade that mediates the splitting of large aggregates into smaller ones.[66] This domain, although not conserved, is sensitive to mutations. Several mutations in the middle domain perturb the in vivo chaperone function of Hsp104, see Table II.[16,67,83] The crowbar model assigns a highly important role to the middle domain and explains the minor role of the N-terminal domain in chaperone activity; the N-terminal domain is dispensable for chaperone function in vivo and in vitro.[9,67,79] However, recent structural studies on Hsp104 locate the middle domain as coiled-coil integrated into to the arrangement of NBD1 and NBD2, not on the outer surface of the hexamer,[8,67] see Figures 1C and 1D. It is noteworthy, that in all disaggregation assays, direct evidence for an aggregate fragmentation activity by Hsp100/ClpB before refolding by Hsp70/40, or accumulation of fragmented

aggregates in the absence of functional Hsp70/Hsp40 is still lacking.

The Substrate Threading Model

An alternative model to explain the disaggregation activity is the "threading mechanism," in which Hsp100/ClpB binds to exposed polypeptide chains or loops of polypeptides on the surface of aggregates and threads them thought the central channel of the hexamer.[5,66,85,96,121,122] The central pore in the available structures of Hsp104 or ClpB is too narrow to allow the translocation of folded proteins, but is sufficiently large (15 Å) to allow the translocation of denatured polypeptide chains and polypeptide loops, suggesting that protein substrates must unfold before or during translocation,[8,66,67] see Figures 1C and 1D. In the threading model, the disaggregation activity of the set of Hsp104/70/40 can be dissected into three different main stages, see Figure 3B. Figure 3B, upper panel: (i) Hsp70/40 binds to unfolded or non-natively polypeptide segments exposed on the surface of aggregates and presents those substrates to Hsp104. Hsp104 itself also recognizes unfolded polypeptides, but efficient chaperone activity by Hsp104 requires the presence Hsp70/40. Figure 3B, middle panel: (ii) Once the polypeptide substrate is bound to Hsp104, it starts to be translocated though the central pore of the hexamer. This process relies on the highly regulated allosteric interplay between NBD1 and NBD2, resulting in an unfolding event that eliminates non-native intermolecular and intramolecular contacts of the polypeptide to the aggregate. The mechanistic details of the allosteric interplay of NBD1 and NBD2 upon substrate translocation are yet not clear, but a tight substrate binding mediated by the Diaphragm loops of the axial channel might be required for stepwise translocation via several cycles of ATP hydrolysis by the hexamer, for detail see (1), (2), and (3) in Figure 3B. Figure 3B, bottom panel: (iii) The unfolded substrate is released at the C-terminal site of Hsp104 and, if required, captured and refolded by Hsp70/40 or transferred to cochaperones such as Sti1 or Cpr7 for subsequent targeting to other chaperones. Therefore, Hsp70/40 may play a role in substrate targeting also downstream of the Hsp104 translocation for assisting the folding of released unfolded proteins, possibly depending on the type of substrate.

Several lines of evidences support the threading model: (i) alanine mutations within the Diaphragm loops of NBD1 and NBD2 ($Hsp104^{Y257A}$ and $Hsp104^{Y662A}$ in Table II), which are located in the central cavity of the Hsp100/ClpB hexamer, show reduced or impaired substrate protein refolding.[5,6,85,97] (ii) Using tryptophan single point mutants of the Hsp104 Diaphragms, $Hsp104^{Y257W}$ and $Hsp104^{Y662W}$, it was possible to demonstrate that the binding of an unfolded polypeptide causes a fluorescence quenching of the tryptophan located in the axial channel indicating that these residues are involved in the binding of substrate proteins to the central cavity of Hsp104.[9] (iii) As the Diaphragm loop of NBD2 of ClpB can be specifically cross-linked to denatured FFL only in the presence of the Hsp70 system, the findings strongly suggests that Hsp70/40 play a major role in substrate transfer towards Hsp100/ClpB.[5] (iv) RepA- or lactalbumin-tagged native green fluorescent protein (GFP) is being unfolded upon incubation with Hsp104 or ClpB. It has been suggested that the RepA or lactalbumin tag is recognized, and the attached GFP is then subsequently unfolded by threading through the central pore of Hsp100/ClpB.[7,14] The crowbar model does not explain the complete unfolding of native GFP. (v) The strongest evidence for substrate translocation was provided by studies on the BAP and HAP mutants of ClpB and Hsp104, respectively, each containing an IGF motif insertion that provides a docking site for the ClpP protease, see HAP in Table II. The engineered mutant forms a complex with ClpP that is observable by TEM.[5] This complex is analogous to the ClpA/ClpP protease complex, in which the ClpP protease is coordinated at the C-terminal exit site of the central channel of the ClpA ATPase hexamer where polypeptide substrates become ejected.[123,124] The $ClpB^{BAP}$/ClpP or $Hsp104^{HAP}$/ClpP complex is active in polypeptide degradation but requires the Hsp70/40 chaperone system. Furthermore, the $ClpB^{BAP}$/Hsp70/40 or $Hsp104^{HAP}$/Hsp70/40 chaperone set is active in protein refolding when ClpP is absent.[5,6] Furthermore, the addition of an inactive ClpP mutant strongly inhibits protein disaggregation by $ClpB^{BAP}$/Hsp70, indicating the formation of a $ClpB^{BAP}$/$ClpP^{inactive}$ complex that is proteolytically inactive and blocks disaggregation.[5] In addition, the combination of $Hsp104^{HAP}$ with a $ClpP^{Trap}$ mutant locks Hsp104 in a substrate-bound state, presumably by impairing substrate release.[6]

