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Letters

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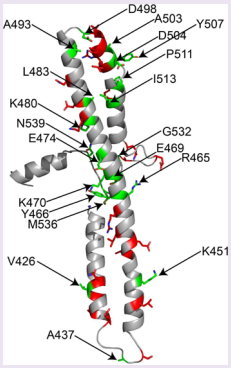
Disparate Mutations Confer Therapeutic Gain of Hsp104 Function

Meredith E. Jackrel,[†] Keolamau Yee,[†] Amber Tariq,[†] Annie I. Chen,[†,‡] and James Shorter*[,†,‡]

[†]Department of Biochemistry and Biophysics, [‡]Cell and Molecular Biology Graduate Group, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, Pennsylvania 19104, United States

Supporting Information

ABSTRACT: Hsp104, a protein disaggregase from yeast, can be engineered and potentiated to counter TDP-43, FUS, or α-synuclein misfolding and toxicity implicated in neurodegenerative disease. Here, we reveal that extraordinarily disparate mutations potentiate Hsp104. Remarkably, diverse single missense mutations at 20 different positions interspersed throughout the middle domain (MD) and small domain of nucleotide-binding domain 1 (NBD1) confer a therapeutic gain of Hsp104 function. Moreover, potentiation emerges from deletion of MD helix 3 or 4 or via synergistic missense mutations in the MD distal loop and helix 4. We define the most critical aspect of Hsp104 potentiation as enhanced disaggregase activity in the absence of Hsp70 and Hsp40. We suggest that potentiation likely stems from a loss of a fragilely constrained autoinhibited state that enables precise spatiotemporal regulation of disaggregase activity.



Hsp104, a hexameric AAA+ protein from yeast, catalyzes the construction and deconstruction of yeast prions.[1−3] Hsp104 is also essential in the reactivation of disordered aggregates that accumulate upon exposure to environmental stress.[4−7] Because yeasts have harnessed infectious amyloids (prions) for adaptive purposes,[8] these tight amyloid-regulatory pathways could potentially be reformulated to counter protein misfolding implicated in neurodegenerative disease in humans.[9−11] Protein misfolding is implicated in numerous neurodegenerative disorders including amyotrophic lateral sclerosis (ALS) and Parkinson's disease (PD).[12,13] In ALS, certain subsets of patients display accumulations of misfolded TDP-43 or FUS.[14] In PD patients, α-synuclein (α-syn) forms highly toxic prefibrillar oligomers and amyloid fibrils that accumulate in Lewy bodies.[12] Treatments for nearly all protein-misfolding disorders remain palliative, and therapeutics that target the underlying causes of these diseases are needed.[9] Accumulations of α-syn amyloid fibrils, as well as TDP-43 and FUS aggregates, are widely considered intractable. No therapeutics are available to eliminate these structures or their precursors. The genes encoding TDP-43 and FUS expression are essential, and thus therapies that reactivate these proteins to their native fold rather than degrading or downregulating them are needed.[15] Due to the complexities of protein misfolding, it remains unclear if small molecule therapeutics can effectively target any of these disorders. Thus, it is crucial to explore alternative therapeutic strategies, such as the rewiring of proteostasis networks by engineering protein disaggregases with enhanced activity.[9]

Hsp104 has only limited ability to recover natively folded TDP-43, FUS, or α-syn from aggregates, preamyloid oligomers, and amyloid fibers, substrates that it does not ordinarily encounter.[9,16,17] Thus, we engineered potentiated Hsp104 variants to counter TDP-43, FUS, and α-syn misfolding.[17−20] Using random mutagenesis, we generated large libraries of Hsp104 variants that we screened for suppression of proteotoxicity in yeast.[17,18] We isolated a series of potentiated Hsp104 variants that suppress TDP-43, FUS, and α-syn aggregation and toxicity, while also restoring nuclear localization of TDP-43 and plasma membrane localization of α-syn in yeast.[17] Potentiated Hsp104 variants are the first disaggregases, or even chaperones, engineered to optimize proteostasis against neurodegenerative disease. These variants also suppress the toxicity and aggregation associated with mutant versions of TDP-43, FUS, and α-syn linked to more severe disease phenotypes.[21] We have also demonstrated that potentiated Hsp104 variants suppress dopaminergic neurodegeneration in a *C. elegans* model of PD.[17] Surprisingly, at certain positions, missense mutation to nearly any amino acid potentiates Hsp104.[17] To the best of our knowledge, generic mutation of specific residues to any amino acid leading to a therapeutic gain-of-function is unprecedented.[9,17] Thus, we sought to better understand the extent to which the Hsp104 middle domain (MD) can be modulated to confer potentiation.

Here, we have employed a modified yeast-based screening assay to isolate a broad series of potentiated Hsp104 variants. We have identified many new variants, and the scope of potentiating mutations is broad and unanticipated. We have genetically mapped the positions at which missense mutations confer Hsp104 potentiation and find that they comprise at least ∼14% of the residues in the MD. However, potentiation is not conferred by *any* mutation in the MD, as we also report a similar number of missense mutations that do not confer potentiation.

