Isolating Potentiated Hsp104 Variants Using Yeast Proteinopathy Models

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Abstract

Many protein-misfolding disorders can be modeled in the budding yeast Saccharomyces cerevisiae. Proteins such as TDP-43 and FUS, implicated in amyotrophic lateral sclerosis, and α-synuclein, implicated in Parkinson’s disease, are toxic and form cytoplasmic aggregates in yeast. These features recapitulate protein pathologies observed in patients with these disorders. Thus, yeast are an ideal platform for isolating toxicity suppressors from libraries of protein variants. We are interested in applying protein disaggregases to eliminate misfolded toxic protein conformers. Specifically, we are engineering Hsp104, a hexameric AAA+ protein from yeast that is uniquely capable of solubilizing both disordered aggregates and amyloid and returning the proteins to their native conformations. While Hsp104 is highly conserved in eukaryotes and eubacteria, it has no known metazoan homologue. Hsp104 has only limited ability to eliminate disordered aggregates and amyloid fibers implicated in human disease. Thus, we aim to engineer Hsp104 variants to reverse the protein misfolding implicated in neurodegenerative disorders. We have developed methods to screen large libraries of Hsp104 variants for suppression of proteotoxicity in yeast. As yeast are prone to spontaneous nonspecific suppression of toxicity, a two-step screening process has been developed to eliminate false positives. Using these methods, we have identified a series of potentiated Hsp104 variants that potently suppress the toxicity and aggregation of TDP-43, FUS, and α-synuclein. Here, we describe this optimized protocol, which could be adapted to screen libraries constructed using any protein backbone for suppression of toxicity of any protein that is toxic in yeast.

Introduction

Yeast proteinopathy models have been developed for protein-misfolding disorders including amyotrophic lateral sclerosis (ALS) and Parkinson’s disease (PD). Expression of the proteins TDP-43 and FUS, which misfold in ALS patients, are toxic and mislocalize to form cytoplasmic aggregates in yeast. Similarly, expression of α-synuclein (α-syn), which is implicated in PD, is toxic and mislocalizes to form cytoplasmic aggregates in yeast. These features recapitulate phenotypes in patients with these disorders. Thus, yeast models provide a useful platform for screening for proteins or small molecules that prevent or reverse these phenotypes. We are interested in the development of proteins that are capable of reversing aggregation and toxicity due to TDP-43, FUS, and α-syn. We focus on Hsp104, an AAA+ protein from yeast that is uniquely capable of disaggregating proteins both from amorphous aggregates and amyloid in yeast, yet it has no human homologue. Hsp104 is finely tuned to disaggregate endogenous yeast prions and has only limited ability to disaggregate substrates implicated in human neurodegenerative diseases, which it never ordinarily encounters. Thus, we aim to engineer enhanced versions of Hsp104 that are able to efficaciously disaggregate these human substrates. To do so, we construct large libraries of Hsp104 variants using error-prone PCR; these libraries can be screened using the yeast proteinopathy models. We have adopted a domain-targeted approach to constructing and screening libraries, as Hsp104 is very large. We initially focused on the middle domain (MD) of Hsp104, though similar approaches can be employed to screen other domains. These models enable screening for disaggregase activity directly, as opposed to alternative techniques such as surface display, which is restricted to use for monitoring binding.

