Structure

Structure of Calcarisporiella thermophila Hsp104 Disaggregase that Antagonizes Diverse Proteotoxic Misfolding Events

Highlights

- The dynamic assembly shows varying tunnel width, helical rise, and domain orientation
- The N-terminal domain and C-terminal tail are not required for CtHsp104 hexamerization
- CtHsp104 rescues TDP-43, polyGlu and α-Syn toxicity in yeast and is well tolerated
- CtHsp104 confers thermotolerance to yeast suggesting collaboration with Hsp70/Hsp40

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In Brief

X-ray crystal and cryo-EM structures of Hsp104 from thermophilic fungus were determined by Michalska et al. Structures reveal left-handed, helical, dynamic assemblies with all domains resolved. This disaggregase robustly antagonizes toxic protein-misfolding events in vivo where yeast Hsp104 is ineffective. Natural Hsp104 variants can provide an untapped therapeutic resource for neurodegenerative diseases.
Structure of Calcarisporiella thermophila Hsp104 Disaggregase that Antagonizes Diverse Proteotoxic Misfolding Events

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https://doi.org/10.1016/j.str.2018.11.001

SUMMARY

Hsp104 is an AAA+ protein disaggregase with powerful amyloid-remodeling activity. All nonmetazoan eukaryotes express Hsp104 while eubacteria express an Hsp104 ortholog, ClpB. However, most studies have focused on Hsp104 from Saccharomyces cerevisiae and ClpB orthologs from two eubacterial species. Thus, the natural spectrum of Hsp104/ClpB molecular architectures and protein-remodeling activities remains largely unexplored. Here, we report two structures of Hsp104 from the thermophilic fungus Calcarisporiella thermophila (ChHsp104), a 2.70 Å crystal structure and 4.0 Å cryo-electron microscopy structure. Both structures reveal left-handed, helical assemblies with all domains clearly resolved. We thus provide the highest resolution and most complete view of Hsp104 hexamers to date. We also establish that ChHsp104 antagonizes several toxic protein-misfolding events in vivo where S. cerevisiae Hsp104 is ineffective, including rescue of TDP-43, polyglutamine, and α-synuclein toxicity. We suggest that natural Hsp104 variation is an invaluable, untapped resource for illuminating therapeutic disaggregases for fatal neurodegenerative diseases.

INTRODUCTION

During severe stress conditions, the capacity of the cell to protect its proteins can become overwhelmed by excessive misfolding and aggregation. However, once normal conditions are restored, the aggregation process is reversed by specialized disaggregases that reactivate proteins trapped in aggregates. These include the hexameric AAA+ protein Hsp104 (Sweeny and Shorter, 2016). In yeast, Hsp104 provides several selective advantages, including: (1) conferring thermotolerance by refolding proteins trapped in heat-induced aggregates (Sweeny and Shorter, 2016); (2) remodeling amyloid fibrils, which empowers yeast to harness protein-based epigenetic elements (prions) for adaptive purposes (Sweeny and Shorter, 2016); (3) promoting degradation of select substrates (Preston et al., 2018; Ruan et al., 2017); (4) regulating material properties of membraneless organelles (Kroschwald et al., 2015); and (5) promoting yeast longevity and fitness by ensuring partitioning of damaged proteins and aggregates to the mother cell during cell division (Sweeny and Shorter, 2016). Protein disaggregases such as Hsp104 are unique among protein-remodeling factors as they can unfold misfolded and aggregated proteins. This disaggre-gase activity offers an intriguing therapeutic strategy to restore proteostasis in humans where it has gone awry, as with neurodegeneration (Jackrel and Shorter, 2015).

Hsp104 forms hexamers that harbor a central channel through which substrate is translocated (Sweeny and Shorter, 2016). Each protomer is composed of five domains: an N-terminal domain (NTD) that has roles in substrate recognition and translocation, two nucleotide-binding AAA+ domains (NBDs 1 and 2, further divided into large [L] and small [S] subdomains) that bind and hydrolyze ATP and translocate substrate, a coiled-coiled middle domain (MD) insertion in NBD1 that enables interdomain communication and collaboration with the Hsp70 chaperone system, and a short C-terminal tail (CTT) that has been reported to be necessary for hexamer formation (Figure 1A) (Sweeny and Shorter, 2016).
Several structures of bacterial ClpBs and eukaryotic Hsp104s have been reported with varying resolution and complex architecture (Table 1). Low-resolution (11–21 Å) cryo-electron microscopy (cryo-EM) single-particle reconstructions of ClpB from *Thermus thermophilus* (*Tt*ClpB) in the apo state or complexed with ADP (Lee et al., 2007) or AMP-PNP (Lee et al., 2003) show flat hexameric rings with a substantial internal cavity. Similar arrangements were reported for low-resolution cryo-EM structures of an *Escherichia coli* ClpB (EcClpB) derivative BAP (ClpB variant engineered to interact with ClpP where S722–N748 of ClpB are replaced with V609–I635 of ClpA) complexed with ClpP (Carroni et al., 2014), and for *Saccharomyces cerevisiae* Hsp104 (ScHsp104) variant Hsp104\(^{N728A}\) complexed with ADP, ATP, or ATP\(_2\)S (Wendler et al., 2007, 2009). Only in one case was an attempt made to obtain reconstruction with no symmetry imposed (Wendler et al., 2009); all other models were generated with C6 symmetry. Recently, much higher-resolution (4–5 Å) cryo-EM structures have been reported for ScHsp104 (Gates et al., 2017; Yokom et al., 2016) and BAP (Deville et al., 2017), which revealed dramatic nucleotide- and substrate-dependent changes in complex architecture. For instance, ScHsp104 adopts an open left-handed helical hexamer in the presence of AMP-PNP (Gates et al., 2017; Yokom et al., 2016) whereas ScHsp104 adopts a closed, right-handed helical hexamer when complexed with ATP\(_2\)S and casein (Gates et al., 2017; Yokom et al., 2016). Additionally, several crystal structures of *Tt*ClpB (Lee et al., 2003), *Ec*ClpB (Carroni et al., 2014) and Hsp104 from *Chaetomium thermophilum* (*Cht*Hsp104) (Heuck et al., 2016) show that proteomers are arranged in left-handed helical filaments that are reminiscent of the discrete helical hexamers observed by cryo-EM. The various hexameric models advanced for Hsp104 may represent different stages of the folding pathway or different functional states of the Hsp104 conformational ensemble. However, the quality of the models varies widely. In some structures, only the NBDs and MDs can be modeled (Carroni et al., 2014; Deville et al., 2017). In two *Ec*ClpB structures, the NTDs have been modeled (PDB: 4D2U, 4D2Q). Thus, to better understand how Hsp104 architecture relates to function, we need a higher-resolution view.

Sequence diversity among Hsp104 orthologs has important functional implications. For instance, ScHsp104 possesses potent amyloid-remodeling activity necessary for inheritance of beneficial yeast prions, whereas bacterial ClpB has limited ability to remodel amyloid (DeSantis et al., 2012). Moreover, single missense mutations within ScHsp104 can profoundly enhance functionality (Jackrel et al., 2014; Tariq et al., 2018). At the same time, the vast majority of sequence space among natural Hsp104s remains unexplored. For example, it is unclear whether there are natural Hsp104 orthologs with divergent enhanced or selective activity against misfolded proteins implicated in neurodegenerative diseases, such as α-synuclein (α-Syn) or TDP-43. Indeed, Hsp104 variants with desirable therapeutic disaggregase activity may have arisen through natural selection as an adaptation to unique proteomes and environmental conditions.

Here, we present data that advance our understanding of these two frontiers of Hsp104 structure and function. We determined the structures of Hsp104 from the thermophilic fungus *Calcarisporiella thermophila* (*Ct*Hsp104) in the presence of ADP by X-ray crystallography (at 2.70 Å) and cryo-EM single-particle reconstruction (at 4.0 Å). Moreover, we establish that *Ct*Hsp104 antagonizes several toxic protein-misfolding events where ScHsp104 is ineffective, including rescue of TDP-43, polyglutamine (polyGlu), and α-Syn toxicity. Thus, we provide evidence for a natural Hsp104 ortholog with enhanced ability to antagonize toxicity linked to neurodegenerative disease.

**RESULTS**

**Crystal Structure of *Ct*Hsp104**

We crystallized a double arginine-finger mutant (R328M/R757M) of *Ct*Hsp104 (*Ct*Hsp104\(^{2R}\)) in complex with ADP. The complex crystallized in the P6\(_3\) space group with a single protein chain in the asymmetric unit. We determined its structure by molecular replacement using individual subdomains of ClpB homologs from *T. thermophilus* and *E. coli* as search probes (see STAR...
Similarly to other eukaryotic Hsp104s, ChtHsp104 consists of five domains: NTD (residues 1–144) followed by a 13-residue-long linker 145–157, NBD1 (residues 158–404 and 530–547), MD (residues 405–529), NBD2 (residues 548–862), and CTT (residues 863–882), part of which remains disordered (Figure 1A). In the crystal structure, both NBDs bind ADP (Figures 1B and 1C). The CTT is enriched in acidic residues and is unique to eukaryotic Hsp104s but somewhat variable among orthologs (Figure S1). Conserved channel-facing loops in the NBDs are disordered, including the two canonical tyrosine-containing loops, B6-B7 and D7-D8, which engage translocating substrate and another channel-facing segment, loop B8-B9 (Gates et al., 2017).

Our structure reveals some notable differences compared with previous Hsp104 crystal structures. These include the resolution and location of the NTD. The NTD is located in close proximity to NBD1 in TtClpB, whereas the NTD-NBD1 linker in ClpB104 is nearly fully extended, positioning the NTD ~30 Å away from NBD1 (Figure 2A). In ChtHsp104 and EcClpB structures, NTDs are disordered. Additionally, we observe helix L2 of the MD of ChtHsp104 is bent while helices L3 and L4 are fused into a single long helix L3, similar to the structure of ChtHsp104 (Heuck et al., 2016) (Figures 1B, 1C, and 2B).

