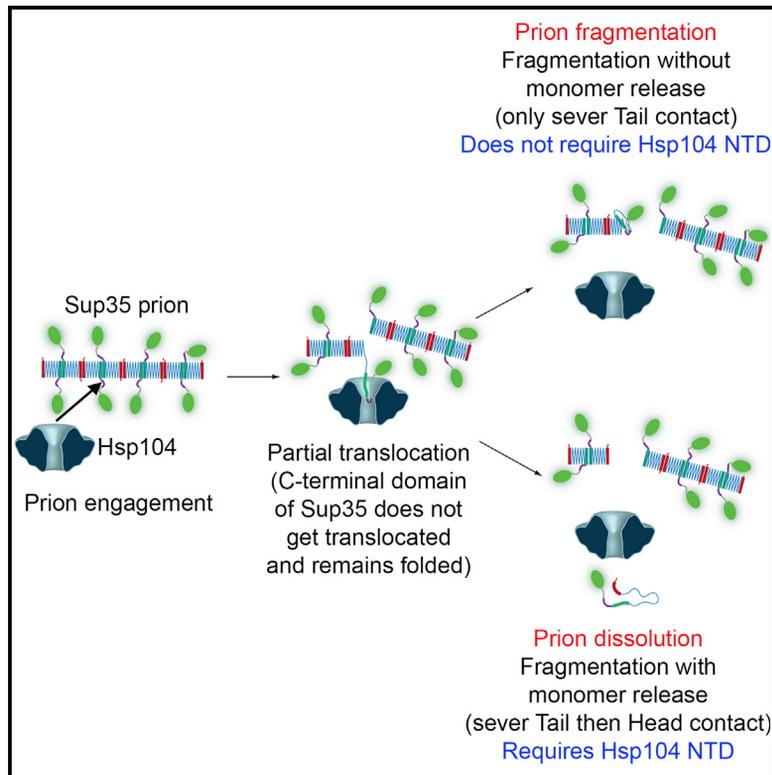


# The Hsp104 N-Terminal Domain Enables Disaggregase Plasticity and Potentiation

## Graphical Abstract



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## In Brief

Sweeny et al. employ small-angle X-ray scattering to reveal that a peristaltic pumping mechanism underpins Hsp104 disaggregase activity. They also define the mechanism by which Hsp104 dissolves Sup35 prions and elucidate that the Hsp104 N-terminal domain enables disaggregase plasticity and potentiation.

## Highlights

- Hsp104 N-terminal domain confers plasticity that is critical for prion dissolution
- Detailed mechanism of how Hsp104 engages, fragments, and dissolves Sup35 prions
- SAXS reconstructions of Hsp104 hexamers reveal peristaltic pumping mechanism
- Hsp104 N-terminal domain is critical for activity of potentiated Hsp104 variants



# The Hsp104 N-Terminal Domain Enables Disaggregase Plasticity and Potentiation

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## SUMMARY

The structural basis by which Hsp104 dissolves disordered aggregates and prions is unknown. A single subunit within the Hsp104 hexamer can solubilize disordered aggregates, whereas prion dissolution requires collaboration by multiple Hsp104 subunits. Here, we establish that the poorly understood Hsp104 N-terminal domain (NTD) enables this operational plasticity. Hsp104 lacking the NTD (Hsp104<sup>ΔN</sup>) dissolves disordered aggregates but cannot dissolve prions or be potentiated by activating mutations. We define how Hsp104<sup>ΔN</sup> invariably stimulates Sup35 prionogenesis by fragmenting prions without solubilizing Sup35, whereas Hsp104 couples Sup35 prion fragmentation and dissolution. Volumetric reconstruction of Hsp104 hexamers in ATP<sub>γ</sub>S, ADP-AIF<sub>x</sub> (hydrolysis transition state mimic), and ADP via small-angle X-ray scattering revealed a peristaltic pumping motion upon ATP hydrolysis, which drives directional substrate translocation through the central Hsp104 channel and is profoundly altered in Hsp104<sup>ΔN</sup>. We establish that the Hsp104 NTD enables cooperative substrate translocation, which is critical for prion dissolution and potentiated disaggregase activity.

## INTRODUCTION

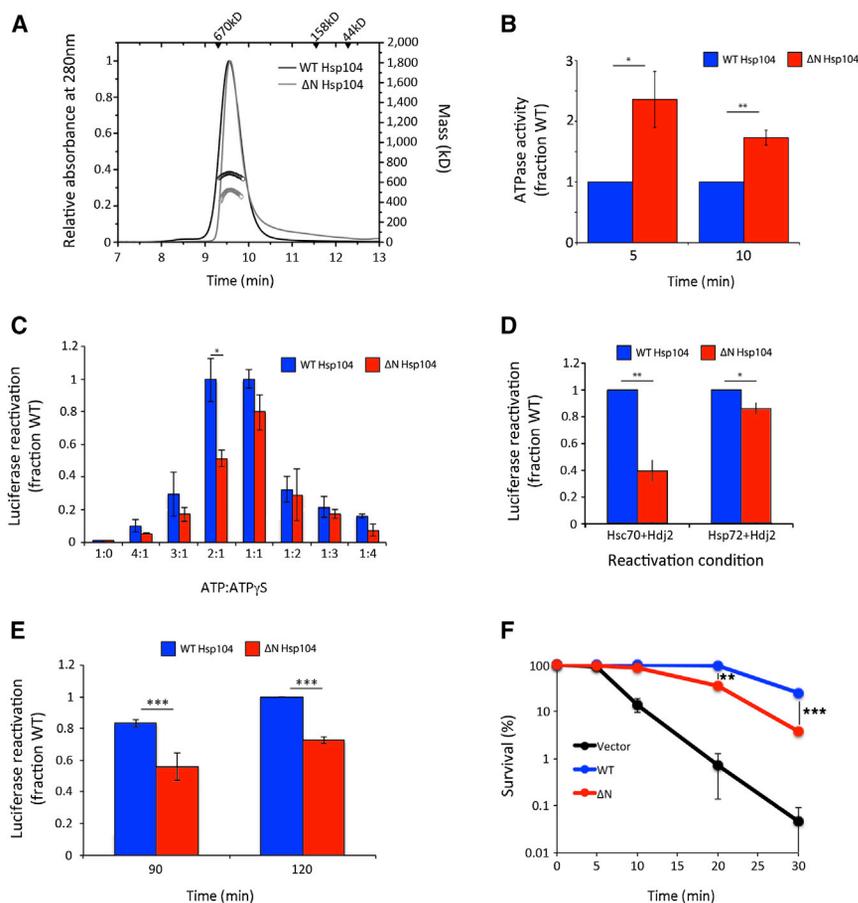
Protein disaggregases hold potential to reverse protein aggregation and amyloidogenesis that underlie several fatal neurodegenerative disorders. Yet their structural and mechanistic basis of action is not understood. In yeast, a hexameric AAA+ protein, Hsp104, couples ATP hydrolysis to dissolution of disordered aggregates, preamyloid oligomers, and amyloid (Shorter, 2008). Curiously, metazoa lack an Hsp104 homolog. Thus, it could be valuable to translate these Hsp104 activities to counter neurodegenerative disease (Jackrel et al., 2014). In yeast, Hsp104

confers two major selective advantages (Shorter, 2008). First, Hsp104 confers tolerance to thermal and chemical stress by re-activating proteins trapped in disordered aggregates. Second, amyloid remodeling by Hsp104 enables yeast to deploy prions for adaptive purposes.

Hsp104 forms dynamic ring-shaped hexamers, which exchange subunits on the minute timescale (DeSantis et al., 2012; Wendler et al., 2007). Hsp104 harbors an N-terminal domain (NTD), two AAA+ nucleotide-binding domains (NBDs) that hydrolyze ATP, and a coiled-coil middle domain (MD) inserted in NBD1. Hsp104 drives protein disaggregation by coupling ATP hydrolysis to partial or complete substrate translocation across its central pore via interaction with conserved tyrosine-bearing pore loops (Shorter, 2008). Yet, the conformational changes of the hexamer and its central channel that drive substrate translocation are poorly resolved. Indeed, the hexameric structure of Hsp104 is unknown, and conflicting models have arisen from cryo-electron microscopy (EM) reconstructions of dysfunctional Hsp104 mutants in a limited number of nucleotide states (Carroni et al., 2014; Lee et al., 2010; Wendler et al., 2007, 2009).

Hsp104 hexamers exhibit mechanistic plasticity and adapt distinct modes of intersubunit collaboration to disaggregate disordered aggregates versus amyloid. To disaggregate disordered aggregates, Hsp104 subunits within the hexamer collaborate noncooperatively via probabilistic substrate binding and ATP hydrolysis (DeSantis et al., 2012). By contrast, to resolve stable amyloid, several Hsp104 subunits within the hexamer cooperatively engage substrate and hydrolyze ATP (DeSantis et al., 2012). How this switch from noncooperative to cooperative mechanism occurs is not understood.

Hsp104 activity is potentiated by specific mutations in the MD (Jackrel et al., 2014). Potentiating mutations enable Hsp104 to dissolve fibrils formed by neurodegenerative disease proteins, including TDP-43, FUS, and  $\alpha$ -synuclein ( $\alpha$ -syn), and mitigate neurodegeneration under conditions where wild-type (WT) Hsp104 is inactive (Jackrel et al., 2014). These mutations reconfigure how Hsp104 subunits collaborate and increase plasticity such that robust disaggregase activity is maintained despite diverse subunit-inactivating events (Jackrel et al., 2014). The precise domain requirements that underpin potentiation as well as operational plasticity are unknown.



**Figure 1. Hsp104 $\Delta$ N Has Reduced Disaggregase Activity**

(A) Size-exclusion chromatography coupled to multiangle light scattering demonstrates that Hsp104 and Hsp104 $\Delta$ N form hexamers. A representative data set from three experiments is shown. (B) Hsp104 $\Delta$ N exhibits elevated ATPase activity compared to Hsp104. Values represent means  $\pm$  SEM (n = 3–4; \*p < 0.05; \*\*p < 0.01, two-tailed t test). (C) Luciferase aggregates were treated with Hsp104 or Hsp104 $\Delta$ N plus various ATP:ATP $\gamma$ S ratios. Values represent means  $\pm$  SEM (n = 3; \*p < 0.05, two-tailed t test). (D) Luciferase aggregates were treated with Hsp104 or Hsp104 $\Delta$ N with ATP plus Hdj2 and Hsp72 or Hsc70 and Hsc70. Values represent means  $\pm$  SEM (n = 3–7; \*p < 0.05; \*\*p < 0.01, two-tailed t test). (E)  $\Delta$ hsp104 yeast expressing luciferase and Hsp104 or Hsp104 $\Delta$ N were shifted to 44°C, treated with cycloheximide, and allowed to recover at 30°C. Luciferase activity (% WT control) was determined. Values represent means  $\pm$  SEM (n = 3; \*\*\*p < 0.001, two-tailed t test). (F)  $\Delta$ hsp104 yeast harboring empty vector or expressing Hsp104 or Hsp104 $\Delta$ N were treated at 37°C for 30 min and then 50°C for 0–30 min. Cells were plated and survival (%) calculated. Values represent means  $\pm$  SEM (n = 3; \*\*p < 0.01; \*\*\*p < 0.001, two-tailed t test).

and Hsp104 $\Delta$ N hexamers coordinate substrate translocation. Indeed, the Hsp104 NTD enables cooperative substrate translocation by Hsp104, which

is critical for potentiated activity and prion dissolution, but not for prion fragmentation.

