



# The elusive middle domain of Hsp104 and ClpB: Location and function<sup>☆</sup>

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## ABSTRACT

Hsp104 in yeast and ClpB in bacteria are homologous, hexameric AAA+ proteins and Hsp100 chaperones, which function in the stress response as ring-translocases that drive protein disaggregation and reactivation. Both Hsp104 and ClpB contain a distinctive coiled-coil middle domain (MD) inserted in the first AAA+ domain, which distinguishes them from other AAA+ proteins and Hsp100 family members. Here, we focus on recent developments concerning the location and function of the MD in these hexameric molecular machines, which remains an outstanding question. While the atomic structure of the hexameric assembly of Hsp104 and ClpB remains uncertain, recent advances have illuminated that the MD is critical for the intrinsic disaggregase activity of the hexamer and mediates key functional interactions with the Hsp70 chaperone system (Hsp70 and Hsp40) that empower protein disaggregation. This article is part of a Special Issue entitled: AAA ATPases: structure and function.

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## 1. Introduction

Hsp104 and ClpB are homologous protein disaggregases, which are classified in the Hsp100 family of proteins [1–9]. This family, in turn, is a member of the AAA+ (ATPases Associated with various cellular Activities) super-family [10–12]. Hsp104, which is found in *Saccharomyces cerevisiae*, has two main functions. First, in collaboration with Hsp70 and Hsp40, Hsp104 confers thermo- and chemical-tolerance to yeast by resolubilizing stress-induced protein aggregates and restoring proteins to native structure and function (Fig. 1a) [13–18]. These aggregates are typically disordered or amorphous in structure [19,20]. Additionally, Hsp104 can directly remodel amyloid and this activity governs prion inheritance in yeast (Fig. 1b) [21–33]. Prions, which are proteins that adopt an infectious amyloid fold, are structurally distinct from disordered aggregates in that they form ordered assemblies with a characteristic ‘cross-β’ structure [20,34–39]. Unlike their mammalian counterparts, yeast prions can confer selective advantages, which are only made possible by the Hsp104-catalyzed remodeling activities that facilitate stable prion inheritance through successive generations [22,27,40–42]. Curiously, Hsp104 is absent from metazoan lineages [43]. Thus, it has been suggested that the ability of Hsp104 to remodel amyloid conformers as well as toxic preamyloid oligomers might even be harnessed, engineered and potentiated for therapeutics against numerous

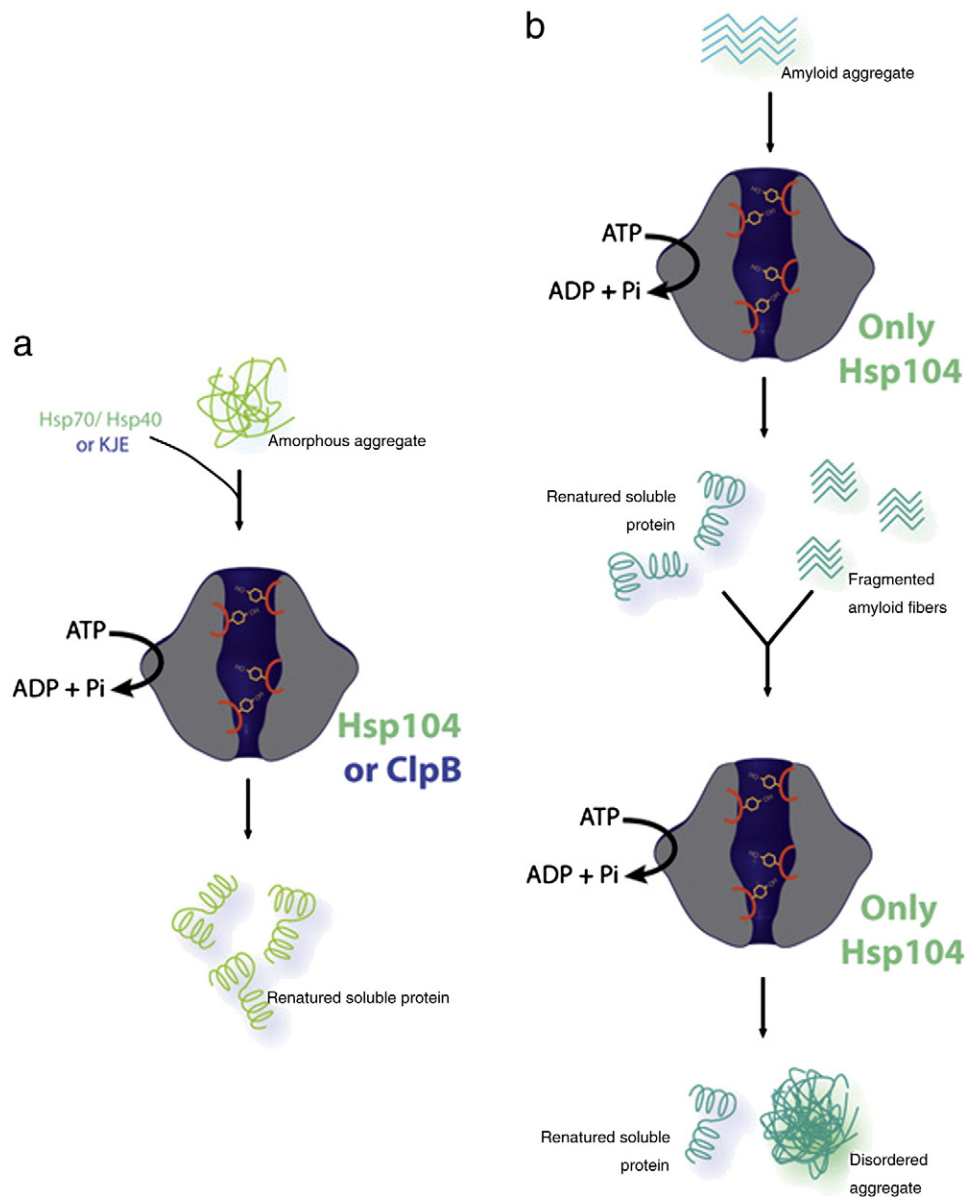
neurodegenerative amyloidoses [6,39,43–45]. Despite sharing over 50% identity with Hsp104, the bacterial protein ClpB does not possess the same dual functionality as Hsp104. Like Hsp104, ClpB is able to disaggregate amorphous substrates in response to environmental stresses that induce widespread protein aggregation [46–50]. However, unlike Hsp104, ClpB appears to be ineffective at remodeling amyloid conformers [21,51,52].

Significant efforts have been made toward gaining a structural understanding of Hsp104 and ClpB. Like many AAA+ proteins, Hsp104 and ClpB are functional as ring-shaped hexamers [1,53,54], which are thought to drive protein disaggregation by directly translocating substrates through their central channel (Fig. 1a, b) [55–60]. However, there is still no general consensus about the gross domain organization within these hexameric molecular machines [53,61–66]. Thus, how the hexamer couples conformational change to generate the mechanical force necessary to drive protein disaggregation continues to remain uncertain. A point of particular contention is the location and orientation of the unique coiled-coil insertion, termed the Middle Domain (MD), within the hexameric assembly. Despite a lack of detailed structural information, a number of studies have recently revealed key mechanistic insights concerning how ClpB and Hsp104 functionally interact with their respective Hsp70 chaperone system (Hsp70 and Hsp40), which is also activated during the stress response [18,67–70]. Here, the elusive MD plays a critical role that mediates the functional interaction between Hsp104/ClpB and the Hsp70 chaperone system [67–69]. In this review, we will first outline the debate concerning the quaternary structural organization of Hsp104 and ClpB and then explore the implications that the MD mediates functional interactions with Hsp70.

