Discovery of Chemical Modulators of a Conserved Translational Control Pathway by Parallel Screening in Yeast

Nuzhat Motlekar,[1] Rogerio Alves de Almeida,[2] Graham D. Pavitt,[2] Scott L. Diamond,[1] and Andrew D. Napper[1,]*

[1]Penn Center for Molecular Discovery, Institute for Medicine and Engineering, and Department of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania.
[2]Faculty of Life Sciences, University of Manchester, Manchester, United Kingdom.
**Current affiliation: Nemours Center for Childhood Cancer Research, Wilmington, Delaware.*

ABSTRACT

Eukaryotic initiation factor 2 (eIF2) B is a guanine nucleotide exchange factor that plays a central role in translation initiation and its control, especially in response to diverse cellular stresses. In addition, inherited mutations in human eIF2B subunits cause a fatal brain disorder commonly called childhood ataxia with central nervous system hypomyelination or leukoencephalopathy with vanishing white matter. In yeast, inhibiting activity of eIF2B up-regulates expression of the transcriptional activator general control nondepressible (GCN) 4. We report here evaluation of high-throughput screening (HTS) using a yeast-based reporter gene assay, in which strains containing either wild-type or a mutant eIF2B were screened in parallel to identify compounds modifying eIF2B-dependent responses. The goals of the HTS were twofold: first, to discover compounds that restore normal function to mutant eIF2B, which may have therapeutic utility for the fatal human disease; and second, to identify compounds that activate a GCN4 response, which might be useful experimental tools. The HTS assay measured cell growth by absorbance, and activation of gene expression via a β-galactosidase reporter gene fusion. Because mutant eIF2B activates GCN4 in the absence of stress inducers, the mutant strain was screened for reduction in GCN4 activation. HTS revealed apparent mutant-selective inhibitors but did not reliably predict selectivity as these hits affected both wild-type and mutant strains equally on dose–response confirmation. Wild-type strain results from the HTS identified two GCN4 response activators, both of which were confirmed to be wild-type selective in dose–response testing, suggesting that these compounds may activate GCN4 by a mechanism that down-regulates eIF2B activity.

INTRODUCTION

The study of cellular responses to environmental stress has provided valuable insight into disease processes and novel approaches for therapeutic intervention. Many such studies have focused on the yeast *Saccharomyces cerevisiae* because of the similarity of its cellular control mechanisms to those in mammalian cells and the relative ease with which it can be manipulated. The response of yeast to a variety of environmental stresses, including amino acid or purine starvation and oxidative stress,[1] is mediated through the transcriptional activator protein general control nondepressible (GCN) 4p (Gcn4p). Gcn4p levels are tightly controlled by modulating the translation of its mRNA[2] and the stability of the protein.[3] Therefore expression of *GCN4* is tightly regulated, a process known as general amino acid control.[2,4]

ABBREVIATIONS: A_{595}, absorbance at 595 nm; ATF4, activating transcription factor 4; CACH, childhood ataxia with central nervous system hypomyelination; DMSO, dimethyl sulfoxide; EC_{50}, 50% effective concentration; eIF2, eukaryotic initiation factor 2; GCN, general control nondepressible; IC_{50}, 50% inhibitory concentration; SDCCG, San Diego Center for Chemical Genomics; VWM, leukoencephalopathy with vanishing white matter.

DOI: 10.1089/adt.2009.0198

In general amino acid control, amino acid starvation results in the accumulation of deacylated tRNAs that bind to and activate the protein kinase Gcn2p. Active Gcn2p phosphorylates the translation initiation factor eukaryotic initiation factor 2 (eIF2) on a conserved serine residue at position 51 of its α-subunit. eIF2 is an essential G protein factor that when bound to GTP recruits initiator methionyl tRNA to the ribosome for each protein synthesis initiation event. Gcn2p-mediated phosphorylation of eIF2 antagonizes this by converting eIF2 into an inhibitor of its own guanine nucleotide exchange factor, eIF2B.[5] This reduces protein synthesis initiation rates in cells. However, the translation of *GCN4* mRNA is specifically stimulated under these conditions, resulting in the downstream transcriptional activation of numerous genes.[6] Many Gcn4p target genes encode amino acid biosynthetic enzymes, including *HIS4*, that enable cells to overcome the starvation imposed (*Fig. 1*, *left* and *middle*). Gcn2p-mediated activation of *GCN4* in response to oxidative stresses appears to be a critical factor allowing yeast cells to avoid damage from diverse oxidants such as hydrogen peroxide and the mutagens cumene hydroperoxide and cadmium.[1,7]

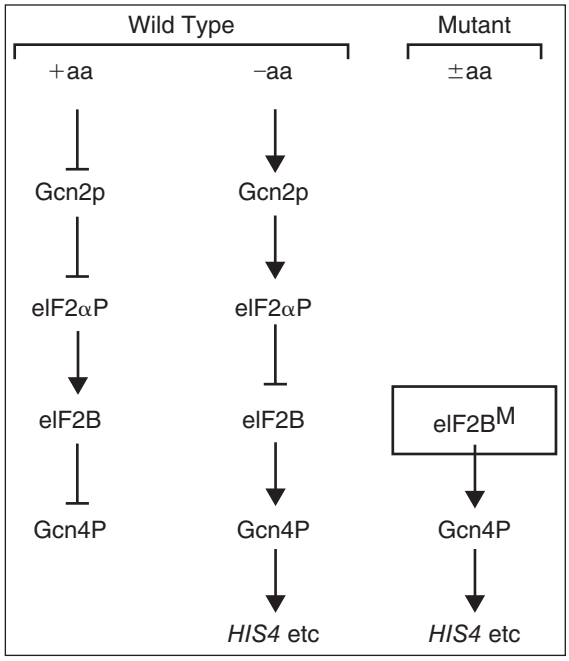

Fig. 1. Signaling to *GCN4* via eIF2B. (**Left**) In wild-type cells amino acids (aa) repress kinase Gcn2p, maintaining low eIF2 phosphorylation (eIF2αP) and active eIF2B. This represses *GCN4* translation. (**Middle**) Starvation for one or more aa activates Gcn2p, inhibiting eIF2B and activating translation of *GCN4* and transcription of its target genes. (**Right**) Mutations in eIF2B (eIF2B^{M}) override this signaling pathway and activate *GCN4* constitutively.

In mammals mGCN2 similarly responds to amino acid starvation to activate translation of activating transcription factor 4 (ATF4). Both *ATF4* and *GCN4* possess upstream open reading frames that limit the flow of scanning ribosomes to the authentic AUG start codon under non-stress conditions, but that allow enhanced *GCN4*/*ATF4* translation under stress situations using a mechanism that requires resumed scanning and re-initiation. Recent studies have shown that mGCN2 may have diverse functions as it is important in the brain for sensing dietary amino acid levels and for long-term memory formation.[8,9]

eIF2B is a multisubunit protein that is required for protein synthesis initiation and its regulation in all eukaryotic cells.[5] It is a complex of five nonidentical subunits (α, β, γ, δ, and ε) that are well conserved from yeast to human. eIF2B appears to be a key mediator of gene-expression control in response to environmental stress. In yeast several stress-response pathways, in addition to those involving Gcn2p, converge on eIF2B, and the events triggered downstream of eIF2B may differ depending on the nature of the stress. For example, during nitrogen starvation amino acid breakdown becomes an important nitrogen source. The breakdown products of this catabolism are fusel alcohols—fermentation-derived higher-order alcohols such as butanol or isoamyl alcohol—that function as potent chemical messengers apparently acting directly on eIF2B to inhibit global translation activation while specifically activating *GCN4* translation. Thus the addition of exogenous fusel alcohols rapidly increases levels of Gcn4p and slows growth.[10] It has been shown that the effect of the fusel alcohols is not mediated through Gcn2p and eIF2 phosphorylation and also that the downstream translational outcome differs from the Gcn2p-mediated response to amino acid starvation.[11]

Activation of *GCN4* may also be induced by specific pharmacologically active agents, giving rise to phenotypic responses similar to those associated with environmental stress. Volatile anesthetics, such as isofluorane or halothane, can reversibly inhibit protein synthesis in both mammalian cells and yeast. These responses are in part caused by eIF2B-dependent mechanisms.[12,13] Notably, the immunosuppressive and anticancer agent rapamycin activates *GCN4*-mediated stress responses through its inhibition of Tor1p and Tor2p, which are the yeast homologs of mammalian target of rapamycin, a mammalian phosphoinositide 3-kinase-related kinase.[14,15] Mammalian target of rapamycin is currently the subject of intense study because inhibitors of this key regulator of cell growth may have broad therapeutic potential.[16–19] An intriguing recent finding is that *GCN4* activation mediates replicative lifespan extension in yeast. Thus, increases in yeast lifespan caused by dietary restriction or treatment with the antibiotic diazaborine—to reduce levels of 60S ribosome subunits—were shown to be dependent on activation of *GCN4*.[20]

Translation of *GCN4* is also derepressed by specific mutations in eIF2B that give rise to the phenotypic responses such as slow growth normally induced upon environmental stress (*Fig. 1*). These yeast eIF2B mutants provide valuable tools to study a rare but devastating human disease. Mutations in human eIF2B genes have recently been found to cause the fatal disorder called childhood ataxia with central nervous system hypomyelination (CACH) or vanishing white matter disease (VWM).[21–24] Disease symptoms are apparently related to a reduction in the catalytic activity of the guanine nucleotide exchange factor activity of eIF2B. CACH/VWM is inherited as an autosomal recessive trait, and progression and symptoms of the disease are exacerbated by febrile infections or minor head trauma, usually leading to death by the age of 2–5 years.[21] Recent evidence implicates an abnormally heightened stress response as the cause of this extreme sensitivity to environmental insults. Up-regulation of the transcriptional activator ATF4 was observed in human embryonic kidney 293 cells containing human eIF2B mutations.[25] Furthermore, primary fibroblasts from CACH/VWM patients showed abnormally high levels of ATF4 in response to stress.[26] These findings extend to yeast, in which dysfunctional eIF2B has been shown to lead to translational activation of *GCN4*, the yeast homolog of human *ATF4*.[23]

