RESEARCH ARTICLE

Potentiating Hsp104 activity via phosphomimetic mutations in the middle domain

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ABSTRACT

Hsp104 is a hexameric AAA+ ATPase and protein disaggregate found in yeast, which can be potentiated via mutations in its middle domain (MD) to counter toxic phase separation by TDP-43, FUS and α-synuclein connected to devastating neurodegenerative disorders. Subtle missense mutations in the Hsp104 MD can enhance activity, indicating that post-translational modification of specific MD residues might also potentiate Hsp104. Indeed, several serine and threonine residues throughout Hsp104 can be phosphorylated in vivo. Here, we introduce phosphomimetic aspartate or glutamate residues at these positions and assess Hsp104 activity. Remarkably, phosphomimetic T499D/E and S535D/E mutations in the MD enable Hsp104 to counter TDP-43, FUS and α-synuclein aggregation and toxicity in yeast, whereas T499A/V/I and S535A do not. Moreover, Hsp104T499E and Hsp104S535E exhibit enhanced ATPase activity and Hsp70-independent disaggregate activity in vitro. We suggest that phosphorylation of T499 or S535 may elicit enhanced Hsp104 disaggregate activity in a reversible and regulated manner.

Keywords: disaggregate; Hsp104; neurodegeneration; ALS; PD; TDP-43

INTRODUCTION

Deleterious protein misfolding and aggregation underpin multiple devastating neurodegenerative disorders including amyotrophic lateral sclerosis (ALS), frontotemporal dementia (FTD), and Parkinson’s disease (PD) (Cushman et al. 2010; Brettschneider et al. 2015; Eisele et al. 2015). In ~97% of ALS cases and ~45% of FTD cases, a predominantly nuclear RNA-binding protein with a prion-like domain, TDP-43, mislocalizes to cytoplasmic inclusions and becomes depleted from the nucleus in degenerating neurons (Neumann et al. 2006; King, Gitler and Shorter 2012; Li et al. 2013; Ling, Polymenidou and Cleveland 2013; Guo and Shorter 2017). However, ALS and FTD can present without TDP-43 pathology (Ling, Polymenidou and Cleveland 2013). Indeed, in ~1% of ALS cases and ~9% of FTD cases, another primarily

In PD, the small, presynaptic, lipid-binding protein α-synuclein (α-syn) forms toxic oligomers and amyloids, which accumulate in cytoplasmic deposits termed Lewy bodies in degenerating dopaminergic neurons (Auluck, Caraveo and Lindquist 2010; Winner et al. 2011; Snead and Eliezer 2014; Abieliovich and Gitler 2016). Although the precise function of α-syn is uncertain, it likely plays important roles in synaptic vesicle trafficking (Gitler and Shorter 2007; Snead and Eliezer 2014; Abieliovich and Gitler 2016). These functions are likely perturbed via sequestration in toxic oligomers and Lewy Bodies. Thus, protein disaggregases that safely disassemble α-syn oligomers and amyloid, and recover functional α-syn could have curative properties in PD and other synucleinopathies (Shorter 2008; Snead and Eliezer 2014; Deyhaz et al. 2015).

In search of therapeutic agents, we have endeavored to tailor Hsp104, a hexameric AAA + protein disaggregate found in yeast, to safely disassemble toxic oligomers, aggregates, and amyloids connected to neurodegenerative diseases such as ALS, FTD, and PD (Shorter 2008, 2016, 2017; Jackrel and Shorter 2015, 2017). Each Hsp104 protomer contains an N-terminal domain, nucleotide-binding domain 1 (NBD1), a middle domain (MD), NBD2, and a short C-terminal domain (Fig. 1A) (Sweeny and Shorter 2016). Hsp104 assembles into asymmetric ring-like or lock-washer hexamers (Wendler et al. 2014; Gates et al. 2017), which extract individual polypeptides from aggregated structures via partial or complete translocation across their central channel (Lum et al. 2004; Shorter and Lindquist 2005a; Haslberger et al. 2008; Lum, Niggemann and Glover 2008; Tessarz, Mogk and Bukau 2008; Castellano et al. 2015; Sweeny et al. 2015). Polypeptide translocation is driven via ratchet-like conformational changes of the hexamer that are coupled to ATP binding and hydrolysis (Yokom et al. 2016; Gates et al. 2017). Hsp104 disaggregate activity can be boosted by additional molecular chaperones, including Hsp110, Hsp70, Hsp40, Hsp42, and Hsp26 (Glover and Lindquist 1998; Kashkar, Duennwald and Lindquist 2005; Haslbeck et al. 2005; Shorter and Lindquist 2008; Sweeny and Shorter 2008; Shorter 2011; Duennwald, Echeverria and Shorter 2012; Sweeny et al. 2015; Kaimal et al. 2017). Hsp104 liberates diverse polypeptides trapped in insoluble phases that accumulate after stress in yeast, and thereby confers large selective advantages (Sanchez and Lindquist 1990; Parsell et al. 1991, 1994; Sanchez et al. 1992, 1993; Glover and Lindquist 1998; Shorter 2008; Wallace et al. 2015). Hsp104 also has a powerful amyloid-dissolvase activity (Pauschkin et al. 1996; Shorter and Lindquist 2004, 2006; DeSantis et al. 2012; DeSantis and Shorter 2012b; Klaips et al. 2014; Sweeny et al. 2015; Pei et al. 2017; Zhao et al. 2017), which enables yeast to utilize and tightly regulate various prions for beneficial purposes (Chernoff et al. 1995; True and Lindquist 2000; True, Berlin and Lindquist 2004; Shorter and Lindquist 2005b; Halfmann, Alberti and Lindquist 2010; Tuite and Serio 2010; Newby and Lindquist 2013; Harvey, Chen and Jarosz 2017; Jarosz and Khurana 2017).