Our protocol is based on two screening steps (Figure 1). First, Hsp104 variants that suppress the toxicity of the disease substrate in yeast are selected. To do so, the Hsp104 variants and disease-associated substrate are cotransformed into Δhsp104 yeast. We employ Δhsp104 yeast to explore Hsp104 sequence space in the absence of wild-type (WT) Hsp104. Importantly, deletion of Hsp104 does not affect α-syn, FUS, or TDP-43 toxicity in yeast, and expression of Hsp104 provides minimal rescue. The yeast is then plated on inducing media to ensure that the toxicity of the substrate is restored after loss of the Hsp104 plasmid. Yeast harboring Hsp104 variants that suppress toxicity of the disease-associated substrate confer growth of the colony. These variants are selected for further analysis, while colonies maintaining variants that do not suppress toxicity die. However, false positives are a substantial problem in this screen. Expression of TDP-43, FUS, and α-syn are highly toxic, which creates a strong selective pressure for the appearance of spontaneous genetic suppressors unrelated to the Hsp104 variant being expressed. Thus, we have used a secondary screen that is also relatively high throughput to eliminate these nonspecific toxicity suppressors. In this secondary screen, selected yeast are treated with 5-Fluoroorotic Acid (5-FOA) to counter select for the Hsp104 plasmid. The strains are then assessed for substrate (TDP-43, FUS, or α-syn) toxicity via spotting assay to ensure that the toxicity of the substrate is restored after loss of the Hsp104 plasmid. Thus, yeast in which toxicity is restored in this secondary screen presumably originally displayed toxicity suppression due to the presence of the Hsp104 variant. These yeast are designated as ‘hits’ and the Hsp104 plasmid should then be recovered and sequenced to identify the
mutations in the Hsp104 gene (Figure 1). Any hits should then be reconfirmed by constructing the mutation independently using site-directed mutagenesis and then retesting for toxicity suppression. The potential applications for this protocol are broad. Using these methods, libraries of any type of protein could be screened for variants that suppress toxicity of any substrate protein that is toxic in yeast.

### Protocol

#### 1. Library Generation

1. To construct libraries of Hsp104 using domain-specific error-prone PCR, first amplify the domain of interest with an error prone DNA polymerase.
2. Purify the PCR product by gel extraction.
3. Perform a megaprimer extension step using a standard site-directed mutagenesis protocol: combine 50 ng template plasmid, 250 ng megaprimer, 200 μM dNTPs, and high-fidelity DNA polymerase in PCR buffer, and dilute to 50 μl total volume with PCR grade water. Run a standard PCR program.
   
   **NOTE:** Specific primers used will vary based on the particular region of the gene that is to be amplified.
   
   1. Following PCR, digest parental template DNA with 1 μl DpnI restriction enzyme for 2 hr at 37 °C.
4. Transform the library by electroporation or other means in ultracompetent *E. coli* and purify it by miniprep.
   
   **NOTE:** Library generation varies substantially based upon the aims of a given experiment. Hsp104 is a very large protein, so it is impractical to randomize the entire gene, which is why we utilize domain-specific error prone PCR. Additionally, the structure of Hsp104 remains poorly understood, making the design of directed libraries challenging. Libraries can be constructed using directed or random approaches to mutagenesis, with the only restriction being that the template plasmid backbone should contain the URA3 gene to allow for 5-FOA counter selection on dextrose media.

#### 2. Transformation of the Hsp104 Library

1. Integrate the disease-associated substrate into W303aΔhsp104 yeast using a standard lithium acetate/PEG transformation protocol. Select single colonies and screen them for toxicity to isolate a strain with high toxicity of the disease associated substrate.
   
   **NOTE:** Use an integrated strain and isolate a single colony to ensure equal expression in all cells. Clone the disease substrate into a plasmid allowing integration of the gene under any marker other than uracil (we use histidine) and the Hsp104 library into the pAG416GAL plasmid (uracil marker). To allow for 5-FOA counterselection, ensure that the library is expressed from a plasmid with the uracil marker. Other yeast strains can also be employed. We have noted a similar toxicity suppression phenotype using W303a and BY4741 yeast strains in both WT and Δhsp104 backgrounds (M.E.J. and J.S., unpublished observations).
2. Transform the Hsp104 library into this strain using the same lithium acetate/PEG transformation protocol. Scale up the transformation appropriately to maintain the sequence space of the library and to preserve the predicted library size.
3. Plate the transformation mixture onto noninducing, selective plates (e.g. SD-His-Ura) using enough plates to ensure growth of a large number of colonies. Use large petri dishes (150 mm) to minimize the number of plates needed. Recovering transformants using plates allows transformation efficiency to be assessed.
4. Transform Hsp104<sup>WT</sup> and vector negative controls in parallel.

