

[13] Screening for Genetic Modifiers of Amyloid Toxicity in Yeast

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Abstract

In recent years the facile, yet powerful, genetics of the baker's yeast *Saccharomyces cerevisiae* has been appropriated for the study of amyloid toxicity. Several models of amyloid toxicity using this simple eukaryotic organism have been developed that faithfully recapitulate many disease-relevant phenotypes. Furthermore, these models have been exploited in genetic screens that have provided insight into conserved mechanisms of amyloid toxicity and identified potential therapeutic targets for disease. In this chapter, we discuss the strengths and weaknesses of yeast models of amyloid toxicity and how experiments with these models may be relevant to amyloid disorders. We suggest approaches for development of new yeast models of amyloid toxicity and provide an overview of screening protocols for genetic modifiers of amyloid toxicity by both random and systematic approaches.

Introduction

Saccharomyces cerevisiae has proven to be an extremely important tool for studying basic cellular processes conserved with higher eukaryotes, including cell division, replication, metabolism, protein folding, and intracellular transport. In fact, almost everything that is known about the fundamental properties of living cells has been learned from the study of *S. cerevisiae* and other model organisms (Fields and Johnston, 2005). The conserved cellular mechanisms explained in these organisms can play a direct role in understanding disease processes. For example, the fundamental knowledge of cell cycle regulation uncovered in yeast has been directly applied toward studies in mammalian systems on cancer biology (Hartwell, 2002; Nurse, 2002). Several factors make yeast the perfect system for biological studies, but the primary reason that work in *S. cerevisiae* has proven so fruitful is the "awesome power of yeast genetics."

Yeast is perhaps the ideal organism for classical genetics, because it can exist in both haploid and diploid states, and it is quite simple to mate haploid strains and to sporulate diploid strains. These features, along with the ease of mutagenesis and the availability of several mutant collections

and open reading frame (ORF) libraries, allow for rapid isolation of genetic modifiers of specific processes or phenotypes. In addition, this ease of genetic manipulation allows for facile dissection of molecular pathways by epistatic analysis. Epistasis is the control of a phenotype by two or more genes by means of a genetic interaction in which one gene masks or suppresses the phenotypic effect of another. This phenomenon can be used to genetically order genes within a pathway and has proven to be a critical technique for determining the order of events in many cellular pathways, such as signaling cascades.

S. cerevisiae is one of the best characterized eukaryotic organisms; in addition to its genome being fully sequenced (Goffeau *et al.*, 1996), *Saccharomyces* genes and proteins are extensively annotated in several genomic and proteomic databases, which contain detailed gene expression profiles, known protein–protein interactions, and predicted orthologs in other organisms. In addition, molecular genetic manipulations, such as DNA transformation, targeted disruption of specific genes, generation of point mutations in cloned genes, and overexpression of proteins of interest can be performed in a matter of days, compared with months or years in other model organisms. The genomic era for yeast has truly arrived with the recent development of arrayed libraries allowing for the systematized testing of deletion and overexpression of most genes in the yeast genome, which permits rapid genomic screening and identification of interacting genes.

Modeling Amyloid Toxicity in Yeast

In recent years, the aggregation and toxicity of amyloid proteins implicated in neurodegenerative disorders has been studied in several nonmammalian organisms: *Drosophila*, *C. elegans*, and *S. cerevisiae*. Although mouse models of disease remain the “gold standard” for the analysis of genetic and pharmacological modification of phenotype/pathology, nonmammalian models provide a useful alternative to the high cost and slow pace of murine strategies. Both *Drosophila* and *C. elegans* have well-characterized nervous systems specialized for sensory, learning, and memory functions. Because of this, several neurodegenerative diseases caused by dominant mutations in single genes have been modeled in these organisms (Link, 2001; Marsh and Thompson, 2004). Many disease-relevant phenotypes are recapitulated in these models, including progressive neurodegeneration, decreased life span, and inclusion body formation. Although *S. cerevisiae* is a much simpler organism, yeast models of amyloid toxicity can tackle both genetic and pharmacological screens with a rapidity and ease not possible in fly and worm models, while still identifying conserved cellular mechanisms critical to toxicity and candidate therapeutic targets (Giorgini *et al.*, 2005; Willingham *et al.*,

2003; Zhang *et al.*, 2005). As with any disease model, it is critical that yeast models faithfully recapitulate many features of the disease being studied. Ultimately, candidate modifier genes or compounds identified using screens in yeast must be validated in mammalian systems, such as cell models or transgenic mouse models.

Two approaches have been taken in modeling aspects of neurodegenerative diseases in yeast: (1) directly studying the function of yeast homologs of human disease genes and (2) studying the phenotypes caused by expressing human disease genes in yeast. The first approach has been used to model aspects of Friedreich's ataxia (FRDA), amyotrophic lateral sclerosis (ALS), and prion disease. Studies with *YFHI* and *SOD1*, the yeast homologs of the human genes implicated in FRDA and ALS pathogenesis, respectively, have helped explain pathogenic mechanisms underlying these diseases (Outeiro and Muchowski, 2004; Puccio and Koenig, 2000). Work in yeast, both with yeast prions and with mammalian PrP protein, has provided insight into the mechanism of prion action (Sherman and Muchowski, 2003). Although not homologous to PrP by sequence comparison and by phenotype, yeast prions behave in an analogous manner to their human counterpart; these proteins can change conformation to form self-propagating, transmittable aggregates. An excellent review of yeast prion biology is provided by Uptain and Lindquist (Uptain and Lindquist, 2002).