## RESULTS

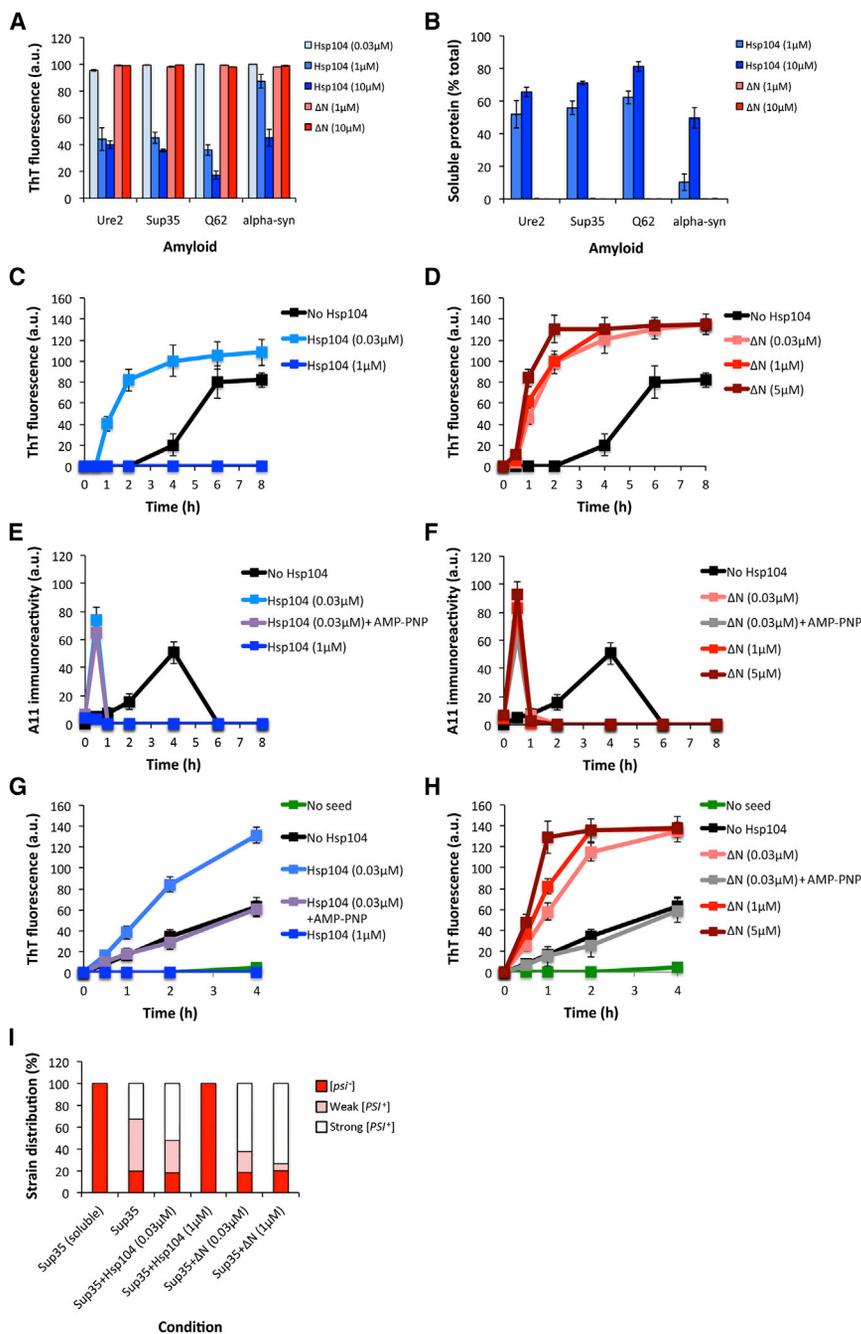
### Hsp104 $\Delta$ N Has Reduced Disaggregase Activity

Deletion of the Hsp104 NTD is reported to have minimal effect on disaggregase functionality (Hung and Masison, 2006; Lum et al., 2008). This is not what we found. Hsp104 $\Delta$ N was hexameric and had elevated ATPase activity (Figures 1A and 1B). Hsp104 and Hsp104 $\Delta$ N solubilized disordered aggregates without Hsp70 and Hsp40 when provided with mixtures of ATP and ATP $\gamma$ S, a slowly hydrolyzable ATP analog (Figure 1C). However, at every ATP:ATP $\gamma$ S ratio tested, Hsp104 $\Delta$ N was slightly less active than Hsp104 (Figure 1C). At 2 ATP:1 ATP $\gamma$ S, Hsp104 was optimally activated, but Hsp104 $\Delta$ N was only ~50% active (Figure 1C). This distinct sensitivity to activation by ATP $\gamma$ S suggests that Hsp104 $\Delta$ N hexamers are tuned differently than Hsp104 hexamers. Specifically, under these conditions, Hsp104 $\Delta$ N hexamers require more ATP $\gamma$ S relative to ATP for maximal disaggregase activity (Figure 1C).

Compared to Hsp104, Hsp104 $\Delta$ N displayed reduced disaggregase activity with different Hsp70s (Figure 1D). This deficit was most pronounced for Hsc70 and significant for Hsp72 (Figure 1D). Hsp104 $\Delta$ N had reduced ability to disaggregate luciferase and confer thermotolerance in vivo (Figures 1E and 1F), despite

Hsp104 harbors an NTD of poorly defined function, which is considered dispensable (Hung and Masison, 2006; Lum et al., 2008). The NTD of ClpB, the *E. coli* Hsp104 homolog, contributes to substrate binding and disordered aggregate dissolution (Barnett et al., 2005). However, several facets of Hsp104 activity are not conserved from ClpB (DeSantis et al., 2012, 2014). Unlike Hsp104, ClpB has limited ability to dissolve amyloid (DeSantis et al., 2012). Thus, whether NTD function is conserved from ClpB to Hsp104 is unclear. Indeed, replacing the Hsp104 NTD with the ClpB NTD disrupts prion propagation (Tipton et al., 2008). Hsp104 lacking the NTD (Hsp104 $\Delta$ N) supports [PSI<sup>+</sup>] (Sup35 prion) inheritance, which requires Sup35 prion fragmentation (Hung and Masison, 2006). Curiously, unlike Hsp104, overexpression of Hsp104 $\Delta$ N does not cure or inefficiently cures [PSI<sup>+</sup>] depending upon genetic background (Hung and Masison, 2006; Park et al., 2014). The direct effects of Hsp104 $\Delta$ N on Sup35 prionogenesis are unknown.

Here, we define critical NTD functions in enabling Hsp104 plasticity and potentiation. Using pure components, we establish that Hsp104 $\Delta$ N dissolves disordered aggregates but not prions. In contrast to Hsp104, which breaks N- and C-terminal intermolecular prion contacts to release soluble Sup35 and eliminate cross- $\beta$  structure, Hsp104 $\Delta$ N only breaks C-terminal intermolecular prion contacts and fragments Sup35 prions without solubilizing Sup35 or resolving cross- $\beta$  structure. These differences reflect profound alterations in how Hsp104



**Figure 2. Hsp104<sup>ΔN</sup> Cannot Dissolve Amyloid and Invariably Promotes Sup35 Prionogenesis**

(A and B) Sup35 or Ure2 prions, or Q62 or  $\alpha$ -syn amyloid, were treated with Hsp104 or Hsp104<sup>ΔN</sup> plus Ssa1, Sse1, and Sis1. Fiber integrity was assessed by ThT fluorescence (A) or sedimentation analysis (B). Values represent means  $\pm$  SEM (n = 3). (C and D) Kinetics of unseeded Sup35 prionogenesis without or with Hsp104 (C) or Hsp104<sup>ΔN</sup> (D) assessed by ThT fluorescence. Values represent means  $\pm$  SEM (n = 3).

(E and F) Unseeded Sup35 prionogenesis plus or minus Hsp104 (E) or Hsp104<sup>ΔN</sup> (F). In some reactions, ATP was replaced with AMP-PNP. At various times, the amount of A11-reactive species was determined. Values represent means  $\pm$  SEM (n = 3).

(G and H) Kinetics of Sup35 prionogenesis seeded by Sup35 prions plus or minus Hsp104 (G) or Hsp104<sup>ΔN</sup> (H) assessed by ThT fluorescence. In some reactions, ATP was replaced with AMP-PNP. Values represent means  $\pm$  SEM (n = 3).

(I) Reactions were performed as in (C) and (D). Reaction products were sonicated and transformed into [psi<sup>-</sup>] cells. The proportion of [psi<sup>-</sup>], weak [PSI<sup>+</sup>], and strong [PSI<sup>+</sup>] colonies was determined. Values represent means (n = 3). See also Table S1.

(Figures 2A and 2B). Strikingly, Hsp104<sup>ΔN</sup> did not release soluble protein (Figure 2B), which helps explain why Hsp104<sup>ΔN</sup> overexpression fails to cure Sup35 prions in some genetic backgrounds (Hung and Masison, 2006). The inability of Hsp104<sup>ΔN</sup> to disaggregate amyloid was not due to reduced binding affinity (see Table S1 available online). Thus, after initial engagement, some aspect of amyloid antagonizes Hsp104<sup>ΔN</sup>, but not Hsp104. We suggest that Hsp104<sup>ΔN</sup> subunits are unable to function in a globally cooperative manner to resolve amyloid.

**Hsp104<sup>ΔN</sup> Only Stimulates Sup35 Prionogenesis**

We investigated the interaction between Hsp104<sup>ΔN</sup> and Sup35 further. Thus, we

titrated Hsp104<sup>ΔN</sup> into de novo Sup35 prionogenesis in vitro, which is very sensitive to Hsp104 concentration. At low concentrations, Hsp104 stimulates spontaneous Sup35 prionogenesis by reducing lag phase and accelerating assembly phase (Figure 2C). At high concentrations, Hsp104 inhibits Sup35 prionogenesis (Figure 2C) (Shorter and Lindquist, 2006). By contrast, even at very high concentrations, Hsp104<sup>ΔN</sup> stimulated spontaneous Sup35 prionogenesis by reducing lag phase and accelerating assembly phase (Figure 2D). Thus, deletion of the Hsp104 NTD drastically alters the concentration-dependent effect of Hsp104 on Sup35 prionogenesis, such that inhibition of prion formation is

similar expression levels to Hsp104. Thus, deletion of the Hsp104 NTD reduces disaggregase activity in vitro and in vivo. Hsp104 dissolves disordered aggregates via a noncooperative mechanism that does not require collaboration between Hsp104 subunits within the hexamer. Indeed, a single active Hsp104 subunit within the hexamer can drive disaggregation (DeSantis et al., 2012). By contrast, amyloid dissolution requires cooperative ATP hydrolysis and substrate binding by several Hsp104 subunits (DeSantis et al., 2012). In contrast to Hsp104, which disaggregated Sup35, Ure2, polyglutamine, and  $\alpha$ -syn amyloid, Hsp104<sup>ΔN</sup> was ineffective even at high concentrations

titrated Hsp104<sup>ΔN</sup> into de novo Sup35 prionogenesis in vitro, which is very sensitive to Hsp104 concentration. At low concentrations, Hsp104 stimulates spontaneous Sup35 prionogenesis by reducing lag phase and accelerating assembly phase (Figure 2C). At high concentrations, Hsp104 inhibits Sup35 prionogenesis (Figure 2C) (Shorter and Lindquist, 2006). By contrast, even at very high concentrations, Hsp104<sup>ΔN</sup> stimulated spontaneous Sup35 prionogenesis by reducing lag phase and accelerating assembly phase (Figure 2D). Thus, deletion of the Hsp104 NTD drastically alters the concentration-dependent effect of Hsp104 on Sup35 prionogenesis, such that inhibition of prion formation is

diminished. Indeed, the absence of the NTD switches Hsp104 to an operating mode that stimulates Sup35 prionogenesis.

