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A histone deacetylase-dependent screen in yeast

Sujith V. W. Weerasinghe [†], Magdalene Wambua, Mary Kay H. Pflum [*]

Department of Chemistry, 5101 Cass Avenue, Wayne State University, Detroit, MI 48202, USA

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ABSTRACT

Histone deacetylase (HDAC) proteins are promising targets for cancer treatment, as shown by the recent FDA approval of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, Vorinostat) for the treatment of cutaneous T-cell lymphoma. To identify additional potent inhibitors and characterize HDAC mutant proteins, there is interest to develop an inexpensive screening method dependent on native substrates. Here, we report the first yeast-based gene reporter screen dependent on the yeast Rpd3, which is a homolog of human class I HDAC proteins. The screen was sensitive to an inactive Rpd3 mutant and various inhibitors in qualitative, agar-based and quantitative, solution-phase formats. Interestingly, inclusion of the lytic enzyme zymolyase enhanced the inhibitor sensitivity of the screen. The gene reporter screen provides a tool to screen Rpd3 mutants and inhibitors of class I HDAC proteins.

1. Introduction

Histone deacetylase (HDAC) proteins catalyze the removal of acetyl groups from acetylated lysines on histone and other protein substrates. The acetylation state of specific lysine residues in histone proteins can alter the chromatin structure and influence eukaryotic gene transcription.[1] Due to their fundamental role in gene expression, HDAC proteins are promising targets for cancer treatment, as shown by the recent FDA approval of the HDAC inhibitor suberoylanilide hydroxamic acid (SAHA, Vorinostat) for the treatment of cutaneous T-cell lymphoma.[2] In addition, HDAC inhibitors are currently in clinical trials to treat various cancers.[3–5] Consistent with their clinical effects, inhibitors of HDAC proteins suppress tumor cell proliferation, induce cell differentiation, and regulate the expression of crucial genes associated with cancer.[6] HDAC inhibitor drugs represent a promising next generation of anti-cancer therapeutics.

Eighteen human HDAC proteins are known and divided into four classes on the basis on mechanism, phylogenetic analysis, and similarity to yeast HDAC proteins.[7] Human HDAC1, HDAC2, HDAC3, and HDAC8 are members of the class I and are homologous to yeast Rpd3 protein.[8–12] Human HDAC4, HDAC5, HDAC6, HDAC7, HDAC9, and HDAC10 belong to class II and are homologues of yeast HDA1 protein.[13–15] The class III proteins include the NAD-dependent Sirtuin proteins, which are related to Sir2 in yeast. HDAC11 is the only member of class IV in humans and is predicted to have diverged very early in evolution.[7,12] Proteins in class I, II, and IV display considerable sequence similarity in their catalytic sites, which suggests that they operate via similar metal-dependent mechanisms.[16–18] Most inhibitors discussed in this work nonspecifically target all 11 metal-dependent HDAC isoforms.[19,20]

Several assays are currently available to screen HDAC inhibitors.[21] The most widely used screen is a commercially available fluorimetric assay that is a convenient and high throughput alternative to protocols utilizing radiolabeled substrates or HPLC methods.[22] However, recent reports have suggested that the non-native fluorophore on the peptide substrate in the fluorimetric assay can lead to false positive hits during screening. Specifically, the ability for resveratrol and other similar compounds to activate the deacetylase activity of the Sirtuin proteins was dependent on the presence of the fluorophore.[23,24] Further, biophysical evidence revealed that the small molecules interacted directly with the fluorophore-labeled peptide substrate.[25] As a result of these reports, development of HDAC screening tools utilizing non-radioactive, native substrates is desirable.

In addition to small molecule screening, the fluorimetric assay has been used to monitor HDAC protein activity. Because structural information on HDAC proteins is limited,[16,17,26–28] site directed mutagenesis has been the used extensively to identify the catalytic active site of many HDAC isoforms. For example, mutagenesis of HDAC1 has revealed residues critical for catalysis.[29,30] The availability of a rapid screening platform to identify active/inactive mutant forms of the HDAC proteins would facilitate their characterization.

Here we report the development of a yeast-based gene reporter system for the screening of HDAC mutant proteins and small molecule inhibitors. Yeast-based screening was used previously to identify small molecule inhibitors of the yeast Sir2 protein.[31,32] The Sir2 inhibitors identified in the screens have been used widely

* Corresponding author. Tel.: +1 313 577 1515; fax: +1 313 577 8822.
E-mail address: pflum@chem.wayne.edu (Mary Kay H. Pflum).
† Present address: Department of Molecular and Integrative Physiology, University of Michigan Medical School, Ann Arbor, MI 48109, United States.

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to inhibit the human Sirtuin proteins,[33,34] which demonstrates the utility of yeast-based screens to identify HDAC inhibitors. Based on the success of the Sir2 inhibitor screen, we envisioned developing a similar yeast screen targeting the metal-dependent yeast HDAC proteins. Yeast Rpd3 shares 60% identity to human class I proteins.[7] With its high sequence similarity, we rationalize that an Rpd3-dependent yeast assay would provide a facile tool for the screening of HDAC protein mutants or inhibitors. Like with the Sir2 inhibitor assays, the yeast Rpd3 screen can be coupled with the fluorimetric assay to provide a powerful approach to mutant and inhibitor characterization.