We report here results of a high-throughput screen designed to discover chemical modulators of stress-response pathways in yeast. We focused specifically on eIF2B, because of its central role in activation of *GCN4* and the identification of human eIF2B mutants as the cause of CACH/VWM disease. A reporter gene assay was developed in which both cell growth and expression of the *GCN4* gene were measured in two yeast strains, wild-type *S. cerevisiae* and an eIF2Bε-R284H mutant that is equivalent to the clinically relevant human eIF2B5-R299H mutation. Both mutant and wild-type strains contain a chromosomally integrated *GCN4*-dependent reporter gene fusion to β-galactosidase. The assay was optimized for HTS by adaptation of a commercially available coupled β-galactosidase–firefly luciferase system for use with yeast cells.[27]

Mutant and wild-type yeast strains were screened in parallel to identify compounds acting specifically on either strain. The parallel screening strategy was designed to eliminate nonspecific compounds directly following HTS and thereby minimize the number of cherry-picks required. A compound that restores normal function to mutant eIF2B should give selective reduction of β-galactosidase activity in the mutant but not the wild-type strain. Such reduction in *GCN4* activation in the strain containing mutant eIF2B might indicate a compound capable of restoring normal function to human eIF2B that would have therapeutic utility against CACH/VWM disease. Conversely, a compound that activates *GCN4* expression through an eIF2B-mediated mechanism should selectively enhance β-galactosidase activity in the wild-type strain. These compounds might be useful chemical probes to study eIF2B-mediated pathways leading to stress responses. Comparison with known modulators of eIF2B such as the fusel alcohols may lead to further insights into the mechanism of action of these agents. Given the recently discovered roles for Gcn4p in protection against oxidative stress and enhancement of yeast replicative lifespan,[7,20] activators of *GCN4* may have potential to forestall the damaging effects of aging and age-related diseases.

This assay represents a novel approach to a target lacking useful chemical probes for mechanistic studies and pharmacological intervention. As such this project warranted significant development effort as it furthered the goals of the National Institutes of Health-funded Molecular Libraries Screening Center Network to address targets in underexplored complex areas of biology. We describe here technical challenges encountered in miniaturizing to a robot-compatible 384-well format and the implementation of a parallel screening strategy to discover modulators of stress-response pathways in yeast.

MATERIALS AND METHODS

Materials

Wild-type yeast cells strain GP4213 (*MATα leu2-3 leu2-112 ura3-52::HIS4–lacZ ino1 gcd6*Δ *gcn2*Δ::*hisG* pAV1265[GCD6 CEN6 LEU2]) and an isogenic *gcd6* mutant strain GP4198 (*MATα leu2-3 leu2-112 ura3-52::HIS4–lacZ ino1 gcd6*Δ *gcn2*Δ::*hisG* pAV1744[gcd6-R284H CEN6 LEU2]) were stored at −80°C as single-use frozen stocks, prepared as described below. YPD medium (10 g/L yeast extract, 20 g/L Bacto Peptone, and 20 g/L glucose), Breathe-Easy™ sealing films (catalog number Z380059), and buffer salts were purchased from Sigma-Aldrich (St. Louis, MO). The 384-well polypropylene V-bottom plates (catalog number 781280) were from Greiner Bio-One (Monroe, NC), and 384-well clear plates (catalog 3702) and white plates (catalog number 3652) were from Corning Life Sciences (Lowell, MA). Protease inhibitor tablets (catalog number 1873580) were from Roche Applied Science (Indianapolis, IN). The Beta-Glo® assay system (catalog number E4780) was from Promega (Madison, WI). Heat seals were applied using a Plate Loc heat sealer (Velocity 11, Menlo Park, CA), and polystyrene lids were from Nunc (Rochester, NY). The compounds tested here were supplied by BioFocus DPI (South San Francisco, CA) from the Molecular Libraries Small Molecule Repository.[28]

Preparation of Yeast Stocks

Yeast strains were taken from storage in a −80°C freezer and streaked onto a YPD plate, and colonies were grown by incubation of the plate for 3 days at 30°C. Cell stocks for the two yeast strains

were prepared by inoculating a single colony into 50 mL of YPD medium in a 250-mL Erlenmeyer flask and growing the culture overnight at 30°C with vigorous shaking (200 rpm). Cell concentration was determined after overnight growth by measurement of absorbance at 595 nm (A_{595}). The cell culture was centrifuged at 3,000 *g* for 5 min, and the pelleted cells were resuspended into YPD medium containing 15% (vol/vol) glycerol to give a cell suspension with $A_{595} = 5$. The cells were aliquoted for single use, frozen on dry ice, and stored at −80°C.

Compound Library

Compounds from the Molecular Libraries Small Molecule Repository were shipped on dry ice from BioFocus DPI as frozen 10 m*M* solutions in dimethyl sulfoxide (DMSO) in heat-sealed 384-well polypropylene plates. Prior to compound plating and shipping, quality control testing at BioFocus DPI confirmed compound solubility in DMSO, the presence of the expected molecular ion in liquid chromatography-mass spectrometry, and >90% purity by evaporative light scattering or ultraviolet absorbance at 214 nm. On arrival, plates were stored in desiccators (<20% relative humidity) at −25°C. After storage for approximately 3 months, plates were allowed to warm to room temperature in desiccators prior to use in HTS assay.

Optimization of Growth Conditions

The yeast growth assay was designed to determine turbidity of the cell suspension after overnight growth by measurement of A_{595}. A serial dilution of yeast cells was used to determine the linear range of absorbance values as a measure of cell density. Yeast cell stock frozen at −80°C was thawed and serially diluted in a clear 384-well plate in YPD medium to give 50 µl per well of dilutions ranging from 1:5 to 1:500. A_{595} was measured using an Envision spectrophotometer (PerkinElmer, Boston, MA).

The optimal time to incubate the yeast suspensions in the HTS assay prior to recording growth was selected by determining a growth time course of over 24 h. For each time point a clear polystyrene 384-well plate was filled with 25 µl per well of YPD medium, and wells designated as blanks were filled with an additional 25 µl of medium in place of yeast cells. Thawed wild-type and mutant yeast cells were diluted 1:500 in YPD medium, and 25 µl of one of the diluted stocks was inoculated to the appropriate wells. Plates were sealed with Breathe-Easy membranes, and the yeast cultures were incubated at 30°C for 4, 8, 16, or 24 h. After incubation, the Breathe-Easy membranes were removed, and A_{595} was measured.

To determine the effect of DMSO, both wild-type and mutant yeast cells were grown for 16 h in the presence of 0–1.6% (vol/vol) DMSO. The growth assay was as described above, except that DMSO was mixed with medium in the assay plate prior to the addition of yeast cells. Wells without added DMSO were included as controls.

HTS Assay

The assay protocol followed here is based on one described previously.[27]

Absorbance assay to record yeast growth. For mutant yeast strain HTS, V-bottom polypropylene 384-well plates were filled with 25 µl of YPD medium using a Multidrop-384 reagent dispenser (Thermo Scientific, Franklin, MA), and an additional 25 µl of YPD medium was added to column 1. Compounds were added by pintool transfer on an EP3 workstation (PerkinElmer). A 384-pin pintool (V&P Scientific, San Diego, CA) was used to transfer 120 nl of a 10 m*M* solution in DMSO into the medium-containing V-bottom growth plates, giving a final concentration of 24 µ*M* of each compound in 0.24% DMSO. Wells in columns 1, 2, 23, and 24 were reserved for controls and blanks and contained 0.24% DMSO but no compound. Thawed mutant yeast cells were diluted 1:500 in YPD medium, and 25 µl was inoculated in all columns except 1 and 23 in the V-bottom growth plates using a Multidrop Micro reagent dispenser (Thermo Scientific). Likewise, 25 µl of diluted wild-type yeast cells was inoculated in column 23 of each V-bottom plate. Plates were sealed with Breathe-Easy membranes, and the yeast cultures were incubated for 16 h at 30°C. Following growth the cell suspension was mixed on the EP3 workstation by 10 aspirate–dispense cycles using 30-µl disposable tips, in which 25 µl of suspension was aspirated from 0.1 mm above the well bottom at a rate of 30 µl/s. Placing the tips as close as possible to the well bottom ensured adequate mixing of cells, while avoiding bubble formation. Immediately following mixing, 5 µl of the cell suspension was used for the luminescence assay (see below). Thirty microliters of the portion that remained was transferred to a clear 384-well plate, and A_{595} was recorded.

Beta-Glo luminescence assay to record β-galactosidase activity. Twenty microliters per well of Z-buffer (82 m*M* disodium hydrogen phosphate, 9 m*M* sodium dihydrogen phosphate, and 0.1% sodium dodecyl sulfate) was added to white 384-well plates using a Multidrop Micro reagent dispenser, followed by 5 µl of cell suspension by tip transfer from V-bottom yeast growth plates (see above). After cells and Z-buffer were mixed and incubated for 20 min at room temperature to permeabilize the cells, Beta-Glo reagent (25 µl) was added using a Multidrop Micro reagent dispenser, and luminescence was measured on an Envision plate reader after a further 90-min incubation at room temperature to allow the luminescence to reach a steady state.

The protocol for wild-type yeast strain HTS was identical to that described above for the mutant strain except that wild-type

yeast was added to all columns except 1 and 23 and mutant yeast was added to column 23 only.

