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Engineering enhanced protein disaggregases for neurodegenerative disease

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ABSTRACT. Protein misfolding and aggregation underpin several fatal neurodegenerative diseases, including Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD). There are no treatments that directly antagonize the protein-misfolding events that cause these disorders. Agents that reverse protein misfolding and restore proteins to native form and function could simultaneously eliminate any deleterious loss-of-function or toxic gain-of-function caused by misfolded conformers. Moreover, a disruptive technology of this nature would eliminate self-templating conformers that spread pathology and catalyze formation of toxic, soluble oligomers. Here, we highlight our efforts to engineer Hsp104, a protein disaggregase from yeast, to more effectively disaggregate misfolded proteins connected with PD, ALS, and FTD. Remarkably subtle modifications of Hsp104 primary sequence yielded large gains in protective activity against deleterious α-synuclein, TDP-43, FUS, and TAF15 misfolding. Unusually, in many cases loss of amino acid identity at select positions in Hsp104 rather than specific mutation conferred a robust therapeutic gain-of-function. Nevertheless, the misfolding and toxicity of EWSR1, an RNA-binding protein with a prion-like domain linked to ALS and FTD, could not be buffered by potentiated Hsp104 variants, indicating that further amelioration of disaggregase activity or sharpening of substrate specificity is warranted. We suggest that neuroprotection is achievable for diverse neurodegenerative conditions via surprisingly subtle structural modifications of existing chaperones.

KEYWORDS. amyloid, EWSR1, FUS, Hsp104, prion, protein engineering, protein disaggregation, TAF15, TDP-43, α-synuclein

ABBREVIATIONS. PD, Parkinson's disease; ALS, amyotrophic lateral sclerosis; FTD, frontotemporal dementia; AD, Alzheimer's disease; α-syn, α-synuclein; WT, wild-type; HD, Huntington's disease; NBD, nucleotide-binding domain; MD, middle domain

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INTRODUCTION

As population demographics shift toward older age groups, several fatal and presently incurable neurodegenerative diseases, including: Alzheimer's disease (AD), Parkinson's disease (PD), amyotrophic lateral sclerosis (ALS), and frontotemporal dementia (FTD) will inevitably increase in prevalence.[1–5] These diseases threaten public health worldwide and present a formidable barrier to living longer, more fulfilling lives. Game-changing therapeutic solutions that attack the cause of these diseases and not merely the symptoms are urgently needed. Indeed, while 'wars' on cancer and heart disease have yielded myriad promising drugs, the same cannot be said for the neurodegenerative diseases where, aside from one notable exception for the treatment of familial amyloid neuropathy,[6–11] the drug pipelines are practically empty. Although each neurodegenerative disease is fundamentally different – some debilitating movement but preserving memory, others destroying memory but sparing movement – a recurring and unifying facet is the accumulation of misfolded protein structures in the brain.[1–5,12–14] These misfolded structures can even become self-templating and propagate disease from single or multiple sites of origination.[2,12–16]

Many of the misfolded proteins found in pathological inclusions, e.g. $\alpha-$synuclein (α-syn) or TDP-43, are expressed in almost all cells, yet only seem to misfold and confer toxicity in specific neurons.[1–5,17] Thus, motor neurons are primarily afflicted in ALS,[3] whereas dopaminergic neurons are selectively devastated in PD.[4,17] In all of these diseases, the proteostasis network collapses and fails to counter specific protein-misfolding events,[18–20] which ultimately overwhelm the system and can even transmit disease.[2,12–16] A key therapeutic advance will come with ability to rescue selectively vulnerable neurons with agents that directly antagonize or reverse the deleterious protein-misfolding events that underpin neurodegeneration.[21,22] Indeed, agents that reverse protein misfolding and restore proteins to native form and function could simultaneously eliminate any deleterious loss-of-function or toxic gain-of-function caused by misfolded conformers.[21,22] Moreover, a disruptive technology of this nature would eliminate self-templating conformers that spread pathology[2,12–16] and catalyze formation of soluble toxic oligomers[23] via secondary nucleation.[12,24] In other contexts, the accumulation of misfolded proteins trapped in inclusions can incur fitness costs and likely also contributes to cancer and aging.[25–30] Hence, agents capable of reactivating misfolded and aggregated proteins could have therapeutic utility beyond neurodegenerative disease.