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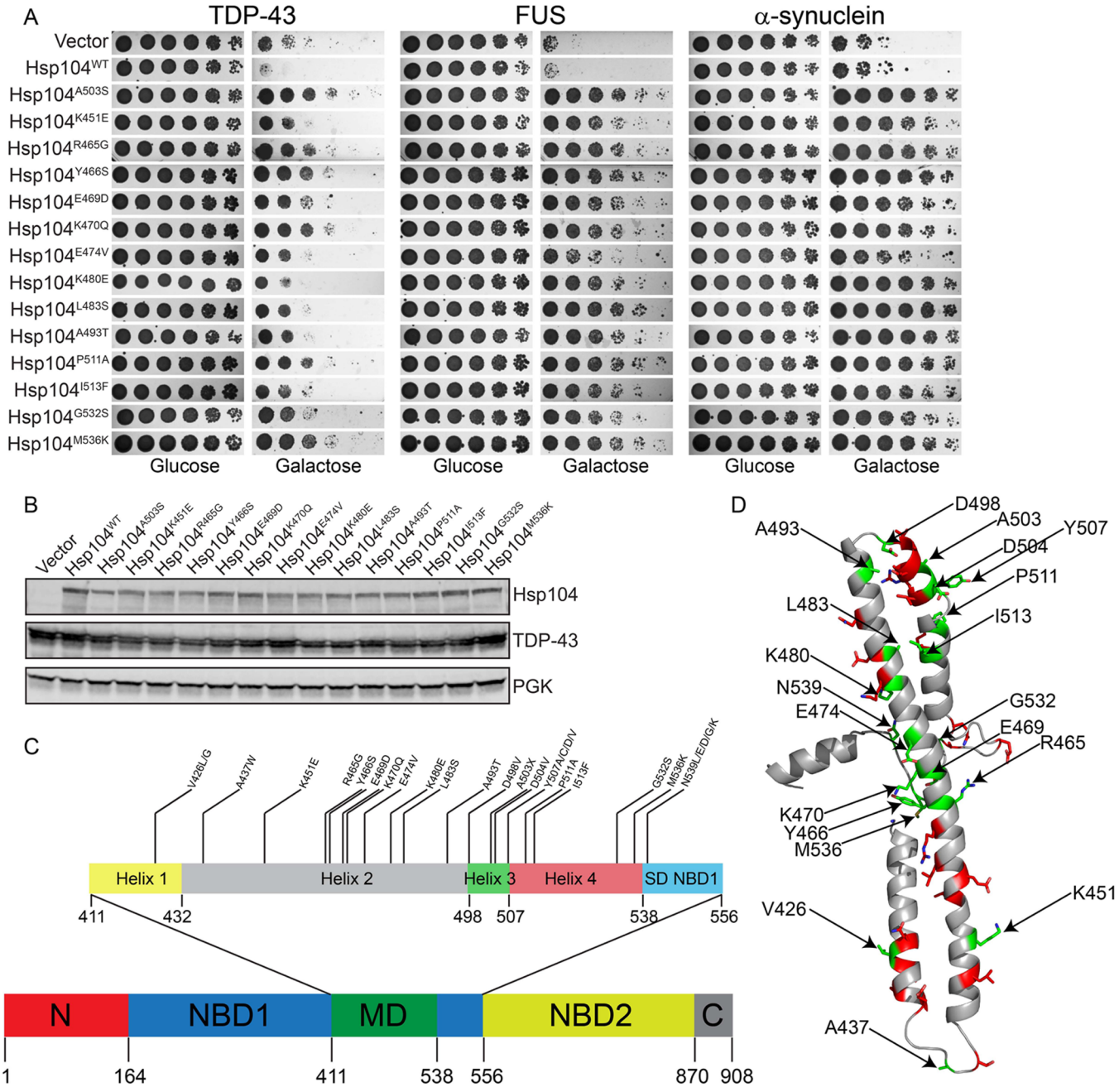

Figure 1. Hsp104 potentiation. Diverse missense mutations in disparate positions throughout the MD and small domain of NBD1 potentiate Hsp104. (A) Δ*hsp104* yeasts integrated with genes encoding TDP-43, FUS, or α-synuclein were transformed with the indicated Hsp104 missense mutants or control. Strains were serially diluted 5-fold and spotted on glucose (off) or galactose (on) media. (B) Selected strains from A were induced for 5 h, lysed, and immunoblotted. 3-Phosphoglycerate kinase (PGK) serves as a loading control. (C) Potentiating mutations are distributed throughout the MD of Hsp104. The MD (green, residues 411−538) is comprised of four helices and is inserted into nucleotide-binding domain 1 (NBD1, blue). Potentiating mutations shown in this figure are further described in this paper and our earlier work.[17] (D) Homology model of the MD and a portion of the small domain of NBD1 of Hsp104, where side chains studied are shown as sticks. Residues where the tested mutations potentiate Hsp104 are shown in green and are numbered. Residues where the tested mutations do not potentiate Hsp104 are shown in red.

Using a series of Hsp104 MD deletion constructs, we have also elucidated the structural requirements for Hsp104 potentiation. To fine-tune Hsp104 variants and enhance their substrate or conformer specificity, it is crucial to accurately understand the mechanistic and structural basis for Hsp104 potentiation. Our studies provide new insight into the basis for Hsp104 potentiation and raise new questions as to why Hsp104 might have evolved to maintain a fragilely constrained autoinhibited state.

RESULTS AND DISCUSSION

Missense Mutations throughout the Middle Domain Potentiate Hsp104. We have previously reported the isolation of a series of potentiated Hsp104 MD variants that suppress the toxicity of TDP-43, FUS, and α-syn.[9,17,21] These mutations were located in MD helix 1, the distal loop between MD helix 1 and 2, and helix 3, as well as the small domain of NBD1 (Supporting Information Table 1).[9,17,21] Using pure-protein biochemistry, we determined that these variants were all potentiated by similar

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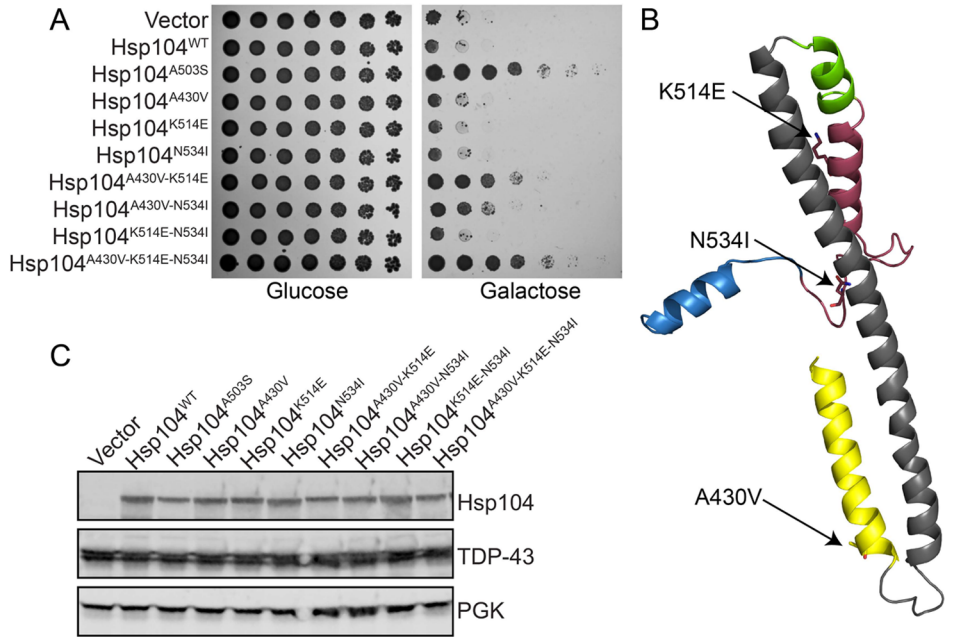