50% Inhibitory Concentration (IC_{50}) and 50% Effective Concentration (EC_{50}) Dose–Response Assay

This assay was identical to the HTS assay, except that 16 twofold dilutions of each compound were tested to give a final concentration range of 100 µ*M* to 3 n*M* in 1% DMSO. Compounds were twofold serially diluted in DMSO as follows: hits selected from HTS were resupplied by BioFocus DPI as frozen 10 m*M* solutions in 23 µl of DMSO. Compounds were arranged 20 per plate in wells A3–A22 of 384-well V-bottom polypropylene plates. For dose–response testing frozen plates were allowed to warm to room temperature in desiccators prior to serial dilution using an EP3 pipetting workstation. DMSO (23 µl) was added to wells A1, A2, A23, and A24, followed by 11 µl per well of DMSO added to the entire plate except row A using a Multidrop Micro reagent dispenser. The compounds were twofold serially diluted by transfer of 11 µl row-by-row from row A to row P using a single row of disposable tips on an EP3 384-tip pipetting head, after which 11 µl was discarded from row P. The resulting dose–response plates contained 16 twofold dilutions (10 m*M*–305 n*M*) of each compound, arranged one compound per column in columns 3–22. Plates were heat-sealed and stored at room temperature for 1–2 days in desiccators prior to use.

HTS Data Analysis

Data were analyzed using ActivityBase (IDBS, Guildford, UK). Each HTS plate contained compounds (25 µ*M* in 0.24% DMSO) in columns 3–22, controls (wild-type cells for wild-type screen and mutant yeast cells for mutant screen) in columns 2 and 24, reference yeast strain (wild-type cells for mutant screen and mutant cells for wild-type screen) in column 23, and blanks (YPD medium) in column 1. Percentage inhibition of growth or percentage inhibition of luminescence was calculated for each compound from the signal in absorbance units or luminescence units, respectively, and the mean of the plate controls and the mean of the plate blanks using Eq. 1:

$$\%\ \text{Inhibition} = 100 \times \{1 - [(\text{signal} - \text{blank mean})/(\text{control mean} - \text{blank mean})]\} \quad (1)$$

The reference yeast strain (column 23) is not used in the calculation. It was used to monitor consistency of cell growth throughout the screen. Percentage inhibition of luminescence was corrected for cell growth using Eq. 2:

$$\text{Corrected \% inhibition} = 100 - [(100 - \%\ \text{inhibition})/(100 - \%\ \text{inhibition of growth})] \quad (2)$$

Results were retrieved from ActivityBase using the SARgen data retrieval tool, arranged in Excel (Microsoft, Redmond, WA), and deposited into PubChem.[29]

IC_{50} and EC_{50} Dose–Response Data Analysis

Dose–response plates contained one compound per column in columns 3–22, controls (wild-type cells for wild-type dose–response plates and mutant yeast cells for mutant dose–response plates) in columns 2 and 24, reference yeast strain (wild-type cells for mutant dose–response plates and mutant cells for wild-type dose–response plates) in column 23, and blanks (YPD medium) in column 1. Each column from 3 to 22 contained 16 twofold dilutions of a single compound, ranging in concentration from 100 µ*M* to 3 n*M*. IC_{50} and EC_{50} values were calculated in an Excel-based ActivityBase template as follows. Percentage activity was calculated for each dilution of each compound from the signal in luminescence units and the means of the plate controls and plate blanks using Eq. 3:

$$\%\ \text{Activity} = 100 \times [(\text{signal} - \text{blank mean})/(\text{control mean} - \text{blank mean})] \quad (3)$$

In the case of inhibitors of luminescence, no dose-dependent effect on growth was observed, so luminescence assay dose–response curves were fit, and IC_{50} values were calculated without correction for cell growth. Conversely, some of the enhancers of luminescence showed a dose-dependent effect on growth, so the luminescence assay percentage activity values at each compound dose were corrected for cell growth as shown in Eq. 4:

$$\text{Corrected \% activity} = \%\ \text{activity}/\%\ \text{growth (relative to growth controls)} \quad (4)$$

Dose–response curves of percentage activity were fit in XLfit (IDBS), using a four-parameter logistic fit (Eq. 205 with maximum percentage activity and minimum percentage activity fixed at 100 and 0, respectively). Results were retrieved from ActivityBase using the SARgen data retrieval tool, arranged in Excel, and deposited into PubChem.[30]

RESULTS

Screening Strategy

The goal of the screening described here was twofold: first, to identify compounds that restore the function of mutant eIF2B in yeast cells; and second, to find activators of *GCN4* in the presence of functional wild-type eIF2B. A reduction in eIF2B function reduces the overall translation rate and tends to cause a reduced rate of growth. It additionally activates translation of the stress-response gene *GCN4*, so that the strain expressing the reduced-function mutant eIF2B has elevated Gcn4p protein levels

and elevated activation of *GCN4* target genes, including *HIS4*.[23,27] Thus any compound that restores mutant eIF2B activity should selectively reduce *GCN4* activation in the mutant yeast strain. Conversely, compounds that activate *GCN4* in an eIF2B-dependent manner should give selective enhancement of reporter gene activity in the wild-type strain.

Modifiers of amino acid metabolism are known to influence signaling to eIF2B/*GCN4*. For example, 3-aminotriazole is an inhibitor of imidazoleglycerol-phosphate dehydratase, encoded in yeast by *HIS3*, which catalyzes the sixth step in histidine biosynthesis. 3-Aminotriazole and other compounds activate the protein kinase Gcn2p. To avoid identifying known or other activators of Gcn2p, which would be indirect modulators of eIF2B, the strains used here were deleted for *GCN2*. In addition, amino acid analogs that can be incorporated into proteins are toxic when yeast are grown in minimal medium. *GCN4*-mediated activation of amino acid biosynthetic enzymes can overcome this toxicity.[23] To avoid selection of toxic amino acid analogs, yeast strains were grown in rich medium (YPD) for this analysis.

Compounds were screened in parallel against two yeast strains, wild-type *S. cerevisiae* and an eIF2Bε-R284H mutant that is equivalent to the clinically relevant human eIF2B5-R299H mutation. Both mutant and wild-type strains contain a chromosomally integrated gene fusion of *HIS4* to β-galactosidase, allowing *GCN4* activation to be monitored using a coupled β-galactosidase–firefly luciferase readout adapted from commercially available reagents.[27] Constitutive down-regulation of eIF2B activity and hence elevated *GCN4* activity are often accompanied by slow growth.[10,23] In this work, loss of eIF2B function in the mutant strain was observed to cause a modest reduction in overnight yeast growth (*Figs. 1* and *2*). However, this change was not statistically significant in the presence of 0.25% DMSO, the DMSO content selected for HTS (*Fig. 3*). Compounds that restore the function of eIF2B in the mutant cells therefore were expected to modestly enhance the growth rate of the mutant yeast strain. Conversely, activators of *GCN4* in the wild-type strain may slow its growth. Thus an HTS strategy was designed in which compounds were screened for their effects on both overnight yeast growth and β-galactosidase activity. The two sets of results were combined by correcting the β-galactosidase data to take account of each compound's effect on growth. In the case of the mutant eIF2B strain this correction was used to reveal compounds that enhanced growth to such a degree that inhibition of β-galactosidase expression was masked. These compounds would show little apparent uncorrected inhibition of β-galactosidase expression due to the increased cell number relative to the plate controls. This analysis also allowed us to exclude toxic compounds that showed apparent inhibition of β-galactosidase activity that was in fact due the lower cell number resulting from growth inhibition or cell killing. In the case of compounds activating *GCN4* in the wild-type strain, growth correction revealed enhancement of luminescence that would otherwise have been masked by the slowed growth that often accompanies *GCN4* activation.

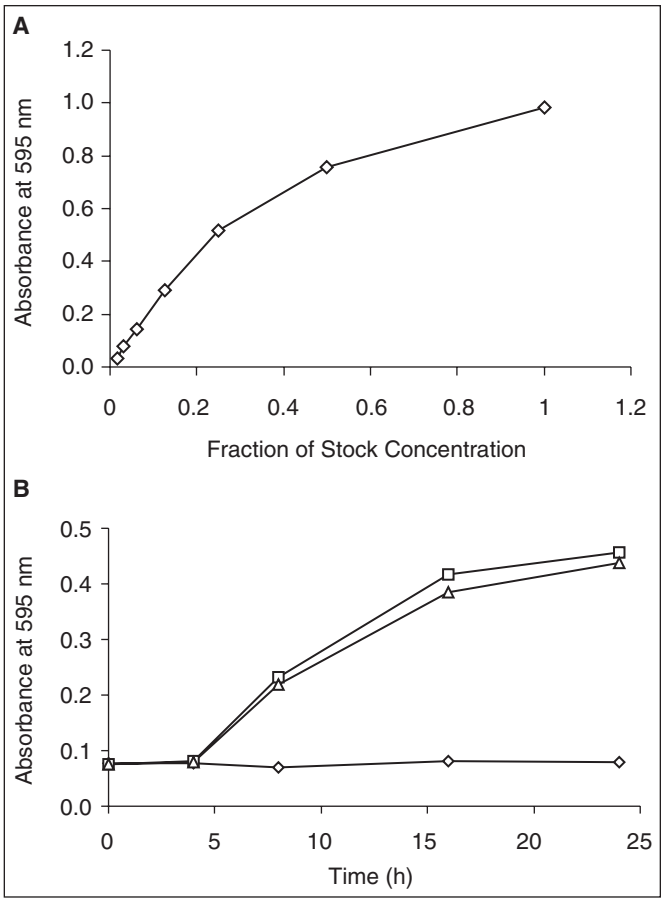

Fig. 2. Time course of yeast growth. (**A**) Dilution of a suspension of mutant strain yeast cell stock used to determine the linear range of A_{595}. (**B**) Growth time course of wild-type (□) and mutant (△) yeast determined by measurement of A_{595} at each time point. Blanks consisting of medium alone (◇) were used to monitor A_{595} in the absence of cell growth. Experimental details are described in Optimization of Growth Conditions in Materials and Methods.

Assay Development and Validation

Before embarking upon HTS, it was necessary to ensure that robotic handling would allow accurate and precise measurements of yeast cell growth and reporter activity and to assess whether the strains were compatible with DMSO, the vehicle used to deliver test compounds.