The very same type of misfolded conformers (e.g., prions or amyloids) that underpin incurable neurodegenerative diseases have, surprisingly, been appropriated during evolution for adaptive purposes elsewhere.[31-46] For example, the same type of prion domain that enables Sup35 and Mot3 to form beneficial prions in yeast[31,33,41] causes TDP-43 and FUS to misfold in ALS,[2,47-51] and hnRNPA1 and hnRNPA2 to misfold in multisystem proteinopathy.[52,53] Indeed, yeast exploit prions for beneficial purposes, including stress resistance and the evolution of new traits in fluctuating environments.[31–35,41,44,45,54] CPEB prions might even encode our own long-term memories.[41,55–60] An important mission is to establish a deep and rigorous mechanistic understanding of how nature has controlled protein misfolding (sometimes even for adaptive purposes) so that we can apply and if necessary re-engineer these natural solutions to counter protein misfolding in disease. Unlocking nature's secrets to mitigate protein misfolding could empower unparalleled opportunities to eradicate neurodegenerative disease. In this review, we highlight our efforts to engineer Hsp104, a protein disaggregase from yeast, to more effectively disaggregate misfolded proteins connected with PD, ALS, and FTD.[61–64]

Applying Hsp104 and Engineered Variants to Deleterious Protein Misfolding

Hsp104 is a hexameric AAA+ ATPase and protein disaggregase from yeast.[65,66] Hsp104

forms a ring-shaped complex that disaggregates protein by coupling ATP hydrolysis to partial or complete substrate translocation across its central channel.[21,67–72] Optimal Hsp104 activity is typically achieved in conjunction with the Hsp110, Hsp70, and Hsp40 chaperone system.[65,73–76] Importantly, Hsp104 is the only cellular agent known to rapidly dissolve stable amyloid fibrils,[73,77,88] which can self-template their own cross-β conformation and encode transmissible phenotypes.[12,15,41] Hsp104 activity has enabled yeast to stably propagate prions and even exploit them for beneficial purposes.[32,33,41,54,89,90] Hsp104 also eradicates toxic soluble oligomers that adopt a generic conformation[23] formed by diverse proteins.[78–80] Thus, amyloid fibrils and toxic oligomers are not intractable and can be eliminated rapidly by Hsp104.[61,73,77–84,87,91]

Inexplicably, Hsp104 has no metazoan ortholog despite being highly conserved in bacteria, plants, fungi, chromista, and protozoa.[39,40,52] The reason for the loss of Hsp104 from metazoan lineages is uncertain, especially as Hsp104 can be expressed safely and broadly in worm, fly, mouse, and rat.[61,78,92–95] However, it has been suggested that the possession of Hsp104's potent disaggregase modality might have incurred a detrimental fitness cost at the divergence of metazoa from protozoa.[96,97] We have hypothesized that Hsp104 could be applied as a disruptive technology to combat protein-misfolding disorders.[21,22,98] Can we add back the powerful disaggregase activity that animals have lost very early in their evolution to impart therapeutic benefit in situations where protein misfolding has caused disease? Applying powerful biochemical activities isolated from the microbial world to solve problems posed by human disease has strong precedent. For example, botulinum neurotoxin variants can achieve therapeutic benefit across a large range of clinical conditions including various movement, urologic, and secretory disorders,[99–101] due to their highly potent and selective ability to cleave SNARE proteins and prevent secretion.[102,103]

Importantly, Hsp104 returns aggregated proteins to native structure and function,[65,66,85] and could simultaneously reverse toxic gain-of-function and loss-of-function phenotypes linked to protein misfolding, as well as eliminate self-templating conformers that spread disease.[13,21,22,98] We have established that Hsp104 disassembles toxic α-syn oligomers and amyloid,[78,84,98] *and* rescues α-syn-induced dopaminergic neurodegeneration in the mammalian substantia nigra.[78] Hsp104 suppresses polyglutamine toxicity in *Drosophila* even when expressed after the onset of polyglutamine-induced degeneration, whereas Hsp70 is ineffective.[92] Thus, Hsp104 is the first disaggregase or chaperone treatment administered after the onset of pathogenic protein-induced degeneration that mitigates disease progression.[92] Moreover, Hsp104 can disassemble amyloid and soluble toxic oligomeric forms of diverse wild-type (WT) and mutant proteins connected to AD, PD, Huntington's Disease (HD) or type II diabetes, including: Aβ42, tau, α-syn, polyglutamine, and amylin.[84,104]

For some substrates, however, Hsp104 is not as active as it is against natural yeast prions, such as those formed by Sup35.[79,80,84,101] Indeed, even some Sup35 prion strains are more resistant to elimination by Hsp104, and this differential sensitivity can drive 'protein only' evolution.[91] Thus, a key goal is to potentiate Hsp104 activity against specific disease-associated substrates via engineering and evolution.[21,22,61,62,98] However, chaperones are difficult targets for protein engineering due to their large size, and protein disaggregases such as Hsp104 have poorly understood structures,[67,82,83,105–108] making rational design challenging. Hence, we employed an unbiased approach to isolate improved Hsp104 variants by screening large libraries of Hsp104 variants to isolate those that rescue yeast models that recapitulate salient features of various neurodegenerative proteinopathies, including protein mislocalization, aggregation, and toxicity.[61,64]

