Potentiated Hsp104 Variants Suppress The Toxicity Of Most Overexpressed Dosage-Sensitive Yeast Genes

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Abstract

Maintenance of optimal gene expression levels is critical for cell viability and homeostasis. However, misregulation of gene expression can and regularly occur. One type of detrimental misregulation involves overexpression of a single gene that can cause organismal death is dosage sensitivity, which is often due to increased concentration of the protein encoded by the gene. Deleterious increases in the expression of specific proteins are associated with various neurodegenerative diseases such as Parkinson's and Alzheimer's Diseases as well as other cellular maladies including various cancers and Down Syndrome. In yeast, it has been estimated that ~20% of genes are toxic when overexpressed. The physicochemical properties and function of a protein seem to dictate whether it will be toxic upon overexpression. However, the mechanism by which individual proteins become toxic when overexpressed is typically unclear, which complicates the development of agents that counter toxicity of diverse dosage-sensitive genes. The overarching goal of this thesis was to rationally engineer a ‘buffer’ that universally mitigates the toxicity of dosage-sensitive genes.

To meet this goal, we turned to Hsp104, a hexameric, ring-shaped AAA+ ATPase and protein-remodeling factor found in yeast, which protects yeast from toxicity associated with aggregated and misfolded proteins induced by chemical, heat, or age-related stress. An engineered variant of Hsp104, Hsp104A503S, displayed potentiated activity and suppressed proteotoxicity of various neurodegenerative disease proteins, including TDP-43, FUS, and α-synuclein in yeast, whereas wild-type Hsp104 was ineffective. Inspired by this striking activity, we determined whether Hsp104A503S could combat the toxicity of diverse yeast dosage-sensitive genes. Surprisingly, Hsp104A503S suppressed the toxicity of nearly 98% of dosage-sensitive genes tested, whereas wild-type Hsp104 rescued none. Expression of Hsp70- or Hsp90-class chaperones also failed to suppress toxicity of the majority of dosage-sensitive genes. To achieve this broad rescue of dosage-sensitive genes, Hsp104A503S required critical tyrosines in pore-loops that engage substrate during protein remodeling and translocation across the central channel of Hsp104. Moreover, ATPase activity at NBD1 or NBD2 was required for Hsp104A503S to alleviate toxicity of dosage-sensitive genes. Rescue of toxicity by Hsp104A503S was not typically due to decreases in toxic protein expression or disaggregation of amyloid. In addition, neither autophagy nor proteasome activity was required for Hsp104A503S to rescue the toxicity of dosage-sensitive genes. Rather, Hsp104A503S effectively prevented the formation of labile, SDS-soluble aggregates, which correlated with alleviation of toxicity. With null mutants, we established that the intrinsic function of several dosage-sensitive kinases and phosphatases was crucial for overexpression toxicity. In vitro functional assays with Ppz1 (a dosage-sensitive protein phosphatase), indicated the phosphatase activity was reduced by Hsp104A503S and not by Hsp104. Lastly, we demonstrated that Hsp104A503S suppressed the toxicity of the potent oncogenic kinase, v-Src, in yeast, decreasing protein levels and kinase activity in yeast. Thus, we suggest that in addition to preventing formation of labile, SDS-soluble aggregates Hsp104A503S can also suppress dosage sensitivity by directly unfolding or otherwise deactivating toxic protein such as Ppz1 and v-Src. These studies establish that potentiated protein-remodeling factors like Hsp104A503S can serve as a powerful buffer that mitigates the toxicity of nearly all dosage-sensitive yeast genes.

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POTENTIATED HSP104 VARIANTS SUPPRESS THE TOXICITY OF MOST
OVEREXPRESSED DOSAGE-SENSITIVE YEAST GENES

Michael Yancey Soo
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For my parents, Joseph and Juliana, and my grandparents, Paul and Jane.
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ABSTRACT

POTENTIATED HSP104 VARIANTS SUPPRESS THE TOXICITY OF MOST OVEREXPRESSED DOSAGE-SENSITIVE YEAST GENES

Michael Yancey Soo
James Shorter, Ph.D.

Maintenance of optimal gene expression levels is critical for cell viability and homeostasis. However, misregulation of gene expression can and regularly occur. One type of detrimental misregulation involves overexpression of a single gene that can cause organismal death is dosage sensitivity, which is often due to increased concentration of the protein encoded by the gene. Deleterious increases in the expression of specific proteins are associated with various neurodegenerative diseases such as Parkinson’s and Alzheimer’s Diseases as well as other cellular maladies including various cancers and Down Syndrome. In yeast, it has been estimated that ~20% of genes are toxic when overexpressed. The physicochemical properties and function of a protein seem to dictate whether it will be toxic upon overexpression. However, the mechanism by which individual proteins become toxic when overexpressed is typically unclear, which complicates the development of agents that counter toxicity of diverse dosage-sensitive genes. The overarching goal of this thesis was to rationally engineer a ‘buffer’ that universally mitigates the toxicity of dosage-sensitive genes.

To meet this goal, we turned to Hsp104, a hexameric, ring-shaped AAA+ ATPase and protein-remodeling factor found in yeast, which protects yeast from toxicity associated with aggregated and misfolded proteins induced by chemical, heat, or age-
related stress. An engineered variant of Hsp104, Hsp104^{A503S}, displayed potentiated activity and suppressed proteotoxicity of various neurodegenerative disease proteins, including TDP-43, FUS, and α-synuclein in yeast, whereas wild-type Hsp104 was ineffective. Inspired by this striking activity, we determined whether Hsp104^{A503S} could combat the toxicity of diverse yeast dosage-sensitive genes. Surprisingly, Hsp104^{A503S} suppressed the toxicity of nearly 98% of dosage-sensitive genes tested, whereas wild-type Hsp104 rescued none. Expression of Hsp70- or Hsp90-class chaperones also failed to suppress toxicity of the majority of dosage-sensitive genes. To achieve this broad rescue of dosage-sensitive genes, Hsp104^{A503S} required critical tyrosines in pore-loops that engage substrate during protein remodeling and translocation across the central channel of Hsp104. Moreover, ATPase activity at NBD1 or NBD2 was required for Hsp104^{A503S} to alleviate toxicity of dosage-sensitive genes. Rescue of toxicity by Hsp104^{A503S} was not typically due to decreases in toxic protein expression or disaggregation of amyloid. In addition, neither autophagy nor proteasome activity was required for Hsp104^{A503S} to rescue the toxicity of dosage-sensitive genes. Rather, Hsp104^{A503S} effectively prevented the formation of labile, SDS-soluble aggregates, which correlated with alleviation of toxicity. With null mutants, we established that the intrinsic function of several dosage-sensitive kinases and phosphatases was crucial for overexpression toxicity. *In vitro* functional assays with Ppz1 (a dosage-sensitive protein phosphatase), indicated the phosphatase activity was reduced by Hsp104^{A503S} and not by Hsp104. Lastly, we demonstrated that Hsp104^{A503S} suppressed the toxicity of the potent oncogenic kinase, v-Src, in yeast, decreasing protein levels and kinase activity in yeast. Thus, we suggest that in addition to preventing formation of labile, SDS-soluble aggregates Hsp104^{A503S} can also suppress dosage sensitivity by directly unfolding or otherwise deactivating toxic protein such as Ppz1 and v-Src. These studies establish
that potentiated protein-remodeling factors like Hsp104$^{A503S}$ can serve as a powerful buffer that mitigates the toxicity of nearly all dosage-sensitive yeast genes.
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1.1 Proteins, Folding, and Misfolding

Proteins are essential building blocks of life and are required for almost every process a living organism must undertake including growth, reproduction, responding to stimuli or threats, and metabolizing nutrients. Other than water, no molecules are more abundant in the cell than proteins (1). Proteins also exhibit incredible diversity. There are about 30,000 protein-coding genes in the human genome, of which over 90% can be alternatively spliced to yield hundreds of thousands of splice variants, each of which could yield a unique protein if translated (2). As such, every organism must carefully control every aspect of the process of protein production from transcribing DNA into RNA, splicing pre-mRNA, and translating the transcript into polypeptide strings featuring 20 amino acids in various combinations, which provide the foundation for virtually every protein on earth.

As proteins are synthesized by the ribosome, the different amino acids within the polypeptide chain interact with each other to form stable interactions, folding the protein into intricate three-dimensional structures to achieve the “native”, active confirmation. However, some proteins are intrinsically disordered, featuring solvent-exposed regions that do not form stable intramolecular interactions (3, 4). Even though the instructions for how each protein folds into its native state are encoded in the primary amino acid sequence, protein folding is a difficult, dynamic process and most proteins do not instantaneously adopt their native structure but take a meandering route (5, 6). The first step of protein folding is a hydrophobic collapse in which the hydrophobic residues quickly bind to each other to form a molten globule that excludes water from the core of
the protein, which maximizes entropy of the water solvent (7-9). The protein then begins to form secondary and tertiary structure, navigating towards the lowest energy conformation, which can be an arduous process with many possible pathways and off-target diversions for a protein to take through a potentially rugged energetic landscape to reach the natively folded state (10) (Fig. 1). For large proteins with multiple domains, this process can be especially difficult because as the protein folds, it forms many intermediates that may not contribute to the final conformation. Moreover, in the exceedingly crowded cytoplasm, the nascent polypeptide is bombarded with different interacting binding partners such as lipids, nucleic acids, small molecules, and other proteins (11).

Once the protein reaches the native state it constantly undergoes conformational changes to execute its function. Protein functionality often demands a dynamic structure that must explore different conformations to maximize activity. These various conformations may only be marginally stable and place the protein at risk for misfolding or aggregation (12). Moreover, thermodynamics dictate that a protein population at equilibrium will feature proteins that are fully folded, partially folded, misfolded, and even completely unfolded following a Boltzmann distribution where the lowest energy states for the protein will have a higher probability of being occupied but all states from native to completely disordered are populated (13). Furthermore, in the human proteome, approximately half of all proteins are predicted to contain intrinsically-disordered regions that are permanently unfolded and nearly a quarter of total proteins are predicted to be completely disordered (3).
Figure 1: Protein-folding energy landscape.
Protein folding takes place within a free energy landscape in which the protein must navigate to reach the native folded state. Intermediates that become trapped in local minima must overcome energy barriers to reach the final conformation. Cellular chaperones in the cell help guide folding towards the native state, prevent aberrant intermolecular interactions that can lead to amorphous aggregate or amyloid fibril formation, and can disaggregate and unfold aggregated proteins to enable refolding to the native state. (Adapted with permission from Elsevier, License Number: 3944940299836) (14)

Because protein folding is such a dynamic process, rife with obstacles, many proteins require the aid of accessory proteins, termed chaperones, which aid in their proper folding (Fig. 1) (15-18). The requirement for chaperones is especially acute if proteins contain multiple domains requiring complex architectural organization. From the moment a nascent polypeptide exits the ribosome, protein chaperones engage the
protein to facilitate folding (19-21). While small, single-domain proteins fold rapidly in vivo and in test tubes, large multi-domain proteins often fold poorly in isolation, generating partially folded intermediates and misfolded conformations, that often expose hydrophobic regions that can promote self-association into aggregates (22). The geometry of the ribosome and speed of protein translation dictates that folding can typically not be completed until the entire sequence has emerged from the ribosomal polypeptide exit channel (20, 21). To promote proper folding, emerging polypeptides are immediately bound by molecular chaperones that associate with the ribosome, including trigger factor in prokaryotes or nascent chain-associated complex (NAC) in eukaryotes (20, 21, 23). These chaperones associate with the nascent protein and prevent formation of aberrant inter- and intra-protein interactions until the polypeptide chain exits the ribosome, thereby preventing improper folding. Chaperones of the classical Hsp70 system, DnaK and DnaJ in bacteria and Hsp70 and Hsp40 in eukaryotes, can also associate with these nascent proteins while still emerging from the ribosome to facilitate folding (24-26). In humans, there are over a dozen Hsp70 and Hsp40 genes, each family member has their own substrate-binding preference and function in protein folding, refolding, disaggregation, and transferring substrates to other downstream processes if the client protein remain non-native (14, 25-27). These downstream processes could include unfolding and refolding by the chaperonin system, GroEL and GroES in bacteria or TRiC in eukaryotes (25, 26), the folding and maturation of clients by the Hsp90 system (28, 29), degradation by the ubiquitin proteasome system (30, 31), or disaggregation of aggregated substrates by the Hsp100 or Hsp110 systems (32-35).
1.2 Gene Overexpression and Its Causes

Cells have developed highly integrated systems for maintaining protein homeostasis to ensure proper synthesis, folding, localization, and turnover in the crowded cellular environment where macromolecular concentration can be as high as 300 mg/ml (most of which is protein) (11, 19). However, misregulation of gene expression can and does occur. One example is the overexpress of a protein-coding gene that can lead to an overabundance of the encoded protein. While many genes are well-tolerated when expressed at high levels natively or are greatly induced in response to stimuli or stress, aberrant overexpression of certain genes can lead to a wide variety of human disease and disorders including Parkinson’s Disease (PD) (36-38), Charcot-Marie-Tooth neuropathy (39), and a multitude of cancers (40, 41). This proteostatic balance is so delicate that the increase in copy number of a single gene encoding α-synuclein, SNCA, can be the sole cause of some cases of PD (36). Aside from gene duplication, increased gene dosage can be due to a variety of sources including aneuploidy, chromosome translocation, activation of transcription factors, and defects in protein degradation systems (42, 43).

1.2.1 Aneuploidy

Most eukaryotic organisms are diploid, possessing two homologous sets of chromosomes, usually one from each parent. During cell division, new sets of chromosomes must be synthesized from the existing chromosome and then segregated equally between daughter cells during cell division. Aneuploidy is the condition when this process is not accurately carried out, resulting in the abnormal increase or decrease in the number of chromosomes. In most cases, the disruption to the cell caused by
aneuploidy is so severe that it leads to cells that are unable to grow, develop, or continue through the cell cycle (44). The cause of the growth defect is likely due to a confluence of factors including broad-spectrum transcriptome perturbations, effects on expression of genes on other chromosomes, and an increased burden to the transcription, translation, chaperone, and protein degradation systems (45).

Increases or decreases in the number of chromosomes can lead to altered organismal fitness likely due to changes in gene expression. Microarray transcriptome profiling in yeast lacking or containing an extra chromosome show that protein expression levels are often correlated with copy number changes of that gene due to aneuploidy (44, 46). In general, organisms are less able to accommodate losses in whole chromosomes, due to decreases in the expression of essential proteins and disruption of protein stoichiometry (47). However, gene dosage increases of most individual genes, does not result in severe growth defects, rather loss of fitness in organisms with extra chromosomes is likely due to the accumulation of multiple small defects, where increased amounts of extra genetic material is correlated with the decreases in proliferation (46). In the case of additional chromosomes, the additive effect of many small changes in protein stoichiometry likely results in loss of fitness. Organisms often attempt to compensate for increases in gene copy and subsequent elevation of transcript levels by decreasing protein expression through suppression of translation or increased degradation (48-51). This dependence on increased protein degradation is supported by findings that show yeast with extra chromosomes are more sensitive to proteasome inhibitors (46) and cancer cells, which are often aneuploid, also exhibit increased sensitivity to proteasome inhibitors (52).

One of the most well-known human examples of aneuploidy, is Trisomy 21 in
which three copies of the twenty-first chromosome (HSA21) instead of two causes Down Syndrome (DS) (53). Even though HSA21 is the smallest chromosome in the human genome, containing only around 300 expressed genes, a single extra copy can lead to widespread physical, developmental, and intellectual deficits (54, 55). The supernumerary chromosome causes a 50% average increase in expression of all genes, but expression is variable on a gene and tissue-specific level with some genes not overexpressed at all and others overexpressed over 1.5-fold (56). Phenotypic mapping has been used to determine the genes that underpin specific defects in DS, but one gene on HSA21 not associated with DS but with other health implications is the Amyloid Precursor Protein gene, APP (55). APP produces a precursor protein that is then cleaved into Amyloid-beta: a peptide that forms fibrillar amyloid plaques in the brains of Alzheimer’s Disease (AD) patients. Interestingly, nearly all brains of deceased DS patients over 30 years of age featured amyloid plaques similar to those found in AD patients and DS patients often present with similar neurological defects such as loss of language skills and ultimately dementia (57, 58). Accumulations of Amyloid-beta plaques and tau tangles developed two to three decades earlier with three-fold higher rate of diagnosed dementia in DS patients compared to the non-DS population (57, 58).

Moreover, another neurodegenerative disease associated gene, Cu/Zn superoxide dismutase (SOD1), is also located on the HSA21. A DS patient with SOD1 mutation leading to ALS was identified, although overexpression of SOD1 is not generally considered to be a mechanism of ALS pathogenesis (59).

Many cancers and tumors with increased growth and proliferation phenotypes feature aneuploidy (60, 61). This correlation is not necessarily causative but can be an indication of the lack of genomic control (62). Indeed, aneuploidy can be both a cause
and an effect of cancer. Several lines of evidence suggest that aneuploidy is not causative of tumorigenesis and is the result of increased rate cell proliferation that can lead to loss of genomic integrity (46, 63, 64). On the other hand, there are clear examples where aneuploidy can be oncogenic, which specific genes that are overexpressed can be transforming (65-69). Studies in yeast have shown that aneuploidy can be advantageous under some circumstances, where it can alter transcription profiles to quickly adapt to changing environmental conditions and stress (70, 71). The instability of the genome in cancer cells may allow them to rapidly explore aneuploid states for phenotypes that would be most beneficial as host cells attack the cancer (72, 73). In cancer, aneuploidy is can be context specific, with overexpression of some genes promoting tumor formation in certain cell types while inhibiting it in others, but the relative chromosomal instability allows rapid access to variant proteome profiles that can be potentially advantageous in specific cancer cell niches and can even confer drug resistance (74). However, the aneuploid nature of many cancer cells might enable evolutionary traps, which predictably shift the population into a druggable space that could be readily eliminated (75).

1.2.2 Chromosome Translocations

Chromosome translocations are another alteration in genomic integrity, which is caused by rearrangement of nonhomologous chromosome that can result in the misregulation of gene expression (76). Chromosome translocations occur when a segment of one chromosome is fused to a nonhomologous chromosome or a new site on the same chromosome (77). Translocations require double-stranded DNA breaks at two separate chromosomal locations, activation of cellular DNA repair machinery, followed by anomalous fusion and repair to create novel chromosomes. Depending on
the location of the chromosomal lesions and the genes that are affected, disruption and misregulation of gene function can occur.

One of the first characterized chromosome translocations was in the Philadelphia chromosome of some chronic myelogenous leukemia (CML) patients, in which the short arms of chromosomes 9 and 22 are switched (78, 79). This translocation created a novel fusion protein due to the fusion of the ABL1 gene, originally on chromosome 9, to BCR on chromosome 22 (80). Further study on BCR-ABL identified the fusion to be an oncogene in which the kinase activity of ABL was constitutively active, stimulating proliferation of myeloid cells leading to CML (81). In other cases, translocations do not result in gene fusions, but instead replace the coding sequence of one gene with another so that the promoter and enhancer sequences of one gene therefore regulate another. The first characterized example of this phenomenon was in Burkitt’s lymphoma patients, in which c-MYC, which regulates the expression of thousands of genes (82, 83), from chromosome 8 was placed under the control of the highly induced immunoglobulin heavy-chain gene promoter region on chromosome 14 (84-86). In a contemporary study, c-MYC was also found to be similarly translocated in mouse plasmacytomas, where it was also placed under control of an immunoglobulin gene promoter (87-90). The result of these translocations was loss of regulated, temporal induction of c-MYC replaced with constitutive, high overexpression, and carcinogenesis.

Since the characterization of the oncogenic translocations in the 1980’s, over 60,000 chromosomal aberrations, of which over 10,000 are gene fusions, have been reported in nearly every cancer type (91). However, chromosome translocation events associated with lymphomas and leukemia can be found in normal individuals and present a real cellular challenge that needs to be overcome because the consequences
10

for failure to combat these can be dire for the organism (92).