#### 3. Screening for Suppression of Proteotoxicity

1. Wash the colonies off the plates using raffinose supplemented dropout media (e.g. SRaff-His-Ura). Use a serological pipette and sterile wooden applicators to loosen the colonies from the plates. Transfer the liquid washes to a 50 ml conical tube and vortex thoroughly to separate any clumps of cells. Dilute to a slightly cloudy culture, OD<sub>600</sub> ~0.025.
   
   **NOTE:** Here raffinose serves to relieve the cells of glucose-mediated repression of induction, thus priming the cells for galactose-mediated induction.
2. Grow the diluted culture overnight in raffinose dropout media with shaking at 30 °C. Grow the Hsp104<sup>WT</sup> and vector controls in parallel.
3. The following morning, plate a range of concentrations onto individual galactose (e.g. SGal-His-Ura) plates (1 μl to 2 ml concentrated culture in 400 μl total volume). Plating a broad range of concentrations will ensure that a plate will have single colonies. Plate the transformation mixture onto noninducing, selective plates (e.g. SD-His-Ura) using enough plates to ensure growth of a large number of colonies. Use large petri dishes (150 mm) to minimize the number of plates needed. Recovering transformants using plates allows transformation efficiency to be assessed.
4. Transform Hsp104<sup>WT</sup> and vector negative controls in parallel.

#### 4. 5-FOA Counterselection and Spotting to Eliminate False Positives

1. Streak out single colonies in duplicate onto double and single dropout media (e.g. SD-His-Ura and SD-His plates) and grow overnight at 30 °C. Repeat with vector and Hsp104<sup>WT</sup> controls. Plating on SD-His prior to 5-FOA increases the efficiency of the 5-FOA step by increasing the likelihood that cells will have lost the Hsp104 plasmid when they are plated on 5-FOA.
2. To prevent plates from drying out, wrap the SD-His-Ura plates in parafilm and store at 4 °C while performing the 5-FOA screen. These plates will eventually be used for sequencing.
5. **Sequencing the Hsp104 Variants by Colony PCR**

1. Scrape ~10 µl yeast from the SD-His-Ura plates in 3 µl 20 mM NaOH in PCR strip tubes and lyse the cells by freeze-thaw. Place the strip tubes in a -80 °C freezer for 10 min or in liquid nitrogen, then incubate at 99 °C in a thermocycler for 10 min. Dilute the strains to 100 µl with PCR grade water.

2. Prepare PCR reactions: 5 µl PCR template, 0.5 µl 100 µM each primer, 0.5 µl 10mM dNTPs, 0.25 µl DNA polymerase in PCR buffer, and make up to 25 µl total volume with PCR grade water. Design primers to amplify the randomized region plus approximately 100 bp on either end. Minimize the size of the amplified region to increase the likelihood of successful amplification. Run a standard PCR program.

3. Analyze samples by agarose gel electrophoresis to confirm amplification of a PCR product of the appropriate size. Repeat PCR if no product is present.

   **NOTE:** PCR failure is typically due to too much yeast being used in step 5.1.

4. Prepare samples for DNA sequencing. Remove PCR primers either by PCR purification or using a PCR product cleanup reagent such as Exonuclease I and Shrimp Alkaline Phosphatase enzyme (ExoSAP-IT) to degrade single stranded DNA. This reagent enzymatically degrades unincorporated primers and dNTPs, allowing for higher throughput.

5. Sequence the PCR products. Be sure to thoroughly analyze sequencing chromatograms for mutations. Mutations may be observed as a mixture of two nucleotides at a given site (Figure 4), as yeast often harbor multiple plasmids.

**Representative Results**

We have constructed a library of Hsp104 variants randomized in the middle domain and screened it for suppression of TDP-43 toxicity. The library was transformed and plated onto glucose and galactose plates (Figure 2) to assess the stringency of the screen. Single colonies were selected and the strains were counter selected using 5-FOA to eliminate the Hsp104 plasmid. These strains were then assessed to confirm that toxicity was due to TDP-43 alone, without the Hsp104 variants. Spotting assays of a subset of the variants selected in the initial screen showed that of the 4 colonies selected, 2 displayed Hsp104-mediated TDP-43 toxicity suppression, 1 was a false positive, and 1 displayed enhanced toxicity was due to TDP-43 alone, without the Hsp104 variants. Spotting assays of a subset of the variants selected in the initial screen showed that of the 4 colonies selected, 2 displayed Hsp104-mediated TDP-43 toxicity suppression, 1 was a false positive, and 1 displayed enhanced toxicity was due to TDP-43 alone, without the Hsp104 variants.