The Intrinsic Unfolding Function of Hsp104

Recent studies have succeeded in demonstrating that Hsp100/ClpB possesses an intrinsic protein remodeling function that can be observed independently of the Hsp70/40 chaperone system.[7,14] As aforementioned, Hsp100/ClpB has been shown to actively unfold proteins carrying a denatured tag, such as fusion proteins containing unfolded α-lactalbumin and natively folded GFP. The unfolding of tagged GFP

can only be observed with wild-type Hsp100/ClpB under special nucleotide conditions such as the presence of the slowly hydrolysable ATPγS or the presence of ATP when the Walker B single mutants $Hsp104^{E285Q}$ or $Hsp104^{E687Q}$ are used.[7,14] Interestingly, by selecting a specific ATP:ATPγS ratio, even reactivation of substrates such as RepA or heat aggregated GFP can be detected in the absence of Hsp70/40.[14] The intrinsic unfolding or disaggregation function by Hsp100/ClpB is experimentally easier to detect, when ATP hydrolysis is slowed down and/or when one of the two types of nucleotide binding domain is locked in the ATP-bound state by mutation. The addition of Hsp70/40 results only in a relatively small enhancement of substrate disaggregation,[14] possibly because Hsp100/ClpB is the rate-limiting factor under these specific conditions. However, the question remains why it is not possible to observe the intrinsic unfolding function of wild-type Hsp104 under native assay conditions. Interestingly, the Hsp104 ATPase is strongly activated by the presence of a polypeptide substrate.[7,9,16,26] The observation of an alteration in the ATP turnover under steady-state ATPase assay conditions supports the existence of a transient substrate interaction of the wild-type chaperone. Hence, an explanation for the lack of experimental evidence for the unfolding and translocation under native assay conditions by $Hsp104^{WT}$ alone might be that polypeptide substrate binding is highly transient due to fast on- and off-rates while ATP is actively hydrolyzed. Substrates might not be captured for a sufficient time span to allow a complete translocation by $Hsp104^{WT}$ alone under physiological steady-state conditions.

Hsp104 ACTIVITY ON AMYLOID PROTEINS

Principal Characteristics of Yeast Prions

Yeast prions are non-Mendelian genetic elements[125] that are inherited from mother to daughter cells by mechanisms involving "protein only". Like mammalian prions, they are dominant phenotypic traits that are self-propagating and infectious. Prions are transmitted upon cell division, by mating between yeast strains or by experimental protein transfection,[126–129] see Figure 4C. The most prominent yeast prions are [PSI^+], [*URE3*], and [RNQ^+], which are generated by cytosolic, amyloidogenic aggregates of the proteins Sup35, Ure2, and Rnq1, respectively.[126,130,131] However, more prion protein candidates exist in yeast.[132] Yeast prion proteins are listed in Table I and do not share sequence homology or phenotypic characteristics but have in common the propensity to misfold and to form amyloid-like aggregates, the propagation of which is dependent on the activity of Hsp104.[133,17,23,44] Both overproduction and inactivation of Hsp104 cause a loss of the [PSI^+] prion state, i.e., prion curing.[44] However, even high levels of Hsp104 do not cure the [RNQ^+] and [*URE3*] prion state.[23,133] Thus, the effect of Hsp104 is dependent on the Hsp104 dosage and on the type of prion protein. Prion propagation also involves the Hsp70/40 system[134] but is strictly dependent only on Hsp104. The strength of the prion phenotype, e.g., strong [PSI^+] versus weak [PSI^+], depends on the number of prion particles per cell, which is directly related to the stability of the prion conformation and the activity or expression level of Hsp104.[135,136] Furthermore, all yeast prions have in common that the addition of guanidinium hydrochloride (GdmCl) to the yeast medium leads to prion curing. GdmCl, used in appropriate amounts, has been shown to act as a specific inhibitor and not as chaotropic agent, causing an inactivation of Hsp104. Accordingly, not only prion propagation, but also thermotolerance, and in vitro chaperone activity, are affected by GdmCl.[12,110,137] It has been shown that trace amounts, such as 500 n*M* GdmCl, act in vitro as uncompetitive inhibitor of Hsp104, reducing K_M and k_{cat} to the same extent, by altering the allosteric regulation of the ATPase of Hsp104.[88] These results demonstrate that small molecules such as GdmCl can affect the delicate allosteric regulation of the ATPase machinery of Hsp104, resulting in a loss of both, the chaperone function and the prion propagation function. Thus, both functions rely on a fully functional allosteric regulation of Hsp104.

Models of Prion Propagation: Conformational Alteration Versus Prion Splitting by Hsp104

While the role of Hsp104 in prion propagation and inheritance is meanwhile established, the exact molecular mechanisms, structural basis, and cellular determinants underlying this function remain poorly understood. Prion propagation by Hsp104 has been attributed to its role in the maintenance of amyloid protein aggregates in the yeast cytosol, thus facilitating their passage from mother to daughter cell during cell division, see Figure 4C. The prion propagation function seems to contradict Hsp104's protective role in thermotolerance, where Hsp104 resolublizes amorphous protein aggregates formed under stress conditions, e.g., heat-shock-induced aggregates, see Figure 4A. However, unlike heat-shock-induced amorphous aggregates, prion aggregates correspond to amyloid-like structures. The pathway of amyloid formation is a self-templating polymerization reaction which results in large, highly stable β-sheet-rich fibrils, see Figure 4B. Accordingly, prion propagation needs to be examined in the context of amyloid formation. Two different models have been proposed to explain the mechanism by