1.2.3. Transcription Factor Activation

Perturbations to transcription factors (TFs) are a common avenue that can lead to gene misregulation and overexpression. TFs are proteins that control which genes are expressed by binding to specific, regulatory DNA sequences near the target genes to stimulate or repress expression (93, 94). Alone or in concert with other proteins, TF binding to DNA influences the engagement of the RNA polymerase II machinery to the target DNA, acting either as an activator that recruits RNA polymerase II to transcribe the gene, or as a repressor that blocks RNA polymerase II binding and thus transcription (94). TF regulation of the genome is essential. TFs control the repertoire of genes that are expressed, which is how every cell in our bodies, despite having the same copies of DNA, can express different genes and have vastly different phenotypes (95, 96). The most common gene mutated in cancer cells is p53, a TF that acts as a tumor suppressor when activated by stress, inducing expression of genes that can arrest cell cycle progression, induce apoptosis, and repair DNA damage (97).

TFs canonically have two separate, modular domains, a DNA-binding domain (DBD) that binds specific DNA sequences corresponding to enhancer, repressor, or promoter regions and a trans-activating domain (TAD) that allows for interaction with other regulatory proteins and the transcription initiation complex (93). TFs may also contain stimulus-response domains, which upon phosphorylation or binding to ligands induces a conformational change that activates or deactivates the TF (98, 99). Mutations to the DBD, TAD, or regulatory regions can lead to changes in the activation of TFs that can therefore drastically change the composition of the transcriptome (100, 101). The DBDs of most TFs are relatively short small regions approximately 20-30 amino acids in
length that bind relatively short DNA sequences that are typically 6-12 bases long \((102)\). The amino acid composition of the DBD dictates the nucleotide sequences it will bind, thus mutations to the DBD will affect the strength and stringency of the interaction, the tolerance for binding modified DNA, and the structural context of the DNA \((103, 104)\). Single amino acid mutations in the DBD can greatly affect the DNA sequences the TF can bind to and thus the genes that are expressed. This effect is especially striking because most TFs are pleiotropic and can influence a large number of genes, one of the most interesting of which is Pdr1 in yeast \((105)\). PDR1, for “pleiotropic drug resistance,” was discovered in a survey of yeast strains where mutations in the gene were discovered to confer resistance to up to 18 different small-molecule drugs with mitochondrial and non-mitochondrial targets, including the fungicides oligomycin, Antimycin A, and cycloheximide \((106, 107)\). Characterization of these PDR1 mutations revealed loss of inhibition and constitutive activation, loss of DNA binding and thus inactivation, changes in the binding preference for DNA leading to a change in genes induced, or perturbations in the TAD that leads to differential protein binding and alterations downstream gene activation \((108, 109)\).

1.2.4. Defects in Protein Degradation

As proteins are being synthesized, they are also constantly turned over, hydrolyzed by proteases back to amino acids to be reused again \((14, 110)\). The rates of degradation vary from minutes to months depending on the individual role of each protein in the cell and are carefully balanced with rate of synthesis \((110)\). Most proteins are degraded by the ubiquitin-proteasome system (UPS) \((111)\) or by lysosomes via autophagy \((112, 113)\). In the UPS, ubiquitin is used as a signaling molecule that marks proteins to be targeted for proteasomal degradation \((14, 110)\). An E1-ubiquitin activating
enzyme primes the ubiquitin, which is then transferred to an ubiquitin-conjugating enzyme that along with a E3-ubiquitin ligase covalently attaches the ubiquitin to a lysine or the N-terminus of a targeted protein (114, 115). Polyubiquitylation can then occur on lysine 48 of ubiquitin to produce a tag to identify proteins for proteasomal degradation (14, 110). The proteasome is an elaborate, barrel-shaped protein holoenzyme consisting of a proteolytic 20S core particle of four stacked heptameric rings that is flanked by a regulatory 19S particle comprise of base and lid substructures, which unfold and deubiquitylate substrates prior to entry into the proteolytic chamber for degradation (116, 117). Proteins can also be degraded by delivery to the lysosome by chaperone-mediated autophagy or macroautophagy. Autophagy is especially crucial for aggregates and misfolded proteins that may not be degraded by the UPS. Chaperone-mediated autophagy (CMA) is the selective degradation of protein substrates in the lysosome (118). In CMA, specific protein substrates are recognized by the heat shock protein, Hsc70, which targets the substrate to the lysosome surface where the protein is then translocated into the lysosomal matrix for degradation (119). In macroautophagy, excess or damaged organelles and entire protein aggregates can be sequestered in double membrane vesicles called autophagosomes, which then fuse with lysosomes for degradation and recycling of biosynthetic constituents (120).

Several human diseases feature increased levels of pathogenic proteins due to deficiencies in the protein degradation pathway including α-synuclein in PD. α-Synuclein is a small, 140-residue, membrane-associated protein found in the pre-synapse of neurons (121, 122). While the normal function of the protein is not fully understood (122), α-synuclein is notable for its formation of beta-sheet rich amyloid fibrils in Lewy neurites in the processes of synaptic neurons and Lewy bodies in cell bodies of neurons,
the defining hallmark of PD and other related neurodegenerative diseases termed
synucleinopathies (123-125). Several uncommon mutations as well as gene duplication
have been discovered to lead to heritable cases of PD (36, 121, 126, 127), but in
general most instances are sporadic (128, 129). Characterization of purified α-synuclein
has shown that it is predominantly unfolded and intrinsically disordered in solution (130,
131). These intrinsically disordered regions present an opportunity to form deleterious
interactions that can lead to aggregation (132, 133). In post-mitotic neurons, where
regulation of homeostasis is crucial, several defects were found in the UPS and
autophagy pathways of PD patients, which included reduced proteasome activity and
downregulation of proteasome components in PD patient tissues, and alterations in
proteasome and autophagy function in a rat models of PD (134). Moreover, α-synuclein
has been demonstrated to be targeted for CMA degradation, and CMA defects have
been described in both familial and sporadic PD (135-137). Additionally, studies with
transgenic animals and neurons expressing α-synuclein have found that proteasome
inhibition resulted in accumulation of α-synuclein in dopaminergic neurons and
accelerated neurodegeneration phenotypes (138-140). Furthermore, other studies in PD
patients have found increases in the number of autophagosomes in affected neurons
(141) as well as decreases in the lysosomal markers suggested accumulation of α-
synuclein in dysfunctional autophagosomes (142, 143).

c-Myc is another example of a UPS substrate in which a failure in degradation
can lead to inappropriate accumulation and ultimately oncogenesis. c-Myc is a proto-
oncogene that encodes a transiently expressed TF with a normally short half-life of
around 30 minutes in cellsb(144, 145). Phosphorylation at threonine 58 and serine 62
are important for ubiquitylation and degradation by the proteasome (146, 147). However,
mutations to Thr58 or Ser62 in c-Myc that prevent phosphorylation or in kinases that phosphorylate these residues can result in a significant decrease in the ubiquitylation and degradation, leading to accumulation of active c-Myc \((148, 149)\). c-Myc activation of downstream pathways can lead to rapid cell proliferation and also enhancement of transformation phenotypes.

1.3 Mechanisms for Dosage Sensitivity Toxicity

The mechanism for toxicity due to gene overexpression and dosage sensitivity has been the subject of much debate. Initially, Hurst and colleagues proposed the “balance hypothesis” in which imbalances in the concentration of subcomponents of protein-protein complexes are deleterious (Fig. 2A) \((150)\). Thus, underexpression or overexpression of protein complex subunits would confer toxicity. Indeed, several lines of evidence suggest that the balance hypothesis helps explain reduced fitness due to underexpression and haploinsufficiency. For example, yeast genes connected with low heterozygote fitness tend to be in protein complexes \((150, 151)\). The specific topological arrangements of protein within a complex is an accurate predictor of underexpression toxicity \((152)\). However, the balance hypothesis has been less able to provide a compelling explanation of overexpression toxicity of yeast genes where many toxic proteins are not components of large protein complexes \((4, 151)\). Moreover, in yeast there is little overlap between genes that are toxic when overexpressed and haplo-insufficient genes \((42)\). With the exception of a small set of essential genes, in which the overexpression phenotype mirrors the deletion phenotype and the overexpressed protein is known to be a member of multi-subunit complexes, altered protein complex stoichiometry is not likely the root cause for most instances of overexpression lethality \((4, 42, 151)\).
Specific regulatory imbalances appear to be the primary cause of overexpression toxicity rather than disruption of protein complex stoichiometry \((4, 42, 151)\). At least \(~80\%\) of yeast genes are not toxic when overexpressed \((4, 42)\). However, proteins that are toxic at elevated concentrations due to overexpression tend to have intrinsically disordered, low-complexity domains \((4)\). Mass-action-driven interaction promiscuity due to intrinsically disordered, low-complexity domains within proteins has emerged as a leading theoretical framework to explain why certain protein-coding genes are toxic when overexpressed \((4)\). In addition, hyperactive gene function (e.g. kinase or phosphatase activity) is likely important for toxicity of a number of dosage-sensitive genes \((4, 42)\). The presence of intrinsically disordered, low-complexity domains within toxic proteins indicates that protein misfolding and potentially aggregation could play a key role in overexpression toxicity \((4, 153)\). Thus, a novel gain-of-function could arise due to toxic soluble oligomer formation or aggregate formation \((4, 154, 155)\). However, intrinsically disordered, low-complexity domains also participate in functional liquid-liquid phase separation (LLPS) events that underpin the formation of various membraneless organelles \((156-159)\). Thus, overexpression could lead to inappropriate or excessive LLPS, which might also be toxic \((160)\). Finally, sequestration of essential proteins or chaperones, and loss of negative feedback and regulation might also contribute to overexpression toxicity \((42, 43, 161, 162)\).

Toxic aggregate formation and accumulation is perhaps the easiest to understand of the mechanisms that cause dosage sensitivity \((4, 153, 154, 155)\). The fact that dosage-sensitive proteins are generally enriched for unfoldedness, long unfolded regions, long transcripts, and slow transcription rates suggests that these proteins are not well folded in their native state and when overexpressed could overwhelm the
proteostasis network and lead to aberrant aggregate formation that the cell cannot abrogate (4). Alternatively, these proteins may adopt soluble but toxic oligomeric conformations like numerous human neurodegenerative disease proteins (163, 164). Indeed, almost all human neurodegenerative disease proteins contain intrinsically disordered regions (155, 165, 166). Aberrant oligomerization and aggregation is thought to be the mechanism for the toxicity of a number of human neurodegenerative diseases, including ALS, PD, AD, and Creutzfeldt–Jakob disease (167-170).

Another hypothesis for overexpression toxicity is that the toxic protein does not aggregate but sequesters essential proteins and chaperones via mass-action-driven interaction promiscuity (4). Dosage-sensitive proteins tend to be enriched in unfolded regions, which likely increases the burden on the endogenous chaperone machinery. In essence, overexpressing certain high-burden protein substrates would bind protein chaperones preventing them from acting on their native substrates, some of which could be essential, resulting in a net loss of function of certain proteins (Fig. 2F). This type of dosage sensitivity is not specific to the function of the protein but could potentially be mimicked by any large influx of highly unfolded protein that demanded the attention of the protein chaperone system. In addition, misfolded and aggregated proteins with exposed hydrophobic regions and other residues not usually solvent accessible in the native conformation could foster aberrant interactions with other soluble proteins, which can remove these proteins from their proper localization or inhibit their functionality (Fig. 2C).

A probable explanation for the mechanism of dosage sensitivity of certain proteins is related to the intrinsic activity of the protein itself. The cell is a fine-tuned machine, which requires all processes to be highly controlled and regulated. For
example, protein kinases phosphorylate substrate proteins and protein phosphatases exist to regulate kinase activity by removing phosphates ligated to substrates. Similarly, fatty acid synthases that create triglycerides to store energy as fat are opposed by lipases that aid in the digestion of the dietary lipids to allow absorption of catabolized nutrients. In almost all cases, pairs or groups of proteins exist that oppose the function of their respective counterparts. However, when the expression of a specific protein is drastically increased, the cell may not be able to regulate this activity because without upregulation of its counteracting partner, the overexpressed protein is now unencumbered in its activity which can lead to defects in growth (Fig. 2D). This regulatory imbalance can lead to anomalous phosphorylation of proteins, abnormal activation of genes by TFs, or deleterious depletion of resources, all of which can be ultimately fatal for the organism.

In this vein, TFs are among the most toxic genes when overexpressed, with more than double the rate of dosage sensitivity compared to the rest of the genome (42). Nearly a third of TFs were reported to be dosage sensitive, which makes sense because TF overexpression can lead to subsequent upregulation of other genes unleashing a cascade of aberrant gene overexpression. Indeed, TF overexpression often results in perturbations of known physiological functions and specific expression activation or repression of their target genes (171). Moreover, TFs often bind to characterized regulatory DNA sequence motifs proximal to their target genes affecting their expression patterns (171). TF overexpression leads to the induction of known targets and shows that overexpression can increase occupancy of TF to their known targets (171). The fact that overexpression of TFs causes growth defects suggests that their increased activity may result in pathway activation and dysregulation that results in deleterious
transcriptome imbalances.

The physicochemical properties and function of a protein seem to dictate whether it will be toxic upon overexpression. However, the mechanism by which individual proteins become toxic when overexpressed has typically not been delineated, and as illustrated above, diverse mechanism may be responsible. This mechanistic diversity complicates the development of agents that counter toxicity of diverse dosage-sensitive genes. Indeed, is it even possible to define a single, wide-reaching solution for genes that are harmful and can cause disease when overexpressed? The answer seemed to be “no” because the mechanisms by which genes might be deleterious when overexpressed are numerous and diverse. However, because Hsp104 is able to unfold many proteins that can be damaged during cellular stress, it seemed to be a promising candidate to pursue.
Figure 2: Possible mechanisms of dosage sensitivity.  
A. Overexpression of one component of a multi-protein complex can lead to loss of stoichiometric balance and formation of nonfunctional intermediates. The phenotype overexpression phenotype for these types of proteins may be the same as gene deletion. B. Overexpression of intrinsically disordered or aggregation-prone proteins could lead to the formation of toxic soluble oligomers or large aggregates. C. Overexpression of toxic aggregation-prone proteins could lead to aggregates that form non-native interactions with soluble protein and remove them from the cytosol. D. Overexpression of a kinase (e.g. kinase in blue) could lead to overactive kinase activity leading to excessive phosphorylation (yellow star) of a substrate (light green) resulting in increased downstream signaling. Without commensurate upregulation of the endogenous phosphatase (dark green), hyperphosphorylation is left unchecked. E. Overexpression of proteins with intrinsically unfolded, low-complexity regions can lead to generation of liquid-liquid phase separations (LLPS) events which may be toxic. F.
Overexpression of difficult-to-fold substrates could lead to sequestration of chaperones and subsequent misfolding of other proteins, some of which could be essential for viability.

**1.4 Hsp104 is a Potent Protein-Remodeling Factor and Protein Disaggregase**

In the brewer’s yeast *Saccharomyces cerevisiae*, Hsp104 is a hexameric, ring-shaped AAA+ (ATPases associated with diverse cellular activities) protein and a potent protein-remodeling factor and protein disaggregase of the Hsp100 family (172, 173). It is an essential protein for yeast to tolerate diverse stresses and is greatly induced following low and high temperature shock and chemical stresses (173). Unlike most protein chaperones like Hsp40, Hsp70, and Hsp90 that facilitate folding of nascent polypeptides, the primary function of Hsp104 is to disaggregate and refold stress-induced, aggregated and misfolded proteins, returning these proteins back to their native form (32, 174, 175). Its aggregated substrates in yeast can include a diverse set of structures from amorphous aggregates formed during heat shock to highly-ordered, fibrillar amyloids of a single species like the Sup35, Rnq1, and Ure2 prions that underpin the non-Mendelian elements \([\text{PSI}^+], [\text{RNQ}^+], \text{and } [\text{URE3}^+], \text{respectively} \) (176-179). However, Hsp104 activity is not restricted to just aggregated substrates. Hsp104 is also a powerful protein-remodeling factor capable of disassembling soluble toxic oligomers formed by diverse proteins (177, 180-182). Hsp104 can also convert intrinsically disordered prion domains into self-replicating prion conformers under certain conditions (177, 180). Finally, Hsp104 can act as an unfoldase, which under some circumstances (e.g. in vitro in the presence of ATP and ATP\(\gamma S\)) unfold natively-folded proteins like GFP provided they are appended to an intrinsically disordered domain (183).

Hsp104 is a member of the Hsp100 family of molecular chaperones, which
contain highly conserved AAA+ domains that utilize the energy from ATP hydrolysis to remodel protein substrates (184, 185). Hsp104 contains two AAA+, nucleotide-binding domains (NBD1 and NBD2), a middle domain (MD) separating the two NBDs, and an N-terminal domain (NTD) and C-terminal domain (CTD) flanking the NBD1 and NBD2 respectively (Fig. 3A). Hsp104 forms barrel-shaped hexamer in vivo, in which the NTD, and the two NBDs form a set of three stacked rings surrounding a hollow central pore through which substrate is translocated (Fig. 4) (186, 187). Recent studies show that the Hsp104 can adopt an asymmetric helical architecture with the AAA+ domains forming a two-turn spiral around the central pore axis (Fig. 3B) (188). The NTD is involved in substrate specification and engagement, and enables optimal disaggregase activity (189). Once engaged, the protein substrate is subsequently bound to conserved substrate-binding tyrosines on pore loops inside the channel in each of the NBDs (190, 191). Hsp104 utilizes ATP hydrolysis to translocate the protein through the central pore thus unfolding it (Fig. 4) (174, 190, 191). The MD can regulate Hsp104 activity by forming autoinhibitory interactions with NBD1 to repress the activity of the protein and by establish inter-protomer bridges across the nucleotide-binding pocket to control ATP hydrolysis and thus Hsp104 function (188, 192, 193). The role of the CTD is not as well understood, but it is require for the hexamerization of Hsp104 (194).
Figure 3: Domain architecture and structure of Hsp104.
A. Domain architecture of Hsp104 with corresponding residues. B. Multiple views from a 3D density map of Hsp104 obtain by Cryo-EM. The domains, shaded the same colors as in part A, are arranged in a left-handed, three-tiered spiral with an asymmetric seam between the first and sixth protomer of the hexamer. Axial and longitudinal dimensions of the whole protein (black) and the central pore (grey) are given. (Adapted with permission from Nature Publishing Group, License Number: 3944951097151) (188).
Figure 4: Model of Hsp104 disaggregation of diverse substrates. Hsp104 in collaboration with Hsp40s and Hsp70s, binds a wide variety of substrates including amorphous aggregates and highly-ordered aggregates through interactions in the N-terminal domain (NTD). The protein substrates are translocated through the central pore of Hsp104 where substrate-binding tyrosines on pore loops in the two nucleotide-binding domains (NBD1, NDB2) powered by ATP hydrolysis provide the locomotive force to unfold and thread the substrate through the hexamer to solution.

In yeast, Hsp104 is crucial for the disaggregation and maintenance of proteins that are associated with aging. Deletion of Hsp104 resulted in an accelerated aging phenotype and reduction in longevity (195). As organisms age, protein homeostasis integrity declines in all aspects from diminished chaperone activity, increased amounts
of misfolded and aggregated protein, and decreased proficiency of protein degradation systems (15, 196-199). This deficiency in maintaining proteostasis combined with increasing amounts of oxidative stress (200, 201) can result in the accumulation of toxic protein that further exacerbates the aging phenotype (202, 203). Yet, despite its seemingly indispensible function in preserving proteostasis and limiting the effects of aging, Hsp104 homologs are not conserved in the metazoan lineage of evolution. Indeed, although conserved in eubacteria, some archaeabacteria, and the vast majority of eukaryotes including all fungi, plants, protozoa, and algae, Hsp104 is conspicuously absent in animals (204, 205). Although some Hsp104 functions are preserved in other molecular chaperones complexes, notably the Hsp110, Hsp70, and Hsp40 system (35), the loss of Hsp104 is highly puzzling considering the myriad benefits it bestows upon the host. Considering the greatest risk factors for human neurodegenerative disease is aging (206), this lack of Hsp104 presented an opportunity for designing and introducing bespoke Hsp104 variants that can target aggregation-prone proteins, especially those implicated in several incurable human neurodegenerative disease (207-210).