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**Fig. 1.** In vitro disaggregase activity of Hsp104 and ClpB. (a) Hsp104 remodels amorphous aggregates via collaboration with Hsp70 and Hsp40, while ClpB remodels these types of aggregates via collaboration with DnaK, DnaJ, and GrpE (KJE). The product of disaggregation of amorphous aggregates is natively folded protein. Hsp104 or ClpB hexamer is shown in gray with the front half cutaway to reveal the axial channel running down the length of the structure. Four pore loops with conserved tyrosine residues are shown in orange. These pore loops are important for substrate binding and threading through the axial channel. (b) Only Hsp104 is able to remodel amyloid aggregates and in vitro this can proceed without the aid of Hsp70 and Hsp40. Products of amyloid disaggregation are soluble natively folded protein and fragmented amyloids. However, for some amyloids (e.g. Sup35 prions) remodeling can continue to generate disordered-type aggregates that Hsp104 cannot remodel and which lack the seeding activity of amyloid [23].

## 2. Two structural models of Hsp104 and ClpB hexamers: whither the middle domain?

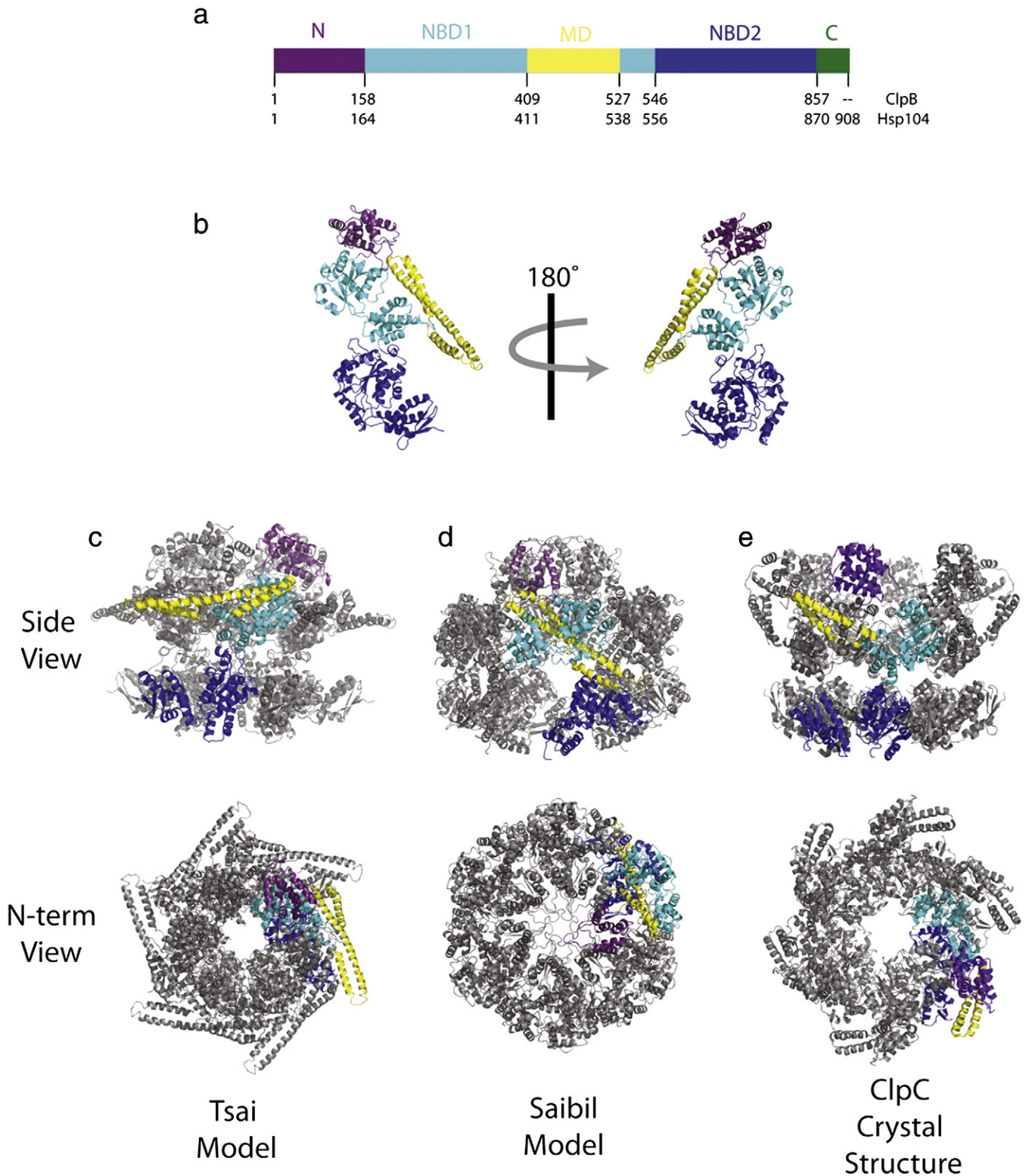
Each Hsp104 or ClpB monomer contains an N-terminal domain, two AAA+ nucleotide-binding domains (NBD1 and NBD2), and a predicted coiled-coil middle domain (MD) that is inserted toward the C-terminal end of NBD1 (Fig. 2a) [1,2,71–73]. Additionally, Hsp104 contains a short C-terminal extension, absent from ClpB, which is required for Hsp104 hexamerization [74]. The tertiary structure of the individual domains was largely resolved in 2003 when a 3.0 Å crystal structure of ClpB (TClpB) from the thermophilic eubacterium, *Thermus thermophilus*, was solved by Tsai and colleagues (Fig. 2b) [63]. These studies revealed that both NBDs adopt a canonical AAA+ fold [63]. The structure of NBD1 agreed with a previously solved structure of this isolated domain [75]. The N-terminal domain, which is the least conserved domain in the disaggregase family, was also

structurally similar to the isolated *Escherichia coli* ClpB N-terminal domain [76]. The MD was revealed as a broken anti-parallel coiled-coil (Figs. 2b, 3b) [63]. For the purpose of this review, we have provided a unified nomenclature to describe regions of the MD in ClpB, TClpB, and Hsp104 (Fig. 3a, b). The MD is less well conserved than NBD1 or NBD2, with Hsp104 and ClpB sharing only ~36% identity. Unfortunately, full-length TClpB did not crystallize in its functional hexameric structure, but rather in a trimeric spiral with protomer–protomer interactions that might, at least in part, reflect crystal contacts rather than native protomer interfaces. In a dynamic, multi-domain molecular machine like Hsp104/ClpB, understanding the relative domain positioning and protomer organization is absolutely critical for understanding the mechanism of action.

