## Screening of Chemical Libraries Using a Yeast Model of Retinal Disease

Benjamin M. Scott<sup>1,2,3</sup>, Leanne E. Wybenga-Groot<sup>4</sup>, C. Jane McGlade<sup>5,6</sup>, Elise Heon<sup>7</sup>, Sergio G. Peisajovich<sup>1</sup>, Belinda S.W. Chang<sup>1,8,9\*</sup>

<sup>1</sup>Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada
<sup>2</sup>Department of Chemistry and Biochemistry, University of Maryland, College Park, MD, USA
<sup>3</sup>Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD, USA
<sup>4</sup>SPARC BioCentre, The Hospital for Sick Children, Toronto, ON, Canada
<sup>5</sup>Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada
<sup>6</sup>Program in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada
<sup>7</sup>Department of Ophthalmology and Vision Science, The Hospital for Sick Children, Toronto, ON, Canada
<sup>8</sup>Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, Canada
<sup>9</sup>Centre for the Analysis of Genome Evolution and Function, University of Toronto, ON, Canada

## Abstract

Retinitis pigmentosa (RP) is a degenerative retinal disease, often caused by mutations in the G protein-coupled receptor rhodopsin. The majority of pathogenic rhodopsin mutations cause rhodopsin to misfold, including P23H, disrupting its crucial ability to respond to light. Previous screens to discover pharmacological chaperones of rhodopsin have primarily been based on rescuing rhodopsin trafficking and localization to the plasma membrane. Here, we present methods utilizing a yeast-based assay to screen for compounds that rescue the ability of rhodopsin to activate an associated downstream G protein signaling cascade. We engineered a yeast strain in which human rhodopsin variants were genomically integrated, and were able to demonstrate functional coupling to the yeast mating pathway, leading to fluorescent protein expression. We confirmed that a known pharmacological chaperone, 9-*cis* retinal, could partially rescue light-dependent activation of a disease-associated rhodopsin mutation (P23H) expressed in yeast. These novel yeast strains were used to perform a phenotypic screen of 4280 compounds from the LOPAC1280 library and a peptidomimetic library, to discover novel pharmacological chaperones of rhodopsin. The fluorescence-based assay was robust in a 96-well format, with a Z'-factor of 0.65, and a signal-to-background ratio of above 14. One compound was selected for additional analysis, but it did not appear to rescue rhodopsin function in yeast. The methods presented here are amenable to future screeens of small molecule libraries, as they are robust and cost effective. We also discuss how these methods could be further modified or adapted to perform screeens of more compounds in the future.

## Introduction

Retinitis pigmentosa (RP) represents a group of hereditary diseases that cause progressive visual degeneration, affecting between 1:3000 to 1:5000 people worldwide.<sup>1</sup> Drug discovery approaches to this disease are particularly challenging due to the large number of RP associated genes, diversity of mutations, and difficulty in characterizing the light-dependent function of proteins.<sup>2-3</sup> Mutations in rhodopsin, a G protein-coupled receptor (GPCR), account for 20 % to 30 % of autosomal dominant RP cases, making rhodopsin a primary drug target.<sup>1</sup> Therefore, a goal of RP research is to discover compounds that function as pharmacological chaperones to correctly stabilize rhodopsin, which may prevent or even reverse vision loss.

The rhodopsin mutation P23H is responsible for the majority of autosomal dominant RP cases in North America.<sup>1</sup> P23H disrupts the extracellular facing N-terminal cap, preventing rhodopsin from forming a stable complex with its natural chromophore 11-*cis* retinal.<sup>4</sup> Consequently, P23H rhodopsin does not efficiently activate the downstream G protein transducin in response to light.<sup>4-5</sup> In addition to this loss of rhodopsin function, overexpression of P23H

rhodopsin *in vitro* causes the protein to accumulate in the ER,<sup>6</sup> reducing plasma membrane localization while activating ER stress pathways.<sup>1</sup> Derivatives of 11-*cis* retinal, collectively called retinal analogs, have shown promise as pharmacological chaperones of P23H rhodopsin by improving biosynthesis, reducing ER retention, and increasing trafficking to the plasma membrane *in vitro*.<sup>7-9</sup> Additional mutations in the rhodopsin N-terminal cap have also been rescued *in vitro* using retinal analogs, indicating potentially broad use of pharmacological chaperones to treat RP.<sup>4</sup> These studies have led to several clinical trials with retinal analogs, which have had mixed results, failing to significantly restore vision.<sup>10-11</sup> Retinal analogs are also inherently light sensitive making them difficult to deliver; moreover, they disassociate from rhodopsin following light exposure, and high concentrations are cytotoxic.<sup>12</sup>