Figure 2. Synergy between mutations in the MD distal loop and helix 4. This synergy yields potentiated Hsp104 variants. (A) Δ*hsp104* yeasts integrated with pAG303GAL-TDP-43 were transformed with each of the indicated pAG416GAL-Hsp104 variants or vector control. Strains were serially diluted 5-fold and spotted on glucose (off) or galactose (on) media. (B) Homology model of the MD and a portion of the small domain of NBD1 of Hsp104. Side chains of the key residues are shown as sticks. (C) Selected strains from A were induced for 5 h, lysed, and immunoblotted. 3-Phosphoglycerate kinase (PGK) serves as a loading control.

mechanistic principles and were typically enhanced in a nonspecific manner, suppressing the aggregation and toxicity of TDP-43, FUS, and α-syn.[17] The potentiating mutations are seemingly dissimilar, and we could determine no unifying features to link them. We hypothesized that additional potentiating mutations likely exist and sought to more comprehensively identify potentiated Hsp104 variants.

We generated an Hsp104 MD library using domain-specific error-prone PCR.[17] We coexpressed this library of variants with TDP-43 in Δ*hsp104* yeast and isolated toxicity suppressors by plating the yeast on galactose-supplemented media. Here, a galactose-inducible promoter controls TDP-43 and Hsp104 expression. Expression was performed at 34 °C, which we predicted would disfavor selection of the previously isolated variants due to their mild toxicity at temperatures of 34 °C and above.[17,21] We uncovered numerous novel potentiating mutations in MD helix 2, as well as several in helix 4, two regions in which we had not identified potentiating mutations before[17] (Figure 1A, Supporting Information Table 1). We confirmed that the potentiated Hsp104 variants do not alter the expression of TDP-43 and that the Hsp104 variants are expressed at similar or lower levels than Hsp104^{WT} (Figure 1B).

We also tested if the toxicity suppression by potentiated Hsp104 variants involves Sse1 or Sse2, the yeast homologues of Hsp110, which functions as a mammalian protein disaggregase.[22] To eliminate this possibility, we confirmed that several variants retain their ability to suppress TDP-43 toxicity in Δ*sse1* and Δ*sse2* yeast strains (Figure S1). Thus, rescue of TDP-43 toxicity in yeast by potentiated Hsp104 variants does not require Sse1 in the same way that Hsp104 requires Sse1 to propagate [*PSI*$^+$] and [*URE3*] in yeast.[23−25]

As with the majority of previously isolated potentiated Hsp104 variants,[17] each of the novel potentiating variants also suppressed the toxicity of FUS and α-syn (Figure 1A). With the identification of these new mutations, we have now identified numerous missense mutations that confer potentiation in every helix of the MD as well as in the small domain of NBD1 (Figure 1C,D). The disparate locations and diversity of mutations that confer a therapeutic gain of Hsp104 function is remarkable and unanticipated.

We also isolated several colonies that harbored more than one Hsp104 mutation. Individual yeast cells can harbor multiple plasmids with the same selection marker. When multiple mutations are identified in a single colony, it is unclear which of the mutations are potentiating. Thus, we constructed each of the mutations as single missense mutants and retested them for suppression of TDP-43 toxicity. For nearly all of the colonies, we identified at least one potentiating mutation (Figure 1A). Many colonies harboring multiple point mutations only harbored a single activating mutation while the other comaintained mutations did not modify TDP-43, FUS, or α-syn toxicity (Supporting Information Table 2). Thus, it is important to note that Hsp104 is not potentiated by *any* mutation in the MD. Indeed, Hsp104 MD missense mutations can be isolated that are neutral or inhibitory.[26]

Curiously, we identified K470Q as an activating variant and K470R as a variant that does not modify toxicity (Figure 1A, Supporting Information Table 2). This finding was surprising because we previously found that conservative mutations such as alanine to glycine or valine potentiate Hsp104.[17] Thus, at some MD positions (e.g., K470), potentiation requires a higher degree of side-chain modification, while at other positions (e.g., A503) virtually any side-chain alteration confers potentiation.

Missense Mutations in the MD Distal Loop and Helix 4 Synergize to Potentiate Hsp104. For each colony identified as a hit that harbored multiple missense mutations, we constructed each of the mutations individually and assessed them for TDP-43 toxicity suppression. With just one exception, each colony harboring multiple mutations contained at least one mutation that conferred potentiation. The exception was one