The overall quaternary structure of Hsp104 and ClpB has been investigated primarily by Cryo Electron Microscopy (Cryo-EM), which has led to recent debate. It is generally agreed that Hsp104 and ClpB

are native hexamers and that oligomerization is promoted by increased protein concentration [53,77–79], low salt [77,80], and the presence of ADP or ATP [53,54,79–81]. Curiously, however, while

nucleotide binding to NBD1 is critical for ClpB hexamerization [80], this situation is reversed in Hsp104 where nucleotide binding to NBD2 is key [53,78,79]. The underlying reason for this switch between NBDs



**Fig. 2.** Predicted structures and hexameric models of Hsp104 and ClpB. (a) Domain organization of one monomer of Hsp104 and ClpB. N-terminal domain (N) shown in purple, Nucleotide binding domain 1 (NBD1) shown in cyan, Middle domain (MD) in yellow, Nucleotide binding domain 2 (NBD2) in dark blue. Only Hsp104 has the short C-terminal extension (C) shown in green. Sequence numbering for ClpB is shown on top and for Hsp104 is shown on the bottom. (b) TClpB crystal structure. Domain coloring corresponds with part (a). A 180° rotation about the vertical axis is shown on the right. (c) The Tsai model for the hexameric quaternary structure of TClpB. The Tsai model, which was initially based on Cryo-EM envelopes generated with TClpB is shown on left. (d) The Saibil model, which used Hsp104 to generate Cryo-EM density, is shown in the middle. (e) The 6.93 Å crystal structure of hexameric, full-length ClpC is shown on the right. The adaptor protein MecA was omitted for clarity. A side view is shown on top and a view down the axial channel from the N-terminus is shown on the bottom. One subunit is colored as described in part (a). The other five subunits are in gray.

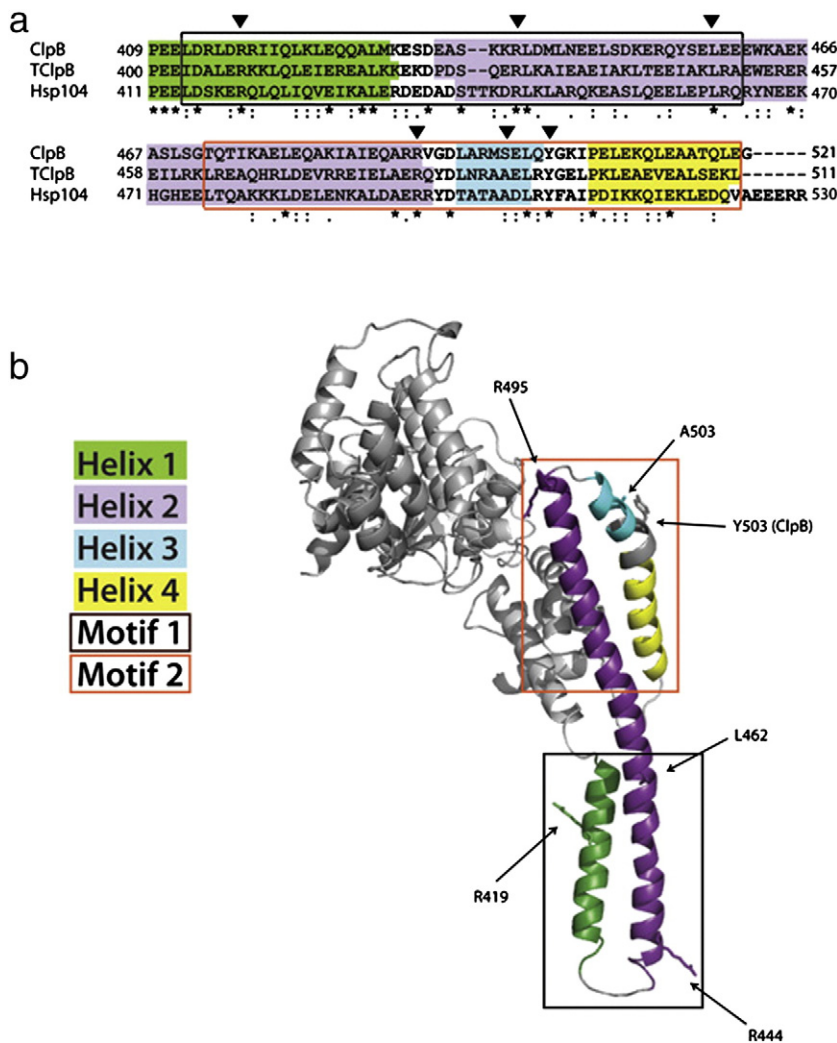


is unknown and continues to remain puzzling and unaddressed. NBD1 contributes the majority of basal ATPase activity in Hsp104 [53,78,79], whereas both NBDs contribute to basal ATPase activity in ClpB [80]. The ATPase activities of both NBDs are required for the full repertoire of protein-remodeling activities and are modulated by allosteric communication within and between NBD1 and NBD2 [73,77,79,80,82–84]. Gross domain position and the protomer-protomer interfacial packing of Hsp104 and ClpB hexamers still remain uncertain. Of particular interest is the position of the coiled-coil MD, which is necessary for disaggregase activity and is unique to the Hsp100 chaperones that function primarily in disaggregation [63,80,85]. In the TClpB crystal structure, this domain was jutting obliquely from the axis of the other domains [63] (Fig. 2b). Thus, in the original Cryo-EM reconstructions of TClpB in the presence of AMP-PNP (a non-hydrolyzable ATP analog) the MD was assigned to protrusions that appeared to emanate from one tier of the hexamer [63] (Fig. 2c, Tsai model).

In subsequent studies, to determine any conformational reorganizations that take place through the ATPase cycle, Cryo-EM envelopes of TClpB in the ADP and apo state were reconstructed as well as the envelope of the Double Walker B TClpB mutant (E271A:E668A) in the presence of ATP [61]. This mutant binds but does not hydrolyze ATP at both NBDs and has increased affinity for substrate [61,86]. In all states, a two-tiered hexamer with an axial channel running through the

center was clearly visible [61]. However, the N-terminal domain was not visible as electron density [61]. In the AMP-PNP-bound state, the TClpB envelope shows clear, well defined protrusions on the outside of the hexamer, which, when the individual domains of the TClpB monomeric crystal structure were fitted as rigid bodies into the density, overlapped partially with predicted MD density [61,63]. It was suggested that the exterior position of the MD might enable it to act as a ‘crowbar’ to pry apart large aggregates [63]. Glover and Lindquist had originally suggested that Hsp104 might possess a ‘crowbar activity’, but did not ascribe this activity to any particular domain [16]. All the other nucleotide states of TClpB (ATP, ADP, and apo) do not have such large protrusions of density that could correlate with a MD projection. It was suggested that this might be due to the inherent mobility of the coiled-coil MD [61]. Indeed, the main difference between the different nucleotide states was the length of these radially extending protrusions [61]. By contrast, the positions of the AAA+ domains remained almost identical in the various nucleotide states [61]. Consequently, these reconstructions do not readily clarify the mechanochemical coupling events that drive substrate translocation through the central channel.

