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Identification of inhibitors of chromatin modifying enzymes using the yeast phenotypic screens

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Abstract

A multitude of enzymes that modify histones and orchestrate nucleosome assembly and remodeling are required for the formation, maintenance and propagation of the transcriptionally repressed chromatin state in eukaryotes. Robust phenotypic screens in yeast *S. cerevisiae* have proved instrumental in identifying these activities and for providing mechanistic insights into epigenetic regulation. These phenotypic assays, amenable for high throughput small molecule screening, enable identification and characterization of inhibitors of chromatin modifying enzymes largely bypassing traditional biochemical approaches.

1. Introduction

1.1. Understanding Chromatin Biology Through Chemical Genetics

1.1.1. Chromatin Structure and Known Chemical Modifiers—Portions of the genome in eukaryotes is maintained in a transcriptionally inactive, or silenced state as a result of the local chromatin structure. The formation and the maintenance of the silenced state is an active process requiring a multitude of enzymes that act on DNA (methylation) and histones (acetylation, phosphorylation, and methylation) (1). Following cell division the silenced state of chromatin is passed onto the daughter cells thus forming a basis for the epigenetic propagation of cellular memory. Faithful transmission of the epigenetic state from mother to daughter plays a key role in many cellular processes in eukaryotes such as mating in yeast (2) or development in multicellular organisms (3). Epigenetic mechanisms also play an important role in the pathogenesis of many human neoplasms (4). The importance of epigenetic regulation in cancer is underscored by the observations that tumor suppressor genes are often silenced rather than mutated and that many dominant oncogenes require epigenetic regulators for their activity. These epigenetic underpinnings of cancer can be exploited as a therapeutic strategy for two reasons. First, since silenced copies of tumor suppressor genes do not harbor genetic mutations, their reactivation in the context of malignant cells may suppress growth or induce death. Second, while transcription factors have traditionally been considered poor drug targets, the enzymatic activities required for their function (e.g. histone acetyl transferases HAT, histone deacetylases HDAC) can be inhibited pharmacologically. Together, these observations point to epigenetic regulation as a major new therapeutic area for cancer.

At the present time our ability to pharmacologically influence epigenetics in cancer cells, and to use this as therapy, is limited by the scarcity of effective small molecule inhibitors of enzymes that control epigenetic states. Classic HDAC inhibitors (e.g. SAHA) and DNA demethylating agents (e.g. deoxy-5-azacytidine) are the only two classes of chromatin

modifying drugs in clinical use. This highlights the need to develop new drugs that target other enzymes involved in the establishment and maintenance of epigenetic states. Traditional approaches to identify enzyme inhibitors rely on high throughput biochemical screens. However, the enzymatic activities and proteins required for epigenetic regulation are extremely well conserved among eukaryotes, which makes drug discovery possible *in vivo* using model organisms.

Yeast is an attractive model system because of its rapid growth rate, ease of genetic manipulation, and because many yeast strains have already been developed to study epigenetics. Using a cell-based screen for compounds that can abrogate silencing at telomeres in yeast we have identified splitomicin, the first inhibitor of Sir2, a major nuclear NAD-dependent histone deacetylase and epigenetic regulator in yeast (5) and a founding member of a broadly conserved class of enzymes, sirtuins (6). Conditional inactivation of Sir2 with splitomicin and its analogues has proved valuable in dissecting chromatin biology in yeast (5,7,8) and mammalian cells (9), and in evaluating inhibition of sirtuins as a therapeutic strategy in cancer (10). Our success in identifying Sir2 inhibitors through phenotypic screens for epigenetic regulators in yeast, suggests that the same strategy can be used for the identification of inhibitors of other enzymes required for propagation of epigenetic memory. In the following sections we provide an overview of silencing in yeast, the enzymatic activities required for efficient silencing, and a description of the silencing assays available. Additionally, we provide a detailed high throughput screening protocol for identifying compounds that disrupt telomeric silencing, a description of the methods employed for characterization of the hits, and an overview of the strategies for identifying the molecular targets of the compounds.