Stimulation of spontaneous Sup35 prionogenesis by low concentrations of Hsp104 is due to two activities (Shorter and Lindquist, 2006). First, Hsp104 reduces lag phase by accelerating formation of prionogenic Sup35 oligomers, which are recognized by an anti-oligomer antibody, A11. This activity requires ATP binding but not hydrolysis by Hsp104. Second, Hsp104 accelerates assembly phase by occasionally fragmenting Sup35 prions to generate additional fibril ends for conformational replication. This activity requires ATP hydrolysis by Hsp104.

We assessed the effect of Hsp104<sup>ΔN</sup> on prionogenic Sup35 oligomer formation. Sup35 slowly formed A11-reactive oligomers that peaked at the end of lag phase (~4 hr) and rapidly disappeared during assembly phase (Figure 2E). High concentrations of Hsp104 prevented formation of A11-reactive species, whereas low concentrations of Hsp104 stimulated their appearance at 30 min, after which A11-reactive oligomers disappeared upon rapid prionogenesis (Figures 2C and 2E) (Shorter and Lindquist, 2006). By contrast, low and high concentrations of Hsp104<sup>ΔN</sup> accelerated A11-reactive oligomer formation (Figures 2D and 2F). This acceleration did not require ATP hydrolysis and was supported by a nonhydrolyzable ATP analog, AMP-PNP (Figures 2E and 2F). Thus, the Hsp104 NTD is not required to accelerate prionogenic oligomer formation, but is essential for high concentrations of Hsp104 to inhibit Sup35 oligomer formation.

To assess how Hsp104<sup>ΔN</sup> affected assembly phase, we titrated it into Sup35 prionogenesis seeded by Sup35 prions. At low concentrations, Hsp104 accelerated seeded Sup35 assembly and was inhibited by AMP-PNP (Figure 2G). At high concentrations, Hsp104 inhibited seeded Sup35 assembly (Figure 2G). By contrast, even at high concentrations, Hsp104<sup>ΔN</sup> accelerated seeded Sup35 prionogenesis (Figure 2H). Acceleration by Hsp104<sup>ΔN</sup> required ATP hydrolysis, and was inhibited by AMP-PNP (Figure 2H). Thus, Hsp104<sup>ΔN</sup> fragments Sup35 prions but is unable to dissolve them (Figures 2A and 2B). Indeed, prion fragmentation and dissolution are uncoupled by deletion of the Hsp104 NTD.

### Hsp104<sup>ΔN</sup> Promotes Formation of Sup35 Prions that Encode Strong [PSI<sup>+</sup>]

Sup35 forms distinct cross- $\beta$  structures or “strains,” which encode distinct [PSI<sup>+</sup>] phenotypes designated “weak” or “strong” to describe the magnitude of Sup35 loss of function. We assessed how Hsp104 and Hsp104<sup>ΔN</sup> altered Sup35 prion strain distribution. Thus, we infected [*psi*<sup>-</sup>] yeast with Sup35 prions formed in the presence of Hsp104 or Hsp104<sup>ΔN</sup>. Sup35 prions formed without Hsp104 gave rise to ~40% strong [PSI<sup>+</sup>] and ~60% weak [PSI<sup>+</sup>] (Figure 2I). Low concentrations of Hsp104 shifted the population toward strong [PSI<sup>+</sup>], ~63% strong [PSI<sup>+</sup>] and ~37% weak [PSI<sup>+</sup>], whereas a higher Hsp104 concentration (1  $\mu$ M) prevented Sup35 prionogenesis (Figure 2I). By contrast, low concentrations of Hsp104<sup>ΔN</sup> (0.03  $\mu$ M) significantly shifted the population toward strong [PSI<sup>+</sup>]: ~76% strong [PSI<sup>+</sup>] and ~24% weak [PSI<sup>+</sup>] (Figure 2I;  $p < 0.05$ , two-tailed t test). Higher Hsp104<sup>ΔN</sup> concentration exacerbated this effect: ~92% strong [PSI<sup>+</sup>] and ~8% weak [PSI<sup>+</sup>] (Figure 2I). Thus, the altered activity of Hsp104<sup>ΔN</sup> accentuates prion strain selection events that favor

strong [PSI<sup>+</sup>]. Indeed, Hsp104<sup>ΔN</sup> “strengthens” [PSI<sup>+</sup>] phenotypes in vivo (Hung and Masison, 2006).

### Hsp104<sup>ΔN</sup> Fragments Sup35 Prions by Selectively Breaking Tail Contacts

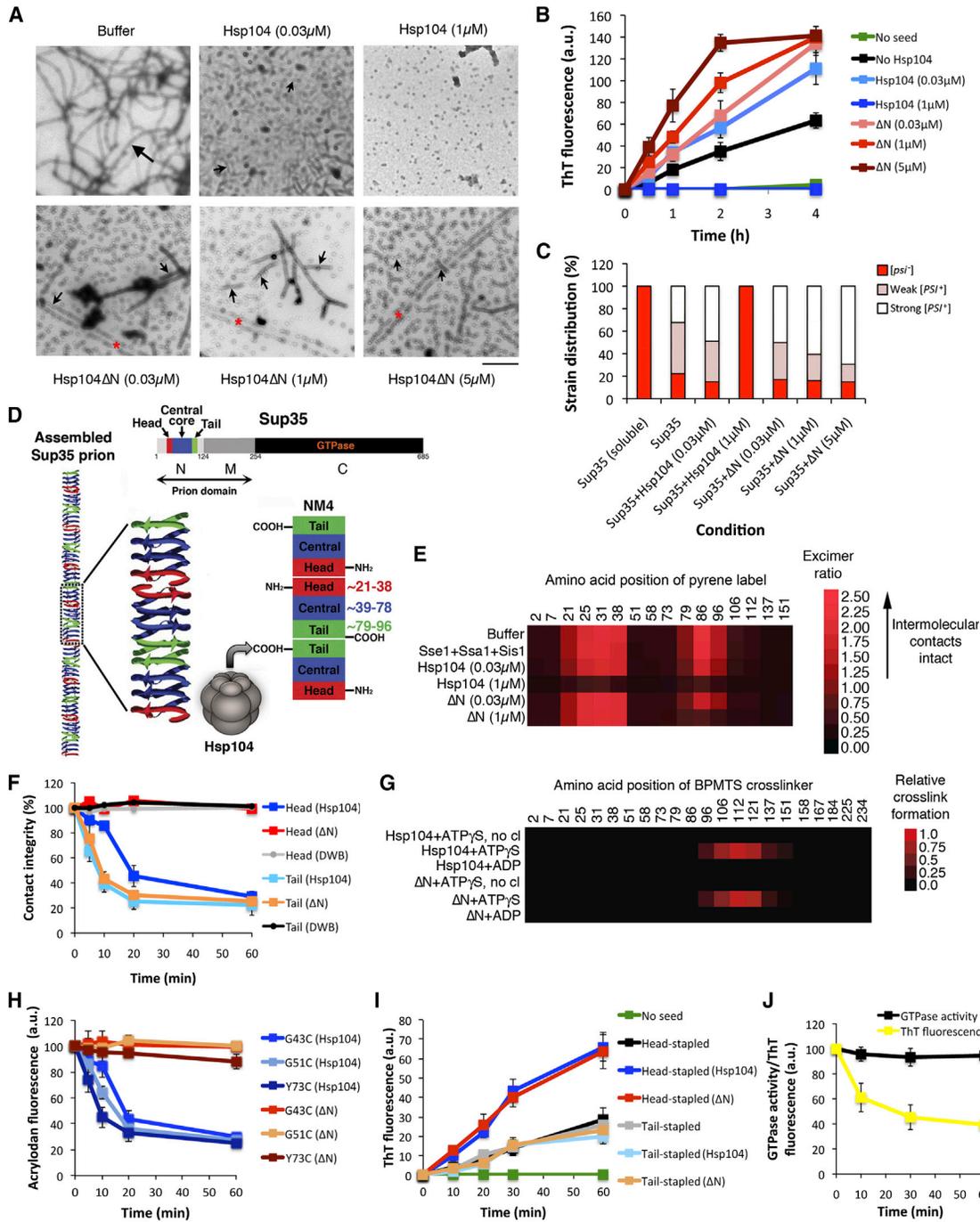
To assess prion-fragmenting activity of Hsp104<sup>ΔN</sup>, we treated Sup35 prions with low or high concentrations of Hsp104<sup>ΔN</sup> or Hsp104. Low concentrations of Hsp104 fragmented Sup35 prions as revealed by EM (Figure 3A), without reducing ThT fluorescence (Figure 2A). Fragmentation was confirmed by the ability of remodeled products to seed Sup35 prionogenesis (Figure 3B) or infect [*psi*<sup>-</sup>] yeast (Figure 3C). High concentrations of Hsp104 eliminated Sup35 prions (Figures 2A and 3A–3C). By contrast, low or high concentrations of Hsp104<sup>ΔN</sup> fragmented Sup35 prions and enhanced their seeding activity without eliminating them (Figures 3A–3C). EM revealed long tracks of closely aligned short fibrils, as though Hsp104<sup>ΔN</sup> had fragmented a long fibril at several positions along its course (Figure 3A, asterisks). Treatment of Sup35 prions with low concentrations of Hsp104 or any concentration of Hsp104<sup>ΔN</sup> amplified strong [PSI<sup>+</sup>] prions (Figure 3C). This effect was most pronounced at high Hsp104<sup>ΔN</sup> concentrations (Figure 3C). Thus, the Hsp104 NTD is essential to dissolve Sup35 prions.

To determine how Hsp104<sup>ΔN</sup> fragments Sup35 prions, we monitored intermolecular prion contacts. We employed the N-terminal prion domain (N, residues 1–121) and MD (M, residues 122–253) of Sup35, termed NM (Figure 3D). We assembled NM prions at 4°C to yield the prion ensemble NM4, which encodes predominantly strong [PSI<sup>+</sup>] (DeSantis and Shorter, 2012). Specifically, we assembled NM4 prions with 17 individual single cysteine NM variants labeled with pyrene at different positions. These pyrene-labeled NM variants retain WT assembly kinetics and infectivity, indicating that pyrene does not significantly alter prion structure (Krishnan and Lindquist, 2005). Upon intermolecular contact formation, pyrene molecules at select positions, in the “Head” or “Tail” (Figures 3D and 3E), form excimers (excited-state dimers) that produce a strong red shift in fluorescence. Excimer fluorescence reports on intermolecular contact integrity, and NM prions are held together by intermolecular Head-to-Head and Tail-to-Tail contacts (Figure 3D) (Krishnan and Lindquist, 2005).