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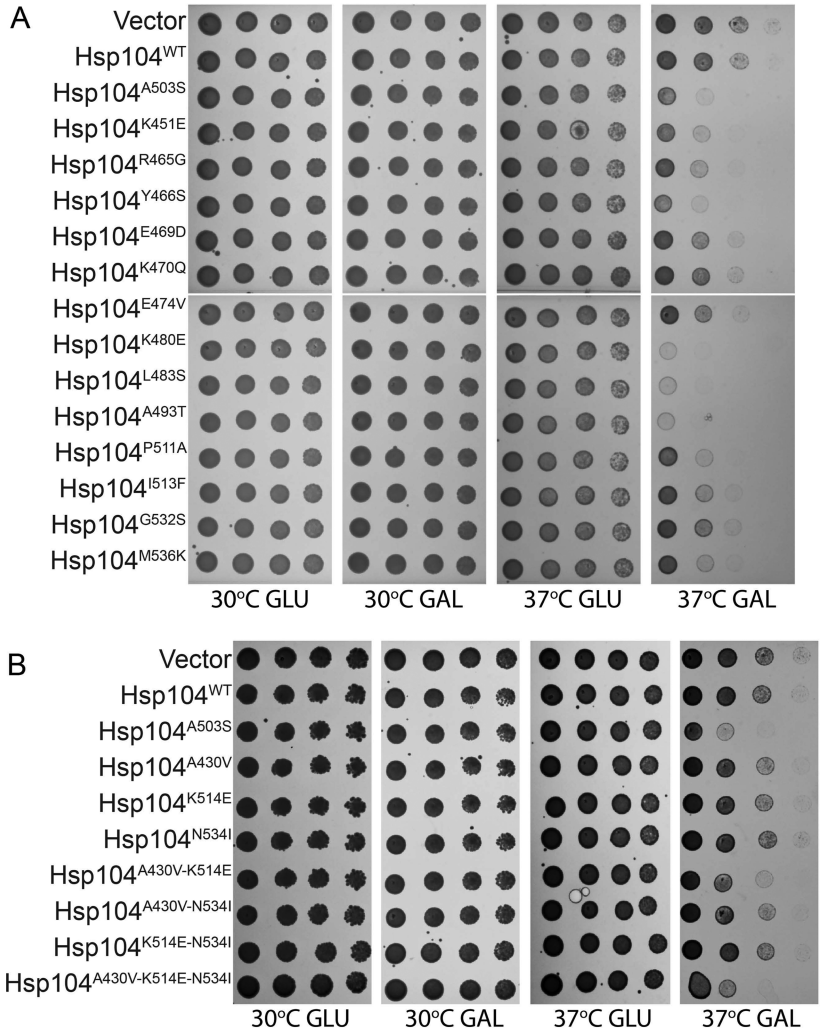

Figure 3. Temperature sensitivity. Novel potentiated Hsp104 variants can display a diminished temperature-sensitive phenotype. (A) W303Δ*hsp104* yeasts were transformed with the indicated 416GAL-Hsp104 plasmid or empty vector control. Yeasts were grown to saturation in synthetic raffinose medium, serially diluted, and spotted onto SD-Ura or SGal-Ura media and incubated at 30 or 37 °C. Plates were analyzed after 2−3 days of growth. (B) Experiments were carried out as in A to test the putative synergistic variants.

colony that harbored three mutations: A430V, K514E, and N534I. None of these missense mutations conferred potentiation individually (Figure 2A, Supporting Information Tables 1 and 2). Therefore, we constructed and tested all four combinations of the three mutations to determine if multiple mutations were required in combination to confer potentiation. Surprisingly, Hsp104$^{A430V-K514E}$ and Hsp104$^{A430V-N534I}$ displayed potentiation, and Hsp104$^{A430V-K514E-N534I}$ displayed even greater activity, whereas the single mutations were inactive (Figure 2A,B). While these mutations do not potentiate Hsp104 independently, in combination they display robust synergy. We confirmed that the Hsp104 variants do not alter expression of TDP-43 and that the Hsp104 variants are expressed at similar or lower levels than Hsp104^{WT} (Figure 2C). We hypothesize that each of these individual mutations might subtly perturb the MD, but insufficiently to relieve autoinhibition. However, in combination, these mutations relieve autoinhibition to potentiate Hsp104 and robustly suppress TDP-43 toxicity.

Second Generation Hsp104 MD Variants Display Reduced Temperature Sensitivity. We predicted that by screening the Hsp104 library at elevated temperatures, this selective pressure might promote isolation of variants with a diminished temperature-sensitive growth phenotype. Hsp104 is not required for yeast growth at 37 °C (Figure 3).[21,27] We also observed no noticeable difference in growth between Δ*hsp104* yeast overexpressing Hsp104 from an exogenous plasmid or vector alone at 30 or 37 °C (Figure 3).[17,21] However, overexpression of potentiated Hsp104^{A503V} results in a growth defect at 37 °C, likely due to promiscuous unfolding of modestly destabilized substrates at this temperature.[17,21] Thus, we tested the novel variants for temperature-sensitivity by expressing them in Δ*hsp104* yeast at 30 and 37 °C. As a control, we compared growth to that of yeast expressing Hsp104^{A503S}, a potentiated variant identified in earlier work as one of the least temperature-sensitive.[17] When overexpressed at 30 °C, none of the variants, including Hsp104^{A503S}, display a growth defect (Figure 3A). When overexpressed at 37 °C, Hsp104^{A503S} displays a moderate growth defect (Figure 3A).[17] Promisingly, many of the newly isolated variants display a diminished temperature-sensitive phenotype as compared to Hsp104^{A503S} (Figure 3A). Hsp104^{R465G}, Hsp104^{E469D}, Hsp104^{K470Q}, Hsp104^{E474V}, and Hsp104^{G532S} display substantially more robust growth than Hsp104^{A503S} at 37 °C, though they still display mild temperature sensitivity relative to the controls (Figure 3A). These variants