In the crystal lattice, molecules are packed along a 6ₐ screw axis to form a left-handed spiral filament with a 16.2-Å rise. This arrangement is similar to the packing previously observed for ChtHsp104 (Heuck et al., 2016), which has ~53% sequence identity with ChtHsp104. In ChtHsp104, the space group was determined as P2₁, with three molecules in the asymmetric unit and twinning (Heuck et al., 2016). However, closer inspection suggests that different interpretations, including one in the P6₃ space group, are possible. The observed helical motif is reminiscent of the architecture displayed in the crystal structure of TtClpB (~52% sequence identity with ChtHsp104) (Lee et al., 2003), where the hexagonal symmetry is slightly violated (Figure 2F), and EcClpB (~49% sequence identity with ChtHsp104) (Carroni et al., 2014). While rather loose packing of the TtClpB molecules creates a wider central tunnel along the filament, the tighter
interprotomer associations in ChtHsp104 and ChtHsp104 fila-
ments leave virtually no open tunnel (Figures 2D–2F).

The CTT
The C-terminal region of Hsp104, including a conserved VLPNH motif (V857IRNH861 in ChtHsp104) and the acidic stretch of resi-
dues unique to eukaryotic orthologs, has been previously named
C-terminal domain (CTD). In ScHsp104, this fragment has been
linked to various functions, including cochaperone recruit-
ment (Abbas-Terki et al., 2001) and hexamerization (Mackay et al.,
2008). Analysis of the available Hsp104 and ClpB structures in-
dicates that the VLPNH motif rather than being an extension
actually belongs to NBD2, whereas the nonglobular fragment,
here termed CTT, corresponds to downstream
/C24
20 amino acids
(Figures 1A and S2). In ChtHsp104, Val857 (Val889 in ChtHsp104)
is part of the e3 β strand while the following section is connected
to NBD2 by interactions with helix E3 and the subsequent E3-e2
loop (Figure S2). This association is enabled by nonconserved
Arg859, which is typically substituted by a proline residue in
other homologs, as seen in ChtHsp104 (Figure S2). In the latter
case, no stabilizing hydrogen bonds can be made and the visible
portion of the C-terminal region following the e3 strand protrudes
from the domain body. A similar arrangement is observed in
TtClpB, in which the e3 strand and its flanking region, though
not identical in sequence, obey the general chemical characteristics (hhPxb
where h = hydrophobic, x = any, P = pro-
line, and b = basic amino acid).

Cryo-EM Single-Particle
Reconstruction of ChtHsp104
Given the apparent nonactive confor-
mation of ChtHsp104 in the crystal, we used
cryo-EM single-particle analysis to inves-
tigate the structure of wild-type (WT)
ChtHsp104 in vitrified solution (Figures 3A
and 3B). The reconstructed 3D map (Fig-
ure 3C), determined from over 220,000
particles, has a resolution of 4.4 Å un-
masked or 4.0 Å masked (Figure 3D).

The NBDs and MD are well resolved at 3–5 Å (Figure 3C). How-
ever, the flexible NTD-NBD1 linker allows several possible NTD
orientations, limiting NTD resolution to
/C24
10 Å(Figure 3C). Further
focus classification on the NTD failed to increase resolution (data
not shown).

Our cryo-EM structure reveals that ChtHsp104 forms an open
one-turn left-handed spiral hexamer, similar to those reported
for ScHsp104 (Yokom et al., 2016) (Figures 3C and 4A–4C).
The Cryo-EM map for all domains is visible in each of the six
protomers (P1–P6) (Figures 4A–4C, S3, and S4; Video S1). In
particular, MDs are resolved well for five protomers (P1–P5),
compared with 3/6 for ScHsp104 maps (Figure S4). All helices
in the NBDs matched well with helical densities in the map (Fig-
ures 3, 4, 5, and S3–S5).

As in ScHsp104, NBD1 and NBD2 from the six protomers form
a helix, with NBD2 from P1 contacting NBD1 of P6 at the seam
(Figure 4A). The MD in P1 reaches across but does not contact
the MD of P6 (Figure 4A). Thus, the ChtHsp104 structure observed
by cryo-EM is significantly more open than that observed in the
crystal. Interestingly, we also found classes (representing ~10% particles) without density for the MD, which shows an even more
open cleft (data not shown).

AAA+ interfaces are similar in the crystal filament and cryo-
EM hexamer, except for the P1–P6 interface, where we observe
an atypical interaction between NBD2 from P1 and NBD1 of P6, as reported for ScHsp104 (Gates et al., 2017; Yokom et al., 2016). This interaction generates the spiral architecture. In P1, only NBD2 is supported by the interaction with NBD1 of P6. Nucleotide occupancy in P1 NBD1 is the lowest of the 12 AAA+ domains, although it varies at each binding site across all six protomers (Figure 5C). Interactions between seam protomers also include an interaction between NBD2 (residues 852–854 from the e2-e3 hairpin) of P1 and loop B4-B5 from P6. In proteors P2-P6, the C-terminal helix E3 (residues 816–835) from protomer p interacts with helix D4 (residues 575–590) in protomer p-1. Likewise, in protomers P2–P6, the MD of protomer p intersects significantly with the MD and NBD1 of protomer p-1. By contrast, in P1 the MD is largely unbound. Two loops in p-1)NBD1 are involved in its interaction with the MD, including loop B4-B5 (residues 225–233) (same residues that in P6 contact the P1 NBD2 at the seam) and loop B3-b2.

**Table 2. Crystallographic Data Processing and Refinement Statistics**

| Processing | Wavelength (Å) | 0.9793 |
| Resolution range (Å) | 30.00–2.70 (2.75–2.70) |
| Space group | P61 |
| Unit cell parameters (Å) | a = 139.17, c = 97.06 |
| Unique reflections | 29,450 (1,467) |
| Multiplicity | 7.3 (7.1) |
| Completeness (%) | 100 (100) |
| <hi> Wilson B factor (Å²) | 23.61 (2.25) |
| Wilson B factor (Å²) | 49.8 |
| R<sub>merge</sub> | 0.096 (above 1) |
| CC<sub>1/2</sub> | 0.794 |
| CC<sup>+</sup> | 0.941 |

**Refinement**

| Resolution (Å) | 30.00–2.70 |
| Reflections work/test set | 29,413/1,496 |
| R<sub>work</sub>/R<sub>free</sub> d | 0.198/0.249 |
| Average B factor (Å²) (no. of atoms) | 74.4 (6,394) |
| Ligands | 82.3 (54) |
| RMSD bond lengths (Å) | 0.008 |
| RMSD bond angles (°) | 1.17 |
| Ramachandran favored (%) | 97.0 |
| Ramachandran outliers (%) | 0 |
| Clashscore e | 7.91 |

**Nucleotide Binding by CTHsp104**

In the crystal structure, protomers interact via canonical AAA+ interfaces: NBD1-NBD1 and NBD2-NBD2 (Figure 1C). In this arrangement, nucleotide-binding sites of both NBDs are occluded by the neighboring protomer, but the ADP ligands are bound exclusively by one NBD chain (Figures 1B and 1C). Specifically, the lower-affinity site in NBD1 (Hattendorf and Lindquist, 2002) shows nucleotide anchored by hydrophobic interactions with the adenine ring and hydrogen bonds between the diphosphoryl group on ADP and the P loop of the NBD (Figures 5A and 5B). The putative arginine fingers from the adjacent protomer, R327, and mutated R328M are located in the same vicinity, but do not participate in nucleotide binding, as seen in the crystal structure (Figure 5B). It is noteworthy that they may only bind to the γ-phosphate of ATP and not necessarily interact with ADP. The side chain of R328M is trapped between helices B9 and B11, whereas the poorly defined R327 appears to stack against helix B11 (Figure 5B). NBD1-NBD1 interactions involve several hydrogen bonds: helix (p-1)B11 interacts with the b3-B6 section (with participation of D239), and loop (p-1)B3-b2 and helix B5 bind to helix C3. R196 from (p-1)B3-b2 also bridges with loop B2-B3 (Figure 5B). The high-affinity site in NBD2 (Hattendorf and Lindquist, 2002) shows the ADP molecule forming a similar pattern of interactions as in NBD1, namely hydrophobic contacts between the base and protein and hydrogen bonds connecting the diphosphoryl group and sugar moiety to the NBD (Figures 5A and 5B). The side chain of the putative (p-1) arginine finger, R757M, is located between helices D12 and D10, more than 8 Å away from the closest oxygen atom of the diphosphoryl moiety (Figure 5B). The protomer-protomer interactions involving NBD2 rely only on two hydrogen bonds between helix (p-1)D4 and helix E1 as well as a single hydrogen bond connecting (p-1)D2 with helix E3. In contrast to NBD1, NBD2L subdomain does not interact with (p-1)NBD2, possibly explaining the high mobility of NBD2 manifesting in poorer electron density and high temperature factors.

The ATP ligand provided with the sample buffer appears to be hydrolyzed as the cryo-EM map is compatible with ADP binding in all NBDs, in a similar arrangement as observed in the crystal structure (Figures 4C and 5S; Video S1). Indeed, nucleotide-binding sites in P5 and two nearby arginine residues from P4 are well resolved in the cryo-EM density. R327M, in particular, comes very close to the ADP molecule (Figure S6).

