

# Applicability of Yeast Genetics to Neurologic Disease

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**A**s advances in gene mapping technology reveal genes associated with neurologic diseases, the need to identify a gene's normal function arises often. Experimental genetics is very useful in identifying a gene's function. It relies on model organisms both because it is not appropriate in humans, and because many processes are remarkably similar among eukaryotes. Many cellular processes have evolved once, and species differences are variations on a theme. Molecular genetic tools available in the yeast *Saccharomyces cerevisiae* provide a means to more rapidly reach an understanding of gene function, yielding substantial insight into the same process in humans. Yeast will never complain of headache or "spells," but do have expansions of trinucleotide repeats, prions, and other processes very much analogous to those underlying many neurologic diseases. In spite of the absence of a nervous system in yeast, yeast genetics has contributed substantial insight into neurologic diseases mechanisms. The real strength of yeast in studying human disease is in genetic analysis of gene function and in providing genetically powerful functional assays.

*Arch Neurol.* 2000;57:1129-1134

The complete DNA sequence of the *Saccharomyces cerevisiae* genome contains approximately 6217 genes. The human genome is 100 times larger, but has only 10 to 12 times as many genes (YGs). Genes are far more tightly spaced in yeast. A third or more of yeast genes have robust homology, and two thirds have a domain with significant homology to human genes. These numbers will increase as more human genes (HGs) become known. Twenty percent of human disease genes found by positional cloning are similar to YGs, and many human genes function well in yeast.<sup>1,2</sup>

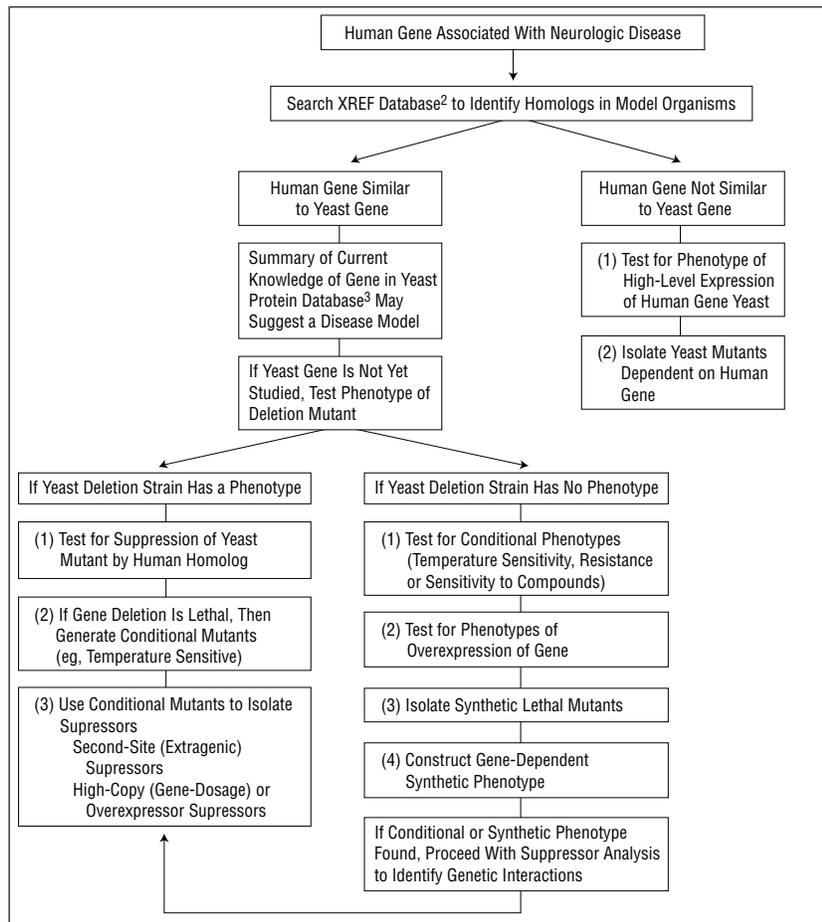
## WHERE TO START WITH YEAST

A search for a gene underlying a neurologic disease may yield a gene of unknown function. A good place to start to identify its function is the XREF database,<sup>2</sup> which catalogs human-similar genes among model organisms. If the newly