As mentioned previously, a second approach to modeling aspects of neurodegenerative disease is expressing a human disease gene in yeast cells. This approach has proven very successful, with development of yeast strains that model aspects of Huntington's disease (HD) and Parkinson's disease (PD). These models recapitulate many disease-relevant phenotypes and have already provided mechanistic insight into the pathology of these diseases. HD is caused by an expansion of a polyglutamine (polyQ) stretch beyond a critical threshold of ~35–40 glutamines in the protein Huntingtin (Htt). Expression of mutant Htt fragments in yeast produces phenotypes reminiscent of HD pathology, including polyQ length-dependent inclusion body formation and toxicity and modulation of this phenotype by perturbation of chaperone levels (Hughes *et al.*, 2001; Krobitsch and Lindquist, 2000; Meriin *et al.*, 2002; Muchowski *et al.*, 2000). Genetic screens using these models have uncovered genes in cellular pathways implicated in HD, including transcriptional regulation, protein folding, vesicle transport, and the kynurenine pathway (Giorgini *et al.*, 2005; Willingham *et al.*, 2003). Although most cases of PD are idiopathic, a small proportion of disease incidence is caused by missense mutations in the α -synuclein gene (Kruger *et al.*, 1998; Polymeropoulos *et al.*, 1997) or triplication of the wild-type locus (Singleton *et al.*, 2003). Consistent with studies in primary cortical

neurons, expression of α -synuclein in yeast leads to formation of cytoplasmic inclusion bodies and cellular toxicity (McLean *et al.*, 2001; Outeiro and Lindquist, 2003). Provocatively, a genome-wide screen for loss-of-function enhancers of α -synuclein toxicity (Willingham *et al.*, 2003) has uncovered many genes involved in vesicular transport and lipid metabolism, pathways implicated in α -synuclein function in mammals (Outeiro and Muchowski, 2004). The relevance of yeast models of amyloid toxicity in regard to various amyloid diseases is discussed in greater detail elsewhere (Outeiro and Muchowski, 2004; Sherman and Muchowski, 2003); here we focus on techniques for generation of yeast models of amyloid toxicity and methods for identification of genetic modifiers of toxicity.

Thus far, the use of yeast models of amyloid toxicity has provided insight into conserved mechanisms of toxicity in several amyloid disorders. This low-cost, facile approach for identification of genetic modifiers can theoretically be applied to any amyloid disorder in which a protein has been associated with pathogenesis. How applicable are findings in yeast to studies in higher eukaryotes? The answer to this question will not be known until genetic modifiers and molecular mechanisms identified in yeast studies are validated by genetic tests in mouse models of neurodegenerative disease (and for that matter, studies in mouse models will not be validated until the proposed disease mechanisms are proven by pharmacological intervention in diseased humans). Nevertheless, there are obvious limitations to studying amyloid toxicity in yeast that are worth mentioning (as there are with any model system). First of all, genes involved in neurodegeneration may not be present in the yeast genome. Second, the toxicity observed in yeast may not be related to that involved in neurodegeneration. In this regard, it is important to consider recent work that has shown that yeast can undergo apoptosis-like cell death in response to several stimuli and that several yeast orthologs of crucial apoptotic regulators exist (Madeo *et al.*, 2004). In addition, it has recently been shown that expression of wild-type α -synuclein or the inherited mutants (A30P, A53T) in yeast triggers several markers of apoptosis and that deletion of a yeast metacaspase gene suppresses many of these apoptosis-like phenotypes (Flower *et al.*, 2005). It is thus possible that toxicity in yeast may be more similar to neurotoxicity than previously thought. Ultimately, any candidate modifier identified in yeast needs to be validated in more physiologically relevant models of neurodegeneration. This chapter is designed as a primer on yeast genetic screens for the amyloid researcher who wishes to exploit this simple organism for molecular genetic studies on the mechanisms of amyloid toxicity. We will not discuss the two-hybrid system, perhaps the most extensively used yeast screening technique, because this has been discussed at length elsewhere (Fields and Sternglanz, 1994).

Genetic Manipulation and Screening

Generating a Yeast Model of Amyloid Toxicity

Developing a yeast model for amyloid toxicity that recapitulates aspects of a particular disease may involve some troubleshooting and will likely require more than merely expressing the implicated protein in yeast cells. Several factors may influence toxicity of expressing your favorite gene (YFG) in yeast, including, but not limited to, promoter strength, plasmid copy number, and protein localization. Other factors to consider when generating constructs are the use of an inducible promoter for gene expression, the ploidy number of the yeast strain (haploid versus diploid), and the addition of a molecular tag (FLAG, GFP, etc.).

Two very useful and versatile expression systems for *S. cerevisiae* have been developed by Martin Funk and colleagues that allow either constitutive or regulated expression of protein over a range of two to three orders of magnitude in several genetic backgrounds (Table I) (Mumberg *et al.*, 1994, 1995). The constitutive expression system consists of 32 expression vectors with which investigators can modulate the level of transcription of YFG by selecting among four promoters (*CYCI*, *ADH*, *TEF*, *GPD*), alter expression level by changing copy number of the plasmid (using either a centromeric or 2μ plasmid), and use the appropriate selectable marker (*HIS3*, *LEU2*, *TRP1*, or *URA3*). All these vectors have multiple cloning sites derived from the Bluescript vectors (Stratagene, La Jolla, CA) containing six to nine unique restriction sites for ease of cloning.