Importantly, the amyloid-dissolvase activity of Hsp104 can be turned against human neurodegenerative disease proteins. Indeed, Hsp104 dissolves diverse amyloids and toxic pre-amyloid oligomers formed by many wild-type and disease-linked mutant proteins connected to human disease, including amyloid-β, tau, α-syn, prion protein, polyglutamine and amylin (Lo Bianco et al. 2008; Liu et al. 2011; Shorter 2011; DeSantis et al. 2012; Duennwald, Echeverria and Shorter 2012). Moreover, Hsp104 mitigates neurodegeneration in animal models of disease (Satyal et al. 2000; Vacher, Garcia-Oroz and Rubinsztein 2005; Perrin et al. 2007; Lo Bianco et al. 2008; Cushman-Nick, Bonini and Shorter 2013). Despite these encouraging advances, effective amyloid dissolution can require high Hsp104 concentrations and Hsp104 is ineffective against TDP-43 and FUS (Lo Bianco et al. 2008; DeSantis et al. 2012; Jackrel and Shorter 2014a; Jackrel et al. 2014a). To circumvent these issues, we have engineered potentiated Hsp104 variants with enhanced disaggregate activity against TDP-43, FUS, and α-syn (Jackrel et al. 2014a, 2015; Jackrel and Shorter 2014a,b, 2015; Sweeny et al. 2015; Torrente et al. 2016).

Engineered Hsp104 variants with enhanced disaggregate activity successfully antagonize TDP-43, FUS, and α-syn aggregation and toxicity in yeast under conditions where Hsp104 is ineffective (Jackrel and Shorter 2014a; Jackrel et al. 2014a, 2015). This suppression of toxicity is accompanied by relocalization of TDP-43 to the nucleus and relocalization of α-syn to the plasma membrane, indicating that protein functionality is likely restored by Hsp104 variants (Jackrel and Shorter 2014a; Jackrel et al. 2014a). Potentiated Hsp104 variants also dissolve TDP-43, FUS, and α-syn fibrils more effectively than Hsp104 in vitro (Jackrel and Shorter 2014a; Jackrel et al. 2014a). Moreover, enhanced Hsp104 variants reverse cytoplasmic aggregation and mislocalization of ALS-linked FUS in mammalian cells (Yasuda et al. 2017) and mitigate dopaminergic neurodegeneration caused by α-syn in a Caenorhabditis elegans model of PD (Jackrel et al. 2014a). Thus, engineered Hsp104 variants may be effective therapeutic agents to be advanced in preclinical studies.

The majority of mutational mutations isolated to date reside in the MD of Hsp104 (Jackrel et al. 2014a, 2015). The MD is an important autoregulatory domain of Hsp104, which encircles the disaggregate and enables interdomain communication between NBD1 and NBD2 and collaboration with Hsp70 (Cashikar et al. 2002; DeSantis and Shorter 2012a, 2014; Lee et al. 2013; Heuck et al. 2016; Yokom et al. 2016; Gates et al. 2017). Potentiating mutations have been found in all four helices of the MD (Jackrel et al. 2015). Some of these mutations may alter inter-protomer contacts between MD helix L1 and MD helix L3 or contacts between the MD and NBD1 (Heuck et al. 2016; Gates et al. 2017). Remarkably, very minor changes in primary sequence can result in enormous changes in disaggregate activity. Indeed, a single missense mutation can potentiate Hsp104 and may be as subtle as removal of a single methyl group (e.g. A503G), removal of a single methylene bridge (e.g. E469D), or addition of a single methylene bridge (e.g. V426I) (Jackrel et al. 2014a, 2015; Jackrel and Shorter 2015). The subtle nature of some potentiating mutations suggests that post-translational modifications of Hsp104 at specific positions in the MD or NBD1 might also potentiate activity. Indeed, several serine and threonine residues throughout Hsp104 can be phosphorylated in vivo, including S78 and S155 in the NTD, S206 and S306 in NBD1, T499 and S535 in the...
Figure 1. Hsp104T499D and Hsp104S535D suppress α-syn toxicity, aggregation, and mislocalization. (A) A map of the different domains of Hsp104 shows the location of serine and threonine residues that can be phosphorylated in vivo. The T499D and S535D potentiating mutations (denoted in red) are found in the MD (green, residues 411–538). The NTD is in pink, NBD1 is in dark blue, NBD2 is in light blue and the C-terminal domain is in brown. (B) Hsp104T499D and Hsp104S535D suppress α-syn toxicity. Phosphomimetic Hsp104 variants in the pRS416GAL-Hsp104 plasmid were transformed into W303a/hsp104 yeast strains integrated with two copies of pAG303GAL-α-syn. The mutant strains were serially diluted 5-fold and spotted in duplicate onto galactose (inducing) and glucose (non-inducing) media. Two negative controls (vector, wild type) and a positive control (Hsp104A503V) were spotted alongside the mutant strains. (C) Hsp104T499D and Hsp104S535D do not reduce α-syn expression. Western blots were conducted for all strains in (B) with 3-Phosphoglycerate kinase (PGK) as a loading control. The strains were induced for 8 h in galactose and lysed prior to western blotting. (D) Hsp104T499A and Hsp104S535A do not suppress α-syn toxicity. Spotting assay performed as in (B). (E) Hsp104T499A and Hsp104S535A do not reduce α-syn expression. Western blots were conducted as in (C). (F) Hsp104T499D and Hsp104S535D suppress α-syn aggregation. The potentiated variants, Hsp104T499D and Hsp104S535D, were transformed into α-syn-YFP yeast. The resulting strains were induced for 8 h in galactose and prepared for fluorescence microscopy. Vector, wild-type Hsp104, and the positive control Hsp104A503V were prepared alongside the mutant strains. (G) Quantification of ∼200–250 cells was performed. The cells were categorized as exhibiting either cytoplasmic aggregates or membrane localization. The graphical representation shows the means ± SEM (n = 3) for the percentage of cells in each category.
exhibit enhanced ATPase and protein-disaggregase activity. We suggest that the phosphorylation of T499 or S535 may enable enhanced Hsp104 disaggregase activity in a reversible and regulated manner.