**Hexamer Flexibility**

Comparison of the crystal and cryo-EM structures highlight dramatic conformational changes in: (1) channel width; (2) helical rise; and (3) NTD, NBD1, and MD orientation (compare Figures 2D–2F, 4C, and 4D). The central channel ranges from the wide pore (25–30 Å) that could accommodate substrate in cryo-EM reconstructions, to a narrow one, as seen in the crystal filaments, which likely represent an inactive state. In all reported structures, the helical rise ranges from 0 to 16 Å within the left-handed spiral hexamers, suggesting a broad spectrum of possible protomer-protomer interfaces, perhaps even a continuum of states between the flat (as reported for some cryo-EM structures) and spiral arrangements (Table 1). This high positional and conformational freedom may enable accommodation of diverse substrates and might promote local refolding of

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substrate secondary structure within the channel. Notably, the orientations of the NTD, NBD1, and MD change drastically between the crystal structure and cryo-EM structure (Figures 4C and 4D). In the crystal structure, the 13-residue linker connecting the NTD with NBD1 is in an extended conformation, placing the NTD/C24 outside of the spiral (Figure 4D). Thus, the NTD interacts with the MD on the next protomer without disrupting NBD-NBD contacts (Figures 1B and 4D). In contrast, in the cryo-EM structure the NTD sits on “top” of the hexamer and contacts neighboring NTDs (Figure 4C). The P6 NTD contacts NBD1 on P1, limiting complex size to a hexamer (Figure 4C). Overall, the hexameric assembly extracted from continuous spiral filament in the crystal shares several features with our cryo-EM model and prior reconstructions of ScHsp104 (Gates et al., 2017; Yokom et al., 2016).

Comparison of ScHsp104 and CtHsp104 Structure
Two previous cryo-EM studies on ScHsp104 have revealed a spiral hexameric architecture, similar to the CtHsp104 structure (Gates et al., 2017; Yokom et al., 2016). Figure S4 shows each extracted protomer side by side for CtHsp104, ScHsp104-AMP-PNP (Yokom et al., 2016), and ScHsp104-ADP (Gates et al., 2017). The NTDs of ScHsp104 in the AMP-PNP and ADP states are visible in P3, P4, and P5, but not for P1, P2, and P6. In CtHsp104, all protomers have defined NTDs; however, they are not as well resolved as the rest of the map, at only ~10 Å (Figure 3C). MD resolution also varies in each state. Interestingly, in ScHsp104-AMP-PNP the MD has a slight downward bend, whereas it is more horizontal in our cryo-EM map and model (Figure S4). These slight variations support the hypothesis that MD conformation is affected by the presence and type of nucleotide.

CtHsp104 Does Not Depend on the NTD or CTT for Hexamerization
To probe the determinants of CtHsp104 oligomerization in solution, we used size-exclusion chromatography and dynamic light scattering. We tested CtHsp104 constructs lacking the NTD (CtHsp104ΔN, residues 153–882) or the NTD and CTT (CtHsp104ΔNC, residues 153–864) with or without mutations in the Walker B motifs (E275Q:E679Q) or mutated arginine fingers (R328M/R757M; Table S1). Under low ionic strength conditions (100 mM KCl) all constructs migrate as hexamers in the presence of ATP. Specifically, the CtHsp104 hexamer is stable at concentrations of 50 μg/mL. Thus, as expected, the NTD is not required for hexamerization of CtHsp104. However, contrary to ScHsp104 (Mackay et al., 2008), the CTT is also not required...
for CtHsp104 hexamerization. Indeed, CtHsp104<sup>DN</sup>, which lacks two-thirds of the previously defined longer CTD, formed hexamers. Thus, CtHsp104 hexamerization is mediated entirely by NBD-NBD interactions.

CtHsp104 Is an ATP-Driven Disaggregase

Biochemical assays were used to explore CtHsp104 functionality. First, we measured the ATPase activity of CtHsp104 and found that it was comparable with ScHsp104 (Figure 6A). Notably, CtHsp104 achieved optimal ATPase activity at 65°C, while ScHsp104 had optimal activity at 42°C (Figure 6B). Thus, CtHsp104 is likely adapted to function at higher temperatures, in keeping with the thermophilic lifestyle of <i>C. thermophila</i>.

We next examined the disaggregate activity of CtHsp104 in vitro. We observed that CtHsp104 robustly disaggregated and reactivated luciferase aggregates, similar to ScHsp104 (Figures 6C and 6D). Both CtHsp104 and ScHsp104 were inactive alone, but were stimulated by Hsp70 and Hsp40, derived from yeast (Ssa1, Sis1, and Ydj1; Figure 6C) or human (Hsc70, Hdj1, and Hdj2; Figure 6D).

Disaggregation of disordered aggregates by Hsp104 can be induced in the absence of Hsp70 and Hsp40 by the presence of a 1:1 mixture of ATP and ATP<sub>G</sub>S (Figure 6E) (DeSantis et al., 2012). However, unlike ScHsp104, CtHsp104 was completely inactive under these conditions (Figure 6E). We performed the same disaggregation experiment at 30°C, 37°C, 42°C, and 55°C to determine whether CtHsp104 may have some Hsp70-independent luciferase disaggregation activity at elevated temperatures where CtHsp104 has enhanced ATPase activity. However, CtHsp104 was unable to reactivate aggregated luciferase in every case (Figure S7A).

One other possibility is that CtHsp104 Hsp70-independent disaggregate activity is induced by a different ratio of ATP/ATP<sub>G</sub>S. For instance, while Hsp70-independent disaggregation by ScHsp104 is optimally induced by 50% ATP<sub>G</sub>S, ScHsp104<sup>A503V</sup> is optimally induced by 20%–30% ATP<sub>G</sub>S (Torrente et al., 2016). However, we could not find an ATP/ATP<sub>G</sub>S mixture that stimulated CtHsp104 disaggregate activity (Figure S7B). Thus, CtHsp104 responds differently than ScHsp104 and EcClpB to mixtures of ATP/ATP<sub>G</sub>S, revealing differences in how CtHsp104 disaggregate activity is regulated.

CtHsp104 Rapidly Disassembles SEVI Amyloids

Next, we asked whether CtHsp104 can disassemble a recalcitrant, ordered amyloid. Thus, we chose SEVI (semen-derived enhancer of virus infection) amyloid fibrils that enhance HIV infection (Castellano et al., 2015). ScHsp104 remodels SEVI fibrils (Figures 6F and 6G) whereas EcClpB does not (Castellano et al., 2015).
et al., 2015). CtHsp104 also rapidly remodeled SEVI fibrils (Figures 6F and 6G). EM revealed that CtHsp104 and ScHsp104 remodeled SEVI fibrils into small, amorphous structures (Figures 6F and 6G). Thus, amyloid remodeling is a general property of eukaryotic Hsp104 orthologs, whereas prokaryotic Hsp104 orthologs exhibit reduced amyloid-remodeling activity (Castellano et al., 2015; DeSantis et al., 2012; Shorter and Lindquist, 2004).

CtHsp104 Complements Thermotolerance Defects of Dhsp104 Yeast

Next, we defined how CtHsp104 functions in yeast. ScHsp104 is essential for acquired thermotolerance in yeast, where it reactivates heat-aggregated proteins (Sweeny and Shorter, 2016). To assess whether CtHsp104 can complement ScHsp104 function in thermotolerance, we transformed Dhsp104 yeast with a plasmid encoding CtHsp104 under the control of the native HSP104 promoter. Transformed yeast was pretreated at 37°C for 30 min to induce Hsp104 expression, then exposed to 50°C heat stress for varying times. CtHsp104 expression conferred thermotolerance to yeast, albeit more weakly than ScHsp104. After a 20-min heat shock, ~40% of yeast expressing CtHsp104 survived compared with ~75% of yeast expressing ScHsp104 (Figure 6H). These observations combined with in vitro studies (Figure 6C) suggest that CtHsp104 can collaborate effectively with ScHsp70 and ScHsp40, dispensing the notion of a stringent species barrier between Hsp104 and Hsp70.

CtHsp104 Rescues Diverse Proteotoxicity Models

Next, we asked whether CtHsp104 could suppress proteotoxicity in yeast associated with expression of several proteins, α-Syn, polyGlu, and TDP-43, involved in human neurodegenerative disease. α-Syn is a lipid-binding protein that normally localizes to the plasma membrane, but mislocalizes to aberrant cytoplasmic inclusions termed Lewy bodies in degenerating dopaminergic neurons in Parkinson's disease (Jackrel and Shorter, 2015). Expanded polyGlu tracts in several proteins are sufficient to cause severe neurodegenerative diseases, including Huntington's disease and the spinocerebellar ataxias (Orr and Zoghbi, 2007), and in yeast overexpression of extended polyGlu tracts (e.g., 103Q) is toxic (Duennwald et al., 2006). TDP-43 is an RNA-binding protein with a prion-like domain that mislocalizes from the nucleus and forms cytoplasmic aggregates in degenerating neurons of amyotrophic lateral sclerosis and frontotemporal dementia patients and also forms cytotoxic inclusions in yeast (Jackrel and Shorter, 2015). Coexpression of ScHsp104 is insufficient to mitigate the aggregation and toxic phenotypes associated with overexpression of any of these proteins (Jackrel et al., 2014) (Figures 7A–7C). However, we have defined a suite of potentiated ScHsp104 variants such as ScHsp104A503S that can potently suppress toxicity associated with overexpression of TDP-43, α-Syn, and FUS in yeast and metazoan (Jackrel and Shorter, 2015) (Figures 7A–7C). We transformed yeast overexpressing TDP-43, α-Syn, or an expanded polyGlu tract (103Q) with CtHsp104 to determine whether CtHsp104 could ameliorate toxicity associated with overexpression of any of these proteins. We observed a modest rescue of TDP-43 toxicity upon CtHsp104 overexpression, whereas ScHsp104 was ineffective and ScHsp104A503S strongly suppressed TDP-43 toxicity (Figure 7A). To our surprise, CtHsp104 robustly rescued α-Syn and polyGlu toxicity (Figures 7B and 7C). Indeed, CtHsp104 was almost as effective as...
ScHsp104A503S in rescuing α-Syn toxicity and similar in efficacy to ScHsp104A503S in rescuing 103Q toxicity (Figures 7B and 7C). By contrast, ScHsp104 was ineffective (Figures 7B and 7C). Furthermore, rescue of TDP-43, α-Syn, and 103Q toxicity by CtHsp104 was not due to decreased expression of these disease proteins (Figures 7A–7C). Thus, unlike ScHsp104, CtHsp104 is naturally endowed with the ability to combat TDP-43, α-Syn, and 103Q toxicity.