1.5 Engineering Potentiated Hsp104 Variants

Encouragingly, Hsp104 is well tolerated in metazoan systems and can collaborate with mammalian Hsp110, Hsp70, and Hsp40 molecular chaperones to remodel aggregated protein (35). Furthermore, wild-type Hsp104 has the ability to remodel the protein aggregates and mitigate the symptoms associated with ALS (211), PD (182), AD (212), and HD (213-216) in animal models of human neurodegenerative disease. Moreover, Hsp104 can disaggregate a diverse panel of amyloid fibrils formed by neurodegenerative disease proteins (181). However, this ability to cure
neurodegenerative disease phenotypes is limited and in some cases, Hsp104 may actually enhance toxicity of some disease-associated proteins (217). To improve on the native function of Hsp104, genetic variants of Hsp104 were systematically generated and screened in yeast for the ability to suppress toxicity of TDP-43 and FUS, each implicated in ALS and Frontotemporal Lobar Degeneration (FTLD), and also α-synuclein, which has an active role in the development of PD (207-210). Compared to Hsp104, which had limited efficacy in suppressing disease protein toxicity, many of the mutants generated were far more effective (207-209).

An uncovered Hsp104 variant that was one the best suppressors of α-synuclein, TDP-43, and FUS toxicity in yeast contained an A503V mutation (Hsp104A503V) (207-210). The A503 is in helix 3 of the coiled-coil, middle domain of Hsp104, which is important for ATPase activity, coordination of NBD1 and NBD2 activity, disaggregation, and forming interactions with Hsp70 that can promote protein disaggregation (192). Given its crucial role in regulating so many processes and interaction, it is perhaps not surprising that previous studies of MD variants found that mutations in this region could be toxic to the organism or also have beneficial gain-of-function phenotypes (218).

Mutational characterization found that nearly all mutations at the 503 position enhanced Hsp104 suppression of toxicity to varying degrees and only A503P had no effect or increased toxicity of the toxic disease-associated proteins (207). Hsp104A503V was able to suppress toxicity and aggregation of α-synuclein, FUS, and TDP-43 in yeast restoring solubility and proper localization, without drastically decreasing toxic protein expression and independent of the unfolded protein response and autophagy pathways (207). Hsp104A503V and Hsp104A503S could also disaggregate preformed α-synuclein, FUS, and TDP-43 fibrils in vitro at concentrations where Hsp104 was inactive (207). Hsp104A503S,
which has very similar activity to Hsp104$^{A503V}$, was tested in a *C. elegans* model of PD (207). In this PD nematode model, human $\alpha$-synuclein is overexpressed in the dopaminergic neurons, resulting in severe neurodegeneration, which worsens as the animals age (219). Hsp104$^{A503S}$ co-expression in the dopaminergic neurons was protective, significantly increasing the number of worms with the complete complement of dopaminergic neurons (207). Biochemical studies of *in vitro* purified Hsp104$^{A503V}$ demonstrated that compared to Hsp104, Hsp104$^{A503V}$ exhibited elevated ATPase rate, superior disaggregation function even without Hsp70 and Hsp40, improved substrate translocation efficiency, and the enhanced ability to unfold soluble protein (207).

The significant efficacy of Hsp104$^{A503S}$ in mitigating the toxicity of several dosage-sensitive human neurodegenerative disease proteins in yeast and $\alpha$-synuclein in a nematode model of PD led us to interrogate the extent of the buffering capacity of Hsp104$^{A503S}$. Thus, we tested Hsp104$^{A503S}$ against a battery of dosage-sensitive yeast proteins to obtain a more global picture of the repertoire of toxic proteins Hsp104$^{A503S}$ can rescue.

1.6. Overarching Goal of Thesis.

The overarching goal of this thesis was to rationally engineer a ‘buffer’ that universally mitigates the toxicity of dosage-sensitive genes, i.e. genes that are toxic when overexpressed. By introducing the A503S mutation into Hsp104, we have engineered an enhanced protein-remodeling factor that antagonizes the toxicity of a remarkable number of dosage-sensitive genes. The results of my studies are presented in the next chapter.
CHAPTER 2: SUPPRESSING DOSAGE LETHALITY WITH HSP104\textsuperscript{A503S}

2.1 Introduction

Hsp104\textsuperscript{A503S} was very effective at suppressing the toxic aggregation phenotypes in our yeast neurodegenerative disease models in which disease-associated proteins (e.g. TDP-43, FUS, or α-synuclein) are overexpressed (207-210). Thus, we investigated the ability of Hsp104\textsuperscript{A503S} to combat dosage sensitivity caused by overexpression of endogenous yeast proteins. We hypothesized that if overexpression of some proteins causes proteostatic stress due to the formation of soluble toxic oligomers, toxic aggregates, or inappropriate liquid-liquid demixing phase transitions, then Hsp104\textsuperscript{A503S} should be able to disassemble these toxic species and suppress the deleterious effect of dosage sensitivity. Dosage-sensitive screens have been used to determine substrates of proteins such as kinases, phosphatase, transcription factors, and other proteins (42, 220), as well as dissect complex pathways such as kinetochore assembly, chromosome segregation, establishing cell polarity, histone regulation of transcription, and cell cycle progression (221-224). We overexpressed genes that caused a growth defect in yeast, to identify genes whose toxicity was suppressed by Hsp104\textsuperscript{A503S}. We predicted these genes would code for protein substrates that Hsp104 could directly bind, disaggregate or remodel, and detoxify. We expected Hsp104 to have a limited capacity to suppress dosage-sensitive proteins because of its restricted ability to combat the most toxic human disease-associated proteins in our yeast models, but Hsp104\textsuperscript{A503S} would be able to suppress a greater number of dosage-sensitive genes because in the same context it has proven to be more effective (207-210). Because dosage-sensitive proteins and many aggregation-prone disease proteins commonly have large portions that are unfolded and disordered (4), we further postulated that Hsp104\textsuperscript{A503S} would engage these
toxic proteins through their exposed, unstructured low-complexity domains, and unfold the complete toxic protein via ATPase-coupled translocation across the central Hsp104 channel. This coupled unfoldase and translocase activity would also unfold monomeric soluble protein, disassemble soluble toxic oligomers, disaggregate aggregated structures, and denature toxic conformers. Moreover, the increased ATPase activity of Hsp104<sup>A503S</sup> enables it to complete its reaction cycle more rapidly (207). Thus, we expected Hsp104<sup>A503S</sup> to be a significantly more robust inhibitor of dosage sensitivity because it would be able to remodel more toxic substrates than Hsp104 per unit time. In essence, we suspected that the hyperactive protein-remodeling activity of Hsp104<sup>A503S</sup> would be extremely beneficial when cells are challenged with the overexpression of a single toxic protein.

2.2 Results

2.2.1 Hsp104<sup>A503S</sup> but Not Hsp104 Suppressed Most Instances of Dosage Sensitivity

We previously screened the FLEXGene overexpression plasmid library (225) for candidate genes that were toxic when overexpressed in the BY4741 yeast strain in both the wild-type background and with HSP104 deleted. We did not find a substantial number of genes with significant difference in toxicity when expressed in the wild-type background compared to Δhsp104. From those hits we created a small library of 198 plasmids containing genes marked as potentially dosage-sensitive (Table 1). Each gene was individually arrayed onto a 96-well tissue culture plate to be used in high-throughput yeast transformations. In this study, each plasmid was individually transformed into Δhsp104 yeast to create strains containing an empty pAG413Gal vector (BYV),
pAG413Gal-Hsp104 (BYW), or pAG413Gal-Hsp104<sup>A503S</sup> (BYA) to determine if either Hsp104 or Hsp104<sup>A503S</sup> overexpression could suppress the toxicity of each induced dosage-sensitive gene (Fig. 5A). The pAG413Gal plasmid system was chosen for expressing Hsp104 because the centromeric element ensures a relatively consistent, low copy number per cell (1-2), the Gal1-10 promoter produces robust inducible expression with galactose-containing media (and strong repression on glucose to enable routine passage of yeast), and the HIS3 gene allowed for positive selection of the plasmid in BY4741 yeast. The toxicity of each dosage-sensitive gene in the BYV, BYW, and BYA yeast strains was scored on a scale of 0 to 5, with 0 corresponding to no toxicity and 5 corresponding to full toxicity and no growth (Fig. 5B). Confirming previous results (207), Hsp104<sup>A503S</sup> strongly suppressed the toxicity of α-synuclein, FUS, and TDP-43 (Fig. 5C).
Table 1: Dosage-sensitive genes screened for Hsp104<sup>A503S</sup> suppression. These 198 genes were selected after a previous genome-wide screen in BY4741 for potential dosage sensitivity. Systematic and standard names are given for each gene.
Figure 5: Protocol for screening and scoring dosage-sensitive genes.

A. An overexpression plasmid library of potentially dosage-sensitive genes was screened to determine which toxic yeast genes could be suppressed by Hsp104 or Hsp104\textsuperscript{A503S} expression. The transfected yeast were serially diluted and plated on Glucose (control) and Galactose-containing (overexpression) plates. Toxic genes that were strongly suppressed (yellow star) by Hsp104\textsuperscript{A503S}, weakly suppressed (red star), or not suppressed at all (blue star) were identified.

B. Genes were scored on a toxicity
scale from 0 to 5, with 5 corresponding to the greatest toxicity (BIK1) and 0 for genes with no toxicity (UTP18). C. Human neurodegenerative disease proteins, TDP-43, FUS, and α-synuclein were expressed with an empty vector, Hsp104, and Hsp104<sub>A503S</sub> coexpression (n = 4 independent transformations).

Surprisingly, Hsp104<sub>A503S</sub> suppressed the toxicity of the overwhelming majority of dosage-sensitive genes (97.5%), whereas Hsp104 overexpression was almost completely ineffective (Fig. 6). In the heat map, red is used to represent toxicity of the overexpressed gene and blue is the suppression of toxicity (“Rescue”) of each gene by Hsp104<sub>A503S</sub>, which is the difference in toxicity from the BYA strain compared to BYV. Only genes with an average toxicity score of 0.75 or greater are shown on this heatmap (120 of 198 screened) and they are sorted by “Rescue” (with the greatest Hsp104<sub>A503S</sub> suppression of toxicity listed first to those with Hsp104<sub>A503S</sub> enhancement of toxicity listed last). There were only three genes of the 120 toxic genes (2.5%) that were not rescued by Hsp104<sub>A503S</sub> (Fig. 6). KAR1 toxicity was not suppressed by Hsp104<sub>A503S</sub>, whereas MUK1 and TRM5 toxicity was slightly enhanced by Hsp104<sub>A503S</sub> (Fig. 6). By contrast, the vast majority of toxic genes (117 of 120 or 97.5%) are suppressed by Hsp104<sub>A503S</sub> (Fig. 6). Indeed, 95% (114 of 120) of cases of dosage sensitivity were rescued by Hsp104<sub>A503S</sub> by a score of 0.5 or more and 72.5% (87 of 120) were rescued by a score of 1.0 or more (Fig. 6). The difference in toxicity is just as stark on the spotting assay plates from which the quantification was obtained. The expression of the 30 most suppressed toxic genes, such as AKL1 and SFI1, results in virtually no growth in BYV or BYW but featured nearly full growth in BYA (Fig. 7). Even in examples where toxicity is more moderate like the top half of the second column of spottings in Fig. 7, the suppression of toxicity by Hsp104<sub>A503S</sub> is glaring and unmistakably obvious. To the best of our knowledge, Hsp104<sub>A503S</sub> is the first agent discovered to rescue such a large proportion of dosage-
sensitive yeast genes.

Figure 6: Hsp104\textsuperscript{A503S} suppresses the toxicity of most dosage-sensitive yeast genes.

Heat map depicting the toxicity of each dosage-sensitive gene in each strain ranked by Hsp104\textsuperscript{A503S} suppression of toxicity from greatest to least. Human neurodegenerative disease gene toxicity is shown at the end of the chart. Red indicates increased toxicity, blue indicates increased rescue by Hsp104\textsuperscript{A503S}, yellow indicates increased toxicity enhancement (n = 3-5 independent transformations)
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**Figure 7: Hsp104\textsuperscript{A503S} but not Hsp104 suppresses the toxicity of most dosage-sensitive yeast genes.**

Representative spotting assays from all toxic genes, sorted by suppression of toxicity by Hsp104\textsuperscript{A503S} from most to least rescue. Heat maps from Fig. 1C were aligned to the corresponding spotting.

To further emphasize the difference between Hsp104 and Hsp104\textsuperscript{A503S} suppression of overexpression toxicity, the toxicity score of each gene in the BYW and BYA strains were compared to the control BYV in a scatter plot. The toxicity score of each gene in the vector control strain was plotted on the x-axis with the toxicity score of the gene in BYW (Fig. 8A) or BYA (Fig. 8B) on the y-axis. The best-fit linear regression of the data for the BYW-BYV comparison has a slope of 0.9290 ± 0.0243, with a coefficient of determination ($r^2$) value of 0.9251 (Fig. 8A). This very strong positive correlation (where the slope and $r^2$ values are each very close to 1) indicates that Hsp104 had an almost no effect on suppressing dosage sensitivity (Fig. 8A). Only a few genes, TPK2, SHE1, KIP3, GIP4, and HSF1, showed a difference in toxicity score of one or more between the BYV and BYW strains (Fig. 8A). Hsp104 weakly rescued the toxicity of HSF1, but enhanced toxicity of TPK2, SHE1, KIP3, and GIP4 (Fig. 6, 7, 8A). In sharp contrast, Hsp104\textsuperscript{A503S} suppressed the toxicity of almost all toxic genes, which is immediately evident in the scatter plot, with most data points featuring a y-coordinate value (Fig. 8B). The best-fit linear regression of the data for the BYA-BYV had a slope of 0.1980 ± 0.0393 and $r^2$ value of 0.1769 (Fig. 8B). The flat slope of the linear regression fit and very low $r^2$ value indicates that gene toxicity in BYV did not correlate with gene toxicity in BYA and that overall despite increasing toxicity of some genes in the BYV strains, Hsp104\textsuperscript{A503S} overexpression was able to suppress toxicity greatly (Fig. 8B). The vast majority of toxic genes were suppressed in BYA (Fig. 6, 7, 8B). Only KAR1, ITT1,
MKS1, and GAT3 exhibited toxicity greater than 2 in BYA (Fig. 6, 7, 8B). For these genes, only KAR1 toxicity was unaffected by Hsp104\textsuperscript{A503S}, whereas Hsp104\textsuperscript{A503S} slightly reduced ITT1 toxicity and moderately reduced toxicity of MKS1 and GAT3 (Fig. 6, 7, 8B). MUK1 and TRM5 were the only two genes in which toxicity was slightly enhanced by Hsp104\textsuperscript{A503S} (Fig. 6, 7, 8B). We also tested the ability of another potentiated Hsp104 variant, Hsp104\textsuperscript{A503V} (207), to rescue all dosage-sensitive yeast genes. Remarkably, the results were very similar to Hsp104\textsuperscript{A503S}, and Hsp104\textsuperscript{A503V} suppressed toxicity of nearly all dosage-sensitive yeast genes (data not shown). Thus, Hsp104\textsuperscript{A503X} mutants (except Hsp104\textsuperscript{A503P}) might all be hyperactivated in a very similar manner and be broad-spectrum inhibitors of overexpression toxicity in yeast.
Figure 8. Overexpression of Hsp104<sub>A503S</sub> but not Hsp104, Ssa1, or Hsp82 suppresses dosage sensitivity.

Scatter plot of toxicity of dosage-sensitive genes in yeast expressing Hsp104 (A), Hsp104<sub>A503S</sub> (B), Ssa1 (C), and Hsp82 (D) on the y-axis compared to the vector control on the x-axis. The best-fit linear regression line is plotted in black.
2.2.2 Ssa1 and Hsp82 Overexpression Do Not Suppress Dosage Sensitivity

To determine the specificity of Hsp104<sup>A503S</sup> dosage suppression, we also generated Δhsp104 yeast strains overexpressing either SSA1 (BYS) or HSP82 (BYH) (Fig. 9A, B). Ssa1 is a yeast Hsp70-family chaperone that is crucial for assisting folding of newly translated protein, preventing misfolding, shuttling aberrantly folded proteins for degradation, and ensuring proper protein transport to its final destination (226, 227). In addition, Ssa1 also collaborates with Hsp104 to disassemble protein aggregates and regulate yeast prions (32, 33, 228, 229). In the absence of Hsp104, Ssa1 combines with Sse1 (Hsp110) and Sis1 (Hsp40) to disaggregate various aggregated structures (35, 230). Despite high cytosolic concentrations of Ssa1 and other Hsp70-class proteins in yeast, Ssa1 expression is greatly induced following heat or environmental stress suggesting that it is crucially needed under crisis conditions (231, 232). Yet, despite the importance of Ssa1 in maintaining proteostasis, we found that Ssa1, much like Hsp104, did not suppress the toxicity of most dosage-sensitive genes (Fig. 8C). There were a few exceptions. For example, Ssa1 potently suppressed toxicity of SQS1, REB1, YDR306C, ESL2, and HSF1 more than Hsp104 and slightly enhanced TDA9 toxicity (Fig. 8C). In general, most cases of overexpression toxicity were unaffected by Ssa1 overexpression where the best-fit linear regression for the BYS-BYV data has a slope of 0.9588 ± 0.0290, with a coefficient of determination (r²) value of 0.9034 (Fig. 8C).

We also tested Hsp82 for its ability to suppress dosage sensitivity. Hsp82 was an appealing candidate to test as a potential regulator of dosage sensitivity because it is an Hsp90-class chaperone that assists in the folding of metastable substrates that have difficulty achieving their final stable conformations (233). Typically, Hsp90-chaperones assist in protein maturation, acting only on proteins that have nearly reached their final
form (234). It also has a vital responsibility in folding aggregation-prone substrates and refolding stress or chemically-denatured proteins back to their native state (233, 235). Moreover, Hsp82 is critical for the correct folding of many regulatory and signaling proteins like kinases and TFs, many of which are dosage-sensitive (236-238). We expected Hsp82 to aid in the folding of some substrates to the native, active form potentially increasing the toxicity of some proteins while preventing misfolding of other proteins and suppressing their toxicity. However, when Hsp82 was overexpressed with the genes in the dosage-sensitive library, it did not affect the dosage sensitivity of many genes (Fig. 8D). Compared to the vector control, Hsp82 slightly enhanced the toxicity of RPH1, PDS1, GIP4, SWH1, AZF1, and SLK19 while subtly suppressing the toxicity of NUP100, SKN7, and ESL2 (Fig. 8D). Overall, however, the best-fit linear regression of the data for the BYH-BYV comparison has a slope of 0.9766 ± 0.0260, with a coefficient of determination ($r^2$) value of 0.9260, which demonstrates almost no meaningful difference between toxicity of genes in the vector control compared to yeast overexpressing Hsp82 (Fig. 8A).

These results support that Hsp104$^{A503S}$ has a unique activity in broad-scale suppression of dosage sensitivity that is not easily replicated by overexpression of other chaperones. Thus, neither Hsp104, Ssa1, nor Hsp82 rescued more than a handful of dosage-sensitive yeast genes. By engineering Hsp104 to enhance protein-remodeling activity via introduction of the A503S mutation in the middle domain, we have generated a powerful buffer able to counter the toxicity of diverse and numerous dosage-sensitive genes.
Figure 9. Ssa1 and Hsp82 are induced in yeast.

Ssa1 (A) and Hsp82 (B) were significantly induced following transformation of a plasmid carrying pAG413Gal-Ssa1 and pAG413Gal-Hsp82 in BY4741Δhsp104. Part B, shows 3 independent transformations and inductions.

2.2.3 Characteristics of Dosage-sensitive Genes

The dosage-sensitive genes that were suppressed by Hsp104<sup>A503S</sup> shared many features with previously described dosage-sensitive genes and fell into several general functional categories that are commonly enriched in dosage-sensitive genes (239): TFs, cytoskeleton, cell cycle/mitosis regulators, kinases, or phosphatases (Fig. 10A). GO (gene ontology) Term analysis for “Function” terms were primarily enriched for TF-associated terms related to DNA binding or interactions with RNA Polymerase II complex (Fig. 10B). GO Term analysis for “Component” terms returned mostly cytoskeletal and cell cycle-related terms (Fig. 10B). Bioinformatic analysis of the dosage-sensitive genes revealed that 7 of 10 most enriched terms were for protein unfoldedness (Fig. 11A), consistent with other studies of dosage-lethality (4). Dosage-sensitive proteins are also highly enriched in linear sequence motifs (4). These linear motifs are short protein sequences that can be recognized by common signaling domains, phosphorylated by Serine/Threonine- or Tyrosine-kinases or mediate binding interactions with protein or phospholipids (240). The enrichment of linear sequence
motifs in dosage-sensitive proteins provides support for the interaction promiscuity hypothesis for dosage sensitivity, which posits that these exposed linear motifs may provide additional modalities for aberrant interactions (4).

