Heat shock protein 104 (HSP104) is a conserved AAA+ protein disaggregate, can disassemble the toxic aggregates formed by different amyloid proteins, and is protective in various animal models associated with amyloid-related diseases. Extensive studies have attempted to elucidate how HSP104 disassembles the aggregated form of clients. Here, we found that HSP104 exhibits a potent holdase activity that does not require energy, prevents the soluble form of amyloid clients from aggregating, and differs from HSP104's disaggregate activity. Using cryo-EM, NMR, and additional biophysical approaches, we found that HSP104 utilizes its small subdomain of nucleotide-binding domain 2 (ssNBD2) to capture the soluble amyloid client (K19 of Tau) independent of its ATP hydrolysis activity. Our results indicate that HSP104 utilizes two fundamental distinct mechanisms to chaperone different forms of amyloid client and highlight the important yet previously unappreciated function of ssNBD2 in chaperoning amyloid client and thereby preventing pathological aggregation.

Proteostasis is crucial in maintaining cellular function (1–3), imbalance of which may lead to numerous human diseases such as Alzheimer’s disease, type 2 diabetes, peripheral amyloidosis, and cystic fibrosis (1, 4, 5). Proteostasis is maintained by an integrated network of different proteins, where molecular chaperones act as the key players for protein folding, refolding, disaggregation, and degradation (1, 6). Chaperones are classified into several families by molecular weight, e.g., HSP100, HSP90, HSP70, HSP60, HSP40, and small HSPs (1). Different chaperones fulfill their individual functions with distinct chaperone activities including (i) holdase activity by, e.g., small HSPs and HSP40 (7); (ii) foldase activity by, e.g., HSP60 (8); and (iii) disaggregate activity by, for example, HSP104 (4, 9). The different activities of chaperones are defined by the different domain compositions and arrangements (10, 11). Thus, structural studies on each individual domain and their cooperations are crucial to reconstitute the complete activity of the whole chaperone and to better understand the function of the chaperone under normal and disease conditions.

HSP104, a member of the HSP100 family, plays an essential role in thermostolerance, prion inheritance, and protein quality control in yeast (9, 12). Unlike its HSP100 homologs from other organisms, HSP104 exhibits a potent disaggregate activity not only on amorphous protein aggregates but also on a variety of pathological amyloid fibrils with cross-β structures such as Tau, Aβ, α-synuclein, and TDP43 (13–18). Moreover, HSP104 can alleviate the proteotoxicity of amyloid aggregates of polyQ, α-synuclein, and TDP43 in different animal models including worms, flies, and rodents (19–21). HSP104 consists of five domains (22). The N-terminal domain (NTD) is involved in client engagement (11, 23). The middle domain (MD) is for interaction with HSP70 and regulation of disaggregate activity (24–26). The two AAA + nucleotide-binding domains (NBD1 and NBD2) drive client translocation by hydrolyzing ATP (27). The C-terminal domain is required for HSP104 self-assembly (28). Recent studies revealed that HSP104 forms a hexamer that arranges in a spiral architecture (29). Based on cryo-EM structures of HSP104 in multiple translocation states, a rotary translocation model has been proposed to explain how HSP104 depolymerizes aggregated client proteins through its central channel upon ATP hydrolysis (30).

In this study, we found that, in addition to disassembling aggregated proteins, HSP104 can act as a holdase to capture the soluble form of amyloid client K19 of Tau and protect it from pathological aggregation.

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This article contains supporting text, Tables S1 and S2, and Figs. S1–S7. To whom correspondence may be addressed: Interdisciplinary Research Center on Biology and Chemistry, Shanghai Institute of Organic Chemistry, Chinese Academy of Sciences, 26 Qiuyue Rd., Shanghai 201210, China. E-mail: lixueming@tsinghua.edu.cn.

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amyloid aggregation. This process only requires the presence of HSP104 and K19 and thus is distinct from the disaggregase activity of HSP104, which requires ATP and co-chaperones (31). By combining multiple biophysical approaches, we revealed that HSP104 utilizes its small subunit of NBD2 to bind the key amyloidogenic core segment of Tau for modulating its amyloid aggregation. Our results provide the structural basis of the interplay between HSP104 and soluble amyloid client, suggesting that HSP104 utilizes distinct strategies to tackle different forms of amyloid clients.