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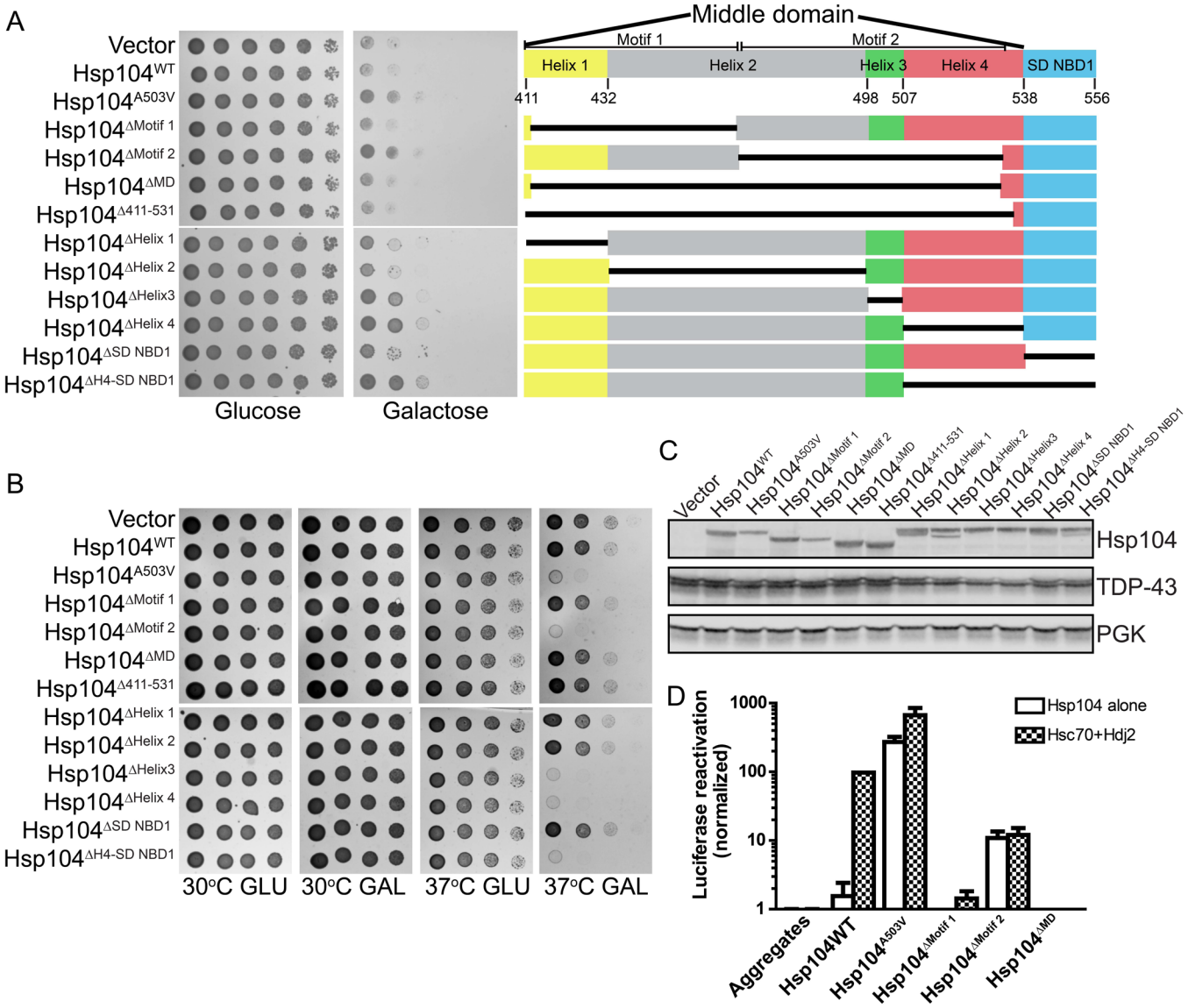

Figure 4. Deletion of Hsp104 MD motifs and helices. Deletion of MD motif 2, helix 3, or helix 4 potentiates Hsp104. (A) W303Δ*hsp104* yeasts integrated with pAG303GAL-TDP-43 were transformed with the indicated 416GAL-Hsp104 deletion construct, empty plasmid, Hsp104^{WT}, or Hsp104^{A503V}. Strains were serially diluted 5-fold and spotted on glucose (off) or galactose (on) media (left). Organization of the middle domain and small domain of NBD1 (right). (B) W303Δ*hsp104* yeasts were transformed with the indicated 416GAL-Hsp104 plasmid or empty vector control. Yeasts were grown to saturation in synthetic raffinose medium, serially diluted, and spotted onto SD-Ura or SGal-Ura media and incubated at 30 or 37 °C. Plates were analyzed after 2−3 days of growth. (C) Selected strains from A were induced for 5 h, lysed, and immunoblotted. 3-Phosphoglycerate kinase (PGK) serves as a loading control. (D) Luciferase aggregates were incubated with the purified Hsp104 variants (0.167 μM hexamer) plus (checkered bars) or minus (clear bars) Hsc70 (0.167 μM) and Hdj2 (0.167 μM). Values represent means ± SEM (n = 3).

also maintain a robust level of toxicity suppression of the disease-associated substrates (Figure 1A). We also tested the synergistic mutations and found that Hsp104$^{A430V-N534I}$ displayed nearly no temperature-sensitive growth phenotype, growing at levels similar to the vector and Hsp104^{WT} controls (Figure 3B). Hsp104$^{A430V-N534I}$ suppresses TDP-43 toxicity (Figure 2B), but less so than Hsp104^{A503S} or the other potentiated synergistic variants. This finding suggests that diminished temperature-sensitivity incurs a cost of diminished overall protective activity against neurodegenerative disease proteins. Nonetheless, Hsp104$^{A430V-N534I}$ does buffer TDP-43 toxicity, indicating that potentiation is not always linked with a temperature-sensitive growth phenotype.