We focused on yeast models of neurodegenerative proteinopathies caused by aberrant TDP-43 and FUS misfolding, e.g. ALS and FTD,[3,109,110] or α-syn misfolding, e.g., PD and multiple system atrophy.[4,17,111,112] In these yeast models, the neurodegenerative disease protein is overexpressed from the

galactose-inducible promoter, which induces protein mislocalization, aggregation, and toxicity idiosyncratic to each specific neurodegenerative disease.[47–49,113–118] Overexpression is a key tool to study the aggregation and toxicity of human neurodegenerative disease proteins in yeast and enables protein misfolding via increasing protein concentration and overwhelming proteostatic buffers.[117,118] Importantly, an established cause of FTD or ALS is increased expression of TDP-43[119] or FUS[120,121] respectively, and elevated α-syn expression causes PD.[122-125] These yeast models have proven to be an extraordinary resource and have enabled the identification of genetic and small-molecule suppressors of TDP-43, FUS, and α-syn toxicity, which can also exhibit therapeutic efficacy against degeneration in the metazoan nervous system, human cells and neurons, and even patient-derived neurons.[49,114,116,126–138] In the case of TDP-43, the yeast model even enabled identification of a common genetic risk factor for ALS: intermediate polyglutamine expansions (27-33 glutamines) in ataxin 2.[128,139–145] Indeed, several aggregation-prone human RNA-binding proteins with prion-like domains, including TAF15, EWSR1, hnRNPA1, and hnRNPA2 have been successfully predicted as neurodegenerative disease genes based on initial studies in yeast.[50–53,146,147] Thus, the power of yeast to elucidate protein-misfolding events and methods to counter them relevant to neurodegenerative disease should not be doubted.[130,137,139]

Importantly, for our purposes, the toxicity of TDP-43, FUS, and α-syn in yeast is maintained in Δ*hsp104* backgrounds,[61] indicating that, unlike polyglutamine toxicity in yeast,[148–150] TDP-43, FUS, and α-syn toxicity does not depend on Hsp104 or Hsp104-dependent prions. Moreover, in the Δ*hsp104* background, overexpression of wild-type (WT) Hsp104 did not rescue TDP-43, FUS, or α-syn toxicity.[61–64] Thus, we could explore Hsp104 sequence space in the absence of WT Hsp104 and be certain that any Hsp104 variants that rescued TDP-43, FUS, and α-syn toxicity were due to a novel therapeutic gain of Hsp104 function.[61–64]

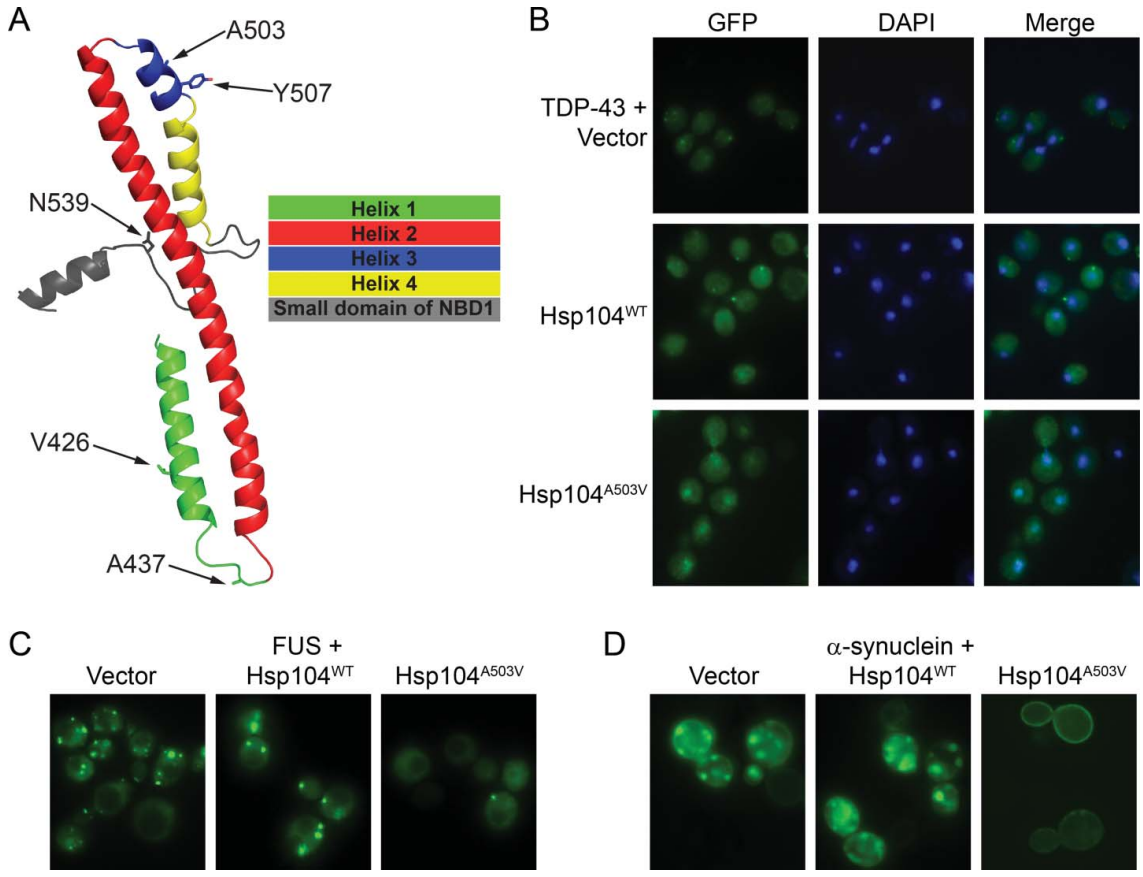
Potentiating Mutations Can be Uncovered in the Middle Domain of Hsp104