MATERIALS AND METHODS
Yeast strains, plasmids and media
Yeast were WT W303a (MATa, can1-100, his3-11, 15, leu2-3, 112, trp1-1, ura3-1, ade2-1) or the isogenic strain W303aΔhsp104 (Jackrel et al. 2014a). Media was supplemented with 2% glucose, raffinose, or galactose as specified. The yeast strains W303aΔhsp104 303GAL-α-syn, FUS, and TDP-43 have been previously described (Jackrel and Shorter 2014a; Jackrel et al. 2014a,b). QuikChange site-directed mutagenesis (Agilent, Santa Clara, CA, USA) was used to create mutations in the pRS416GAL-Hsp104 plasmid and all mutations were confirmed by DNA sequencing.

Yeast transformation and spotting assays
Yeast transformations were performed using standard polyethylene glycol and lithium acetate procedures (Gietz and Schiestl 2007). For the spotting assays, yeasts were grown to saturation in raffinose supplemented dropout media overnight at 30°C. The saturated overnight cultures were serially diluted 5-fold, and a 96-bolt replicator tool (frogger) was used to spot the strains in duplicate onto both glucose and galactose dropout plates. These plates were grown at 30°C and imaged after 72 h to assess suppression of disease-protein toxicity.

Western blotting
Transformed phosphomimetic mutants and controls were grown overnight in raffinose media. The overnight cultures were diluted to an OD of 0.3 (A600nm = 0.3) and grown in galactose-supplemented media at 30°C. α-Syn samples were induced for 8 h, while FUS and TDP-43 samples were induced for 5 h. Samples were then normalized to an OD of 0.6. The pelleted cells were resuspended in 0.1 M NaOH for 5 min and then pelleted again and resuspended in 1x SDS sample buffer. The samples were then boiled and separated by SDS-PAGE (4%-20% gradient, Bio-Rad, Hercules, CA, USA), and then transferred to a PVDF membrane (Millipore). The following primary antibodies were used: anti-GFP monoclonal (Roche Applied Science, Penzberg, Germany), anti-FUS polyclonal (Bethyl Laboratories, Montgomery, TX, USA), anti-TDP-43 polyclonal (Proteintech, Rosemont, IL, USA), anti-Hsp104 polyclonal (Enzo Life Sciences, Farmingdale, NY, USA) and anti-PGK monoclonal (Invitrogen, Carlsbad, CA, USA). Blots were imaged using a LI-COR Odyssey FC Imaging system.

Toxicity assay
Phosphomimetic mutants along with applicable controls were transformed into W303aΔhsp104 yeast. The strains were grown overnight in raffinose dropout media at 30°C with shaking. The saturated cultures were spotted in duplicate onto two sets of SD-Ura and SGal-Ura plates. One set of plates was placed at 37°C and the other at 30°C. Both sets of plates were analyzed for toxicity after 72h.

Fluorescence microscopy
Microscopy samples were grown and induced as they were for immunoblotting. For TDP-43 samples, cells were harvested, fixed in 1 mL 70% ethanol, and immediately pelleted. The cells were then washed 3 times with cold PBS and resuspended in 15 ul of Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories, Burlingame, CA, USA). α-Syn and FUS samples were imaged live. All cells were imaged at 100x magnification using a Leica-DM-IRBE microscope. Analysis of cells was performed in ImageJ. Approximately 200–250 cells were quantified for each sample in three independent trials.

RESULTS
Hsp104ΔT499D and Hsp104ΔS535D suppress α-syn toxicity, aggregation and promote its plasma membrane localization
A number of serine and threonine residues in Hsp104 can be phosphorylated in vivo, including T87 and S155 in the NTD, S206 and S306 in NBD1, T499 and S535 in the MD, and S577, S578, and S768 in NBD2 (Fig. 1A) (Albuquerque et al. 2008; Holt et al. 2009; Swaney et al. 2013). To assess whether serine or threonine phosphorylation at these positions might enhance Hsp104 activity, we introduced single phosphomimetic aspartate or glutamate substitutions at these positions in Hsp104. We then determined whether these phosphomimetic Hsp104 variants could antagonize α-syn toxicity in yeast. Upon expression from the inducible galactose promoter, α-syn accumulates in cytoplasmic aggregates and is toxic to yeast thereby recapitulating the phenotype of degenerating dopaminergic neurons in PD patients (Outeiro and Lindquist 2003). This yeast model of α-syn aggregation and toxicity has been tremendously valuable in identifying novel genetic and small-molecule suppressors of α-syn toxicity, which have translated to worm, fly, mouse, and patient-derived neuronal models of PD (Cooper et al. 2007; Tardiff and Lindquist 2013; Tardiff et al. 2013; Caraveo et al. 2014, 2017; Jackrel et al. 2014a; Khurana et al. 2017).