Growth assay. To allow sufficient time to discern test compound effects on yeast growth, the assay was designed to measure cell density after overnight incubation with each compound. In addition to the importance of ensuring that the yeast cells remained in an exponential growth phase, it was also necessary to confirm the linearity of the absorbance readings used to measure cell turbidity. Above a certain threshold, light scattering causes the correlation between cell density and absorbance to deviate from linearity.[31] A standard curve of serially diluted yeast stock showed that absorbance values up to approximately 0.4 are directly proportional to cell density (*Fig. 2A*). Overnight growth curves confirmed that 16-h growth of a 1:1,000 dilution of the frozen yeast stocks gave absorbance values within the linear range of detection (*Fig. 2B*). Sensitivity of yeast cell growth to DMSO was also assessed as several reports have described effects of DMSO on yeast cells.[32] As shown in *Fig. 3* there was no significant effect on growth at 0.25% DMSO, but a reduction of almost 25% in the presence of 1% DMSO. Interestingly, growth of the mutant yeast was consistently repressed more strongly than that of wild-type yeast. To prevent inconsistencies and false-positives due to growth suppression, we limited the DMSO concentration in the HTS to 0.25%.

β-Galactosidase assay. Measurement of β-galactosidase activity after overnight yeast growth showed that, as previously observed,[27] the mutant eIF2B strain gave at least twofold higher luminescence than the wild-type (*Fig. 4*). The rate of light output reached a steady state after 90 min at room temperature, allowing for a single end-point read after this time in the HTS. The observed difference between mutant and wild-type luminescence defined the assay signal window in the screen for compounds that restore function to mutant eIF2B; the goal was to identify compounds that reduced the mutant strain β-galactosidase activity towards the lower activity of the wild-type strain. Conversely, an increase in β-galactosidase activity in the wild-type strain was expected to reveal compounds that activate *GCN4* in the presence of wild-type eIF2B. Both yeast strains were included in each HTS plate to monitor the magnitude and consistency of the luminescence values. Thus, one control column in mutant HTS plates contained wild-type yeast, and likewise wild-type plates contained a control column of mutant yeast.

To ensure well-to-well consistency of the luminescence readings, a critical parameter that had to be carefully optimized was the mixing and transfer of the cell suspensions from the yeast growth plate to the β-galactosidase assay plate. After overnight growth the yeast cells needed to be resuspended to ensure efficiency of transfer of a small volume of cells for the luminescence assay. Mixing in a flat-bottom clear plate using 30-µl tips on the EP3 liquid handling workstation tended to concentrate the cells to the rims of the well; it was found to be extremely difficult to

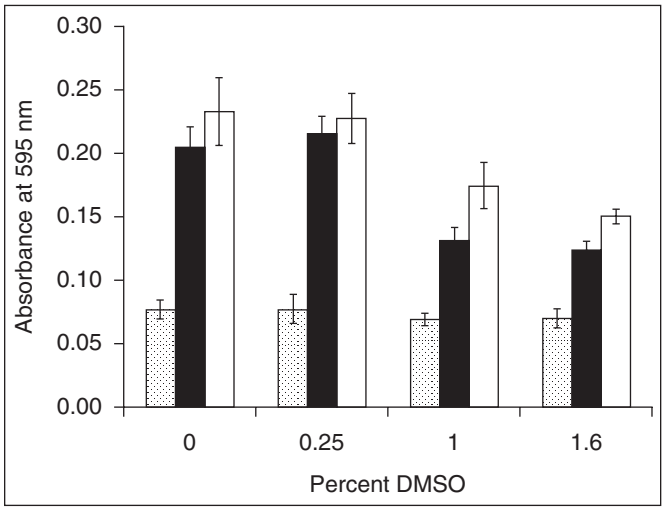

Fig. 3. Effect of DMSO on growth of wild-type and mutant yeast cells. Cell growth after 16 h was determined by measurement of A_{595} of a suspension of wild-type cells (open columns), mutant cells (solid columns), or medium blank (stippled columns) as described in the section entitled Optimization of Growth Conditions in Materials and Methods. Data are mean values, and error bars represent ± 1 SD of 16 replicate wells.

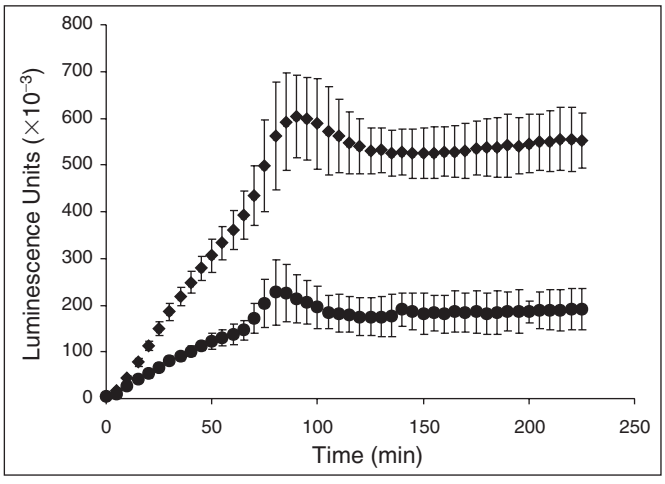

Fig. 4. Time course of luminescence signal. Wild type (●) and mutant (◆) yeast strains were grown overnight and mixed with Beta-Glo reagent as described in the section entitled HTS Assay in Materials and Methods. DMSO was added by pintool in place of test compounds. Luminescence was read at 5-min intervals for 225 min. Data are mean values, and error bars represent ± SD of 16 replicate wells. In this experiment there was no significant difference in growth rates between mutant and wild-type strain, and the data shown have not been corrected for cell growth.

get uniform mixing in these plates. Therefore, we decided to grow cells in V-bottom polypropylene plates. Efficient mixing necessitated slow aspiration of the cell suspension with each pipette tip a fraction of a millimeter above the well bottom, and this in turn required very precise adjustment of the EP3 head to ensure uniform alignment of the tips across an entire 384-well plate. Using V-bottom plates we were able to achieve robust growth and reproducible mixing, leading to consistent results in the luminescence assay as shown in the pilot HTS plate in *Fig. 5*.

Screening and Hit Confirmation

A total of 27,199 compounds were screened once at a concentration of 24 μ*M* against both the eIF2B mutant and the corresponding wild-type yeast strain for their effects on cell growth and expression of the β-galactosidase reporter. Most of the yeast growth plates gave robust *Z*'-factors, whereas a substantial number of the luminescence assay plates gave a *Z*'-factor below the 0.5 value that is often used as an indicator of a robust HTS assay (*Fig. 6*). Based on the mean coefficients of variance of the plate controls, which varied between 14.6% and 20.4% for the four sets

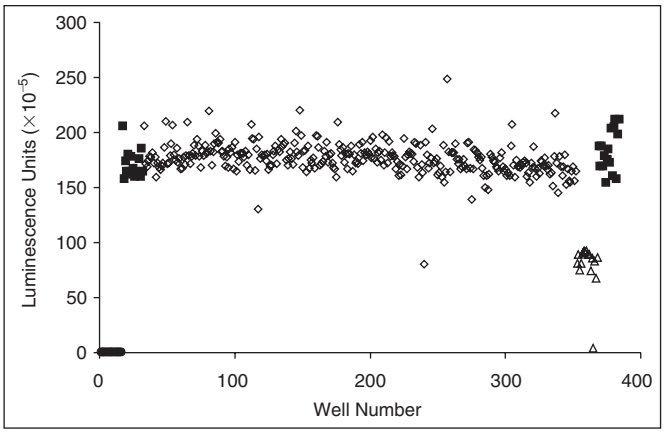

Fig. 5. Mutant strain luminescence assay validation plate. Mutant control (■), wild-type control (△), blank (●), and test (◇) wells were assayed for β-galactosidase expression as described in the section entitled HTS Assay in Materials and Methods.

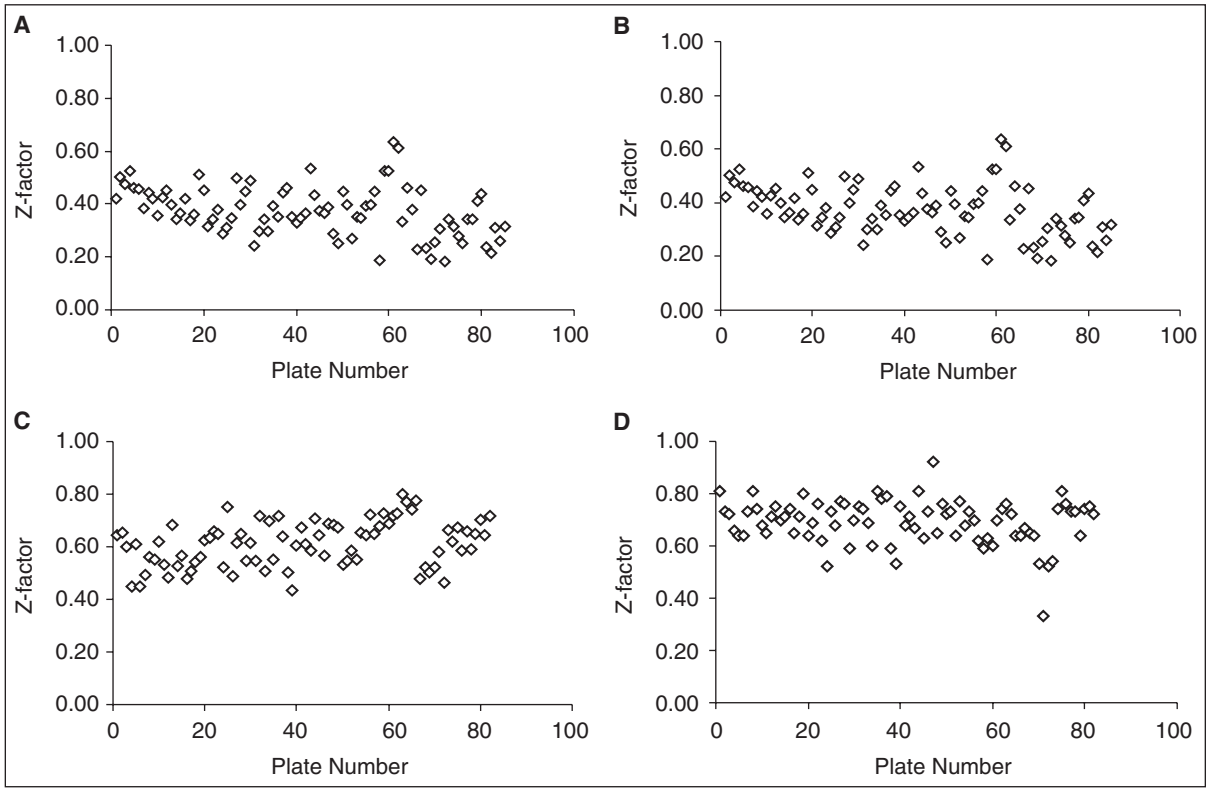

Fig. 6. HTS plate *Z*'-factors: (**A**) mutant luminescence assay, (**B**) wild-type luminescence assay, (**C**) mutant growth assay, and (**D**) wild-type growth assay. Experimental protocols are described in the section entitled HTS Assay in Materials and Methods.