**Results**

**HSP104 exhibits the holdase activity distinct from its disaggregate activity**

HSP104, acting as a disaggregate of amyloid fibrils, has been intensively investigated (13, 32), although we found that HSP104 can efficiently modulate the amyloid aggregation of K19 (33), the three-repeat isoform that forms the fibril core of Tau. As shown by the thioflavin T (ThT) florescence kinetic assay in Fig. 1, A and C, HSP104 modulated K19 aggregation in an ATP-independent manner. The error bars denote means ± S.D. with n = 3. B and D, the TEM images of K19 fibrils with or without HSP104 in the ThT assays. The samples with the lowest concentration of HSP104 (K19:HSP104 = 125:1) illustrated some disperse bundles of mature fibrils. The samples with the highest concentration of HSP104 (K19:HSP104 = 5:1) exhibited almost no fibrils structures but small and amorphous aggregates. E and F, comparison of HSP104 WT with HSP104A503V, HSP104N728A, and HSP104ΔN with respect to K19 aggregation modulated by HSP104 in the presence of 1% (w/w) seeds. The ThT value was taken at the time point of 45.04 h from the ThT kinetics curves. The error bars denote means ± S.D. with n = 3. *** indicates significant difference at α = 0.01 by LSD test.
HSP104 chaperones amyloid client as a holdase

To further exclude the contribution of the disaggregase activity in modulating K19 aggregation, we prepared three HSP104 variants, i.e. A503V, N728A, and the N-terminal truncation (∆N). A503V possesses hyperactivity of ATP hydrolysis and enhanced disaggregase activity (32). N728A exhibits impaired ATP hydrolysis (27). ∆N eliminates its client recognition indispensable for its amyloid disaggregase activity (11, 23). The ThT results showed that no significant difference was observed between HSP104 WT and the three variants with respect to the chaperone activity of HSP104 against K19 aggregation (Fig. 1, E and F).

We next measured the binding affinity between HSP104 and K19 by bio-layer interferometry (supporting methods). As shown in Fig. S2, HSP104 binds to K19 with an equilibrium dissociation constant (K_{D}) of 1.08 µM. Taken together, our results demonstrate that HSP104 possesses a previously unappreciated holdase activity to bind the soluble form of K19 and modulate its aggregation, which is distinct from the disaggregase activity driven by ATP and concerted with co-chaperones.

The cryo-EM structure of HSP104^{N728A}

We next sought to determine the structure of HSP104 by using cryo-EM 3D reconstruction. We prepared WT and different mutations of HSP104 with/without nucleotide substrates (ATP, ATPγS, AMP-PNP, and ADP). Among them, the sample of HSP104^{N728A} mutant incubated with ATPγS showed the best homogeneity under cryo-EM. Thus, the HSP104^{N728A} sample was imaged on a Titan Krios microscope for single-particle 3D reconstruction. A total of 890 micrographs yielded 103,860 particles. These particles were subjected to 2D and 3D classifications, resulting in the reconstruction with no imposed symmetry of the complex at 6.78 Å resolution on the basis of the Fourier shell correlation value of 0.143 by 69,537 particles (Fig. 2A and Fig. S3 and Table S2). The final map comprises 74% of the single-particle data after sorting by 2D classification. The 3D classification identified no additional conformations. Thus, the structural model built from the final density map represents the predominant conformer of HSP104^{N728A} under this condition.

HSP104^{N728A} features an asymmetric hexamer which consists of three different layers: formed by the NTD, NBD1&MD, and NBD2, respectively (Fig. 3A and B) (29, 30). The cryo-EM density of NBD1 and NBD2 presents the highest resolution of 6 Å from local resolution estimation, whereas the NTD and MD are highly flexible with reduced cryo-EM density in three protomers (9–13 Å for NTD of P2 to P4 and 9–11 Å for MD of P3 to P5) (Fig. S3G). The density of six nucleotides was visualized in the pockets of NBD1 and NBD2. Notably, the orientation and the comparative position of NTD, MD, and the small subunit of NBD2 (ssNBD2) among different protomers are relatively diverse (Fig. 2C). The NTD rotates 60° clockwise from P2 to P3 and 110° anticlockwise from P3 to P4 (Fig. S4C). The MD helices L1 and L2 of P3 is 25° offset from those of P5 (Fig. 2, C and D). The intrinsically dynamic nature of NTD and MD within the hexamer was believed to be important for client recognition and regulation of HSP104 disaggregase activity (11, 23, 24, 30, 34). Intriguingly, unlike the ssNBD1, which is buried inside the hexamer, ssNBD2 is located on the outer side of the hexamer with conformational flexibility among protomers similar to that of NTD and MD (Fig. 2, C and E, and Fig. S4D).