2. Materials and methods

2.1. Yeast strains and plasmids

The *FT5 Δrpd3::HIS3* yeast strain and pJK1621 reporter plasmid used in this study were generous gifts of Dr. Kevin Struhl.[35] YEplac112-Rpd3-LexA-FLAG and YEplac112-Rpd3H150/H151A-LexA-FLAG expression plasmids were generated as follows: Rpd3-LexA-FLAG or Rpd3H150/H151A-LexA-FLAG PCR fragments were generated from YEplac112-Rpd3-LexA or YEplac112-Rpd3H150AH151A-LexA (from Dr. Kevin Struhl)[35] using the CGGG GTACCATGGTATATGAAGCAACACCTTTTGATCCG forward and CTGG TTAATTATAAATTAGCCATGGCCTTTATCATCATCATCTTTATAATCCAG CCAGTCGCCGTTGCG reverse primers, which contain the FLAG epitope. Generated PCR fragments were cloned into Nco1 digested YEp112–FLAG (from Dr. Kevin Struhl)[35] using homologous recombination. Generation of plasmids was confirmed by DNA sequencing.

2.2. β-galactosidase activity screen

For the agar assay, *FT5 Δrpd3::HIS3* yeast cells transformed with the pJK1621 reporter alone, the pJK1621 reporter and YEplac112-Rpd3-LexA-FLAG expression plasmid, or the pJK1621 reporter and YEplac112-Rpd3H150/H151A-LexA-FLAG expression plasmid were plated on selection media (CSM-Ura-His for pJK1621 alone or CSM-Trp-Ura-His for the others) containing 0.5% dextrose with or without small molecule. Cells were grown for 48 h at 30 °C and then overlaid with X-gal solution (0.25 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma), 6% DMF, 0.1% SDS, 0.5 M KPO_4 pH 7.0). Blue color development was monitored visually, with best results observed in less than 4 h at 30 °C.

For the solution-phase assay, overnight cultures of the transformed cells (described above) were diluted to 0.1 absorbance at OD_{600} with the appropriate media containing 0.5% dextrose (1 mL total volume) and then incubated without (2% DMSO control) or with small molecule for 6 h. Small molecule final concentrations are as listed in the Figures. The OD_{600} was then measured. Cells were harvested by centrifugation from equal culture volumes (typically 500–800 μL) and then permeabilized by vortexing for 30 s in 500 μL of permealization buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 2 mM $MgSO_4$, 0.27% β-mercaptoethanol, pH 7.0) containing 25 μL of 0.1% SDS and 20 μL of chloroform. The β-gal substrate 2-nitrophenyl β-D-galactopyranoside (ONPG, Sigma, 100 μL of 4 mg/mL solution) was added to each solution and the reaction was incubated at 30 °C for not more than 1 h. Once yellow color developed, the reaction was quenched by adding 100 μL of 1 M Na_2CO_3. The reactions were centrifuged to remove cell debris and absorbance at OD_{420} was measured of the soluble fraction. Normalized β-galactosidase activity units were calculated using the following equation, units = $(OD_{420} \times 1000)/(OD_{600} \times T \times V)$, where T = ONPG incubation time in min and V = the volume in μL of the soluble fraction used to obtain the OD_{420}.[36,37]

2.3. Immunoprecipitation and histone deacetylase assay

Whole cell extracts were prepared from *FT5 Δrpd3::HIS3* yeast cells transformed with the pJK1621 reporter alone, the pJK1621 reporter and YEplac112-Rpd3-LexA-FLAG expression plasmid, or the pJK1621 reporter and YEplac112-Rpd3H150/H151A-LexA-FLAG expression plasmid. Specifically, the yeast were grown overnight with shaking at 30 °C in 5 mL of appropriate selection media (CSM-Ura-His or CSM-Trp-Ura-His). After centrifugation to collect the cells, glass beads equal to the packed volume of the cell pellet were added and the cell/glass bead mixture was resuspended in 1 mL of yeast lysis buffer (20 mM HEPES pH 7.9, 150 mM, NaCl, 10 mM, 10% glycerol) with 1× protease inhibitor cocktail set V (Calbiochem). Cells were vortexed for 30 s and kept on ice for additional 45 s. This cycle was repeated 6–8 times to complete the lysis. After the last vortex cycle, the sample was incubated on ice for 2 min. The extract was collected and centrifuged to remove cell debris. The soluble fraction was either used immediately or stored at −80 °C.