α-Syn overexpression is toxic to yeast (Fig. 1B) and is an established cause of PD (Singleton et al. 2003). This α-syn toxicity cannot be buffered by wild-type Hsp104 (Fig. 1B). Likewise, the phosphomimetic aspartate variants Hsp104ΔT499D, Hsp104ΔS535D, Hsp104ΔS206D, Hsp104ΔS306D, Hsp104ΔS577D, Hsp104ΔS578D, and Hsp104ΔS768D were unable to rescue α-syn toxicity despite...
being robustly expressed (Fig. 1B and C). In contrast, Hsp104T499D and Hsp104S535D strongly suppressed α-syn toxicity to a level similar to the canonical potentiated Hsp104 variant, Hsp104A503V (Fig. 1B). This rescue was achieved without any reduction in α-syn expression (Fig. 1C). Very similar results were obtained with phosphomimetic glutamate variants. Thus, only Hsp104T499E and Hsp104S535E rescued α-syn toxicity (data not shown). Altogether, we find phosphomimetic mutations at T499 or S535 in the MD potentiate Hsp104 activity, whereas single phosphomimetic mutations at T87 or S155 in the NTD, or S206 or S306 in NBD1, or S577, S578, or S768 in NBD2 do not.

Several positions in the MD can be mutated to diverse amino acids and confer potentified Hsp104 activity (Jackrel et al. 2014a). For example, A503 in helix L3 of the MD can be mutated to any amino acid, except proline, and enable Hsp104 to strongly suppress α-syn, FUS, and TDP-43 toxicity (Jackrel et al. 2014a). To confirm that enhanced Hsp104 activity was due to the phosphomimetic mutation at T499 or S535 rather than a general effect of any mutation, we generated the T499A and S535A variants. Unlike Hsp104T499D or Hsp104S535D, neither Hsp104T499A nor Hsp104S535A rescued α-syn toxicity despite robust expression (Fig. 1D and E). Thus, unlike A503, not any mutation at T499 or S535 potentiates Hsp104 activity. Indeed, we also assessed T499I, a mutation that when combined with G217S causes Hsp104 to be toxic (Schirmer et al. 2004). Hsp104T499I exhibits mildly reduced ATPase activity in vitro, but confers thermotolerance in vivo (Schirmer et al. 2004). Like Hsp104T499A, Hsp104T499I was unable to rescue α-syn toxicity (data not shown). Moreover, we have previously shown that Hsp104T499V does not rescue α-syn toxicity (Jackrel et al. 2014a). Thus, only select missense mutations at the T499 position confer potentified activity.

α-Syn accumulates in cytoplasmic aggregated structures in yeast that are not affected by expression of Hsp104 (Fig. 1F and G). Indeed, ∼70% of cells present with cytoplasmic α-syn foci in the vector control or in the presence of Hsp104 (Fig. 1F). In contrast, the potentiated variant, Hsp104A503V, antagonizes formation of cytoplasmic α-syn foci and enables α-syn to localize to the plasma membrane in ∼90% of cells (Fig. 1F and G). The MD phosphomimetic variants, Hsp104T499D and Hsp104S535D, also suppressed the formation of cytoplasmic α-syn aggregates and enabled α-syn localization to the plasma membrane in ∼80% and ∼50% of cells, respectively (Fig. 1F and G). Thus, Hsp104T499D and Hsp104S535D are not as effective as Hsp104A503V in suppressing cytoplasmic α-syn aggregation. Regardless, the level of protection against cytoplasmic α-syn aggregation conferred by Hsp104T499D and Hsp104S535D is sufficient to potently mitigate α-syn toxicity (Fig. 1B and D).

Hsp104T499D and Hsp104S535D suppress TDP-43 toxicity, aggregation and promote its nuclear localization

Next, we assessed the same single phosphomimetic aspartate or glutamate Hsp104 variants against TDP-43 aggregation and toxicity in yeast. TDP-43 is a mostly nuclear protein that shuttles between the nucleus and cytoplasm, but aggregates and mislocalizes to the cytoplasm of degenerating motor neurons in ALS and cortical neurons in FTD (Guo and Shorter 2017). Overexpression of TDP-43 in yeast replicates these phenotypes of cytoplasmic aggregation and toxicity (Johnson et al. 2008, 2009). Indeed, TDP-43 overexpression is connected to FTD (Gitcho et al. 2009). This yeast model of TDP-43 aggregation and toxicity has empowered the discovery of key genetic and small-molecule modifiers of TDP-43 toxicity, which have translated to fly, mouse, and neuronal models of ALS/FTD (Elden et al. 2010; Armakola et al. 2012; Tardiff et al. 2012; Kim et al. 2014; Jackrel et al. 2014a; Becker et al. 2017). Indeed, this yeast model sparked the discovery of intermediate-length, polyglutamine expansions (∼27–33 glutamines) in ataxin 2 as a common risk factor for ALS (Elden et al. 2010; Lee et al. 2011a, b; Yu et al. 2011; Auburger et al. 2017).