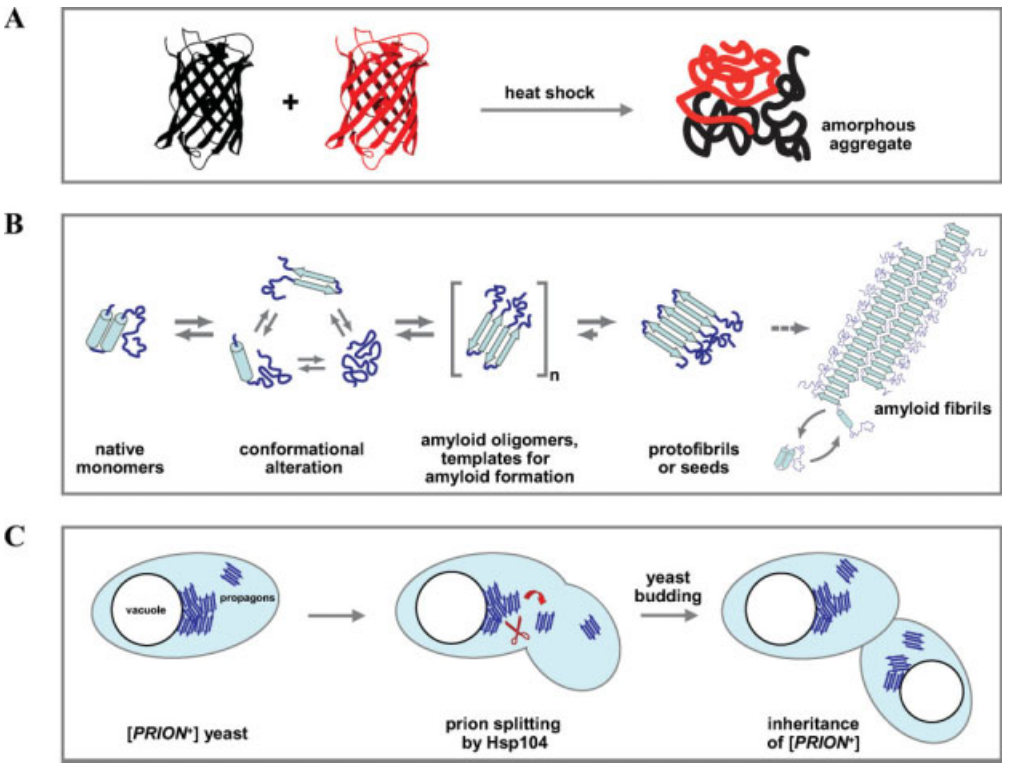

FIGURE 4 Mechanism of nonspecific aggregation, amyloid formation, and prion propagation. (A) Nonspecific aggregation upon heat-induced conformational destabilization: various proteins are destabilized and undergo unspecific, non-native interactions resulting in their aggregation and precipitation. (B) Model of amyloid formation. Amyloidogenic proteins, such as prion proteins, form highly ordered β-sheet-rich fibrils via oligomeric/protofibrilar intermediates. Once oligomers have been formed, monomers are continuously added leading to the formation of stable, fibrillar structures. (C) Prion propagation in yeast depends on the activity of Hsp104 producing prion propagons by mechanical splitting. The prion seeds or propagons are inherited to the next generation through cytoplasmatic transduction, i.e., cytoduction.

which Hsp104 mediates prion propagation in yeast.[84,109,138] Both models are based on the amyloid formation pathway and on the ability of Hsp104 to remodel and/or to induce breakage of preformed protein aggregates, i.e., the disaggregase activity.

The first model hypothesizes that Hsp104 induces a conformational alteration of the native state of a prion protein and catalyzes the formation of an intermediate aggregation competent state, see Figure 5A. The molecular basis of this effect is not clear, but Hsp104 might act on the native monomeric prion/amyloid protein and alter its structure by temporarily unfolding it via translocation through the hexamer. However, Hsp104 might also offer a specific binding site for the amyloidogenic conformation of the monomer and, thus, stabilize this species by altering the equilibrium with the native monomer on the pathway of amyloid formation. From a kinetic point of view, Hsp104 has been suggested to reduce the barrier of free energy for the transition form the native to an amyloidogenic intermediate folding state, see[1] in Figure 6. Once formed, these folding intermediates can either be captured in pre-existing prion aggregates in [*PRION*$^+$] cells or revert back into the native state in the absence of prion particles, such as in [*prion*$^-$] cells. Thus, Hsp104 may catalyze the aggregation by promoting the formation of an aggregation-prone amyloidogenic intermediate.[44,84] This model describes the growth in size of prion aggregates as a function of Hsp104 but it does not explain how the number of prion particles is amplified. It has been suggested that prion particles might break into smaller pieces naturally upon growth once they exceed a certain length.[84] However, it became evident that the limiting step in prion propagation or in prion loss of yeast is determined by the number of prion aggregates per cell rather than by their size or growth rate.[135,140] Moreover, recombinant yeast prion proteins do