Expressed wild type and mutant FLAG-tagged Rpd3 proteins were immunoprecipitated from the whole cell extracts (200 μg of total protein) by incubating with 15 μL of anti-FLAG agarose beads (Sigma) in buffer F (20 mM HEPES at pH 7.6, 1 mM EDTA, 150 mM NaCl, 20% glycerol) at 4 °C for 2 h. The deacetylase activity of immunoprecipitated proteins was measured using the Fluor de Lys™ fluorescence activity assay kit (Biomol), as described.[38] Briefly, the immunoprecipitates were resuspended in HDAC assay buffer (25 μL; 50 mM Tris/Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$). After 30 min of incubation, the Fluor de Lys™ substrate (25 μL; 100 μM final concentration) containing 2% DMSO alone or 100 μM trichostatin A in 2% DMSO was added and incubated at 37 °C for 45 min with shaking. Developer (50 μL) was added and the reaction was incubated for 5 min with shaking. The fluorescence signal at 465 nm was measured after excitation at 360 nm using a GENios Plus plate-reading fluorimeter (Tecan). The percentage HDAC activity was determined by dividing the fluorescence intensity of the reaction with mutant Rpd3 or no protein with that of wild type Rpd3. For western blot analysis, immunoprecipitated proteins were separated by 10% SDS-PAGE, transferred to a PVDF membrane (Immobilon P^{SQ}), and probed with LexA antibodies (Santa Cruz).

3. Results

The HDAC-dependent screen is based upon a gene reporter assay in yeast reported by the Struhl laboratory.[35] As shown in Figure 1, the assay monitors expression of the *LACZ* gene from a reporter controlled by an intact CYC1 promoter and 4 LexA DNA binding sites. Because CYC1 promotes a basal level of transcription, cells transformed with the reporter alone express the *LACZ* gene (Fig. 1A). The HDAC-dependent screen involves expressing the yeast HDAC protein Rpd3 as a LexA-FLAG fusion protein (Rpd3-LexA-FLAG). In the presence of the reporter, LexA recruits Rpd3 to the *LACZ* gene via binding the LexA DNA binding sites, which results in deacetylation of the nucleosomal histones and reduction in *LACZ* gene expression (Fig. 1B). In contrast, if Rpd3 in the LexA-FLAG fusion is catalytically inactive or incubated with a small molecule inhibitor, the nucleosomal histones will remain acetylated and accessible to the transcription machinery, causing expression of *LACZ* gene (Fig. 1C). The *LACZ* gene encodes the enzyme β-galactosidase (β-gal), which can hydrolyze the substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal), resulting in a blue color. Therefore, by observing the color of the yeast cell, the screen will monitor inhibition of Rpd3 activity by a small molecule or mutation.

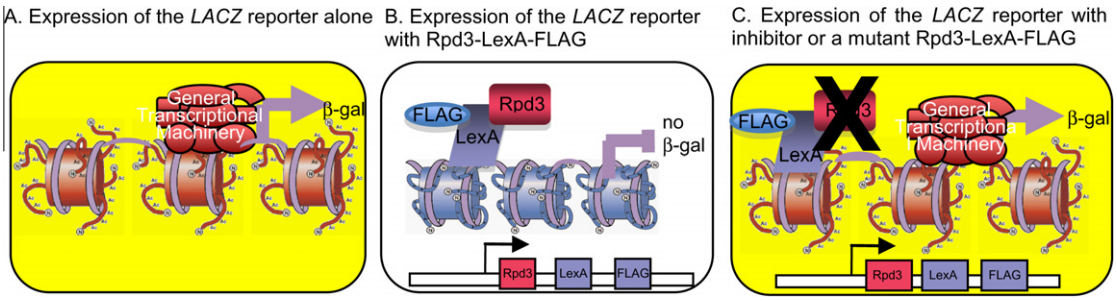

Figure 1. Schematic diagram of yeast-based *LACZ* gene reporter screen. (A) The promoter displays basal *LACZ* expression and β-gal activity, resulting in a colored cell. (B) The presence of the expression plasmid for the Rpd3-LexA-FLAG fusion (bottom construct) results in the repression of *LACZ* gene expression due to deacetylation by Rpd3, resulting in minimal β-gal activity and color. (C) Inactivation of the deacetylase activity of the Rpd3-LexA-FLAG fusion due to mutation or inhibition results in expression of the *LACZ* gene, β-gal activity, and a colored cell.

3.1. Qualitative HDAC-dependent screen

To test the yeast gene reporter screen, the *LACZ* reporter plasmid, pJK1621,[35] was transformed alone or with an expression plasmid encoding Rpd3-LexA-FLAG into the *FT5 Δrpd3::HIS3* yeast strain. The *RPD3* gene has been deleted in the yeast strain to reduce possible complications from endogenous Rpd3. After growth of the cells on agar, the β-gal activity due to *LACZ* gene expression was measured qualitatively by overlaying the colony with a solution containing the X-Gal substrate. As shown in Figure 2, yeast transformed with the *LACZ* reporter plasmid alone showed a high level of β-gal activity and a blue colored cell due to basal *LACZ* gene expression (Fig. 2, column A). As expected, yeast cells transformed with the Rpd3-LexA-FLAG fusion showed a minimum level of β-gal activity and reduced blue color (Fig. 2, column C), indicating that recruitment of Rpd3 suppresses the expression of the reporter gene. To assure that the HDAC activity of Rpd3 is responsible for the reduced *LACZ* expression, cells transformed with the reporter and a catalytically inactive Rpd3H150/151A-LexA-FLAG fusion were also tested. The expectation was that the inactive mutant would not influence *LACZ* gene expression. As expected, cells containing the catalytically inactive Rpd3H150/151A-LexA-FLAG fusion maintained similar levels of β-gal activity compared to the reporter alone, as observed by the similar blue color (Fig. 2, column E vs A). In total, these results suggest that the yeast gene reporter is dependent on Rpd3 activity, which is consistent with previous results from the Struhl lab.[35]