47 of the 120 dosage-sensitive yeast genes were determined to have human homologs by sequence homology (Table 2). Of these, 10 yeast genes, HRD1, FKH1, FHL1, HSF1, KIP1, KIP3, PBS2, SMP1, TUB2, and RSP5, have human homologs with OMIM (Online Mendelian Inheritance in Man) annotated disease associations, which are varied including renal cell carcinoma, mental retardation, and anemia (Table 2).

Of the 120 toxic genes, the localization of 91 proteins was reported in a global analysis of localization in yeast where each gene was C-terminally GFP tagged in its original chromosomal location (241). The localization profile of the dosage-sensitive proteins was similar to those of all proteins in general (Fig. 11B), with about half of the toxic proteins (45 of 91, 49.5%) reported as localized to the cytoplasm compared to studies that estimated 47% all proteins to be cytoplasmic in proteome-wide studies (242). However, there are some differences from reported subcellular compartmentalization of yeast proteins. The biggest outlier is the enrichment of nuclear proteins in the dosage-sensitive set, 43 of 91 (47.3%) annotated genes (Fig. 11B), which is greater than the 27% reported for the entire yeast proteome (242). In large part, this is due to overrepresentation of transcription factors in the dosage-sensitive gene library, with accounted for nearly 20% (21 of 120) of toxic genes in the library we created (Fig. 10A). In contrast, genes in the mitochondria and exocytc networks (ER, Golgi, and secretory pathways), reported to be approximately 13% of the proteome each respectively (242), were underrepresented in our dosage-sensitive gene set (Fig.11B). The large proportion of genes that are localized to the cytoplasm, nucleus, or both and
relatively few number of genes localized to membrane isolated organelles allows Hsp104$^{A503S}$ to easily engage these substrates.
Figure 10. Features of dosage-sensitive genes suppressed by Hsp104<sub>A503S</sub>.
A. Toxic genes with toxicity as shown in Fig. 1C grouped by function. B. The Gene Ontology Term Finder reveals significant enrichment of genes with DNA-binding/Transcription Factor associated “Function” terms and cytoskeletal or nuclear “Component” terms.
Figure 11. Dosage-sensitive proteins are enriched for predicted disorder or unfoldedness and generally localized to the nucleus or cytoplasm.

A. Dosage-sensitive gene set was compared to the rest of the genome for features that were correlated with dosage sensitivity. A cross-validation experiment was used to determine the predictiveness of each feature for gene dosage sensitivity. The mean area under a receiver operating characteristic (ROC) curve for each of the cross-validation experiments was plotted for each feature. 7 of the top 10 most correlated terms are associated with predicted intrinsic protein disorder (IUcount, ANCHORcount, Flnumaa, Intrinsic.Disorder.GlobPlot, IUmaxrun, Intrinsic.Disorder, DisEMBL.COILS, Flmaxrun), the others refer to protein length, enrichment for linear motifs (ELMcount), and high Asparagine content. Analysis performed by Oliver King. B. Each dosage-sensitive gene with a reported localization (91 of 120) from a global study was counted and the distribution by localization terms are given (241). The localization categories are not mutually exclusive with over 30 assigned two or more localization including 16 that were reported as having both cytoplasmic and nuclear localization.
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<td>Human genes associated with the specific disorder are shown in bold if more than one homolog exists for a yeast gene. Analysis performed by Oliver King.</td>
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<td>2.2.4 Hsp104&lt;sup&gt;A503S&lt;/sup&gt; Does Not Typically Rescue Dosage Sensitivity by Reducing Protein Expression</td>
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<td>One possible explanation for the broad-spectrum rescue of overexpression toxicity was that Hsp104&lt;sup&gt;A503S&lt;/sup&gt; simply reduced toxic protein expression. However, previous studies established that Hsp104&lt;sup&gt;A503S&lt;/sup&gt; rescues TDP-43 and α-synuclein toxicity without affecting their expression level in yeast (207-210). Indeed, we confirmed that Hsp104&lt;sup&gt;A503S&lt;/sup&gt; rescued TDP-43 and α-synuclein toxicity without affecting their expression level (Fig. 12A, B). However, we did observe a modest reduction in FUS expression by</td>
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Hsp104\textsuperscript{A503S}, which had also been observed previously (208). Thus, Hsp104\textsuperscript{A503S} can rescue toxicity of human neurodegenerative disease proteins without affecting their expression, but would the same be true for the yeast dosage-sensitive proteins?

We analyzed the expression levels of 18 dosage-sensitive yeast proteins to determine if Hsp104\textsuperscript{A503S} was lowering toxic protein expression. Utilizing Gateway cloning technology, dosage-lethal genes were inserted into a modified pAG413Gal-ccdb destination vector with an N-terminal 6x hemagglutinin (HA) tag upstream of the gateway cloning sequence. The HA-fusions were transformed into and expressed in BYV, BYW, and BYA strains. 18 HA-fusions that produced growth phenotypes similar to that of untagged protein were chosen for quantitative Western Blot analysis (Fig. 12A).

Although, curiously, addition of the HA tag to Glc7 enabled Hsp104 to rescue toxicity in addition to Hsp104\textsuperscript{A503S} (Fig. 12A). Triose-phosphate dehydrogenase, Tdh1, is not toxic when overexpressed and was used as a control in the analysis (Fig. 12A). Hsp104\textsuperscript{A503S} coexpression with Tdh1 resulted in a slight (~12%), but not statistically significant, decrease in protein expression (Fig. 12B). Of the dosage-sensitive proteins, 4 of 18 (Glc7, Bni4, Tbf1, and Fkh1) showed no decrease in expression in BYA compared to BYV or BYW, indicating that Hsp104\textsuperscript{A503S} can rescue toxicity without affecting toxic protein expression level as with TDP-43 and \alpha-synuclein (Fig. 12B) (207, 209, 210). 12 of 18 (Ppz1, Bik1, Hsf1, Kap95, Gip3, Skn7, Nam8, Ark1, Clb3, Swh1, Nab3, and Hms1) dosage-sensitive proteins showed a slight to modest decrease in expression level (~12-36%) in the BYA strain compared to the BYV control, but none of these differences were statistically significant (Fig. 12B). Moreover, Hsp104 slightly to modestly reduced protein expression level (by ~7-34%) to a similar extent as Hsp104\textsuperscript{A503S} for five of these dosage-sensitive proteins (Ppz1, Bik1, Kap95, Skn7, and Ark1), but conferred no rescue of
toxicity (Fig. 12A, B). Therefore, slight to modest reduction of protein expression level is not sufficient to rescue toxicity in these cases. However, Hsp104<sup>A503S</sup> significantly decreased expression of Akl1 (~60%) and Slk19 (~62%) compared to the vector and Hsp104 controls (Fig. 12B). Thus, reduced protein levels of Akl1 and Slk19 by Hsp104<sup>A503S</sup> could contribute to the reduction in toxicity. Furthermore, when the decreases in expression of each gene was plotted against the suppression of toxicity by Hsp104<sup>A503S</sup>, there was no correlation between the two variables (r<sup>2</sup> = 0.0329), indicating that decreased protein expression does not provide a general explanation for rescue of toxicity by Hsp104<sup>A503S</sup> (Fig. 13). These studies indicate that typically Hsp104<sup>A503S</sup> does not rescue overexpression toxicity by reducing protein expression.
A

Untagged

Vector Hsp104 Hsp104

TDH1 GLC7 PPZ1 BIK1 HSF1 KAP95 GIP3 SKN7 BNI4 TBF1 NAM8 ARK1 CLB3 FKH1 AKL1 SWH1 NAB3 SLK19 HMS1

HA-Tagged

Vector Hsp104 Hsp104

TDH1 GLC7 PPZ1 BIK1 HSF1 KAP95 GIP3 SKN7 BNI4 TBF1 NAM8 ARK1 CLB3 FKH1 AKL1 SWH1 NAB3 SLK19 HMS1

B

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**P-values:**

- **P<0.01**
- **P<0.05**

Legend:

- 100% Increase
- 50% Increase
- No Change
- 50% Decrease
- 100% Decrease
Figure 12. Hsp104<sup>A503S</sup> expression reduces the expression of some dosage-sensitive genes.

A. N-terminal 6xHA tagged dosage-sensitive genes were transformed in yeast carrying an empty vector or galactose-inducible Hsp104 or Hsp104<sup>A503S</sup>. The toxicity of untagged dosage-sensitive proteins (left) was similar to HA-tagged (right). 

B. Expression levels of HA-tagged dosage-sensitive proteins and human neurodegenerative disease-associated proteins, α-Synuclein, TDP-43, and FUS were measured by Western blot analysis. α-Synuclein was C-terminally tagged with 6x-HA, TDP-43 and FUS were untagged and detected with antibody to endogenous epitope. Representative blots for the loading control, Pgk1, and tagged protein, HA, are shown to the left. Quantification of the Western blots are given to the right. HA-tagged protein expression normalized to the vector control strain. 

Blue indicates decrease in expression, red indicates increase in expression. (Mean ± s.e.m., n = 3-5 independent transformations, Two-way ANOVA using Fisher’s LSD Test, * P< 0.05, ** P<0.01, black * only statistically significant difference between BYV-BYA, red * statistically significant difference between BYV-BYA and BYW-BYA).
Figure 13. Hsp104<sup>A503S</sup> reduction of protein levels not correlated with suppression of toxicity.

Scatter plot of HA-tagged protein toxicity suppression by Hsp104<sup>A503S</sup> expression versus HA-tagged protein expression levels in the Hsp104<sup>A503S</sup> strain. The best-fit linear regression is plotted in black.

2.2.5 Dosage-sensitive Proteins Do Not Form Amyloids in Yeast

A primary function of Hsp104 in yeast is to disaggregate proteins that accumulate in amyloid structures and disordered aggregates (32, 172, 180, 243, 244). Thus, we
assessed whether HA-tagged toxic proteins formed SDS-resistant amyloid structures, and if Hsp104 or Hsp104$^{A503S}$ prevented their assembly. 4 of the 18 proteins tested (Akl1, Hms1, Nab3, and Nam8) were among the top 179 candidates in a study that identified yeast proteins with putative prion domains, a type of intrinsically disordered, low-complexity domain enriched in glutamine, asparagine, tyrosine, and glycine residues (245). However, none of these candidates formed bona fide prions in yeast (245). Using semi-denaturing detergent agarose gel electrophoresis (SDD-AGE) analysis with the standard 2.0% SDS (Fig. 14) and lowered, less stringent 0.5% SDS (data not shown), we did not detect the presence of high-molecular weight smears indicative of amyloid in lysates from BYV, BYW, and BYA yeast expressing the dosage-sensitive proteins (Fig. 14). For Akl1, Hms1, Nab3, and Nam8 these findings were consistent with a previous study, which also found these proteins did not assemble into SDS-resistant structures detected by SDD-AGE (245). By contrast, the yeast prion protein, Rnq1, readily formed these structures in wild-type yeast (Fig. 14). These results were not unexpected in the vector control, which lacks endogenous Hsp104 that is essential in the formation and maintenance of amyloid-based prion states in yeast (180). However, these findings confirm that these proteins are not forming toxic amyloid conformers in yeast. Likewise, the human neurodegenerative disease-associated proteins also did not require the formation of amyloid to be toxic (Fig. 14). Thus, Hsp104$^{A503S}$ does not need to utilize its amyloid-disaggregase activity to suppress the deleterious growth defect caused by dosage-sensitive yeast genes, TDP-43, FUS, or $\alpha$-synuclein.
Figure 14. HA-tagged dosage-sensitive proteins do not form large, SDS-resistant aggregates or amyloid.
Representative SDD-AGE of HA-tagged dosage-sensitive proteins expressed in yeast with vector control (V), Hsp104 (W), and Hsp104<sup>Asp35</sup> (A). The lack of a distinct high molecular weight smear show that these proteins do not form SDS-resistant, aggregated species consistent with amyloid formation. Rnq1-YFP readily formed SDS-resistant, high molecular weight species in wild-type BY4741 yeast with Hsp104 (+), but not in a BY4741 Δhsp104 strain (-) in which Hsp104 is disrupted.
2.2.6 Hsp104\textsuperscript{A503S} Coexpression Abrogates Aggregate Formation

Next, we assessed whether Hsp104\textsuperscript{A503S} might prevent the formation of a less stable, aggregated protein species to counter toxicity. Thus, we developed a filter retention assays with milder conditions (0.1% SDS and 0.1% Triton X-100) than used for SDD-AGE to determine if detergent-labile, less stable aggregates were formed in BYV that were prevented or dissolved in BYA with Hsp104\textsuperscript{A503S} expression. We used a vacuum-based dot blot apparatus to apply the lysates to two consecutively stacked membranes; first, a 0.2µm cellulose acetate (CA) membrane that trapped large aggregates but allowed structures smaller than the pore size to pass through and bind to the second nitrocellulose (NC) membrane underneath (Fig. 15).
Figure 15. Filter Retention Assay.
The Filter retention assay was used to isolate, large aggregates formed in yeast. Lysates from BYV, BYW, and BYA yeast expressing HA-tagged proteins were applied to two stacked membranes: first, the 0.2µm cellulose acetate (CA) filter that traps large aggregates but allows soluble proteins to pass through to the nitrocellulose (NC) membrane.

We observed detectable aggregate formation (i.e. retention by the CA membrane) by all dosage-sensitive toxic proteins in the BYV background (Fig. 16A, B). Increasing the concentration of SDS to 2% with or without boiling the lysates resulted in almost complete dissolution of aggregate species on the CA membrane (Fig. 17A, B). Thus, in general these protein aggregates were not very stable and were detergent soluble, indicating they are more likely to be more labile, disordered aggregates. The
only exceptions were Glc7, Bik1, Kap95, Nam8, and Gip3, which appeared to form some aggregated structures that were SDS-resistant at room temperature (Fig. 17B). Importantly, non-toxic proteins, Adh1, Amd1, and Tdh1 did not form aggregated structures detected by this assay (Fig. 17C, D). Interestingly, Kar1, a dosage-sensitive protein whose toxicity is not suppressed by Hsp104 or Hsp104<sup>A503S</sup> does not form significant amounts of aggregated protein. However, in general, the presence of these aggregated structures correlates with toxicity. Indeed, the toxic human neurodegenerative disease proteins, TDP-43, FUS, and α-synuclein also formed aggregated structures in the BYV background that were trapped by the CA membrane (Fig. 16A, B).

Consistent with previous studies (207, 208), Hsp104 did not affect the aggregation of TDP-43, FUS, and α-synuclein (Fig. 16A, B), and is unable to rescue their toxicity (Fig. 5C). Likewise, Hsp104 did not significantly reduce the amount of aggregated HA-tagged Glc7, Ppz1, Kap95, Gip3, Skn7, Bni4, Tbf1, Ark1, Fkh1, Clb3, Akl1, Swh1, Kip1, Nab3, 14 of the 18 dosage-sensitive yeast proteins tested (Fig. 16A, B). Hsp104 is also unable to rescue the toxicity of these proteins except for HA-tagged Glc7 (Fig. 12A). Thus, the rescue of HA-tagged Glc7 toxicity by Hsp104 is likely not related to alterations in Glc7 aggregation. Interestingly, Hsp104 significantly reduced the aggregation of HA-tagged Bik1, Hsf1, Nam8, and Slk19 (Fig. 16A, B), but did not suppress their toxicity (Fig. 12A). Thus, preventing aggregation to this extent alone appears insufficient to eliminate toxicity for these proteins.

Consistent with previous studies (207, 208), Hsp104<sup>A503S</sup> prevented aggregation of TDP-43, FUS, and α-synuclein (Fig. 16A, B), and rescued their toxicity (Fig. 12C). Strikingly, Hsp104<sup>A503S</sup> also significantly reduced aggregate formation by 14 of 18
dosage-sensitive proteins (Ppz1, Bik1, Hsf1, Gip3, Skn7, Tbf1, Nam8, Ark1, Fkh1, Clb3, Swh1, Kip1, Nab3, and Skl19) compared to the vector control (Fig. 16A, B). It also reduced aggregate formation by Glc7, Kap95, Bni4, and Akl1 (Fig. 16A, B), but here the difference compared to the vector control was not statistically significant, although the reduction in aggregates for Glc7 and Bni4 was significant compared to Hsp104 (Fig. 16A, B). Hsp104^{A503S} rescued the toxicity of all of these HA-tagged proteins (Fig. 12A). However, Hsp104^{A503S} was unable to prevent the toxicity of HA-tagged Kar1 (Fig. 17C, D), a highly toxic protein(246). Thus, aggregate dissolution may generally be utilized by Hsp104^{A503S} to suppress protein toxicity. Collectively, these findings indicate that rescue of overexpression toxicity by Hsp104^{A503S} is typically accompanied by a reduction in the amount of toxic protein entering SDS-soluble aggregates.

With 6 of the 18 HA-tagged proteins, Glc7, Ppz1, Bni4, Fkh1, Clb3, and Kip1, Hsp104^{A503S} reduced aggregate formation significantly more compared to Hsp104 (Fig. 16A), confirming its enhanced activity compared to Hsp104. In all cases tested and quantified, Hsp104^{A503S} reduced aggregate formation more effectively than Hsp104 (Fig. 16A, B). Moreover, Hsp104 significantly reduced the aggregation of HA-tagged Bik1, Hsf1, Nam8, and Skl19 (Fig. 16A, B), but did not rescue their toxicity (Fig. 8A). These findings suggest that the mechanism by which Hsp104^{A503S} prevents protein aggregation to rescue toxicity is different from Hsp104, which can also impede the aggregation of some toxic proteins but without alleviating toxicity (Fig. 12A, 16A, B). Thus, Hsp104^{A503S} may prevent the formation of particularly toxic aggregated structures, whereas Hsp104 only prevents the formation of benign aggregated structures and not toxic species. That is, aggregates formed in the presence of Hsp104 possess a different toxic structure (or ‘strain’), whereas those formed in the presence of Hsp104^{A503S} do not. The ability of
Hsp104 to promote different ‘strains’ of protein aggregates with different phenotypic properties (e.g. toxicity) has been observed previously in the context of Sup35 prions (247) and polyglutamine aggregation (217). Alternatively, Hsp104\textsuperscript{A503S} may also prevent the co-aggregation of other proteins that contributes to toxicity, whereas Hsp104 may not. Thus, aggregates formed in the presence of Hsp104 may sequester other essential proteins and continue to confer toxicity. Further experiments are required to distinguish between these possibilities.

Finally, it is also possible that Hsp104\textsuperscript{A503S} exerts additional effects on soluble forms of toxic proteins, which are not exerted by Hsp104. For example, Hsp104\textsuperscript{A503S} may disassemble toxic soluble oligomers, whereas Hsp104 may not. Alternatively, Hsp104\textsuperscript{A503S} may preferentially recognize the unfolded stretches of toxic proteins and unfold, remodel, or otherwise inactivate the entire toxic protein, whereas Hsp104 may not. Previous studies suggest that Hsp104\textsuperscript{A503V} recognizes shorter unfolded segments of proteins than Hsp104, and also promotes their rapid unfolding and inactivation in situations where Hsp104 has no effect (207, 208). Thus, we anticipate that in addition to preventing toxic aggregation, Hsp104\textsuperscript{A503S} also has a direct effect on soluble forms of the toxic protein, which contributes to rescue of toxicity.
Figure 16: Hsp104^{A503S} prevents aggregation of dosage-sensitive proteins.

A. The bar graph shows the quantification of aggregated protein detected by filter retention assay as a ratio of aggregated (bound to the cellulose acetate) to flowthrough soluble protein (bound to nitrocellulose). The values for the Hsp104 and Hsp104^{A503S} expressing strains were normalized to the vector control strain (Mean ± s.e.m., n = 3-5 independent transformations, Two-way ANOVA using a Fisher’s LSD Test, * P < 0.05, ** P <0.01, *** P < 0.0001, black * indicates statistical difference compared to BYV, red * indicates statistical difference between BYV-BYA and BYW-BYA with number of * only for BYV-BYA comparisons, P < 0.05 for all BYW-BYA comparisons, blue * indicates statistical differences between BYW-BYA comparisons only). B. Representative filter retention blots showing the aggregation state of HA-tagged proteins bound to the cellulose acetate (CA) and nitrocellulose (NC) membranes in the vector control, Hsp104, and Hsp104^{A503S} strains.
Figure 17. High concentrations of SDS or boiling dissolve aggregates of dosage-sensitive proteins.