TDP-43 toxicity was not rescued by Hsp104 or by any of the aspartate or glutamate phosphomimetic variants in the NTD, NBD1, or NBD2 (Fig. 2A). In contrast, the MD phosphomimetic variants, Hsp104T499D and Hsp104S535D, mitigated TDP-43 toxicity (Fig. 2A). Hsp104T499E and Hsp104S535E also rescued TDP-43 toxicity (data not shown). Rescue of TDP-43 toxicity by Hsp104T499D and Hsp104S535D was similar to that achieved by Hsp104A503V and was not due to gross reductions in TDP-43 expression levels (Fig. 2A and B). In contrast, Hsp104T499A, Hsp104T499I, Hsp104T499V and Hsp104S535A did not rescue TDP-43 toxicity (Fig. 2C and D; data not shown) (Jackrel et al. 2014a). Thus, phosphomimetic mutations at T499 or S535 enhanced Hsp104 activity against TDP-43, whereas alanine, valine or isoleucine substitutions at T499 or alanine substitution at S535 did not.

We determined that Hsp104T499E and Hsp104S535E also suppressed TDP-43 aggregation and mislocalization (Fig. 2E and F). In yeast expressing Hsp104 or the vector control, TDP-43 was found in the nucleus in ∼35% of cells, whereas ∼65% had cytoplasmic TDP-43 aggregates (Fig. 2E and F). Conversely, in yeast cells expressing Hsp104T499D or Hsp104S535D, TDP-43 was found in the nucleus in ∼65% of cells, whereas ∼35% had cytoplasmic TDP-43 aggregates (Fig. 2E and F). Collectively, these findings establish that Hsp104T499D and Hsp104S535D are potent suppressors of TDP-43 aggregation and toxicity. Importantly, Hsp104T499E and Hsp104S535E also restore TDP-43 localization to the nucleus.

Hsp104T499D and Hsp104S535D suppress FUS toxicity and aggregation

We next assessed phosphomimetic Hsp104 variants against FUS proteotoxicity. FUS overexpression in yeast induces cytoplasmic FUS aggregation and toxicity, thereby mimicking events in degenerating neurons in ALS or FTD with FUS pathology (Ju et al. 2011; Sun et al. 2011; Harrison and Shorter 2017). Moreover, elevated FUS expression is also connected to neurodegenerative disease (Sabatelli et al. 2013; Dini Modigliani et al. 2014). This yeast model of FUS proteinopathies has enabled discovery of several genetic modifiers of FUS toxicity, which have translated to fly, mammalian cell and neuronal models of ALS and FTD (Sun et al. 2011; Daigle et al. 2013; Jackrel et al. 2014a; Bardana et al. 2015; Daigle et al. 2016; Yasuda et al. 2017).

As with α-syn and TDP-43, FUS toxicity was not buffered by wild-type Hsp104 or by any of the aspartate or glutamate phosphomimetic variants in the NTD, NBD1 or NBD2 (Fig. 3A). However, Hsp104T499D and Hsp104S535D strongly mitigated FUS toxicity and were just as effective as Hsp104A503V (Fig. 3A). These potentiated variants also mildly reduced FUS protein levels and this effect was most pronounced for Hsp104A503V (Fig. 3B). Hsp104T499E and Hsp104S535E also strongly rescued FUS toxicity (data not shown). In contrast, Hsp104T499A, Hsp104T499I, Hsp104T499V and Hsp104S535A did not rescue FUS toxicity (Fig. 3C and D; data not shown) (Jackrel et al. 2014a). Thus, rescue of FUS toxicity was enabled by phosphomimetic mutations at T499 or S535, whereas other substitutions at T499 or S535 explored here were ineffective.

Hsp104T499D and Hsp104S535D also reduced cytoplasmic FUS aggregation in yeast (Fig. 3E and F). Indeed, over 80% of cells...
Hsp104T499D/E but not Hsp104S535D/E reduces yeast growth at 37°C

Potentiated Hsp104 variants with mutations in the MD such as Hsp104A503V can confer a temperature-sensitive growth defect whereby yeast grow normally at 30°C but exhibit attenuated growth at 37°C (Fig. 4) (Jackrel and Shorter 2014a,b; Jackrel et al. 2014a). This toxicity at 37°C likely reflects off-target effects such as promiscuous binding and unfolding of essential proteins (Schirmer et al. 2004; Jackrel and Shorter 2014a,b; Jackrel et al. 2014a). Thus, we assessed whether Hsp104T499D/E...
Figure 3. Hsp104T499D and Hsp104S535D antagonize FUS toxicity and aggregation. (A) Hsp104T499D and Hsp104S535D antagonize FUS toxicity. Phosphomimetic variants in the pRS416GAL-Hsp104 plasmid were transformed into W303a Δhsp104 yeast strains integrated with pAG303GAL-FUS. The mutant strains were serially diluted 5-fold and spotted in duplicate onto galactose (inducing) and glucose (non-inducing) media. Two negative controls (vector, wild type) and a positive control (Hsp104A503V) were spotted alongside the mutant strains. (B) Hsp104T499A and Hsp104S535A do not suppress FUS toxicity. Spotting assay performed as in (A). (C) Hsp104T499D and Hsp104S535D do not suppress FUS toxicity. Western blots were conducted for all strains in (A) with 3-Phosphoglycerate kinase (PGK) as a loading control. The strains were induced for 5 h in galactose and lysed prior to western blotting. (D) Hsp104T499A and Hsp104S535A do not reduce FUS expression. Western blots were conducted as in (B). (E) Hsp104T499D and Hsp104S535D suppress FUS aggregation in yeast. The potentiated mutations T499D and S535D were transformed into FUS GFP-tagged yeast. The resulting strains were induced for 5 h in galactose and prepared for fluorescence microscopy. Vector, wild type, and the positive control Hsp104A503V were prepared alongside the mutant strains. (F) Quantification of approximately 200–250 cells was performed. The cells were categorized as containing multiple foci, a single focus, or no foci. The graphical representation shows the means ± SEM (n = 3) for the percentage of cells in each category.