To establish the sensitivity of the screen to small molecule inhibition, the cells containing the *LACZ* reporter without or with either Rpd3-LexA-FLAG or Rpd3H150/151A-LexA-FLAG were incubated with the HDAC inhibitor trichostatin A (TSA) prior to X-Gal overlay. The expectation was that the TSA would significantly influence *LACZ* expression in the cells containing the Rpd3-LexA-FLAG fusion, but not the others. TSA-treated cells expressing the *LACZ* reporter and wild type Rpd3-LexA-FLAG showed elevated β-gal activity and blue color (Fig. 2, column D) compared to the cells without TSA (Fig. 2, column C). In addition, the extent of blue color was similar to that observed with the reporter alone (Fig. 2, lane A) or with the inactive Rpd3-LexA-FLAG fusion (Fig. 2, column E). The data indicate that the expression of *LACZ* reporter gene is sensitive to TSA. As critical controls, TSA treatment had no effect on cells expressing *LACZ* reporter plasmid with or without the inactive Rpd3-LexA-FLAG fusion (Fig. 2, columns B and F), indicating that the effect of the endogenous HDAC activity on the expression of the reporter is negligible. In total, the screen is TSA-dependent, suggesting that it is appropriate for small molecule inhibitor screening.

As a critical control, the enzymatic activities of the Rpd3-LexA-FLAG fusion proteins used in the yeast-based screen were assessed. The fusion proteins were immunoprecipitated using the attached C-terminal FLAG tag and enzymatic activities were monitored using the commercially available fluorimetric HDAC assay (Biomol). As expected, the immunoprecipitated Rpd3-LexA-FLAG fusion displayed significant HDAC activity (Fig. 3, column 2), while the Rpd3H150/151A-LexA-FLAG fusion displayed activity comparable to the no protein control (Fig. 3, columns 1 and 4). In addition, the TSA-sensitivity of the immunoprecipitated proteins was assessed. As expected, the immunoprecipitated Rpd3-LexA-FLAG

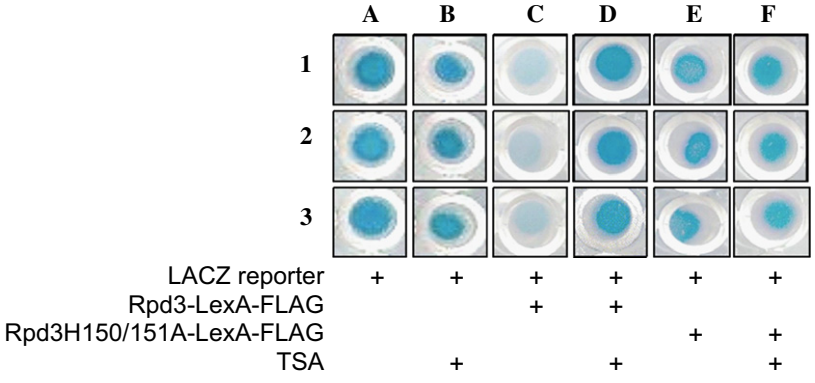

Figure 2. The *LACZ* reporter plasmid, pJK1621, was transformed with or without wild type Rpd3 or catalytically inactive Rpd3H150/151A mutant as LexA-FLAG fusions into the *FT5 Δrpd3::HIS3* yeast strain. Transformed cells were grown with or without TSA (100 μM) for 48 h in 96-well plates and were overlaid with the X-Gal substrate. β-gal activity was observed qualitatively by blue color formation after 3 h of incubation. The components of each experiment are indicated below each column. The experiment was carried out in triplicate (rows 1, 2, and 3).