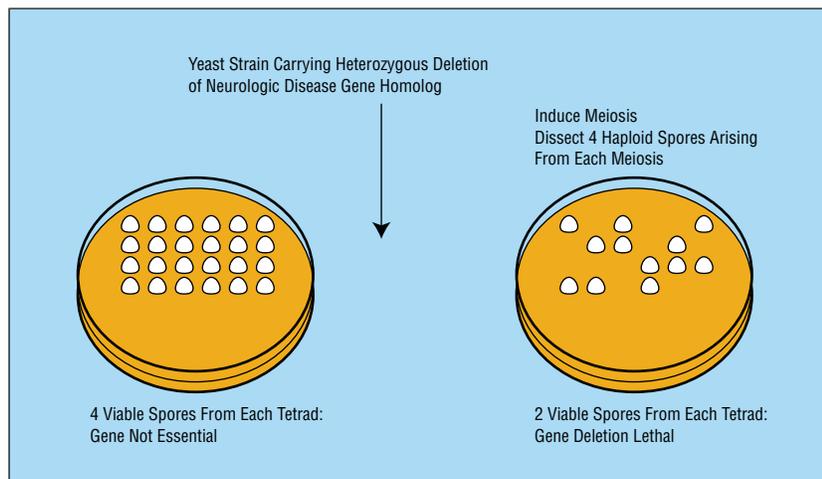
found HG is similar to a yeast gene, then a detailed summary of current knowledge about the YG can be found at the Yeast Protein Database.<sup>3</sup> This may immediately suggest a disease model (**Figure 1**).

If an HG is similar to a yeast gene of unknown function, yeast genetics may help reveal its function. The first test is to determine whether deletion of the gene causes a phenotype. Any YG can be deleted because efficient homologous recombination allows precise manipulation of chromosomes. To test for a deletion phenotype, a diploid strain heterozygous for gene deletion is constructed and induced to complete meiosis (**Figure 2**). The 4 meiotic products of 1 cell are microdissected into a row on an agar plate. If only 2 grow into colonies, then deletion of the gene is lethal. If all 4 grow, the gene is not essential. In this case, the 2 meiotic products carrying the gene deletion will sometimes have a distinctive phenotype. By the time you read this, a deletion strain for every YG should be commercially available. The *Saccharomyces* Genome Deletion Project<sup>4</sup> is

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**Figure 1.** Flowsheet of an algorithm for using yeast genetics to study the function of a human gene newly associated with a neurologic disease. Steps are discussed in the text. Web addresses for databases are given in the references.



**Figure 2.** Method for determining whether a yeast gene is essential. A diploid strain heterozygous for a gene deletion is induced to undergo meiosis. Each cell yields 4 haploid spores associated in a tetrad. Tetrads are separated using a dissecting microscope and micromanipulated such that the 4 spores of each tetrad are placed in a row. If only 2 spores per tetrad yield colonies, then deletion of the gene is lethal. If 4 grow, the gene is not essential.

making strains that carry a precise, marked deletion of individual genes, with over 5100 YGs currently represented. Deletion strains are used to test whether an HG can function-

ally replace its yeast homolog, as has been shown for well over 70 genes, providing functional assays to aid in testing models for disease mechanisms.

## CONDITIONAL ALLELES AND SUPPRESSORS TO FIND FUNCTIONAL INTERACTIONS

If a gene is essential, it is useful to generate conditional alleles, such as temperature-sensitive mutants that grow at 30°C, but not at 37°C (restrictive temperature) (**Figure 3**). The terminal phenotype after shift to the restrictive temperature may be informative. For example, yeast defective in DNA replication arrest before mitosis as large budded cells. Conditional alleles are also used to isolate suppressors (**Figure 4**). Suppression occurs when one mutant phenotype is corrected by a second genetic change that compensates for the first. For example, suppression of a temperature-sensitive mutant would allow growth at the restrictive temperature. Suppression may result either from mutation of a second gene,<sup>5</sup> or by expression of a gene on a plasmid.<sup>6</sup> Suppressors can identify direct protein interactions, or genes acting in the same or a parallel pathway.