A. Representative filter assay using lysates boiled in sample buffer with 2% SDS at 99°C for 5 minutes.

B. Representative filter retention assay with lysates treated with 2% SDS sample and incubated at 20°C for 5 minutes.

C. Filter retention assay with non-dosage-sensitive proteins (Adh1, Amd1, and Tdh1), which do not form aggregates in BYV, BYW, or BYA strains. The non-suppressed dosage-sensitive protein, Kar1, also does not form in BYV, BYW, or BYA strains.

D. Spotting assays of BYV, BYW, and BYA yeast expressing HA-tagged Adh1, Amd1, Tdh1, and Kar1.
2.2.7 Defining Substrate Binding and ATPase Modalities Needed For Hsp104^{A503S} to Suppress Overexpression Toxicity.

To elucidate the mechanistic requirements for suppression of overexpression-induced toxicity, we introduced several deactivating mutations to Hsp104^{A503S}, targeting the residues of Hsp104^{A503S} critical for effective substrate binding and remodeling, as well as ATP hydrolysis (Fig. 18A). Alanine substitutions to each of the two pore loops tyrosines, Y257 (in NBD1) and Y662 (in NBD2), severely dampen substrate binding at each pore loop and translocation through the central pore of Hsp104 (174, 248, 249). Glutamine substitutions to each of the two Walker B motif glutamates, E285 (in NBD1) and E687 (in NBD2), abolishes ATP hydrolysis but not binding, allowing the formation of hexamers that can engage and bind substrate but not disassemble them (250). Thus, we constructed seven Hsp104^{A503S} variants to define substrate-binding and ATPase modalities necessary to rescue overexpression toxicity: Hsp104^{Y257A:A503S} (termed PL1, ‘pore loop NBD1’, which is dysfunctional in substrate binding to the NBD1 pore loop), Hsp104^{A503S:Y662A} (termed PL2, ‘pore loop NBD2’, which is dysfunctional in substrate binding to the NBD2 pore loop), Hsp104^{Y257A:A503S:Y662A} (termed DPL, ‘double pore loop’, which is dysfunctional in substrate binding to the NBD1 and NBD2 pore loops), Hsp104^{E285Q:A503S} (termed WB1, ‘Walker B motif NBD1’, which is dysfunctional in ATP hydrolysis at NBD1), Hsp104^{A503S:E687Q} (termed WB2, ‘Walker B motif NBD2’, which is dysfunctional in ATP hydrolysis at NBD2), Hsp104^{E285Q:A503S:E687Q} (termed DWB, ‘Double Walker B’, which is dysfunctional in ATP hydrolysis at NBD1 and NBD2), and Hsp104^{Y257A:E285Q:A503S:Y662A:E687Q} (termed DPL DWB, ‘Double Pore Loop Double Walker B’, which is dysfunctional in substrate binding and ATP hydrolysis at NBD1 and NBD2). These pore loop and ATPase Hsp104^{A503S} variants were robustly expressed in Δhsp104
yeast (Fig. 18B). These Hsp104<sup>A503S</sup> variants were screened against the dosage-sensitive gene library.

**Figure 18. Mutations of Hsp104<sup>A503S</sup> that reduce substrate binding and ATPase activity are well expressed.**

The Y257A and Y662A mutations inactivate the substrate-binding pore loops and the E285Q and E687Q Walker B mutations prevent ATP hydrolysis but not ATP binding in NBD1 and NBD2 respectively. **A.** On the structural model of Hsp104, the blue spheres depict the location of the substrate-binding tyrosines along the central pore and the red spheres the Walker B glutamates that bind ATP. **B.** Western blots of Δhsp104 yeast expressing Hsp104<sup>A503S</sup> pore loop and Walker B mutants show robust expression of all seven mutants.
As anticipated from previous studies on Hsp104^{A503V} (251), rescue of toxicity of the human neurodegenerative disease proteins, TDP-43, FUS, and α-synuclein, by Hsp104^{A503S} was severely impaired in PL1, PL2, DPL, DWB, and DWBDPL (Fig. 19). However, WB1 and WB2 displayed partial ability to rescue (Fig. 19) (251). Thus, both substrate-binding pore loops are critical for rescue of TDP-43, FUS, and α-synuclein toxicity (251). By contrast, ATP hydrolysis at NBD1 or NBD2 is sufficient for partial rescue of TDP-43, FUS, and α-synuclein toxicity, but ATP hydrolysis at both NBDs is required for complete rescue (Fig. 19) (251). These findings suggest that the protein-remodeling activity, protein disaggregase activity, or both are required for Hsp104^{A503S} to rescue TDP-43, FUS, and α-synuclein toxicity in yeast (251).

For most dosage-sensitive yeast proteins, functional pore-loop tyrosine residues and ATP hydrolysis by both NBDs were very important for full dosage suppression provided by Hsp104^{A503S} (Fig. 19). Interestingly, as with the human neurodegenerative disease proteins, single Walker B variants of Hsp104^{A503S}, WB1 or WB2, were partially or fully active against diverse toxic proteins (Fig. 19). Thus, in many cases, ATP hydrolysis at NBD1 or NBD2 is sufficient to rescue overexpression toxicity. By contrast, single pore-loop variants, PL1 or PL2, were typically more inactivating and allowed the rescue of relatively few toxic proteins (Fig. 19). As expected, the DPL, DWB, and DPLDWB variants were almost completely inactive in rescuing overexpression toxicity (Fig. 19). These findings suggest that the protein-remodeling activity, protein disaggregase activity, or both are required for Hsp104^{A503S} to rescue the toxicity of most dosage-sensitive genes in yeast.
Figure 19. Pore loop and Walker B mutations greatly diminished Hsp104<sup>A503S</sup> suppression of dosage-sensitive gene toxicity.

Spotting assay quantification of yeast co-expressing dosage-sensitive proteins and Hsp104<sup>A503S</sup> mutants: Y257A (PL1), Y662A (PL2), Y257A:Y662A (DPL), E285Q (WB1), E687Q (WB2), E285Q:E687Q (DWB), Y257A: E285Q: Y662A:E687Q (DPL DWB). Red indicates increased toxicity, blue indicates increased rescue, yellow indicates increased toxicity enhancement (n = 2-4 independent transformations).

When each variant is directly compared to Hsp104<sup>A503S</sup> for dosage suppression, several notable features emerge. Alanine mutations to the individual substrate-binding
tyrosines, PL1 (Y257A) and PL2 (Y662A), each almost completely abolished A503S potentiation of Hsp104 (Fig. 20A, B). Of the 86 toxic genes that were suppressed by Hsp104 A503S by a score of 1 or more, only 11 of these genes were still suppressed by that level when Y257A was introduced: RSP5, CHD1, KAP122, SFB3, PDS1, RTG3, ASE1, NFI1, YDR306C, HSF1 and ECM9 (Fig. 20A, 21A, 22A). For Y662A, there are six genes that fit this criteria: SEC31, CHD1, KAP122, HSF1, TUB2, and HSF1; and seven genes for DPL: SEC31, KAP122, CHD1, PDS1, HSF1, ECM9, and YDR306C (Fig. 20B, 21A, 22A). PDS1, KAP122, HSF1, and CHD1 were the only genes rescued by all three of the pore-loop variants (Fig. 20C, 21A, 22A). This strongly indicates that substrate binding and especially the 662-position Tyrosine is crucial for Hsp104 A503S potentiation, and loss of substrate binding at either tyrosine effectively nearly ablates Hsp104 A503S-mediated suppression of dosage sensitivity. The few toxic proteins that were rescued by PL1, PL2, or DPL variants might interact with the NTD of Hsp104 A503S, which is also able to engage substrates and plays a key role Hsp104 potentiation (189). Alternatively, the rescue of these toxic proteins might be mediated by an indirect effect.

Intriguingly, single Walker B motif mutations, WB1 and WB2, were generally much more effective at suppressing overexpression toxicity than the pore loop variants (Fig. 19, 20D, E). Of the 86 toxic genes that Hsp104 A503S suppressed by a score of 1 or more, the WB1 mutants of Hsp104 A503S suppressed the toxicity of 47 genes by a score of 1 or more and WB2 mutant suppressed 45 genes in the same manner. Surprisingly, the set of genes suppressed are almost completely overlapping. 43 are suppressed by both WB1 and WB2 (43 of 49 unique genes, 87.8%), with only BBP1, RPI1, STE4, and SUI1 uniquely rescued by WB1 and AFT2 and PAM1 suppressed only by WB2 (Fig. 21). This remarkable result suggest that that certain substrates are specifically more amenable to
suppression by Hsp104\textsuperscript{A503S} than others and that full ATPase activity is required for other substrates. In the case of MHP1, ARK1, NFI1, and AKL1, these very toxic genes were suppressed by both WB1 and WB2 nearly to the same extent as the fully ATPase-competent version, Hsp104\textsuperscript{A503S} (Fig. 22B). The DWB and DPLDWB strains show almost no suppression of toxic genes in comparison to Hsp104\textsuperscript{A503S} (Fig. 20F, G). Here too, any suppression of toxicity likely stems from passive chaperone activity of the Hsp104\textsuperscript{A503S}, which could involve the pore loops and NTD for DWB, and likely just the NTD for DPLDWB. The best-fit linear regression fit data from these scatter plots (Fig. 20) illustrate the lack of suppression of PL1, PL2, DPL, DWB, and DPLDWB in preventing dosage sensitivity and the relative robustness of WB1 and WB2 (Table 2). These data suggest that the increased effectiveness of Hsp104\textsuperscript{A503S} requires substrate binding to both NBD pore loops and ATPase activity at both NBDs. However, Hsp104\textsuperscript{A503S} activity is more sensitive to mutations that disrupt substrate binding at either NBD than mutations that disrupt ATPase activity at either NBD (251).
Figure 20. Pore loop mutations more significantly decrease Hsp104<sup>A503S</sup> potentiation than Walker B motif mutations. Scatter plots of the suppression of each dosage-sensitive protein in Hsp104<sup>A503S</sup> (x-axis) compared to suppression by each pore loop or Walker B variant in Hsp104<sup>A503S</sup> (y-axis): A. Hsp104<sup>Y257A,A503S</sup> (PL1); B. Hsp104<sup>A503S,Y662A</sup> (PL2); C. Hsp104<sup>Y257A,A503S,Y662A</sup> (DPL); D. Hsp104<sup>E285Q,A503S</sup> (WB1); E. Hsp104<sup>E285Q,A503S,Y662A</sup> (WB2); F. Hsp104<sup>E285Q,A503S,E687Q</sup> (DWB); G. Hsp104<sup>E285Q,A503S,Y662A,E687Q</sup> (DPL DWB). The blue line in A-G corresponds to y=1, i.e. suppression of toxicity of 1 in the mutant strains.

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Figure 21. Hsp104\textsuperscript{A503S} with Walker B motif mutations suppresses dosage sensitivity of many genes.
A heat map depicting the genes suppressed by single Walker B motif mutants of Hsp104\textsuperscript{A503S} (WB1 and WB2). Suppression is scored as in Figure 20 with blue indicating increased rescue. Black boxes are for suppression scores below 1.

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<td>Hsp104\textsuperscript{A503S} DWB</td>
<td>0.223 ± 0.0732</td>
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<td>Hsp104\textsuperscript{A503S} DPL DWB</td>
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Table 3. Only single Walker B mutants of Hsp104\textsuperscript{A503S} are consistent suppressors of diverse dosage-sensitive genes.
The slope and coefficient of determination ($r^2$) values based on the best-fit linear regressions for the scatter plots shown in Figure 16 comparing Hsp104\textsuperscript{A503S} suppression of toxicity to each of the pore loop and Walker B motif mutants.

Notably, mutations to the pore loops and Walker B motifs were not only null in suppressing toxicity of some dosage-sensitive genes, but actually enhanced toxicity of many genes (Fig. 19, 20A-G). Even with the single Walker B Hsp104\textsuperscript{A503S} mutants that retain some rescue activity, 10-15% of genes fall below the x-axis indicating negative suppression scores or enhanced toxicity (Fig. 20D, E). For the PL1, PL2, DPL, DWB, DPL DWB Hsp104\textsuperscript{A503S} mutants, the toxicity of up to 50% of genes was enhanced. Some genes such as CDH1 exhibited greatly enhanced toxicity when co-expressed with PL1, PL2, DPL, DWB, or DPLDWB, but were still rescued by WB1 or WB2 (Fig. 20A-G, 22C). Other genes, such as CST6 exhibited greatly enhanced toxicity when co-expressed with PL1, PL2, DPL, DWB, or DPLDWB, but toxicity was unaffected by WB1 or WB2 (Fig. 20A-G, 22C). These synthetic lethal phenotypes are intriguing and may stem from
incomplete or partial remodeling of substrates to more toxic conformation by these Hsp104 variants. Alternatively, these defective Hsp104\textsuperscript{A503S} variants may hinder the activity of molecular chaperones that would ordinarily engage these toxic substrates and reduce toxicity.

Figure 22. Mutations of Hsp104 that reduce substrate binding and ATPase activity diminish Hsp104\textsuperscript{A503S} suppression of dosage-sensitive gene toxicity.  
A. Representative serial dilution spottings show that despite mutations to pore loops the toxicity of PDS1 and KAP122 were still partially suppressed.  
B. Single Walker B mutants (WB1 and WB2) were still able to suppress the toxicity of 4 very toxic genes: MHP1, ARK1, NF1, and AKL1.  
C. CDH1 and CST6 over expression were slightly toxic in the vector, Hsp104, or Hsp104\textsuperscript{A503S} strains, but in the PL1, PL2, DPL, DWB, and DPL DWB strains these two genes were very toxic when expressed, showing an enhancement of toxicity. WB1 and WB2, were able to provide partial rescue of toxicity for CDH1 and CST6.
2.2.8 Autophagy and Proteasome Activity Are Not Required for Hsp104<sup>A503S</sup> Activity

Next, we sought to determine if the rescue of overexpression toxicity by Hsp104<sup>A503S</sup> was due to increased activity of Hsp104<sup>A503S</sup> alone or whether it also required downstream degradation pathways. We screened three yeast deletion mutants in which autophagy and proteasome activities were perturbed to ascertain if Hsp104<sup>A503S</sup> leveraged these pathways to inhibit proteotoxicity and if these deletions would affect Hsp104<sup>A503S</sup> suppression. We disrupted the genes encoding Atg8, which is crucial for autophagy induction and an essential component of autophagosomes (252-255), Rpn4, a transcription factor that promotes proteasome gene expression, and deletion reduces the level of the proteasome (256-258), and Ubr2, an E3 ubiquitin-ligase that targets Rpn4 and promotes its degradation, which when deleted leads to increase proteasome activity due to increase Rpn4 levels (259). Yeast with deletions or disruptions of ATG8, RPN4, and UBR2 are viable and were used to screen the dosage-sensitive gene library. The candidate genes were overexpressed in ∆atg8∆hsp104 (autophagy-deficient), hsp104∆ubr2 (proteasome-induced), and ∆hsp104∆rpn4 (proteasome-reduced) yeast with Hsp104<sup>A503S</sup> or a pAG413Gal-ccdB vector control (Fig. 23).
Figure 23. Dosage-sensitive protein toxicity and suppression by Hsp104$^{A503S}$ is not typically affected in autophagy or proteasome-perturbed yeast strains. ATG8, UBR2, and RPN4 were knocked out with HSP104 deletion were screened for dosage-sensitive protein toxicity with a vector control and Hsp104$^{A503S}$. Spotting assays were quantified as in Figure 6 using the rubric in Figure 5B. Red indicates increased toxicity, blue indicates suppression of toxicity in the $\Delta hsp104$ background, and yellow indicates enhancement of toxicity in the $\Delta hsp104$ background ($n = 2-4$ independent transformations).

Hsp104$^{A503S}$ suppressed the toxicity of TDP-43, FUS, and $\alpha$-synuclein in the
Δhsp104Δatg8, Δhsp104Δubr2, and Δhsp104Δrpn4 backgrounds. These findings are consistent with previous studies that found Hsp104A503S suppression of TDP-43, FUS, and α-synuclein toxicity did not require autophagy (207). We do observe a slight enhancement of toxicity of several proteins in the double mutant strains, especially TDP-43 and FUS in the Δhsp104Δrpn4 strain where proteasome activity is reduced, however, rescue by Hsp104A503S is still robust (Fig. 23). Surprisingly, the Hsp104A503S-mediated suppression of dosage sensitivity was generally unaffected by these genetic disruptions.

When the Hsp104A503S suppression of the toxicity of each gene in the autophagy-defective and proteasome-perturbed yeast strains was plotted against Hsp104A503S suppression of the same gene in Δhsp104 yeast, the extent of suppression was remarkably similar (Fig. 24A-C). The slopes of the linear regression fits for the Hsp104A503S toxicity suppression in Δhsp104Δatg8, Δhsp104Δubr2, and Δhsp104Δrpn4 versus Δhsp104 were 0.806, 0.815, and 0.613 with coefficient of determination ($r^2$) values of 0.590, 0.596, and 0.402 respectively, indicating high concordance in rescue. The lesser linear regression slope and correlation values for the Δhsp104Δrpn4 to Δhsp104 strain comparisons indicate that proteasome function may have an effect on gene toxicity itself because Hsp104A503S suppression is still very potent (Fig. 23). These data suggest that neither autophagy nor the proteasome are strictly required for Hsp104A503S to rescue overexpression toxicity.

Alterations to the autophagy and proteasome systems did slightly affect the toxicity of genes in the Δatg8Δhsp104, Δhsp104Δrpn4, and Δhsp104Δubr2 strains expressing just the empty vector. The slopes of the linear regression fit for the toxicities of the dosage-sensitive genes in the Δhsp104Δatg8, Δhsp104Δubr2, and Δhsp104Δrpn4 strains compared to the Δhsp104 strain with only the vector control were 0.898, 0.908, 0.898, 0.908, and 0.898 respectively.
and 0.738 with coefficient of determination ($r^2$) values of 0.667, 0.665, and 0.432 for the respective comparisons (Fig. 24D-F). These comparisons show that when proteasome and autophagy pathways are affected, the basal toxicity of individual genes are generally very similar to the $\Delta hsp104$ strain but with some variability. The slope of nearly 1 and relatively high correlation for the $\Delta hsp104\Delta atg8$, $\Delta hsp104\Delta ubr2$ plots indicate that gene toxicity is generally very similar between these strains and $\Delta hsp104$. The decrease in the correlation of the gene toxicity in the $\Delta hsp104$ and $\Delta hsp104\Delta rpn4$ comparison suggests that proteasome activity reduction can have a substantial effect on the toxicity of the genes when overexpressed (Fig. 24F). However, Hsp104$^{A503S}$ suppression of toxicity is still very robust in $\Delta hsp104\Delta rpn4$ yeast (Fig. 23), indicating that Hsp104$^{A503S}$ can still suppress the deleterious effects of dosage sensitivity even when proteasome function is limited.

Some individual dosage-sensitive genes were significantly affected by ATG8, UBR2, or RPN4 deletion. Some notable examples are CDH1 and CLB6, two genes whose toxicity in the $\Delta hsp104\Delta atg8$ and $\Delta hsp104\Delta ubr2$ background were enhanced (Fig. 23, 24D, E, 25A). As expected, Hsp104$^{A503S}$ expression still robustly suppressed the toxicity of CDH1 and CLB6 (Fig. 23, 25A). CDH1 toxicity was also increased in the $\Delta hsp104\Delta rpn4$ yeast strain and likewise Hsp104$^{A503S}$ suppression of toxicity was still effective, whereas CLB6 toxicity was unaffected when RPN4 was deleted. Curiously, ITT1 and HSF1 toxicities were abolished in the $\Delta hsp104\Delta rpn4$ strain (Fig. 24F). Another set of outliers are genes such as MRN1 and AKL1, which were less toxic when overexpressed in $\Delta hsp104\Delta atg8$, $\Delta hsp104\Delta ubr2$, and $\Delta hsp104\Delta rpn4$ strains (Fig. 23, 24E-F, 25A). As with CDH1 and CLB6, Hsp104$^{A503S}$ expression still rescued the dosage sensitivity of MRN1 and AKL1 (Fig. 23, 25B). However, the toxicity of some genes was

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enhanced in the Δhsp104Δatg8, Δhsp104Δubr2 and Δhsp104Δrpn4 strains that Hsp104A503S co-expression was not able to fully suppress (Fig. 23). Notably, Hsp104A503S was not able to fully suppress toxicity of TPK3 and STE12 in the Δhsp104Δatg8 and Δhsp104Δrpn4 backgrounds (Fig. 23, 24A, B, 25C); however, genes like TPK3 and STE12 that are not suppressed were the rare exceptions. Overall, despite some examples, these data indicate that Hsp104A503S is rectifying dosage sensitivity-associated toxicity directly and independent of autophagic and proteasomal function.
Figure 24. Dosage-sensitive protein suppression by Hsp104<sup>A503S</sup> is not greatly affected in autophagy and proteasome-perturbed yeast strains.
The suppression of dosage-sensitive protein toxicity by Hsp104<sup>A503S</sup> in hsp104Δ (x-axis) plotted against Hsp104<sup>A503S</sup> suppression in A. Δ hsp104Δatg8 yeast; and B. Δ hsp104Δrpn4 (y-axis). The toxicity of dosage-sensitive genes in the Δhsp104 (x-axis) plotted against C. hsp104Δatg8 yeast; and D. Δ hsp104Δrpn4 (y-axis).