of plates, we selected compounds exceeding a threshold of 50% deviation from the plate control mean for further evaluation. The modest *Z'*-factors suggested that a substantial number of the putative hits would be false-positives and that weak compounds might be lost in the assay noise. Nevertheless, the combination of the 50% hit threshold and the 24 μ*M* screening concentration was expected to reveal actives with the desired IC_{50} or EC_{50} values below 10 μ*M*. Analyses of the actives inhibiting mutant luminescence and those enhancing wild-type luminescence are shown in *Tables 1* and *2*, respectively.

Screening for inhibitors of reporter gene function. Without correction for cell growth, 355 actives inhibited mutant luminescence, a hit rate of 1.35% (*Table 1*). Assuming a normal distribution of the data, 108 compounds at random (0.4%) would be expected to exceed the 50% inhibition threshold, based on this threshold being 2.7-fold greater than the SD of the HTS data.[33] Therefore, even taking into account the expected scatter of the data, the mutant luminescence assay gave a relatively large number of hits. After correction for cell growth, this number increased to 540. Although 75 compounds appeared to be toxic based on their inhibition of yeast growth and were removed from the original list of 355, a further 260 compounds in addition to the original 355 showed enhanced cell growth such that growth correction revealed inhibition of luminescence, so these were included as active. Similar analysis of the wild-type luminescence assay used as the counterscreen revealed 448 compounds that gave >30% inhibition after correction for the effect of each compound on wild-type cell growth. Comparison of the mutant and wild-type lists of actives revealed 146 compounds that inhibited luminescence in both the mutant and wild-type strains; exclusion of these compounds left 394 hits that appeared selective for the mutant strain. Analysis of activation results (*Table 2*) revealed 420 compounds that gave >50% enhancement of wild-type luminescence prior to growth correction. Growth correction increased this number substantially, but a number of compounds caused severe growth inhibition suggestive of toxicity. Compounds that caused >50% inhibition of growth were judged to be toxic and excluded from further analysis, leaving 985 compounds that gave 50% activation in the wild-type strain after growth correction. Of these, 896 appeared to be selective for the wild-type and were selected for retesting. The 1,290 actives, consisting of 394 inhibitors of mutant luminescence and 896 activators of wild-type luminescence, from the consolidated list were tested in a dose–response assay consisting of 16 twofold dilutions starting at a top concentration of 100 μ*M*. As in the HTS, overnight cell growth and β-galactosidase expression was determined with both mutant and wild-type yeast.

Table 1. Summary of Mutant Inhibition HTS Results

Active Compound Classification	Number of Inhibitors		Hit Rate (Inhibition of Mutant)
	>50% Inhibition of Mutant	>30% Inhibition of Wild-Type	
Primary HTS actives	355	403	1.35%
Actives removed after growth correction	75	114	
Actives revealed after growth correction	260	159	
Total actives after growth correction	540	448	2.05%
Actives common to mutant and wild-type	146	146	
Mutant-selective actives	394	N/A	1.46%

Primary HTS actives are defined as compounds that gave >50% inhibition of β-galactosidase activity in the mutant strain, >30% inhibition in the wild-type strain, or both. Actives removed after growth correction are either (1) compounds that gave >50% inhibition of β-galactosidase activity in the mutant strain but <50% inhibition after correction for cell growth as described in Materials and Methods or (2) compounds that gave >30% inhibition of β-galactosidase activity in the wild type strain but <30% inhibition after correction for cell growth. Conversely, actives revealed after growth correction are either (1) compounds that gave <50% inhibition of β-galactosidase activity in the mutant strain but >50% inhibition after correction for cell growth as described in Materials and Methods or (2) compounds that gave <30% inhibition of β-galactosidase activity in the wild-type strain but >30% inhibition after correction for cell growth. Total actives after growth correction is the number of primary HTS actives less the number of actives removed after growth correction plus the number of actives revealed after growth correction. Actives common to mutant and wild-type are the subset of total actives after growth correction that gave >50% inhibition of β-galactosidase activity in the mutant strain and >30% inhibition in the wild-type strain. Mutant-selective actives are the subset of total actives after growth correction that gave >50% inhibition of β-galactosidase activity in the mutant strain and <30% inhibition in the wild-type strain. The number of mutant-selective actives (394) equals the total actives in the mutant strain after growth correction (540) minus the actives common to mutant and wild-type (146).

Table 2. Summary of Wild-Type Activation HTS Results

Active Compound Classification	Number of Activators		Hit Rate (Activation of Wild-Type)
	>50% Activation of Wild-Type	>30% Activation of Mutant	
Primary HTS actives	420	751	1.54%
Actives removed after growth correction	82	175	
Actives revealed after growth correction (<50% inhibition of growth)	647	898	
Total actives after growth correction	985	1,474	3.61%
Actives common to wild-type and mutant	89	89	
Wild type-selective actives	896	N/A	3.28%

Primary HTS actives are defined as compounds that gave >50% activation of β-galactosidase activity in the wild-type strain, >30% activation in the mutant strain, or both. Actives removed after growth correction are either (1) compounds that gave >50% activation of β-galactosidase activity in the wild-type strain but <50% activation after correction for cell growth as described in Materials and Methods or (2) compounds that gave >30% activation of β-galactosidase activity in the mutant strain but <30% activation after correction for cell growth. Conversely, actives revealed after growth correction are either (1) compounds that gave <50% activation of β-galactosidase activity in the wild-type strain but >50% activation after correction for cell growth as described in Materials and Methods or (2) compounds that gave <30% activation of β-galactosidase activity in the mutant strain but >30% activation after correction for cell growth. Compounds revealed as active after growth correction that gave >50% inhibition of growth were judged to be toxic and were excluded from the list of actives. Total actives after growth correction is the number of primary HTS actives minus the number of actives removed after growth correction plus the number of actives revealed after growth correction (<50% inhibition of growth). Actives common to wild-type and mutant are the subset of total actives after growth correction that gave >50% activation of β-galactosidase activity in the wild-type strain and >30% activation in the mutant strain. Wild-type-selective actives are the subset of total actives after growth correction that gave >50% activation of β-galactosidase activity in the wild-type strain and <30% activation in the mutant strain. The number of wild-type-selective actives (896) equals the total actives in the wild-type strain after growth correction (985) minus the actives common to wild-type and mutant (89).

Only 17 out of 394 putative inhibitors of mutant strain luminescence gave an IC_{50} <100 μ*M* against the mutant strain, a retest rate of 4.3%. Interestingly, 13 of the confirmed actives were compounds from the original 355 mutant luminescence actives prior to correction for cell growth, and only four additional compounds were found in the additional 260 compounds selected from the HTS as inhibitors of mutant luminescence after correction for apparent enhancement of cell growth. Furthermore, none of the additional 260 compounds showed concentration-dependent enhancement of overnight cell growth, suggesting that the elevated cell number values in the HTS did not represent reproducible enhancement of growth. Thus the luminescence HTS results prior to growth correction were in fact more reliable predictors of activity (or lack thereof) on dose–response confirmation than the growth-corrected results. Testing of the putative mutant-selective luminescence inhibitors in dose–response against the wild-type strain revealed that all the confirmed active compounds gave similar IC_{50} values against both mutant and wild-type cells; thus none of the compounds was selective for the eIF2B mutant. Results are shown in *Table 3*, and IC_{50} curves for the most potent compounds are given in *Fig. 7*. To ensure that compounds acting selectively on mutant eIF2B had not been missed in the hit selection process, two more groups of samples were tested in dose–response: first, compounds that were actives in the mutant luminescence assay before correction for cell growth but were just under the 50% inhibition threshold after correction; and second, compounds that appeared to be potent but nonselective inhibitors in the original HTS. All of the confirmed actives from these selections showed similar IC_{50} values against the mutant and wild-type strains. *Table 3* also includes luciferase enzyme inhibition data reported by the San Diego Center for Chemical Genomics (SDCCG).[34] Comparison of our results with the biochemical assay data from the SDCCG shows that the activity of all but five of the confirmed hits in the cell-based screen may be accounted for by inhibition of the luciferase enzyme in the Beta-Glo reagent. The fact that the active compounds are luciferase inhibitors accounts for their equal potency in both wild-type and mutant screens.