Hsp104 is comprised of 5 domains: an N-terminal domain, nucleotide-binding domain 1 (NBD1), a coiled-coil middle domain (MD), NBD2, and a short acidic C-terminal domain.[67,81] We focused our libraries on the coiled-coil MD, which is comprised of 4 α-helices and facilitates optimal ATPase activity, communication between NBD1 and NBD2, intrinsic disaggregase activity, and interactions with Hsp70 during disordered aggregate dissolution.[67,82,151–154] Importantly, the MD is less conserved than the 2 NBDs, indicating that it can withstand various missense mutations without eliminating disaggregase functionality.[67] Indeed, the MD can even tolerate large protein insertions (e.g., insertion of lysozyme between Asn467 and Glu468 in MD helix 2) or helix replacements and yet still maintain Hsp104 disaggregase activity.[106,152,153] Moreover, previous studies suggested that Hsp104 MD variants can have unexpected gain-of-function phenotypes, including rescue of polyglutamine aggregation and toxicity.[106,150,155]

Remarkably, we uncovered a large number of MD variants that enabled Hsp104 to rescue the aggregation and toxicity of TDP-43, FUS, and α-syn in yeast.[61] These mutations were located throughout the MD in helix 1 (e.g., V426L), the distal loop between helix 1 and 2 (e.g., A437W), and helix 3 (e.g., A503S, Y507C)[61] (**Figure 1A** and **Table 1**). We also uncovered potentiating mutations in the small domain of NBD1 (e.g., N539K) immediately adjacent to the C-terminal end of the MD[61] (**Figure 1A**). Interestingly, we recently discovered that the N-terminal domain of Hsp104 (residues 1-157) is essential for the potentiated activity of $Hsp104^{A503V}$ and $Hsp104^{A503S}$.[156]

Typically, these potentiated Hsp104 variants reduced aggregation and toxicity of TDP-43, FUS, and α-syn in yeast without reducing their expression level or inducing a heat shock

FIGURE 1. Potentiated Hsp104 variants suppress aggregation and mislocalization of disease proteins in yeast proteinopathy models. (**A**) Homology model of the MD and a portion of the small domain of NBD1 of Hsp104. Side chains of indicated residues are shown as sticks. (**B**) Fluorescence microscopy of yeast coexpressing fluorescently tagged TDP-43 and $Hsp104^{WT}$, $Hsp104^{A503V}$, or vector. Cells are stained with DAPI to visualize nuclei (blue). TDP-43 only exhibits nuclear localization upon coexpression of potentiated $Hsp104^{A503V}$. (**C**) Fluorescence microscopy of cells coexpressing FUS-GFP and $Hsp104^{WT}$, $Hsp104^{A503V}$, or vector. Yeast coexpressing potentiated $Hsp104^{A503V}$ display fewer cytoplasmic FUS foci. (**D**) Fluorescence microscopy of cells coexpressing α-synuclein-YFP and $Hsp104^{WT}$, $Hsp104^{A503V}$, or vector. Yeast coexpressing potentiated $Hsp104^{A503V}$ display fewer cytoplasmic α-syn foci, and α-syn only accumulates at the plasma membrane upon coexpression of $Hsp104^{A503V}$.



response.[61] The expression of potentiated Hsp104 variants was also similar or lower to the expression of WT Hsp104, which failed to rescue TDP-43, FUS, and α-syn aggregation or toxicity.[61,63] Moreover, neither the unfolded protein response nor autophagy was required for potentiated Hsp104 variants to rescue toxicity.[61] Remarkably, aggregates were now cleared from the majority of cells, and the correct localization of the neurodegenerative disease protein could be restored.[61,63] Thus, potentiated Hsp104 variants resolved cytoplasmic TDP-43 aggregates and restored TDP-43 to the nucleus[61,63] (**Figure 1B**). A major goal in ALS therapeutics is to achieve this phenotype in degenerating motor neurons.[3] For FUS, cytoplasmic aggregates were dissolved, but FUS remained cytoplasmic and did not return to the nucleus because the yeast nuclear-import machinery fails to decode the FUS PY-NLS[49,114] (**Figure 1C**). Several suppressors of FUS toxicity in yeast have been uncovered in