Figure 25. Toxicity of some dosage-sensitive genes are affected by deletion of ATG8, UBR2, and RPN4.
Representative spotting assays of dosage-sensitive genes in Δatg8Δhsp104, Δhsp104Δrpn4, and Δhsp104 yeast with vector control or Hsp104<sup>A503S</sup>. A. CDH1 and CLB6 displayed enhanced toxicity in the Δhsp104Δatg8 and Δhsp104Δrpn4 strains compared to Δhsp104 yeast. Hsp104<sup>A503S</sup> was still able to suppress the toxicity. B. MRN1 and AKL1 both displayed a decrease of toxicity in the Δhsp104Δatg8 and Δhsp104Δrpn4 with the empty vector. C. The suppression of TPK3 and STE12 toxicity by Hsp104<sup>A503S</sup> was diminished in the Δatg8Δhsp104 and Δhsp104Δrpn4 strains.

2.2.9 Hsp104<sup>A503S</sup> Buffering Capacity Extends Beyond a Single Gene But Not To Entire Chromosomes

Because of the tremendous ability of Hsp104<sup>A503S</sup> to suppress the toxicity of a single overexpressed gene, we were inspired to determine the dosage-sensitive protein buffering capacity of Hsp104<sup>A503S</sup> by simultaneously co-expressing multiple dosage-
sensitive genes in yeast. We selected four genes, AKL1, RSP5, MRN1, and SFB3, to test in combination because of their differing toxicity scores and functions in the cell. AKL1 is a serine-threonine kinase in the Ark1-family of yeast kinases that regulates actin dynamics in cytoskeletal organization and endocytosis (260, 261). AKL1 was one of the most toxic gene in the library with a highest score of 5 in BYV and BYW but no toxicity in BYA (Fig. 6). RSP5 is an E3 ubiquitin ligase with many cellular functions in yeast, one of which is its essential role in targeting proteins misfolded by heat shock for proteolysis (261, 262). RSP5 only caused a mild growth defect with a score of 1.7 in BYV and no toxicity in BYA (Fig. 6). MRN1 is an RNA-binding protein that interacts with chromatin-remodeling complexes and is important for mRNA maturation in yeast (263, 264). MRN1 caused a moderate growth defect, score of 3.5, with full rescue by Hsp104^{A503S} (Fig. 6). SFB3 (formerly LST1) is a Sec24p-family protein that dimerized with Sec23p to form part of the COPII vesicle coat that targets vesicles from the endoplasmic reticulum to the Golgi, including those that contain large oligomeric proteins and difficult protein cargoes (265, 266). SFB3 has moderate toxicity in BYV with a score of about 3 that is almost completely suppressed in BYA (Fig. 6). We found that Hsp104^{A503S} was indeed able to suppress the toxicity of several pairs of co-expressed, unrelated, dosage-sensitive genes (Fig. 21A). Toxic individually, the combinations of AKL1 + RSP5, MRN1 + RSP5, SFB3 + RSP5, AKL1 + MRN1, and AKL1 + SFB3 were all very toxic to yeast expressing the vector control or Hsp104, but expression of Hsp104^{A503S} provided very robust suppression of these five pair-wise combinations of dosage-sensitive genes (Fig. 26A). These results indicate that Hsp104^{A503S} can have the capacity to buffer the toxicity of at least two genes simultaneously that have completely unrelated functions and possibly different mechanisms of dosage sensitivity.
Next, we tested whether Hsp104$^{A503S}$ could buffer growth defects in aneuploid yeast strains bearing entire chromosomal duplications. In this way, we could determine if there was an upper limit to the buffering capacity of Hsp104$^{A503S}$. Specifically, we employed yeast strains that contained discrete, characterized, single chromosome duplications (267). However, Hsp104$^{A503S}$ expression was unable to suppress the toxicity of most aneuploid strains despite only very mild growth defect caused by each additional chromosome (Fig. 26B). Only against disome XII, did we observe a modest growth enhancement by Hsp104$^{A503S}$ (Fig. 26B). Despite the ability to suppress the toxicity of different pairs of unrelated genes, Hsp104$^{A503S}$ may not be able to suppress the growth defect caused by a single extra chromosome. Thus, Hsp104$^{A503S}$ appears well equipped to counter toxicity caused by the acute overexpression of one or two toxic genes. However, Hsp104$^{A503S}$ is unable to buffer toxicity connected with a subtler upregulation (~1.5-2-fold) in the expression of hundreds of yeast genes, with the possible exception of chromosome XII.
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Glucose (Off)  Galactose (On)

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Glucose (Off)  Galactose (On)
2.2.10 Some Catalytically-inactive Dosage-Sensitive Genes Lose Toxic Phenotype

To gain substrate-specific insight into Hsp104$^{A503S}$ dosage sensitivity suppression, we focused on several toxic protein substrates for further analysis. We sought to determine if the overexpression growth defect was connected to the enzymatic activity of the toxic protein. Thus, we focused on protein kinases and phosphatases, where it seems probable that excessive protein phosphorylation or dephosphorylation due to overexpression could readily lead to regulatory defects and toxicity.

First, we focused on Ark1 and Akl1, which are members of the same family of serine-threonine kinases that regulate actin cytoskeleton dynamics in yeast (260, 268). The sequence identity in the kinase region of ARK1 and AKL1 is about 40%, with divergent C-terminal regions that are implicated in regulation of function (260). The C-terminal regions of both proteins are predicted to be highly disordered and aggregation-prone (Fig. 28C, D). Hsp104$^{A503S}$ rescue of Akl1 toxicity is accompanied by a reduction on Akl1 expression and slightly reduced Akl1 aggregation (Fig. 12, 16), whereas Hsp104$^{A503S}$ rescue of Ark1 is accompanied without an effect on protein expression but with reduced Ark1 aggregation (Fig. 12, 16). However, was the kinase activity of Akl1 and Ark1 require for their toxicity?

We generated catalytically-inactive mutants of these dosage-sensitive kinases Ark1$^{K56A}$ (260), and Akl1$^{K78A}$ (260) to determine if catalytic function was necessary for
dosage sensitivity (Fig. 27A). Ark1\textsuperscript{K56A} and Akl1\textsuperscript{K78A} were not toxic when overexpressed (Fig. 27B) despite high levels of expressed proteins (Fig. 29), demonstrating that kinase activity is a necessary component of dosage sensitivity for these kinases. These findings suggest that Hsp104\textsuperscript{A503S} may also exert a direct effect on soluble Akl1 and Ark1, which might reduce their kinase activity. Since Hsp104\textsuperscript{A503V} can unfold soluble substrates that bear an intrinsically unfolded domain, such as RepA-GFP (207, 208), we suggest that Hsp104\textsuperscript{A503S} might also unfold Akl1 and Ark1 to reduce kinase activity and rescue toxicity. Further experiments are required to test this possibility.

Next, we focused on Glc7 and Ppz1, which are related serine-threonine protein phosphatases with about 60% sequence identity in their catalytic regions (269). Ppz1 differs from Glc7 with a long stretch of amino acids in the N-terminal region of the protein that is predicted to be unfolded natively but participates in interactions with binding partners (Fig. 27A, 28A,B) (270). Hsp104\textsuperscript{A503S} rescues toxicity of Glc7 and Ppz1 without significantly affecting their expression level (Fig. 12), but does reduce their aggregation (Fig. 16). However, was the phosphatase activity of Glc7 and Ppz1 needed for their overexpression toxicity? To answer this question we generated Glc7\textsuperscript{H65K} (271), and Ppz1\textsuperscript{R451L} (272) phosphatase-dead variants (Fig. 27A).

Ppz1\textsuperscript{R451L} was robustly expressed but not toxic (Fig. 22B, 29), demonstrating that Ppz1 phosphatase activity was critical for overexpression toxicity. Interestingly, Glc7\textsuperscript{H65K} was also robustly expressed but still toxic in BYV or BYW (Fig. 22B, 29). Thus, phosphatase activity was not required for Glc7 toxicity. Despite being catalytically inactive, Glc7\textsuperscript{H65K} might still have the ability to form protein-protein interactions that are deleterious to yeast that are possibly disrupted by Hsp104\textsuperscript{A503S}. Indeed, perhaps the aggregation of Glc7 (Fig. 16) is sufficient for toxicity, which is modestly reduced by
Hsp104\textsuperscript{A503S}. However, Hsp104 also rescues HA-tagged Glc7 and Glc7\textsuperscript{H65K} toxicity (Fig. 12A, 27B), but does not affect aggregation of HA-tagged Glc7 (Fig. 16A). Thus, reduced aggregation of Glc7 does not appear to be required for rescue of Glc7 toxicity. Perhaps disruption of aberrant Glc7 protein-protein interactions by Hsp104\textsuperscript{A503S} and aberrant HA-tagged Glc7 protein-protein interactions by Hsp104 is critical to rescue toxicity. Further experiments are needed to test this possibility.

Next, we explored whether the intrinsically disordered region of Ppz1 (Fig. 22A) was required for Ppz1 toxicity. Thus, an N-terminal truncation mutant of Ppz1 (Ppz1\textsuperscript{Δ1-344}) was constructed to investigate the importance of the intrinsically disordered region for dosage sensitivity. This unfolded region is also required for endogenous Ppz1 function (270). Ppz1\textsuperscript{Δ1-344} remained toxic in yeast (Fig. 27B), and thus the disordered region was not essential for toxicity. Toxicity of this construct was reduced by introduction of the R451L mutation, demonstrating that the phosphatase activity of Ppz1 is critical for toxicity (Fig. 27B). Hsp104\textsuperscript{A503S} (but not Hsp104) rescued the toxicity of Ppz1 and Ppz1\textsuperscript{Δ1-344} (Fig. 27B). Thus, the disordered region of Ppz1 is not required for overexpression toxicity and is not required for rescue of toxicity by Hsp104\textsuperscript{A503S}. We suggest that Hsp104\textsuperscript{A503S} might also target soluble Ppz1 to rescue toxicity, possibly inhibiting phosphatase activity via binding site occlusion or forced unfolding. Increased protein turnover or degradation was not a cause of loss of toxicity because protein expression levels for Ppz1\textsuperscript{Δ1-344} and Ppz1\textsuperscript{Δ1-344\textsuperscript{R451L}} were substantial and consistent between strains (Fig. 29).
Figure 27. Deactivated dosage-sensitive proteins can lose toxicity. 
A. Catalytically inactive versions of dosage-sensitive proteins, Glc7<sup>H65K</sup>, Ppz1<sup>R451L</sup>, Ark1<sup>K56A</sup>, and Akl1<sup>K78A</sup> and truncation mutants, Ppz1Δ1-344 and Ppz1Δ1-344<sup>R451L</sup>, were generated to determine importance of protein function to dosage sensitivity. 
B. Representative serial dilution spottings with wild-type and null genes both untagged and HA-tagged.
Figure 28. Ppz1, Ark1, and Akl1 are all predicted to have large unfolded stretches. FoldIndex prediction of the foldedness of a protein based on hydrophobicity and net charge (273). Predictions are provided for A. Glc7; B. Ppz1; C. Ark1; and D. Akl1.
Figure 29. Kinase- and Phosphatase-inactive proteins were expressed in Vector control, Hsp104, and Hsp104^{A503S} strains.

A. Representative Western blots of HA-tagged Glc7, Ppz1, Ppz1Δ1-344, Akl1, and Ark1 with their respective null mutants in Δhsp104 yeast expressing an empty vector, Hsp104, or Hsp104^{A503S}. Pgk1 was used as the loading control.

2.2.11 Hsp104^{A503S} Directly Decreases Ppz1 Phosphatase Activity in vitro

To determine if Hsp104^{A503S} can target and deactivate soluble, active protein, we purified Ppz1 from *E. coli* (274, 275) (Fig. 30A). We then assessed Ppz1 phosphatase activity in the presence or absence of Hsp104 or Hsp104^{A503S}. The phosphatase-dead variant, Ppz1^{R451L}, was also purified and used as a negative control for Ppz1 (Fig. 30A). Purified Ppz1 and Ppz1^{R451L} were mixed with purified Hsp104 or Hsp104^{A503S} and para-nitrophenyl phosphate (pNPP) substrate to determine Ppz1 phosphatase activity in assay conditions similar to those used to test unfolding of RepA-GFP by Hsp104 (183)
pNPP is a chromogenic, artificial substrate for many phosphatases, including serine/threonine phosphatases like Ppz1. Phosphatases hydrolyze the pNPP, liberating a phosphate and leaving a para-nitrophenol that absorbs at 405 nm. Hsp104 incubated with Ppz1 slightly decreased the activity of the phosphatase by approximately 15% compared to Ppz1 alone. Remarkably, Hsp104\textsuperscript{A503S} substantially decreased Ppz1 activity by over 75% (Fig. 3B, C). Indeed, Ppz1 alone and Ppz1 incubated with Hsp104 had activity levels that were within error, whereas, Ppz1 incubated with Hsp104\textsuperscript{A503S} had activity that was similar to the negative control, catalytically-inactive Ppz1\textsuperscript{R451L} (Fig. 3B, C). The \textit{in vitro} inhibition of Ppz1 by Hsp104\textsuperscript{A503S} demonstrates that there is a direct interaction between Hsp104\textsuperscript{A503S} with functional Ppz1 that is sufficient to decrease its phosphatase activity, which is necessary for dosage sensitivity.
Figure 30. Hsp104A503S suppresses Ppz1 phosphatase activity in vitro. 
A. Coomassie stain of E. coli purified Ppz1 and Ppz1R451L separated by SDS-PAGE. B. 0.7 μM purified recombinant Ppz1 was incubated with buffer control or 4mM ATP and 2.1 μM hexameric Hsp104, or Hsp104A503S. Phosphatase activity was measured with 10 μM pNPP substrate over the course of 15 minutes at room temperature in a microplate.
reader. Phosphatase activity of Ppz1\textsuperscript{R451L} with 4 mM ATP was also measured. Phosphatase activity normalized to Ppz1 activity at 15 minutes. (Mean ± s.e.m., n = 4 technical replicates). C. Phosphatase activity after 15 minute was normalized to Ppz1 activity in buffer control (Mean ± s.e.m., n = 4 technical replicates, one-way ANOVA using Fisher’s LSD multiple comparisons test, **** = p < 0.0001).

2.2.12 Hsp104\textsuperscript{A503S} Ablates v-Src Toxicity in Yeast

Based on the ability of Hsp104\textsuperscript{A503S} to combat the toxicity of dosage-sensitive genes associate with diverse neurodegenerative diseases as well as diverse dosage-sensitive yeast genes, we investigated its ability to suppress the phenotype caused by an overexpressed oncogene. Oncogenes are cancer-associated genes that are mutated or misregulated, which often leads to hyperactivation or overexpression that can contribute to malignancy. We employed v-Src, a Rous sarcoma virus gene encoding a tyrosine kinase that is oncogenic in a variety of cell types chickens (276). However, in S. cerevisiae, v-Src expression causes the opposite phenotype--stalled cell cycle progression leading to large unbudded cells, presumably due to tyrosine phosphorylation that is hypothesized to cause aberrant downstream signaling resulting in disruption of mitosis (277, 278). v-Src is a specific client of Hsp90 chaperones, which interact with the nascent v-Src protein to stabilize the protein, ensure proper folding, and insertion into the plasma membrane, thus activating the protein (277). Deletion of yeast Hsp90 gene, HSC82, abolishes v-Src toxicity, decreases v-Src expression levels, and decreases the amount of tyrosine phosphorylation (277).

Remarkably, like with yeast dosage-sensitive genes, Hsp104\textsuperscript{A503S} (but not Hsp104) co-expression robustly suppressed the very severe v-Src growth defect (Fig. 31A). Unlike with most dosage-sensitive yeast genes, Hsp104\textsuperscript{A503S} expression significantly reduced the protein expression levels of v-Src by over 60% (Fig. 31B, C).
Hsp104\textsuperscript{A503S} overexpression also resulted in an approximately 40% decrease of phosphotyrosines in yeast (Fig. 31D, E). These results mirror phenotypes observed in yeast with HSC82 deletions, in which v-Src toxicity is also suppressed. Hsp104\textsuperscript{A503S} overexpression might act in a similar manner as HSC82 deletion, by directly opposing the activity of Hsp90, which stabilizes, folds, and directs v-Src to the plasma membrane. Thus, Hsp104\textsuperscript{A503S} may remediate v-Src toxicity in yeast by unfolding or preventing the folding of functional v-Src, which can then facilitate protease digestion. The reduction of cytosolic v-Src, may then decrease the amount of potentially deleterious tyrosine phosphorylation.
Figure 3.1. Hsp104<sup>AS03S</sup> suppresses v-Src toxicity and activity in yeast.

A. Representative serial dilution spotting with v-Src co-expressed with an empty vector, Hsp104, or Hsp104<sup>AS03S</sup>. B. Western blot detecting induced Hsp104 and v-Src protein levels in Δhsp104 yeast expressing an empty vector, Hsp104, or Hsp104<sup>AS03S</sup>. Pgk1 was used as the loading control. C. Western blot detecting induced phosphotyrosine protein levels in Δhsp104 yeast expressing an empty vector, Hsp104, or Hsp104<sup>AS03S</sup>. Pgk1 was used as the loading control. D. Quantification of Pgk1-normalized v-Src expression in the
vector, Hsp104 and Hsp104^{A503S} strains (Mean ± s.e.m., n = 4-6 independent transformations and inductions, One-way ANOVA using Fisher’s LSD Test, **** = p < 0.0001). 

E. Quantification of Pgk1-normalized phosphotyrosine expression in the vector control, Hsp104 and Hsp104^{A503S} strains (Mean ± s.e.m., n = 4-6 independent transformations and inductions, One-way ANOVA using Fisher’s LSD Test, *** = p < 0.001, **** = p < 0.0001).

2.3 Discussion

In this chapter, we show that Hsp104^{A503S} displays unprecedented efficacy as a suppressor of dosage sensitivity. Hsp104^{A503S} suppressed the toxicity of the vast majority, over 95%, of all genes tested, from the very toxic to those with more moderate toxicity. We determined that Hsp104^{A503S} was not simply depressing the protein expression levels to rescue dosage sensitivity but likely had a more active role. We found that while dosage-sensitive genes were not producing proteins that formed amyloid, they did form large, SDS-soluble aggregates that were associated with toxicity. Typically, Hsp104^{A503S} was able to prevent the formation of these aggregates whereas Hsp104 was not, although there were exceptions as discussed above. We probed the ATPase and substrate binding modalities of Hsp104^{A503S} and discovered that it is more sensitive to mutations that impede substrate binding at either NBD than mutations that disrupt ATPase activity at either NBD. Single mutations to either substrate binding loop almost completely nullified Hsp104^{A503S} potentiation, but mutations to Walker B motif residues in individual NBDs were well tolerated. We revealed that Hsp104^{A503S} suppression of dosage sensitivity was not dependent on autophagy pathways or proteasome function. By interrogating its buffering capacity, we found that Hsp104^{A503S} was able to suppress several combinations of two dosage-sensitive genes expressed on different plasmids, but that it was ill equipped to counter the more subtle and system-wide proteome disruption caused by aneuploidy. To gain more granular insight into the
process by which Hsp104\textsuperscript{A503S} prevents dosage sensitivity, we purified a dosage-sensitive protein, Ppz1, and demonstrated that Hsp104\textsuperscript{A503S}, but not Hsp104, could greatly diminish Ppz1 phosphatase activity \textit{in vitro}, which is essential for the toxic overexpression phenotype \textit{in vivo}. Lastly, we show that Hsp104\textsuperscript{A503S} was able to robustly suppress the toxicity of a potent oncogene, v-Src, and decrease the abundance of protein and its activity, which may restore proper cycle progression.