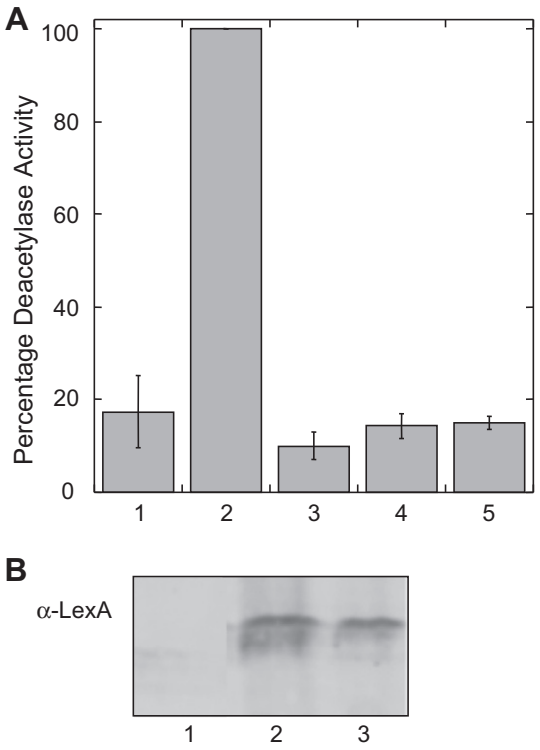

Figure 3. (A) Deacetylase activity of Rpd3-LexA-FLAG fusions. Rpd3 (columns 2 and 3) or Rpd3H150/151A (columns 4 and 5) were expressed in *FT5 Δrpd3::HIS3* yeast strain as LexA-FLAG fusion proteins and immunoprecipitated using their C-terminal FLAG tag and anti-FLAG-agarose resin (Sigma). A no protein control was included in column 1. The immunoprecipitated proteins were used in fluorescence HDAC assays either in the absence (columns 2 and 4) or presence (columns 3 and 5) of 100 μM trichostatin. The mean percent activities of three independent trials was compared with that of the wild type protein (100%—column 2) with standard error shown (Table S1). (B) The immunoprecipitated proteins were separated by SDS–PAGE and probed with a LexA antibody (Santa Cruz) to ensure protein expression. Lane 1: no protein control; lane 2: Rpd3-LexA-FLAG; lane 3: Rpd3H150/151A-LexA-FLAG.

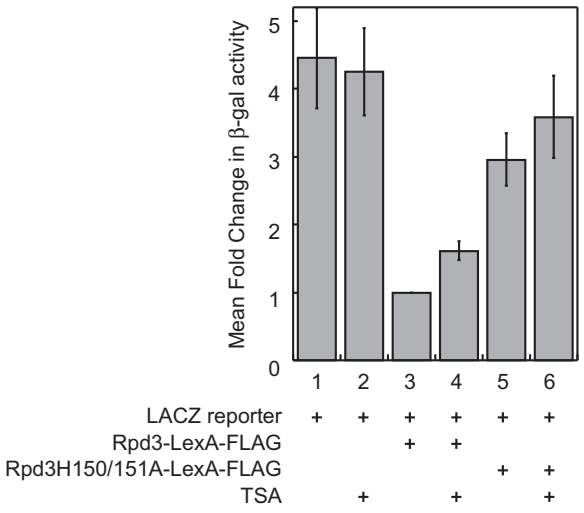

Figure 4. The yeast-based screen is quantitative. The reactions were grown as described in Figure 2 for 6 h at 30 °C and β-gal activity was measured quantitatively by measuring absorbance at 420 nm. Relative fold change in β-gal activity was normalized to the Rpd3-LexA data (set to 1.0) and the mean of three trials is shown with standard error (Table S2).

fusion was inactivated by TSA (Fig. 3, column 3), while the inactive mutant was unaffected (Fig. 3, column 5). The immunoprecipitation experiment confirms that the yeast-based screen is dependent on active Rpd3 and sensitive to inhibitors.

3.2. Quantitative HDAC-dependent screen

We next tested the possibility of quantitatively monitoring Rpd3-LexA-FLAG activity using the β-gal substrate 2-nitrophenyl β-D-galactopyranoside (ONPG) by detecting the production of yellow color at 420 nm. In this case, the assay was performed in liquid format, making it amenable for larger scale screening efforts. Consistent with the agar-based X-Gal format, yeast cells transformed with *LACZ* reporter plasmid alone showed high levels of β-gal activity (Fig. 4, column 1), which was unaffected by TSA treatment (Fig. 4, column 2). The presence of the Rpd3-LexA-FLAG fusion suppressed the expression of *LACZ* gene, resulting in a 4.4 ± 0.7-fold reduction in β-gal activity (Fig. 4, compare columns 1 and 3). In addition, the presence of catalytically inactive Rpd3H150/151A-LexA-FLAG elevated β-gal activity by 3.0 ± 0.4-fold compared to wild type Rpd3-LexA-FLAG (Fig. 4, column 5), which was also unaffected by TSA treatment (Fig. 4, column 6). We next tested the influence of TSA in the presence of the Rpd3-LexA-FLAG fusion. The β-gal activity was elevated 1.6 ± 0.1-fold after TSA treatment (Fig. 4, column 4), indicating that the screen is sensitive to TSA inhibition. Consistent with the X-Gal data, the quantitative analysis confirmed that the yeast gene reporter screen is Rpd3-dependent and sensitive to inhibitors. We note that the human HDAC1-LexA fusion protein[35] produced weak LACZ repression in the ONPG assay (data not shown), which is consistent with the previous reports demonstrating that recombinant human HDAC1 protein from yeast is inactive.[39]