## APPROACHES IF GENE DELETION HAS NO OBVIOUS PHENOTYPE

Some mutations show a phenotype only under certain conditions, found by empiric testing of growth conditions or the effect of different compounds.<sup>7</sup> Some mutants lack a phenotype because of a redundant gene or pathway. For example, yeast have 2 *RAS* genes. Deletion of either one results in no remarkable phenotype, but deletion of both is lethal. Lethality requiring 2 mutations is called “synthetic lethality.” Synthetic lethal mutants, found by established methods (**Figure 5**), may reveal redundant pathways or direct protein interactions. A related way to generate a phenotype is to isolate yeast with lethal mutations that are suppressed by an HG.<sup>8</sup> The nature of such mutants may reveal a previously unknown function. Expression of an HG in yeast sometimes yields a dominant phenotype, as with the proapoptotic genes discussed below.<sup>5,6</sup>

## SYNTHETIC PHENOTYPES

If something about the function of an HG is known, it is useful to construct a synthetic phenotype depen-

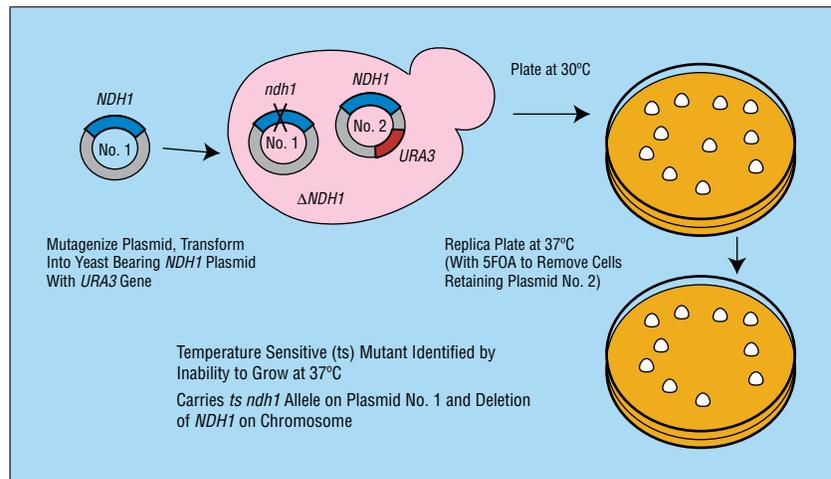
dent on that function in yeast, providing an assay for genetic studies. It is particularly easy to create a synthetic phenotype if the gene of interest alters transcription of another gene, either directly as a transcription factor, or indirectly through a signaling pathway. To construct such a phenotype, a promoter that responds to the gene of interest is used to drive expression of a reporter gene. Many reporters are available in yeast, including genes allowing selection for or against expression, and genes that change yeast colony color. The 2-hybrid system depends on synthetic phenotypes by expressing reporter genes in response to interaction of 2 proteins. Human promoter elements can be used in constructing synthetic phenotypes, as for the *p53* tumor suppressor gene, discussed below.<sup>9</sup>