TABLE 1. Summary of potentiating Hsp104 mutations. The mutations that potentiate Hsp104 activity, their location, and their properties are listed based on[61,63,107]

Potentiating Mutations	Middle domain helix	Properties
V426G	1	Suppresses toxicity of FUS
V426L	1	Suppresses toxicity of TDP-43, FUS, and α-syn; Does not modify EWSR1 toxicity; Restores nuclear TDP-43 and clears FUS and α-syn aggregates
A437W	Loop between 1 and 2	Suppresses toxicity of TDP-43, FUS, and α-syn; Enhances EWSR1 toxicity
D498V	3	Suppresses toxicity of FUS and α-syn; Does not modify TDP-43 toxicity; ATPase similar to WT Hsp104; Does not require Hsp70 and Hsp40 for disaggregase activity; Hsp70 and Hsp40 do not stimulate disaggregase activity
A503X*	3	Suppress toxicity of TDP-43, FUS, and α-syn
A503V	3	Suppresses toxicity of TDP-43, FUS, and α-syn; Enhances EWSR1 toxicity; Elevated ATPase, disaggregase, translocase, and unfoldase activity; Does not require Hsp70 and Hsp40 for disaggregase activity; Restores nuclear TDP-43 and clears FUS and α-syn aggregates
A503S	3	Suppresses toxicity of TDP-43, FUS, TAF15, and α-syn; Enhances EWSR1 toxicity; Elevated ATPase, disaggregase, and unfoldase activity; Does not require Hsp70 and Hsp40 for disaggregase activity; Suppresses neurodegeneration in *C. elegans* PD model
A503V-DPLF	3 (Plus pore loop Y257F in NBD1 and Y662F in NBD2)	Suppresses toxicity of TDP-43, FUS, and α-syn; Enhances EWSR1 toxicity; Elevated ATPase, disaggregase, and unfoldase activity; Does not require Hsp70 and Hsp40 for disaggregase activity; Suppresses neurodegeneration in *C. elegans* PD model
D504V	3	Suppresses toxicity of TDP-43, FUS, and α-syn
D504C	3	Suppresses toxicity of FUS, and α-syn; Does not modify TDP-43 toxicity; ATPase activity similar to WT Hsp104; Elevated disaggregase and unfoldase activity; Does not require Hsp70 and Hsp40 for disaggregase activity; Hsp70 and Hsp40 do not stimulate disaggregase activity
Y507A		Suppresses toxicity of TDP-43, FUS, and α-syn; Does not require Hsp70 and Hsp40 for disaggregase activity
Y507D		Suppresses toxicity of TDP-43, FUS, and α-syn
Y507V	3	Suppresses toxicity of FUS, and α-syn; Does not modify TDP-43 toxicity; Elevated ATPase and disaggregase activity; Does not require Hsp70 and Hsp40 for disaggregase activity
Y507C	3	Suppresses toxicity of TDP-43, FUS, and α-syn; Enhances EWSR1 toxicity; Elevated ATPase, disaggregase, and unfoldase activity; Does not require Hsp70 and Hsp40 for disaggregase activity
N539L/E/D/G	Small domain NBD1	Suppresses toxicity of FUS
N539K	Small domain NBD1	Suppresses toxicity of TDP-43, FUS, and α-syn; Does not modify EWSR1 toxicity

*X = any amino acid except A or P.

genome-wide screens,[49,114] but none of these cleared FUS aggregates, indicating a novel mode of rescue by potentiated Hsp104 variants.[61,63] Potentiated Hsp104 variants cleared cytoplasmic α-syn inclusions and restored plasma membrane localization of α-syn[61,63] (**Figure 1D**). Achieving these phenotypes in patients would provide a game-changing solution for PD. Importantly, 2 potentiated Hsp104 variants, Hsp104^{A503S} and Hsp104$^{DPLF\text{-}A503V}$ (where substrate-engaging pore-loop tyrosines, Y257 and Y662 are mutated to F), rescued α-syn-induced dopaminergic neurodegeneration in a *C. elegans* model of PD.[61] Thus, potentiated Hsp104 variants confer neuroprotective phenotypes in the context of the metazoan nervous system. Next, it will be important to assess the efficacy of potentiated Hsp104 variants in mammalian models of PD[78] as well as TDP-43- and FUS-related ALS.[157,158] Nonetheless, we have established the first ever example of engineered disaggregases rescuing neurodegeneration in the metazoan nervous system. Thus, general neuroprotection via activated protein disaggregases may be achievable for a range of neurodegenerative diseases.