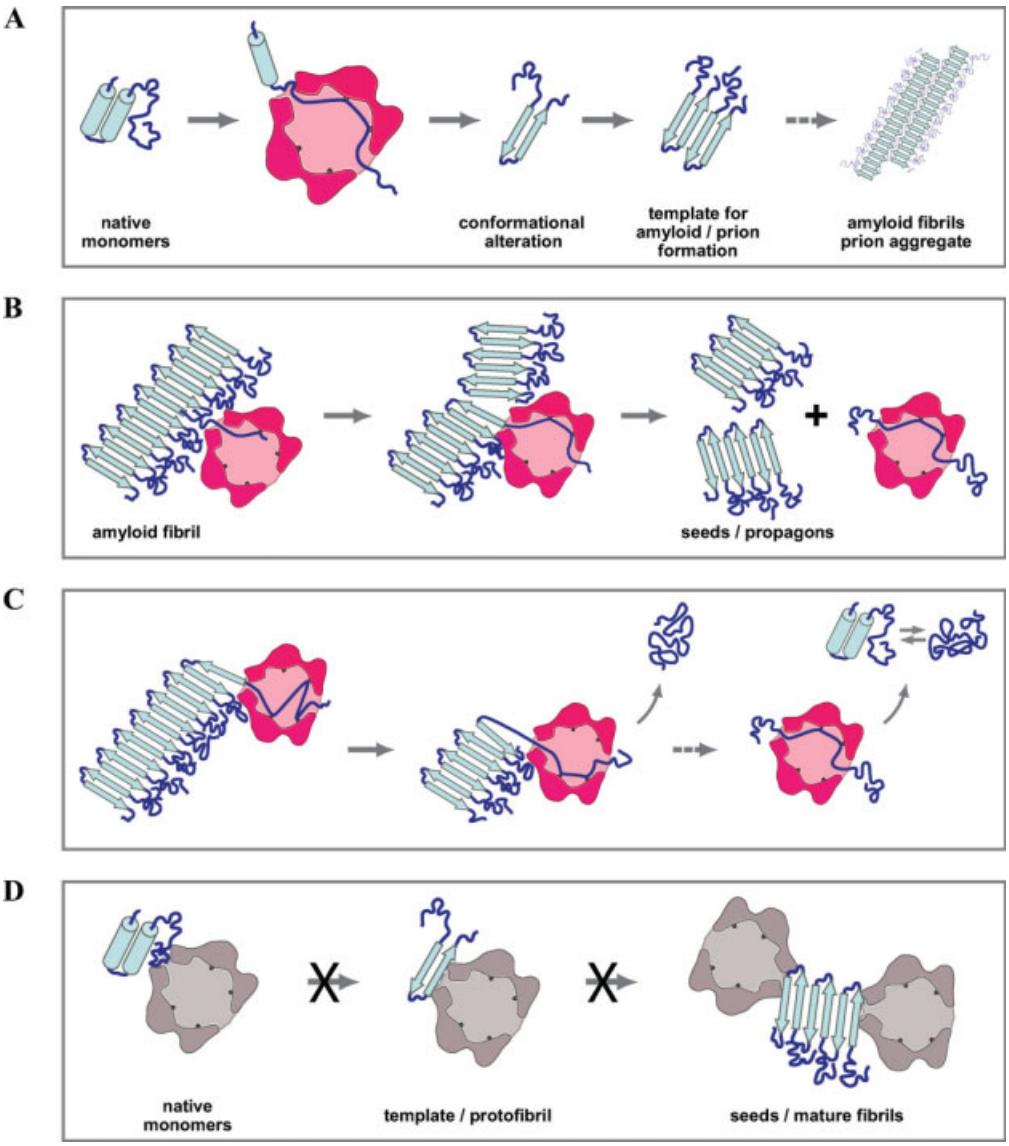

FIGURE 5 Mechanism of Hsp104 in amyloid formation. (A) The conformational alteration hypothesis consists of the interaction of Hsp104 with native protein, leading to structural alterations that prime the formation of prion/amyloid structures. This type of interaction requires ATP and low concentrations of active Hsp104. (B) Mechanical fibril splitting activity of Hsp104. Hsp104 actively produces amyloid seeds or prion propagons out of mature fibrils. By increasing the number of seeds, amyloid formation is accelerated. This type of interaction requires ATP and high concentrations of active Hsp104. (C) Analogous to (B), Hsp104 may attack prion/amyloid fibrils by the end, leading, after several cycles of binding, translocation, and monomer release, to a complete dissociation of the fibril. Complete dissociation only occurs if the on-rate of amyloid formation is lower than the dissociation rate by Hsp104 or if released monomers are removed from the amyloid formation reaction by attaining their native, nonamyloid folding state. (D) Suppression of amyloid formation by Hsp104 blocking several species on the pathway of amyloid formation. Hsp104 inhibits amyloid formation by sequestering the templating and/or seeding amyloid species from the pathway and/or by protecting monomers from aggregation. Hsp104 is depicted in gray since the nucleotide state or ATPase activity does not to seem to be relevant for this type of interaction.

not require Hsp104 to aggregate or become incorporated in pre-existing prion particles or amyloid fibrils in vitro.[141–143]