This structural model of the hexamer was challenged by Cryo-EM reconstructions of Hsp104 by Saibil and colleagues [64–66]. In these studies, Cryo-EM reconstructions were generated of Hsp104 lacking its N-terminal domain,  $\Delta$ N-Hsp104, and an NBD2 sensor-1 Hsp104



**Fig. 3.** Middle domain nomenclature. (a) An alignment of the MD from *E. coli* ClpB, *T. thermophilus* ClpB, and *S. cerevisiae* Hsp104. Helix 1 is colored in green, helix 2 is colored in purple, helix 3 is colored in light blue, and helix four is colored in yellow. Motif 1 (also called wing 2) is boxed in black while motif 2 (also called wing 1) is boxed in orange. Arrowheads denote key residues discussed in the text. (b) Close up of the MD in the TClpB crystal structure. Each helix and motif is colored as indicated in part (a). NBD2 is omitted for clarity. Arrows point to side chains (shown as sticks) of key residues discussed in the text.

mutant, Hsp104<sup>N728A</sup>, which has slowed hydrolysis in NBD2 and is able to catalyze disaggregation of disordered aggregates in the absence of Hsp70 and Hsp40, but is unable to remodel amyloid [77,82]. Contrary to the TClpB reconstruction, Hsp104<sup>N728A</sup> had a structured N-terminal domain and presented as a three-tiered hexamer with no oblique MD protrusion [64–66]. The central cavity of Hsp104<sup>N728A</sup> was also much larger than that observed with TClpB and the modeled protomer–protomer packing was unlike that of typical AAA+ structures [64–66]. It was hypothesized that the enlarged cavity might serve as an adaptation necessary to remodel large aggregated structures [64–66]. A TClpB-homology model of Hsp104 was fitted as a rigid body into the electron density with each domain connected by a flexible linker (Fig. 2d, Saibil model) [64–66]. The resulting fits placed the MD intercalated within NBD1 and NBD2, rather than projecting out into solution (Fig. 2d) [64–66]. This physical proximity of the MD to both NBDs, which is also partially supported by fluorescence proximity studies of ClpB [87], might help explain how the MD mediates communication pathways between NBD1 and NBD2 [73].

The cryo-EM reconstructions of Hsp104<sup>N728A</sup> with ATP $\gamma$ S (a slowly hydrolysable ATP analog), ATP and ADP were also generated [65,66]. Hsp104<sup>N728A</sup> displayed large nucleotide-dependent domain reorganizations, with the ATP $\gamma$ S state having the most expanded central cavity [65,66]. These domain movements displaced the substrate-binding tyrosine loops in the central channel and triggered a peristaltic motion that provides a clear structural basis for N- to C-terminal substrate translocation [65,66]. Asymmetric reconstructions of Hsp104<sup>N728A</sup> with ATP $\gamma$ S and ATP provided unprecedented insight into disaggregase activity [65,66]. These reconstructions suggested a sequential mechanism of ATP hydrolysis in NBD1 coupled to clockwise handover of substrate in the NBD1 ring and a coordinated handover between NBD1 and NBD2 [65,66]. Thus, the first detailed structural picture of the mechanochemistry that underpins protein disaggregation emerged [4,65,66].

It remains unclear why the Cryo-EM reconstructions of TClpB and Hsp104<sup>N728A</sup> would be so disparate. Could the differences simply reflect a fundamental difference in hexamer architecture for the prokaryotic and eukaryotic enzymes? Or could the different reconstructions reflect the different protein preparations for the Cryo-EM? For example, glutaraldehyde fixation was employed for the TClpB studies [61,63], whereas fixative was omitted for the Hsp104 studies [64,65]. The story takes another twist when Tsai and colleagues presented Cryo-EM reconstructions of a double Walker B Hsp104 mutant (E285A:E687A) in the presence of ATP and  $\Delta$ N-Hsp104 in the presence of ATP $\gamma$ S with and without glutaraldehyde fixation [62]. Both sets of envelopes appeared very similar, indicating that fixation might not be an issue [62]. Intriguingly, and in contrast to their prior TClpB reconstructions, there was a striking absence of additional mass density on the outside of the  $\Delta$ N-Hsp104 or Hsp104<sup>E285A:E687A</sup> hexamer [62]. Indeed, the Cryo-EM envelopes of the different Hsp104 variants constructed by the different groups are remarkably similar, particularly for  $\Delta$ N-Hsp104 plus ATP $\gamma$ S (especially when either reconstruction is subjected to a 180° vertical rotation) [62,64,65]. This similarity among Hsp104 envelopes might even suggest that TClpB hexamers possess a subtly distinct architecture. After all, Hsp104 is equipped to remodel amyloid, whereas ClpB is not [21,51,52], so perhaps subtle structural differences might be anticipated. However, despite the absence of lateral projections emanating from the Hsp104 hexamer, the authors placed the MD outside of the hexamer walls in their atomic structure fitting as with TClpB [62]. Thus, it is still unclear whether the MD of Hsp104 and ClpB is located inside or outside of the hexamer. It should also be noted that no reconstructions have been presented with full-length wild-type Hsp104.

In an effort to visualize the MD during Cryo-EM, an Hsp104 chimera was generated with T4 lysozyme inserted into the MD [62].

Because the lysozyme was visible as density on the outside of the hexamer, it was suggested that the MD must also be located on the exterior [62]. However, density corresponding to the MD itself on the outside of the hexamer could still not be readily visualized even with this artificial construct [62]. It is extremely probable that this large-scale (19 kDa) insertion disrupts the native quaternary structure of Hsp104, especially since the insertion was located in a helical region of the MD (between residues N467 and E468 of helix 2 (Fig. 3a, b)) and not in a predicted loop region. Indeed, although this chimera possesses some disaggregation activity against non-native substrates *in vitro*, it should be noted that its ATPase activity was elevated, hexamerization was perturbed, disaggregase activity was dysregulated and the functionality was not assessed *in vivo* [62,69]. Thus, it is not clear whether the two models can really be distinguished with this insertion variant despite its partial functionality in some settings. Rather, the ability of the Hsp104 to accommodate such a large insertion and retain some activity indicates an extraordinary plasticity of the hexamer.

Additionally, the structure of Hsp104 and ClpB has been investigated using biochemical techniques other than Cryo-EM. Engineered disulfide cross-links in ClpB that covalently link NBD1 to motif 2 of the MD (Fig. 3a, b) have shown that these two domains are closely associated and that movement of the MD is crucial for disaggregation [63,67]. Unfortunately, these cross-links do not differentiate between the two models because the MD is closely associated with NBD1 in both proposed structures [61–66]. Importantly, the MD is inaccessible to three monoclonal antibodies that recognize native MD epitopes [73] in Hsp104 hexamers, but is exposed in Hsp104 monomers [65]. These data suggest that the MD becomes shielded upon hexamerization but remains accessible and solvated when Hsp104 is monomeric [64–66]. This observation might explain why monomeric TClpB crystallized with the MD jutting away from the axis of the NBDs. However, in contrast to these results, Lee et al. inserted a short StrepII tag (WSHPQFEK) into the MD (between residues N467 and E468 of helix 2 (Fig. 3a, b)) of Hsp104 and employed dot blots to determine whether the tag was exposed in monomeric and hexameric StrepII-tagged Hsp104 [62]. These studies suggested that the StrepII tag was accessible in monomeric and hexameric forms of Hsp104 [62]. Unfortunately, however, it is uncertain whether this epitope tag is truly innocuous or partially disruptive. Moreover, these data are difficult to assess because it is not clear whether monomeric and hexameric Hsp104 were spotted on the same dot blot [62]. Thus, one cannot be certain from the presented data whether the signal intensities for monomeric and hexameric conformers are directly comparable [62].