Hsp104T499E, Hsp104S535D or Hsp104S535E might also confer this phenotype. None of the variants were toxic at 30°C (Fig. 4). In contrast, expression of Hsp104T499D or Hsp104T499E was toxic at 37°C, indicating that these variants have off-target effects (Fig. 4). Hsp104T499D and Hsp104T499E toxicity at 37°C was similar to Hsp104A503V (Fig. 4). Despite these off-target effects in the absence of disease protein, Hsp104T499D and Hsp104T499E potently rescue α-syn, TDP-43, and FUS toxicity. In contrast, Hsp104S535D and Hsp104S535E were not toxic to yeast at 37°C and resembled wild-type Hsp104 (Fig. 4). Thus, Hsp104S535D and Hsp104S535E exhibit potentiated activity but minimal off-target toxicity, which are attractive features for therapeutic protein disaggregases to be advanced to preclinical studies.

Location of T499 and S535 in Hsp104 hexamers

Next, we mapped the potentiating phosphomimetic mutations onto the structure of Hsp104 hexamers bound to ADP...
T499 is a poorly conserved residue and is typically a leucine or a large aromatic residue in other homologs (Fig. 5A). T499 is found at the start of helix L3 in the MD and is anticipated to be readily accessible to kinases and phosphatases (Fig. 5B) (Yokom et al. 2016; Gates et al. 2017). Generative Regularized ModelS of proteINs (GREMLIN) analysis of Hsp104 suggest that T499 interacts and coevolves with A503 in helix L3 (Fig. 5C) (Ovchinnikov, Kamisetty and Baker 2014). Indeed, the structure of Hsp104 hexamers in the presence of ADP suggests that T499 interacts with A503 in the same protomer via a backbone hydrogen bond (Fig. 5C) (Gates et al. 2017). Mutation of A503 to any amino acid except proline potentiates Hsp104 (Jackrel et al. 2014a), which may be due to disruption of the interaction with residue T499. Likewise, mutation of T499 to aspartate or glutamate, but not alanine, valine, or isoleucine likely also perturbs the interaction with A503 leading to potentiating activity. T499 may also make an intramolecular contact with E494 in helix L2 of the MD in protomer 4 (Gates et al. 2017). Interestingly, T499 also contacts MD residues V426 and K429 in the neighboring protomer (Fig. 5C). Mutations at V426 can also potentiate Hsp104 activity (Jackrel et al. 2014a, 2015). Thus, remodeling this network of interactions within the MD and between MDs in adjacent protomers likely yields potentiated activity.

Like T499, S535 is a poorly conserved residue and is typically proline or arginine in other homologs (Fig. 5A). S535 resides in the linker between helix L4 in the MD and the C-terminal remainder of NBD1 and is likely accessible to kinases and phosphatases (Fig. 5B) (Yokom et al. 2016; Gates et al. 2017). Intriguingly, S535 makes intraprotomer contacts with E494 in helix L2 of the MD (Fig. 5D) (Gates et al. 2017). S535D and E496D potentiate Hsp104 activity perhaps due to alteration of this intraprotomer MD contact (Jackrel et al. 2015).

Hsp104T499E and Hsp104S535E exhibit enhanced ATPase and protein-disaggregase activity

Next, we purified Hsp104T499E and Hsp104S535E and assessed how their biochemical activity compared to Hsp104. First, we established that these phosphomimetic MD variants display elevated ATPase activity (Fig. 6A). Hsp104T499E exhibited ATPase activity ∼4-fold higher than Hsp104, whereas Hsp104S535E was ∼2-fold higher (Fig. 6A). Second, we tested the protein disaggregation and reactivation activity of Hsp104T499E and Hsp104S535E variants using denatured luciferase aggregates as a model substrate (Glover and Lindquist 1998). While Hsp104 requires Hsp70 and Hsp40 for luciferase reactivation (Glover and Lindquist 1998), Hsp104T499E and Hsp104S535E do not (Fig. 6B). In the absence of Hsp70 and Hsp40, Hsp104S535E was slightly less active than Hsp104 with Hsp70 and Hsp40, whereas Hsp104T499E was ∼6-fold more active (Fig. 6B). The addition of Hsp70 and Hsp40 to Hsp104S535E variants increased luciferase reactivation by ∼6-fold, whereas Hsp104T499E was stimulated only slightly further (Fig. 6B). Hsp104T499E is almost fully active without Hsp70 and Hsp40 (Fig. 6B). In contrast, Hsp104S535E was greatly stimulated by Hsp70 and Hsp40 (Fig. 6B). These observations indicate differences in the mechanism of potentiation by T499E and S535E, which likely reflects the rearrangement of different structural contacts (Fig. 5C and D). Nonetheless, the potentiating T499E and S535E mutations increase disaggregase activity in the absence or presence of Hsp70 and Hsp40.