HSP104 utilizes ssNBD2 to bind K19

To identify the domain of HSP104 involved in K19 binding, we constructed a series of HSP104 variants containing either a single domain or a combination of different domains and measured the chaperone activity of each variant against K19 aggregation, respectively (Fig. 3A). Notably, only the variants that contain NBD2 (i.e. HSP104 WT, ∆N, ∆NC, and NBD2) exhibit...
edited obvious chaperone activities against K19 aggregation (Fig. 3, B–E), indicating that NBD2 may bind K19 and account for the modulation of K19 aggregation (Fig. S1, C and D).

To further map the binding interface of NBD2 and K19, we performed cross-linking experiments coupled with MS using the cross-linker bis(sulfosuccinimidyl) suberate (BS3). As shown in Table 1 and Fig. 6B, two cross-linked pairs between NBD2 and K19 were identified, including Lys774 (HSP104) linked with Lys311 (K19) and Lys839 (HSP104) with Lys343 (K19). Intriguingly, the two linked sites of NBD2 are both located within ssNBD2 (residues 772–870). Thus, we subcloned the ssNBD2 and tested its effect on K19 aggregation. We found that ssNBD2 alone exhibited potent chaperone activity against K19 aggregation either in the presence or absence of heparin (supporting methods), recapitulating the activity of the full-length HSP104 (Fig. 4 and Figs. S1, E and F, and S5). On the other hand, deletion of ssNBD2 from full-length HSP104 (HSP104 1–774) abolished the chaperone activity of HSP104 against K19 aggregation (Fig. 4A and Fig. S6). The results indicate that HSP104 utilizes its ssNBD2 to capture the soluble form of K19 and modulate K19 aggregation. Thus, HSP104 adopts distinctive domains to bind clients for the holdase and disaggregase activity. The former involves the ssNBD2, whereas the latter is mainly fulfilled by the NTD (23, 31).

Furthermore, we performed the NMR titration experiments to identify the region of soluble K19 for ssNBD2 binding. By titrating ssNBD2 to 15N-labeled monomeric K19 (Fig. S7), we found that resonance of residues Val306–Thr319 exhibited remarkable intensity drops (I/I0 = 0.45), indicating the direct binding of ssNBD2 to the Val306–Thr319 region of K19 (Fig. 5A). This result is consistent with the cross-linking experiment, which shows that Lys711 and Lys743 of K19 are involved in the
interaction between NBD2 and K19 (Fig. 6B and Table 1). Notably, the primary ssNBD2 binding sites of K19 include the 306VQIVYK311 segment, which is the key amyloidogenic segment of K19 as well as other isoforms of Tau (e.g., Tau40 and K18) (15, 33). These data explained the potent chaperone activity of ssNBD2 against K19 aggregation.

In addition, titration of full-length HSP104 to 15N-labeled K19 consistently showed that residues Val306–Thr319 of K19 are involved in the binding with HSP104 (Fig. 5B). As a control, titration of HSP104 MD, which cannot interfere with K19 aggregation, resulted in no significant change of the NMR spectra (Fig. 5C). Taken together, the results revealed that HSP104 utilizes its ssNBD2 to capture K19 via the region containing its highly amyloidogenic segment 306VQIVYK311 (33).