The NTD of Hsp104 has roles in substrate recognition and intersubunit collaboration within hexamers, and deletion of the Hsp104 NTD inhibits potentiated activity against neurodegenerative disease proteins (Sweeny et al., 2015). Hence, we tested whether CtHsp104D showed α-Syn and polyGlu toxicity. CtHsp104D suppressed α-Syn and polyGlu toxicity as well as CtHsp104WT (Figure 7D). Thus, the NTD of CtHsp104 is dispensable for its ability to rescue α-Syn and polyGlu toxicity in yeast.
Figure 7. CtHsp104 Rescues Diverse Proteotoxicity Models

(A–C) Δhsp104 yeast transformed with plasmids encoding galactose-inducible TDP-43 (A) or α-Syn-YFP (B) and the indicated galactose-inducible FLAG-tagged Hsp104 were serially diluted 5-fold and spotted onto glucose (expression off) or galactose (expression on). (C) Wild-type yeast transformed with plasmids encoding galactose-inducible 103Q-CFP and the indicated galactose-inducible FLAG-tagged Hsp104 were serially diluted 5-fold and spotted onto glucose (expression off) or galactose (expression on).

(D) Δhsp104 yeast expressing α-Syn-YFP or 103Q-CFP and CtHsp104WT or CtHsp104DN were serially diluted 5-fold and spotted onto glucose (expression off) or galactose (expression on).

(E) Lysates from yeast expressing 103Q-CFP and the indicated Hsp104 were subjected to SDD-AGE and probed for endogenous Rnq1 prions using a polyclonal anti-Rnq1 antibody. The same lysates were subjected to SDS-PAGE and western blotting for total Rnq1 and PGK to confirm equal loading of samples (bottom).

(F) CtHsp104 does not exhibit reduced growth at 37°C. Hsp104 variants were expressed in the 416GAL vector in Δhsp104 yeast in the absence of any disease protein. The strains were serially diluted 5-fold, spotted in duplicate onto galactose (inducing) and glucose (non-inducing) media, and analyzed at both 30°C and 37°C.
Rescue of polyGlu Toxicity Is Not Simply due to the Elimination of Rnq Prions

Expression of polyGlu proteins is only toxic in yeast that also harbor the prion [RNQ\(^+\)] (Duenwald et al., 2006). Overexpression of polyGlu in [mq\(^-\)] strains (which lack Rnq1 prions) is nontoxic (Duenwald et al., 2006). Therefore, it is possible that ChHsp104 could rescue polyGlu-associated toxicity either by directly antagonizing polyGlu misfolding and aggregation or through disruption of [RNQ\(^+\)] inheritance. To assess the latter possibility, we monitored the conformational state or Rnq1, the protein determinant of [RNQ\(^+\)], in yeast expressing 103Q and Hsp104 variants using semi-denaturing detergent agarose gel electrophoresis (SDD-AGE). In 103Q yeast transformed with an empty vector, we observed SDS-resistant Rnq1 polymers indicative of Rnq1 prions (Figure 7E). By contrast, an isogenic [mq\(^-\)] strain was resistant to polyGlu toxicity and lacked SDS-resistant Rnq1 polymers (Figures 7C and 7E). Overexpression of ScHsp104 had little effect on SDS-resistant Rnq1 polymers (Figure 7E). SDS-resistant Rnq1 polymers were reduced in strains expressing ScHsp104\(^{AS03S}\), ChHsp104, and ChHsp104\(^{4AN}\) but were still present compared with the [mq\(^-\)] control, which entirely lacked these species (Figure 7E). Thus, it is unlikely that ChHsp104 or Hsp104\(^{4AS03S}\) rescue of 103Q toxicity is solely due to [RNQ\(^+\)] disruption.

ChHsp104 antagonizes several toxic protein-misfolding events where ScHsp104 is ineffective, including rescue of TDP-43, polyGlu, and \(\alpha\)-Syn toxicity. Remarkably, ChHsp104 achieved this rescue without exhibiting any off-target activity at 37°C, unlike potentiated Hsp104 variants such as Hsp104\(^{AS03V}\) or Hsp104\(^{4AS03S}\), which are toxic to yeast at 37°C (Figure 7F). Thus, ChHsp104 confers enhanced protection against neurodegenerative disease proteins without toxic side effects, a valuable feature for a potential therapeutic agent. These results suggest that natural Hsp104 variation is an invaluable, untapped resource for illuminating therapeutic disaggregases for fatal neurodegenerative diseases.

DISCUSSION

The AAA+ protein disaggregate Hsp104 is conserved in all non-metazoan eukaryotes and eubacteria, although attention has historically been focused on Hsp104 from S. cerevisiae and the bacterial ortholog CipB. Here, we present the highest-resolution crystal structure combined with cryo-EM single-particle reconstruction of the spiral hexamer in vitrified solution of a eukaryotic Hsp104 ortholog from the thermophilic fungus C. thermophila. The main body of ChHsp104 hexamers is composed of the two AAA+ NBDs that are encircled by the six MDs, forming a ring-like unit in the crystal and interrupted ring in solution. The “top” site, decorated by NTDs that are highly flexible, can adopt very different orientations (Figures 1, 2, and 3), and a “bottom” site renders the location of the CTT. Comparisons of our crystal and cryo-EM structures highlight dramatic conformational changes in (1) channel width, (2) helical rise, and (3) NTD, NBD1, and MD orientations (Figures 2D–2F, 4C, and 4D).

The structures presented here add to a range of structural data demonstrating that Hsp104 forms a highly dynamic assembly. The channel width ranges from the wide pore observed (diameter ~25–30 Å) in cryo-EM reconstructions that appears to be ready to accept substrate to a very narrow one (diameter ~8–10 Å), as seen in the crystal filaments, which may represent an inactive state. The helical rise ranges from 0 to 16 Å for all structures, suggesting that Hsp104 may exist in many stable or transient states that evolved to accommodate many protein sequences (DeSantis et al., 2012). Notably, the relative orientations of the NTD, NBD1, and MD change dramatically between our crystal structure and cryo-EM structure. In the crystal the NTD is located away from the hexamer, allowing the MD to intercalate between NBD1 and the NTD. By contrast, in the cryo-EM structure the NTDs are located over the central channel of the hexamer and MDs wrap around adjacent NBD1s, consistent with previous reports (Yokom et al., 2016). We suggest that these conformational rearrangements are involved in the formation and function of Hsp104 hexamers and suggest a possible “breathing motion” in the hexamer, complementing the large conformational changes among ATP hydrolysis documented by others (Gates et al., 2017; Sweeney et al., 2015; Sweeney and Shorter, 2016; Yokom et al., 2016). Overall our findings support the conformational flexibility of Hsp104, and we suggest that such flexibility may be a generic feature of AAA+ proteins, facilitating torque generation to translocate substrate (van den Boom and Meyer, 2018; Zehr et al., 2017).

The cryo-EM structure shows that ADP occupancy differs across all six protomers (Figure 5C). ATP binding and hydrolysis drive conformational changes, which presumably enable translocation of the protein through the central channel of the hexameric assembly. The cryo-EM structure provides some indication about conformational differences between ADP-bound and ADP-free states, as illustrated in Figure S8. Two helices (C1 and C3) of NBD1, which are adjacent to the nucleotide-binding site, are directly connected to the MD. In our map several bulky side chains in this area are moderately resolved, e.g., D384 close to the ADP binding site, Y353, D391, H356, and H357 in the middle of the two helices, and R399, R469, and Y458 close to where they contact the MD. While at ~4-Å resolution we cannot be fully certain of side-chain placements and potential salt bridges, the \(\alpha\) helices themselves are well resolved in all protomers, hence we are certain of their position (within ~1 Å positional error). Moreover, the crystal structure solved at 2.70-Å resolution provides more details of this network of interactions that connects MD, C1, C3, and the nucleotide-binding site. Severing these interactions will likely affect ATP binding and hydrolysis (Jackrel et al., 2015; Lipinska et al., 2013).

To compare the conformations of the C1 and C3 helices for ADP-bound and ADP-less states, we aligned the protomers P1 (lowest ADP occupancy) and P4 (highest ADP occupancy) by aligning all residues in the NBD1 except for the C1 and C3 helices. The superposition is shown in Figure S8B. The two helices in protomer P1, the ADP-less-like state, are highlighted in bright red and can be seen to be extending outward from the ADP site, whereas the helices in protomer P4, the ADP-bound state, highlighted in bright blue, can be seen to be drawn in toward the ADP molecule (possibly because of the D384 residue). The difference in positions can be expressed roughly as a shift of ~10 Å and rotation of ~20°. The connection of these helices to the MD in turn appears to result in the MD of P1 being in a more “open” position (more outward from the center of the complex) compared with the MD domain in protomer P4.
The “bottom” of the hexamer is much more open and featureless, with CTTs extending from NBD2 toward the outer edges of the assembly, about 25 Å from the central channel exit. Our structures indicate that CTT is not a structural domain and show that the portion of previously identified CTD belongs to NBD2 (Figure S2). Notably, CTT remains disordered in both crystal and cryo-EM structures, indicating that this element does not participate in assembly stabilization. Indeed, deletion of 24 C-terminal residues does not impair hexamerization (Table S1). Two acidic motifs (DEDM and EDED) in the CTT are somewhat conserved among Hsp104s (Figure S2), and for ScHsp104 have been implicated in cochaperone binding and ATPase activity (Abbas-Terki et al., 2001; Mackay et al., 2008). Possibly in CtHsp104 it also modulates interactions with helper proteins such as Sti1, in analogy to similar motifs found in Hsp70 and Hsp90 (Abbas-Terki et al., 2001; Yu et al., 2015). Indeed, C-terminal acidic residues of Hsp104 are critical in curing some Sup35 prion variants, as are cochaperones such as Sti1, which interact with this region of Hsp104 (Gorkovskiy et al., 2017; Zhao et al., 2017). Thus, these acidic motifs may indirectly facilitate processing and folding of substrates by recruiting downstream cochaperones.