DISCUSSION

The subtle nature of some potentiating mutations in Hsp104 led us to hypothesize that post-translational modifications of Hsp104 at specific positions in the MD might also potentiate activity. Indeed, several serine and threonine residues throughout Hsp104 can be phosphorylated in vivo (Albuquerque et al. 2008; Holt et al. 2009; Swaney et al. 2013). Here, we have introduced phosphomimetic aspartate or glutamate residues at these positions and assessed Hsp104 activity. Phosphomimetic mutations at T87 and S155 in the NTD, S206 and S306 in NBD1, or S577, S578 and S768 in NBD2 did not enhance Hsp104 activity. Remarkably, phosphomimetic mutations at two positions in the MD, T499 and S535, enabled Hsp104 to rescue TDP-43, FUS, and α-syn aggregation and toxicity in yeast. Moreover, Hsp104T499E and Hsp104S535E exhibit enhanced ATPase activity and protein-disaggregase activity in vitro. We suggest that phosphorylation of T499 or S535 may enable enhanced Hsp104 disaggregase activity in a reversible and regulated manner. Thus, enhanced disaggregase activity could be unleashed and restrained at specific locations or in response to specific environmental cues in vivo. Understanding this regulation could also inform strategies to engineer transient bursts of enhanced disaggregase activity in therapeutic settings.

How do the T499D/E and S535D/E mutations potentiate Hsp104? Mutation to a negatively charged residue is important for Hsp104 activity even in the absence of Hsp70. However, Hsp104T499D/E but not Hsp104S535D/E reduces yeast growth at 37°C. Interestingly, T499 lies in helix L3 of the MD and has coevolved and likely alters interactions with A503 in the same protomer, but not alanine, valine, or isoleucine at T499 do not enhance activity. In contrast, alanine, valine, or isoleucine at T499 do not enhance activity. Thus, T499D/E and S535D/E mutations potentiate Hsp104 by Hsp70 and Hsp40 for luciferase reactivation (Glover and Lindquist 1998). While Hsp104 requires Hsp70 and Hsp40, Hsp104S535E was ∼6-fold more active (Fig. 6B). The addition of Hsp70 and Hsp40 to Hsp104S535E variants increased luciferase reactivation by ∼6-fold, whereas Hsp104T499E was stimulated only slightly further (Fig. 6B). Hsp104T499E is almost fully active without Hsp70 and Hsp40 (Fig. 6B). In contrast, Hsp104S535E was greatly stimulated by Hsp70 and Hsp40 (Fig. 6B). These observations indicate differences in the mechanism of potentiation by T499E and S535E, which likely reflects the rearrangement of different structural contacts (Fig. 5C and D). Nonetheless, the potentiating T499E and S535E mutations increase disaggregase activity in the absence or presence of Hsp70 and Hsp40.

The subtle nature of some potentiating mutations in Hsp104 led us to hypothesize that post-translational modifications of Hsp104 at specific positions in the MD might also potentiate activity. Indeed, several serine and threonine residues throughout Hsp104 can be phosphorylated in vivo (Albuquerque et al. 2008; Holt et al. 2009; Swaney et al. 2013). Here, we have introduced phosphomimetic aspartate or glutamate residues at these positions and assessed Hsp104 activity. Phosphomimetic mutations at T87 and S155 in the NTD, S206 and S306 in NBD1, or S577, S578 and S768 in NBD2 did not enhance Hsp104 activity. Remarkably, phosphomimetic mutations at two positions in the MD, T499 and S535, enabled Hsp104 to rescue TDP-43, FUS, and α-syn aggregation and toxicity in yeast. Moreover, Hsp104T499E and Hsp104S535E exhibit enhanced ATPase activity and protein-disaggregase activity in vitro. We suggest that phosphorylation of T499 or S535 may enable enhanced Hsp104 disaggregase activity in a reversible and regulated manner. Thus, enhanced disaggregase activity could be unleashed and restrained at specific locations or in response to specific environmental cues in vivo. Understanding this regulation could also inform strategies to engineer transient bursts of enhanced disaggregase activity in therapeutic settings.

How do the T499D/E and S535D/E mutations potentiate Hsp104? Mutation to a negatively charged residue is important for Hsp104 activity even in the absence of Hsp70. However, Hsp104T499D/E but not Hsp104S535D/E reduces yeast growth at 37°C. Thus, T499 and S535 are not like A503, where any amino acid aside from alanine or proline elicits elevated disaggregase activity. Interestingly, T499 lies in helix L3 of the MD and has coevolved and interacts with A503. Mutation of T499 to aspartate or glutamate likely alters interactions with A503 in the same protomer, but also with E494 in helix L2 of the same protomer (in protomer 4) and V426 in helix L1 of the MD in the adjacent protomer (Gates et al. 2017). Remodeling these interactions by T499D/E yields a potentiating Hsp104 variant with highly elevated disaggregase activity even in the absence of Hsp70. However, Hsp104T499D/E also exhibits off-target toxicity, which is disadvantageous for further development as a therapeutic disaggregate. In contrast,
Figure 5. Location of potentiating phosphomimetic MD mutations in Hsp104. (A) Clustal Omega alignment of a portion (residues 494–540) of the MD/NBD1 from Saccharomyces cerevisiae Hsp104 with S. cerevisiae Hsp78, Schizosaccharomyces pombe Hsp104, Chlamydomonas reinhardtii Hsp104, Arabidopsis thaliana Hsp101, Monosiga brevicollis Hsp104, Thermus thermophilus ClpB, and Escherichia coli ClpB. T499 and S535 are indicated with arrowheads and highlighted in yellow. Consensus symbols: ‘∗’ denotes fully conserved residue, ‘:’ denotes conservation of residues with strong similarity, ‘.’ indicates the conservation of residues with weak similarity. (B) Homology model of the MD (green) and a portion of the small domain of NBD1 (dark blue) of Hsp104, where T499 and S535 side chains are shown as sticks. T499 is found in helix L3 and S535 is in the linker between helix L4 and the C-terminal portion of the small domain of NBD1 (dark blue). (C) View of protomers 3 (green) and 4 (grey) of Hsp104 bound to ADP (Gates et al. 2017) showing the positions of T499 and A503 in helix L3 of the MD of protomer 3 and V426 and K229 in helix L1 of protomer 4. The GREMLIN score for the interaction between T499 and A503 is indicated. (D) View of protomer 3 of Hsp104 bound to ADP (Gates et al. 2017) showing the positions of S535 in the linker between helix L4 of the MD and ND81 and E469 in helix L2 of the MD of the same protomer.