1.1.2. Yeast silent chromatin and enzymatic activities—Silent chromatin occurs at three distinct sites in the yeast genome: silent mating-type loci (HML and HMR), telomeres and at ribosomal RNA genes (rDNA) (11). The formation of silent chromatin, best understood at the silent mating type loci and telomeres, depends on DNA elements or silencers. These silencers are located in close proximity to genes they regulate and contain binding sites for several DNA binding proteins including Rap1, Abf1 and the origin recognition complex. These DNA binding proteins, through protein-protein interactions, recruit the SIR (silent information regulator) complex (Sir2–4). Once nucleated at the silencers, the SIR complex is thought to spread long the chromatin through binding of Sir3 and Sir4 to the hypoacetylated NH₂ – terminal tails of histones H3 and H4. The NH₂ – terminal tails of histones H3 and H4 are kept in the hypoacetylated state through the action of Sir2 and this activity is critical for the formation of silent chromatin. Sir2 deacetylase activity is also required for silent chromatin formation at rDNA. However, at rDNA Sir2 is part of different protein complex, which does not include Sir3 or Sir4.

The formation of silent chromatin leads to transcriptional repression at the silent mating types and at subtelomeric regions. The mating type of yeast cell is determined by mating type information (MAT_a or MAT_α) present at the mating locus (MAT) on chromosome III (12). The ability of a and α cells to respond to mating pheromones and mate depends on expression of the haploid-specific and MAT_a or MAT_α specific genes controlled by MAT_a or MAT_α transcription factors. In addition to being present at the active MAT locus, a copy of the MAT_α gene exists in a silent state at the silent HML locus and a copy of MAT_a exists at the silent HMR locus on the same chromosome (12). Normal diploid a/α state is defined by the coexpression of MAT_a and MAT_α information from the active MAT loci. However, the loss of silencing at the HMR and HML loci can create a pseudo diploid a/α state in a haploid cell by allowing the expression of MAT_a or MAT_α from the silent mating type loci. Accordingly, loss of silencing at the HML and HMR loci creates a mating defect and cells

become insensitive to mating pheromones. The response of MATa cells to alpha factor can be used as an assay for scoring derepressions at the silent HMR locus.

Silencing at the rDNA locus has two important effects. It represses transcription of the ribosomal RNA gene or an inserted reporter gene (13), and it suppresses recombination between the tandem copies of rRNA genes (14).

Beside the NAD-dependent deacetylase activity of Sir2, which is required for the silenced state at all silenced sites in yeast genome (15), other nuclear process including DNA replication (16), nucleosome assembly (17) and remodeling machinery (18) and several other histone modifying enzymes also participate in silent chromatin formation and maintenance. Intriguingly, the main role for several histone modifying enzymes, such as Dot1 (19), Set1 (20,21), and Sas2 (22–24), which are required for efficient silencing at telomeres, appears to be in limiting heterochromatin spreading. In their absence, the SIR complex extends beyond its normal boundaries and the redistribution of the SIR complexes weakens silencing at the native sites. While detailed mechanistic insight into how each of these and other enzymes contribute to the epigenetic memory is still lacking, it is clear that silencing of reporter genes at telomeres is a very sensitive readout of the local chromatin state.

1.2. Silencing assays in yeast

Robust phenotypic assays have been developed for examining each of the silenced loci in the yeast genome (25). In the following sections we will discuss the assays that are suitable for high throughput small molecule screening. Table 1 lists the strains used to assay silencing at the telomeres, silent mating loci, and rDNA.

1.2.1. Silencing at the telomeres—When a URA3 gene is introduced in the vicinity of a telomere its transcription is repressed (26). However, the transcriptional repression of a telomeric URA3 gene is overcome in medium lacking uracil. Low uracil growth conditions up regulate the transcription factor Ppr1, which transactivates URA3 and other genes required for uracil biosynthesis (28). Deletion of PPR1 can be therefore be used to modulate the strength of URA3 transcription. In the absence of PPR1, URA3 expression is largely dictated by the local chromatin state and is insensitive to the lack of uracil in cells. Accordingly, in the S288C strain background, the Δppr1 URA3-TEL (VII-L) reporter strain will grow poorly in media without uracil. In the ppr1 strain, perturbed silencing at the telomeres permits sufficient URA3 transcription for growth in medium lacking uracil. In addition to positive selection, the URA3 telomeric reporter strain can also be employed in a negative selection screen using 5-Fluoroorotic Acid (5-FOA). When silencing is perturbed the URA3 gene products convert the nontoxic 5-FOA into 5-Fluorouracil, which is highly toxic.