Several missense mutations in TDP-43 or FUS cause ALS,[3,159,160] whereas specific α-syn mutations cause PD.[17,161,162] Many of these variants cause aggressive, early-onset forms of disease.[3,17,159-162] Enhanced proteotoxicity often stems from an increased intrinsic propensity of the mutant protein to misfold in isolation,[48,163] e.g., as with TDP-43^{M337V} and α-syn^{A53T}, or to mislocalize in vivo, e.g. as with FUS^{P525L} and FUS^{R521C}.[3,157,164–166] A subset of the potentiated Hsp104 variants, Hsp104^{A503V}, Hsp104^{A503S}, Hsp104^{A503G}, Hsp104^{V426L}, Hsp104^{A437W}, Hsp104^{Y507C}, Hsp104^{N539K}, and Hsp104$^{DPLF\text{-}A503V}$, were tested against yeast models expressing disease-linked: TDP-43 (TDP-43^{A315T}, TDP-43^{Q331K}, and TDP-43^{M337V} [167,168]); FUS (FUS^{P525L} and FUS^{R521C} [3,157,164-166]); and α-syn (α-syn^{A53T} and α-syn^{E46K} [17,161,162]). Remarkably, potentiated Hsp104 variants suppressed toxicity and aggregation of these disease-linked forms of TDP-43, FUS, and α-syn.[63]

Initially, these Hsp104 variants appeared to be potentiated against all neurodegenerative disease substrates, but this was not the case.[63] We also tested their activity against EWSR1, another RNA-binding protein with a prion-like domain implicated in ALS and FTD, which aggregates and is toxic in yeast.[3,50,146,147,169] None of the potentiated Hsp104 variants suppressed EWSR1 aggregation or toxicity in yeast, and instead enhanced toxicity except for Hsp104^{V426L} and Hsp104^{N539K}, which had no effect like WT Hsp104.[63] This result was surprising as EWSR1 is closely related to FUS in terms of primary sequence and domain architecture.[170] Moreover, potentiated Hsp104 variants could rescue TAF15 toxicity in yeast.[63] TAF15 is also an RNA-binding protein with a prion-like domain implicated in ALS and FTD that is closely related to FUS and EWSR1.[50,146,147] We could rationalize these findings as in vitro Hsp104^{A503S} but not WT Hsp104 could disaggregate preformed FUS and TAF15 fibrils, but not EWSR1 fibrils.[63] Thus, EWSR1 fibrils appear to be refractory to potentiated Hsp104 variants in vitro and in vivo.[63] Further engineering of Hsp104 appears to be necessary to antagonize EWSR1 aggregation and toxicity.

Potentiated Hsp104 Variants Typically Have Elevated ATPase Activity

To assess the mechanism by which potentiating mutations enhance Hsp104 activity, we explored their biochemical properties. Typically, potentiated Hsp104 variants exhibited ~2-4-fold elevated ATPase activity than Hsp104, indicating that potentiation might stem from an ability to undergo more rapid rounds of ATP binding and hydrolysis.[61] However, enhanced ATPase activity was not always a feature of potentiated Hsp104 variants. For example, Hsp104^{D498V} and Hsp104^{D504C} exhibited ATPase activity similar to Hsp104.[61] Interestingly, Hsp104^{D498V} and Hsp104^{D504C} were more selective in their ability to rescue the yeast neurodegenerative disease models. They could both rescue FUS and α-syn toxicity in yeast, but failed to rescue TDP-43 toxicity.[61] These data suggest that elevated ATPase activity of potentiated Hsp104 variants is critical to rescue toxicity of a broad spectrum of

neurodegenerative disease proteins, and that reducing ATPase activity in this context promotes selectivity for specific substrates.

Potentiated Hsp104 Variants Do Not Require Hsp70 and Hsp40 for Disaggregation

Rescue of toxicity by enhanced Hsp104 variants could be a consequence of an altered mechanism of disaggregation compared to Hsp104. Thus, we assessed disaggregase activity against disordered luciferase aggregates in vitro. Hsp104 failed to disaggregate luciferase unless supplemented with Hsp70 or Hsp40. In striking contrast, Hsp70 and Hsp40 were not required for any of the potentiated Hsp104 variants to reactivate aggregated luciferase.[61] Typically, in the absence of Hsp70 and Hsp40, potentiated Hsp104 variants were ~3-9-fold more active than Hsp104 in the presence of Hsc70 (an Hsp70) and Hdj2 (an Hsp40).[61] Surprisingly, these findings suggest that absolute dependence on Hsp70 and Hsp40 hinders Hsp104 from rescuing α-syn, FUS, and TDP-43 toxicity.