Although the preceding system is extremely versatile, expression of YFG using an inducible and regulatable system may be desirable, especially if the protein is strongly toxic to yeast or if you wish to compare growth in mutant strains with and without protein expression. The regulatable vectors mentioned previously take advantage of the *GALI* promoter, which is tightly repressed by glucose (GLU) and is strongly induced by galactose (GAL) (Johnston and Davis, 1984). In addition, *GALI* is an extremely strong promoter, capable of inducing protein expression over 1000-fold in GAL, such that the protein of interest can constitute up to 0.8% of total cell protein (Schneider and Guarente, 1991). Funk and colleagues generated deletion variants of the *GALI* promoter that contain either one and one-half (GALS) or two (GALL) of the three upstream activator sequences (UAS) required for full induction of the *GALI* promoter by galactose (Mumberg *et al.*, 1994; West *et al.*, 1984), allowing for inducible expression of heterologous proteins over a range of two to three orders of magnitude. As with the constitutive expression vectors, the set of GAL-inducible vectors included both centromeric and 2μ plasmids, several

TABLE I
 CONSTITUTIVE AND INDUCIBLE VECTORS FOR EXPRESSION OF HETEROLOGOUS
 PROTEINS IN YEAST

Origin of replication	Selectable marker	Name	Promoter type	Strength
p41X-CEN6/ARSH4				
p42X-2-micron	P4X3-HIS3	CYC1	Constitutive	Weak
	P4X4-TRP1	ADH	Constitutive	Intermediate
	P4X5-LEU2	TEF	Constitutive	Strong
	P4X6-URA3	GPD	Constitutive	Very strong
		GAL1	Inducible	Weak
		GALL	Inducible	Strong
		GALS	Inducible	Very strong

From [Mumberg et al. \(1994, 1995\)](#).

choices for selectable markers (*HIS3*, *LEU2*, *TRP1*, or *URA3*), and a versatile multiple cloning site with six to nine unique sites. To clone YFG into the plasmid of choice, one can either use standard cloning techniques or PCR-based homologous recombination in yeast ([Longtine et al., 1998](#); [Reid et al., 2002](#)). It is worth noting that several yeast gene deletion strains grow slowly in GAL media and that this must be considered when analyzing deletion strains that seem to enhance toxicity (reduce growth) of an YFG under inducing (+GAL) conditions.

In general, when constructing a yeast model of toxicity, it is desirable that expression of YFG cause toxicity; if toxicity is not observed, low expression levels may be the cause. It has previously been observed that increasing the expression levels of both α -synuclein and a mutant Htt fragment in yeast increases toxicity ([Outeiro and Lindquist, 2003](#); [Meriin et al., 2002](#)). Fortunately, the expression systems described previously allow expression of YFG over a broad range. Integration of YFG into the yeast genome may also increase toxicity of the construct while decreasing variation in expression levels ([Outeiro and Lindquist, 2003](#)); techniques for integrating YFG by homologous recombination are described in detail in a previous volume of this series: *Volume 194: Guide To Yeast Genetics and Molecular and Cell Biology—Part A* ([Rothstein, 1991](#)). Another method for obtaining or increasing toxicity of YFG is the use of signal peptides, such as nuclear localization signals or secretion signals, to modify the cellular localization of YFG. The use of such signals in yeast models of amyloid toxicity has been observed to increase the severity of the resulting phenotypes seen in yeast ([Hughes et al., 2001](#)). Another factor to consider

is whether to use a haploid or diploid yeast strain for the model, because ploidy of the cell may lead to differences in phenotype and toxicity because of YFP expression. Finally, it is of utmost importance to construct a valid control for toxicity experiments, because measurement of toxicity is always relative. In the case of polyQ expansion disease models, a non-expanded polyQ control is an obvious choice. For toxicity models expressing another mutant protein, expression of the wild-type protein may be a good control. In some cases, the best control may be a scrambled version of the toxic construct, thus ensuring that overexpression of a similar protein with the same localization does not produce toxicity.

A final factor to consider when generating a construct for YFG is the use of molecular tags. It is highly recommended to generate a fusion with a fluorescent protein (i.e., GFP, RFP, CFP, YFP) if possible, this greatly simplifies imaging of protein localization by microscopy, since this technique requires no additional processing in yeast. This is of special importance when dealing with protein aggregation diseases, because it is important to monitor whether the protein of interest forms inclusion bodies. It may also be desirable to fuse your heterologous protein to epitope tags (i.e., MYC, FLAG, HA), because antibodies are readily available for these epitopes that allow immunoblotting and immunoprecipitation of the protein. However, it is important to validate that the tag used does not influence various properties of *YFG*. In the end, many variables should be tested when generating a yeast model of amyloid toxicity. Proper planning and a little patience will result in the development of the optimal model for your particular needs.

Obtaining Gene Mutations in Yeast

The heart of any genetic screen is scanning a collection of mutants that provides thorough genomic coverage of the organism of interest. These gene mutations can either be generated directly by mutagenesis or can be obtained in mutant collections. In yeast, several methods are available for random generation of forward mutations (the change of a gene from the wild-type to a mutant form), including chemical mutagenesis, exposure to ultraviolet (UV) light, and insertional mutagenesis. The widely used alkylating agents ethylmethane sulfonate (EMS) and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induce high frequencies of base-pair substitutions and low levels of lethality to yeast (Kohalmi and Kunz, 1988). Although these mutagens almost exclusively produce transition mutations at G-C base pairs, this specificity is not a problem for most applications. A wider range of mutations can be generated using treatment of cells with UV light that induces both transitions and transversions and also

can produce frameshift mutations, most often by single nucleotide deletion (Kunz *et al.*, 1987; Lee *et al.*, 1988). More recently, transposon-insertion libraries have been used as mutagens in yeast. One approach is to use bacterial transposons to mutagenize genomic yeast DNA in *Escherichia coli*, and then shuttle this DNA back into yeast *en masse*. Insertion alleles of genes can then replace their chromosomal counterparts by homologous recombination (Kumar *et al.*, 2002). Insertion alleles provide the added benefit that the insertion site (gene of interest) is easy to identify by PCR amplification or plasmid rescue. Detailed protocols and excellent reviews of these mutagenesis techniques are found in a previous edition of this series, *Volume 350: Guide To Yeast Genetics and Molecular and Cell Biology—Part B* (Kumar *et al.*, 2002; Lawrence, 2002).