We have additionally established a range of activities for CtHsp104 in vitro and in vivo. CtHsp104 functionally complements ScHsp104 in acquired thermotolerance in yeast, and remodels SEV1 amyloid fibrils in vitro. Intriguingly, CtHsp104 is able to robustly suppress toxicity of diverse proteins associated with neurodegenerative diseases, including α-Syn, polyGlu, and TDP-43, similar to previously defined potentiated Hsp104 variants (Jackrel et al., 2014, 2015). However, the biochemical activity of CtHsp104 more closely resembles wild-type ScHsp104 rather than potentiated Hsp104s (e.g., no elevated ATPase or disaggregase activity) (Jackrel et al., 2014; Tariq et al., 2018; Torrente et al., 2016). Thus, the ability of CtHsp104 to potently rescue TDP-43, polyGlu, and α-Syn toxicity may result from differences in substrate recognition between CtHsp104 and ScHsp104. These potential differences in substrate recognition were not due to the NTD of CtHsp104, as CtHsp104K4 could rescue polyGlu and α-Syn toxicity in yeast as effectively as CtHsp104. An interesting direction for future studies will be to determine the factors underlying substrate recognition by CtHsp104.

Our results establish that a natural Hsp104 ortholog can robustly antagonize proteotoxic misfolding where ScHsp104 is inactive, which has important implications in engineering disaggregases for therapeutic modalities. Sequence variation among homologous proteins is recognized as a valuable reservoir for rapid exploration of a protein sequence space in directed evolution studies (Cramer et al., 1998). Recent studies have demonstrated that molecular evolution techniques such as ancestral protein reconstruction can improve the pharmacologic properties of a protein-based drug (Zakas et al., 2017). Therefore, it will be important to determine whether other Hsp104 homologs are capable of suppressing proteotoxicity where ScHsp104 cannot, which may further illuminate the design of therapeutic disaggregases.

The loss of Hsp104 from metazoan lineages was abrupt (Erives and Fassler, 2015). The reason for this loss is unknown. It is also not known why Hsp104 is not naturally potentiated. One possible explanation is that Hsp104 may have been lost by a negative selection event, since potentiated Hsp104s can have deleterious side effects, e.g., promiscuous unfoldase activity that causes increased temperature sensitivity, and disrupted prion inheritance (Jackrel and Shorter, 2015). However, here we have established that the Hsp104 ortholog CtHsp104 is naturally endowed with a range of therapeutic protein-remodeling activities while being well tolerated by host organisms such as yeast and seemingly devoid of discernible deleterious effects. Thus, it will be very interesting to understand the basis of this possible alternative mode of enhanced activity and to discover whether similar activity is seen in other Hsp104 orthologs.

STAR METHODS

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DATA AND SOFTWARE AVAILABILITY

The Hsp104 crystal structure factors and atomic coordinates have been deposited in the PDB under accession code PDB: 6A6Y, and the atomic coordinates and the cryo-EM map have been deposited in the PDB/EMD under accession codes PDB: 6D00 and EMD-7782, respectively.

SUPPLEMENTAL INFORMATION

Supplemental Information includes eight figures, one table, and two videos and can be found with this article online at https://doi.org/10.1016/j.str.2018.11.001.
ACKNOWLEDGMENTS

We thank Korrie Mack and JiaBei Lin for comments on the manuscript, the SBC staff for help with data collection, Xiang Zhang and Mingliang Jin for help in screening grids at National Center for Protein Science Shanghai, which was funded by the CAS-Shanghai Science Research Center High-End User Project. This work was supported by NIH grants R01GM099836 (to J.S.), GM046458 and GM115586 (to A.J.), P41GM103832, R01GM079429, U54GM103297 and S10OD21600 (to W.C.), NIAID contracts HHSN272201200026C and HHSN272201700060C to the Center of Structural Genomics of Infectious Diseases (to A.J.), NIH training grants T32GM071399 and F31NS101807 (to Z.M.M.) and the US Department of Energy, Office of Biological and Environmental Research, under contract DE-AC02—6CH11357. J.S. was also supported by a Muscular Dystrophy Association Research Award (MDA277268), the Life Extension Foundation, the Packard Center for ALS Research at Johns Hopkins University, and Target ALS. L.M.C. was supported by an NSF Graduate Research Fellowship DGE-0822. M.E.J. was supported by an ALS Research at Johns Hopkins University, and Target ALS. L.M.C. was supported by a Muscular Dystrophy Association Research Award (MDA277268), the Life Extension Foundation, the Packard Center for ALS Research at Johns Hopkins University, and Target ALS. L.M.C. was supported by an NSF Graduate Research Fellowship DGE-0822. M.E.J. was supported by a Target ALS Springboard Fellowship. E.C. was supported by a Blavatnik Family Fellowship.

AUTHOR CONTRIBUTIONS

K.M. performed X-ray structure determination; K.Z. and K.M. performed cryo-EM experiments; K.Z. and G.P. performed cryo-EM structure determination; Z.M.M., L.M.C., L.J.M., M.E.J., and E.C. performed biochemical and yeast experiments; C.H.-S. expressed, purified, and characterized proteins for biophysical studies and cryo-EM; L.B. expressed, purified, and crystallized protein; R.J. cloned the genes for crystallography, cryo-EM, and biophysical characterization; J.S., W.C., and A.J. designed experiments, analyzed data, and together with K.M., K.Z., and Z.M.M. wrote the paper.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: May 1, 2018
Revised: September 9, 2018
Accepted: November 1, 2018
Published: December 27, 2018

REFERENCES


288 domain 1 (NBD1) and middle (M) domain in Hsp100 disaggregase unleashed protein plasticity and potentiation. Mol. Cell 75, 2061–2072.


## STAR★METHODS

### KEY RESOURCES TABLE

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**Critical Commercial Assays**

- PiColorLock Phosphate Detection: Innova/Expedeon, Cat# 303
- Luciferase assay reagent: Promega, Cat# E1483

**Deposited Data**

- CryoEM structure of CtHsp104: This paper, EMD-7782/PDB: 6D00
- X-ray crystal structure of CtHsp104<sup>2R</sup> complex with ADP: This paper, PDB: 6AZY

**Experimental Models: Organisms/Strains**

- W303a (MATα, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, ade2-1) (Schirmer et al., 2004) N/A
- W303aΔhsp104 (MATα can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, ade2-1, hsp104::KanMX) (Schirmer et al., 2004) A3224

**Oligonucleotides**

- CtHsp104, 153-882 construct was created using forward primer - TACTTCCAATCCAATGCCGCAGAGGAGGCGTACGAGG and reverse primer - TTATCCACTTCCAATGTTAGATTTCCATATCCTCGTCTTCAACCATG: This paper N/A
- CtHsp104, 153-864 construct was created using forward primer - TACTTCCAATCCAATGCCGCAGAGGAGGCGTACGAGG and reverse primer - TTATCCACTTCCAATGTTAGATTTCCATATCCTCGTCTTCAACCATG: This paper N/A

**Recombinant DNA**

- pMCSG68 (Kim et al., 2011) N/A
- CtHsp104 in pMCSG68: This paper N/A
- CtHsp104<sup>R328M/R757M</sup> in pMCSG68: This paper N/A
- CtHsp104<sup>AN</sup> in pMCSG68: This paper N/A
- CtHsp104<sup>AN,LC</sup> in pMCSG68: This paper N/A
- CtHsp104<sup>AN,N2R</sup> in pMCSG68: This paper N/A
- ScHsp104 in pNOTAG (Jackrel et al., 2014) N/A
- pE-SUMO: LifeSensors N/A
- Ssa1 in pE-SUMO: This paper N/A
- Hsc70 in pE-SUMO: This paper N/A
- Sis1 in pE-SUMO: This paper N/A
- Ydj1 in pE-SUMO: This paper N/A
- Hdj1 in pE-SUMO: This paper N/A
- Hdj2 in pE-SUMO: This paper N/A
- pAG416GAL-Hsp104-FLAG: This paper N/A
- pAG416GAL-Hsp104<sup>Δ503-508</sup>-FLAG: This paper N/A
- pAG416GAL-Hsp104<sup>Δ503-508</sup>-FLAG: This paper N/A
- pAG416GAL-ChHsp104-FLAG: This paper N/A
- pAG303GAL-TDP43 (Johnson et al., 2009) N/A
- pAG303GAL-α-Syn-YFP (Gitler et al., 2008) N/A

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Requests for resources, reagents, and further information should be directed to, and will be fulfilled by Andrzej Joachimiak (andrzejj@anl.gov) or James Shorter (jshorter@pennmedicine.upenn.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAIL

Yeast Strains and Media
All yeast strains were WT W303a (MATa, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, ade2-1) or the isogenic strain W303a Δhsp104 (Schirmer et al., 2004). Yeast were grown in rich media (YPD) or in synthetic media lacking L-histidine and uracil, and containing 2% glucose (SD-His-Ura), raffinose (SRaf-His-Ura) or galactose (SGal-His-Ura).

METHOD DETAILS

Plasmids
The gene encoding ChHsp104 was amplified from C. thermophila cDNA (a gift from Dr. Adrian Tsang, Concordia University) via PCR and inserted into pMCSG68 vector (Kim et al., 2011). The R328M/R757M double mutant (ChHsp1042R) was created using modified PIPE cloning previously described (Klock and Lesley, 2009). The presence of both mutations was confirmed by sequencing at University of Chicago Cancer Research DNA Sequencing Facility. The expression vectors were transformed into E. coli BL21-Gold (DE3) cells (Agilent). The N- and C-terminal truncations were produced from the full-length gene using the following set of primers: the 153-882 construct was created using forward (TACTTCCAATCCAATGCCGCAGAGGAGGCGTACGAGG) and reverse (TTATCCACTTCCAATGTTAGATTCCATATCCTCGTCTTCAACCATG) primers and the 153-864 construct was created using
The column was pre-equilibrated with buffer containing 20 mM HEPES-KOH pH 7.5, 20 mM KCl (mutants) or 100 mM KCl (WT), 2 mM ATP pH 8.0, 2 mM MgCl2, and 4 mM DTT to 0.5 mg/mL, 1 mg/mL and 5 mg/mL.