This elevated disaggregase activity even in the absence of Hsp70 differentiates potentiated Hsp104 variants from hyperactive ClpB (the *E. coli* homolog of Hsp104) variants bearing specific MD mutations. In contrast to potentiated Hsp104 variants, hyperactive ClpB variants still require the Hsp70 chaperone system for robust disaggregase activity.[171] This key difference likely reflects the more stringent requirement for Hsp70 for ClpB disaggregase activity compared to Hsp104.[107]

In most cases, potentiated Hsp104 activity could be stimulated even further by supplementation with the Hsp70 chaperone system.[61] Thus, potentiated Hsp104 variants can still collaborate with the Hsp70 chaperone system, but do not absolutely require it for the disaggregation of disordered aggregates. Interestingly, once again the 2 exceptions were $Hsp104^{D498V}$ and $Hsp104^{D504C}$. The luciferase disaggregase activity of $Hsp104^{D498V}$ and $Hsp104^{D504C}$ was not stimulated further by the Hsp70 chaperone system.[61] These data suggest that the ability of potentiated Hsp104 variants to collaborate with Hsp70 is critical to rescue toxicity of a broad spectrum of neurodegenerative disease proteins, and that reducing collaboration with Hsp70 promotes selectivity for specific substrates.

Potentiated Hsp104 Variants Exhibit Enhanced Translocase and Unfoldase Activity

We also established that potentiated Hsp104 variants had enhanced activities against various soluble model substrates. Thus, potentiated variants translocated the intrinsically disordered soluble substrate, FITC-casein, more rapidly across their central channel.[61] This accelerated substrate-translocation rate likely enables potentiated Hsp104 variants to avoid kinetic traps during translocation and exert additional force to unfold stable substrates. Indeed, potentiated Hsp104 variants were also enhanced unfoldases, and unfolded GFP bearing a long ($RepA_{1-70}$) or short (6-HIS-TEV) unfolded tag more rapidly than Hsp104 in the presence of ATP.[61,63] In fact, Hsp104 did not unfold these substrates at all in the presence of ATP, but instead required a mixture of ATP and ATPγS.[61,63] Even then, Hsp104 unfolded these substrates less effectively and at a slower rate than the potentiated Hsp104 variants.[61,63] Importantly, neither Hsp104 nor the potentiated Hsp104 variants unfolded untagged GFP, and thus the potentiated variants do not unfold any protein.[61,63] Collectively, these data suggest that potentiated Hsp104 variants are enhanced unfoldases that are intrinsically primed to recognize and rapidly unfold substrates bearing even short unfolded tags and unlike Hsp104 do not have to wait for regulatory events to initiate unfolding (simulated here by ATP:ATPγS mixtures).

Potentiated Hsp104 Variants Exhibit Altered Subunit Collaboration

Using a mutant doping strategy,[104] we also revealed that a potentiated Hsp104 variant, $Hsp104^{A503V}$, promoted protein disaggregation by employing a different mechanism of intersubunit collaboration compared to Hsp104.

Indeed, the Hsp104^{A503V} hexamer possessed greater plasticity and maintained robust disaggregase activity in the presence of a wider variety of subunit-inactivating events. For example, an Hsp104^{A503V} subunit that binds but cannot hydrolyze ATP and engages substrate will stimulate the disaggregase activity of an adjacent Hsp104^{A503V} subunit within the hexamer. By contrast, in Hsp104, a single subunit with these properties inactivates the entire hexamer.[104] This increased resistance of Hsp104^{A503V} hexamers to subunit-inactivating events likely empowers facile resolution of recalcitrant substrates. Importantly, this altered activity enabled potentiated Hsp104 variants to disaggregate diverse neurodegenerative disease substrates in vitro, including α-syn, FUS, TDP-43, and TAF15 fibrils, under conditions where Hsp104 was inactive.[61,63]

Degeneracy of Potentiating Mutations at Specific MD Positions

Our findings establish that the Hsp104 MD plays a critical role in regulating Hsp104 function.[61,63] Remarkably, missense mutations to diverse residues at specific and disparate positions within the MD (e.g., A503, Y507, N539) conferred a therapeutic gain of function.[61] Indeed, A503 could be mutated to any residue except proline to yield potentiated Hsp104 variants capable of rescuing TDP-43, FUS, and α-syn toxicity in yeast.[61] Thus, potentiated disaggregase activity is enabled by loss of amino acid identity at specific positions in the MD rather than by mutation to a specific residue or class of residue. This finding indicates that Hsp104 disaggregase activity is usually tightly constrained, but can be unleashed by even very subtle changes to side chains at specific positions. The ability to attain such a wide-reaching set of gain of therapeutic functions via such minor changes in primary sequence, e.g. by adding a single methylene bridge (V426L) or by removing a single methyl group (A503G) is without precedent. It also suggests that drug-like small molecules that bind to the correct region of the MD might also enhance Hsp104 activity.[154] Moreover, post-translational modification of Hsp104 in specific regions of the MD could act as a switch to elicit potentiated activity in a reversible manner. Our findings suggest that the regulatory constraints placed on Hsp104 are simply too tight to counter TDP-43, FUS, and α-syn misfolding and toxicity under certain conditions. Thus, we discover an unanticipated and inimical limitation in existing disaggregase functionality. The MD can be viewed as a capacitor braced to unleash Hsp104 activity. Potentiating mutations likely destabilize autoinhibitory interactions that dampen Hsp104 activity or induce structural rearrangements that mimic or enable allosteric activation perhaps akin to the effect of Hsp70 binding the Hsp104 MD.[154] It remains unclear how diverse conservative and nonconservative mutations can result in this phenotype. Mutation of specific residues might subtly perturb hexamer structure, possibly promoting enhanced flexibility, altered channel properties, and stable population of the potentiated state.