Although several mutant collections, or libraries, are available for yeast, the most powerful collection of mutant strains is the yeast gene knockout (YKO) collection. The YKO set was developed by an international consortium and is commercially available from several sources (Winzeler *et al.*, 1999). The YKO set is an array of ~4850 viable gene deletion strains generated by systematically deleting each predicted gene in yeast using a “knockout,” or targeted disruption, method. During the gene-disruption process, a unique DNA sequence tag or “molecular bar code” was introduced into the genome for each deletion strain (Johnston, 2000). This allows rapid determination of each strain by PCR amplification of the bar code followed by DNA sequencing. In addition, because the deletion strains are arrayed in microtiter plates, not only can individual strains be identified by position in the array, but the entire library can be manipulated systematically *en masse* using robotics. This set of strains can also be pooled for growth competition assays among the strains (whole-genome parallel analysis) in response to environmental factors; the abundance of each deletion strain is quantified by determining levels of the associated molecular bar code using hybridization to an oligonucleotide array of the complementary bar-code sequences (Giaever *et al.*, 2002; Winzeler *et al.*, 1999). One drawback to the YKO deletion set is that second site mutations exist in the set; by one estimate ~6.5% of the strains contain such mutations (Grunenfelder and Winzeler, 2002). In addition, as much as 8% of the strains may be aneuploid, complicating phenotypic analysis in these strains (Hughes *et al.*, 2000). The most comprehensive selection of YKO collections is available from Open Biosystems (Huntsville, AL). The YKO collections are available as haploid strains [Mat A (BY4741 background) and Mat α (BY4742)], homozygous diploid knockouts (BY4743), and also heterozygous diploids (BY4743). The loss-of-function genetic screening techniques presented herein are based on the use of these YKO collections.

Loss-of-Function Screens

All published screens using yeast models of amyloid toxicity have been performed with loss-of-function mutant sets, specifically the YKO collection. This approach consists of transforming your construct of interest into all the strains of the deletion set, either individually or *en masse* in pools (protocols are described in the following). If the construct being used drives expression of YFG by a GAL-inducible (*GALI*) promoter, then transformants are plated on media containing GLU (on which expression is repressed). On growth, the colonies are transferred to GAL-containing plates by replica plating that induces expression of YFG. In the wild-type strain, if YFG is toxic, little to no growth will be observed on GAL media; if on the other hand, expression of YFG is not toxic, the yeast will grow normally on GAL media (Fig. 1). In the case of a toxic construct, the gene deletion collection can be screened for deletions that eliminate, or suppress, toxicity. These loss-of-function mutants, or suppressors, are deletions of genes required for toxicity in yeast. Because genetic inhibition of a suppressor's activity alleviates toxicity, the respective gene products are excellent candidate therapeutic targets for small molecule inhibitors, assuming the gene is conserved in humans. Thus, loss-of-function suppressor screens of amyloid toxicity in yeast are predicted to isolate not only genes required for toxicity but can also help identify targets for rational drug design.

Enhancer screens with nontoxic constructs can be equally rewarding and may provide great insight into the mechanisms of amyloid toxicity. As stated previously, if YFG is not toxic to wild-type yeast, colonies will grow normally on both GLU media and GAL media. In the case of a nontoxic construct, the gene deletion collection can be screened for deletions that enhance toxicity. These enhancer strains represent deletions in genes required for suppression of toxicity and identify pathways that are sensitive to expression of the amyloid protein of interest. Therefore, genes identified in loss-of-function enhancer screens represent pathways somehow disrupted or perturbed by expression of YFG.

Overexpression Screens

An alternate approach to genetic screening relies on overexpression of gene products, an approach that has identified genes involved in many cellular functions (Rine, 1991). A gene overexpression screen is the converse of a loss-of-function screen; the object is to alter the phenotype of choice by increased gene dosage. An overexpression suppressor will rescue the original phenotype; in the case of a yeast model of amyloid toxicity, such a suppressor would relieve toxicity. Enhancement of the phenotype