Other telomeric reporter strains have been developed and include the ADE2-TEL (V-R) strain (26). The ADE2 telomeric reporter strain relies on a straightforward assay based on ADE2 expression and colony color. Strains expressing a wild type copy of ADE2 produce white colonies and strains with silenced or deleted ADE2 form red colonies. The ADE2-TEL (V-R) strain stochastically switches the ADE2 gene from transcriptionally repressed to transcriptionally active and this process leads to variegated expression of the ADE2-TEL (V-R) gene. As the colonies grow, the transcriptional state of the telomere is passed on to daughter cells and red and white sectors appear in the colony (*see Note 1*²). The amount of

¹Red-pigmented colonies do not grow as robustly as normal white colonies. The use of YEPD media instead of C media will help minimize the disparity.

²The degree of Red/White sectoring is not uniform among colonies of the same strain.

sectoring in the colony is directly related to the transcriptional state of the ADE2 gene, and if silencing is perturbed colonies appear with less red sectoring (*see* Note 3). In addition to the URA3 and ADE2 reporter strains, other telomeric reporters, such as strains utilizing HIS3, or TRP1 reporters, are also available (reviewed in (25)) and can be easily adapted to screens for small molecules with antisilencing properties.

1.2.2. Silencing at HMR and HML Loci—Silencing at the HMR locus is assayed using a TRP1 reporter gene integrated at the HMR locus. In a cell with intact silencing, the TRP1 gene will not be expressed and the cell will be unable to grow in media lacking tryptophan (C –Trp). When silencing at the HMR locus is disrupted, the TRP1 gene will be expressed and the strain will grow in C –Trp media. Testing silencing at the HML locus relies on the mating pheromone α -factor. When two haploid yeast cells, of opposite mating type, contact each other they secrete cell type specific mating pheromones (2). MATa cells secrete α -factor, a modified hydrophobic peptide, and MAT α cells secrete α -factor, an unmodified soluble peptide. Typically α -factor is used in experiments utilizing a mating pheromone because it can be dissolved in liquid media and it is easily obtained commercially. α -Factor induces MATa cells to arrest in the G₁ phase of the cell cycle and undergo morphological changes, known as shmooing, in preparation for cell and nuclear fusion. MATa cells with aberrant silencing at the HML locus are insensitive to α -factor and do not arrest in G₁ or shmoo.

1.2.3. Silencing at the rDNA—Yeast ribosomal DNA (rDNA) consists of a 9.1 kilobase region of DNA, containing a 5S rRNA gene and a 35S pre-rRNA gene, which is repeated 100–200 times along chromosome XII. Silencing at the rDNA is assayed with a strain that utilizes a URA3 reporter, with a minimal TRP1 promoter (mURA3), integrated at the rDNA repeats (13). Use of the mURA3 instead of native URA3 improves URA3 silencing and permits scoring by positive and negative selection. In addition to silencing, recombination at the rDNA locus can be measured through the loss rate of an ADE2 gene integrated into the rDNA (29).

1.3. Screening Strategy

1.3.1. Primary screen—The use of a telomeric URA3 reporter as a primary screen has several advantages over other available reporter systems. Primarily, silencing at the telomere is a very sensitive readout of chromatin state (*see* Note 4) that is influenced by the products of many genes. Furthermore, the loss of silencing-induced activation of the URA3 reporter can be scored as growth in medium lacking uracil, or as toxicity in medium containing 5-FOA. The design of a high throughput screen utilizing selection for growth has an advantage in that it requires the compound inhibit a relevant target at concentrations that do not perturb other cellular processes (*see* Note 5).

1.3.2. Secondary Assays—Compounds identified in the primary screen that promote growth in C –Ura, or demonstrate toxicity in 5-FOA are subjected to secondary screens with the goal of identifying compounds that specifically inhibit silencing. This is carried out by comparing toxicity of compounds in medium with and without 5-FOA, and by examining

³Development of the red pigmentation is greatly improved by placing plates at 4°C for several days after colonies have formed.

⁴We have noted that derepression of telomeric reporters can be achieved by at least fivefold lower concentrations of the Sir2 inhibitor splitomicin, than those required for derepression of the reporters at the silent mating loci.