**Discussion**

Despite that HSP104 was initially identified in yeast as the key chaperone for thermo-tolerance and prion propagation (35, 36), it drew an increasing general interest because of its potent disaggregase activity to various pathological amyloids and its protective role in a variety of models of neurodegenerative diseases (13, 19–21, 32, 37). Previous studies mainly focused on the molecular mechanism underlying the disaggregase activity of HSP104 (11, 29, 30, 38), whereas in this study, we found that HSP104 can perform as a holdase to bind the soluble form of amyloid clients and prevent them from aggregation. Several chaperones have been reported to possess more than one chaperone activity (39). For example, HSP70 exhibits holdase, foldase, and disaggregase activities (39, 40). However, HSP70 utilizes a similar client recognition mechanism for different chaperone activities (41, 42). In contrast, HSP104 recognizes distinct forms of amyloid clients and utilizes two fundamentally different mechanisms to fulfill distinctive chaperone activities.

As summarized in Fig. 6A, HSP104 hexamer recognizes the aggregated form of amyloid clients by its NTD in corporation with its co-chaperones and transfers clients across the central channel formed by the two NBDs fueled by ATP hydrolysis for disaggregation of the clients (30). In contrast, as for the soluble form of amyloid clients, HSP104 uses its solvent-exposed ssNBD2 to capture clients and prevents them from aggregation without requirement of ATP binding and hydrolysis. Although HSP104 can fulfill its holdase activity by ssNBD2 alone, it

**Table 1**

Identified cross-linked peptides between HSP104 and K19

| CL    | Enz   | E value | Mass  | ∆     | Pr | S | E | Res | Sequence          |
|-------|-------|---------|-------|-------|----|---|---|-----|------------------|
| BS³   | Trypsin | 6.04E⁻¹⁵ | 2902.706 | 1.559237 | Pr1 | NBD2 | 766 | 777 | 774 | ISSIVIFKNLSR      |
| BS³   | Trypsin | 4.68E⁻¹³ | 2812.591 | −0.818817 | Pr1 | NBD2 | 831 | 843 | 839 | LIONETINKLALR     |

**Figure 4. The ssNBD2 of HSP104 modulates K19 amyloid aggregation.** A, comparison of HSP104 WT with its variants ssNBD2 and HSP104 1–774 in modulating K19 (100 μM) amyloid aggregation. The error bars denote means ± S.D. with n = 3. *** indicates significant difference at α = 0.01 by LSD test. N.S. stands for not significant. B, the ThT kinetics of K19 aggregation (100 μM) with 1% (w/w) seeds in the presence of HSP104 ssNBD2 with different molar ratios of K19:ssNBD2 as indicated. The error bars represent means ± S.D. with n = 3. C, the representative TEM images of K19 fibrils with or without the presence of ssNBD2. The samples were collected from the assays shown in B at the time point of 49.99 h. Scale bars represent 500 nm.
remains unclear whether this holdase activity is involved in the process of disaggregation of aggregated clients. Because ssNBD2 is located at the exit of HSP104 spiral ring where the translocated polypeptide is supposed to be released, it is possible that ssNBD2 is involved in stabilizing the translocating polypeptide and facilitating the release of the polypeptide until it entirely passes through the channel. As the small subdomain of a typical AAA+/H11001 nucleotide-binding domain, ssNBD2 contains a conserved sensor-2 motif (Arg826) and is involved in ATP binding (43). Our work suggests that the amyloid-binding surface of ssNBD2 contains both hydrophobic and charged patches (Fig. 6, B–D). Intriguingly, a combination of hydrophobic and electrostatic interactions was previously found to mediate client binding by HSP90 and Spy (44–47). Thus, ssNBD2 may utilize a similar strategy for client binding. Our work reveals the mechanism of such a small domain in reducing amyloid aggregation, which may help to understand the function of its parent protein HSP104 and shed light on the development of inhibitors for potential therapeutics of amyloid-related diseases.