## RELEVANCE TO THE STUDY OF NEUROSCIENCE

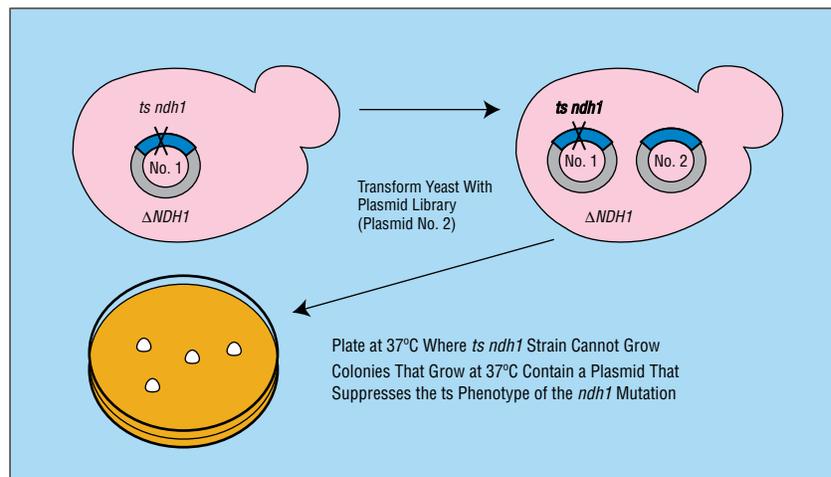
### Friedreich Ataxia

Positional cloning identified frataxin as the gene mutated in Friedreich ataxia, the most common inherited ataxia.<sup>10</sup> Ninety-eight percent of patients with Friedreich ataxia have expansion of a GAA trinucleotide repeat in the first frataxin intron, causing reduced expression, while a few harbor point mutations. The frataxin sequence is similar to a YG of previously unknown function, now called *YFH1* (yeast frataxin homolog). Yeast frataxin homolog deletion yields a strain unable to grow on nonfermentable carbon sources, indicating defective oxidative phosphorylation. Iron is accumulated in mitochondria to high levels in *yfh1* mutants and in Friedreich ataxia cells. Excess mitochondrial iron is thought to underlie the respiratory defect by generating oxidizing free radicals through Fenton-like reactions. Iron sulfur proteins acting in respiration, including aconitase, are particularly sensitive to free radicals, and reduced levels are found both in yeast *yfh1* mutants and in Friedreich ataxia cells, while products of lipid peroxidation are increased. This has focused attention on free radical scavengers as therapeutic agents.

Frataxin and Yph1 are both localized in mitochondria. Normal hu-



**Figure 3.** Generation of temperature-sensitive alleles of an essential gene. The neurologic disease gene homolog (*NDH1*) is used as an artificial example. A strain with deletion of *NDH1* requires plasmid-borne *NDH1* on plasmid No. 2 to grow. Plasmid No. 1, bearing another copy of *NDH1*, is mutagenized *in vitro*, and transformed into this strain. As transformants grow, some cells in each colony spontaneously lose plasmid No. 2. Colonies are replica plated to a plate (at 37°C) containing 5-fluoroorotic acid (5FOA), which kills cells with *URA3*, thereby allowing only cells that have lost plasmid No. 2 to grow. Colonies that do not grow on 5FOA at 37°C have sustained an *ndh1* mutation on plasmid No. 1 that is temperature sensitive.



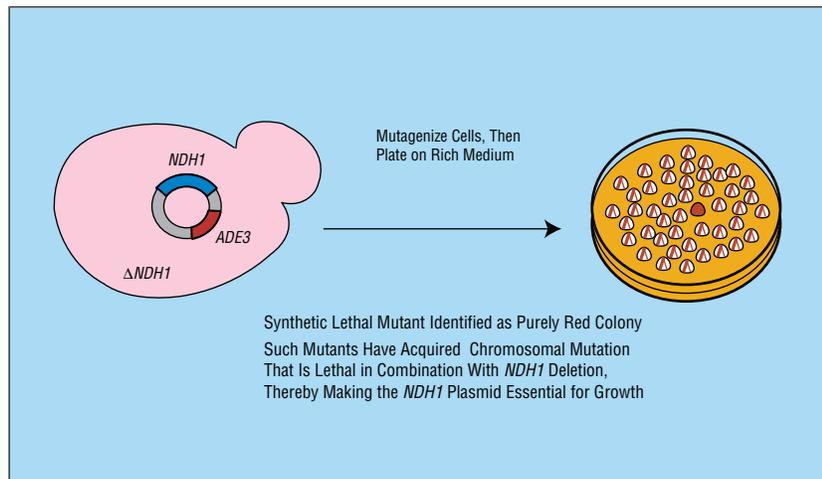
**Figure 4.** Isolating suppressors using a temperature-sensitive mutant. A library of plasmids constructed to overexpress genes is transformed into the temperature-sensitive (*ts*) *ndh1* mutant described in Figure 3. Transformants are plated at 37°C, where the temperature-sensitive mutant cannot grow. Colonies that grow at 37°C contain a plasmid that suppresses the phenotype of the *ndh1* mutation. In this strain, the neurologic disease gene homolog (*NDH1*) on the chromosome is deleted. Suppressors can also arise by mutations that have occurred in another gene in the yeast genome. No. 1 and No. 2 refer to plasmids.