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**Figure 1:** Flow-chart for isolating potentiated Hsp104 variants. Hsp104 libraries (URA3 marker, GAL1 promoter) are transformed in yeast containing the disease substrates (HIS3 marker, GAL1 promoter) and screened for toxicity suppressors by plating on inducing media. Potential hits are then screened again using a 5-FOA counterselection step to eliminate nonspecific toxicity suppressors. Variants selected in this second step are then sequenced by colony PCR. Please click here to view a larger version of this figure.
Figure 2: Screening for toxicity suppressors. Yeast cotransformed with pAG303GAL-TDP-43 and the pAG416GAL-Hsp104 library were plated onto glucose (repressing) or galactose (inducing) media. TDP-43 is highly toxic, so very few Hsp104 variants are capable of suppressing this toxicity, and this screen is very stringent. Single colonies are selected as hits from the galactose plate for the 5-FOA secondary screen. Both large and small colonies are typically obtained, but we have not observed any trends that dictate how colony size correlates with activity.

Figure 3: 5-FOA secondary screen. Yeast treated with 5-FOA to counter select for the Hsp104 plasmid were assessed by spotting assay. Strains were grown in SRaff-His media, serially diluted 5 fold, and spotted in duplicate onto SD-His and SGal-His plates. Hsp104_{A503V} is a true hit that we have previously verified and show here as a control along with vector alone. Rows 3 and 4 are library hits that retain TDP-43 toxicity following loss of the Hsp104 suppressor. Row 5 shows a false positive, where TDP-43 is no longer toxic, so a nonspecific toxicity suppressor is present. Row 6 shows a strain that is more toxic than TDP-43 typically is, possibly due to 5-FOA toxicity. This strain could still be sequenced but may not be a valid hit.
Figure 4. Analyzing sequencing results. Selected yeast often contains multiple plasmids. Therefore, following sequencing of the colony PCR products, care must be taken to ensure any mutations are not overlooked in the chromatograms. In this chromatogram, two potential mutations (denoted by *) could be overlooked. In the first instance, there is a mixture of A and T and in the second, a mixture of T and C.
Discussion

Here we present our approach to isolating potentiated Hsp104 variants that suppress the toxicity of disease-associated substrates using yeast proteopathy models. Using this approach, large libraries of variants can be screened in high throughput, with the only limitation being the number of variants that pass the 5-FOA secondary screen. By performing these steps in 96 well format, we routinely screen up to 200 hits at a time in the 5-FOA step over the course of 1-2 weeks. The number of hits obtained during the initial screening step will vary largely depending upon substrate toxicity and the makeup of each particular library. When screening hits, it is essential to carefully analyze all sequencing chromatograms since mixtures of plasmids are typically present in each cell.

We have sometimes noted a large percentage of Hsp104\(^{\text{WT}}\) being isolated as ‘hits’. This is likely due to the presence of spontaneous genetic suppressors or very weak activity. To circumvent this problem, we have found that screening at elevated temperatures (e.g. 34 °C) can decrease the percentage of ‘hits’ that are Hsp104\(^{\text{WT}}\). It is also essential to re-clone any sequenced hits to independently assess the activity of any novel variants and to ensure plasmid purity. Once new hits are identified, they can be assessed for suppression of aggregation in yeast and characterized biochemically.

We have used these methods to isolate potentiated Hsp104 variants against TDP-43, FUS, and α-synuclein and verified their activity using pure protein biochemistry assays\(^{17}\). Ultimately, these methods could be used to screen protein libraries against any substrate of interest that is toxic in yeast.

Disclosures

The authors have nothing to disclose.

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