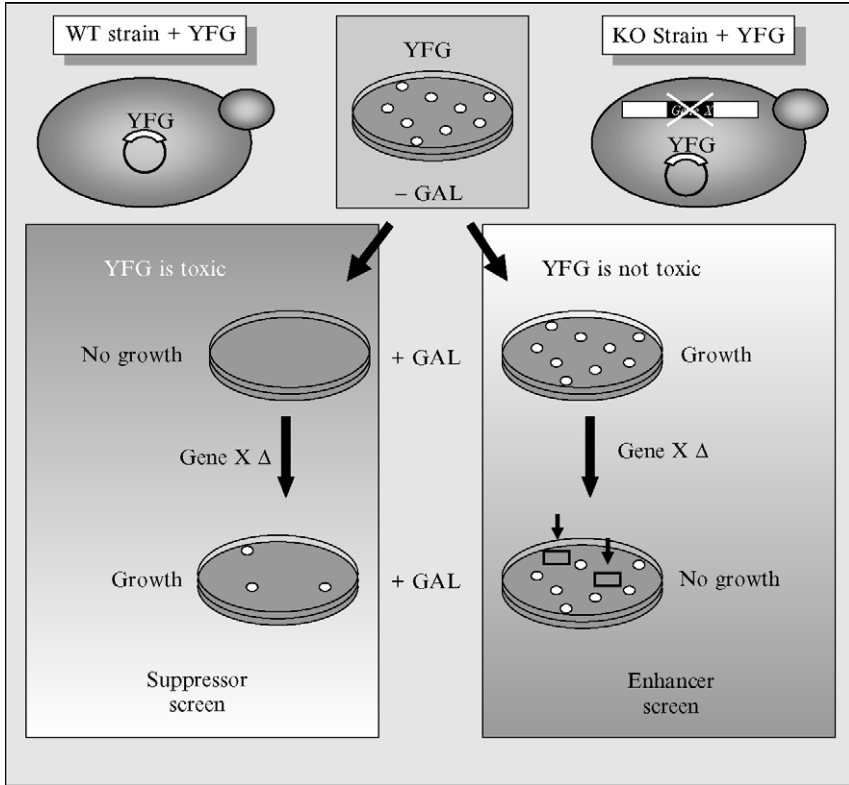


FIG. 1. An overview of a loss-of-function screen for suppressors or enhancers of amyloid toxicity in yeast. A plasmid containing YFG under the control of a GAL-inducible promoter is transformed into wild-type yeast cells and the YKO deletion set. If YFG is toxic to wild-type yeast cells, then the YKO set can be screened for gene deletions (i.e., Gene X Δ) that restore growth or suppress toxicity. If on the other hand, YFG is not toxic to wild-type cells, then the YKO set can be screened for gene deletions that enhance toxicity of YFG.

would exacerbate its severity, or in our example, increase toxicity (Forsburg, 2001). Identification of both types of genetic modifiers leads to a better understanding of the pathways disrupted by the toxic protein or activated by its presence. Although random clone libraries have been available for quite some time for overexpression studies in yeast, only recently has an array of overexpression constructs for most (>90%) of the yeast genome become available (Yeast ORF Collection [YOC], Open Biosystems, Huntsville, AL). The YOC is a collection of more than 5500 constructs each containing an individual *S. cerevisiae* open reading frame (ORF). Each construct is under

the control of the *GALI* promoter and contains Protein A and 6xHis domains together with a HA epitope tag. This collection is arrayed in 96-well microtiter plates, allowing systematic transformation into yeast models and individual analysis of the effect of candidate gene overexpression on amyloid toxicity. The clones may also be pooled and transformed *en masse* into the yeast model for a more traditional overexpression library screen.

The screening approach with the YOC overexpression set is quite similar to that described previously for the YKO set. A double-transformation of YFG and the pooled YOC library into a wild-type yeast strain is performed (Fig. 2). Transformants are plated on media containing GLU; once grown,

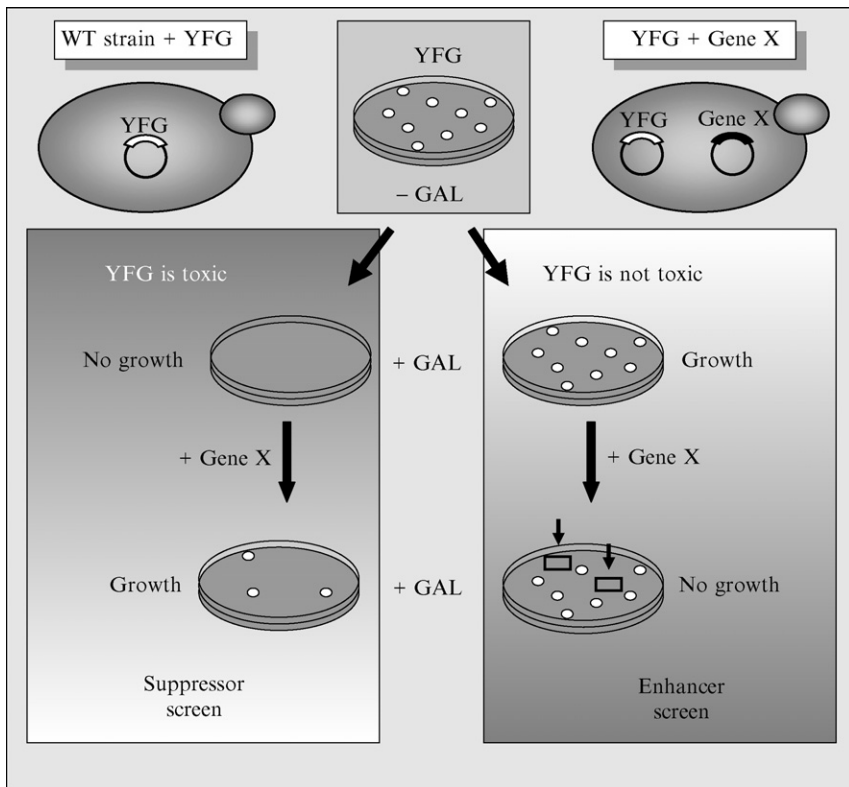


FIG. 2. An overview of an overexpression screen for suppressors or enhancers of amyloid toxicity in yeast. A plasmid containing YFG under the control of a GAL-inducible promoter is transformed into wild-type yeast cells alone or in combination with an ORF from a clone library (i.e., Gene X). If YFG is toxic to wild-type yeast cells, then the ORF library can be screened for a gene that when overexpressed restores growth or suppresses toxicity. If on the other hand, YFG is not toxic to wild-type cells, then the ORF library can be screened for a gene that when overexpressed enhances toxicity of YFG.

the colonies are transferred to GAL-containing plates by replica plating, thereby inducing expression of both YFG and the clones from the YOC library. If YFG is toxic to wild-type cells, little to no growth will be observed on GAL media, and suppressing colonies can be selected. These will represent colonies overexpressing a gene that suppresses the toxicity of YFG. If, on the other hand, expression of YFG is not toxic, the yeast will grow normally on GAL media, and enhancers of toxicity can be identified. An added benefit of overexpression screens is that mammalian cDNA libraries can be exploited. For example, in a genetic screen for suppressors of amyloid toxicity in yeast, a human cDNA library could be used, allowing investigators to directly test genetic interactions between YFG and human genes from tissues affected by the disease.