Screening for activators of reporter gene function. Out of 896 putative activators of wild-type luminescence, only two showed dose-dependent enhancement of luminescence, a retest rate of

Table 3. Confirmed Active Inhibitors of Mutant Strain Luminescence

Compound Number	PubChem SID[a]	% Inhibition				IC_{50} (μM)[b]		% Inhibition of Luciferase at 10 μM[c]
		Before Growth Correction		After Growth Correction				
		eIF2B Mutant	Wild-Type	eIF2B Mutant	Wild-Type	eIF2B Mutant	Wild-Type	
Mutant-selective (before and after growth correction to % inhibition)[d]								
1	14745497	73.2	26.5	74.4	29.6	6 ± 0.47	3.69 ± 0.9	96.9
2	14744660	77.4	7.6	78.5	15.2	6.61 ± 0.6	3.61 ± 0.51	94.2
3	14722165	82.4	−9.1	85.1	4.1	7.38 ± 0.13	3.87 ± 2.41	95.3
4	7976637	67.0	27.9	66.2	9.5	13.7 ± 2	17.9 ± 13.5	80.3
5	4259689	58.3	−17.9	51.3	−36.3	14.8 ± 1.8	13.1 ± 2.4	7.5
6	14745243	51.1	−11.4	51.1	−6.8	16.5 ± 4.1	16.4 ± 1.6	30.4
7	4244098	66.4	−0.9	74.2	−8.1	16.7 ± 0.6	10.2 ± 4.1	63
8	844378	54.3	15.5	50.6	−25.5	26.3 ± 2.3	31.2 ± 5.6	16
9	14724006	72.4	−39.2	70.7	−74.0	33.8 ± 8.4	35.5 ± 14.3	90
10	862355	65.9	14.8	65.9	22.1	59.3 ± 6	44.1 ± 12.8	−6
11	4251901	53.1	21.7	57.0	24.9	75.2 ± 3.5	92.6 ± 12.8	39.1
12	4250979	69.0	11.4	67.9	10.0	85.3 ± 1.2	98.7 ± 1.9	57.5
13	7976887	70.4	10.0	78.6	25.7	92.2 ± 3.3	>100	39.5
Mutant-selective (after growth correction to % inhibition)								
14	7974876	64.0	42.8	57.9	24.1	19.7 ± 1.1	12 ± 8.1	73.9
15	14726364	39.7	14.1	57.8	13.1	81.6 ± 5.9	84.2 ± 20.3	26.5
16	14743463	43.0	−1.7	52.5	3.7	84.4 ± 5.7	65.4 ± 21.6	3.6
17	4264128	45.8	28.6	51.5	12.6	86 ± 0.4	>100	−2.4
Mutant-selective (before growth correction to % inhibition but not after correction)								
18	14744764	55.8	3.3	41.9	−36.3	15 ± 1.4	15.9 ± 2.7	37.6
19	4257725	51.1	0.7	37.8	0.1	18 ± 1.6	14.7 ± 4.3	37.3
20	14743794	52.6	0.6	47.6	−30.2	18.4 ± 0.2	11.1 ± 6.6	11.5
21	4262348	54.2	28.6	45.8	3.4	20.7 ± 2.5	17.4 ± 2.4	51.1
22	4258477	51.5	35.9	50.0	19.5	36.4 ± 1.1	18.2 ± 10.5	42.4
23	14743208	50.3	22.3	44.8	22.8	42.2 ± 2.3	61.1 ± 33.7	33.6
Nonselective[e]								
24	14746372	91.87	87.41	90.71	88.10	4.45 ± 0.28	2.78 ± 2.04	−4.5
25	14739728	85.29	72.39	80.88	64.42	12.2 ± 2	6.14 ± 4.32	84.9

[a]Substance ID used to retrieve biological data and chemical properties from PubChem.
[b]IC_{50} values are expressed as the mean of three independent determinations ± SD.
[c]See http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=1006.[34]
[d]Mutant-selective defined as >50% inhibition of mutant luminescence and <30% inhibition of wild-type luminescence.
[e]Nonselective defined as >50% inhibition of mutant luminescence and >30% inhibition of wild-type luminescence.

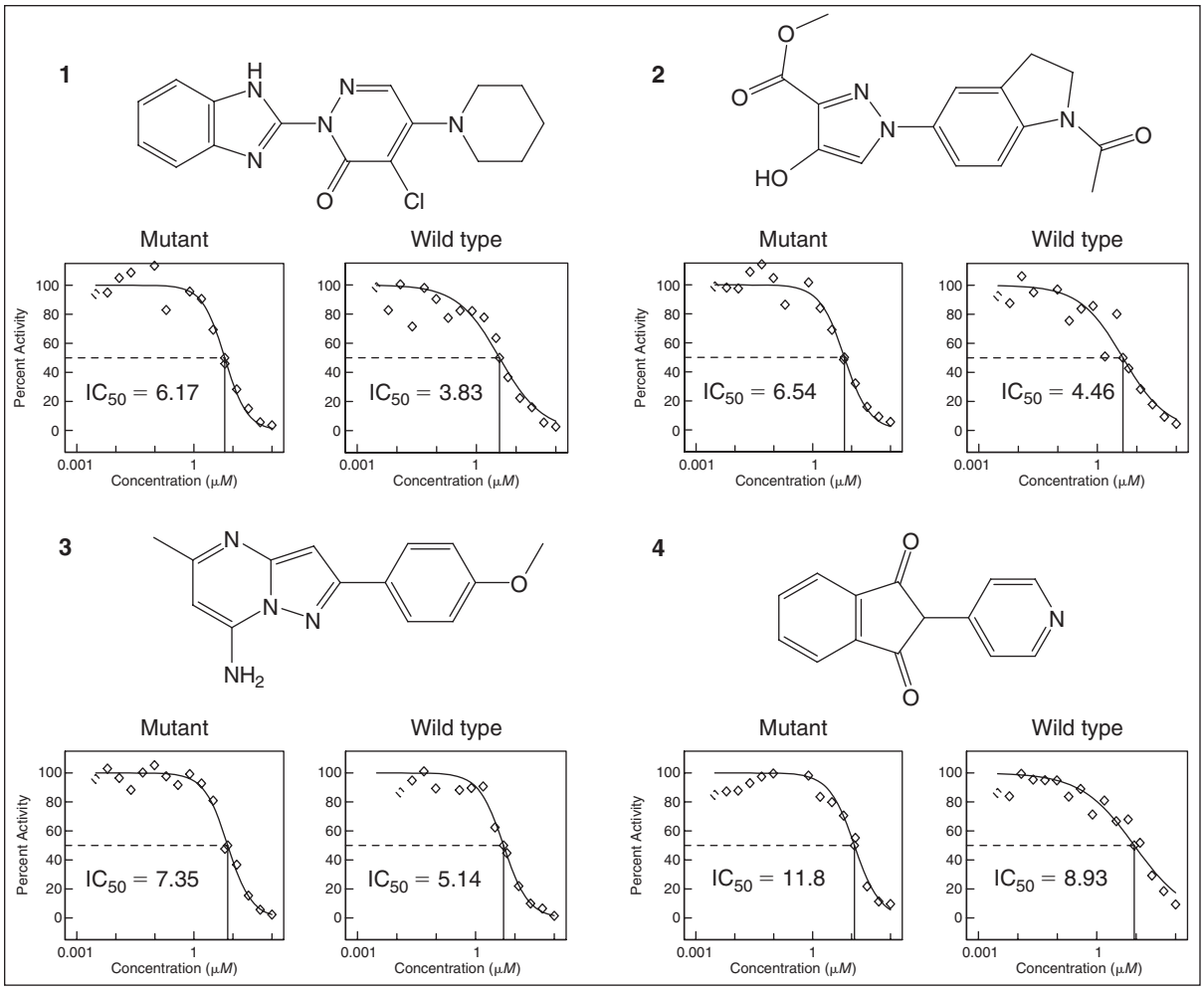

Fig. 7. Selected dose–response curves and IC_{50} values against mutant and wild-type luminescence for the most potent confirmed inhibitors of mutant strain luminescence. Compound numbers refer to those listed in Table 3. The assay was performed as described in the section entitled IC_{50} and EC_{50} Dose–Response Assay in Materials and Methods. Curve fitting and calculation of IC_{50} values were as described in the section entitled IC_{50} and EC_{50} Dose–Response Data Analysis in Materials and Methods. IC_{50} values are expressed in micromolar; each value corresponds to the specific curve shown and does not equal the mean values reported in Table 3: for compound **1**, (**left**) mutant and (**right**) wild-type, 6.17 and 3.83, respectively; for compound **2**, 6.54 and 4.46, respectively; for compound **3**, 7.35 and 5.14, respectively; for compound **4**, 11.8 and 8.93, respectively; for compound **5**, 12.6 and 13.9, respectively; and for compound **6**, 16.6 and 18.2, respectively. None of the compounds showed a dose-dependent effect on yeast growth, so the data have not been corrected for cell growth.

0.2%. Both confirmed actives were compounds from the original 420 actives prior to correction for cell growth; none was confirmed from the additional 476 compounds selected from the HTS as activators of wild-type luminescence after correction for inhibition of cell growth. Both actives showed modest concentration-dependent inhibition of overnight cell growth, up to 19% and 10% inhibition, respectively, at the highest concentration tested (100 μ*M*). Therefore, EC_{50} values were calculated from a curve fit to the data after correction for cell growth. Both active compounds were confirmed to be selective enhancers of luminescence in the wild-type strain, suggesting that they are in fact activating *GCN4* via an eIF2B-mediated mechanism. Results are shown in *Table 4*, and EC_{50} curves are given in *Fig. 8*. Inspection of the luciferase enzyme inhibition data from the SDCCG[34] showed that neither of the confirmed enhancers had any significant effect on luciferase enzyme activity.