The prion splitting model suggests that Hsp104 partially disaggregates [*PRION*$^+$] complexes into smaller aggregates, thus, increasing the number of prion particles, i.e., seeds or propagons. These particles are transferred to daughter cells, where they can nucleate further aggregation, ensuring the propagation of prion aggregates from one generation to another,[84,109,138] see Figures 4C and 5B. Consequently, in this model Hsp104 converts amyloid structures into self-propagating prions. The prion splitting activity suggests that Hsp104 attacks amyloid fibrils in the middle of the structures and pulls out single subunits by binding and actively translocating the unstructured polypeptide segments decorating the surface of amyloid fibrils in an ATP-dependent process, as indicated in Figure 5B. However, in principle, Hsp104 could also attack the prion structures at the ends of the amyloid fibrils, see Figure 5C, where the β-sheet core structures are accessible. The latter activity would only result in fibril dissociation and not in fibril splitting, i.e., there would be no seeding of amyloid formation. This function would certainly not maintain a prion phenotype in yeast. It is plausible that both prion/amyloid fibril splitting and prion/amyloid fibril dissociation take place in parallel, but only the prion splitting function generates propagons to maintain of the prion state. Accordingly, the maintenance of prions in yeast can be explained by a fast on-rate of amyloid formation, i.e., the growth of prion aggregates, which is, due to the high number of prion seeds, faster than the dissociation rate achieved by Hsp104. Several lines of evidence support this hypothesis: (i) Hsp104 function has been shown to be required for the generation of propagons but not for the conversion of soluble proteins into prion complexes.[109,112,144,145] (ii) Overexpression of Hsp104 in yeast partially solubilizes Sup35 aggregates.[138] (iii) A reduction of Hsp104 in vivo levels by down-regulation results in the formation of larger prion aggregates.[109] (iv) Time-lapse microscopy of yeast cells shows that Hsp104 is involved in the remodeling of prion aggregates; existing Sup35-GFP prion aggregates are dynamic structures in wild-type yeast cells but become immobile upon Hsp104 inhibition.[111] (v) The ability of Hsp104 to propagate prions is dependent on the stability of the prion aggregates; i.e., highly stable prion particles are resistant to fragmentation by Hsp104 and are inefficiently propagated. For example, amyloid fibrils generated in vitro at 4°C are not very stable and not tightly packed; however, after in vivo transfection, they are easily propagated by Hsp104, resulting in a strong [*PSI*$^+$] phenotype. In contrast, amyloid fibrils generated at 37°C are more stable and tightly packed, and are more resistant towards Hsp104-mediated fragmentation and replication, resulting in a weak [*PSI*$^+$] phenotype.[135,136] Hence, the prion phenotype directly correlates with the stability of the corresponding amyloid fibrils, affecting the replication efficiency by Hsp104. (vi) In vitro, Hsp104 has been shown to break down amyloid fibrils of Sup35 and Ure2 into smaller fragments that have the capacity of seeding amyloid formation in vitro and inducing a prion state by in vivo transfection.[19,24] Interestingly, long incubation with Hsp104 has been shown to give rise to different types of products; the Hsp104 reaction products of Ure2 fibrils are highly efficient in seeding and propagation of the [*URE3*] prion irrespective of the Hsp104 concentration, but Sup35 fibrils are inactivated by excess Hsp104, and the end product of the reaction does not efficiently function as an amyloid seed or as [*PSI*$^+$] prion.[24] Accordingly, Sup35/[*PSI*$^+$] prion particles might be more sensitive to excess Hsp104 than Ure2/[*URE3*] prion particles. This might explain the observed differences in prion curing of [*PSI*$^+$] and [*URE3*] upon Hsp104 overexpression in vivo. (vii) Finally, the addition of GdmCl inhibits Hsp104 and thus blocks the generation of prion propagons in vivo, without affecting the ability of existing prion aggregates to grow larger via recruitment of soluble prion protein. However, these aggregates are diluted out in the growing yeast culture by asymmetric cell divisions while the mother cells retain the prion aggregate.[111,146] These effects of GdmCl on prion maintenance were recently used to determine the number of [*PSI*$^+$] propagons which was shown to be 300–1300 per yeast cell, depending on the [*RNQ*$^+$] state of the cell.[140]

In conclusion, Hsp104 might exert several activities on prion proteins and their aggregates in vivo, but the prion splitting activity dominates other effects so that only the resulting prion propagation function is apparent. Hsp104-mediated fragmentation of prion/amyloid aggregates appears to correspond to an incomplete disaggregation reaction, as indicated in Figures 3B and proposed in Figures 5B and 5C. By utilizing a chimeric Hsp104/ClpB construct with the mutant ClpP^{Trap}, which is an analogous experimental setup to the work on HAP/Clp^{Trap} that provided evidence for the translocation of substrates by Hsp104,[6] Tipton et al. have shown that prion propagation in yeast is accompanied by the binding of prion proteins to the chimeric HAP/Clp^{Trap} complex (The combination of the HAP mutant with Clp^{Trap} serves to trap substrates in a locked translocation-complex with Hsp104.[6]). As this was only observed with the [*PSI*$^+$] state but not with the [*psi*$^-$] state, the presented data strongly suggest that prion protein translocation through the Hsp104 hexamer is taking place only in yeast cells containing prion particles being subjected to prion propagation. Interestingly, this complex formation of chimeric HAP/Clp^{Trap} and prion protein also requires Hsp40 (yeast Sis1) acting