Methods

Pooling the YKO Library Strains

Screening the pooled YKO strain set offers the benefit of random screening of mutants without *a priori* bias in selection. In addition, transformation of the pooled strains is quite simple and does not require expensive equipment. One drawback is that the variable growth rates and transformation efficiencies of the deletion strains within the pools may allow certain strains to become overrepresented and others to be underrepresented in the final transformation. To reduce competition among the gene deletion strains, we recommend dividing the YKO set into five pools, four for the strains with normal growth rates (~50 plates total), and a fifth pool for all the slow-growing strains (two plates, which are grouped separately in the YKO sets).

1. Inoculate 100 μl of YPD media in 96-well microtiter plates with cells from the YKO stocks by using a multichannel pipetter or a pinning tool (see “[Systematic Approaches to Genetic Screening](#)”). Thaw YKO frozen stocks two to four plates at a time on ice, pin 1–5 μl into YPD media, and immediately return stocks to -80° . To ensure that cross-contamination of strains does not occur, be very careful when handling these plates.
2. Incubate the plates at 30° for 3–4 days, until the cultures are saturated ($\text{OD}_{600} \sim 5.0\text{--}10.0$; $1\text{--}2 \times 10^8$ cells/ml).
3. Pool strains using a multipipetter to remove cultures from wells and pipette into a sterile solution basin. The large pools will consist of ~12–13 plates, or approximately 125 ml of culture.
4. Centrifuge at 1500g for 5 min at 4° , discard media.

5. Resuspend cell pellet in 1/10th volume YP-media + 5% DMSO (~12.5 ml).
6. Distribute into 250- μ l aliquots in cryovials, and store at -80° .

High-Efficiency Yeast Transformation

This method is a modification of the lithium acetate (LiAc) TRAF0 method (Gietz and Schiestl, 1996). Variations of this protocol have been used extensively for high-efficiency transformation of libraries into yeast for two-hybrid and three-hybrid screens. This protocol can be used either for transforming a pool of YKO deletion strains with a plasmid encoding a GAL-inducible YFG or transforming a parental strain with a GAL-inducible cDNA library. This protocol yields transformation efficiencies of up to 2.2×10^7 transformants/ μ g of plasmid DNA.

1. Inoculate 50 ml of YPD from a single colony or an aliquot of the pooled YKO strains and incubate overnight at 30° with shaking.

2. Harvest cells when density reaches 5×10^6 cells/ml culture by centrifugation in a table-top centrifuge at 1500g for 5 min at 4° . A hemocytometer may be used to calculate the cell number from an appropriate dilution of the culture. When determining cell number, count cells with equal size buds as two cells; cells with an obvious larger mother cell and smaller bud, should be counted as a single cell. Although the relationship between cell number and OD₆₀₀ is strain specific, an estimate of $\sim 1.0\text{--}2.0 \times 10^7$ cells/ml at OD₆₀₀ of 1.0 may be used.

3. Pour off media, and wash cells with 10 ml sterile water. Centrifuge at 1500g for 5 min at 4° .

4. Resuspend in 10 ml of 100 mM LiAc (sterile), and refrigerate overnight at 4° .

5. Centrifuge at 1500g for 5 min.

6. Pour off supernatant and resuspend in sterile water to a final volume of 1 ml.

7. Pipette 100- μ l aliquots of the cells into Microfuge tubes.

8. Pellet cells by centrifugation at 1500g for 5 min at 4° , and remove water with a micropipetter.

9. Layer on each pellet in the following order:

- a. 240 μ l 50% PEG

- b. 36 μ l 1.0 M LiAc

- c. 5 μ l salmon sperm DNA (10 mg/ml)

- d. 0.1–1.0 μ g plasmid DNA (in a total of 79 μ l sterile water); 360 μ l total

10. Vortex vigorously for 1 min to ensure cell pellet is resuspended.

11. Incubate at 30° for 30 min.
12. Heat shock at 42° for 30 min in a water bath.
13. Pellet cells by centrifugation at 1500g for 5 min at 4°, and remove transformation mix with a micropipetter.
14. Resuspend cells in 100 μ l of sterile water, and plate each aliquot onto a large (15-cm diameter) GLU plate with appropriate selection.
15. Incubate the plates at 30° for 2–3 days.
16. Replicate plate onto GAL plates with appropriate selection.
17. For isolation of suppressors of toxicity, identify colonies that grow on both GLU and GAL plates. To isolate deletion strains that enhance toxicity, identify colonies that grow normally on GLU, but weakly on GAL.
18. Streak modifying deletion strains onto GLU plates with appropriate selection to obtain colonies for further testing.

Amplification and Sequencing of Molecular Barcode

Once individual strains of interest from the YKO set have been identified above, the identity of the strain is determined by PCR amplification of the molecular barcode or TAG, followed by DNA sequencing and identification of the gene deletion strain using the database at the *Saccharomyces* Genome Deletion Project web page (see later).