Deletion of MD Helix 3 or 4 Confers Hsp104 Potentiation. We have isolated numerous potentiated Hsp104 variants with missense mutations dispersed throughout all four helices of the MD as well as the small domain of NBD1. It is perplexing that mutation at such disparate positions should activate the protein so profoundly. Thus, we were curious if deletion of entire helices or motifs, or perhaps even deletion of the entire MD, might also potentiate Hsp104. In the *E. coli* homologue of Hsp104, ClpB, deletion of motif 1 (helix 1 and a portion of helix 2), motif 2 (a portion of helix 2, helix 3, and helix 4), or the entire MD confers a hyperactive state, which is highly toxic to *E. coli* at regular growth temperatures.[28,29] While Hsp104 and ClpB are often assumed to function by the same mechanism,[30] we have established several key mechanistic differences between Hsp104 and ClpB.[16,26] Thus, we constructed a comprehensive series of deletion constructs to elucidate the MD requirements for Hsp104 potentiation. We

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constructed Hsp104$^{\Delta Motif\ 1}$, Hsp104$^{\Delta Motif\ 2}$, and Hsp104$^{\Delta MD}$, which eliminate motif 1 (Δ412−459), motif 2 (Δ460−524), or the entire MD (Δ412−524), respectively (Figure 4A, right). For comparison, we also constructed Hsp104$^{\Delta 411-531}$, which has been characterized *in vitro*.[31] Plasmids harboring the Hsp104 variants were cotransformed in Δ*hsp104* yeast integrated with the TDP-43 gene. Hsp104$^{\Delta Motif\ 2}$ suppresses TDP-43 toxicity, while neither Hsp104$^{\Delta Motif\ 1}$, Hsp104$^{\Delta MD}$, nor Hsp104$^{\Delta 411-531}$ suppresses TDP-43 toxicity (Figure 4A, left). This finding is in striking contrast to ClpB, in which deletion of either motif or the entire MD confers a hyperactive state.[28,29,32] Thus, the requirements for Hsp104 potentiation are strikingly different from the requirements for generating hyperactive ClpB variants, indicating profound differences between Hsp104 and ClpB.

We next deleted each helix of the Hsp104 MD and tested for suppression of TDP-43 toxicity. Supporting our findings with deletion of entire motifs, deletion of helices 1, 2, or the small domain of NBD1 does not potentiate Hsp104, while deletion of helices 3 or 4 confers potentiation (Figure 4A). Similar results were obtained for FUS and α-synuclein (Figure S2). We also tested the variants for temperature sensitivity and, consistent with our results in suppression of TDP-43 toxicity, we found that deletion of motif 2, helix 3, or helix 4 confers a temperature-sensitive growth phenotype (Figure 4B). We confirmed that expression of the Hsp104 deletion constructs does not alter TDP-43 expression levels and found that the deletion constructs were expressed at similar or lower levels than Hsp104^{WT} (Figure 4C).

To further assess the activity of the deletion constructs, we purified Hsp104$^{\Delta Motif\ 1}$, Hsp104$^{\Delta Motif\ 2}$, and Hsp104$^{\Delta MD}$ and tested the variants for reactivation of chemically denatured luciferase *in vitro*. We found that deletion of motif 1 or the entire middle domain inactivates Hsp104, while deletion of just motif 2 preserves Hsp104 activity (Figure 4D). However, Hsp104$^{\Delta Motif\ 2}$ retains only ∼15% activity in comparison to Hsp104 in the presence of Hsc70 and Hdj2 (Figure 4D). However, Hsp104$^{\Delta Motif\ 2}$ displays similar activity in the presence or absence of Hsc70 and Hdj2 (Figure 4D), whereas Hsp104 is inactive without Hsc70 and Hdj2. Thus, our results reveal a key feature governing Hsp104 potentiation. Potentiation depends strongly on obviation of the requirement for Hsp70 and Hsp40 for disaggregase activity, while total activity is not as important. Collectively, these data establish that, surprisingly, the MD of Hsp104 regulates Hsp104 activity differently than the MD of ClpB regulates ClpB activity.[26,28,29,32]

We have demonstrated that Hsp104, an AAA+ protein disaggregase from yeast, can be mutated at disparate positions to relieve autoinhibition and confer Hsp104 potentiation. Potentiated Hsp104 variants suppress the toxicity of diverse substrates implicated in ALS and PD, whereas Hsp104 is ineffective. We have comprehensively illustrated the sequence-specific requirements for Hsp104 potentiation via mutations in the MD and have uncovered a diverse array of potentiating mutations. Perplexingly, mutation at any of 20 different residues located throughout the MD and small domain of NBD1 (a 145 amino acid stretch) confer Hsp104 potentiation (Supporting Information Table 1). Additionally, at many of these positions, mutation to diverse classes of residues, including both conservative and nonconservative substitutions, confers potentiation.[17] We are aware of no other examples in which mutation at such disparate positions to both conservative and nonconservative substitutions leads to a therapeutic gain-of-function.[9,17] It is perplexing that mutation of at least ∼14% of residues in the MD can confer this effect. However, Hsp104 autoinhibition is not relieved by simply mutating *any* residue.[26] We have identified an additional 25 residues where mutation does not confer potentiation (Supporting Information Table 2, Figure 1D). We suggest that Hsp104 has evolved to maintain an autoinhibited state, in which it is restrained from generically unfolding all substrates. However, this autoinhibited state is fragile, as seemingly subtle but diverse changes to side chains can transform Hsp104 into its potentiated form.

Using a series of MD deletion constructs, we have further elucidated the structural requirements for Hsp104 potentiation. Deletion of the MD hyperactivates ClpB.[28,29,32] However, in the Hsp104 backbone, the requirements for potentiation are more complex and deletion of the entire MD does not confer potentiation. Rather, deletion of helix 3, helix 4, or both confers potentiation. These findings suggest that the requirements for Hsp104 potentiation are strikingly different from the requirements for hyperactivation of ClpB. In the future, it will be important to develop a complete map of potentiating mutations covering every position in the MD and small domain of NBD1, as we currently have mapped only ∼30% of this region. Our studies provide new insights into the basis for Hsp104 potentiation and provide clues as to how Hsp104 might be fine-tuned to enhance specificity. We suggest that potentiating mutations in the MD relieve an autoinhibitory function of this domain or mimic an allosteric event that enhances disaggregase activity in the absence of Hsp70 and Hsp40. Independence from Hsp70 and Hsp40 might be particularly important if the functionality of Hsp70 and Hsp40 has been compromised by excessive sequestration in protein aggregates.[33] It will be interesting to more thoroughly elucidate the structural requirements that dictate which MD residues yield potentiated states via missense mutation and which cannot. We hypothesize that potentiation likely stems from structural perturbation and partial loss of the coiled-coil structure of the MD, because the diverse range of potentiating mutations would be highly unlikely to all introduce a similar increased structural stability or specific structural feature. The idea that structural destabilization is the underlying basis for potentiation is supported by the lower expression levels of potentiating versus nonpotentiating variants (Figure 4C).