Size exclusion chromatography (SEC) was performed on a Superdex-200 10/300GL column using AKTAxpress (GE Healthcare). 20 mM imidazole, and 10 mM 30 mL of LB-phosphate media containing 10 g tryptone, 5 g yeast extract, 5 g NaCl (BioShop), 40 mM K2HPO4 (BioShop), 0.5% glucose (Sigma Aldrich), 150 mg ampicillin (BioShop) per liter was inoculated with 100 µL of overnight starter E. coli culture expressing CtfHsp104²R and was grown at 37°C, 200 rpm. After 16 h, large scale cultures were inoculated by adding 30 mL small scale overnight culture to 1 L LB-phosphate media. Cultures were grown at 37°C, 190 rpm to OD600 1.0, and cooled to 18°C prior to overnight induction with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at 18°C, 180 rpm. Cells were harvested via centrifugation at 6,500 rpm, 4°C for 10 minutes and resuspended in 30 mL lysis buffer (50 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 20 mM imidazole, and 10 mM β-mercaptoethanol (β-ME), plus 1 protease inhibitor cocktail tablet (Complete ULTRA, Roche, Indianapolis, Indiana, USA)). Resuspended cells were stored at –80°C prior to protein purification.

Frozen cells were thawed, supplemented with lysozyme (final concentration 1 mg/mL) and lysed on ice for 1 h. Lysate was sonicated on ice with 4 s bursts, followed by a 20 s pause, for 4 min. Cells were then centrifuged at 13,000 rpm for 90 min followed by filtration through 0.45 µm syringe filters. Clarified lysate was manually purified using the vacuum assisted purification system and Sepharose High Performance Nickel Beads (GE Healthcare Life Sciences, Piscataway, NJ, USA) on ice. Column was washed with water, and pre-equilibrated with lysis buffer (50 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, 20 mM imidazole, and 10 mM β-ME) and protein was eluted with buffer containing 250 mM imidazole pH 8.0. Fractions containing CtfHsp104²R were pooled and digested with recombinant His₂-tagged TEV protease, at the ratio 1 mg/25 mg of target protein. The completeness of the His₂-tag digestion was verified by SDS-PAGE and CtfHsp104²R was dialyzed overnight against desalting buffer (50 mM HEPES-NaOH pH 8.0, 500 mM NaCl, 5% (v/v) glycerol, and 10 mM β-ME) at 4°C to remove the His₂-tag. The resulting protein carries the N-terminal SNA sequence artifact. The CtfHsp104²R protein was concentrated to 1.5 mL on Amicon Ultra-15 centrifugal concentrators (Millipore, Bedford, MA, USA) and further purified using size exclusion chromatography (SEC) on Superdex 200 column (GE Healthcare Life Sciences, Piscataway, NJ, USA) pre-equilibrated with desalting buffer to remove the His₂-tagged TEV protease and other small molecular weight contaminants. The CtfHsp104²R fractions containing monomeric protein were then concentrated using Amicon Ultra-15 concentrators (Millipore, Bedford, MA, USA) and buffer-exchanged with a crystallization buffer containing 20 mM HEPES-NaOH pH 8.0, 200 mM KCl, 4 mM dithiothreitol (DTT). Glycerol was then added to a final concentration of 10% prior to cryo-cooling the protein in liquid nitrogen and storage at –80°C.

Preparation of CtfHsp104 and Deletion Mutants for Cryo-EM, SEC and DLS

The 30 mL of LB-phosphate media culture of CtfHsp104 and deletion mutants were prepared as described above. After 16 h, large scale cultures were prepared by adding 30 mL small scale overnight culture to 1 L LB-phosphate media, prepared as above. Cells were grown at 37°C, 200 rpm to OD600 1.0, and cooled to 15°C prior to their induction with 0.5 mM IPTG. Cultures were allowed to grow at 15°C, 180 rpm overnight. Cells were harvested via centrifugation at 4,500 rpm, 4°C for 20 min and resuspended in 35 mL lysis buffer containing 20 mM HEPES-KOH pH 7.57, 20 mM KCl (mutants) or 100 mM KCl (WT), 5% (v/v) glycerol, 20 mM imidazole pH 8.0, 2 mM MgCl₂, 0.5 mM ATP pH 8.0 and 10 mM β-ME. Resuspended cells were stored at ~80°C prior to their purification. Frozen cells were thawed and sonicated on ice with 2 s bursts, followed by a 20 s pause, for 3 min. Cells were then centrifuged at 13,000 rpm for 90 min followed by filtration through 0.45 µm syringe filters. Clarified lysate was manually purified as described above. The nickel column was pre-equilibrated with lysis buffer and eluted with elution buffer containing 250 mM imidazole pH 8.0. Collected fractions were digested with recombinant His-tagged TEV protease, 1 mg for every 25 mg of target protein overnight. Sample was concentrated down to 1.5 mL using Amicon Ultra-15 100K concentrators (Millipore, Bedford, MA, USA) and further purified via size exclusion chromatography (see below). The fractions containing CtfHsp104 hexamer (~664kDa WT) were then collected and concentrated using 100K concentrators and buffer-exchanged with a buffer containing 20 mM HEPES-KOH 7.57, 20 mM KCl (mutants) or 100 mM KCl (WT), 2 mM MgCl₂, 2 mM ATP pH 8.0 and 4 mM DTT.

The samples were further characterized biophysically to determine conditions that support oligomerization. Due to the addition of nucleotide, the final CtfHsp104 concentration (60.9 mg/mL) was determined via the colorimetric, bicinchoninic acid (BCA) protein assay @562nm (Pierce, ThermoFisher Scientific). Dynamic light scattering (DLS) (DynaPro Plate Reader, Wyatt) was used to identify dilutions suitable for cryo-EM single particle imaging. The DLS experiments were performed at 23°C. CtfHsp104, CtfHsp104ΔN, CtfHsp104ΔN,ΔC, CtfHsp104ΔN,ΔC,ΔW and CtfHsp104ΔN,ΔC,ΔW (Table S1) were diluted using 20 mM HEPES-KOH pH 7.57, 20 mM KCl (mutants) or 100 mM KCl (WT), 2 mM ATP pH 8.0, 2 mM MgCl₂, and 4 mM DTT to 0.5 mg/mL, 1 mg/mL and 5 mg/mL.

Size Exclusion Chromatography of CtfHsp104 and Deletion Mutants

Size-exclusion chromatography (SEC) was performed on a Superdex-200 10/300GL column using AKTAxpress (GE Healthcare). The column was pre-equilibrated with buffer containing 20 mM HEPES-KOH pH 7.5, 20 mM KCl (mutants) or 100 mM KCl (WT), 2 mM ATP pH 8.0, 2 mM MgCl₂, and 4 mM DTT to 0.5 mg/mL, 1 mg/mL and 5 mg/mL.
5% glycerol, 20 mM imidazole, pH 8.0, 0.5 mM ATP pH 8.0, 2 mM MgCl₂, and 10 mM β-ME. The column was calibrated with premixed protein standards, including catalase (230 kDa), ferritin (440 kDa), and thyroglobulin (660 kDa). A 1.5 mL protein sample was injected into the column. The chromatography was carried out at room temperature at a flow rate of 1.5 mL/min. The calibration curve of Kav versus log molecular weight was prepared using the equation Kav=(Ve−Vo)/(Vt−Vo), where Ve is the elution volume for the protein, Vo is the column void volume, and Vt is the total bed volume. The CtHsp104, CtHsp104D̴N, CtHsp104D̴N:2R migrate the SEC column as hexamers (Table S1).

Protein Crystallization
CtHsp104D̴N variant was screened for crystallization using a Mosquito nanoliter liquid handler (TTP Labtech, Cambridge, MA) using the sitting drop vapor diffusion technique in 96-well CrystalQuick plates (Greiner Bio-one, Monroe, NC). Prior to setting up crystallization, CtHsp104D̴N was diluted to 15 mg/mL and incubated with 5 mM ADP and 10 mM MgCl₂ for 10 min at 40 °C. For each condition, 0.5 μL of protein and 0.5 μL of crystallization formulation were mixed; the mixture was equilibrated against 135 μL of the crystallization solution in each reservoir well. The crystallization screens MCSG-1-4 ((Anatrace, Maumee, OH)) were used for screening at 4°C and 16°C. Crystals appeared under MCSG-2 condition containing 0.2 M calcium acetate, 0.1 M MES-NaOH pH 6.0, and 10% propanol at 4°C.

X-ray Data Collection, Structure Determination and Refinement
Prior to flash-freezing in liquid nitrogen, CtHsp104D̴N crystals were cryo-protected in mother liquor supplemented with 35% glycerol. The X-ray diffraction data collection was carried out at the Structural Biology Center 19-ID beamline at the Advanced Photon Source, Argonne National Laboratory. The data were collected at 0.9726 Å wavelength. The diffraction images were processed with the HKL3000 suite (Minor et al., 2006). Intensities were converted to structure factor amplitudes in the Ctruncate program (French and Wilson, 1978; Padilla and Yeates, 2003) from the CCP4 package (Winn et al., 2011).

The structure was solved by molecular replacement using Phaser (McCoy et al., 2007) with T7ClpB (PDB: 1QVR, (Lee et al., 2003)) and E. coli (PDB: 1KHY) as search models. Specifically, the 1QVR template was divided into 6 domains. First, NBD1 (residues 153-330) and NBD2 (residues 331-396, 511-540) were identified. This solution was used as a starting point to search for CTT (residues 756-850) and subsequently to search MD (residues 397-510) and NTD (with 1KHY as a model). The resulting solution was morphed into the electron density map (Terwilliger et al., 2012) and rebuilt in Buccaneer (Cowtan, 2006). An additional round of Phaser with Buccaneer-derived structure used as a fixed partial solution enabled the localization of additional residues (residues 543-578, 588-636, 651-686, 700-709, 740-755). In the next step, morphing and autobuilding was used (Adams et al., 2013; Terwilliger et al., 2008). Finally, iterative manual structure corrections in Coot (Emsley and Cowtan, 2004) and crystallographic refinement in Phenix (Afonine et al., 2012) was applied to improve the model. The refinement protocol included optimization of TLS parameters, with the protein chain divided into 5 groups. In the final model, 810/882 (92%) of residues were included, corresponding to the following fragments: Ser2-Asp75, Glu80-Gly144, Ala156-Ala247, Gly251-Ala282, Ala288-Leu647, Gly661-Leu721, Thr738-Ile863 (Figure 1). In ordered segments, the majority of amino-acid side chains are well defined in the electron density. The missing segments are disordered in the crystal lattice. The data collection, processing and refinement statistics are given in Table 2.