⁵The choice of a positive or negative selection strategy for the primary screen should be based on the characteristics of the chemical libraries being assayed. If the library is enriched for toxic compounds, (e.g. 20–25% of compounds in the National Cancer Institute repository exhibit toxicity), a primary screen using positive selection, which assays for stimulation of growth in medium lacking uracil, is expected to be more efficient than a screen for toxicity in 5-FOA medium. Libraries that are not enriched for toxic compounds can be efficiently screened for toxicity in 5-FOA as a primary screen followed by dose response evaluations of toxicity in C medium and growth enhancement in medium lacking uracil as secondary screens.

the ability of compounds to stimulate growth in medium lacking uracil. A dose response curve using the same concentration of drug employed in the primary screen and several two-fold dilutions is carried out in complete medium, medium containing 5-FOA and medium lacking uracil. The concentration of an ideal antisilencing drug that inhibits growth by 50% (IC_{50}) is expected to be at least 2–4 fold lower in medium containing 5-FOA relative to the IC_{50} in C-medium. The dose response curve in medium lacking uracil is expected to identify the concentration of drug that activates the reporter and stimulates growth. Growth inhibition at the higher drug concentrations for compounds that activate a telomeric reporter may indicate inhibition of an essential activity that is important for telomeric silencing, or indicate inhibition of an unrelated essential target (known as off target activity).

In order to demonstrate that a compound interferes specifically with the state of chromatin, and does not solely promote transactivation of the URA reporter, silencing of other reporters at telomeres and at loci other than the telomeres should be characterized. For this purpose, the sectoring assay using a strain containing ADE2 at the telomere can be used. The presence of heterochromatin at sites other than the telomeres can be examined by monitoring activation of a TRP1 reporter integrated at the HMR locus or the responsiveness of MATa cells to alpha-factor can be used to assay silencing of the endogenous HML locus. Furthermore, both silencing and recombination rates at rDNA can be assayed using available strains. A URA3 reporter strain has been developed for assaying silencing, and strains have been developed with ADE2 integrated at the rDNA to determine the recombination rate by measuring loss of the ADE2 gene.

1.4. Characterizing Hits

1.4.1. Identification of Molecular Targets—Identification of the targets of compounds identified through cell based screens presents one of the major challenges of chemical genetics. Traditional target identification strategies rely on biochemical affinity methods such as purification of the target protein using a drug affinity matrix. One of the major drawbacks of this approach is the need for ligand modification, which is often time consuming, and can lead to loss of drug activity. Furthermore, this approach is limited to compounds that have a relatively high affinity to their corresponding target. Beside the affinity purification methods, the large body of preexisting knowledge of yeast epigenetic regulation and the facile genetics offers additional means for target identification. The ideal drug, upon binding the target protein, mimics either a loss of function or a gain of function mutation in the corresponding gene. This premise serves as a basis for identifying drug targets by *drug-mutant* matching approaches. An antisilencing drug that inhibits a specific chromatin modifying enzyme is expected to 1) recreate the effect of a point mutation in the corresponding enzyme that abrogates its enzymatic activity *in vitro*, 2) recreate the same global or local chromatin alterations, 3) change the global transcriptional profile, and 4) replicate the synthetic interaction profiles of their corresponding loss of function mutants.

1.4.2. *In vitro* Inhibition of Known Enzymatic Activities Important for Silencing—Several of the genes that affect telomeric silencing in yeast encode proteins that have defined *in vitro* enzymatic activities. While the published assays (e.g. enzymatic assays for Dot1, Set1, Sir2 and Sas2 are described in references (19,20,23,30)) may not be suitable for high throughput screening, the number of compounds that need to be characterized in this step is expected to be limited, which makes the *in vitro* testing for enzyme inhibition the most straightforward approach for evaluating whether the antisilencing compounds inhibit these known enzymatic activities.

1.4.3. Histone Modifications in Drug Treated Cells and Silencing Mutants—A deficiency of histone modifying enzymes is expected to lead to local (i.e. telomeric) or

global alterations in specific histone posttranslational modifications (PTM). The ability of compounds to alter histone PTM can be evaluated using a panel of antibodies specific to different modifications of histone proteins using Western blots, for global, or chromatin immunoprecipitation for local telomere alterations respectively. Antibodies targeting more than 40 different histone modifications (methylation, acetylation of different lysine residues) are commercially available.