**Experimental procedures**

**Proteins expression and purification**

All HSP104 constructs were cloned into a pPROEX HTb vector (Invitrogen) with an N-terminal His<sub>6</sub> tag and a following tobacco etch virus protease cleavage site. The plasmid encoding full-length HSP104 (pGALSc104b) was a gift from Dr. James Shorter. The HSP104<sup>Δ501V</sup> and HSP104<sup>N728A</sup> were generated by site-directed mutagenesis. All of the constructs were confirmed by DNA sequencing. HSP104, HSP104<sup>Δ501V</sup>, and HSP104<sup>N728A</sup> were expressed and purified as reported previously (48).
The truncations of HSP104 residues 164–908 (HSP104ΔN), 164–870 (HSP104ΔNC), 1–774 (HSP104 1–774), 164–538 (HSP104 ΔD1M), 411–538 (HSP104 ΔMD), 556–870 (HSP104 NBD2), and 772–870 (HSP104 ssNBD2) were generated by PCR and subcloned between the BamHI and XhoI sites of pPROEX-HTb vector. Plasmid constructions were confirmed by DNA sequencing. All of the truncated HSP104 variants were expressed in codon-optimized *Escherichia coli* BL21-Codon-Plus cells. Cultures were grown at 37 °C to an approximate *A*$_{600}$ of 0.6 and were induced with 500 μM isopropyl-β-D-thiogalactopyranoside overnight at 16 °C. The cells were lysed by a high-pressure homogenizer in 40 mM HEPES-KOH, pH 7.4, 500 mM KCl, 20 mM MgCl$_2$, 5% (w/v) glycerol, 20 mM imidazole, 2 mM β-mercaptoethanol, and complete protease inhibitor mixture (1 EDTA-free tablet/50 ml). Cell debris was removed by centrifugation (15,000 × g, 45 min, 4 °C), and the supernatant was loaded on a nickel column (GE Healthcare). The column was then washed with 5 volumes of nickel buffer (40 mM HEPES-KOH, pH 7.4, 500 mM KCl, 20 mM MgCl$_2$, 5% (w/v) glycerol, 40 mM imidazole, and 2 mM β-mercaptoethanol). Proteins were eluted with nickel buffer plus 350 mM imidazole. Peak fractions were collected, and the buffer was exchanged into 40 mM HEPES-KOH, pH 7.4, 500 mM KCl, 20 mM MgCl$_2$, 2.5% (w/v) glycerol, and 1 mM DTT. The proteins were further purified by gel filtration using a Superdex 200 or 75 column (GE Healthcare). The gene of human K19 of Tau was constructed in a vector of pNG2 with the restriction sites of NdeI and BamHI. The resulting construct was verified by DNA sequencing (GENEWIZ, Inc. Suzhou, China). The expression of K19 was induced in *E. coli* BL21 (DE3) by addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside with the *A*$_{600}$ of 0.4–0.6 and grew overnight at 20 °C. The cells were then harvested by centrifugation at 4,000 rpm for 20 min at 4 °C. The cell pellets were resuspended in the lysis buffer of 50 mM Tris-HCl, pH 8.0, 1 mM DTT, 1 mM PMSF, and complete protease inhibitor mixture (1 EDTA-free tablet/50 ml). The sample was then homogenized by a high-pressure homogenizer and subsequently boiled for 30 min, followed by addition of NaCl to a final concentration of 500 mM. The soluble extract was isolated by centrifugation at 15,000 × g for 30 min and was dialyzed against 25 mM NaH$_2$PO$_4$, pH 6.0, 1 mM DTT at 4 °C overnight. The dialysate was loaded on a HighTrap HP SP 5-ml column (GE Healthcare) and eluted by a linear gradient of up to 60% buffer B (25 mM NaH$_2$PO$_4$, pH 6.0, 1 mM NaCl, and 1 mM DTT). The eluted proteins were further loaded to a Superdex-75 column (GE Healthcare) in a buffer of 50 mM Tris-HCl, pH 8.0, 150 mM KCl, and 1 mM DTT for further purification. The purified Tau protein was concentrated to 40 mg ml$^{-1}$ and stored at −80 °C.