To further explore the screen quantitatively, we assessed the inhibitor dose dependency. Specifically, cells expressing Rpd3-LexA-FLAG were treated with different concentrations of TSA and the β-gal activity was measured using ONPG as the substrate. As shown in Figure 5, TSA showed dose dependent inhibition of HDAC activity. Specifically, while 1 μM TSA treatment showed a 1.2 ± 0.03-fold elevated β-gal activity compared to the TSA-untreated sample (Fig. 5, column 2), 10 μM, 18 μM and 100 μM TSA

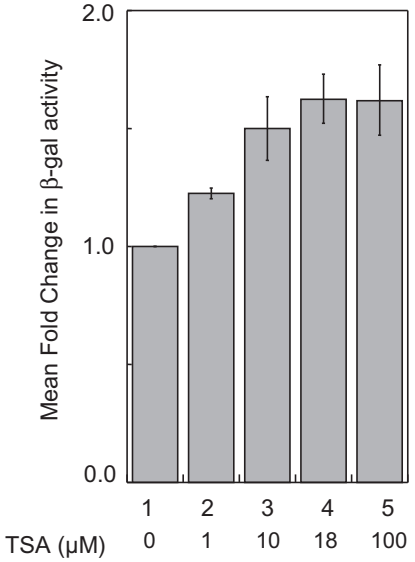

Figure 5. HDAC inhibition by TSA is dose dependent. Transformed yeast cells (see Fig. 2) were grown without or with increasing concentrations of TSA and β-gal activity were measured quantitatively as described. Relative fold change in β-gal activity was calculated with respect to TSA-untreated cells and the mean fold change of four trials is shown with standard error (Table S3).

treatments showed a 1.5 ± 0.1-fold, 1.6 ± 0.1-fold and 1.6 ± 0.1-fold elevated β-gal activity, respectively (Fig. 5, columns 3–5). Collectively, data obtained from quantitative analysis suggests that the yeast gene reporter screen is capable of detecting dose dependent HDAC inhibition.

3.3. Optimization of reporter screen to increase the inhibitor sensitivity

The β-gal activity could be elevated up to 1.6-fold by inhibiting Rpd3-LexA-FLAG activity with TSA (Fig. 4, column 4). However, the presence of catalytically inactive Rpd3H150/151A mutant elevated β-gal activity up to 3.0-fold compared to wild type Rpd3 (Fig. 4, column 5). We speculate that the lower levels of β-gal activity observed with TSA treatment compared to the inactive Rpd3 mutant may be due to the small molecule cell permeability issues in yeast, as has been previously reported.[40,41]

The yeast cell envelope is a protecting capsule that plays a major role in controlling the permeability properties of the cell. The cell wall of a yeast cell envelope is remarkably thick (100–200 nm) and its major structural constituents are polysaccharides (80–90%), mainly glucans and mannans.[42] Both β-2,6 and β-1,3 linked glucans provide strength to the cell wall, forming a microfibrillar network. Digestion of the yeast cell wall by lytic enzymes, such as zymolyase, is necessary for many experimental procedures, including spheroplasting, immunofluorescence, transformation, and protein purification.[43–45] The main enzymatic activities of zymolyase are β-1,3 glucanase and β-1,3 glucan laminaripentaohydrolase, which hydrolyze glucose polymers at β-1,3 glucan linkages,[46] leading to cell wall digestion.

We hypothesized that weakening of the yeast cell wall by treating with zymolyase would facilitate cellular uptake of small molecules, which will augment the HDAC inhibition and β-gal activity. To explore this hypothesis, yeast cells transformed with the *LACZ* reporter Rpd3-LexA-FLAG fusion were incubated with TSA and increasing amounts of zymolyase prior to measuring *β-gal* activity. As shown in Figure 6, zymolyase enhanced the β-gal activity of the cells in a dose dependent manner. Specifically, while cells incubated with TSA and 1 U/mL of zymolyase showed the same level of β-gal activity compared to TSA alone (1.7-fold and 1.8-fold, Fig. 6, columns 1 and 2), treatment with 13 U/mL or 50 U/mL of zymolyase augmented the β-gal activity to 2.6-fold or 3.5-fold, respectively (Fig. 6, columns 3 and 5). Collectively, the increased β-gal activity observed in the presence of zymolyase confirms that the weakening of the yeast cell wall facilitates cellular uptake of small molecules. Importantly, we show that zymolyase treatment increases the sensitivity of the gene reporter screen towards HDAC inhibitors.