1.4.4. Matching of Transcriptional Effects—Silencing in yeast occurs at three locations: telomeres, silent mating loci and rDNA. While the loss of SIR2 affects silencing at all three sites, the effect of inactivating other chromatin modifying enzymes may be restricted to specific locations. In addition to known sites affected by silencing, proteins required for silencing also affect transcription, directly or indirectly, at other locations in the genome. As a result, the global transcriptional changes in drug treated wild type cells are expected to be highly correlated with the changes observed in cells with mutations in proteins required for transcription at various loci. The gene which, when deleted, creates a transcriptional profile most highly correlated with the transcription profile created by drug treated wild type cells should be further analyzed as potentially encoding for a drug target. Large numbers of yeast mutants have been subjected to transcriptional profiling (31,32), and the data are readily available.

The definite proof that the mutant affects a putative target will rely on:

- a. Inhibition of the proteins *in vitro* activity for targets with *in vitro* biochemical activity,
- b. Demonstrating specific binding of the drug to its target if no biochemical activity has been ascribed to the protein,
- c. Obtaining a mutant protein through *in vivo* screening for alleles that confer drug resistance and have alterations in drug binding or are resistant to enzyme inhibition *in vitro*.

2. Materials

2.1. Positive Selection Screen

1. Strain UCC2210 Mat α ade2d::hisG his3d200 leu2d0 lys2d0 met15d0 trp1d63 ura3d0 adh4::URA3-TEL (VII-L) ppr1::HIS3 (*see* Note 6)
2. YEPD medium (For 2 L of media): 20 g bacto yeast extract (cat. no. DF0127-17-9, Fisher), 40 g bacto peptone (cat. no. S71604, Fisher), 40 g dextrose (cat. no. BP350-1, Fisher).
3. Complete synthetic (C) medium (For 2 L of media): 2.9 g yeast nitrogen base without amino acids and ammonium sulfate (cat. no. DF0335-15-9, Fisher), 10 g ammonium sulfate (cat. no. A938-500, Fisher), 40 g dextrose (cat. no. BP350-1, Fisher), and amino acids (omit Uracil for C –URA media) according to the table below.

⁶The sensitivity of strain UCC2210 can be increased by deletion of the pleiotropic drug resistance genes PRD3 and PDR1, as well as the ergosterol biosynthesis gene ERG6.

Amino Acid	Grams	Cat. No.	Vendor
adenine (6-aminopurine)	1	A-8626	Sigma
L-arginine	1	BP370-100	Fisher
L-aspartic acid	5	BP374-100	Fisher
L-glutamic acid	5	BP378-100	Fisher
L-histidine	1	BP382-100	Fisher
L-isoleucine	4	BP384-100	Fisher
L-leucine	4	BP385-100	Fisher
L-lysine	3	L9037	Sigma
L-methionine	1	BP388-100	Fisher
L-phenylalanine	2.5	BP391-100	Fisher
L-serine	20	BP393-100	Fisher
L-threonine	10	BP394-100	Fisher
L-tryptophan	4	BP395-100	Fisher
L-tyrosine	3	BP396-100	Fisher
uracil	1	U-1128	Sigma
L-valine	7.5	BP397-100	Fisher

4. Flat bottom 96 well microplate (cat. no. 12-565-311, Fisher).
5. Compound library and a positive control, such as splitomicin (*see* Note 7), known to abrogate silencing in yeast.
6. 30°C incubator.
7. Microplate reader (e.g. Molecular Devices VERSAMAX Tunable Microplate Reader).
8. Distributing cells and compounds to 96 well plates is greatly simplified with the use of an automated 96-channel pipettor (e.g. a PlateMate or similar programmable device).

2.2. Negative Selection Screen

1. Strain UCC3503 Mat α ura3-52 lys2-801 ade2-101 leu2-d1 his3-d200 adh4::URA3-TEL (VII-L) ADE2-TEL (V-R)
2. C +5-FOA media is C media as described in section 2.1. Step 3 with 2 g 5-FOA (cat. no. F9001, Zymoresearch) added to 2 L of C media.