**Thioflavin T fluorescence assay**

The ThT assay for amyloid aggregation of K19 of Tau was performed in a buffer containing 50 mM Tris-HCl, 150 mM KCl, and 0.03% (w/w) NaN$_3$ at pH 8.0. HSP104 WT and variants

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Figure 6. A model proposed for HSP104 to chaperone different forms of amyloid clients and mapping of surface properties on ssNBD2. **A**, the schematic diagram shows that on one hand, HSP104 hexamer recognizes the aggregated form of amyloid clients by its NTD in cooperation with co-chaperones and extracts individual polypeptides from the aggregated structures. The polypeptides are translocated through the central channel of HSP104 hexamer by a ratchet-like mechanism coupled with ATP binding and hydrolysis (30). On the other hand, HSP104 can bind the soluble form of amyloid clients by its solvent-exposed ssNBD2 and holds it from aggregation, which is independent of ATP hydrolysis. The asterisk indicates that the holdase activity may be involved in the process of the disaggregation. **B**, the residues Lys$^{774}$ and Lys$^{839}$ identified to cross-link with K19 are shown as red spheres in the ribbon diagram of HSP104 ssNBD2. **C**, the hydrophobicity of the ssNBD2 surface calculated by Chimera (60). **D**, surface potential of ssNBD2 calculated by the PyMOL Molecular Graphics System (version 1.7.0.0, Schrödinger, LLC).
were premixed with K19 at substoichiometric ratios between 5:1 and 125:1 (K19:HSP104). 60 μl of premixed solution was added to each well in a 384-well black flat-bottomed nonbinding plate (Thermo Fisher Scientific). The final concentrations of the mixed solution were 100 μM K19, 40 μM heparin (Sigma, CAS: 9041-08-1), 50 μM ThT, 1% (w/w) K19 fibril seeds (the K19 fibril seeds were obtained by 15-s sonication) and in the absence or presence of different concentrations of HSP104 (49).

The plate was covered with a clear polyolefin sealing tape (Thermo Fisher Scientific) to prevent evaporation and placed in a FLUOstar Omega microplate reader (BMG Labtech). Samples were shaken using 600 rpm at 37 °C, and fluorescence was measured using 440- and 485-nm filters for excitation and emission, respectively. The samples were measured in triplicate with average fluorescence values and standard deviation plotted. At least three independent biological repeats were performed. The data statistical analysis was performed in SAS/STAT version 9.3 (SAS Institute Inc., Cary, NC) using the PROC GLM according to least significant difference (LSD) test at α = 0.05 or 0.01.

**BS3 cross-linking and MS analysis**

The purified HSP104 and K19 were cross-linked with BS3 (Thermo Fisher Scientific, 21585). The spacer arm length of BS3 is 11.4 Å. Cross-linking was performed in 40-μl reactions containing 20 μM HSP104 NBD2, 80 μM K19, and 800 μM BS3. The reactions were incubated for 30 min at room temperature and quenched by adding 5.4 μl of 1 M Tris-HCl, pH 8.0. The proteins were precipitated with acetone and subjected to trypsin (Promega) digestion. LC-MS/MS analyses of the digested samples were performed on an ultra HPLC EASY-nLC 1000 system coupled with an online Q-Exactive HF-X mass spectrometer (Thermo Fisher Scientific). The pLink software (50) was used to identify cross-linked peptides, and the results were filtered by applying a 5% FDR cutoff at the spectral level and then an E value cutoff at 10^-6.

**NMR spectroscopy**

NMR experiments were conducted on a Bruker 900 MHz spectrometer equipped with a cryogenic TXI probe at 25 °C. 1H-15N-Labeled K19 was dissolved at a concentration of 50 μM in 20 mM HEPES-KOH, 190 mM KCl, 10 mM MgCl2, 2.5% (w/v) glycerol, pH 7.0, containing 10% (v/v) D2O in the presence or absence of the HSP104 WT, MD, and ssNBD2, respectively. For 1H-15N heteronuclear single-quantum correlation measurements, the spectra were collected with 16 scans per transient and complex points of 2048 × 160. NMR spectra were processed with the program NMRPIPE (51) and analyzed with Sparky (52). Chemical shift changes (Δδ) were calculated using the following equation, \( \Delta \delta = \text{SQRT}((\Delta \delta^{1H^3})^2 + 0.17(\Delta \delta^{15N})) \), whereas \( \Delta \delta^{1H} \) and \( \Delta \delta^{15N} \) are the chemical shift differences of amide proton and amide nitrogen between free and bound state of the protein, respectively.