In totality, these finding establish Hsp104\textsuperscript{A503S} as a potent suppressor of dosage sensitivity in yeast. Without the aid of the autophagy and proteasome pathways, Hsp104\textsuperscript{A503S} disrupts aggregation formation and can directly inhibit protein activity to rescue protein toxicity due to overexpression.
CHAPTER 3: CONCLUSIONS AND FUTURE DIRECTIONS

3.1 Conclusions

We demonstrate that Hsp104^{A503S} is an effective agent for combating toxicity associated with protein overexpression. The suppression of over 95% of dosage-sensitive genes by Hsp104^{A503S} is unprecedented in scope. Of the 120 genes that had a toxicity score above 0.75, Hsp104 rescued the dosage sensitivity of all but 3 genes, KAR1, MUK1, and TRM5. Moreover, the effect of Hsp104^{A503S} co-expression on dosage sensitivity was extremely robust. Of the 48 moderate-to-high toxicity genes with scores of 2.5 or above, Hsp104^{A503S} reduced the toxicity of these genes by 2 or more for all but 2 genes, ITT1 and KAR1, proving that it is able to suppress toxicity of even the most toxic genes.

We generated HA-tagged fusions to probe the expression and aggregation phenotypes of the overexpressed, dosage-sensitive proteins in yeast when Hsp104, Hsp140^{A503S}, or a vector control was also expressed. Western Blot analysis showed that although Hsp104^{A503S} had a slight effect on protein expression, the extent of rescue was not correlated to the decrease of protein expression. Thus, limiting the amount of protein expressed was not the sole means of preventing protein overexpression toxicity. Likewise, none of 18 dosage-sensitive proteins tested formed amyloid—although some did form detergent-soluble, unstructured aggregates that were readily dissolved by Hsp104^{A503S}. The prevention or dissolution of these labile aggregates was correlated to suppression of dosage sensitivity. In some cases, Hsp104 could reduce this aggregation without affecting toxicity, which indicates that non-aggregated, soluble protein species may be causing toxicity. Hsp104^{A503S}, but not Hsp104, may be able to further denature
these soluble toxic species—potentially through exposed hydrophobic regions that are endemic to dosage-sensitive proteins, and a common modality for forming aberrant inter-protein interactions that can lead to aggregation. This enhanced ability of Hsp104<sup>AS03S</sup> (compared to Hsp104) to unfold soluble substrates is a feature consistent with previous studies (207, 208) and may be leveraged to prevent protein overexpression-induced toxicity.

To determine if other cellular pathways were involved or necessary in the Hsp104<sup>AS03S</sup> suppression of dosage sensitivity we screened in several yeast knockout mutant strains where autophagy and proteasome function were affected. Deletion of ATG8, which disrupted autophagosome formation, UBR2, which induced proteasome activity, and RPN4, which reduced proteasome function, did not alter that ability of Hsp104<sup>AS03S</sup> to suppress dosage sensitivity in yeast. Although the basal toxicity of some dosage-sensitive proteins were affected in the autophagy-deficient and proteasome-perturbed strains, Hsp104<sup>AS03S</sup> expression heartily suppressed dosage lethality in almost all cases. These results strongly suggest that protein degradation pathways are not adopted by Hsp104<sup>AS03S</sup> to quell toxicity due to protein overexpression.

Next, we sought to elucidate the characteristics of Hsp104<sup>AS03S</sup> that were most important for its potentiation against dosage-sensitive proteins. Mutational analysis showed that while substrate binding to pore loops was indispensable for Hsp104<sup>AS03S</sup> to rescue toxicity of diverse proteins, the potentiated protein was still very effective when mutations to single NBDs ablated ATP hydrolysis. Moreover, mutations to the individual NBD did not seem to greatly affect the repertoire of proteins that Hsp104<sup>AS03S</sup> could suppress. WB1 and WB2 variants could prevent the toxicity of an almost completely
overlapping set of genes, indicating that perhaps certain protein substrates are less taxing on Hsp104\(^{A503S}\), not requiring full activity.

We found that Hsp104\(^{A503S}\) can attenuate the toxicity of at least two different genes with different functions when they are overexpressed simultaneously. The robust suppression of the combination of AKL1, one of the most toxic genes, and SFB3, a moderate-high toxicity gene, was even more striking because two proteins are involved in completely different processes and localized to different compartments in the cell. Akl1, is a cytoplasmic kinase that is involved in actin cytoskeleton organization (261) and Sfb3 is a part of the COP II vesicle coat assembly that targets vesicles from the ER to the Golgi (265, 266). Yet even when both were overexpressed in the same yeast, Hsp104\(^{A503S}\) was able to suppress the toxicity of both genes.

To gain more insight into the mechanism of dosage sensitivity and how Hsp104\(^{A503S}\) may act to inhibit it, we generated and expressed inactivated versions of several toxic genes. We showed that dosage sensitivity was dependent on the enzymatic activity of Ppz1, Ark1, and Akl1. Thus, we wondered whether Hsp104\(^{A503S}\) might engage soluble toxic proteins and inactivate them, perhaps by forced unfolding as with model RepA-GFP substrate (207, 208). We demonstrated that purified, functional Ppz1 was inhibited by Hsp104\(^{A503S}\) \textit{in vitro}, significantly decreasing Ppz1 phosphatase activity far more than Hsp104. Thus, Hsp104\(^{A503S}\) can directly inhibit Ppz1. We suggest that in addition to preventing formation of labile, SDS-soluble aggregates Hsp104\(^{A503S}\) can also suppress dosage sensitivity by directly unfolding or otherwise deactivating the toxic protein.
However, further experiments are needed to determine if Hsp104$^{A503S}$ is fully unfolding Ppz1 to inactivate it or whether it is only partially engaging the substrate—enough to sterically hinder the active site and thus inhibit phosphatase activity. The potentially enlightening experiment would be to include with Hsp104$^{A503S}$ a modified version of GroEL, GroEL$^{D87K}$, a ‘trap’ variant that can bind denatured substrates but not release them due to its deficiency in ATP hydrolysis (279). Binding of Ppz1 to “trap” form of GroEL would indicate that the phosphatase is indeed unfolded and will help clarify if it is fully unfolded by Hsp104$^{A503S}$. Likewise, the unfolding activity of Hsp104 can be coupled to proteolytic degradation to demonstrate substrate translocation through the central pore (280, 281). We can generate the HAP variant of Hsp104$^{A503S}$, in which the G739, S740, K741 residues are mutated to IGF, to foster physical interaction with the bacterial protease, ClpP (249). In this complex, the Hsp104$^{A503S}$-HAP would engage and unfold substrate, directly translocating the substrate into the ClpP proteolytic chamber where it is degraded into small peptide fragments (282). SDS-PAGE with Coomassie staining or immunoblotting would provide a clear picture of whether Ppz1 was unfolded by Hsp104$^{A503S}$ and degraded by ClpP. Pairing Hsp104$^{A503S}$ with either GroEL$^{D87K}$ or ClpP would provide more convincing evidence that Hsp104$^{A503S}$ is indeed unfolding toxic substrates via polypeptide translocation.

Finally, we show that Hsp104$^{A503S}$ can even suppress the activity of a human oncogene that is dosage sensitive in yeast. Although in chickens, v-Src promotes oncogenesis, in yeast, it has the opposite effect of stalling cell cycle progression(278). However, Hsp104$^{A503S}$ strongly suppressed this toxic phenotype in yeast. Thus proving that in principle it (and perhaps other engineered protein unfoldases) may have widespread applications in a variety of human diseases connected with protein overexpression.
Paradoxically, if $\text{Hsp104}^{A503S}$ is able to identify and unfold toxic, dosage-sensitive substrates in nearly every instance, how does it avoid other soluble proteins? Hints of this lack of specificity are present in the ever so slight defect in growth caused by $\text{Hsp104}^{A503S}$ expression alone in the BYA strain, suggesting a basal level of $\text{Hsp104}^{A503S}$ promiscuity in substrate selection which can be deleterious. This slight toxicity might have exerted negative selection pressure for any amino acid at the 503 position but alanine in yeast, which would explain why such a beneficial mutation against overexpression toxicity was not adopted in yeast. Moreover, this mild toxicity is more severe when yeast are grown at 37ºC and under mild thermal stress, whereas Hsp104 is not toxic in these conditions. Our findings suggest that toxicity due to overexpression of a single yeast gene is a rare form of stress, which may not have been a strong selective force dictating Hsp104 sequence space. The inability for yeast to fully regulate internal temperature makes them particularly susceptible to environmental changes that induce protein-folding stress that could induce deleterious $\text{Hsp104}^{A503S}$ activity. These potential off-target effects leading to a decrease in growth could be remedied by iterative mutations of $\text{Hsp104}^{A503S}$ to derive variants with enhanced activity towards specific substrates of interest, while limiting interactions with other substrates and thereby eliminating the growth defect.

3.2 Future directions

The surprising efficacy of $\text{Hsp104}^{A503S}$ in combating nearly every dosage-sensitive protein suggests that $\text{Hsp104}^{A503S}$ can potentially be leveraged to treat many other diseases caused by protein overexpression. Beyond the use in treating neurodegenerative disease such as AD, PD, HD, and ALS which feature accumulations of aggregated protein, any disease in which protein regulation is lost and aberrant
overexpression promotes disease pathogenesis would be a viable candidate for treatment with Hsp104$^{A503S}$. For example, many cancers feature overexpression of protein including transcription factors, growth factors, and metabolic enzymes, all of which could be targeted by Hsp104$^{A503S}$ for activity suppression. The promising result of Hsp104$^{A503S}$ decreasing v-Src toxicity in yeast leads to the next steps of determining whether it can be effective in cell culture and animal models of cancer to limit cellular proliferation, return cells back to a more differentiated state, or counter oncogenic phenotypes (reverse EMT, decrease telomerase expression, decrease colony formation, restore contact inhibition, etc).

Yet, expressing Hsp104 in human patients is not without substantial challenges, the primary of which is the high likelihood that expressing foreign yeast proteins in humans would lead to a significant and potentially deadly immune response. Limited expression of Hsp104 in the brain may be more easily tolerated due to Central Nervous System (CNS) immune privilege, in which destructive, inflammatory T-cell responses common in the periphery are dampened in certain areas of the CNS (283). The effectiveness of Hsp104$^{A503S}$ in reversing cancer phenotypes presents an exciting proof-of-principle to inform future work. If shown to be effective against models of cancer, other proteins with lower immunogenicity such as human protein-remodeling factors or unfoldases like Hsp70 and Hsp110 could be developed and engineered to gain activity akin to Hsp104$^{A503S}$ in suppressing dosage sensitivity and prevent oncogenesis. Retroviral delivery or CRISPR-Cas9 editing to introduce engineered human proteon-remodeling factors to throttle the effects of overexpressed and aggregated protein in targeted cell types would be an exciting next step.

Two chaperones that were tested in this study, Ssa1 and Hsp82, were not
effective at suppressing protein overexpression toxicity. However, we only overexpressed wild-type versions of these respective proteins with a moderate increase in protein levels in a background that already feature substantial expression levels of each. That neither wild-type Ssa1 nor Hsp82 were active against dosage-sensitive proteins should not disqualify them for future studies because wild-type Hsp104 was also unable to suppress dosage sensitivity in the same system. Rather, the results from this study suggest that engineered mutants of Hsp82 or Ssa1 might be uncovered that are effective against a wide spectrum of dosage-sensitive proteins. Moreover, chaperones of the Hsp40 or Hsp110 families could be tested for elevated dosage sensitivity suppression capacity. Like Ssa1 and Hsp82, Hsp40 and Hsp110 family chaperones are also conserved in humans and any gains made in potentiating these enzymes in yeast could be directly translatable to humans. Furthermore, these studies would be particularly illuminating to determine the substrate recognition and binding potential of these chaperones. It would be interesting to learn if engineered version of these proteins also had a broad substrate repertoire like Hsp104$^{A503S}$ or if they were narrower, tailored to specific substrates or classes of substrates.

In this study, Ppz1 phosphatase activity was shown to be essential for its dosage sensitivity in yeast and we demonstrate that Hsp104$^{A503S}$ directly inhibited the phosphatase activity of Ppz1 in vitro, which we proposed was the mechanism by which Hsp104$^{A503S}$ curbed Ppz1 toxicity, but is this what actually happens cells? An interesting next step would be to test to see if protein phosphorylation in yeast overexpressing Ppz1 was affected by coexpression of Hsp104$^{A503S}$. A proteomics approach utilizing mass spectrometry could accurately identify proteins whose phosphorylation state were altered by Ppz1 expression and whether Hsp104$^{A503S}$ expression could affect the
quantity or distribution of protein modifications. This would provide more direct evidence that Hsp104\textsuperscript{A503S} attenuation of Ppz1 activity in vivo was the mechanism by which it suppressed Ppz1 toxicity. A phosphoproteomics approach could also be employed to test the many other kinases and phosphatases that are dosage-sensitive (Fig. 10A) to determine if the hyperactivity of these proteins are also abrogated by Hsp104\textsuperscript{A503S} as a means of limiting toxicity associated with overexpression of these proteins.

It is particularly interesting to note that not all members of a class of proteins are dosage-sensitive. For example, in yeast, there are 3 members of the ARK1 kinase family: ARK1, AKL1, and PRK1. Ark1 and Akl1 were two of the most toxic dosage-sensitive proteins in our system. Despite sharing over 70% sequence identity with ARK1 in the kinase domain, having overlapping function, and similar architecture with an intrinsically unfolded C-terminal region (268), PRK1 overexpression in our system was not toxic. More than the isolated function of proteins must then determine dosage sensitivity. Likewise, Ppz2 and Ppz1 are highly similar proteins, sharing 94% sequence identity in the phophatase domain of the protein with 43% sequence identity elsewhere and even boasting overlapping functions (269) and yet only Ppz1 is toxic when overexpressed. How is it that genes with such similar functions and features, even sharing high sequence homology could be drastically different in toxicity when overexpressed? Characterizing the difference between Ppz1 and Ppz2 or Ark1 and Prk1 could reveal the factors that are most important for determining whether a gene is dosage-sensitive. Moreover, understanding these differences could be useful in determining how the deleterious effects of overexpression can be mitigated by cells with or without potentiated Hsp104.

The toxicity of the main outliers in this study are also worthy of consideration.
ITT1 and KAR1 are two very toxic genes that are almost completely resistant to Hsp104<sup>A503S</sup> activity and TRM5 is a gene whose toxicity is enhanced by Hsp104<sup>A503S</sup> expression. Of the 48 moderate-to-high toxicity genes with scores of 2.5 or above, ITT1 and KAR1 were the two genes that were not substantially rescued, although ITT1 toxicity was slightly decreased by Hsp104<sup>A503S</sup> expression. ITT1 (Initiation of Translation Termination) encodes a protein that can regulate the efficiency of translation termination (284). Overexpression of ITT1 was shown to decrease the efficiency of translation termination, presumably by increasing binding to polypeptide chain release factors which inhibits translation termination and results in significant increases in read-through of nonsense and legitimate stop codons (284). The read-through of stop codons might lead to the increase production of unfolded or misfolded proteins that exceeds the buffering capacity of Hsp104<sup>A503S</sup>. Unlike with other dosage sensitive proteins, which Hsp104<sup>A503S</sup> directly engage to suppress its deleterious effects, ITT1 overexpression may produce misfolded substrates that Hsp104<sup>A503S</sup> bind instead of Itt1, leaving it to continue promoting read-through of stop codons and the production of spurious protein products. Indeed, Hsp104<sup>A503S</sup> was also typically unable to buffer toxicity conferred by aneuploidy, which likely involves modest overproduction of hundreds of gene products. Explanation for why the toxicity Kar1, a protein involved spindle pole body formation and duplication during karyogamy and mitosis (285), is not rescued by Hsp104<sup>A503S</sup> is not as clear, especially when so many of the other dosage-sensitive are also spindle pole body components or otherwise involved in cell division. Likewise, how Hsp104<sup>A503S</sup> expression enhances the toxicity of TRM5, which encodes a tRNA m<sup>1</sup>G37 methyltransferase (286), is also not clear.

Is there a unifying theme to what makes genes toxic when overexpressed?
Several studies hypothesize that dosage-sensitive proteins are involved in large complexes with specific stoichiometry that is disrupted by overexpression (287), but that theory has been largely discredited for dosage sensitivity caused by overexpression—although it is still likely the cause of growth disruption for gene underexpression or haploinsufficiency (42). The findings of this study suggest that some dosage-sensitive proteins are toxic because its elevated enzymatic activity is deleterious and not the result of a passive process such as the balance hypothesis would suggest (239, 288). When mutated to the inactive form, Ppz1, Ark1, and Akl1 lost their dosage sensitivity toxicity phenotype. This finding suggests that the normal function of some proteins is essential for dosage sensitivity and Hsp104A503S can prevent this by decreasing protein activity levels potentially though unfolding soluble protein (Fig. 32A).

Other recent studies also suggest that mass-action-driven interaction promiscuity through interactions with intrinsically disordered regions within proteins is the most likely explanation for the dosage sensitivity of overexpressed proteins. This theory is also supported within the context of our hyperactivity model because aberrant interactions with catalytically active dosage-sensitive proteins may result in detrimental consequences. For example, Ppz1 has an intrinsically unfolded N-terminal region that is essential for protein function which can be myristoylated and is thought to be important for forming protein-protein interaction to specify function (270). Overexpression of Ppz1 could lead to overwhelming the regulation of this protein, especially in the intrinsically unfolded region, resulting formation of unregulated promiscuous interactions with non-native client proteins. Ppz1 may then dephosphorylate non-canonical regulatory sites resulting in unwanted activation or inhibition of crucial enzymes. However, we found that this region of Ppz1 was not required for overexpression toxicity. Nonetheless, the high
enrichment of intrinsically unfolded regions and linear motifs in dosage-sensitive proteins is a likely modality for these toxic proteins to form deleterious protein-protein interactions.

Exposed disordered regions in dosage-sensitive proteins provide low specificity binding surfaces that could also foster aberrant and promiscuous interactions that can lead to toxic aggregate or oligomer formation, sequestration of essential proteins through non-native interactions, diversion of chaperones, and even formation of liquid-liquid phase separations (LLPS). Hsp104\(^{A503S}\) is well adapted to also tackle each of these possibilities. Hsp104\(^{A503S}\) was shown to be very effective (much more so than Hsp104) at dissolving dosage sensitivity-associated protein aggregates in cells and also in vitro (Fig. 12A, B) (207, 208), so if dosage-sensitive proteins were forming aggregates, Hsp104\(^{A503S}\) would be more capable of dissolving them (Fig 32B). Moreover, the enrichment in linear motifs and intrinsically disordered regions in dosage-sensitive proteins, which can cause toxic oligomer or aggregate formation, can also facilitate promiscuous interactions with non-native binding partners and even trapping these proteins within aggregated structures. Hsp104\(^{A503S}\) can mediate the release these speciously bound proteins by disrupting oligomeric complexes and dissolving aggregates (Fig. 33C). However, if dosage-sensitive proteins are diverting other molecular chaperones from their intended substrates, Hsp104\(^{A503S}\) can provide an additional buffer for these toxic proteins (Fig. 33D). Hsp104\(^{A503S}\) is capable of refolding aggregated and denatured substrates, even without collaboration of Hsp40 and Hsp70 co-chaperones in vitro (207, 208), its elevated activity against misfolded and aggregation-prone substrate, would unencumber the proteostasis network.

Lastly, the intrinsically-disordered regions endemic to dosage-sensitive proteins could be undergoing inappropriate and toxic LLPS (160). Indeed, in addition to the high
propensity of these toxic proteins to have intrinsically-disordered regions compared to the genome, another highly common feature are prion-like domains (Fig. 11A). Prion-like domains feature high polar uncharged amino acid and glycine content (289), which can also drive LLPS (157, 158, 290-292). Recent work has shown that for the dosage-sensitive protein, Mip6, toxicity was correlated with induction of a LLPS and growth was restored when the foci were dissolved (160). This work suggest that SDS-soluble aggregates we detected in the filter retention (Fig 16A, B) may actually be labile, phase-separated liquid aggregates and Hsp104^{A503S} was able to suppress the toxicity of these proteins by fully dispersing these droplet structures, which may be correlated with toxicity (Fig. 32E).