1. Pick colony of interest into 20 μ l of 20 mM NaOH in a tube appropriate for a thermocycler.
2. Lyse colonies at 94° for 20 min using a thermocycler.
3. Centrifuge at 1500g for 5 min at 4° to pellet cell debris.
4. Use 3 μ l of supernatant lysate for PCR amplification.
Per 25- μ l reaction:
 - a. 3 μ l yeast genomic lysate
 - b. 0.5 μ l Forward primer (5 pmol)
 - i. 5' GCCTCGACATCATCTGCCAG 3'
 - c. 0.5 μ l Reverse primer (5 pmol)
 - ii. 5' CGGTGTCGGTCTCGTAG 3'
 - d. 0.5 μ l dNTP mix (10 mM)
 - e. 0.75 μ l MgCl₂ (50 mM)
 - f. 2.5 μ l PCR reaction buffer (10 \times)
 - g. 0.1 μ l Taq polymerase (1 unit)
 - h. 17.15 μ l H₂O
5. Amplify TAG with thermocycler as follows:
 - a. 94° for 2 min
 - b. 35 cycles of:

- i. 94° for 15 sec
 - ii. 55° for 15 sec
 - iii. 72° for 30 sec
- c. 72° for 10 min
- d. Cool to 4°
6. Purify and sequence the amplified product by standard methods.
7. Identify gene deletion strain using TAG sequence at the *Saccharomyces* Genome Deletion Project web page: http://sequence-www.stanford.edu/group/yeast_deletion_project/deletions3.html

High-Throughput Transformation Protocol

Because of the high level of false positives isolated in these screens (see “[Systematic Approaches to Genetic Screening](#)”), all candidate modifier YKO strains and YOC plasmids must be retested. In the case of the YKO strains, each strain should be freshly retrieved from the frozen stock and retransformed with the construct of interest. In the case of the YOC plasmids, genetic interactions with YFG can be confirmed by double transformation into the parental strain. The following protocol is a simple method for performing up to 96 transformations at a time, allowing for rapid retesting of candidate modifiers. Modified from the “One step transformation of yeast in stationary phase” protocol ([Chen *et al.*, 1992](#)).

One Step Buffer (prepare fresh)

- 0.2 M Lithium acetate
- 40% Polyethylene glycol (PEG) 3350
- 100 mM Dithiothreitol (DTT); add fresh from 1 M frozen stock

1. Add 100 μ l of YPD media per well of a 96-well microtiter plate using a multichannel pipetter.
2. Inoculate media with a single colony of the strain of interest.
3. Grow cells 1–2 days at 30° until the cultures reach stationary phase ($OD_{600} \sim 5.0$ – 10.0 ; 1 – 2×10^8 cells/ml).
4. Centrifuge plate(s) in a table-top centrifuge for 5 min at 1200g.
5. Remove as much media as possible using a multichannel pipetter, being careful not to disturb the cell pellet.
6. Wash cell pellet by resuspending in 50 μ l of sterile water using a multichannel pipetter.
7. Centrifuge plate(s) in a table-top centrifuge for 5 min at 1200g.
8. Remove as much water as possible using a multichannel pipetter, being careful not to disturb the cell pellet. Prepare transformation cocktail

by mixing 2 ml of One Step Buffer, 100 μl of 10 mg/ml sheared, boiled salmon sperm DNA (1.0 mg), and 20 μg of plasmid.

9. Add 20 μl of the transformation cocktail per well using a multichannel pipetter.

10. Resuspend cells thoroughly in the transformation cocktail by pipetting up and down 10–20 times.

11. Incubate at 45° for 30 min in a water bath.

12. Use multichannel pipetter to spot 5 μl of cells per well onto the appropriate selective plate. Allow spots to absorb into media before placing in oven. Drying plates ahead of time speeds this process.

13. Incubate at 30° for 2 days.

14. Growth spots represent a mixture of many transformed cells; to obtain colonies derived from individual transformants, streak out a sampling of the cells onto a fresh selective plate.

Spotting Assays

Spotting assays are a simple, yet powerful, method to measure relative toxicity or growth between strains expressing heterologous proteins (Fig. 3). The method that follows is designed for 96-well microtiter plates and is well suited for high-throughput testing.

1. Add 100 μl of appropriate selective media + raffinose (RAF) per well of a 96-well microtiter plate using a multichannel pipetter.

2. Inoculate with a single colony from desired transformant. Because of variation in copy number it is advisable to test several transformants per

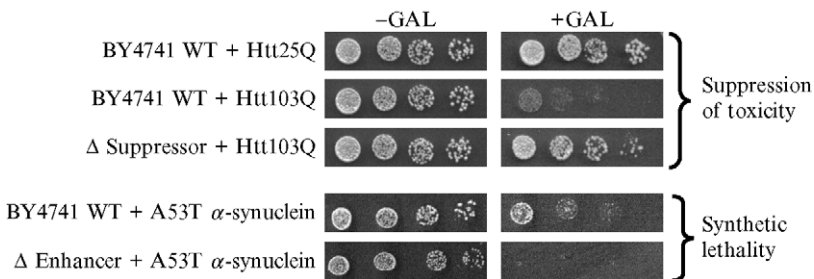


FIG. 3. Example of using spotting assays to measure the relative effect of genetic modifiers on amyloid toxicity. Assuming YFG is under the control of a GAL-inducible promoter, a sample of cultures in log phase is serially diluted and plated on both GLU and GAL plates. In the top example, a loss-of-function suppressor of toxicity is observed to relieve the growth defect caused by Htt103Q. In the bottom example, a loss-of-function enhancer is observed to increase toxicity of A53T α -synuclein.

clone. Incubate at 30° for 1–2 days until the cultures reach stationary phase ($OD_{600} \sim 5.0\text{--}10.0$; $1\text{--}2 \times 10^8$ cells/ml).