man frataxin complements *YFH1* deletion, while a point mutant allele from a patient with Friedreich ataxia only partially complements the yeast mutant. Characterization of genetic suppressors of *yfh1* mutants (Figure 1), will reveal genetic interactions that should provide insight into frataxin function. An interesting complication to suppressor analysis is that *yfh1* mutants rapidly lose mitochondrial DNA, probably secondary to mitochondrial dysfunction. Because suppressors will not replace lost mitochondrial DNA, creative selection schemes will be required to take this into account. If mi-

tochondrial DNA loss also occurs in Friedreich ataxia, any attempts at gene therapy will be similarly complicated.

### Batten Disease

Juvenile neuronal ceroid lipofuscinosis, or Batten disease, is a good example of how yeast can aid the study of a lethal neurodegenerative disease.<sup>7</sup> Positional cloning identified *CLN3* mutations in patients with Batten disease. The *CLN3* sequence does not reveal its function. A YG, *BTN1*, is 39% identical to human *CLN3*. Although the *BTN1* deletion strain has



**Figure 5.** Isolating synthetic lethal mutants starting with a nonlethal mutation,  $\Delta$ NDH1. A colony color marker is used to indicate the presence of a plasmid bearing NDH1. In this case, ADE3 causes cells bearing the plasmid to be red.<sup>8</sup> Some cells lose the plasmid, yielding white sectors in red colonies. A mutation in a gene that is lethal in combination with  $\Delta$ NDH1 makes the NDH1 gene on the plasmid essential. Plasmid-dependent colonies are easily identified as purely red colonies among colonies with white sectors. Secondary screens not described here are used to confirm that mutations are synthetically lethal with the deletion of NDH1. NDH1 indicates neurologic disease gene homolog.

no obvious phenotype, an extensive search identified resistance to the chloramphenicol breakdown product d-(-)-threo-2-amino-1-[p-nitrophenyl]-1,3-propanediol (ANP). Human CLN3 reestablishes ANP sensitivity in the yeast *btn1* mutant, and the degree of ANP resistance imparted by different alleles correlates well with severity of Batten disease. This suggests that the function mediating ANP sensitivity and the function lost in Batten disease are the same. Btn1 is localized in the vacuole, and Cln3 in the lysosome, the human equivalent of the yeast vacuole. In *btn1* mutants, the vacuole is abnormally acidic during initial growth, suggesting that dysregulation of lysosomal pH causes the lysosomal protein accumulation that is a hallmark of Batten disease. This yeast model system will be very useful in determining how loss of CLN3 leads to lysosomal pH dysregulation and protein accumulation, and in testing candidate therapeutic agents.

### Amyotrophic Lateral Sclerosis

Fifteen to twenty percent of patients with familial Amyotrophic Lateral Sclerosis (ALS) carry a mutation of *SOD1*, initially suggesting that ALS is due to decreased clearance of superoxide radicals. Human *SOD1* can complement the yeast *sod1* mutant. However, complementing ac-

tivity of ALS mutant *SOD1* alleles does not correlate with disease severity<sup>11</sup>; this yeast assay, therefore, does not reflect the function associated with disease. Some Sod1 mutant proteins retain normal superoxide dismutase activity, yet still induce ALS. Motor neuron disease is absent in mice lacking *SOD1*. These results indicate that ALS is not due to reduced superoxide dismutase activity, but rather to a dominant gain of function of mutant *Sod1*. When this gained function is better understood, it may be possible to link it to a phenotype in yeast to create a more useful functional assay.