High concentrations of Hsp104 disrupted Head (residues 21–38) and Tail (residues 79–96) contacts of NM4 prions, whereas the low Hsp104 concentration also disrupted both contacts, but to a lesser extent (Figure 3E). By contrast, Hsp104<sup>ΔN</sup> only disrupted Tail contacts even at high concentrations (Figure 3E). Thus, the NTD is not required for Hsp104 to break the Tail contact, but is critical to break the Head contact and dissolve Sup35 prions.

### Hsp104 Breaks the Tail Contact and then the Head Contact of Sup35 Prions

To understand the selective breakage of Tail contacts by Hsp104<sup>ΔN</sup>, we tracked NM4 prion remodeling kinetics. The “double Walker B” (DWB, E285Q:E687Q) Hsp104 mutant, which can bind but not hydrolyze ATP, failed to break Head or Tail contacts (Figure 3F). At early times (0–10 min), Hsp104 and



**Figure 3. Hsp104<sup>ΔN</sup> Fragments Sup35 Prions by Selectively Breaking Tail Contacts**

(A) Sup35 prions were treated with buffer, Hsp104, or Hsp104<sup>ΔN</sup> plus Sse1, Ssa1, and Sis1 for 1 hr and processed for EM. Note the long fibrils in buffer control (large arrow), shorter fibrils (small arrows) in the presence of Hsp104<sup>ΔN</sup> or Hsp104 (0.03 μM), and absence of fibrils with Hsp104 (1 μM). Asterisks denote long tracks of closely aligned short fibrils. Scale bar, 0.5 μm.

(B) Sup35 prions were left untreated, sonicated, or treated with His<sub>6</sub>-Hsp104 or His<sub>6</sub>-Hsp104<sup>ΔN</sup> plus Sse1, Ssa1, and Sis1 for 1 hr. Reactions were depleted of His<sub>6</sub>-Hsp104 or His<sub>6</sub>-Hsp104<sup>ΔN</sup> and used to seed (2% wt/wt) Sup35 prionogenesis assessed by ThT fluorescence. Values represent means ± SEM (n = 3).

(C) Sup35 prions were treated as in (A), and reaction products were sonicated and transformed into [*psi*<sup>-</sup>] cells. The proportion of [*psi*<sup>-</sup>], weak [*PSI*<sup>+</sup>], and strong [*PSI*<sup>+</sup>] colonies was determined. Values represent means (n = 3).

(D) Sup35 harbors a C-terminal GTPase domain (residues 254–685, black), a charged middle domain (M, residues 124–253, dark gray), and a prionogenic N-terminal domain (N, residues 1–123, light gray). Within N, prion recognition elements make homotypic intermolecular contacts, and Sup35 prions are maintained by Head-to-Head (red) and Tail-to-Tail (green) contacts. A central core (blue) is sequestered by intramolecular contacts. Head, central core, and Tail position are shown for NM4 prions. Hsp104 engages Sup35 prions C-terminal to the Tail contact.

(legend continued on next page)

Hsp104<sup>ΔN</sup> broke the Tail contact (G86C), whereas the Head contact (G31C) remained intact (Figure 3F). At later times (20–60 min), Hsp104 severed Head contacts, but Hsp104<sup>ΔN</sup> did not (Figure 3F). Thus, Hsp104 breaks the Tail and then the Head contact to remodel NM4 prions. This temporal separation suggested that Hsp104 and Hsp104<sup>ΔN</sup> engage NM4 prions C-terminal to the Tail contact and then exert a directional pulling force that first breaks the Tail contact (Figure 3D). However, Hsp104<sup>ΔN</sup> is unable to melt cross-β structure N-terminal to the Tail and releases after the Tail contact is broken but before the Head contact is broken. By contrast, Hsp104 breaks the Tail contact and continues to translocate NM sequence along a C- to N-terminal vector, thereby melting cross-β structure of the central core and then breaking the Head contact. To test this model, we performed three experiments.

First, we assessed where Hsp104 and Hsp104<sup>ΔN</sup> initially engage NM4 prions. Thus, single cysteine NM variants labeled with a cleavable thiol-specific UV-activatable 13Å crosslinker, benzophenone-4-carboxamidocysteine methanethiosulphonate (BPMTS), were assembled into NM4 prions. BPMTS-labeled NM variants retain WT assembly kinetics and infectivity (Figures S1A and S1B), indicating that BPMTS does not affect prionogenesis. BPMTS-labeled NM4 prions were incubated with Hsp104 or Hsp104<sup>ΔN</sup> plus ATP<sub>γ</sub>S (to favor binding) or ADP (to disfavor binding) and crosslinked. Neither Hsp104 nor Hsp104<sup>ΔN</sup> was recovered without crosslinking or with ADP (Figure 3G). By contrast, with ATP<sub>γ</sub>S, Hsp104 and Hsp104<sup>ΔN</sup> were recovered only when BPMTS was attached at positions 96, 106, 112, 121, 137, and 151 (Figure 3G). Thus, Hsp104 and Hsp104<sup>ΔN</sup> initially engage NM4 prions C-terminal to the Tail contact, in a region spanning residues 96–151 (Figure 3D). Subsequently, Hsp104<sup>ΔN</sup> breaks the Tail contact and fragments the prion, but is unable to release soluble NM, which requires unfolding the central core and severing the Head contact, a task accomplished by Hsp104.

Second, we tracked the central core between the Head and Tail. Thus, we employed single cysteine NM variants bearing acrylodan labels at G43C, G51C, or Y73C, which lie in the central core (Figure 3D). Sequestration of labeled sites from solvent in the assembled prion increases acrylodan fluorescence at these positions (Krishnan and Lindquist, 2005). Hsp104<sup>ΔN</sup> failed to alter acrylodan fluorescence of NM4 prions (Figure 3H). Thus, Hsp104<sup>ΔN</sup> does not remodel the central core. By contrast,

Hsp104 reduced acrylodan fluorescence at these positions, indicating that the central core was remodeled and exposed to solvent (Figure 3H). Hsp104-driven unfolding of the central core was not concerted but occurred in a stepwise manner. Thus, the Y73 position displayed changes prior to G51 and G43, indicating that Hsp104 remodels C-terminal portions of the central core prior to N-terminal portions (Figure 3H). Thus, Hsp104 breaks the Tail contact and then unfolds the central core by pulling on its C-terminal end.

Third, we assembled NM prions from single cysteine NM variants that were stapled together at the Head (N21C) or Tail (G96C) contact by an 11Å crosslinker 1,4-bis-maleimidobutane (BMB) (Krishnan and Lindquist, 2005). NM4 prions stapled at the Head contact could be fragmented by Hsp104 and Hsp104<sup>ΔN</sup> and were more potent seeds than untreated NM4 prions (Figure 3I). By contrast, NM prions stapled at the Tail contact could not be fragmented by Hsp104 or Hsp104<sup>ΔN</sup> and seeded NM assembly just as well as untreated prions (Figure 3I). Thus, Hsp104 cannot break the Head contact until after the Tail contact has been broken.

NM lacks the C-terminal GTPase domain of Sup35 (Figure 3D). Does Hsp104 need to unfold the C-terminal GTPase domain to dissolve Sup35 prions? Full-length Sup35 retains similar GTPase activity in the prion and soluble state (Krzewska et al., 2007). Thus, to assess whether the Sup35 C-terminal domain was unfolded during Hsp104-catalyzed prion dissolution, we included GroEL<sub>TRAP</sub>, which captures unfolded protein and prevents refolding. Hsp104 disassembled Sup35 prions, but GTPase activity was unchanged (Figure 3J), indicating that Hsp104 dissolves Sup35 prions without unfolding the C-terminal GTPase domain. Likewise, Hsp104 did not unfold GFP during dissolution of NM-GFP prions (Figures S1C and S1D). Thus, Hsp104 selectively resolves N-terminal prion structure without unfolding the appended C-terminal domain.

### Hsp104<sup>ΔN</sup> Has Impaired Translocation and Unfoldase Activity

The inability to resolve cross-β structure or break Head contacts of NM4 prions suggested that Hsp104<sup>ΔN</sup> might be defective in substrate translocation and unfolding. Indeed, FITC-casein degradation and RepA<sub>1-70</sub>-GFP unfolding assays confirmed that Hsp104<sup>ΔN</sup> has impaired translocation and unfoldase activity (Figures S1E and S1F).

(E) NM4 prions carrying pyrene labels at the indicated single site were treated with Hsp104 or Hsp104<sup>ΔN</sup> plus Sse1, Ssa1, and Sis1 for 1 hr. The ratio of excimer to nonexcimer fluorescence ( $I_{465nm}/I_{375nm}$ ) was then determined. Values represent means (n = 3).

(F) NM4 prions carrying pyrene labels in the Head (G31C) or Tail (G86C) were incubated with Hsp104, Hsp104<sup>ΔN</sup>, or Hsp104<sup>DWB</sup> (1 μM) plus Sse1, Ssa1, and Sis1 for 0–1 hr. At various times, the ratio of excimer to nonexcimer fluorescence ( $I_{465nm}/I_{375nm}$ ) was determined and compared to the zero time point to determine contact integrity (%). Values represent means ± SEM (n = 3).