It is perplexing that a poorly conserved region of Hsp104, the MD, plays a crucial role in regulating Hsp104 activity. Poor sequence conservation is often thought to reflect the unimportance of that region. For Hsp104, the opposite is true. It has been postulated that poor sequence conservation of the MD might have allowed ancestral forms of Hsp104 to rapidly acquire new functions.[27] Indeed, the mutability of the MD may have provided the capacity for ancestral forms of Hsp104 to rapidly evolve and promote yeast survival through environmental stress and simultaneously regulate the inheritance of diverse prion-based, bet-hedging devices that can be beneficial in some environments.[8,11,34,35] These diverse activities, encompassing disordered aggregate reactivation, prion fragmentation, and prion dissolution place different physical demands on Hsp104, which has resulted in the evolution of a disaggregase with increased operational plasticity in comparison to ClpB, which functions primarily in stress tolerance and disordered aggregate reactivation.[16]

Why is Hsp104 not naturally potentiated? Potentiated Hsp104 variants can display defects in the propagation of potentially beneficial prions.[36] Moreover, it seems probable that the temperature-sensitive growth defect has likely selected against naturally potentiated variants, even though they can antagonize

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the excessive aggregation and toxicity caused by the overexpression of a single protein in yeast, such as TDP-43, FUS, or α-syn.[17,21] Thus, it would seem that elevated levels of a single, aggregation-prone, toxic protein is an unusual stress for yeast, which has not been a significant selection pressure governing Hsp104 primary sequence and activity. Hsp104 appears to have evolved to exist in a naturally and fragilely constrained autoinhibited state, which likely enables precise spatiotemporal regulation of disaggregase activity. Thus, disaggregase activity would only be elicited when or where it is needed, perhaps by the Hsp70 chaperone system[37] or by natural prion substrates.[1,16] It is curious that Hsp104 has not acquired secondary mutations to make accessing the potentiated state improbable. Thus, the MD likely serves as a switch that maintains Hsp104 in the autoinhibited state but allows for the nonrepressed state to be rapidly accessed. However, this regulation appears too tight to enable Hsp104 to antagonize the excessive aggregation and toxicity caused by the overexpression of a single protein in yeast, such as TDP-43, FUS, or α-syn.[9,17,21] It remains possible that the proteomes and environmental challenges faced by other species might have created selective pressures that enabled evolution of naturally potentiated Hsp104 variants. Thus, it will be fascinating to compare the activity of Hsp104 orthologues from different species with disparate proteomes that inhabit diverse environments. Indeed, a naturally occurring Hsp104 variant could display enhanced activity that eradicates the aggregation of human neurodegenerative disease proteins. Regardless, our work suggests that optimization of the MD may prove critical to engender activity and specificity against noncognate substrates connected with human disease.

MATERIALS AND METHODS

Yeast Strains, Media, and Plasmids. All yeasts were W303aΔ*hsp104* (*MATa, can1−100, his3−11,15, leu2−3,112, trp1−1, ura3−1, ade2−1*).[7] Yeasts were grown in a rich medium (YPD) or in synthetic media lacking the appropriate amino acids. Media were supplemented with 2% glucose, raffinose, or galactose. Vectors encoding TDP-43, FUS, and α-syn (pAG303GAL-TDP-43, pAG303GAL-FUS, pAG303GAL-α-syn-YFP, and pAG304Gal-α-syn-YFP) were from A. Gitler and M. Duennwald.[38−40] pRS416GAL-Hsp104 variants have been described previously.[17] All mutations were constructed using QuikChange site-directed mutagenesis (Agilent) and confirmed by DNA sequencing. To assess the importance of Hsp110 for suppression of toxicity, we used BY4741Δ*sse1* and Δ*sse2* yeast strains from B. Johnson.

Yeast Transformation and Spotting Assays. Yeasts were transformed according to standard protocols using polyethylene glycol and lithium acetate.[41] The Hsp104 MD library generated by error-prone PCR has been described previously.[17] This library was screened to isolate suppressors of TDP-43 toxicity as described,[17,18] but in this work selection was carried out at 34 °C. Single colonies were selected, grown, and screened to confirm toxicity suppression was Hsp104 dependent.[17,18] For each verified hit, the missense mutations were constructed using QuikChange site-directed mutagenesis (Agilent) and confirmed by DNA sequencing.

For the spotting assays, yeasts were grown to saturation overnight in raffinose-supplemented dropout media at 30 °C. Cultures were serially diluted and spotted in duplicate onto synthetic dropout media containing glucose or galactose. Plates were analyzed after growth for 2−3 days at 30 °C.

Assessing Toxicity of Hsp104 Variants. W303Δ*hsp104* yeasts were transformed with the indicated 416GAL-Hsp104 plasmid. Yeasts were diluted and grown to saturation overnight in raffinose-supplemented dropout media at 30 °C. Cultures were serially diluted and spotted in duplicate onto SD-Ura or SGal-Ura media and incubated at 30 or 37 °C. Plates were analyzed after 48−72 h of growth.

Immunoblotting. Yeasts were grown and induced in galactose containing medium for 5 h. Cultures were normalized to $A_{600\ nm}$ = 0.6; 6 mL of cells were harvested and treated in 0.1 M NaOH for 5 min at RT. Cell pellets were resuspended into 100 μL 1 × SDS sample buffer and boiled. Cleared lysates were separated by SDS-PAGE (4−20% gradient, BioRad) and transferred to a PVDF membrane. Membranes were blocked using LI-COR blocking buffer for 1 h at RT. Primary antibody incubations were performed at 4 °C overnight. Antibodies used: anti-TDP-43 polyclonal (Proteintech), anti-Hsp104 polyclonal (Enzo Life Sciences), and anti-PGK monoclonal (Invitrogen). Blots were processed using the LI-COR Odyssey Fc Imaging system.