⁷The use of splitomicin is useful for gauging the performance of the assay and to determine the stopping point of the assay. Care must be taken in a positive selection screen when scoring plates for growth. At early timepoints, such as 18–36 hours, splitomicin will permit cells to grow in C-Ura media and untreated cells will not have grown appreciably. At timepoints nearer 48 hours, the stochastic nature of silencing will lead to a small percentage of cells that develop spontaneous derepressions of the reporter gene and as this small population divides it will quickly reach a high density. Incubations longer than 48 hours will allow growth to be observed in some, though not all, negative control wells. Thus, scoring at later time points allows greater sensitivity but may lead to increase rate of false positive hits. In order to minimize false positives, growth should be scored at a timepoint when wells treated with splitomicin have an OD of 0.25–0.4 (on a Molecular Devices VERSAMAX Tunable Microplate Reader) and untreated wells have an OD below 0.25. Splitomicin can be used as a positive control at 10 μ M in 3.1.1., 3.1.2., 3.2.2., 3.2.3., 3.2.4., and 3.2.5. For the dose response assays in 3.2.1., a range of splitomicin concentrations from 0.1 to 50 μ M should be analyzed. Colonies growing on agar containing 10 μ M splitomicin appear white. Splitomicin should be diluted in media immediately prior to use as it rapidly hydrolyzes in aqueous solutions (half life 1–8 hours depending on pH).

3. See required materials for Positive Selection (2.1.).

2.3. Dose Response Assay

1. Strain UCC3503 Mat α ura3-52 lys2-801 ade2-101 leu2-d1 his3-d200 adh4::URA3-TEL (VII-L) ADE2-TEL (V-R) or Strain UCC2210 Mat α ade2d::hisG his3d200 leu2d0 lys2d0 met15d0 trp1d63 ura3d0 adh4::URA3-TEL (VII-L) ppr1::HIS3.
2. C media, C -URA media, and C +5-FOA media as described above.
3. See required materials for Positive Selection (2.1.).

2.4. α -Factor Resistance Assay

1. Strain BY4741 Mat a his3 leu2 met15 ura3.
2. C media (described in 2.1.).
3. α -Factor (cat. no. Y1001, Zymoresearch).
4. See required materials for Positive Selection (2.1.).

2.5. HMR Silencing Assay

1. Strain YJB959 MATa ura3-1 ade2-1 his3-11 leu 2-3,112 can 1-100 trp1-1 HMR::TRP1
2. C media prepared without tryptophan (see 2.1. Step 3)
3. See required materials for Positive Selection (2.1.).

2.6. Red/White Colony Formation

1. Strain UCC3503 Mat α ura3-52 lys2-801 ade2-101 leu2-d1 his3-d200 adh4::URA3-TEL (VII-L) ADE2-TEL (V-R)
2. YEPD agar (For 2 L of media): 20 g bacto yeast extract (cat. no. DF0127-17-9, Fisher), 40 g bacto peptone (cat. no. S71604, Fisher), 40 g dextrose (cat. no. BP350-1, Fisher), 40 g bacto agar (cat. no. DF0140-07-4, Fisher).
3. YEPD agar plates containing drug at the IC₅₀ concentration and plates without drug.

2.7. rDNA Silencing

1. Strain S26 Mat α his3 Δ 200 leu2 Δ 1 ura3-167 RDN1::Ty1-mURA3.
2. C-Ura media, or +5-FOA media as described above in Section 2.2. Step 2.
3. See required materials for Positive Selection (2.1.).

3. Methods

3.1. Primary Screens

3.1.1. Positive Selection Screen

1. Grow a culture of the assay strain in YEPD media to saturation (approximately 2×10^8 cells/mL).
2. Harvest cells by centrifugation and wash once with water.

3. Resuspend washed cell pellet in C-media prepared without uracil (referred to as C – URA) to a density of 5×10^4 cells/mL.
4. Deliver 135 μ L of cell suspension to each well of a 96 well plate.
5. The compounds to be assayed should be diluted with water to $10 \times$ the desired assay concentration. Generally stock concentrations of compounds should be between 50 to 150 μ M, solubility permitting.
6. Deliver 15 μ L of the compound stocks to the yeast cells.
7. The total volume of each well will be 150 μ L, with cells at a density of 5×10^4 and the compounds to be assayed at final concentrations in the range of 5 to 15 μ M (*see* Note 7).
8. Incubate the plates at 30°C for 24–48 hours (*see* Note 8).
9. Score growth by reading the optical density (OD) of the wells at 660nm on a microplate reader (Molecular Devices VERSAMAX Tunable Microplate Reader) (*see* Note 9).