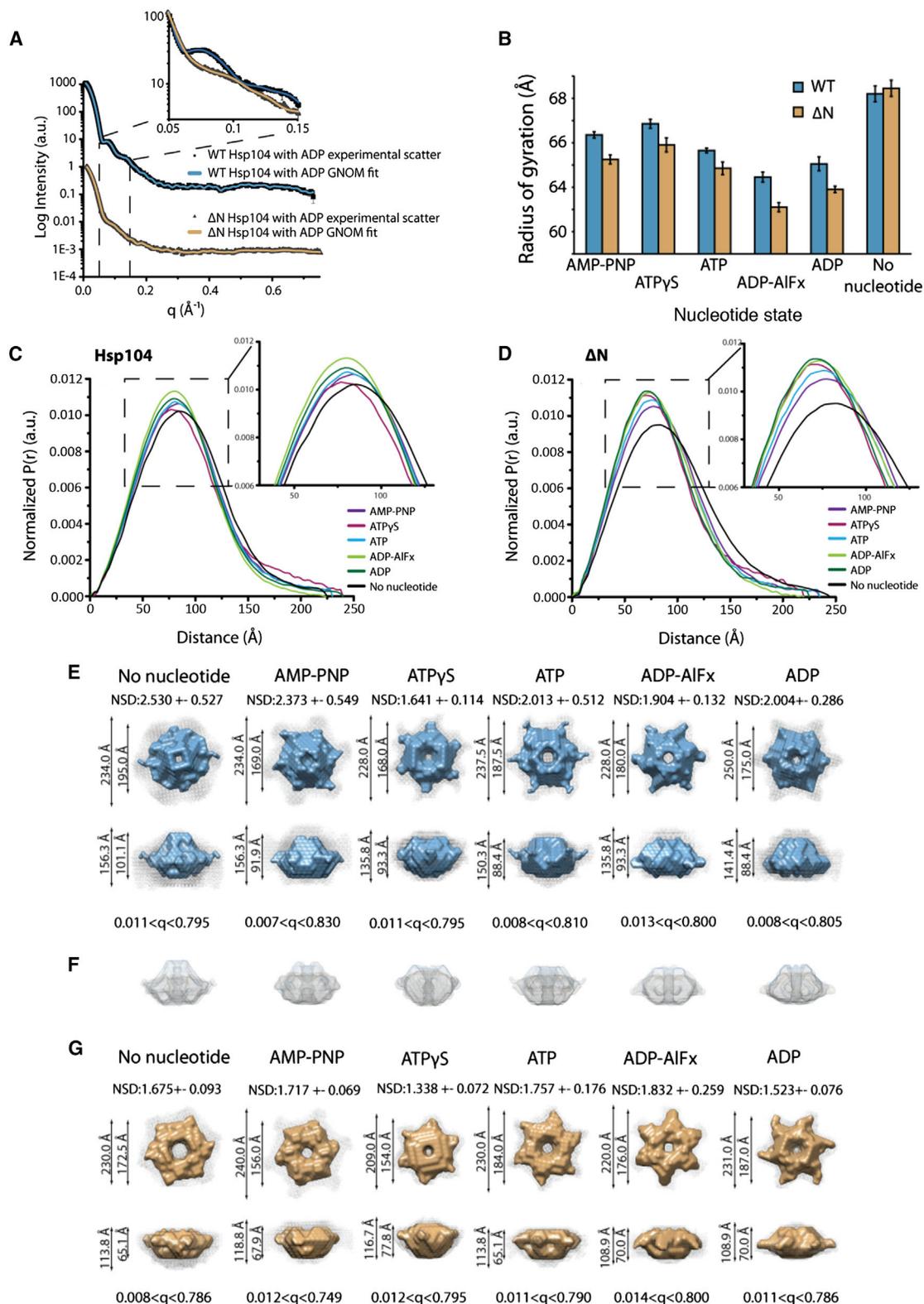
(G) Mapping contact sites between NM4 prions and Hsp104+ATP<sub>γ</sub>S, Hsp104+ADP, Hsp104<sup>ΔN</sup>+ATP<sub>γ</sub>S, and Hsp104<sup>ΔN</sup>+ADP by site-resolved BPMTS crosslinking. Heatmap displays positions where Hsp104 was crosslinked to NM4 prions. No cl (no crosslinking control). Values represent means (n = 3).

(H) NM4 prions carrying acrylodan labels in the central core (G43C, G51C, or Y73C) were treated with Hsp104 or Hsp104<sup>ΔN</sup> (1 μM) plus Sse1, Ssa1, and Sis1 for 0–1 hr. At the indicated times, acrylodan fluorescence was measured. Values represent means ± SEM (n = 3).

(I) NM prions (2.5 μM monomer) crosslinked by BMB in the Head (N21C) or Tail (G96C) were left untreated or treated with His<sub>6</sub>-Hsp104 (1 μM) or His<sub>6</sub>-Hsp104<sup>ΔN</sup> (1 μM) plus Sse1, Ssa1, and Sis1 for 1 hr. Reactions were depleted of His<sub>6</sub>-Hsp104 or His<sub>6</sub>-Hsp104<sup>ΔN</sup> and used to seed (2% wt/wt) NM prionogenesis assessed by ThT fluorescence. Values represent means ± SEM (n = 3).

(J) Sup35 prions were treated with Hsp104 (1 μM) plus GroEL<sub>TRAP</sub>, Sse1, Ssa1, and Sis1 for 0–1 hr. At various times, GTPase activity and ThT fluorescence were measured. Values represent means ± SEM (n = 3).

See also Figure S1.



**Figure 4. NTD Deletion Alters ATPase-Driven Conformational Changes of Hsp104 Hexamers**

(A) Representative scattering profiles for Hsp104 and Hsp104<sup>ΔN</sup> (intensity versus momentum transfer,  $q$  [ $\text{\AA}^{-1}$ ]). Profiles are arbitrarily scaled on the y axis for better visualization. Inset shows enlargement of regions where Hsp104 consistently differs from Hsp104<sup>ΔN</sup> in all nucleotide states. Experimental data are overlaid by GNOM fit.

(legend continued on next page)

### NTD Deletion Alters ATPase-Driven Conformational Changes of Hsp104 Hexamers

To understand the differences between Hsp104 and Hsp104<sup>ΔN</sup> at a structural level, we examined changes in shape of Hsp104 and Hsp104<sup>ΔN</sup> hexamers through the ATPase cycle in solution using small- and wide-angle X-ray scattering (SAXS/WAXS). X-ray scattering at very low angles provides information about particle size and shape. Hsp104 and Hsp104<sup>ΔN</sup> were hexameric and monodisperse in solution and ideally suited for SAXS (Figure 1A; and see calculated mass of particle [MM by Qr] in Table S2). We measured scattering at multiple concentrations and different beamlines and obtained very similar results (Table S2). Guinier analysis confirmed the absence of aggregation or other concentration-dependent effects (Figure S2A) (Volkov and Svergun, 2003). Thus, we determined structural parameters of Hsp104 and Hsp104<sup>ΔN</sup>, including maximum dimension ( $D_{\max}$ ) and radius of gyration ( $R_g$ ) in six nucleotide states—AMP-PNP, ATP $\gamma$ S, ATP, ADP-AIF<sub>x</sub> (ATP hydrolysis transition state mimic), ADP, and no nucleotide—to simulate the steps of the ATP hydrolysis cycle. Data were measured to a  $q_{\max}$  of  $\sim 0.7$ – $0.8 \text{ \AA}^{-1}$  to yield a nominal resolution limit ( $2\pi/q_{\max}$ ) of 7.6–8.4 Å.

Raw scattering profiles ( $[I(q)]$  versus  $q$ , where  $q = 4\pi[\sin\theta]/\lambda$ ) and GNOM (Svergun, 1992) fits to experimental data revealed large differences between Hsp104 and Hsp104<sup>ΔN</sup> (Figure 4A). Distinctive features in the low  $q$  region present in Hsp104, but not Hsp104<sup>ΔN</sup> (corresponding to the NTD), are highlighted (Figure 4A, inset). In the absence of nucleotide, Hsp104 and Hsp104<sup>ΔN</sup> hexamers have their largest  $R_g$  and  $D_{\max}$  (Figure 4B; Tables S2 and S3). Addition of nucleotide decreased  $R_g$  and  $D_{\max}$  (Figure 4B; Tables S2 and S3). For Hsp104 and Hsp104<sup>ΔN</sup>, AMP-PNP and ATP $\gamma$ S elicited larger spatial properties (Figure 4B; Tables S2 and S3). ADP-AIF<sub>x</sub>, which mimics the ATP hydrolysis transition state, where ADP and Pi are bound, yielded the smallest  $R_g$  and  $D_{\max}$  values for Hsp104 and Hsp104<sup>ΔN</sup> (Figure 4B; Tables S2 and S3). The spatial properties of Hsp104 and Hsp104<sup>ΔN</sup> then expand slightly upon Pi release in the ADP state (Figure 4B; Table S3). Thus, Hsp104 and Hsp104<sup>ΔN</sup> hexamers contract upon ATP hydrolysis and expand upon ATP binding (Figure 4B; Tables S2 and S3), indicating a pumping mechanism to drive substrate translocation.

Structural changes of Hsp104 and Hsp104<sup>ΔN</sup> hexamers were evident in the real-space pairwise distance distribution function, or  $P(r)$ , which represents the distances between pairs of atoms within a given volume. As with  $R_g$ , the apparent redistribution of interatomic vectors in the  $P(r)$  curves demonstrates that nucleotide addition and identity induce specific changes in shape for Hsp104 and Hsp104<sup>ΔN</sup> hexamers (Figures 4C and 4D). How the

Hsp104 hexamer responds to a given nucleotide, both in terms of the magnitude and the specific effect, differs in the absence of the NTD (Figures 4C and 4D).

To visualize these changes, we employed the ab initio modeling program GASBOR (Svergun et al., 2001) to derive volumetric reconstructions of the averaged solution shape of Hsp104 and Hsp104<sup>ΔN</sup> hexamers with each nucleotide. GASBOR employs simulated annealing to match experimental scattering data with an ensemble of beads corresponding to the composition of the particle. For each nucleotide, GASBOR calculations were performed ten times using scattering data to  $q_{\max}$  of 0.7–0.8 Å<sup>-1</sup> (nominal resolution limit of 7.6–8.4 Å). GASBOR calculations using  $q_{\max}$  truncated to 0.5 Å<sup>-1</sup> or calculations with the program DAMMIN/F yielded similar results. We imposed 6-fold symmetry based upon cryo-EM analyses of Hsp104 (Wendler et al., 2007). Superposition of each GASBOR solution for Hsp104 and Hsp104<sup>ΔN</sup> (Figures S2B and S2C) revealed consensus that was confirmed by the normalized spatial discrepancy (NSD) between independent calculations. NSD indicates the degree of discrepancy between the same relative position between any two structures (Volkov and Svergun, 2003). NSD values indicated little deviation between independent calculations, and reconstructions from different synchrotron trips yielded similar results (Figures 4E–4G; Table S2).

The outputs of each GASBOR calculation were averaged to provide filtered and unfiltered densities using DAMAVER (Figures 4E–4G) (Volkov and Svergun, 2003). Hsp104 shape reconstructions were oriented using Hsp104<sup>ΔN</sup> hexamers, which when overlaid indicated where density for the missing NTD would fit (Figure 4F). The general dimensions of Hsp104 and Hsp104<sup>ΔN</sup> particles determined by SAXS agree with Hsp104<sup>N728A</sup> and Hsp104<sup>ΔN</sup> cryo-EM reconstructions (Wendler et al., 2007), and the central channel through which substrate is translocated is resolved (Figures 4E–4G). Thus, we can identify conformational changes that enable Hsp104 and Hsp104<sup>ΔN</sup> to couple ATPase activity to protein disaggregation.