Cryo-Electron Microscopy Data Acquisition
The samples were initially screened at the Advanced Electron Microscopy facility at the University of Chicago, National Center for Protein Science Shanghai and data collection was performed at the National Center for Macromolecular Imaging at SLAC-Stanford. Two microliter samples of CtHsp104 hexamer (0.5 mg/mL) in a buffer 100 mM KCl, 2 mM MgCl₂, 2 mM ATP pH 8.0 and 4 mM DTT were applied onto glow-discharged 200-mesh R2/1 Quantifoil grids. The grids were blotted for 3 s and rapidly cryo-cooled in liquid ethane using a Vitrobot Mark IV (Neumann et al., 2006). The samples and images were recorded using Talos Arctica cryo-electron microscope (Neumann et al., 2006) operated at 200 kV and then imaged in a Titan Krios cryo-electron microscope (Neumann et al., 2006) with GIF energy filter (Gatan) at a magnification of 130,000 x (corresponding to a calibrated sampling of 1.06 Å per pixel). Micrographs were recorded with a Gatan K2 Summit direct electron detector, where each image is composed of 30 individual frames with an exposure time of 6 s and a dose rate of 5.3 electrons per second per Å². A total of 3,186 video stacks were collected with a defocus range of 0.8-2.8 μm.

Single Particle Image Processing and 3D Reconstruction
All micrographs were motion-corrected using MotionCor2 (Zheng et al., 2017) and CTF-corrected using Gctf (Zhang, 2016). Initial particles data set was picked manually using EMAN2 (Tang et al., 2007), which yielded ∼3,000 particles from 50 micrographs. 25 2D-class averages, determined by RELION (Scheres, 2012), were used to generate an initial model in EMAN2. Then, the initial model was used for template-automated particle picking in EMAN2, yielding a ∼377,000-particle data set from the selected 2,716 micrographs. Then, particle coordinates were imported to RELION, where the 2D/3D classification and 3D refinement were performed. After removing poor 2D class averages by 2D classification for 3 times, a total data set of ∼260,000 particles was used for 3D refinement, generating the 4.1 Å map. Further 3D classification with C1 symmetry using the 4.1 Å map with 60 Å low-pass filter as an initial model was used to remove a bad class with 13.5% of the particle set. Next, final 3D refinement was performed using 224,915 particles, 4.4 Å map without mask and 4.0 Å map with mask were achieved.
Segmentation and Fitting

The reconstructed 3D density map was segmented using Segger (Pintilie and Chiu, 2012) and UCSF Chimera (Pettersen et al., 2004) generating a rough initial estimate of the 6 protomers expected in the assembly. Six copies of the crystallographic monomer structure were then rigidly fitted to the map by alignment to each of the 6 segmented regions. Secondary structure elements matched well between the fitted models and the map, especially in the NBDs. The NTD in the crystal model was well outside the cryo-EM density, since it has a drastically different position in the crystallographic structure. The NTD in the cryo-EM density is at a lower resolution, and secondary structures are not clearly visible as in the NBDs. Local exhaustive search (using the Fit to Segments tool in Segger) was thus used to determine the position and orientation of each of the 6 protomers in the density; moderate Z-scores (2-3) indicated reasonable confidence in the final placements. These placements also agreed with previous cryo-EM maps (Yokom et al., 2016). The 6 independently fitted protomers were then joined into one model (chains P1 - P6), also taking the ADP ligands from the crystal model (chains A-F).

Each of the 6 rigidly fitted protomers however, appeared to have a slightly different conformation in the cryo-EM density. Hence we then applied the Molecular Dynamics Flexible Fitting, or MDFF (Trabuco et al., 2008). We applied the method in 10 independent runs, to be able to calculate the uncertainty in the resulting model (Video S2). Each run consisted of 10⁴ minimization steps followed by 10⁵ molecular dynamics steps, after which the model stopped deforming significantly. The resulting 10 structures were then input into ProMod (Pintilie et al., 2016) to produce a probabilistic model, which measures the uncertainty at each atom position given the 10 possible results.

Proteins for Biochemical Assays

Untagged Hsp104 from S. cerevisiae was expressed and purified as described previously (DeSantis et al., 2014; Jackrel et al., 2014; Torrente et al., 2016). CbHsp104 was purified from BL21(DE3)RIL cells grown in 2xYT broth supplemented with 25 μg/mL chloramphenicol and 100 μg/mL ampicillin. Protein expression was induced at an OD600 of 0.4-0.6 with 1 mM IPTG for 16 h at 15°C. Cells were harvested by centrifugation, 4000 g, 20 min), 20 mg/mL pepstatin A, and complete protease inhibitor (Roche), and lysed by incubation with hen egg lysozyme followed by sonication. Lysate was clarified by centrifugation at 16,000 rpm for 20 min and loaded onto Ni-NTA resin. The resin was washed with 10 volumes of wash buffer (40 mM HEPES-KOH pH 7.4, 500 mM KCl, 20 mM MgCl2, 2.5% glycerol, 20 mM imidazole, 2 mM β-ME) supplemented with 5 μM pepstatin A and complete protease inhibitor tablets (Gitter et al.). All purification steps were carried out at 4°C. Cells were lysed by incubation with 1 mg/mL hen egg lysozyme and sonication. Lysate was clarified by centrifugation at 16,000 rpm for 20 min and loaded onto Ni-NTA resin. The resin was washed with 10 volumes of wash buffer (40 mM HEPES-KOH pH 7.4, 500 mM KCl, 20 mM MgCl2, 2.5% glycerol, 20 mM imidazole, 2 mM β-ME) supplemented with 5 μM pepstatin A and complete protease inhibitor (Roche). Protein was eluted in wash buffer supplemented with 350 mM imidazole. TEV protease was added to eluted protein, and the sample was dialyzed against wash buffer containing no imidazole for 4 h at room temperature followed by ~16 h at 4°C. After dialysis and cleavage, the protein was loaded onto a second Ni-NTA column to remove the His6 tag and uncleaved protein. Eluted CbHsp104 was pooled, concentrated, and exchanged into high salt storage buffer (40 mM HEPES-KOH pH 7.4, 500 mM KCl, 20 mM MgCl2, 10% glycerol, and 1 mM DTT). A portion was used immediately for biochemical assays, and the remainder was flash cooled in liquid nitrogen and stored at -80°C until further use.

Ssa1, Hsc70, Sis1, Ydj1, and Hdj2 (in pE-SUMO (Life Sensors)) were expressed as N-terminally His6-SUMO-tagged proteins in BL21(DE3)RIL cells. Transformed cells were grown at 37°C in LB media supplemented with 25 μg/mL chloramphenicol and 100 μg/mL ampicillin to an OD600 ~0.5. Cultures were cooled to 15°C, and expression was induced with 1 mM IPTG for 16 h. Cells were harvested, resuspended in lysis buffer (50 mM HEPES pH 7.5, 750 mM KCl, 5 mM MgCl2, 10% glycerol, 20 mM imidazole, 2 mM β-ME, 5 μM pepstatin A, and complete protease inhibitor (Roche), and lysed by incubation with hen egg lysozyme followed by sonication. Lysates were clarified by centrifugation (16,000 x g, 20 min, 4°C), and incubated with Ni-NTA resin for 90 min at 4°C. Resin was washed with 10 column volumes of wash buffer (50 mM HEPES pH 7.5, 750 mM KCl, 10 mM MgCl2, 10% glycerol, 20 mM imidazole, 1 mM ATP, 2 mM β-ME) and eluted with 2 column volumes of elution buffer (wash buffer+300 mM imidazole). To cleave the His6-SUMO tag, Ulp1 was added at a 1:100 molar ratio, and imidazole was removed by dialysis against wash buffer. After dialysis, protein was loaded onto a 5 mL HisTrap column (GE Healthcare) and eluted with a linear imidazole gradient (20-350 mM) over 40 column volumes. Fractions containing cleaved protein were pooled, concentrated, and purified further by Resource Q (Ssa1, Hsc70, Ydj1, and Hdj2) or Resource S (Sis1 and Ydj1) ion exchange chromatography.

ATPase Assay

CbHsp104 (0.042 mM hexamer) was incubated with ATP (1 mM) for 5 min at the indicated temperatures in luciferase-refolding buffer (LRB): 25 mM HEPES-KOH pH 7.4, 150 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT. ATPase activity was assessed by the release of inorganic phosphate, which was determined using a malachite green phosphate detection kit (Innova). Background hydrolysis was determined at time zero and subtracted.

Luciferase Reactivation Assay

Luciferase reactivation was performed as described (DeSantis et al., 2012). To assemble aggregates, firefly luciferase (Sigma; 100 μM) in LRB (25 mM HEPES-KOH pH 7.4, 150 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT) plus 6 M urea was incubated at 30°C for 30 min. The sample was then rapidly diluted 100-fold into LRB. Aliquots were snap-cryo-cooled and stored at -80°C until use. Aggregated luciferase (100 nM) was incubated with CbHsp104 (0.167 μM hexamer) with ATP (5.1 mM) and an ATP regeneration system (1 mM creatine phosphate, 0.25 μM creatine kinase (Roche)) in the presence or absence of Hsp70 (either Ssa1 or Hsc70, 0.167 μM), and Hsp40 (either 0.073 μM each Hdj1 and Hdj2 or 0.073 μM each Sis1 and Ydj1) for...
90 min at 25 °C. After 90 min, luciferase activity was assessed with a luciferase assay system (Promega). Recovered luminescence was monitored using a Tecan Safire plate reader. In some reactions, Hsp70 and Hsp40 were not present, and CtHsp104 concentration was increased to 1 μM hexamer, and ATP was replaced by a 1:1 mixture of ATP and ATPγS (to a total nucleotide concentration of 5 mM).