### Ataxia Telangiectasia

There is much in common between yeast and human responses to DNA damage. Ataxia telangiectasia due to mutation of *ATM* is characterized by progressive ataxia, cancers including brain tumors, immune deficits, and sensitivity to DNA damage. Atm is closely related to yeast Mec1 and Tel1 protein kinases. Mutation of *ATM* or *MEC1* results in loss of several cell cycle checkpoints, making cells unable to respond to DNA damage by blocking DNA replication or mitosis while damage is repaired. Checkpoints are stress responses that normally protect genomic integrity. Damage to DNA normally causes Atm to phosphorylate the Chk2 protein kinase, as well as other targets

including p53. Chk2 then phosphorylates Cdc25C, blocking its key role in initiating mitosis by dephosphorylation of Cdc2. This signaling pathway is quite similar in yeast, where DNA damage causes the Atm homolog, Mec1, to phosphorylate Rad53/Spk1. In fact, human Chk2 was found because the yeast system predicted existence of a human homolog of Rad53/Spk1.<sup>12</sup> Cancer cells have checkpoint defects, and are sensitive to chemotherapy partly because they cannot halt cell cycle progression when DNA is damaged. Better understanding of checkpoints may lead to new ways to increase sensitivity of cancer cells to chemotherapy.

### Trinucleotide Repeat Expansion

Trinucleotide repeat expansion (TRE) causes a dozen or more neurodegenerative disorders, including Huntington disease, myotonic dystrophy, and inherited ataxias. Two major questions are "How does TRE occur?" and "How does TRE cause disease?" Yeast genetics has begun to open the door to the mechanism by which TRE occurs, often during replication of the lagging DNA strand. Some TREs, such as CTG, fold back on themselves to form hairpin structures. When Okazaki fragments containing hairpins are joined, the new daughter strand has an expanded repeat because of the extra repeats in the hairpin. No human genes involved in the mechanism of generating TRE are known. Recently, *S cerevisiae rad27* mutants have been shown to expand trinucleotide repeats at a greatly increased rate.<sup>13</sup> Rad27 and its human homolog, FEN-1, are nucleases that trim primers from Okazaki fragments during DNA replication. Other genes involved in expanding repeats will be found by identifying further yeast mutants that expand repeats, and by isolating suppressors of the repeat expansion phenotype of *rad27* mutants. Such genetic studies are difficult or impossible in humans. Large TREs causing human disease seem to occur during meiosis. Both mitotic and meiotic expansions can be studied using yeast genetics.

The TREs occur both in coding and untranslated regions. There-

fore, the mechanisms by which TREs cause disease are diverse. An example where yeast may help is the voltage-sensing, pore-forming subunit, CACNA1A, of the voltage-dependent calcium channel. Mutations in CACNA1A underlie 3 different diseases. Short expansions (20-30 CAG repeats) enlarge a stretch of polyglutamine in the intracellular carboxyterminal region, and cause spinocerebellar ataxia type 6.<sup>14</sup> CACNA1A missense mutations have been shown to underlie familial hemiplegic migraine, while nonsense CACNA1A truncation mutations cause familial episodic ataxia type 2. The mechanism by which the 3 different types of mutation lead to different disorders is unclear. Yeast *Cch1* is similar to human CACNA1A. Deletion of *CCH1* blocks calcium uptake in response to mating pheromone signaling, leading to cell death.<sup>15</sup> It remains to be tested whether human CACNA1A complements deletion of *CCH1*. If so, and if phenotypes can be linked to the different mutant alleles, then suppressor analysis should help identify interactions relevant to the different disorders. Yeast genes known to interact genetically with *CCH1* include *MID1* and *CDC1*. It remains to be determined whether human homologs of these genes are involved in disease.