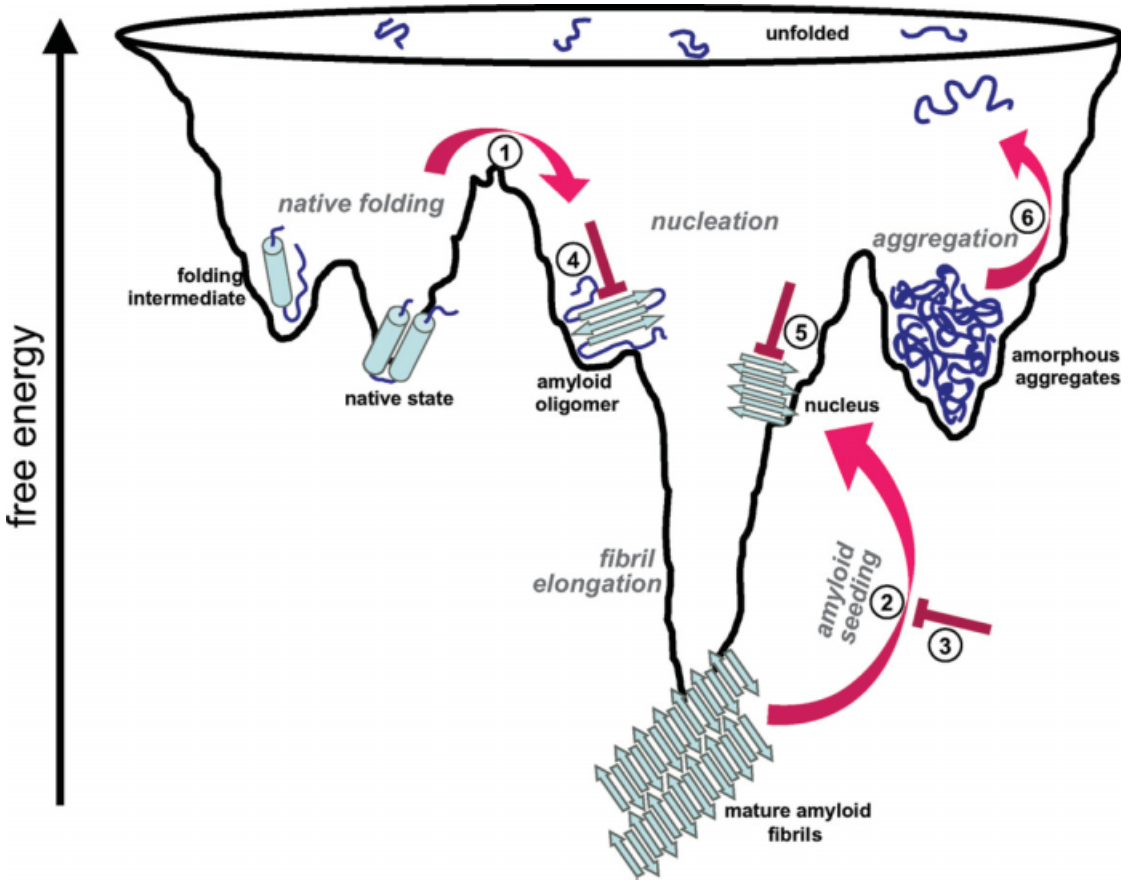

FIGURE 6 Hsp104 interacts with several species on the pathway of amyloid formation and aggregation. Hsp104, whose interaction sites indicated in red, can enhance amyloid formation by (1) mediating the transition from a native to an amyloid structure by the conformational alteration hypothesis, and by (2) generating amyloid seeds via mechanical splitting. However, depending on the type of amyloid and its intrinsic stability, Hsp104 can also inhibit fibrillization by interacting with intermediates on the pathway of amyloid formation, see (3), (4), and (5). Nevertheless, Hsp104 also possesses unfolding activity (6) that targets amorphous aggregates; this requires Hsp70/40 for efficient disaggregation and renaturation. The figure integrates the function of Hsp104 modulating protein conformations into the theory of the protein folding and amyloid formation by Jahn and Radford.[139]

upstream of Hsp104, which appears to play a role in prion particle transfer towards Hsp104.[18] Accordingly, the prion propagation function is most likely caused by a substrate threading reaction of Hsp104 resulting in a splitting of prion particles into seeds. However, the splitting model for prion propagation proposes that Hsp104 interacts with flexible ends of subunits in the middle of the fibril and, upon extraction of this single subunit, the fibrils break into smaller fragments. One way to rationalize this model is that Hsp104 interacts preferentially with extended cross-β-sheet structures found, for example, on the surface of amyloid fibrils. However, the extreme stability of amyloid fibrils in comparison with nonspecific aggregates, such as those formed as a result of heat- or chemical-induced aggregation, argues against this model. The amyloid fibril represents one of the most stable structure on the protein level and exhibits strong resistance towards denaturation or disassociation by proteases, acids, and chemical denaturants. If the ability of Hsp104 to generate fibril fragments was dependent on its ability to interact with exposed loops of the fibril subunits, then Hsp104 should neither exhibit any disaggregation activity nor promote prion propagation of amyloid forming proteins lacking exposed termini or partially folded structures in the fibrillar state. This hypothesis could be tested using amyloid fibrils lacking exposed ends or partially exposed structures or loops as substrates. Several small peptides have been shown to form cross-β amyloid fibrils of diameters equal to the length of the peptide and thus could be used to test this model.[147]

Alternatively, a brief treatment of amyloid fibrils with proteases to remove any exposed strands and loops should generate fibrils without putative Hsp104 binding sites, which would in turn be resistant to Hsp104 fragmentation.

Acceleration and Inhibition of Amyloid Formation by Hsp104

In vitro assays showed that the fibril splitting function is not the only activity of Hsp104 on amyloid proteins. Low concentrations (<0.5 μg/mL) of Hsp104 have been reported to accelerate amyloid formation in vitro in an ATP-dependent fashion,[19] which would support the templating model (Figure 5A). However, in vitro aggregation studies also have consistently demonstrated that at high Hsp104 concentrations (>10 μg/mL) and regardless of the sequence or fold of the aggregating protein, Hsp104 inhibits amyloid formation. This has been rationalized by the fact that at low concentrations, the prion/amyloid splitting function of Hsp104 dominates, whereas at high concentration its inhibitory effects override this activity, see case studies below. Recent studies with amyloid-β and α-synuclein provided new insight into how Hsp104 can block the fibrillization of amyloidogenic proteins by targeting multiple intermediates on the pathway of amyloid formation,[26,29] see Figure 5D and (3), (4), and (5) in Figure 6. Hsp104 might inhibit amyloid formation by binding to the monomeric protein and by stabilizing its non-amyloidogenic native state. However, this type of interaction would require an amyloid protein-to-Hsp104 ratio close to 1:1 in order to efficiently block amyloid formation. Alternatively, Hsp104 could bind specifically to the polymerization sites - or ends - of aggregation intermediates or fibrils, thereby blocking fibril elongation by hindering the addition of monomers (Figure 5D). This mechanism is consistent with the ability of Hsp104 to block the fibrillization of Aβ42 at substoichiometric ratios of 1000:1 in an ATP-independent manner.[26] Current studies often lack detailed data on the binding specificity of Hsp104 regarding amyloidogenic structures or peptide sequences. Thus, the mechanistic details of the Hsp104 interaction with amyloid monomers, protofibrils, or fibrillar seeds remain understood. It is not clear, whether Hsp104 recognizes a "general β-amyloid fold" on fibrillar structures or preferentially interacts with a common sequence or cluster of amino acids in amyloidogenic proteins, as shown for Sup35-derived peptides by Glover and coworkers.[9] The lack of sequence or structural homology among the amyloid forming proteins argues against the latter hypothesis. Furthermore, whether Hsp104 targeting and stabilization of aggregation intermediates occurs in vivo remains unclear. The consequences of such interactions, if present in vivo, on amyloid toxicity still need to be elucidated. Although the protective effect of Hsp104 against toxicity of amyloidogenic proteins in cellular and animal models would appear to argue against this model, it is plausible that Hsp104 binding to toxic aggregation intermediates remodels their structure and renders them nontoxic. This may occur via a structural alteration by Hsp104 that targets these intermediates towards proteolysis or towards other molecular chaperones, or by simply diverting them to some off-pathways that do not lead to amyloid formation.