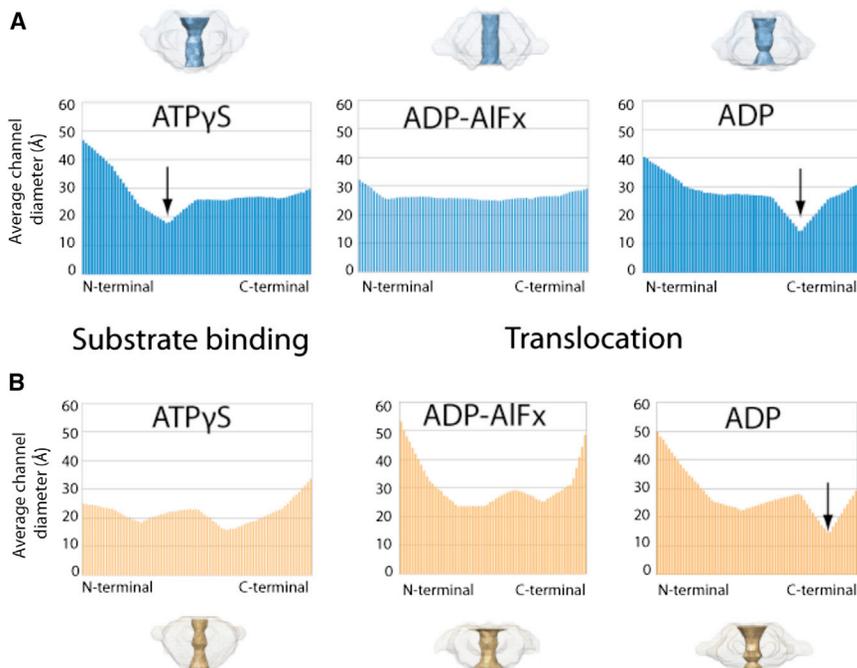
Hsp104 and Hsp104<sup>ΔN</sup> hexamers undergo large conformational changes between nucleotide states (Figures 4E–4G). Hsp104 and Hsp104<sup>ΔN</sup> hexamers have similar width, but Hsp104<sup>ΔN</sup> is shorter in height (Figures 4E and 4G). Two distinctive features change in each nucleotide state: (1) the placement of a projection of density on the hexamer exterior, along the plane of the largest dimension; and (2) the diameter and contours of the central channel (Figures 4E–4G, 5A, and 5B). The exterior projection is evident in the  $P(r)$  as a small population of large vectors that start around 175 Å (Figures 4C and 4D). Hsp104 and Hsp104<sup>ΔN</sup> have dynamic projections that shift from a more

(B)  $R_g$  of Hsp104 and Hsp104<sup>ΔN</sup> with the indicated nucleotide calculated by GNOM. These values closely match the Guinier approximations for  $R_g$  (Table S2). Values represent means  $\pm$  SEM ( $n = 3$ –7).

(C and D) Real-space shape information for Hsp104 and Hsp104<sup>ΔN</sup>. Normalized  $P(r)$  curves (density distribution plots) generated by GNOM for Hsp104 (C) and Hsp104<sup>ΔN</sup> (D) in the presence of AMP-PNP, ATP $\gamma$ S, ATP, ADP-AIF<sub>x</sub>, ADP, and no nucleotide.  $P(r)$  plots are normalized to the area under the curve and overlaid to show differences between nucleotide states. Insets display the  $P(r)$  peak, which reveals differences between nucleotide states. Compared to Hsp104, Hsp104<sup>ΔN</sup> has large spatial extent in the absence of nucleotide relative to its nucleotide-bound states.

(E–G) Averaged ab initio GASBOR volume reconstructions of Hsp104 (E) and Hsp104<sup>ΔN</sup> (G). Filtered density is solid blue (Hsp104) or orange (Hsp104<sup>ΔN</sup>) overlaid with the unfiltered average shown in gray mesh. NSD of averaged models and  $q$  range used for reconstructions are shown plus average particle dimensions. (F) Overlay of Hsp104 and Hsp104<sup>ΔN</sup> average reconstructions for each state, which was used to orient particles. Reconstructions are oriented with the N terminus pointing toward the top of the page.

See also Tables S2 and S3, Figure S2, Movie S1, and Movie S2.



**Figure 5. Channel Motions of Hsp104 and Hsp104 $\Delta$ N Hexamers**

(A and B) Cut-away side views of Hsp104 (A) and Hsp104 $\Delta$ N (B) hexamers in ATP $\gamma$ S, ADP-AIF $_x$ , or ADP. The channel was reconstructed using the filtered average volumes for each nucleotide state. Bar graphs display the average channel diameter (Å) of each z slice starting from the N terminus. Each bar represents 1 Å, and the number of bars represents the length of the channel that is closed for 360°. Substrate binds in the ATP $\gamma$ S state and is translocated from the N-terminal entrance to the C-terminal exit. The Hsp104 channel exhibits a peristaltic wave motion: dilation at the N-terminal entrance (in ATP $\gamma$ S) followed by a contraction of the N-terminal end of the channel (in ADP-AIF $_x$ ) and finally a shift in the location of a constriction from the N- to the C-terminal region (in ADP, arrow). The Hsp104 $\Delta$ N hexamer displays defects in the peristalsis motion, especially at the N-terminal channel entrance, which fails to contract in the ADP-AIF $_x$  and ADP states.

See also [Movie S1](#) and [Movie S2](#).

N-terminal position (in no nucleotide, AMP-PNP, ATP $\gamma$ S, and ATP) to a more C-terminal position (in ADP-AIF $_x$  and ADP) upon ATP hydrolysis (Figures 4E–4G, 5A, and 5B; [Movie S1](#) and [Movie S2](#)). Rudimentary rigid body domain fitting reveals that the volumetric envelopes readily accommodate six Hsp104 monomers (Figure S2D). The external projection is likely the MD in accord with cryo-EM models of HAP plus ATP $\gamma$ S and ClpP (Figure S2D) ([Carroni et al., 2014](#)). Thus, our SAXS reconstructions resolve controversy surrounding MD location generated by cryo-EM studies ([Lee et al., 2010](#); [Wendler et al., 2007, 2009](#)). The change in position of the external projection (Figures 4E–4G) is consistent with the MD located on the surface of the hexamer, which can move from an N-terminal, horizontal position to a C-terminally tilted position ([Carroni et al., 2014](#); [DeSantis et al., 2014](#)).

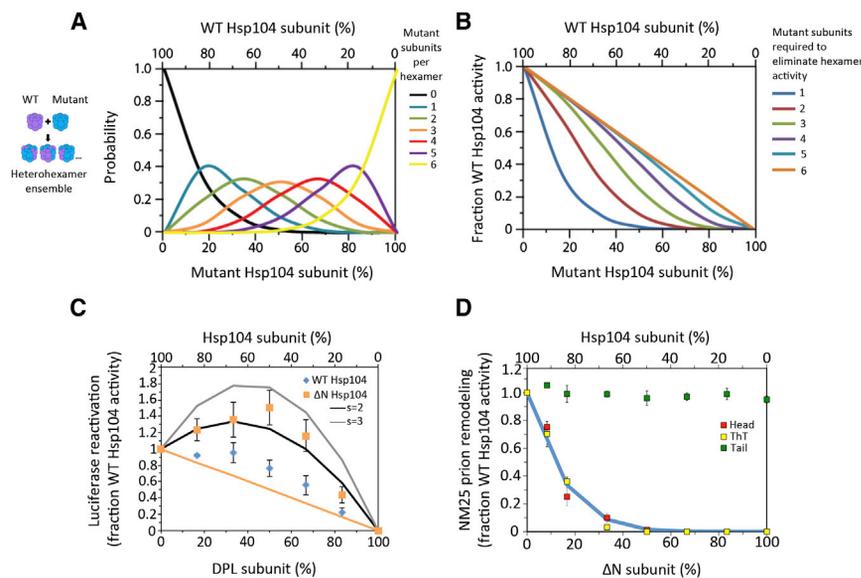
To disaggregate substrates, Hsp104 translocates proteins through its central channel. Thus, we focused on the central channel (Figures 5A and 5B). The changes in shape of the Hsp104 channel are reminiscent of a peristaltic wave: there is dilation at the site of substrate entrance followed by a wave of constriction that travels in the direction that substrate is being pumped. Substrate enters through the N-terminal entrance and can be expelled from the C-terminal exit ([Shorter, 2008](#)). Accordingly, in the ATP $\gamma$ S state where Hsp104 initially engages substrate, the N-terminal channel entrance of Hsp104 is open and dilated with a diameter of  $\sim$ 45–50 Å (Figure 5A). In the ATP $\gamma$ S state, there is a region of constriction (channel diameter  $\sim$ 18 Å) after the N-terminal opening (Figure 5A, arrow), and C-terminal to this constriction the channel is  $\sim$ 25–30 Å in diameter. The channel constricts to a diameter of  $\sim$ 25–30 Å across its entire length with the transition state mimic ADP-AIF $_x$  (Figure 5A), the state with the smallest  $R_g$  (Figure 4B). Thus, upon ATP hydrolysis the Hsp104 channel constricts (Figure 5A). In the ADP state,

a C-terminal point of constriction becomes apparent (Figure 5A, arrow), which likely helps expel substrate from the C-terminal exit. Thus, cycles of ATP binding and hydrolysis drive a peristaltic pumping motion of the Hsp104 hexamer, which likely drives directional substrate translocation ([Movie S1](#)). The peristaltic pumping motion likely underpins how Hsp104 transduces energy from ATP hydrolysis to conformational change and substrate remodeling using physical force.

NTD deletion grossly perturbs this peristaltic pump motion (Figure 5B; [Movie S2](#)). In ATP $\gamma$ S, the Hsp104 $\Delta$ N channel is narrow in diameter ( $\sim$ 16–30 Å) compared to Hsp104, and there is no dilation at the N-terminal entrance (Figures 5A and 5B). Thus, it is more difficult for substrate to access the Hsp104 $\Delta$ N channel in the binding-competent ATP $\gamma$ S state. In ADP-AIF $_x$ , the Hsp104 $\Delta$ N channel is dilated at N- and C-terminal ends, and only a central portion of the channel is  $\sim$ 25–30 Å in diameter, unlike Hsp104, where the channel aperture is  $\sim$ 25–30 Å across its whole length (Figures 5A and 5B). Thus, the “power stroke” motion elicited by ATP hydrolysis in Hsp104 is profoundly altered in Hsp104 $\Delta$ N. In ADP, the Hsp104 $\Delta$ N and Hsp104 channels change in diameter in a similar manner along their length, although the N-terminal channel entrance is more dilated in Hsp104 $\Delta$ N (Figures 5A and 5B). The more dilated Hsp104 $\Delta$ N channel in ADP-AIF $_x$  might allow substrate to escape the channel. These channel defects help explain why Hsp104 $\Delta$ N is defective in translocation, unfolding, and disaggregation.

### Hsp104 $\Delta$ N hexamers Operate Differently Than Hsp104 Hexamers

The profound alterations in the Hsp104 $\Delta$ N channel (Figures 4E–4G, 5A, and 5B) indicated that Hsp104 $\Delta$ N and Hsp104 might coordinate substrate translocation differently. Indeed, Hsp104 $\Delta$ N hexamers appear unable to process substrate in a subglobally



**Figure 6. Altered Substrate Handling by Hsp104<sup>ΔN</sup> Hexamers Precludes Prion Dissolution**

(A) Theoretical Hsp104 hexamer ensembles containing zero (black), one (blue), two (green), three (orange), four (red), five (purple), and six (yellow) mutant subunits as a function of fraction mutant subunit.

(B) Theoretical activity curves where one or more (blue), two or more (red), three or more (green), four or more (purple), five or more (light blue), or six mutant subunits (orange) ablate Hsp104 hexamer activity.

(C) Luciferase aggregates were treated with Hsp104 (blue markers) or Hsp104<sup>ΔN</sup> (orange markers), Hsp72, and Hdj2 plus increasing fractions of Hsp104<sup>DPL</sup> (blue markers) or Hsp104<sup>ΔNDPL</sup> (orange markers) subunits. Luciferase reactivation was determined (fraction WT Hsp104 activity). Values represent means ± SEM (n = 3–4). Theoretical disaggregate activity if six DPL subunits ablate hexamer activity (orange line). Theoretical curves are shown wherein adjacent pairs of WT:WT (or ΔN:ΔN) or WT:DPL (or ΔN:ΔNDPL)

subunits confer hexamer activity, while adjacent DPL (or ΔNDPL) subunits have no activity. Each adjacent WT:DPL (or ΔN:ΔNDPL) pairs have a stimulated activity (s), and the effect of s = 2 (black curve) or s = 3 (gray curve) is shown.