3.1.2. Negative Selection Screen—Conducting a negative selection screen in 5-FOA media is carried out in the same fashion as the positive selection screen, but it is necessary to use a strain that is wild type for PPR1. Incubate plates for 24–48 hours at 30°C (*see* Note 7,⁸ and ¹⁰).

3.2. Secondary Screens

3.2.1. Dose Response Assay

1. Prepare cells as in the positive selection screen (3.1.1.), and resuspend in the appropriate media (*see* Note 11).
2. Deliver 135 μ L of cell suspension to each well of a 96 well plate.
3. Prepare 1:2 serial dilutions of the compound of interest, spanning a range of concentrations above and below the concentration used in the primary screen.
4. Deliver 15 μ L of compound dilutions to cells (*see* Note 7).
5. Incubate at 30°C for 24–48 hours and read the optical density (*see* Note 9 and ¹⁰).

3.2.2. α -Factor resistance (HML silencing)

1. Prepare cells as in the positive selection screen (3.1.1.) and resuspend in C media containing 5 μ M α -Factor.
2. Distribute 135 μ L of cell suspension into each well of a 96 well plate and add 15 μ L of the compounds of interest at the desired concentration (*see* Note 7,¹² and ¹³).

⁸Place plates inside a clean plastic bag, or seal with parafilm to prevent evaporation during the incubation period.

⁹In a positive selection screen, an OD between 0.25 and 0.4 indicates growth.

¹⁰In a negative selection screen, an OD below 0.25 indicates death.

¹¹For the dose response assays, resuspend cells in C media, C-Ura media and C+5-FOA media, and conduct the dose response assay in each type of media.

¹²When assaying the compounds for silencing at the mating loci and ADE-TEL (VR) locus, use the concentration of drug determined to best stimulate growth in C -Ura media in section 3.2.1. When assaying compounds for rDNA silencing use the IC₅₀ concentration from the dose response curve in C-Ura media, for a positive selection screen, or the IC₅₀ in 5-FOA, for a negative selection screen.

¹³In addition to growth, loss of silencing is also expected to abrogate the ability of MATa cells to shmoo, which can be scored using a microscope.

3. Incubate the plate(s) at 30°C for 12–24 hours, and read the optical density at 660 nm.

3.2.3. HMR silencing assay

1. Prepare cells as in the positive selection screen (3.1.1.) and resuspend in C media prepared without tryptophan.
2. Distribute 135 µL of cells to each well of a 96 well plate, and deliver 15 µL of the compounds of interest (*see* Note 7 and ¹²).
3. Incubate the plate(s) at 30°C for 12–36 hours, and read the optical density at 660 nm.

3.2.4. Red/White Colony Formation Assay—The Red/White colony formation assay can be carried out using 96 well plate format. Grow cells in YEPD liquid to saturation.

1. Dilute and plate cells for singles (*see* Note 14) on YEPD agar and YEPD agar containing the drug of interest.
2. Grow colonies at 30°C for 24–48 hours and transfer plates to 4°C for 72 hours to develop red pigmentation (*see* Note 1, ², ³, and ⁷).

3.2.5. rDNA Silencing Assay

1. The S26 strain (Mat α his3 Δ 200 leu2 Δ 1 ura3-167 RDN1::Ty1-mURA3) can be used in a positive selection screen in medium lacking uracil as described in 3.1.1., or in a negative selection screen in medium containing 5-FOA as described in 3.1.2.

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¹⁴For 96 well format, plate 5–10 cells per well.

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Table 1

Yeast Strains Used in Silencing Assays

Assay	Relevant Genotype	Selection Criteria	Strain
Telomeric Silencing	ppr1-Δ URA3-TEL (VII-L)	C -Ura media	UCC2210 (26)
	URA3-TEL (VII-L)	C +5-FOA media	UCC3503 (26)
	ADE2-TEL (V-R)	Colony color	UCC3503 (26)
HML Silencing	Mat a	Sensitivity to α-factor	
HMR Silencing	hmrΔ::TRP1/HMR	C -Trp media	YJB959 (27)
rDNA Silencing	RDN1::Ty1-mURA3	C -Ura or C +5-FOA	S6 (13)