Case Studies of Hsp104 Activity on Amyloid Proteins

Hsp104 exerts its specific functions on all amyloid proteins tested so far. Its substrates comprise natural endogenous substrates, such as Sup35 and Ure2 from yeast, but also exogenous substrates, such as human amyloidogenic proteins, see Table I. Since no Hsp104 ortholog was identified in humans, the studies combining Hsp104 and human proteins might be considered as artificial with no practical or physiologic relevance. However, human amyloidogenic proteins are the best understood model substrates for elucidating the functions of Hsp104, and more importantly, studies involving the function of Hsp104 on these proteins provide very valuable insight into the mechanisms of disease development, toxicity of amyloid proteins and reversion of toxicity by molecular chaperones.

[PSI$^+$]/Sup35

Incubating preformed Sup35NM fibrils in vitro with Hsp104 yields short fibril fragments that are active in seeding the Sup35NM amyloid formation in vitro. The fibril fragmentation reaction takes place within 30–60 min and requires an ATP regenerating system and fully functional Hsp104,[19] see Figure 5B. The observed fibril fragmentation activity of Hsp104 is independent from Hsp70/40, but Sup35 fibrils that were pre-assembled in the presence of Hsp70/40 are more efficiently fragmented by Hsp104.[148] However, others did not observe fibril fragmentation by Hsp104 but rather found that full length Sup35 fibrils assembled in the presence of Hsp104 possess an increased seeding capacity.[20] These differences in the Hsp104's effects might be due to variations in sample preparation and assay conditions. The amyloidogenic region of the NM domain of Sup35, Sup35,[5–8,12,29,32–34,41–53] served in NMR experiments to identify the oligomeric species of Sup35[5–8,12,29,32–34,41–53] preferentially interacting with Hsp104. These hexameric/tetrameric oligomers were found to disassemble in the presence of Hsp104.[21,22] Beyond the fibril remodeling activity, Hsp104 has been shown to accelerate Sup35 amyloid fibril formation when added at

low concentrations (~0.17–0.5 μg/mL) to an on-going fibrillization experiment.[19,20,24,149] At low Hsp104 concentrations and in the presence of ATP, the fibrillization of Sup35NM and full length Sup35 was enhanced. The nucleation phase was dramatically shortened[19,24] and/or the final Thioflavin T (ThT) fluorescence values were higher than those of the control samples.[20] This enhancement of fibrillization supports the model of conformational alteration by Hsp104 for prion formation, see Figure 5A. Alternatively, it has been suggested that Hsp104 itself offers a catalytic surface for molten prion domains to attain an amyloid nucleating state.[24] However, at high concentrations (>2.9 μg/mL), Hsp104 inhibits amyloid formation.[19–21,24] Thus, the functions of Hsp104, fibril breakage and acceleration or inhibition of amyloid formation, depend on its concentration. Accordingly, Hsp104 appears to have the capacity to exert multiple effects on the formation and amplification of Sup35 amyloid structures.

[URE3]/Ure2

Analogous to Sup35, it has been demonstrated that Hsp104 also catalyzes the fragmentation of mature Ure2 fibrils into smaller fragments, which are active in the seeding of amyloid formation in vitro and also active as prion in vivo.[24] Melki and coworkers have shown that Hsp104 converts mature Ure2 fibrils into species that are less resistant to sodium dodecyl sulfate (SDS) treatment and that are not retained in cellulose acetate filters with 0.20-μm pore size, unlike the fibrillar Ure2 starting material.[25] Furthermore, Hsp104 also accelerates Ure2 amyloid fibril formation in vitro when added at low concentrations[24,25] and inhibits Ure2 amyloid formation at high concentrations, similar to its effect on Sup35.[24]

Alzheimer's Disease/Aβ42

A fusion of human amyloid-β (Aβ42) peptide with the C-terminal domain of Sup35 (MRF) has been used to develop a yeast based reporter system of Alzheimer's disease.[40] The expression of the Aβ42-MRF fusion results in a [PSI^+]-like phenotype and in the formation of SDS stable oligomers of Aβ42-MRF. The phenotype and the formation of oligomers were influenced by endogenous Hsp104 in yeast. Deletion of the *HSP104* gene decreases the amount of Aβ42-MRF oligomers and slightly suppresses the [PSI^+]-like phenotype. Hsp104 also coimmunoprecipitated with Aβ42-MRF indicating an interaction.[40] Interestingly, the size and structural properties of preformed amyloid fibrils or oligomers from human Aβ42 peptide are not influenced by Hsp104 in vitro, even at higher concentrations.[26] However, Hsp104 inhibits the fibrillization of monomeric and protofibrillar forms of Aβ42 in a concentration-dependent, but ATP-independent, manner. Similar to the findings for Sup35 and Ure2, Hsp104 delays the lag phase of amyloid formation. However, Aβ42 seems to be more sensitive to Hsp104 and a significant inhibition can be observed at Hsp104 concentrations as low as 1 μg/mL, at a molar ratio of 1000 to 1, suggesting a preferential interaction of Hsp104 with aggregation intermediates (e.g., oligomers, protofibrils, small fibrils). However, Hsp104 was found to associate with monomers but also with protofibrils of Aβ42. Since Hsp104 shows an inhibitory effect at all stages of Aβ42 aggregation, it might therefore target multiple intermediates on the pathway to amyloid formation,[26] see Figure 5D.