To further evaluate the optimized yeast gene reporter screen, the proper controls were repeated in the presence of zymolyase (20 U/mL). Specifically, the *LACZ* reporter plasmid, pJK1621, was transformed with or without the LexA-FLAG fused to wild type Rpd3 or catalytically inactive Rpd3 H150/151A into *FT5 Δrpd3::HIS3* yeast strain and the β-gal activity was measured quantitatively. As shown in Figure 7, cells transformed with *LACZ* reporter plasmid alone showed the same high levels of β-gal activity in the presence or absence of TSA (Fig. 7, columns 1 and 2), consistent with the data obtained without zymolyase (Fig. 4). While recruitment of Rpd3-LexA-FLAG reduced β-gal activity (Fig. 7, column 3), inhibition by TSA restored that activity by 2.7-fold (Fig. 7, column 4). It is important to note that only 1.6-fold elevation of β-gal activity was observed with the treatment of 100 μM TSA alone (Fig. 4), indicating that zymolyase treatment results in greater sensitivity to TSA. Interestingly, the presence of catalytically inactive Rpd3H150/151A-LexA-FLAG elevated β-gal activity to 4.1-fold (Fig. 7, column 5), which is greater than the 3.0-fold increase observed without zymolyase (Fig. 4). The enhanced β-gal activity observed in all reactions (compare Figs. 4 and 7) suggests that zymolyase facilitates the permeability of yeast cell and release of β-galactosidase.

To establish the yeast gene reporter screen as a general tool, we tested two other known HDAC inhibitors, apicidin and SAHA. Unlike TSA, apicidin (100 μM, Fig. 8, column 2) and SAHA (2 mM, Fig. 8, column 4) did not show the expected elevated levels of β-gal activity compared to untreated cells (Fig. 8, column 1). We wondered if issues of cell permeability explained the lack of β-gal activity,[47,48] because apicidin and SAHA are potent nM inhibitors of class I HDAC proteins.[19] To test the issue of cell

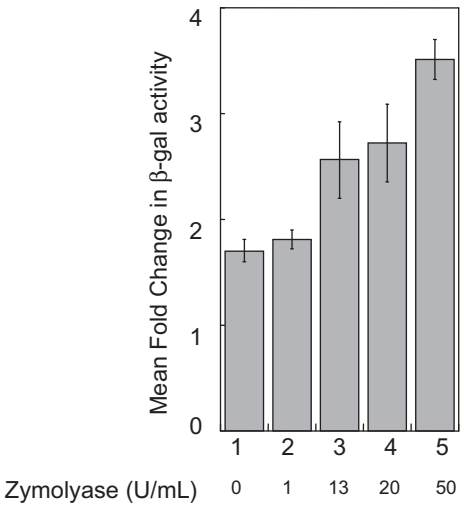

Figure 6. The influence of zymolyase on TSA dependence. Transformed cells were grown as described in Figure 2 along with the indicated amounts of zymolyase (Zymo Research, Inc.) before β-gal activity was measured as described. An untreated control was performed at each zymolyase concentration to determine the mean fold change, although only the control for the reaction without zymolyase is shown in lane 1. Mean relative fold change of at least three trials is shown with standard error (Table S4).

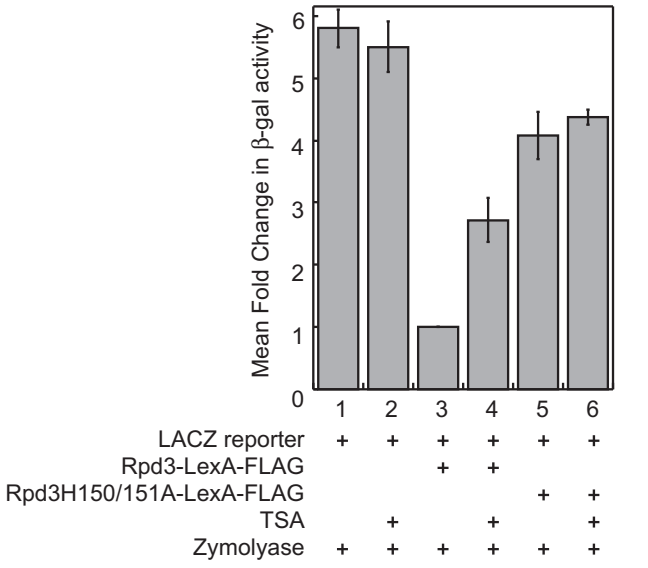

Figure 7. Zymolyase enhances the small molecule sensitivity of the screen. Transformed cell (see Fig. 2) were grown in the presence of zymolyase (20 U/mL) and with or without TSA (50 μM) before β-gal activity was measured quantitatively as described. Mean relative fold change of at least three trials is shown with standard error (Table S5).

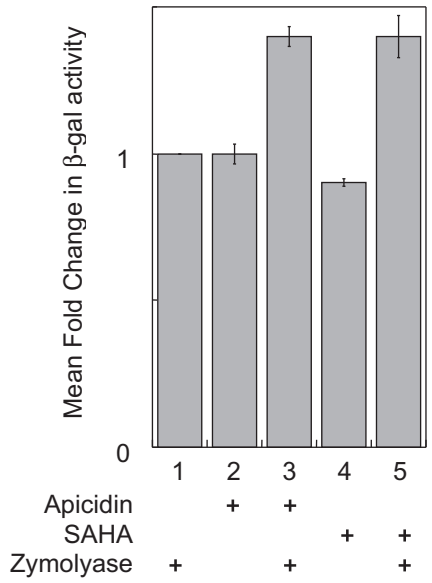

Figure 8. Inhibition of Rpd3-LexA-FLAG activity by apicidin and SAHA. Transformed cells (see Fig. 2) were grown with or without apicidin (100 μM) or SAHA (2 mM) in the presence or absence of zymolyase (100 U/mL) before β-gal activity was measured as described. Mean relative fold change of at least three trials is shown with standard error (Table S6).