The ability for Hsp104^{A503S} to suppress the toxicity of nearly every dosage-sensitive gene opens up the possibility that Hsp104^{A503S} can be used to treat many human diseases in which increased gene dosage can be a contributor to disease pathology. Moreover, the multitude of potential mechanisms by which Hsp104^{A503S} can employ to overcome proteotoxicity in diseases in which proteostatic integrity is lost, provides great flexibility in its application to treat neurodegenerative disease such as AD, PD, HD, and ALS. Further study will be required to determine whether or not Hsp104^{A503S} or related engineered proteins can be translated in clinical settings to provide lasting impact in treating human diseases.
Figure 32. Hsp104\textsuperscript{A503S} may suppress dosage sensitivity in many distinct ways. 
\textbf{A.} A protein can cause dosage sensitivity when overexpression leads to decreased regulation and in increased activity of the protein resulting be spurious and off-target effects. Hsp104\textsuperscript{A503S} may bind the overexpressed toxic protein through exposed disordered regions, unfold it, and reduce deleterious activity. \textbf{B.} Some proteins when overexpressed will form oligomeric species or aggregates that have a toxic gain-of-function. Hsp104\textsuperscript{A503S} can bind and dissolve these oligomers and aggregates to allow them to refold to their native form. \textbf{C.} Dosage sensitive proteins are enriched in linear motifs and intrinsically-disordered regions, which may be able to form low specificity, promiscuous interactions with non-native binding partners or trap essential proteins in aggregates. Hsp104\textsuperscript{A503S} can unfold these misfolded proteins and release their binding partners. \textbf{D.} Dosage sensitive proteins can also have the indirect effect of diverting other molecular chaperones from their intended substrates. Hsp104\textsuperscript{A503S} can provide an additional buffer for these proteins that are misfolded and aggregation-prone, thereby empowering the proteostasis network. \textbf{E.} The intrinsically disordered regions and prion-like domains enriched in dosage-sensitive proteins can also form intermolecular interactions to drive LLPS events that are correlated with dosage sensitivity. Hsp104\textsuperscript{A503S} can mediate the dispersion of these phase-separated droplets.
CHAPTER 4: METHODS

4.1 96-well Plate Yeast Transformation

Modified from published protocols (293). 250-500 ng of selected plasmid DNA from the FLEXgene library (~5,500 genes and ORF’s in a galactose-inducible expression pBY011 plasmid system, (294)) were individually plated into the wells of 96-well cell culture plates (Denville T1096). For each 96-well plate of DNA to be transformed into yeast, 200ml of YPD were inoculated with an overnight culture of yeast at OD_{600} of 0.1 and grown with shaking (250 rpm for all steps) at 30 °C until OD_{600} measured 0.8-1.0. Yeast cultures were pelleted by centrifugation (5 minutes at 1500 g for all steps), supernatant was decanted, and the pellet was resuspended in 10 ml of sterile water. The yeast slurry was pelleted by centrifugation, washed in 0.1 M lithium acetate (LiOAc) in TE (10 mM Tris, pH 7.5 and 1mM EDTA) and pelleted by centrifugation. The yeast was then resuspended in 7ml 0.1M LiOAc/TE and incubated for 15 minutes with shaking at 30 °C. β-mercaptoethanol was added to a final concentration of 0.1M and incubated for 15 minutes with shaking at 30 °C. Boiled and chilled, sonicated salmon sperm (Agilent 201190) was added to yeast slurry to 3% final concentration. 50 µl yeast slurry was dispensed into to each well by multichannel pipette or liquid handling robot. 125 µl transformation mix (0.1M lithium acetate, 40% PEG 3350, 10% DMSO) was added to each well by multichannel pipette or liquid handling robot, mixed thoroughly, and then the plates were incubated for 30 minutes at room temperature. Yeast were then heat shocked for 30 minutes at 42 °C and pelleted by centrifugation. The PEG solution was forcefully decanted over a liquid waste receptacle and 100 µl appropriate selective, synthetic media supplemented with glucose was added to each well. Yeast were pelleted by centrifugation, the supernatant was decanted, and
200 µl synthetic media supplemented with glucose was added to each well. Plates were incubated for 48 hours at 30 ºC to allow for selection and growth of transformed yeast. Small white yeast colonies should be visible at the bottom of each well.

4.2 Yeast Spotting Assays

Transformed yeast were resuspended by vigorous pipetting and 5-10µl suspension was added to raffinose-supplemented synthetic media in 96-well tissue culture plates (200 µl per well) then grown overnight at 30°C without shaking. Cultures were 5-fold serially diluted into new 96-well tissue culture plates with sterile water and spotted using a 96-bolt blot replicator (V&P Scientific Cat. No. VP 404) onto 2% agar-solified synthetic media with glucose or galactose in Omnitrays (Thermo 242811). Plates were grown inverted for 2 days at 30°C or 3 days at room temperature, photographed with a Canon SD1200 IS digital camera on an enlargement stand, and scored by the scale given in Fig. 1B.

4.3 Analysis of Dosage-sensitive Gene Features

Dosage-sensitive gene functions (Fig. 10A, B) and component Gene Ontology (GO) terms were obtained using the SGD Gene Ontology Slim Mapper

http://yeastgenome.org/cgi-bin/GO/goSlimMapper.pl

Dosage sensitive gene feature enrichment correlation (Fig. 11A) was performed by Oliver King as described (4). Briefly, for each feature tested, the correlation was determined for the gene to that feature. A tenfold cross-validation experiment was used to determine the predictiveness of each feature to the dosage-sensitive genes. The mean area under a receiver operating characteristic (ROC) curve for each of the cross-
validation experiments was used to determine the enrichment of each feature in the
dosage-sensitive gene set. The following features were used:

- From the SGD table protein.properties.tab, but with counts of each AA changed
to percentages, to remove most (but maybe not all) of their correlation with
  PROTEIN.LENGTH:
    - PI (isoelectric point)
    - CAI (codon adaptation index (codonw.sourceforge.net))
    - PROTEIN.LENGTH (number of AAs in protein)
    - CODON.BIAS (codon bias index (codonw.sourceforge.net))
    - PCT.ALA
    - PCT.ARG
    - PCT.ASN
    - PCT.ASP
    - PCT.CYS
    - PCT.GLN
    - PCT.GLU
    - PCT.GLY
    - PCT.HIS
    - PCT.ILE
    - PCT.LEU
    - PCT.LYS
    - PCT.MET
    - PCT.PHE
    - PCT.PRO
    - PCT.SER
    - PCT.THR
    - PCT.TRP
    - PCT.TYR
    - PCT.VAL
    - FOP.SCORE (frequency of optimal codons (codonw.sourceforge.net))
    - GRAVY.SCORE (hydropathicity of protein)
    - AROMATICITY.SCORE (Frequency of aromatic amino acids: Phe, Tyr, Trp)
    - VERIFIED (1 if Verified, 0 if Uncharacterized or Dubious ORF)

- PRD.LLK (Prion-like AA comp score form earlier version of PLAAC)
- FInumaa (FoldIndex: number of disordered residues)
- FLmeanhydro (FoldIndex: mean hydropathy score)
- Flmeancharge (FoldIndex: mean charge)
- Flmeancombo (FoldIndex: disorder score for whole protein)
- Flmaxrun (FoldIndex: longest run of consecutive disorder)
- IUcount (IUpred: number of disordered residues)
- IUmaxrun (IUpred: longest run of consecutive disorder)
- IUmean (IUpred: mean of per-residue disorder scores)
- ANCHORcount (ANCHOR: number of disordered binding regions)
• ELMcount (number of Eukaryotic Linear Motifs)

• 1 for Gene Ontology membership in following categories, 0 otherwise
  o GO.regulation.of.biological.process (GO:0050789)
  o GO.transcription (GO:0006350)
  o GO.signaling (GO:0023052)

• From BIOGRID 2.0.5.29
  o Essential (1 if gene deletion is toxic (need ref; hom or het?))
  o y2h.total (total number interactions by Y2H (from BIOGRID 2.0.5.29))
  o y2h.unique (number of distinct proteins with Y2H interaction)
  o y2h.essential.total (total number of interactions with essential proteins by Y2H)
  o y2h.essential.unique (number of distinct essential proteins with Y2H interaction)
  o count.het.sensitive (number of conditions in which heterozygous deletion was sensitive)
  o count.hom.sensitive (number of conditions in which homozygous deletion was sensitive (ref?))
  o TAP.FILTERED (protein filtered out from TAP interactome (for being too sticky?)
  o count.sga.hits (number of synthetic genetic associations (Costanzo and Boone 2009)
  o sga.universe (if part of universe of tested genes in above study)
  o HAS.HUMAN.ORTH (1 if protein had human ortholog, 0 otherwise)
  o HAS.DISEASE.ORTH (1 if protein had human ortholog associated with OMIM disease, 0 otherwise)

• The following features are from Vavouri et al Cell 2009
  o Dosage.sensitive ## Toxic due to overexpression in Sopko et al 2006
  o Intrinsic.Disorder..GlobPlot.
  o Intrinsic.Disorder..DisEMBL.REM.
  o Intrinsic.Disorder..DisEMBL.COILS.
  o Number.of.binary.protein.interactions
  o 5..UTR.length..bp.
  o Translation.rate
  o Upstream.conservation..fraction.
  o Transcription.rate
  o Protein.half.life..mins.
  o Upstream.noncoding.region..bp.
  o mRNA.half.life..mins.
  o Protein.abundance
  o Aggregation.load
  o 3..UTR.length..bp.
  o Aggregation.score..TANGO.score.
  o Responsiveness
  o Noise..DM.

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- Aromaticity.score
- Expression.divergence
- Recombination.rate
- Number.of.protein.complex.interactions
- Frequency.of.optimal.codons
- mRNA.abundance
- Codon.bias
- Ka.Ks
- Underwrapping

4.4 Immunoblotting

Yeast were grown to logarithmic growth phase in synthetic media with raffinose, then pelleted by centrifugation, resuspended in galactose-containing media, and incubated overnight (16 hours) at 30 °C with rotation. Cultures were pelleted, washed with sterile water, and treated with 0.05 M NaOH for 10 min at room temperature. Yeast suspensions were pelleted and then resuspended in sample buffer (60mM TrisHCl, pH 6.8, 5% glycerol, 2% SDS 200, 4% beta-mercaptoethanol, 0.0025% bromophenol blue) and boiled for 5 minutes. Cleared lysates were separated by SDS-PAGE (Bio-Rad Cat. No. 3450010 Criterion Tris-HCl Precast Gels, 4–20% gradient) and transferred onto a PVDF membrane (EMD Millipore IPFL00010 Immobilon-FL) by semi-dry transfer (BIO-RAD Cat. No. 1703940). Membranes were blocked (Odyssey 927-40000 PBS Blocking Buffer) overnight at 4°C. Primary antibodies were diluted in blocking buffer and incubated with blots for 1 hour at room temperature with rocking. Blots were washed 4 times for 10 minutes in PBS-T (0.01 M phosphate buffer, pH 7.4, 0.0027 M potassium chloride, 0.137 M sodium chloride, 0.1% Tween-20) and incubated with secondary antibodies for 1 hour at room temperature with rocking. Blots were washed 4 times for 10 minutes in PBS-T, twice for 5 minutes in PBS. Blots were visualized using LI-COR Odyssey Model 9120 or Fc Imaging systems and analyzed with LI-COR Image Studio software.
Antibodies used were:

- anti-Hemagglutinin (HA) monoclonal (Roche 11583816001, 0.4µg/ml final concentration)
- anti-Hsc82 (Abcam ab30920, 1:1000)
- anti-Hsp70 (Abcam)
- anti-TDP-43 polyclonal (Proteintech 10782-2-AP, 1:1000)
- anti-FUS polyclonal (Bethyl A300-302A, 1:2000)
- anti-Hsp104 polyclonal (Enzo Life Sciences ADI-SPA-1040, 1:1000)
- anti-3-phosphoglycerate kinase (Pgk1) monoclonal (Novex Part# 459250, 1:1000)
- anti-Src antibody (Abcam ab16885, 1:250)
- anti-phosphotyrosine (Millipore 05-321, 1:1000)
- IRDye680RD Donkey Anti-Rabbit (LICOR 926-68073, 1:7500)
- IRDye800CW Donkey Anti-Mouse (LICOR 925-32212, 1:7500)

4.5 Semi-denaturing Detergent Agarose Gel Electrophoresis (SDD-AGE)

SDD-AGE was conducted with modification as reported (Halfmann, JoVE 2008).

The HA-tagged proteins were transformed into BYV, BYW, and BYA yeast. Transformants were grown in Raffinose-containing synthetic media to logarithmic growth phase in 96-well deepwell plates (Eppendorf Cat. No. 0030502302), then yeast were pelleted by centrifugation (1500 g for 5 minutes) and resuspended in Galactose-containing synthetic media at an optical density (OD_{600}) of 0.100. Following overnight (16 hour) induction, yeast were pelleted by centrifugation, washed with sterile water, pelleted, and then resuspended in spheroplasting solution (1.2 M D-sorbitol, 0.5 mM MgCl, 220 mM Tris, pH 7.5, 50 mM β-ME and 0.5 mg/ml Zymolyase 100T) and incubated for 1 hour at 30°C with occasional shaking to keep the yeast suspended in solution. Spheroplasts were pelleted by centrifugation (500 rcf for 5 minutes) and resuspended in lysis buffer (100 mM Tris, 7.5, 500 mM NaCl, and 1% Protease Inhibitor cocktail (Sigma P8215), 2mM EDTA, and 2mM PMSF). The suspensions were vortexed at high speed for 1 minute and then snap-frozen in liquid nitrogen. 4X sample buffer (2X TAE, 20% glycerol, 2 or 8% SDS, 10% β-ME, and 0.0025% bromophenol blue) was
added to lysates and incubated for 5 minutes at room temperature. Samples were loaded onto a 1.5% Agarose gel in 1X TAE and 0.5% or 2% SDS in a horizontal slab electrophoresis apparatus tray. Samples were run at 5 V per cm of terminal distance in a 4 °C cold room until dye front was 1.5 cm from the end of the gel. The samples were then transferred by vertical capillary action overnight onto a nitrocellulose membrane as reported (Halfmann, JoVE 2008). The membranes were blocked overnight in Licor Blocking Buffer and then incubated with anti-HA monoclonal primary diluted in blocking buffer (1:1000) for 1 hour, washed 4 times in PBS-T for 10 minutes each, incubated with anti-Mouse IRDye 800CW secondary antibody in blocking buffer (1:7500) for 1 hours, washed 4 times for 10 minutes in PBS-T, twice for 5 minutes in PBS. Proteins were detected LI-COR Odyssey Models 9120 and Fc and analyzed with LI-COR Image Studio software.

4.5 Filter Retention Assay

Lysates from yeast expressing HA-tagged proteins were prepared as for SDD-AGE. 4X sample buffer (2X TAE, 20% glycerol, 0.4% SDS, 10% β-ME, and 0.0025% bromophenol blue) was added to lysates and incubated for 5 minutes at room temperature. Samples were applied onto two stacked membranes using a Minifold I 96 well spot-blot array system (GE Healthcare 10447850): on top, the 0.2μm pore size cellulose acetate (Whatman OE66 Cat No. 10404129) that bound large aggregates with 0.45μM nitrocellulose membrane (Thermo 88018) below to bind protein flowthrough. A piece of Whatman 3MM CHR (GE Healthcare 3030-221) filter paper was placed on the bottom of the stack to facilitate sealing of each well. 20-100 μl of prepared lysates were applied directly to the membranes. Care was taken not to over load the membranes and thus disrupting flow of sample and wash buffer through the membrane. While still in the
spot-blot apparatus, each sample well on the membrane was washed three times with 200 µl PBS-T. The blots were removed from the apparatus and washed again with PBS-T for 10 minutes with rocking and then blocked with Odyssey Blocking Buffer (PBS) overnight at 4 °C with rocking. Next, the blots were incubated with anti-HA monoclonal primary diluted in Licor Blocking Buffer (1:1000) for 1 hour at room temperature with rocking. After four 10-minute washes in PBS-T with rocking, membranes were incubated with anti-Mouse IRDye 800CW secondary antibodies in Licor Blocking Buffer (1:7500) for 1 hour at room temperature with rocking. Membranes were washed 4 times for 10 minutes in PBS-T, twice for 5 minutes in PBS, all with rocking, and finally visualized using LI-COR Odyssey Model 9120 or Fc Imaging systems and analyzed with LI-COR Image Studio software.

4.6 Generation of yeast deletion strains (with Korrie Mack)

PCR-based gene disruptions was performed to knockout Hsp104 from BY4741-based atg8Δ and rpn4Δ yeast strains (Dharmcon YSC6273-201930575, YSC6273-201935103, YSC6273-201921616 respectively). Forward and reverse primers were designed with 5' overhangs that were complementary to the ORF of the Hsp104 gene and a 3' section that were complementary to Hygromycin B phosphotransferase gene. PCR-amplification was used to produce a DNA fragment that was transformed into yeast that utilized homologous recombination to replace the Hsp104 ORF with the Hygromycin B phosphotransferase gene. Transformants were plated on YPD agar with 300 µg/ml Hygromycin B to select for yeast with the gene insertion. PCR was used to confirm gene disruption and Western blot analysis used to determine loss of Hsp104 expression.
4.7 Ppz1 Protein Purification

GST-PPZ1 and GST-PPZ1^{R451L} were purified from BL21-CodonPlus(DE3)-RIL E. coli (Agilent Cat. No. 230245) as reported with modifications (275). Briefly, bacteria transformed with the pGEX6P1-PPZ1 or pGEX6P1-PPZ1^{R451L} were grown in LB with 0.5 mM MnCl$_2$ at 37 °C with rotation overnight. The following day, the cultures were diluted in LB with 0.5 mM MnCl$_2$ and incubated at 37 °C with shaking (250 rpm) to OD$_{600}$ 0.4. Cultures were then chilled to 15 °C and induced with 0.5 mM IPTG overnight, approximated 16 hours. Cells were pelleted by centrifugation, resuspended in 10 pellet volumes sonication buffer (50 mM Tris-HCl, pH 7.5, 0.2 mM EGTA, 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 2 mM dithiothreitol, 2 mM phenylmethysulfonyl fluoride, and ceOmplete protease inhibitor cocktail (Roche Cat. No.11836153001)). Cell suspensions were sonicated three times for 30 seconds with 90% power on ice. Lysates were clarified by centrifugation (20 minutes, 16,000 rcf) and the supernatant was bound to sonication buffer equilibrated Glutathione Sepharose (GE Healthcare 17075601) for 2 hours at 4 °C with rotation. Resin was washed with 30 resin volumes of sonication buffer and GST-fusion proteins were eluted with 20mM Glutathione in sonication buffer. Eluted protein was buffer exchanged into cleavage buffer (50 mM Tris HCl, pH 8.0, 100 mM NaCl, 1 mM EGTA, 1 mM DTT). To cleave GST moiety, 2 units Prescision Protease (GE Healthcare 27-0843-01) were added for every 100 mg of eluted protein and incubated for 4 hours at 4 °C. The cleavage reaction was then incubated to cleavage buffer equilibrated Glutathione Sepharose for 30 minutes to removed cleaved GST and Prescision Protease, which is also GST-tagged. Elution fractions were pooled and concentrated to 3 mg/ml.
4.8 Ppz1 Phosphatase Assay

0.7 µM purified Ppz1 or Ppz1^{R451L} was mixed with either 2.1 µM hexameric Hsp104 or Hsp104^{A503S} (purified as reported)(207) and 4 mM ATP (pH 7.5) in Buffer A (20 mM Tris-HCl pH 7.5, 100 mM KCl, 5 mM DTT, 0.1 mM EGTA, 10% glycerol, 20µg/ml BSA, 0.005% Triton X-100, 10 mM MnCl₂). 10mM pNPP reagent was added, at the concentration recommended in manufacturer instructions (BioAssay Systems Cat. No. POPN-500). Absorbance at 405nm was measured every 30 seconds for 15 minutes by Tecan Safire2 at room temperature.
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