Parkinson's Disease/α-Synuclein

In vivo, overexpression of α-synuclein in yeast leads to the formation of inclusions and causes toxicity.[39] Yeast contains endogenous Hsp104, interestingly, overexpression of Hsp104 does not show any significant effect on α-synuclein aggregation and toxicity.[63] In the mammalian system, the lentiviral coexpression of Hsp104 and α-synuclein in the rat *substantia nigra* results in a significant reduction of α-synuclein aggregation and in a protection against α-synuclein-induced loss of dopamingergic neurons in a concentration-dependent manner.[29] In vitro, Hsp104 partially disassembles α-synuclein fibrils in an ATP-dependent reaction.[29] Moreover, it has also been reported to possess the capacity to remodel and disassemble the oligomeric and protofibrillar forms of α-synuclein. Unlike the observations for yeast prion proteins, Hsp104 does not accelerate α-synuclein amyloid formation but inhibits its aggregation and fibril formation at high concentrations of >10 μg/mL Hsp104.[29]

Huntington's disease/PolyQ

Hsp104 has been shown to be required for the aggregation of polyglutamine (polyQ), or of huntingtin with specific polyQ repeat, in a yeast model system of Huntington's disease.[27] However, this property is dependent on the length of the polyQ repeats. Only long constructs >70Q form aggregates in yeast,[27,150] and the aggregation of longer polyQ constructs is the less dependent on Hsp104.[35,151] Beyond aggregation, Meriin et al. reported that polyQ toxicity in yeast is dependent on Hsp104.[150] Both the deletion of *HSP104* and the expression of the dominant negative K218T/K620T double mutant reduce polyQ toxicity.[36] Overexpression of Hsp104 increases the number of small fluorescent foci in yeast, i.e., the number of visible aggregates of polyQ.[27] Other groups have observed a resolubilization of polyQ aggregates and a

reduced polyQ toxicity upon overexpression of Hsp104 alone[152] or in conjunction with sHsps.[63] Interestingly, polyQ aggregation and toxicity depend on the presence of Rnq1 aggregates, i.e., the [RNQ^+] prion state.[36,37,150] Even the protective effect of Hsp104 overexpression against polyQ toxicity was found to be dependent on the actual prion state, [PSI^+] and/or [RNQ^+].[36] These data indicate that Hsp104 indeed converts amyloid structures into self-propagating prions, as proposed by the prion splitting model, see Figure 5B; however, the huntingtin/polyQ model system also requires an existing yeast prion state in the cell in order to be maintained as amyloid aggregate, demonstrating that existing prion aggregates from another protein species may serve as a template for amyloid formation of polyQ.

In animal and cellular models of Huntington's disease, Hsp104 protects against polyQ toxicity and reduces its aggregation. Expression of Hsp104 in *C. elegans* was shown to suppress the formation of GFP-polyQ inclusions and to reverse the retardation in growth rates induced by polyQ aggregation.[32] The expression of Hsp104 in mammalian cells,[43] in the brain of a transgenic mouse model of Huntington's disease employing a MoPrP promoter for specific expression in brain,[33] and in striata of the brain of rats after lentiviral injection[34] protects against polyQ toxicity and reduces the number of polyQ inclusions.

Transmissible Spongiform Encephalopathies/Prion Protein

Hsp104 has been shown to promote the conversion of mammalian prion protein PrPc (cellular, soluble) into PrPres (protease resistant). However, this process was shown to be independent from ATP or Hsp104-ATPase-mutations.[38] In a transmissible spongiform encephalopathy (TSE) mouse model, Hsp104 showed no enhancement or protective effects. Hsp104 was expressed in the brain, the spinal cord, and the cerebellum using a neuron-specific promoter. After infection with prions by injection of brain homogenate from ME7 mice, the recipient mice developed TSE symptoms independent from Hsp104 expression.[153] Accordingly, these experiments suggest that Hsp104 does not cure or protect the animals from mouse prion disease.

CONCLUSIONS

It had previously been suggested that Hsp104 recognizes common structural features in the aggregated state of amyloid forming proteins. More recent evidences allow a more differentiated understanding. Experimental data on the different substrates demonstrate that the ability of Hsp104 to catalyze or inhibit prion and amyloid formation appears to be strongly dependent on the individual stability and structural properties of the aggregates as well as on the primary sequence and structure of the native amyloidogenic protein. The degrees to which Hsp104 can dissociate and reverse protein aggregation are furthermore dependent on the Hsp104 concentration, on the presence of ATP, and may require the active cooperation of other chaperones. In vitro Hsp104 appears to exert different functions on amyloid proteins and their structures, some of which can be attributed to the classical disaggregation activity and may involve a substrate threading mechanism. Other activities, such as the inhibition of amyloid formation, require only Hsp104 but neither ATP nor cochaperones. In vivo Hsp104 appears to mostly fulfill a function in protein disaggregation and prion/amyloid fragmentation, which results in aggregate clearance and prion propagation, respectively. Therefore, the interplay between these different mechanisms (enhancement and suppression of aggregation, disaggregation, and amyloid/prion fragmentation) must be considered in future studies aimed at dissecting the molecular mechanisms underlying Hsp104 activities both in vivo and in vitro.

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