3. Read the optical density of the cultures using a plate reader, and then individually adjust each culture to OD_{600} 0.4.

4. Incubate at 30° for 3 h. This incubation allows the yeast cells to exit lag phase and begin actively dividing (log phase) before plating.

5. Perform serial fivefold dilutions of cultures in water (1:5, 1:25, 1:125, 1:625) in 96-well microtiter plates (diluting 20 μl of cells into 80 μl of water is convenient). Because yeast cells fall out of solution quickly, make sure that cells are fully resuspended when making dilutions.

6. Plate 5 μl of each dilution onto both GLU and GAL selective plates to compare growth of the strains with and without expression of YFG. Make sure to include a transformant containing a control construct on each plate for reference. It is critical that the plates are extremely dry or else the spots may run. Dry the plates at room temperature for 2–4 days, or 30 min in a sterile vacuum hood.

7. Incubate at 30° for 2 days (GLU plates) or 3 days (GAL plates). Relative toxicity is determined by comparisons of growth between the clones of interest and controls on GAL and GLU media (under inducing and repressed conditions).

Systematic Approaches to Genetic Screening

Although pooled approaches to screening are simple to perform and allow random isolation of genetic modifiers without *a priori* bias, this approach has several drawbacks and limitations that array approaches can eliminate. Perhaps the largest obstacle to this screening method is the large number of false positives isolated during transformation. During a recent loss-of-function screen, we found that >80% of modifiers isolated were false positives, likely because of second site mutations not related to the bar-coded gene knockout (Giorgini *et al.*, 2005). Such mutations may be due to the process of DNA transformation itself, which is mutagenic. If the promoter being used is at all “leaky,” there is strong selective pressure within the pool for any second site mutation that relieves toxicity. Because the pool transformation approach requires screening individual transformants, any second site mutations affecting toxicity will confound the true effect of the deletion strain or clone being tested. On the other hand, if transformations are performed with the arrayed strains or ORF, each “spot” of transformants derived from the 96-well microtiter plates will represent dozens, perhaps hundreds, of transformants. By testing these transformants *en masse*, the confounding effect of second site mutations is greatly reduced. Furthermore, analysis of transformants *en masse*

also reduces clone to clone differences in expression level of YFG because of variability in plasmid copy number, which can also lead to false positives.

Another major benefit of keeping the strains and ORFs ordered in arrays during testing is that the clones are readily identifiable by their position in the 96-well plate, which eliminates the need for PCR amplification of the bar code or plasmid isolation of the ORF and subsequent DNA sequencing, which can become quite costly when done on a large scale. Systematic testing of arrayed strains or ORFs can be performed either manually using 96- or 384-pin manual pinning tools (V&P Scientific, Inc., San Diego, CA) or with the aid of robotics, such as the Biomek FX Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA). Even without the benefit of automation, the availability of the YKO and YOC sets, combined with manual pinning tools, allows for rapid systematic genomic screening in yeast.

Automated screening has been combined with several new genomic techniques in yeast, including construction of protein–protein interaction (PPI) networks in yeast using the two-hybrid system (Ito *et al.*, 2000; Uetz *et al.*, 2000), synthetic genetic array (SGA) analysis (Tong *et al.*, 2001), and diploid-based synthetic lethality analysis on microarrays (dSLAM) (Pan *et al.*, 2004). The PPI network approach has been used to generate a protein interaction map for the yeast genome (Schwikowski *et al.*, 2000) and can be used to generate PPI networks for disease genes (Giorgini and Muchowski, 2005; Goehler *et al.*, 2004). SGA analysis is a method for systematic construction of double mutants, in which a query mutation (i.e., YFG integrated into the yeast genome) is mated to the YKO deletion set. Because YFG is integrated into the yeast genome, expression levels of YFG are much more consistent, reducing the number of false positives in screens for enhancers and suppressors of toxicity. In addition, bringing together the gene deletion strain of interest and YFG is far simpler by mating than by individual transformation of each strain. Finally, the dSLAM method is another array-based analysis using the YKO set in which heterozygous strains are converted to haploid YKO strains immediately before genomic profiling, eliminating variation in growth rate among the strains, making the set more amenable to manipulation as a population. All of these rapid automated techniques, and others in development, make the use of yeast genomics an extremely powerful and informative approach for explanation of the mechanisms of amyloid toxicity.

Conclusion

In this chapter, we have only scratched the surface of the plethora of genomic and proteomic strategies available in yeast that can be applied toward studies of amyloid toxicity. Approaches from simple, classical genetics (i.e., epistatic analysis to order genes in pathways or networks)

to complicated, data-intensive gene profiling experiments with microarrays, can be used to exploit these yeast models to their fullest potentials. In the end, a combination of several approaches in yeast and mammalian models is required for validation of interesting genetic pathways and for subsequent application of this information toward explaining amyloid disease. It is worth noting that results from loss-of-function screens can be nicely complemented by the use of RNA interference in mammalian cell models of disease. Nonetheless, the “Golden Age” of yeast genomics is on us in earnest (Fields and Johnston, 2005; Johnston, 2000), and the application of these genomic techniques in studies of amyloid toxicity using yeast and other model organisms is leading to an explosion of exciting new information of potential relevance to amyloid disease.

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