Another major difference between the hexameric models is the positioning of the putative arginine fingers [61–66]. Typically, an arginine finger is an arginine residue that coordinates the  $\gamma$ -phosphate of a bound nucleotide and is a recurring characteristic of AAA+ family members [10]. In many AAA+ proteins the arginine residue is located distal to the nucleotide-binding site and is provided by the adjacent subunit of the oligomer [10,88,89]. Arginine fingers contribute to ATP hydrolysis through stabilization of the transition state [10]. When the MD is positioned on the exterior of the hexamer the arginine-finger residues are positioned in a canonical position reaching into the nucleotide-binding site of an adjacent protomer [10,61–63]. By contrast, when the MD is intercalated between NBDs the arginine fingers are in a non-canonical position [64,65]. However, it should be noted that the arginine finger is not conserved in all members of the AAA+ family, and it is not clear if the position of this motif in the oligomeric structure is stringently conserved among all the different clades of the family. For example, in some crystal structures, the predicted arginine finger of HslU, an Hsp100 and AAA+ family member with only one NBD, is approximately 8 Å from the nucleotide-binding site [90]. In this regard, it is interesting to note that the missense mutation, R444M, in the distal loop region between helices

1 and 2 in the MD (Fig. 3a,b) reduces ATPase activity of Hsp104 and impairs thermotolerance in a dominant-negative manner and disrupts amyloid remodeling functionality [64]. This deficiency may suggest a close contact between the distal loop of the MD and the NBD2 [64]. Thus, it is possible that other conserved arginines might fulfill the role of the arginine finger in Hsp104.

Finally, a crystal structure of another Hsp100 family member from *Bacillus subtilis*, ClpC, was recently solved and has weighed in on the debate [91] (Fig. 2e). Like ClpB and Hsp104, ClpC is an AAA+ disaggregase involved in modulation of stress response [92] and protein quality control [93]. Typically, ClpC passes disaggregated substrates to the chambered protease, ClpP, for degradation [91–94]. While ClpC is natively a hexamer, it requires an adaptor protein, MecA, to oligomerize and form an active enzyme [95,96]. By employing a variant with four loop deletions and two ATPase-obliterating mutations, a 6.93 Å crystal structure of hexameric ClpC in complex with the adaptor protein MecA was obtained [91]. Interestingly, the packing within the NBD1 ring corresponded well with that proposed for TClpB [61,63,91] (Fig. 4). Additionally, the MD of ClpC, which is approximately half the length of the MD of ClpB [96], was also jutting out and away from the NBDs and was distinctly located on the outside of the hexamer [91]. These data provide independent support for the TClpB hexameric model. However, the coiled-coil domain of ClpC is considerably shorter than that of Hsp104 or ClpB and must interact with MecA so that ClpC can hexamerize [91]. Thus, the MD of ClpC plays a very different role to the MD of ClpB or Hsp104 where it is dispensable for hexamerization [69,80].

In closing this section, we suggest that further biochemical characterization and alternative techniques are urgently needed to discern which hexameric model is correct or whether a revised model is required for Hsp104 and ClpB. It is probable that both models are partially correct because Hsp104 and ClpB are large, oligomeric machines that possess significant domain plasticity [62,63,69]. Potentially, the MD might be able to occupy both structural conformations depending on the stage of the disaggregase cycle. Two factors are likely to have greatly increased the difficulty in obtaining high-resolution structural information on the Hsp104 or ClpB hexamer. First, the hexamer is highly dynamic and monomers are exchanged on the minute timescale [97,98]. Second, individual subunits or domains likely adopt different conformations within the hexamer [65,66]. Future studies employing a full gamut of complementary techniques will be essential to refine our understanding of the hexameric structure of Hsp104 and ClpB.

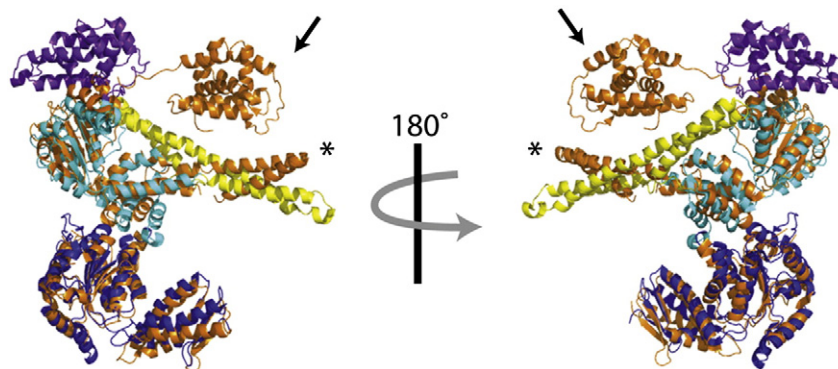
### 3. The function of the MD

Despite the ambiguity in MD location, several studies have probed MD function. For instance, in ClpB, deletion of the MD causes a loss of

thermotolerance function [80]. Furthermore, partial truncations of the ClpB MD cause decreased ATPase activity, hexamerization defects, and impaired disaggregation [99]. Even point mutations in the MD have been implicated in altered ATPase activity, loss of thermotolerance activity, and/or destabilization of the hexamer [64,67,85,100]. The dynamic mobility of the MD also appears critical for Hsp104/ClpB function, as crosslinks that hinder MD flexibility reduce or ablate disaggregation activity of ClpB [63,67]. Additionally, the MD appears to be involved in facilitating NBD1 and NBD2 communication [73]: when motif 2 is covalently attached to NBD1, ATPase activity in NBD2 increases by ~30-fold [87]. Clearly, the structural integrity and relative mobility of the MD must be maintained for full disaggregase functionality.

At first glance, the MD appears to differentiate Hsp100 proteins that possess disaggregase activity from other Hsp100s. The disaggregases: ClpB and ClpC [98,101,102] in bacteria, Hsp104 and Hsp78 [103–106] in yeast, as well as Hsp101 [107–110] in plants, all contain a coiled-coil insertion toward the C-terminal end of NBD1. ClpE is another bacterial homologue that contains a coiled-coil insertion [111,112]. Genetic studies have implicated ClpE as part of the cellular disaggregation machinery, but this has yet to be demonstrated with pure protein biochemistry [113]. While there are several examples of Hsp100 proteins that do not contain a MD and also do not possess disaggregase activity [114], the correlation between MD and disaggregase function is not quite so straightforward. For instance, ClpV, another bacterial Hsp100 protein, also contains a predicted MD insertion between its two AAA+ domains but does not possess any disaggregation activity against substrates *in vivo* [115]. Additionally, ClpA, another Hsp100 and AAA+ protein with two NBDs per monomer found in *E. coli* (but not in yeast), displays disaggregase activity against disordered aggregates but does not harbor a coiled-coil MD [82,116]. Indeed, in collaboration with its adaptor protein, ClpS, ClpA disaggregates substrates and delivers them to ClpP for degradation [116]. ClpA disaggregates substrates without any need for the Hsp70 chaperone system [114,116], indicating that Hsp70 is not absolutely required for disaggregation *per se*. Importantly, however, the Hsp70 chaperone system inhibits protein disaggregation by ClpA [116]. In this way, DnaK prevents degradation of aggregated substrates by ClpAP in *E. coli* and permits their disaggregation and reactivation by ClpB [116]. Since the MD is a major distinguishing feature of ClpB compared to ClpA, these studies provided the first hint that the MD might contribute to collaboration with Hsp70.