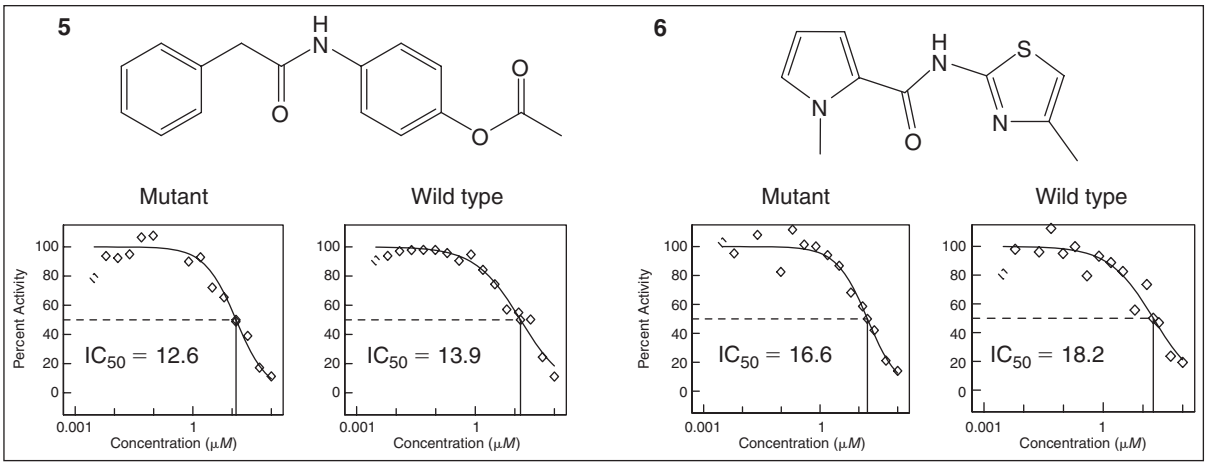

Fig. 7. (*Continued*)

Table 4. Confirmed Active Enhancers of Wild-Type Strain Luminescence

Compound Number	PubChem SID[a]	% Enhancement				EC_{50} (μ*M*)[b]	
		Before Growth Correction		After Growth Correction			
		Wild-Type	eIF2B Mutant	Wild-Type	eIF2B Mutant	Wild-Type	eIF2B Mutant
26	4261321	52.37	24.14	93	14.2	27.66 ± 7.12	>100
27	865813	63.71	−3.37	142	44.94	2.83 ± 1.47	>100

[a]Substance ID used to retrieve biological data and chemical properties from PubChem.
[b]EC_{50} values are expressed as the mean of three independent determinations ± SD.

DISCUSSION

The screening described here achieved our second goal, to find activators of *GCN4* in the presence of functional wild-type eIF2B, but not our first, to identify compounds that restore the function of mutant eIF2B in yeast cells. Overall, HTS and dose–response confirmation successfully identified two compounds that appeared to cause a stress response resulting in *GCN4* activation, but none that reduced *GCN4* activation by selectively restoring mutant eIF2B function.

Preliminary studies confirmed our expectation of a high inhibition rate for the β-galactosidase reporter assay. The parallel wild-type assay thus served as a counterscreen to aid in the identification of specific inhibitors of the mutant eIF2B yeast strain by eliminating nonspecific hits such as luciferase inhibitors or modulators of the expression or activity of β-galactosidase working by a non–eIF2B-related mechanism. Conversely, the mutant eIF2B assay served as a counterscreen to identify compounds that selectively enhance wild-type luminescence by eliminating nonspecific enhancers of the luminescent readout.

In the search for compounds that selectively restore mutant eIF2B function, no mutant growth enhancement was observed on dose–response testing, and all the compounds inhibiting mutant luminescence were equally potent against wild-type. The fact that we found active compounds with IC_{50} values ranging from 2 to 100 μ*M* against both mutant and wild-type suggests that the HTS would have identified selective compounds if they were present in the compound collection. Given the complex biology, the chances of finding a compound capable of restoring function to mutant eIF2B may be very low, necessitating the screening of several hundred thousand compounds. A successful outcome from such a large screen requires an efficient process of elimination to focus attention on hits with the desired activity profile. In this regard the HTS reported here was not as efficient as originally expected. A sizeable number of compounds appeared

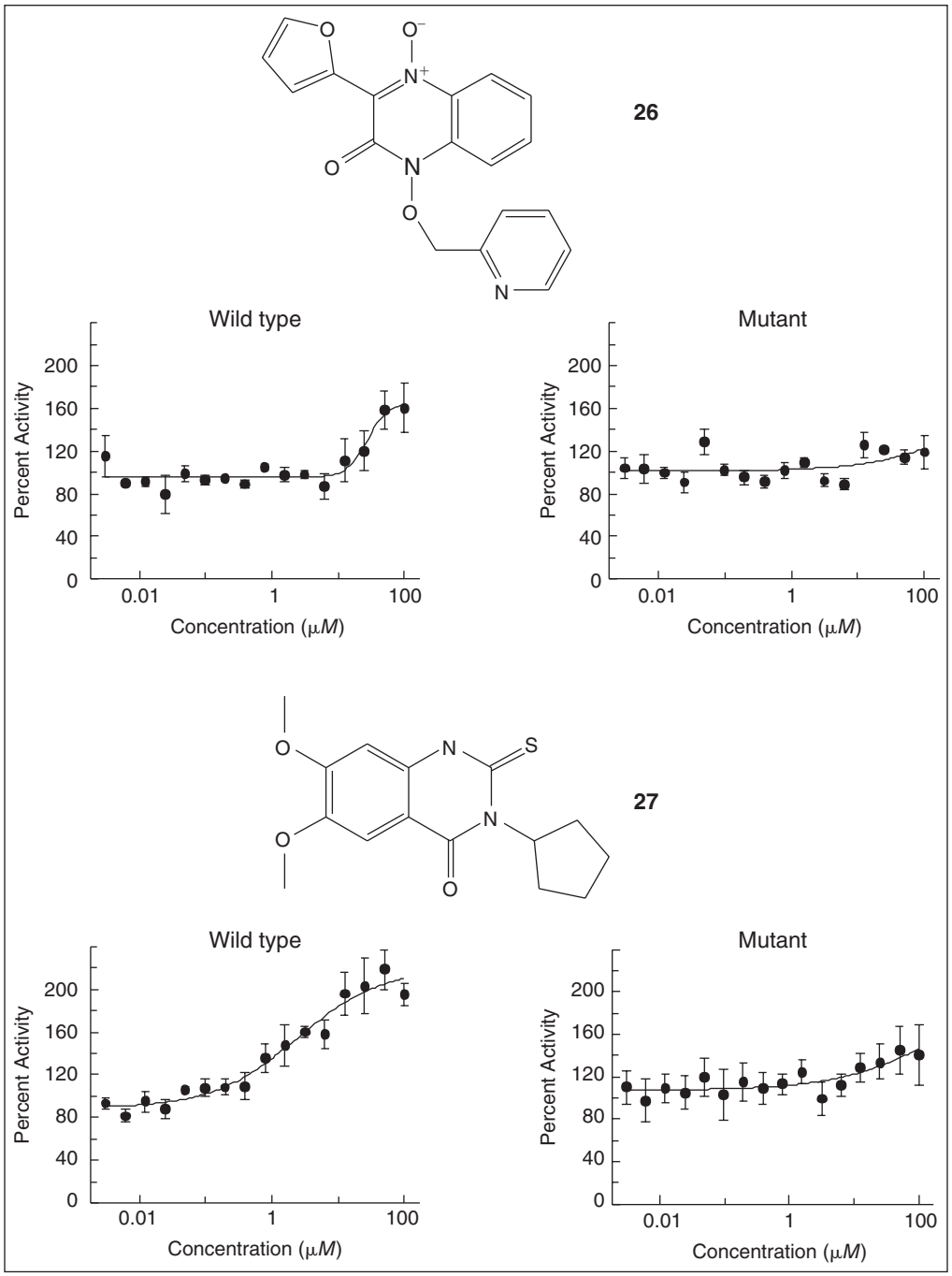

Fig. 8. Selected dose–response curves and EC_{50} values against wild-type and mutant luminescence for those compounds confirmed to be selective enhancers of wild-type yeast luminescence. Compound numbers refer to those listed in Table 4. The assay was performed as described in the section entitled IC_{50} and EC_{50} Dose–Response Assay in Materials and Methods. Correction for cell growth, curve fitting, and calculation of EC_{50} values were as described in the section entitled IC_{50} and EC_{50} Dose–Response Data Analysis in Materials and Methods. Each data point is the mean of three wells; error bars represent 1 SD from the mean. EC_{50} values are expressed in micromolar; each value corresponds to the specific curve shown and does not equal the mean values reported in Table 4: for compound **26**, (**left**) wild-type and (**right**) mutant, 27.5 and >100, respectively; for compound **27**, 2.4 and >100, respectively.

from the HTS to be selective but were found to be nonselective on dose–response testing (*Table 3*). Even compounds such as 2 and 3 that gave 75–85% inhibition of mutant luminescence and 0–15% inhibition of wild-type luminescence (with or without growth correction) gave almost identical IC_{50} values on retesting. Furthermore, the retest rate for both inhibition of mutant strain luminescence and enhancement of wild-type luminescence was very low. In both cases <5% of the compounds that exceeded the

threshold of 50% deviation from the HTS controls were active in dose–response testing.

The luminescence inhibition results suggest that, despite considerable optimization effort, the assay is not sufficiently robust to allow for reliable predictions to be made from single HTS data points obtained at one compound concentration. Such single-point data are usually sufficient when screening one parameter; true actives can be identified among a sizeable number of false-positives. However, in a complex multiparameter study such as that described here elimination of apparently selective false-positives at the HTS stage may require replicates at a single concentration or testing of multiple concentrations. An alternative is to select a large number of primary hits from a single HTS and postpone correlation of multiple readouts until dose–response testing. Use of the luminescence assay alone (*i.e.*, without data correction based on a parallel growth assay) as the primary HTS might allow for an assay design that avoids the cell transfer step on the EP3 workstation. This step was a significant source of assay variability and required very careful optimization. Adequate mixing of the cells with the Beta-Glo reagent would nevertheless remain critical to obtaining robust data; thus growth of the yeast in round-bottom white plates might be required so that the Beta-Glo reagent could be added directly to the yeast culture. Based on the results of our 27,199-compound HTS, screening of a large compound library (300,000–500,000 compounds) against the wild-type and eIF2B mutant yeast strains in separate HTS campaigns would produce many thousands of hits, requiring extensive cherry-picking and dose–response profiling to identify true hits among false-positive compounds. Therefore, it may be more efficient to obtain duplicate data on each compound in parallel HTS to improve the robustness of the correlation of the parallel datasets than to postpone this correlation until after dose–response confirmation.