Protein Purification and Luciferase Reactivation Assays. Potentiated Hsp104 variants were purified as described.[17] Hsp104 concentrations refer to the hexamer concentration. Firefly luciferase was purchased from Sigma while Hsc70 and Hdj2 were purchased from Enzo Life Sciences. Luciferase reactivation assays were performed as described[16,17] using 50 nM aggregated luciferase with 0.167 μM Hsp104 hexamer in the presence or absence of 0.167 μM Hsc70 and Hdj2.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acschembio.5b00765.

Supporting Tables 1 and 2, Figures S1 and S2 captions (PDF)

AUTHOR INFORMATION

Corresponding Author

*E-mail: jshorter@mail.med.upenn.edu.

Author Contributions

M.E.J. and J.S. conceived and designed the experiments. M.E.J., A.I.C, and J.S. contributed key reagents. M.E.J., K.Y., and A.T. performed the experiments. M.E.J. and J.S. analyzed the data. M.E.J. and J.S. wrote the paper.

Notes

The authors declare no competing financial interest.

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Supplementary Materials

Disparate mutations confer therapeutic gain of Hsp104 function

Meredith E. Jackrel[1], Keolamau Yee[1], Amber Tariq[1], Annie I. Chen[1,2], and James Shorter[1,2]*

[1]Department of Biochemistry and Biophysics, [2]Cell and Molecular Biology Graduate Group, Perelman School of Medicine at the University of Pennsylvania, Philadelphia, PA 19104, U.S.A.

*Correspondence: jshorter@mail.med.upenn.edu

Running title: Diversity of enhanced Hsp104 variants

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Supplementary Figure 1. Hsp110 is not required for Hsp104-mediated suppression of TDP-43 toxicity. BY4741 WT, Δ*sse1,* and Δ*sse2* yeast strains were transformed with 413GAL TDP-43 and the indicated Hsp104 missense mutants or control. Strains were serially diluted fivefold and spotted on glucose (off) or galactose (on) media.

Supplementary Figure 2. Deletion of MD motif 2, helix 3, or helix 4 potentiates Hsp104. W303Δ*hsp104* yeast integrated with pAG303GAL-FUS (left) or pAG303GAL-α-syn + pAG304GAL-α-syn (right) was transformed with the indicated 416GAL-Hsp104 deletion construct, empty plasmid, $Hsp104^{WT}$, or $Hsp104^{A503V}$. Strains were serially diluted fivefold and spotted on glucose (off) or galactose (on) media.

Supplementary Table 1. Potentiating Hsp104 mutations.
The mutations that potentiate Hsp104 activity, their location, and their identification are listed.

Potentiating Mutations	Middle domain helix	Source
V426L/G	1	Jackrel et al., 2014
A437W	Distal loop	Jackrel et al., 2014
K451E	2	This paper
R465G	2	This paper
Y466S	2	This paper
E469D	2	This paper
K470Q	2	This paper
E474V	2	This paper
K480E	2	This paper
L483S	2	This paper
A493T	2	This paper
D498V	3	Jackrel et al., 2014
A503X*	3	Jackrel et al., 2014
D504V	3	Jackrel et al., 2014
Y507A/C/D/V	3	Jackrel et al., 2014
P511A	4	This paper
I513F	4	This paper
G532S	4	This paper
M536K	4	This paper
N539L/E/D/G/K	Small domain NBD1	Jackrel et al., 2014
A430V-K514E	Distal loop and 4	This paper
A430V-N534I	Distal loop and 4	This paper
A430V-K514E-N534I	Distal loop and 4	This paper

*X = any amino acid except A or P

Supplementary Table 2, Mutations in the Hsp104 MD that do not potentiate activity.
The MD mutations do not that potentiate Hsp104 activity, their location, and their identification are listed.

Non-potentiating Mutations	Middle domain helix	Source
Q425R	1	This paper
E427K	1	This paper
A430V	Distal loop	This paper
E432D	Distal loop	This paper
S439T	Distal loop	This paper
L447I	2	This paper
L455S	2	This paper
E457K	2	This paper
L459S	2	This paper
R463K	2	This paper
K470R	2	This paper
K481R	2	This paper
D484G	2	This paper
N488I	2	This paper
T499V	3	Jackrel et al., 2014
A500V	3	Jackrel et al., 2014
T501V	3	Jackrel et al., 2014
A502V	3	Jackrel et al., 2014
L505V	3	Jackrel et al., 2014
R506V	3	Jackrel et al., 2014
K514E	4	This paper
Q523R	4	This paper
E528G	4	This paper
A531V	4	This paper
N534I	4	This paper

Fig. S1. Jackrel et al.

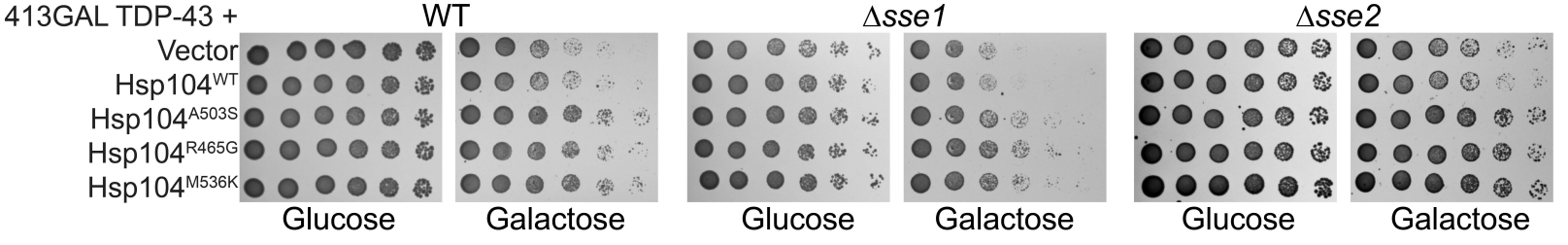

Fig. S2. Jackrel et al.