Hsp104 and ClpB require collaboration with Hsp70 and Hsp40 chaperones for successful disaggregation of amorphous aggregates, both *in vitro* and *in vivo* [13,16,47,49,117,118]. Hsp70 and Hsp40 can also ameliorate the amyloid-remodeling activity of Hsp104 [24,28,44,51,119]. In yeast, members of the Hsp70 chaperone family (e.g. Ssa1, Ssb1) and Hsp40 family (e.g. Ydj1 and Sis1) have been



**Fig. 4.** Overlay of ClpB and ClpC monomers. ClpB and ClpC monomers were aligned by their AAA+ domains using Pymol. The resulting RMSD was 3.4. Domains for ClpB are colored as in Fig. 2 with the N domain in purple, NBD1 in cyan, MD in yellow, and NBD2 in dark blue. All domains in ClpC are colored in orange. Note the drastically different position of the ClpB N-terminal domain (marked with an arrow) and the different angle of the ClpC MD (marked with an asterisk).



identified as being able to collaborate with Hsp104 [16,24]. In bacteria, the homologous DnaK and DnaJ (along with the nucleotide exchange factor GrpE) (KJE) collaborate with ClpB [46,48,49]. The exact mechanisms by which Hsp70 and Hsp40 collaborate with Hsp104 are largely unresolved. However, two key functions are commonly ascribed. First, Hsp70 is proposed to act upstream of Hsp104 or ClpB by interacting with the aggregate and shuttling substrate into the axial channel of Hsp104 or ClpB (Fig. 1a) [16,58,67,98,120–122]. Second, Hsp70 is proposed to act downstream of Hsp104 and promotes refolding of unfolded polypeptides once they emerge from the axial channel [16,49,82,86].

The Hsp70 chaperone system, however, may fulfill a third role by modulating the ATPase activity of Hsp104/ClpB in a manner that promotes the successful disaggregation of disordered aggregates [2,82,114]. Indeed, mixing of ClpB with KJE results in a synergistic increase in global ATPase activity [114]. Moreover, the requirement for Hsp70 to disaggregate certain disordered aggregates can be bypassed altogether by addition of specific mixtures of ATP:ATPγS (3:1 for Hsp104 and 1:1 for ClpB), or by introducing specific Walker A (e.g. K620T in Hsp104), Walker B (e.g. E279Q or E678Q in ClpB) or sensor-1 (e.g. N728A in Hsp104) mutations into either NBD1 or NBD2 [82–84,114,123]. Thus, part of the function of Hsp70 might be to 'set' Hsp104 ATPase activity in a mode in which predominantly one NBD is rapidly hydrolyzing ATP. This mode of ATPase activity can be sufficient to promote the disaggregation of disordered aggregates [65,82,114,123]. However, it precludes amyloid remodeling by Hsp104 [82]. Further studies are needed to define the precise mechanisms by which Hsp70 promotes protein disaggregation by Hsp104. Moreover, it has long remained unclear how the functional interaction between Hsp104 and Hsp70 is mediated and what domains of either protein are required. Recent studies, however, ascribe a critical role for the MD in promoting communication with the Hsp70 system.

#### 4. The role of the MD in Hsp70 chaperone collaboration

Interestingly, it has long been clear that Hsp104 is unable to collaborate with the bacterial Hsp70 chaperone system [16]. Likewise, ClpB is unable to collaborate with the yeast Hsp70 chaperone system [106]. This situation is often referred to as the 'species-specific' interaction between Hsp104 and Hsp70 [2,68,69]. However, the term 'species specific' should not be taken too literally. Hsp104 is able to collaborate effectively with the mammalian Hsp70 chaperone system [44,84,124] and multiple eukaryotic Hsp104 homologues can complement the thermotolerance function of Hsp104 in yeast (but interestingly not always the prion propagation function of Hsp104) [125–128], whereas ClpB cannot [51,70]. Rather, it seems that eukaryotic Hsp100s are unable to collaborate with prokaryotic Hsp70, and likewise prokaryotic Hsp100s cannot collaborate with eukaryotic Hsp70. Within prokaryotes, TClpB cannot collaborate with DnaK from *E. coli* [129]. Moreover, Ssa1 and DnaJ can collaborate with Hsp104, whereas DnaK and Ydj1 cannot [16]. Thus, the key specificity determinant appears to be the Hsp70 chaperone rather than the Hsp40.

Yet, defining whether or how Hsp70 or Hsp40 might interact directly with Hsp104 has proven remarkably difficult. Initial evidence suggested that Hsp104 coimmunoprecipitates with Ydj1, but not Sis1, from yeast extracts [16]. Using pure proteins, a weak physical association has been observed between hexameric ClpB and DnaK [129]. In another study, DnaJ was found not to interact with ClpB, whereas the substrate-binding domain of DnaK was required for an interaction with ClpB [130]. This interaction was disrupted by deletion of the N-terminal domain of ClpB and was partially disrupted by deletion of portions of the coiled-coil MD of ClpB [130]. These data indicated that DnaK might interact with the N-terminal domain and MD of ClpB [130]. However, it is not clear whether these interactions

reflect specific functional interactions between ClpB and DnaK that are required for protein disaggregation, or whether they reflect the chaperone activity of DnaK, which would recognize and bind transiently unfolded portions of ClpB.

The first functional evidence for the interaction between the MD and the Hsp70 chaperone system was uncovered by characterization of specific helix 3 MD mutants (e.g. Y503D; Fig. 3a,b) [67] (Table 1). ClpB<sup>Y503D</sup> was unable to collaborate with KJE in protein disaggregation [67]. However, ClpB<sup>Y503D</sup> formed hexamers, had elevated ATPase activity and translocated unfolded polypeptides [67]. The specific defect in ClpB<sup>Y503D</sup> was pinpointed to an inability of KJE to shuttle aggregated substrates to the NBD1 channel loop [67]. Thus, it was concluded that the MD functions to couple the interaction with Hsp70 to translocation of aggregated substrates [67]. Yet, a direct interaction between helix 3 of the MD and DnaK was not observed [67]. Rather, helix 3 of the MD displays conformational flexibility that is necessary to regulate a direct interaction between helix 3 of the MD and NBD1 [67]. Mutations in helix 3 (e.g. Y503D) cause this interaction to become dysregulated and consequently ATPase activity becomes elevated to such an extent that substrate handover from KJE to the ClpB channel can no longer be co-ordinated [67]. Thus, these studies revealed that helix 3 of the MD indirectly controls the functional interaction with Hsp70 through a direct interaction with NBD1.