Hsp104S535D/E is not toxic. Hsp104S535E disaggregase activity is not as elevated as Hsp104T499E in the absence or presence of Hsp70, but is substantially higher than Hsp104. S535D/E likely disrupts an intraprotomer interaction with E469 in helix L2 of the MD. Hsp104L469D is also an enhanced Hsp104 variant, which like Hsp104S535D/E exhibits reduced off-target toxicity (Jackrel et al. 2015). Thus, Hsp104S535D/E and Hsp104L469D are interesting variants to advance in preclinical studies as they potently rescue proteotoxicity with minimal side effects.

Under what conditions are T499 and S535 phosphorylated in vivo? One study found that Hsp104 may be phosphorylated at T499 and S535 under conditions of DNA damage stress (Albuquerque et al. 2008). These observations raise the possibility that Hsp104 might get phosphorylated at T499 or S535 as part of a stress response to elicit enhanced disaggregase activity, which is cytoprotective. Once the stress has passed, dephosphorylation of T499 or S535 would restore Hsp104 activity to basal levels. In this way, yeast may utilize phosphorylation of T499 or S535 to unleash enhanced Hsp104 disaggregase activity exactly when or where it is needed in a reversible and regulated manner.

Curiously, T499 and S535 are not very well conserved residues. Indeed, of the 4950 Hsp104 species variants assessed in our GREMLIN analysis, ~1.7% had T at position 499. Indeed, L, Y or F were most commonly found at position 499 (Fig. 5A). Likewise, only ~13% of Hsp104 species variants had S at position 535 (Fig. 5A). At this position, P or R was the most commonly found residue. Thus, this potential method of Hsp104 regulation via phosphorylation of T499 or S535 may be idiosyncratic to a restricted number of species. However, position 499 is frequently tyrosine in ~16% of species, and thus could be regulated in a similar manner via tyrosine phosphorylation. Further studies are needed to reveal under what exact conditions Hsp104 is phosphorylated at T499 or S535 in yeast. The identity of Hsp104 kinases and phosphatases also needs to be delineated. Indeed, it will be of great interest to reconstitute the regulation of potentiated Hsp104 disaggregase activity in vitro via the addition of defined kinases and phosphatases. Other post-translational modifications may also unleash enhanced Hsp104 activity in a regulated manner. For example, the MD of Hsp104 can be modified via ubiquitylation, succinylation, and acetylation, which
The size of the ionic shell and the negative charge of phosphomimetic mutations accurately phenocopy serine and threonine phosphorylation events and have been extremely informative (Su and Tsai 2017; M.K. was supported by NIH grants DP2OD002177, R01GM099836, a Muscular Dystrophy Association Research Award (MDA277268), the Life Extension Foundation, a Linda Montague Pechinik Research Award, the Packard Center for ALS Research at Johns Hopkins University, and Target ALS Springboard Award, J.S. was supported by NIH grants K12GM081259 and K22NS09131401 NIH, K.L.M. was supported by an NSF graduate research fellowship (DGE-1321851), M.E.J. was supported by an AHA post-doctoral fellowship and a Tar- get ALS Springboard Award, J.S. was supported by NIH grants DP2OD002177, R01GM099836, a Muscular Dystrophy Association Research Award (MDA277268), the Life Extension Foundation, a Linda Montague Pechinik Research Award, the Packard Center for ALS Research at Johns Hopkins University, and Target ALS. Conflict of interest. None declared.

**REFERENCES**


**Figure 6.** Hsp104<sup>T499E</sup> and Hsp104<sup>S535E</sup> exhibit enhanced ATPase and protein-disaggregase activity. (A) Hsp104<sup>T499E</sup> and Hsp104<sup>S535E</sup> exhibit elevated ATPase activity. Values represent means ± SD (n = 2). (B) Hsp104<sup>T499E</sup> and Hsp104<sup>S535E</sup> exhibit elevated disaggregase activity. Luciferase aggregates were incubated with Hsp104 variant (0.167 μM) plus ATP (5 mM) in the presence (blue bars) or absence (red bars) of Hsc70 (0.167 μM) and Hsp2 (0.167 μM) for 90 min at 25 °C. Values represent means ± SEM (n = 4).


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