(D) NM25 prions were treated with Hsp104, Sse1, Ssa1, and Sis1 plus increasing fractions of Hsp104<sup>ΔN</sup>. Remodeling was monitored by Head contact integrity (for G31C-pyrene-labeled NM25 prions, red markers), ThT fluorescence (for unlabeled NM25 prions, yellow markers), or Tail contact integrity (for G96C-pyrene-labeled NM25 prions, green markers). Values represent means ± SEM (n = 3). Theoretical disaggregate activity if 1 Hsp104<sup>ΔN</sup> subunit ablates hexamer activity (blue line).

See also Figure S1.

or globally cooperative manner required for prion dissolution (DeSantis et al., 2012). We utilized a mutant subunit doping strategy to generate heterohexamer ensembles and determine whether subunit collaboration was altered with respect to substrate handling in Hsp104<sup>ΔN</sup> (Figures 6A and 6B) (DeSantis et al., 2012).

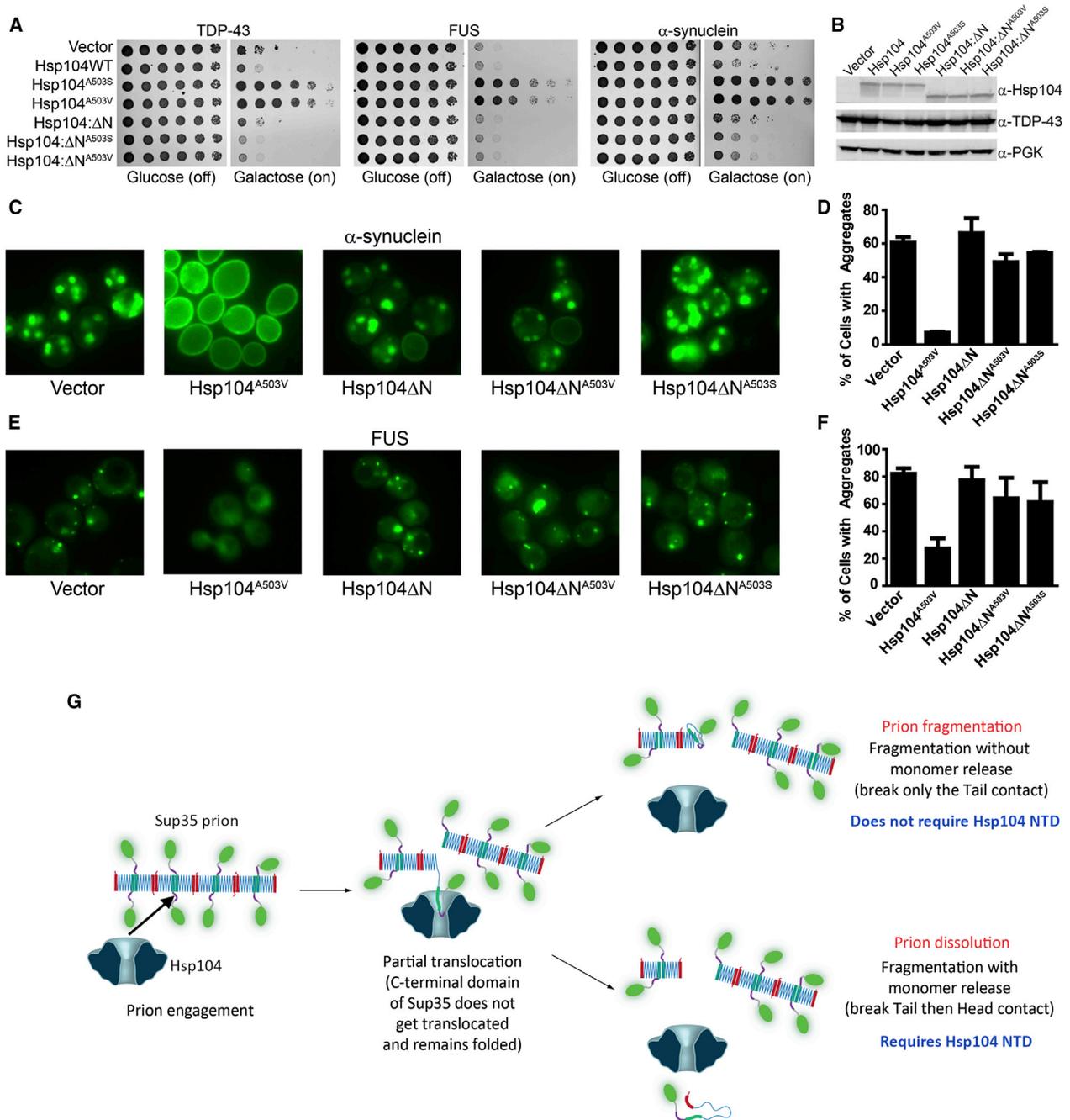
To define how Hsp104<sup>ΔN</sup> subunits coordinate substrate binding during disordered aggregate dissolution, we employed the “double pore loop” (DPL, Y257A:Y662A) mutant. DPL has normal ATPase activity but harbors Y257A and Y662A mutations in substrate-binding pore loops, which impair substrate translocation (DeSantis et al., 2012). We assembled heterohexamer ensembles of Hsp104 and Hsp104<sup>DPL</sup>, or Hsp104<sup>ΔN</sup> and Hsp104<sup>ΔNDPL</sup>, and assessed disaggregate activity against disordered luciferase aggregates. Hsp104 and Hsp104<sup>ΔN</sup> hexamers responded very differently to DPL subunits (Figure 6C). Hsp104<sup>DPL</sup> subunits caused a roughly linear decline in Hsp104 luciferase reactivation activity, indicating probabilistic substrate handling (Figure 6C) (DeSantis et al., 2012). By contrast, Hsp104<sup>ΔNDPL</sup> subunits stimulated Hsp104<sup>ΔN</sup> activity and only inhibited when the average number of Hsp104<sup>ΔNDPL</sup> subunits per hexamer exceeded 4 (Figure 6C). We could model this behavior if we imposed rules whereby an Hsp104<sup>ΔNDPL</sup> subunit stimulates the activity of an adjacent Hsp104<sup>ΔN</sup> subunit by ~2-fold but exerts an inhibitory effect if it is adjacent to a mutant subunit (Figure 6C) (DeSantis et al., 2012). Thus, Hsp104<sup>ΔN</sup> subunits cooperate negatively with respect to substrate binding. Addition of up to 4 substrate-binding defective subunits within the Hsp104<sup>ΔN</sup> hexamer stimulates activity against disordered aggregates. Thus, the NTD is essential for cooperative substrate handling by the Hsp104 hexamer.

### Hsp104<sup>ΔN</sup> Subunits Inhibit Prion Dissolution by Hsp104 Hexamers

The negative cooperativity of Hsp104<sup>ΔN</sup> subunits with respect to substrate binding likely precludes prion dissolution by Hsp104<sup>ΔN</sup>, which requires multiple subunits within the hexamer to work together (DeSantis et al., 2012). To assess how Hsp104<sup>ΔN</sup> subunits affected prion remodeling by Hsp104, we doped Hsp104<sup>ΔN</sup> subunits into Hsp104 hexamers and assessed ability to (1) break Head and Tail prion contacts, and (2) dissolve NM25 prions (NM prions formed at 25°C). Tail contact severing was unaffected by Hsp104<sup>ΔN</sup> subunits, whereas a single Hsp104<sup>ΔN</sup> subunit per Hsp104 hexamer inhibited Head contact severing and elimination of amyloid structure (Figure 6D). Thus, all six Hsp104 subunits must possess the NTD for globally cooperative prion dissolution.

### Hsp104<sup>ΔN</sup> Is Not Potentiated by Mutations in the MD

Hsp104 disaggregate activity is potentiated by specific mutations in the MD, which enable Hsp104 to dissolve TDP-43, FUS, and α-syn fibrils and mitigate neurodegeneration under conditions where Hsp104 is inactive (Jackrel et al., 2014). We tested whether potentiating MD mutations, A503S and A503V, could overcome defects in cooperativity caused by NTD deletion. Unlike their full-length counterparts, Hsp104<sup>ΔN-A503V</sup> and Hsp104<sup>ΔN-A503S</sup> could not rescue TDP-43, FUS, or α-syn toxicity in yeast, despite robust expression (Figures 7A and 7B). Moreover, Hsp104<sup>ΔN-A503V</sup> and Hsp104<sup>ΔN-A503S</sup> failed to rescue α-syn or FUS aggregation in yeast, unlike Hsp104<sup>A503V</sup> (Figures 7C–7F). Thus, the NTD is essential for potentiation of the Hsp104 hexamer by specific MD mutations.



**Figure 7. Deletion of the Hsp104 NTD Inhibits Hsp104 Potentiation**

(A)  $\Delta$ *hsp104* yeast integrated with galactose-inducible TDP-43, FUS, or  $\alpha$ -syn was transformed with the indicated Hsp104 variant or vector. Strains were serially diluted 5-fold and spotted on glucose (off) or galactose (on) media.

(B) Selected yeast from (A) were induced for 5 hr, lysed, and immunoblotted. 3-phosphoglycerate kinase (PGK) serves as a loading control.

(C) Fluorescence microscopy of cells expressing  $\alpha$ -syn-YFP plus indicated Hsp104 variant or vector.

(D) Quantification of  $\alpha$ -syn aggregation. Values represent means  $\pm$  SEM (n = 2).

(E) Fluorescence microscopy of cells expressing FUS-GFP plus indicated Hsp104 variant or vector.

(F) Quantification of FUS aggregation. Values represent means  $\pm$  SEM (n = 2).

(G) Model of Sup35 prion fragmentation versus dissolution by Hsp104. Hsp104 initially engages Sup35 prions in a region (residues 96–151; purple) C-terminal to the Tail contact (dark green). Directional pulling on N-terminal cross- $\beta$  structure leads to partial translocation and breakage of the Tail contact and Sup35 prion fragmentation. Further translocation breaks Central Core contacts (blue) and the Head contacts (red), resulting in monomer release. Thus, Sup35 prions are fragmented with or without monomer release. The Sup35 C-terminal domain remains folded throughout. Hsp104 <sup>$\Delta$ N</sup> can break the Tail but not the Central Core or Head contacts, thus fragmenting Sup35 prions without solubilizing Sup35.

## DISCUSSION

We have established that the Hsp104 NTD is essential for nucleotide-dependent conformational changes that enable productive hexamer cooperativity, plasticity, and potentiation. Reconstruction of Hsp104 hexamers in solution via SAXS revealed conformational changes that drive a peristaltic pumping motion triggered by ATP hydrolysis and completed by release of Pi. This peristaltic pumping motion likely drives directional substrate translocation through the N-terminal channel entrance, across the central channel, and out the C-terminal exit, but is grossly perturbed in Hsp104<sup>ΔN</sup>.