### Yeast and Apoptosis

Apoptosis, implicated in many neurodegenerative diseases, seems not to occur in yeast. Yeast have no genes for caspase proteases or several other genes central to apoptosis in multicellular organisms. Nevertheless, human proapoptotic Bax and Bak kill yeast by a mechanism related to apoptosis.<sup>5,6</sup> Presence of multiple Bcl2/Bax family members makes it difficult to study action of any 1 member in human cells. Killing of yeast by Bax, in the absence of any related genes, suggests that Bax has intrinsic killing activity, arguing against the model that Bax kills only indirectly by inhibiting Bcl2. Bax and Bak mutants that do not kill mammalian cells do not kill yeast. Human antiapoptotic Bcl2 blocks Bax-induced death in yeast. Bax causes release of cytochrome *c* from mitochondria, which can be blocked by Bcl2 in both yeast

and human cells. This indicates that some apoptotic targets exist in yeast, making it possible to use yeast genetics to find them. By isolating yeast mutants resistant to Bax killing, the F<sub>0</sub>F<sub>1</sub>-ATPase mitochondrial proton pump was found necessary for Bax-induced cell death.<sup>5</sup> Bax and Bcl2 localize to the outer mitochondrial membrane in both yeast and human cells. Similarly, Bax inhibitor 1 was selected from a human complementary DNA library by its ability to suppress bax-induced killing in yeast.<sup>6</sup> Bax inhibitor 1 blocks Bax-induced apoptosis in human cells as efficiently as Bcl2.

Caspases are being studied using a synthetic phenotype in yeast.<sup>16</sup> A fusion protein with a transcription factor at one end and a membrane-bound protein at the other, separated by a caspase cleavage site, is inactive as a transcription factor in yeast. Caspase cleaves the fusion protein, freeing the transcription factor to enter the nucleus and activate a reporter gene. This assay can be used to identify caspases and caspase activators and inhibitors. In this way, yeast genetics has been fruitfully applied to apoptosis, a process that apparently does not occur in yeast.

### Prions in Yeast

Yeast have epigenetic traits, such as [PSI<sup>+</sup>], similar to prion-mediated human diseases. [PSI<sup>+</sup>] is a non-Mendelian trait that suppresses nonsense mutations by reducing the efficiency of translation termination at stop codons. Inheritance of the [PSI<sup>+</sup>] trait is mediated by a prionlike conformation of the translation release factor Sup35. The [PSI<sup>+</sup>] phenotype can be cured by protein denaturants. Overexpression of Sup35 induces the [PSI<sup>+</sup>] phenotype and accumulation of cytoplasmic Sup35 aggregates resembling amyloid fibers. Efficient conversion of soluble Sup35 to fibrillar Sup35 in vitro is induced by preformed Sup35 fibers.<sup>17</sup> This strongly supports the seed-dependent polymerization model proposed for human prion diseases. It should be possible to use yeast to find approaches to block polymerization, hopefully leading to new therapeutic approaches to human prion diseases.