In wild-type yeast, the two activating compounds enhanced β-galactosidase luminescence linked to *GCN4* expression and slowed growth, similar to the behavior of agents such as fusel alcohols that are known to activate *GCN4* expression.[10] The fact that the luminescence-enhancing compounds were inactive in the yeast strain containing the reduced-activity mutant eIF2B probably reflects the fact that *GCN4* is already activated in these cells. As the protein kinase gene *GCN2* was deleted from the screen strain, the activator compounds cannot be functioning by stimulating eIF2α kinase activity. Therefore these compounds must be acting downstream and could be directly targeting eIF2B. Both active compounds appear quite selective for the *GCN4*-β-galactosidase screen. At the time of writing, compound **26** had been tested in a total of 260 assays reported to PubChem,[35] and the only activity confirmed in dose–response testing was an IC_{50} of 34 μ*M* against the serine protease complement factor C1s.[36] From 243 assays[37] compound **27** had been reported as an agonist of the dopamine D1 receptor with an EC_{50} of 4.5 μ*M*[38] and an inhibitor of 15-lipoxygenase with an IC_{50} of 15.8 μ*M*.[39] A search in SciFinder (Chemical Abstracts Service, Columbus, OH) revealed that neither **26** nor **27** had been reported in the literature. Further studies will be required to explore the mechanism of action and utility of these compounds.

ACKNOWLEDGMENTS

The work reported here was supported by the National Institutes of Health Molecular Libraries Screening Center Network (grant U54HG003915-02) and grant ELA 2005-008C5 from the European Leukodystrophy Association.

AUTHOR DISCLOSURE STATEMENT

No competing financial interests exist.

REFERENCES

1. Shenton D, Smirnova JB, Selley JN, Carroll K, Hubbard SJ, Pavitt GD, *et al.*: Global translational responses to oxidative stress impact upon multiple levels of protein synthesis. *J Biol Chem* 2006;281:29011–29021.
2. Hinnebusch AG: Mechanisms of gene regulation in the general control of amino acid biosynthesis in *Saccharomyces cerevisiae*. *Microbiol Rev* 1988;52:248–273.
3. Kornitzer D, Raboy B, Kulka RG, Fink GR: Regulated degradation of the transcription factor Gcn4. *EMBO J* 1994;13:6021–6030.
4. Hinnebusch AG: Translational regulation of GCN4 and the general amino acid control of yeast. *Annu Rev Microbiol* 2005;59:407–450.
5. Pavitt GD: eIF2B, a mediator of general and gene-specific translational control. *Biochem Soc Trans* 2005;33:1487–1492.
6. Natarajan K, Meyer MR, Jackson BM, Slade D, Roberts C, Hinnebusch AG, *et al.*: Transcriptional profiling shows that Gcn4p is a master regulator of gene expression during amino acid starvation in yeast. *Mol Cell Biol* 2001;21:4347–4368.
7. Mascarenhas C, Edwards-Ingram LC, Zeef L, Shenton D, Ashe MP, Grant CM: Gcn4 is required for the response to peroxide stress in the yeast *Saccharomyces cerevisiae*. *Mol Biol Cell* 2008;19:2995–3007.
8. Costa-Mattioli M, Gobert D, Harding H, Herdy B, Azzi M, Bruno M, *et al.*: Translational control of hippocampal synaptic plasticity and memory by the eIF2alpha kinase GCN2. *Nature* 2005;436:1166–1173.
9. Hao S, Sharp JW, Ross-Inta CM, McDaniel BJ, Anthony TG, Wek RC, *et al.*: Uncharged tRNA and sensing of amino acid deficiency in mammalian piriform cortex. *Science* 2005;307:1776–1778.
10. Ashe MP, Slaven JW, De Long SK, Ibrahimo S, Sachs AB: A novel eIF2B-dependent mechanism of translational control in yeast as a response to fusel alcohols. *EMBO J* 2001;20:6464–6474.
11. Smirnova JB, Selley JN, Sanchez-Cabo F, Carroll K, Eddy AA, McCarthy JE, *et al.*: Global gene expression profiling reveals widespread yet distinctive translational responses to different eukaryotic translation initiation factor 2B-targeting stress pathways. *Mol Cell Biol* 2005;25:9340–9349.
12. Palmer LK, Rannels SL, Kimball SR, Jefferson LS, Keil RL: Inhibition of mammalian translation initiation by volatile anesthetics. *Am J Physiol Endocrinol Metab* 2006;290:E1267–E1275.

13. Palmer LK, Shoemaker JL, Baptiste BA, Wolfe D, Keil RL: Inhibition of translation initiation by volatile anesthetics involves nutrient-sensitive GCN-independent and -dependent processes in yeast. *Mol Biol Cell* 2005;16:3727–3739.
14. Cherkasova VA, Hinnebusch AG: Translational control by TOR and TAP42 through dephosphorylation of eIF2alpha kinase GCN2. *Genes Dev* 2003;17:859–872.
15. Kubota H, Obata T, Ota K, Sasaki T, Ito T: Rapamycin-induced translational derepression of GCN4 mRNA involves a novel mechanism for activation of the eIF2 alpha kinase GCN2. *J Biol Chem* 2003;278:20457–20460.
16. Corradetti MN, Guan KL: Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? *Oncogene* 2006;25:6347–6360.
17. Crespo JL, Hall MN: Elucidating TOR signaling and rapamycin action: lessons from *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* 2002;66:579–591.
18. Inoki K, Ouyang H, Li Y, Guan KL: Signaling by target of rapamycin proteins in cell growth control. *Microbiol Mol Biol Rev* 2005;69:79–100.
19. Tsang CK, Qi H, Liu LF, Zheng XF: Targeting mammalian target of rapamycin (mTOR) for health and diseases. *Drug Discov Today* 2007;12:112–124.
20. Steffen KK, MacKay VL, Kerr EO, Tsuchiya M, Hu D, Fox LA, *et al.*: Yeast life span extension by depletion of 60s ribosomal subunits is mediated by Gcn4. *Cell* 2008;133:292–302.
21. Fogli A, Boespflug-Tanguy O: The large spectrum of eIF2B-related diseases. *Biochem Soc Trans* 2006;34:22–29.
22. Leegwater PA, Vermeulen G, Konst AA, Naidu S, Mulders J, Visser A, *et al.*: Subunits of the translation initiation factor eIF2B are mutant in leukoencephalopathy with vanishing white matter. *Nat Genet* 2001;29:383–388.
23. Richardson JP, Mohammad SS, Pavitt GD: Mutations causing childhood ataxia with central nervous system hypomyelination reduce eukaryotic initiation factor 2B complex formation and activity. *Mol Cell Biol* 2004;24:2352–2363.
24. van der Knaap MS, Leegwater PA, Konst AA, Visser A, Naidu S, Oudejans CB, *et al.*: Mutations in each of the five subunits of translation initiation factor eIF2B can cause leukoencephalopathy with vanishing white matter. *Ann Neurol* 2002;51:264–270.
25. Li W, Wang X, Van Der Knaap MS, Proud CG: Mutations linked to leukoencephalopathy with vanishing white matter impair the function of the eukaryotic initiation factor 2B complex in diverse ways. *Mol Cell Biol* 2004;24:3295–3306.
26. Kantor L, Harding HP, Ron D, Schiffmann R, Kaneski CR, Kimball SR, *et al.*: Heightened stress response in primary fibroblasts expressing mutant eIF2B genes from CACH/VWM leukodystrophy patients. *Hum Genet* 2005;118:99–106.
27. Alves de Almeida R, Burgess D, Shema R, Motlekar N, Napper AD, Diamond SL, *et al.*: A *Saccharomyces cerevisiae* cell-based quantitative beta-galactosidase assay compatible with robotic handling and high-throughput screening. *Yeast* 2008;25:71–76.
28. NIH Molecular Libraries Small Molecule Repository. http://mlsmr.glpg.com/MLSMR_HomePage/ (accessed February 27, 2009).
29. AID 688: PubChem bioassay: yeast eIF2B assay. http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=688 (accessed February 27, 2009).
30. AID 792: PubChem bioassay: eIF2B IC50 against mutant and WT yeast. http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=792 (accessed February 27, 2009).
31. Warringer J, Blomberg A: Automated screening in environmental arrays allows analysis of quantitative phenotypic profiles in *Saccharomyces cerevisiae*. *Yeast* 2003;20:53–67.
32. Murata Y, Watanabe T, Sato M, Momose Y, Nakahara T, Oka S, *et al.*: Dimethyl sulfoxide exposure facilitates phospholipid biosynthesis and cellular membrane proliferation in yeast cells. *J Biol Chem* 2003;278:33185–33193.
33. Moroney MJ: *Facts from Figures*. Penguin, London, 1956.
34. AID 1006: PubChem bioassay: counter screen for luciferase-based primary inhibition assays. http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=1006 (accessed February 27, 2009).
35. SID 4261321: PubChem bioactivity analysis. http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?q=sids&reqid=1055952122768554026 (accessed February 27, 2009).
36. AID 787: PubChem bioassay: complement factor C1s IC50 from mixture screen. http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=787 (accessed February 27, 2009).
37. SID 865813: PubChem bioactivity analysis. http://pubchem.ncbi.nlm.nih.gov/assay/assay.cgi?q=sids&reqid=1100756580204387424 (accessed February 27, 2009).
38. AID 858: PubChem bioassay: allosteric modulators of D1 receptors. http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=858 (accessed February 27, 2009).
39. AID 887: PubChem bioassay: qHTS assay for inhibitors of 15-hLO (15-human lipoxygenase). http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcassay&term=887 (accessed February 27, 2009).

Address correspondence to:
Andrew D. Napper, Ph.D.
Nemours Center for Childhood Cancer Research
1701 Rockland Road
Wilmington, DE 19803

E-mail: napper@medsci.udel.edu

and

Graham D. Pavitt, Ph.D.
Faculty of Life Sciences
The Michael Smith Building
The University of Manchester
Oxford Road
Manchester M13 9PT
United Kingdom

E-mail: graham.pavitt@manchester.ac.uk