**Semen-Derived Enhancer of Virus Infection (SEVI) Remodeling**

SEVI remodeling was performed as previously described (Castellano et al., 2015). SEVI fibrils (20 μM monomer) were incubated with ScHsp104 or CtHsp104 (3 μM hexamer) in LRB buffer in the presence of ATP (5 mM) and an ATP regeneration system (0.1 mM ATP, 0.02 mg/mL creatine kinase, 10 mM creatine phosphate). Samples were incubated at 37 °C for the duration of the experiments. At various time points, aliquots were removed, added to a 96-well plate containing a solution of 25 μM ThT in LRB buffer. ThT fluorescence characteristics were measured on a Tecan Safire² microplate reader with excitation and emission filters set to 440 nm and 482 nm, respectively. To assess fibril morphology by negative stain EM, reaction aliquots were spotted on Formvar carbon-coated grids (EM Sciences) and stained with 2% uranyl acetate. Samples were visualized using a JEOL-1010 electron microscope.

**Yeast Strains and Media**

All yeast strains were WT W303a (MATa, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, ade2-1) or the isogenic strain W303a Δhsp104. Yeast were grown in rich media (YPD) or in synthetic media lacking the appropriate amino acids. Media was supplemented with 2% glucose, raffinose or galactose.

**Yeast Transformation and Spotting Assays**

Yeast were transformed according to standard protocols using polyethylene glycol and lithium acetate (Gietz and Schiestl, 2007). For spotting assays, yeast were grown to saturation overnight in synthetic raffinose dropout media at 30 °C. Cultures were serially diluted 5-fold and spotted in duplicate onto synthetic dropout media containing glucose or galactose. Plates were analyzed after growth for 2-3 days at 30 °C.

**Thermotolerance**

Thermotolerance assays were performed essentially as described (Schirmer et al., 1994), with some modifications. W303a Δhsp104 yeast were transformed with plasmids bearing either Hsp104 from S. cerevisiae or C. thermophila under the native HSP104 promoter (e.g. pRS313HSE-ScHsp104-FLAG or pRS313HSE-CtHsp104-FLAG), or an empty vector control. Transformants were selected, grown to saturation in yeast minimal media SD-His, and then diluted to OD600=0.2 in SD-His. Yeast were allowed to double at 30 °C (~4 h), after which cultures were normalized. Cells were then heat shocked at 50 °C for 0-60 min and cooled for 2 min on ice. Cultures were diluted 1000-fold, plated on SD-His, and plates were incubated at 30 °C for 2-3 days to observe viable colonies.

**Semi-Denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE)**

SDD-AGE was performed as described (Halfmann and Lindquist, 2008). To prepare lysates, yeast strains were grown to saturation in raffinose dropout media (SRaff-His-Ura) at 30 °C, then transferred to 10 mL galactose media (SGal-His-Ura) and grown for an additional 16h to induce polyGlu and Hsp104 expression. Following overnight induction, cells were normalized, washed with sterile water, and resuspended in spheroplasting solution (1.2 M D-sorbitol, 0.5 mM MgCl₂, 20 mM Tris pH 7.5, 50 mM β-ME, and 0.5 mg/mL Zymolyase 100T) and incubated for 1 hour at 30 °C with intermittent shaking. Spheroplasts were pelleted by centrifugation (500xg for 5 min) and resuspended in lysis buffer (100 mM Tris pH 7.5, 500 mM NaCl, 10 mM β-ME, Protease inhibitor cocktail (Sigma P8215), 10 mM EDTA, 2 mM PMSF, and 0.10% Triton-X 100). This suspension was vortexed at high speed for 2 minutes to lyse spheroplasts. Lysates were combined with 4X sample buffer (2X TAE, 20% glycerol, 8% SDS, 10% β-ME, and 0.0025% bromophenol blue) and incubated for 5 minutes at room temperature. Samples were loaded onto a 1.5% agarose gel in TAE buffer containing 0.1% SDS. The gel was run at 3 V/cm gel length for 5 h. Proteins were transferred to a nitrocellulose membrane by capillary transfer overnight. The nitrocellulose membrane was blocked and probed for Rnq1 conformers with an anti-Rnq1 polyclonal antibody.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

All data points in each graph are mean ± SEM, and the n is a biological replicate when referring to yeast experiments or an independent experimental trial when referring to biochemical experiments. No statistical significance testing was employed in this study.
Supplemental Information

Structure of *Calcarisporiella thermophila* Hsp104 Disaggregase that Antagonizes Diverse Proteotoxic Misfolding Events

Karolina Michalska, Kaiming Zhang, Zachary M. March, Catherine Hatzos-Skintges, Grigore Pintilie, Lance Bigelow, Laura M. Castellano, Leann J. Miles, Meredith E. Jackrel, Edward Chuang, Robert Jedrzejczak, James Shorter, Wah Chiu, and Andrzej Joachimiak
Figure S1. Sequence alignment of Hsp104s and TtClpB related to CtHsp104 five domains section and Figure 1. Color bars show domain ranges as in Figure 1A.
Figure S2. Structure of C-terminal region of CtHsp104 related to CCT and Figure 1. (A) C-terminal region of CtHsp104 shown in 2DFo-mFc electron density map contoured at 1.2σ level. Hydrogen bonds are shown as red lines. Protomer \( p \) is colored grey and protomer \( p-1 \) is colored pink. (B) Superposition of CtHsp104\textsuperscript{2R} (grey and pink) with ChtHsp104 (green, (Heuck et al., 2016)) and TtClpB (yellow, (Lee et al., 2003)). (C) Sequence alignment of C-terminus of CtHsp104, ChtHsp104, ScHsp104 and TtClpB (Prepared with ESPript (Gouet et al., 2003)).
Figure S3. Structure of individual CftHsp104 protomers resolved by cryo-EM related to single particle reconstruction of CftHsp104 section and Figures 3 and 4. Hexameric complex fitted to cryo-EM map (top left), and pairs of individual protomers arranged so as to show interactions (top row). On the top row, the black arrow points to the MD domains from P6, P4 and P2 which are in different position with respect to the protomers P5, P3 and P2. These different positions may be dependent on nucleotide binding or other arrangements of NBD1 and NBD2 based on the position of the protomer in the complex. The bottom row shows the protomers expanded but in the same orientation they have in the complex. The relative step along the spiral is also indicated. Similar steps are seen for P5, P4 and P3 with respect to P6, however bigger steps are seen for P2 and P1.
Figure S4. Comparison of individual CtHsp104 and ScHsp104 protomers resolved by cryo-EM related to single particle reconstruction of CtHsp104 and segmentation and fitting sections and Figure 4. Linearly arranged protomers 1 through 6 from three cryo-EM data sets: CtHsp104 (top), ScHsp104-AMP-PNP (Yokom et al., 2016), and ScHsp104-ATP (Gates et al., 2017). An arrow indicates rise along helical axis from one protomer to the next.
Figure S5. Nucleotide binding sites in CtHsp104 protomers related to single particle reconstruction of CtHsp104 and nucleotide binding by CtHsp104 sections and Figure 5. Nucleotide is highlighted in red.
Figure S6. Sample cryo-EM density maps related to nucleotide binding by CfHsp104 section and Figures 5 and S5. Top: Protomers 4 and 5 (left) and a close up on the nucleotide-binding pocket (right). The density for the ADP molecule bound to P5 is clearly visible, as are two interacting arginine residues (196 and 327) from P4. Note that the latter are not as well resolved in other protomers, which have lower relative ADP-occupancy. Bottom: Three fragments of the density showing resolved helices and beta sheets in P4, as expected at ~4 Å resolution.
Figure S7. CtHsp104 disaggregate activity cannot be induced by mixtures of ATP and ATPγS in the absence of Hsp70 and Hsp40 related to CtHsp104 is an ATP-driven disaggregate section and Figure 6. (A) Luciferase disaggregation by the indicated Hsp104 in the presence of 50% ATPγS at the indicated temperatures. Values represent means ± SEM (n=3), and are normalized to ScHsp104 at 30°C. (B) Luciferase disaggregation by the indicated Hsp104 in the presence of varying amounts of ATPγS at 25°C. Values represent means ± SEM (n=3).
Figure S8. The effect of ADP on MD conformation related to cryo-EM structure and Figures 4 and 5. (A) The NBD1 ADP binding site is directly adjacent to helices C1 (residues 339-357) and C3 (residues 384-402) (bright blue for emphasis, with several resolved side chains), which is in turn with the MD domain (green). Hence, binding of ADP and subsequent hydrolysis potentially directly affects the conformation of the MD. (B) The same ADP-affected region shown for two protomers, P4 (with the highest ADP occupancy for NBD1), and P1 (the lowest ADP occupancy). The presence of ADP favors a conformation where the two helices C1/C3 (bright blue) are pulled towards the ADP, in turn pulling the MD domain inward as well. Crystal structure is most similar to protomer 4, i.e. corresponding to high ADP occupancy state.
Supplementary Table

Table S1. Molecular weight of \textit{CtHsp104} constructs as determined by SEC and oligomerization state related to size exclusion chromatography of \textit{CtHsp104} and deletion mutants section and Figure 1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Protein construct (AA range)</th>
<th>SEC ( M_r ) (kDa)</th>
<th>MW monomer</th>
<th>Expected MW Hexamer</th>
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<tr>
<td>\textit{CtHsp104}</td>
<td>1-888</td>
<td>613.6</td>
<td>98.7</td>
<td>592.2</td>
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<tr>
<td>\textit{CtHsp104}^{4N}</td>
<td>153-888</td>
<td>522.0</td>
<td>81.8</td>
<td>490.9</td>
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<tr>
<td>\textit{CtHsp104}^{4N,ΔC}</td>
<td>153-864</td>
<td>521.5</td>
<td>79.8</td>
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<td>\textit{CtHsp104}^{4N,DBW}</td>
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<td>\textit{CtHsp104}^{4N,2R}</td>
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