The established connection between the MD and Hsp70 [67] stimulated efforts to utilize chimeras of Hsp104 and ClpB to determine which domain(s) of Hsp104 would enable ClpB to function in yeast (Fig. 5) [51,68,69]. Thus, each domain (N, NBD1, MD and NBD2) was treated as a module and chimeras were constructed that had various combinations of modules from either ClpB or Hsp104 [51,68,69] (Fig. 5). For example, the chimera 444B contains the N-terminal domain, NBD1 and MD of Hsp104 plus NBD2 of ClpB. In an initial study, 444B and <sub>44B</sub> (444B with the N-terminal domain deleted) could function in thermotolerance and prion propagation, indicating that either the N-terminal domain, NBD1 or the MD of ClpB might preclude activity in yeast [51]. Subsequent studies revealed that the MD plays a critical role [68]. Thus, BB4B was able to partially complement Hsp104 in thermotolerance (Fig. 5) [68], but was inactive in propagation of the yeast prion [PSI<sup>+</sup>] [51]. Moreover, BB4B and a similar construct could now collaborate with eukaryotic Hsp70 to promote the disaggregation of disordered aggregates in vitro (Fig. 5) [68,69]. Thus, the MD of Hsp104 enables ClpB to functionally interact with eukaryotic Hsp70.

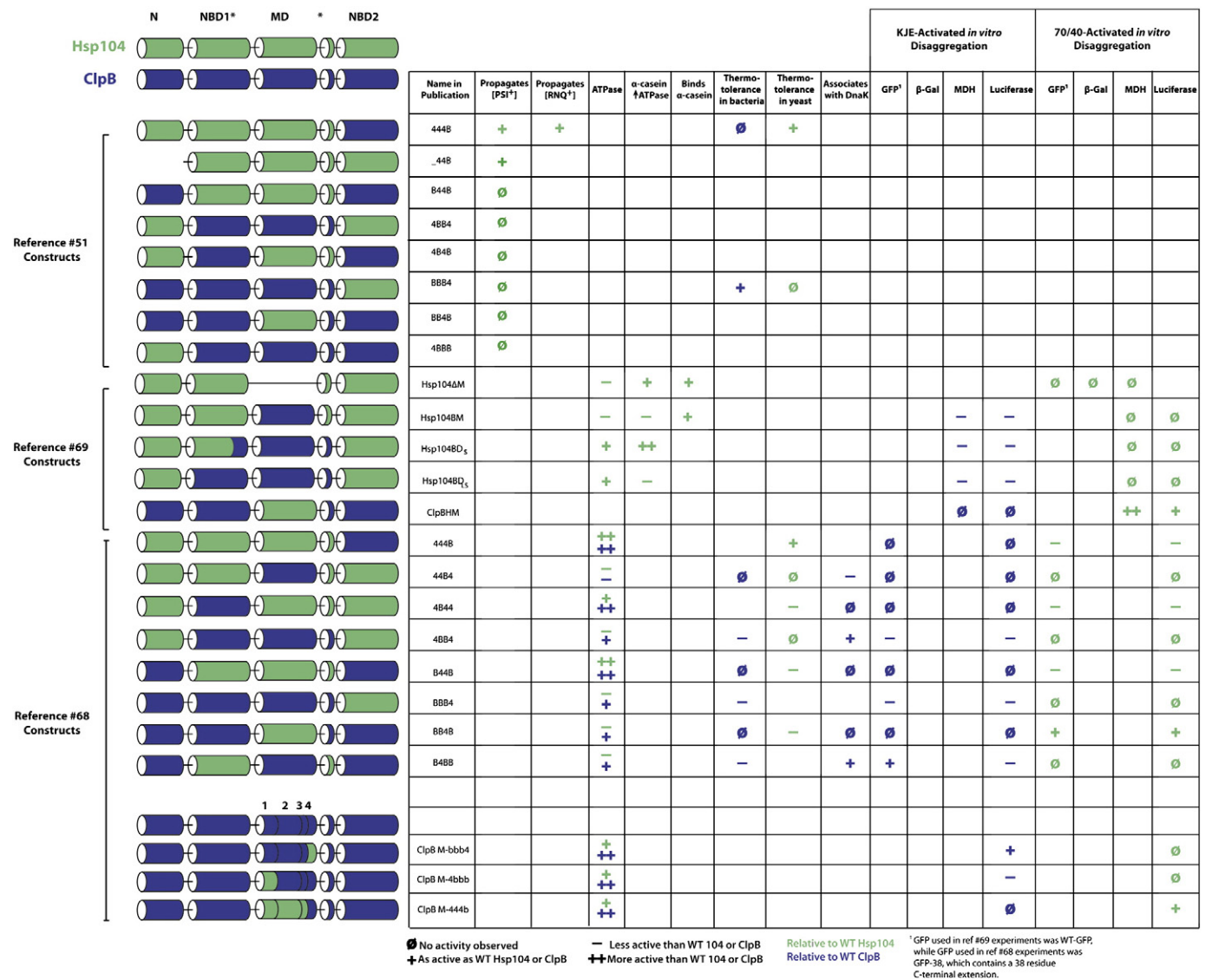
Curiously, however, the converse domain transplant was not nearly as successful (Fig. 5). Thus, 44B4 could not collaborate with KJE in vitro or in *E. coli* (Fig. 5) [68]. Another study found that 44B4 had ATPase activity, formed hexamers and could weakly collaborate with KJE in disaggregation in vitro [69]. Thus, simply transplanting the MD of ClpB into Hsp104 does not enable Hsp104 to collaborate with DnaK in the same way that ClpB can [68]. Rather, additional sequences appeared to be required. The addition of the NBD1 of ClpB helped restore activity to wild-type ClpB levels [68]. Thus, 4BB4 was active in vitro with KJE and in *E. coli* [68]. Curiously, 44BB has not been studied. Collectively, these data assert that the MD of Hsp104 is extremely important for a functional interaction with eukaryotic Hsp70 in the disaggregation of disordered aggregates [68,69]. However, the situation with ClpB is more complex as the MD of ClpB does not enable or only minimally enables Hsp104 to interact functionally with KJE [68,69]. Surprisingly, no mechanistic explanation for this disparity has been advanced [68,69]. Moreover, although these studies [68,69] strengthen the functional interaction between the MD and Hsp70, there is still no compelling evidence for a direct interaction between the MD and Hsp70 that is required for protein disaggregation. A hint is provided by a bacterial two-hybrid system in which the MD of ClpB appears to confer (in some constructs) the ability to interact physically with DnaK, although it remains possible that this interaction might still be indirect [68]. Even in this experiment,

**Table 1**  
Selected MD missense mutants. Activity relative to wild-type (WT) Hsp104 is shown in green symbols and activity relative to WT ClpB is shown in blue symbols. ++ indicates that mutant displayed greater activity than WT, + indicates that the mutant displayed comparable activity to WT, - indicates that mutant displayed less activity than WT, while ∅ indicates that no activity was observed for the mutant tested. Blank cells indicate that the mutant was not tested for this activity. Please note that this is not an exhaustive table of tested MD mutations. For a more exhaustive listing of characterized MD missense mutants, see Barends and colleagues review [4].