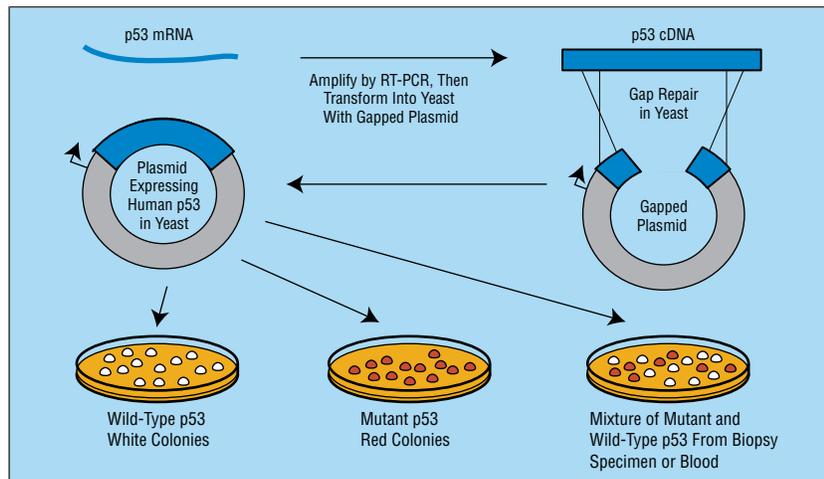
## RELEVANCE TO THE PRACTICE OF NEUROLOGY AND APPLICATIONS

### Synthetic Functional Assays of Human Genes in Yeast

When screening for disease-associated mutations, direct gene sequencing does not distinguish normal variations (polymorphisms) from important mutations. Human genes can be linked to a phenotype in yeast to develop functional assays for mutation detection. Such a yeast functional assay is commercially available for p53, the most commonly mutated gene in human cancers. Nearly all cancer-associated p53 mutations affect its activity as a transcription factor. A reporter gene driven by human p53 binding elements is a sensitive detector of p53 function, changing yeast colony color from red to white.<sup>9</sup> This assay can be applied to tumor biopsy specimens or blood. Basically, human p53 mRNA is amplified by reverse transcription and polymerase chain reaction, and transformed into yeast along with a linear gapped expression plasmid (**Figure 6**). The gapped plasmid is constructed to express p53 in yeast, but has most of the p53 gene removed to create a gap. Efficient homologous recombination links the amplified p53 DNA to p53 flanking sequences in the gapped expression plasmid. The resulting yeast express p53 from the person being screened, and colony color reveals whether it is functional. Heterozygotes for p53 mutation, as in the cancer-prone Li-Fraumeni Syndrome, show half red and half white colonies. Functional assays for other genes associated with neurologic diseases could be equally useful. In addition to mutation screening, functional assays can be used for suppressor analysis.

### Using Yeast to Find New Therapeutic Agents

As the function of disease-associated genes is better understood, candidate therapeutic targets become apparent. One approach to find drugs acting through a specific target takes advantage of both yeast genetics and DNA chip technology.<sup>18</sup> This method is based on the premise that an ef-



**Figure 6.** Yeast functional assay for human p53 mutations. p53 Messenger RNA (mRNA) is amplified by reverse transcription (RT) and polymerase chain reaction (PCR) from blood or a biopsy specimen. Resulting p53 complementary DNA (cDNA) is mixed with a gapped plasmid containing only the flanks of the p53 gene and transformed into yeast. Efficient homologous recombination in yeast fills the gap with p53 cDNA, yielding colonies expressing p53. The bent arrow indicates an active yeast promoter. The yeast strain contains a reporter gene that makes colonies white when activated by normal p53. Red colonies identify nonfunctional p53.

fective and specific pharmacologic inhibitor of a target protein will cause approximately the same genome-wide alterations in gene expression as does deletion of the target gene. For example, if calcineurin is chosen as an appropriate drug target, then RNA from a yeast strain with the calcineurin gene deleted is hybridized to a chip bearing an array of all YGs. This shows the relative abundance of all mRNAs in the absence of calcineurin. Then drugs are screened for those that cause the wild-type yeast expression pattern to mimic that of the calcineurin mutant. To validate that the drug acts by inhibiting calcineurin, the expression pattern in the calcineurin deletion strain with and without drug treatment is compared. If the drug acts by inhibiting calcineurin, then the expression pattern of the calcineurin deletion strain will be largely unaffected by drug treatment, whereas a marked change would occur if the drug acts through a different target. This strategy correctly identified calcineurin and FK506 binding protein as targets of the immunosuppressant drug FK506, and calcineurin and cyclophilin as targets of cyclosporin A. Both the common and the specific targets of the 2 drugs were effectively identified. Although it is theoretically possible to use a DNA chip with human genes and human deletion mutants, the prospect of generating a collection of human deletion strains analogous to

the yeast deletion strains is daunting at best. This potentially powerful approach, therefore, is best applied in yeast in cases where yeast have a gene similar to the human target of interest. Specificity can probably be improved if a yeast strain is used in which the intended target gene has been replaced by its homologous human gene.

Only a few examples have been discussed here, but other diseases have and will continue to benefit from yeast genetic studies. For example, apoptosis is thought to be an important mechanism of cell death in stroke as well as in the neurodegenerative diseases discussed here. Caspase inhibitors are under investigation as therapeutic agents for stroke and other neurologic disorders. As described above, yeast are already being used to identify new caspase inhibitors. Success in using yeast to find therapeutic agents depends on the accuracy of disease models, and on the creativity of the investigator in developing yeast functional assays that reflect gene functions relevant to disease.

Accepted for publication November 8, 1999.

Supported by Research Project grant RPG-97-166-01-MBC from the American Cancer Society, New York, NY.

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