Mutant doping revealed negative cooperativity between substrate-binding pore loops in Hsp104<sup>ΔN</sup> hexamers. Remarkably, Hsp104<sup>ΔN</sup> hexamers containing ~1–4 subunits that cannot engage substrate outperform Hsp104<sup>ΔN</sup> hexamers in disaggregation of disordered aggregates. Thus, the NTD regulates substrate binding and prevents nonproductive competition for substrate binding by pore loops. This finding helps explain why Hsp104<sup>ΔN</sup> is less active than Hsp104 in disaggregating disordered aggregates. Although subunit cooperativity is not essential for disordered aggregate dissolution (DeSantis et al., 2012), it is necessary for optimal activity and adaptable hexamer function. This deficiency in Hsp104<sup>ΔN</sup> hexamer cooperativity due to defects in conformational changes results in deregulated ATPase activity, reduced disaggregase, unfoldase, and translocase activity, and an inability to dissolve stable amyloid, even in the presence of potentiating mutations. Hsp104<sup>ΔN</sup> is slightly less able to collaborate with Hsp70 and Hsp40 (Figure 1D), which might also contribute to reduced amyloid dissolution. However, amyloid dissolution by Hsp104 does not typically require Hsp70 and Hsp40 (DeSantis et al., 2012); thus we suggest that altered subunit cooperativity is the major defect limiting amyloid dissolution by Hsp104<sup>ΔN</sup>.

Cryo-EM reconstructions of Hsp104 have fueled controversy, and a clear picture of how Hsp104 drives protein disaggregation has not emerged from these studies. Controversy has been compounded by the use of only dysfunctional Hsp104 mutants in a limited number of nucleotide states: only ATP<sub>γ</sub>S, ATP, and ADP have been explored (Carroni et al., 2014; Lee et al., 2010; Wendler et al., 2007, 2009). It is difficult to relate these findings to WT Hsp104. To provide an independent view, we employed SAXS, a powerful method to study structural changes of AAA+ proteins in solution (Chen et al., 2010). SAXS is performed in solution, under conditions where Hsp104 is active, eliminating issues caused by freezing or fixation in cryo-EM. We reconstructed Hsp104 and Hsp104<sup>ΔN</sup> in AMP-PNP, ATP<sub>γ</sub>S, ATP, ADP-AIF<sub>x</sub> (hydrolysis transition state), ADP, and apo states to a nominal resolution of 7.6–8.4 Å. Thus, we provide the highest-resolution and most comprehensive set of volume envelopes for Hsp104 (which has not been studied by cryo-EM) and Hsp104<sup>ΔN</sup> hexamers to date. By studying Hsp104 in various nucleotides, we uncover hexameric states that are likely populated during its natural ATPase cycle. We revealed a peristaltic pumping motion of the central channel that drives directional substrate translocation, which is profoundly altered in Hsp104<sup>ΔN</sup>. This finding helps explain several functional deficits of Hsp104<sup>ΔN</sup>. However, pore shape is unlikely to be the only determinant of

substrate translocation, and it is critical to define the location of the substrate-binding pore loops in each nucleotide state. Future studies will fit atomic models of Hsp104 monomers into these SAXS envelopes and will be constrained by X-ray footprinting data (DeSantis et al., 2014).

We have elucidated the mechanism of Sup35 prion severing and dissolution by Hsp104. Hsp104 engages Sup35 prions by binding to a region spanning amino acids 96–151 (Figure 7G, purple regions). Hsp104 then exerts a directional pulling force that selectively unfolds cross-β structure N-terminal to this binding site, but does not unfold domains C-terminal to this binding site (Figure 7G). This partial translocation mechanism enables Hsp104 to dissolve Sup35 prions without unfolding the C-terminal GTPase domain (Figure 7G). Thus, Hsp104 rapidly releases functional, folded protein from the prion to rapidly cure the loss-of-function [*PSI*<sup>+</sup>] phenotype (Paushkin et al., 1996).

After engaging the prion, Hsp104 resolves cross-β structure N-terminal to its binding site in three steps: (1) the Tail-to-Tail contact is broken (Figure 7G, dark green regions), (2) the central cross-β core is unfolded (Figure 7G, blue regions), and (3) the Head-to-Head contact is broken to release soluble Sup35 (Figure 7G, red regions). This sequence was confirmed by covalently stapling the Tail-to-Tail or Head-to-Head contact with BMB. Thus, Hsp104 severed prions with a covalent Head-to-Head contact by breaking the Tail-to-Tail contact, but could not fragment prions with a covalent Tail-to-Tail contact.

Hsp104<sup>ΔN</sup> is specifically defective in the second and third steps of this process. Hsp104<sup>ΔN</sup> engages the same binding site on Sup35 prions and breaks the Tail-to-Tail contact. However, Hsp104<sup>ΔN</sup> is unable to unfold the central core or break the Head-to-Head contact. Thus, Hsp104<sup>ΔN</sup> is capable of fragmenting but not dissolving Sup35 prions (Figure 7G). The ability of Hsp104<sup>ΔN</sup> to fragment but not dissolve Sup35 prions explains why it can propagate [*PSI*<sup>+</sup>], but not readily eliminate it at high concentrations in vivo (Hung and Masison, 2006). Indeed, in vitro, Hsp104<sup>ΔN</sup> operates in a way that only stimulates Sup35 prionogenesis and selectively amplifies strong [*PSI*<sup>+</sup>] prions.

Curiously, Hsp104<sup>ΔN</sup> overexpression very slowly cures [*PSI*<sup>+</sup>] in some genetic backgrounds but not others (Park et al., 2014). It was proposed that Hsp104<sup>ΔN</sup> promotes Sup35 prion dissolution via a “trimming” activity that solubilizes Sup35 only from the ends of prion fibrils (Park et al., 2014). Our findings provide a rationale for this proposed activity. Selective cleavage of the Tail-to-Tail contact by Hsp104<sup>ΔN</sup> could liberate soluble Sup35 at the subset of fibril ends where the Tail-to-Tail contact holds the final monomer to the fibril. However, we did not observe dissolution of Sup35 from assembled prions by Hsp104<sup>ΔN</sup> in vitro. Released monomers could be rapidly converted to the prion form by fibril ends or Hsp104<sup>ΔN</sup> may not access fibril ends in vitro. In vivo, other factors not reconstituted here might prevent this reassociation or selectively target Hsp104<sup>ΔN</sup> to Sup35 prion fibril ends.

Mechanisms distinct from prion dissolution have been proposed to explain [*PSI*<sup>+</sup>] curing by Hsp104 overexpression including inhibition of Sup35 prion fragmentation (Winkler et al., 2012). Based on colocalization studies, it was proposed

that the Hsp104 NTD mediated binding to large NM-YFP aggregates and displaced Ssa1, thereby perturbing prion fragmentation (Winkler et al., 2012). However, overexpression of the Hsp104 NTD alone does not cure  $[PSI^+]$  (Hung and Masison, 2006). It is unclear whether the colocalization reflects direct binding, as Hsp104 and Hsp104<sup>ΔN</sup> bind pure Sup35 prions with similar affinity. Moreover, these results are uncorroborated with native untagged proteins, and large NM-YFP aggregates are not disseminated prions. Importantly,  $[PSI^+]$  curing kinetics by Hsp104 overexpression are inconsistent with inhibition of prion fragmentation (Park et al., 2014). This mechanism also fails to explain why Hsp104<sup>ΔN</sup> cures  $[PSI^+]$  in some genetic backgrounds (Park et al., 2014).

We have established that the NTD is essential for potentiation of Hsp104 activity by specific MD mutations. Unlike their full-length counterparts, neither Hsp104<sup>ΔN-A503V</sup> nor Hsp104<sup>ΔN-A503S</sup> rescued TDP-43, FUS, or  $\alpha$ -syn toxicity in yeast. Potentiating mutations at the A503 position of the MD likely promote an allosteric activation step that enhances Hsp104 ATPase, unfoldase, and disaggregase activity (Jackrel et al., 2014). These effects are ablated by NTD deletion. We conclude that optimal Hsp104 functionality depends on the NTD, which enables hexamer plasticity and potentiation.

## EXPERIMENTAL PROCEDURES

### Proteins

Proteins were purified using standard protocols. For more details, see Supplemental Experimental Procedures.

### Size-Exclusion Chromatography

Absolute molecular weights of apo hexamers of Hsp104 and Hsp104<sup>ΔN</sup> (15  $\mu$ M monomer) were determined using multiangle light scattering coupled with refractive interferometric detection and a TSK4000 size-exclusion column.

### NTPase Activity

Hsp104 ATPase and Sup35 GTPase activity was assessed as described (DeSantis et al., 2012; Krzewska et al., 2007).

### Protein Disaggregation

Luciferase disaggregation and reactivation in vitro and in vivo were as described (DeSantis et al., 2012). Amyloid and prion disaggregation was as described (DeSantis et al., 2012). For more details, see Supplemental Experimental Procedures.

### Thermotolerance

Yeast thermotolerance was assessed as described (DeSantis et al., 2012).

### Sup35 Prionogenesis and Transformation

Sup35 prionogenesis in vitro and transformation were performed as described (DeSantis and Shorter, 2012; Shorter and Lindquist, 2006). For more details, see Supplemental Experimental Procedures.

### Site-Resolved Pyrene and Acrylodan Fluorescence

Pyrene and acrylodan fluorescence were monitored as described (Krishnan and Lindquist, 2005).

### Site-Resolved BPMTS Crosslinking

Single cysteine NM variants (10  $\mu$ M) bearing BPMTS at the indicated position were assembled into prions with agitation at 1,400 rpm (Eppendorf thermomixer) in the dark. Crosslinking was elicited by UV irradiation at 365 nm for 20 min. Samples were processed for reducing SDS-PAGE and immunoblot. For more details, see Supplemental Experimental Procedures.

### SAXS/WAXS

X-ray scattering data were collected at beamline 4-2 at Stanford Synchrotron Radiation Laboratory (SSRL, Menlo Park, CA) and beamline X9 at the National Synchrotron Light Source (NSLS, Upton, NY). Data were collected and analyzed as described (DeSantis et al., 2014). Shape reconstructions of the hexamer were generated using GASBOR (Svergun et al., 2001). Six-fold symmetry was imposed. Reconstructions were averaged and filtered using DAMAVER and converted to volume envelopes using SITUS (Volkov and Svergun, 2003; Wriggers et al., 1999). For more details, see Supplemental Experimental Procedures.

### Mutant Doping Studies

Mathematical modeling and mutant doping studies were as described (DeSantis et al., 2012).

### Yeast Proteinopathy Models

Yeast strains integrated with galactose-inducible TDP-43, FUS, or  $\alpha$ -syn were transformed with the indicated galactose-inducible Hsp104 variant or vector. Toxicity, aggregation, and expression were assessed as described (Jackrel et al., 2014).

### ACCESSION NUMBERS

The SAXS data have been deposited in BIOISIS, an open-access database dedicated to the study of biological macromolecules by SAXS (<http://www.bioisis.net>). The accession codes are available upon request from the authors.

### SUPPLEMENTAL INFORMATION

Supplemental Information includes two figures, three tables, two movies, and Supplemental Experimental Procedures and can be found with this article at <http://dx.doi.org/10.1016/j.molcel.2014.12.021>.

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