Mutation	Reference #	Forms hexamers?	ATPase activity?	Able to translocate?	Disaggregate amorphous aggregates in vitro?	Disaggregate amyloid aggregates in vitro?	Functional in thermotolerance?	Functional in [PSI <sup>+</sup> ] propagation?
ClpB <sup>Y503D</sup>	67	Yes	++	+	∅	n/a	∅	n/a
Hsp104 <sup>R419M</sup>	64	Yes	-			∅	-	
Hsp104 <sup>R444M</sup>	64	Yes	-			∅	-	
Hsp104 <sup>R495M</sup>	64	Yes	++			∅	-	
Hsp104 <sup>L462R</sup>	134						+	∅
Hsp104 <sup>A503V</sup>	85, 134–136		++				+	-

44B4 displayed a signal that was only slightly higher than background, indicating that the MD of ClpB is insufficient to confer an interaction with DnaK [68]. Here too, there is still the concern that apparent

specificity in the bacterial two-hybrid system might stem from the chaperone activity of DnaK rather than from a direct, functional interaction that is required for disaggregation activity. Further studies in



**Fig. 5.** Summary of ClpB and Hsp104 chimera activity. Cartoon representation of the domain chimeras utilized in references [51,68,69]. Green cylinders represent Hsp104 domains and blue cylinders represent ClpB domains. Activity relative to wild-type (WT) Hsp104 is shown in green symbols and activity relative to WT ClpB is shown in blue symbols. ++ indicates that the chimera displayed greater activity than WT, + indicates that the chimera displayed comparable activity to WT, - indicates that chimera displayed less activity than WT, while ∅ indicates that no activity was observed for the chimera tested. Blank cells indicate that chimera was not tested for this activity. [PSI<sup>+</sup>] propagation, [RNQ<sup>+</sup>] propagation, ATPase activity, increase in ATPase activity upon binding α-casein, the ability to bind α-casein, conferred thermotolerance in *E. coli*, conferred thermotolerance in *S. cerevisiae*, and association with DnaK as measured by a bacterial 2-hybrid system were tested. Additionally, DnaK, DnaJ, and GrpE and Hsp70/40 mediated disaggregation of Green fluorescent protein (GFP), β-galactosidase (β-Gal), and malate dehydrogenase (MDH) were tested.



this system, which pinpoint the exact residues in ClpB and DnaK that are necessary for interaction might be informative.

To determine the precise region within the MD that is required for collaboration with DnaK or Hsp70 another series of ClpB chimeras were generated [68]. Thus, the MD of these chimeras was comprised of various combinations of helices 1, 2, 3 or 4 from Hsp104 or ClpB (Fig. 5). These studies indicate that helices 2 and 3 of the ClpB MD are important for a functional interaction with DnaK, whereas helices 1, 2 and 3 of the Hsp104 MD are important for an interaction with eukaryotic Hsp70 [68]. However, a single missense mutation in helix 2 that disrupted the functional interaction with DnaK could not be identified [68]. By contrast, the Y503D mutation in helix 3 of the ClpB MD ablates any collaboration with DnaK, even though this region does not interact with DnaK directly [67]. This latter finding raises the possibility that all the chimeric proteins that fail to interact with DnaK in protein disaggregation [68,69] might only fail to do so because, in the chimera, the communication between NBD1 and helix 3 of the MD (that is necessary for collaboration with KJE) is disrupted in a manner akin to ClpB<sup>Y503D</sup>. That is, perhaps due to unanticipated, long-range structural effects, the chimera is unable to support the appropriate communication between NBD1 and helix 3 of the MD, which is paramount for collaboration with KJE. Thus, chimera dysfunction might not reflect loss of a direct interaction between DnaK and the MD per se. Rather, chimeras might be unable to collaborate with KJE because of an indirect, long-range structural effect that disrupts communication between NBD1 and helix 3 of the MD. Further studies are needed to resolve these issues and to pinpoint the exact sites where DnaK and ClpB might interact directly.

## 5. Hsp70-Independent functions of the MD

Interestingly, amyloid remodeling by Hsp104 does not absolutely require Hsp70 and Hsp40 in vitro [21,23,24,44,131–133], which suggests that the disaggregation of ordered aggregates may proceed by a different reaction mechanism than amorphous aggregate remodeling. This independence from Hsp70 also provides an opportunity to assess the function of the MD toward the intrinsic disaggregase activity of Hsp104. The MD plays an important role in this intrinsic amyloid-remodeling activity of Hsp104, as individual missense mutations in conserved MD arginines (R419, R444 or R495) ablate the ability of Hsp104 to remodel infectious amyloid forms of Sup35 [64] (Table 1). Thus, the MD plays a critical role in disaggregase activity even in the absence of Hsp70 [64].

Consistent with these in vitro observations, several mutations in the MD of Hsp104 impair prion propagation in vivo [134–136]. Intriguingly, some of these mutations impair propagation of the yeast prion [PSI<sup>+</sup>], which is comprised of infectious amyloid forms of Sup35, but have no effect on thermotolerance. MD mutants that fall in this class include L462R and A503V (Fig. 3a,b) [85,134–136] (Table 1). The mechanistic explanation for the ability of these specific MD mutants to promote the disaggregation of stress-induced disordered aggregates while simultaneously being unable to promote the amyloid-remodeling events required for prion propagation is unknown. Nonetheless, these data hint that Hsp104 might exploit subtly different mechanisms to remodel amyloid versus disordered aggregates, and that the MD plays a key role in these different mechanisms.

## 6. Concluding remarks

In closing, we note that despite several important advances described above, many aspects of the role of the MD in Hsp104 and ClpB activity remain elusive. First, the location of the MD within the hexameric assembly remains a point of debate [61–66,91]. Second, although the MD appears to control the functional collaboration with the Hsp70 chaperone system and even confers some ability to enable chimeric disaggregases to traverse the *S. cerevisiae* – *E. coli* species

barrier (in one direction at least), precisely how this is achieved and whether the MD interacts with Hsp70 directly is still unclear [51,67–69]. Finally, single missense mutations in the MD of Hsp104 can have dominant gain-of-function mutations with highly unexpected biological consequences [85]. Perhaps one of the most surprising examples is provided by A503V [85,134,135]. This mutant confers a growth defect at 37 °C when expressed at high levels, but surprisingly, is more active than wild-type Hsp104 in thermotolerance [85]. Furthermore, Hsp104<sup>A503V</sup> cannot support propagation of the yeast prion [PSI<sup>+</sup>] [85,134]. Yet, remarkably, Hsp104<sup>A503V</sup> is considerably more effective than wild-type Hsp104 in antagonizing polyglutamine aggregation and toxicity in yeast models of Huntington's disease [135]. This surprising therapeutic gain-of-function suggests that MD mutations should be intensively explored in our efforts to identify novel Hsp104 variants with enhanced disaggregase activity against specific proteins that misfold, aggregate and confer toxicity in several increasingly prevalent and presently untreatable neurodegenerative disorders [6,39,43].

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