**EM data acquisition and processing**

Negative-staining samples were prepared using 2% (w/v) uranyl acetate. A drop of 4 μl of purified HSP104N728A at a concentration of 0.01 mg ml^-1 was applied to a glow-discharged holy carbon EM grid covered with a thin layer of carbon film (Beijing Zhongjingkeyi Technology Co. Ltd). The grid was stained by 2% (w/v) uranyl acetate solution for 1 min, blotted using a filter paper, and then washed with buffer and uranyl acetate sequentially. The negative-stained grid was then transferred into an FEI Tecnai T12 electron microscope operated at an acceleration voltage of 120 kV to manually collect micrographs using a Gatan US4000 4k × 4k CCD camera.

For cryo-EM sample preparation, the HSP104N728A (1.1 mg ml^-1) was incubated with ATPγS (2 mM) for 30 min at 25 °C in a buffer containing 20 mM HEPES-KOH, pH 7.4, 150 mM KCl, 20 mM MgCl2, 2% (w/v) glycerol, and 1 mM DTT. A drop of 3.5 μl of sample was applied to a glow-discharged Quantifoil holy carbon grid (R1.2/1.3, 300 mesh). After waiting for 30 s, the grid was blotted for 3.0 s (at 100% humidity and 8 °C) and plunged into liquid ethane cooled by liquid nitrogen using FEI Vitrobot IV. The samples were observed using an FEI Titan Krios microscope operated at 300 kV, equipped with a Gatan K2 Summit camera. UCSF Image4 was used for data collection under a defocus range of 2.0–3.0 μm and nominal magnification of ×22,500, corresponding to pixel size 0.66 Å of super-resolution counting mode (53). Each micrograph was dose-fractionated to 32 frames with 0.25-s exposure time in each frame. The dose rate was 8.2 counts per physical pixel per second, and the total dose was ~ 50 e/Å².

**Image processing and model building**

For the cryo-EM data set, a total of 890 super-resolution micrographs were 2 × 2 binned, yielding an image stack with a pixel size of 1.32 Å. Motion correction was performed using the MotionCorr program, which output motion-corrected integrated images for further processing (54). CTFIND3 was used to determine defocus parameters (55). A total of 185,690 particles were semiautopicked and extracted by RELION1.4 with a pixel size of 2.64 Å (56). All subsequent 2D and 3D image analyses were performed with RELION 1.4 (56) with no symmetry imposed. After several rounds of 2D classification, those particles in the classes with fuzzy class averages were considered as bad particles and excluded from further analysis. Finally, 103,860 particles were selected for further 3D classification. A previously determined cryo-EM 3D reconstruction of AMP-PNP–HSP104 (EMD-8267) (29), low-pass-filtered to 60 Å, served as an initial model for 3D classification and refinement. After two rounds of 3D classification, 69,537 particles were selected and subjected to 3D autorefinement. Then particle polishing and further 3D autorefinement were applied, which resulted in a final map at 6.78 Å resolution estimated with the gold-standard Fourier shell correlation 0.143 criterion (57). Local resolution was estimated using Resmap (58). The final map was used for all rigid-body docking and flexible fitting of atomic structures. The structural model was built first by fitting the cryo-EM structures of the HSP104-ADP (Protein Data Bank code 5VY8) (30), and the individual N-terminal domains of HSP104-AMPPNP (Protein Data Bank code 5KNE) (29) to the EM density map using UCSF Chimera (59). Flexible fitting was further refined using phenix.real_space_refine (60) with secondary structure constraints. Manual modeling of the small molecular were performed in Coot (61). All figures of the struc-
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tures were generated by either UCSF Chimera (59) or PyMOL (version 1.7.0.0 Schrödinger, LLC).

Data availability

The atomic coordinate and cryo-EM maps of HSP104*NT28A are available at the Protein Data Bank and Electron Microscopy Data Bank databases. The accession numbers are 6AHF and EMD-9625, respectively.

Author contributions—X. Z., D. L., and C. L. writing-original draft; X. Z. and C. L. writing-review and editing; L. Z. and F. L. methodology; X. L. and C. L. supervision; C. L. funding acquisition; X. Z. purified proteins and carried out biochemical analyses, performed the EM experiments, and determined Hsp104 structure with help of L. Z. and F. L.; S.Z. performed the NMR experiment.; J.L. and C.Z. purified part of amyloid proteins; X. L. supervised the cryo-EM experiments.

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