permeability, zymolyase was included during inhibitor incubation. In the presence of zymolyase, elevated levels of β-gal activity were observed with apicidin (1.4 ± 0.03-fold, Fig. 8, column 3) and SAHA (1.4 ± 0.07-fold, Fig. 8, column 5). These results confirm that zymolyase treatment enhances the sensitivity of the yeast gene reporter screen to small molecule inhibitors. Collectively, the data indicate that the yeast gene reporter screen is well suited for screening HDAC inhibitors.

4. Discussion

Yeast assays have been used successfully as initial screening tools for the discovery of small molecule modulators.[49–51] Most notably, yeast screens were used to identify small molecule inhibitors of the NAD-dependent Sirtuin HDAC proteins.[31,32,52–54] Despite the utility, no yeast-based screen against metal-dependent HDAC proteins has been reported. We report here development of a yeast-based gene reporter screen centered on the class I yeast homolog Rpd3. The screen is dependent on Rpd3 deacetylase activity and sensitive to the small molecule inhibitors TSA, apicidin, and SAHA. The screen was validated in both a qualitative, agar-based format and a quantitative, solution-based format, making it practical for many applications. In total, the yeast-based gene reporter screen is a tool for testing small molecule inhibitors and mutant HDAC proteins for activity.

The yeast screen adds to the available tools for testing HDAC small molecule inhibitors. While the fluorimetric assay widely used in the field is easy to use and high throughput, it suffers from the use of non-native substrates, which can result is false positives, and high cost (from $1 to $4 per reaction).[38] As a result, there is interest to develop alternative HDAC inhibitor screening methods that use HDAC activity from inexpensive lysates or cells and involve native substrates. The reported yeast screen employs native histone substrates in the cell-based assay, which will avoid artifacts from non-natural peptide substrates. In addition, by relying on commercially available and relatively inexpensive reagents, we estimate the assay to cost several cents per reaction. With the yeast screen available for initial hit identification, the fluorimetric assay can be used to confirm hits and determine IC_{50} values towards discovery of new HDAC inhibitors from small molecule libraries.

One limitation of the yeast-based screen is the difficulty for small molecules to penetrate the yeast cell wall. In fact, the problem of cell permeability likely explains the relative high concentrations of inhibitors required to influence β-gal activity. For example, while TSA inhibits Rpd3 and human class I HDAC proteins at nM concentrations,[19,55] μM concentrations of TSA were required to influence β-gal activity in the screen (see Fig. 4). Despite the issue of cell permeability, yeast-based assays have been used previously to screen libraries of small molecules.[31,32,49,53,56] In each of these cases, μM concentrations of small molecules were used successfully. However, methods to augment the cell permeability of yeast to small molecules would be useful. To increase the sensitivity of yeast gene reporter screen to small molecules, we tested zymolyase. Because zymolyase is routinely used for partial digestion of yeast cell wall, we wondered if including zymolyase in the screen would augment sensitivity. We found that cells treated with TSA and zymolyase showed an increased level of β-gal activity compared to that of TSA alone (2.7-fold vs 1.6-fold, respectively). In addition, we found that zymolyase enhanced detection of poorly cell permeable inhibitors, such as apicidin and SAHA. To our knowledge, this is the first report of a lytic enzyme enhancing the sensitivity of a yeast-based reporter screen to small molecule inhibitors. We intend to explore further the general use of zymolyase to overcome the cell permeability concern of yeast-based screens.

The yeast reporter system is also appropriate for screening Rpd3 mutants for catalytic activity. Identification of inactive mutants has been helpful to characterize the residues involved in activity for yeast and human HDAC proteins.[30,35,57,58] The availability of a gene reporter screening platform will facilitate the identification of additional mutants. For example, catalytically active mutants of kinases have been exploited as tools in cell biology experiments and inhibitor identification studies.[59,60] In addition, inactive mutants have been used to create dominant negative conditions.[61] A screening platform for HDAC protein mutants can be used to create similar cell biology tools and inhibitor design paradigms.

In summary, we developed a yeast gene reporter screen dependent on histone deacetylase Rpd3 activity that is sensitive to inhibitors. To our knowledge, the screen is the first yeast-based method sensitive to class I HDAC proteins. The yeast gene reporter screen relies on native substrates in a cell-based assay and can be coupled with the commercially available fluorimetric assay to rigorously characterize identified inhibitors with IC_{50} values. In addition, the screen can be used to test Rpd3 mutants for enzymatic activity. The availability of multiple complimentary tools for HDAC activity screening will facilitate development of new genetic and chemical tools targeting HDAC proteins.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.08.045.

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