Why Is WT Hsp104 Not Naturally Potentiated?

Why is WT Hsp104 not naturally potentiated? It would seem beneficial to be able to counter the excessive aggregation and toxicity caused by the overexpression of a single protein in yeast.[61,63] However, WT Hsp104 is unable to confer this activity.[61,63] Thus, it seems probable that the stress caused by the overexpression of a single, aggregation-prone, toxic protein is an unusual challenge for yeast that has not featured as a significant selective pressure sculpting Hsp104 primary sequence and activity. Rather, Hsp104 activity has likely been tuned during evolution to refold diverse aggregated proteins that accrue after a range of mild to severe environmental stresses.[65,66,172,173] Moreover, Hsp104 activity is also likely adapted to propagate various beneficial yeast prions.[31,33,34,41,46,90]

With regard to these 2 important activities, the potentiated Hsp104 variants uncovered in our screen display deficits. Thus, even though Hsp104^{A503V} can confer thermotolerance to

high temperatures (e.g., 50°C) just as well or even better than Hsp104,[61,155] Hsp104^{A503V} is toxic to yeast when overexpressed under conditions of very mild thermal stress (e.g. 37°C).[61,155] Moreover, Hsp104^{A503V} overexpression is benign under non-stressful conditions.[61,155] We suggest that under mild stress conditions, many yeast proteins populate mildly destabilized or metastable states that are inappropriately recognized and unfolded by potentiated Hsp104^{A503V}, which causes toxicity.[63] By contrast, WT Hsp104 is likely tuned to ignore such substrates.[63] Hsp104^{A503V} also displays defects in propagation of the beneficial [*PSI*$^+$] prion.[174] Indeed, Hsp104^{A503V} can even display synthetic lethality with strong [*PSI*$^+$] variants.[150] Thus, the alterations in Hsp104 activity caused by potentiating mutations might preclude stable maintenance of beneficial prions states, such as [*PSI*$^+$], which would in turn inhibit revelation of cryptic variation and rapid evolution of new traits in response to environmental stress.[32,33,35,41,45,46] These 2 differences between WT Hsp104 and potentiated Hsp104 in yeast likely explain why the potentiated forms were not fixed during evolution.

Even so, the possibility remains that the proteomes of other organisms might present challenges and selection pressures that necessitated more potentiated versions of Hsp104. Thus, it will be of great interest to compare the activity of Hsp104 orthologues from eukaryotic and prokaryotic species with diverse proteomes. For example, a naturally occurring variant of Hsp104 could display enhanced activity able to even more effectively counter the aggregation of human neurodegenerative disease proteins.

Further Engineering of Potentiated Hsp104 Variants

Using rational design and directed evolution, it will be important to isolate potentiated Hsp104 variants that are specific for single proteins (e.g., FUS) to minimize any potential off-target effects.[61,62] It will also be of interest to isolate conformer-specific potentiated Hsp104 variants. Ideally, Hsp104 variants could be isolated that antagonize only toxic misfolded species, for example: Hsp104 variants that rapidly resolve toxic soluble oligomers but not amyloid fibrils, or Hsp104 variants that resolve toxic amyloid strains and not benign strains. In principle, Hsp104 could be potentiated against any protein or any misfolded conformer, which might find important applications not only in therapeutics but also in the purification of irksome recombinant proteins for valuable basic or pharmaceutical purposes.

Broader Implications for Engineering Other Chaperones or Disaggregases

Reactivation of the disease-associated proteins to their non-pathogenic states suggests that Hsp104 variants and indeed other therapeutics that achieve this goal may provide a highly promising and potentially successful strategy for halting and reversing the progression of devastating neurodegenerative diseases. Potentiation of Hsp104 activity to achieve this goal required only subtle modifications to the existing disaggregase.[61,63] Indeed, engineering and directed evolution of the activity of other molecular chaperones, including GroEL, Hsp70, ClpX, and Spy, has revealed that minor changes in primary sequence (often a single missense mutation) can suffice to drastically alter substrate specificity or enhance global chaperone activity.[175–179] Indeed, minor alterations in primary sequence in naturally occurring chaperone homologs can also radically change activity.[180] It will be of great interest to engineer and enhance human molecular chaperones to counter specific protein-misfolding events related to neurodegenerative disease. In particular, engineering the human Hsp110, Hsp70, and Hsp40 disaggregase system,[65,73–76] to more effectively disaggregate disease substrates is an important goal. Since small changes in primary sequence at specific positions can greatly enhance chaperone activity[61,63,175–180] it appears plausible to isolate therapeutic small molecules that elicit similar enhancements in chaperone activity against disease substrates.[181,182]

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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