Based on the poor suitability of retinal analogs as drug candidates, non-retinal pharmacological chaperones have been investigated in several recent compound screening campaigns. A mammalian cell-based assay that quantifies rhodopsin trafficking to the plasma membrane<sup>12</sup> has contributed to the discovery of non-retinal compounds from diverse chemical families that rescue P23H rhodopsin trafficking, but not signaling function, *in vitro*.<sup>13-15</sup> The diabetic drug metformin was also shown to improve both rhodopsin expression and trafficking *in vitro*, but counterintuitively increased photoreceptor cell death in a rat model.<sup>16</sup> The authors believed this failure was due to the inherent instability of P23H rhodopsin, resulting in destabilized photoreceptor outer segments.<sup>16</sup> This is consistent with other studies that indicate P23H instability, not ER retention, is the primary mechanism contributing to RP pathogenesis.<sup>17-</sup>

Pharmacological chaperones that rescue both the trafficking and signaling function of other GPCRs have been discovered by using cell-based screening assays of downstream G

protein activation.<sup>19-21</sup> Many of these compounds were originally discovered as antagonists, but have shown remarkable versatility in rescuing multiple pathogenic variants of the same GPCRs.<sup>22-23</sup> The most successful pharmacological chaperones of GPCRs, such as that of gonadotropin-releasing hormone receptor (GnRHR) and vasopressin 2 receptor (V2R), are peptidomimetics.<sup>24</sup> This is likely because the natural ligands of these GPCRs are peptides and peptidomimetics can diffuse into cells and mimic protein-protein interactions. Pharmacological chaperones acting on V2R, which are neither peptidomimetics nor act as antagonists, have also been discovered.<sup>25</sup> Thus, there may be pharmacological chaperones yet to discover that rescue mutant GPCR function without disturbing natural ligand interactions.

Rhodopsin differs from most GPCRs as it is covalently bound to its retinal chromophore, and is activated by light rather than a ligand, which presents unique challenges in discovering compounds that rescue rhodopsin signaling function. We previously developed a yeast-based system which functionally links human rhodopsin to the yeast mating pathway.<sup>26</sup> This system has the advantages of being rapid to screen and more cost effective compared to mammalian cell-based methods, while enabling quantification of rhodopsin-dependent G protein signaling in response to light by using a fluorescent reporter gene.

In this study, we extended yeast-based assays of rhodopsin function to screen for pharmacological chaperones that rescue rhodopsin's ability to activate a downstream G protein signaling pathway, acting as a proxy for rescued light-dependent function. Similar yeast-based phenotypic assays have been used to discover compounds acting on human GPCRs,<sup>27</sup> and various yeast-based models of human diseases have been used for compound screening assays.<sup>28-30</sup> We show that light-dependent signaling of yeast-expressed P23H rhodopsin can be partially rescued with high concentrations of retinal chromophore, a known pharmacological chaperone.

Furthermore, we describe engineered yeast strains that enable rapid quantification of G protein activation by rhodopsin in a cellular context. Our work outlines a simple method of screening for compounds that rescue rhodopsin signaling function without requiring light exposure.

## **Materials and Methods**

#### **Yeast Strain Engineering**

Parent yeast strain BS017 was used for all strain engineering. Suppl. Table S1 contains all strain genotypes. Yeast genome modifications were conducted using homologous recombination of selectable markers. The selectable marker HIS3 was inserted at the NotI/SbfI sites in plasmid pBS600,<sup>26</sup> creating plasmid pBS603. Homology regions identical to the 800 base pairs on either side of the yeast SST2 ORF were inserted at restriction enzyme sites NheI/PspOMI and SbfI/KpnI. The constitutive promoter pTDH3 was inserted at sites PspOMI/XhoI, and human rhodopsin (RefSeq NM\_000539.3) was inserted at the AarI sites. One of four human rhodopsin gene variants was introduced: wild-type, or contained the mutation P23H, or mutations E113Q M257Y ("EM"), or mutations P23H E113Q M257Y ("P23H EM") to create the final plasmids (Suppl. Fig. S1). The QuikChange site-directed mutagenesis protocol (Agilent) and using PfuUltra II Fusion HS DNA Polymerase (Agilent) were used to introduce desired mutations into rhodopsin. Additional plasmid constructs were created where yeast-optimized GFP was added to the C-terminus of a rhodopsin variant lacking a stop codon, via the pBS603 BamHI site. After digesting pBS603 with PmeI, yeast strain BS017 was transformed with 1 µg of the linearized cassette DNA and plated on SD-HIS selective media (BioShop Canada). Successful recombination events were confirmed by PCR and flow cytometry.

#### Yeast Microscopy

Yeast strains expressing human rhodopsin C-terminally tagged with GFP were grown to log phase in SD-Complete media (BioShop Canada), then plated on glass slides treated with 1 mg/mL concanavalin A (L7647, Sigma-Aldrich) in sterile phosphate buffered saline (AM9625, Invitrogen) supplemented with 1 mmol/L CaCl<sub>2</sub> and 1 mmol/L MgCl<sub>2</sub>. Images were acquired using a Leica TCS SP5 confocal microscope.

# Yeast Light Activation Assay and Pharmacological Rescue of Plasmid Expressed Human P23H Rhodopsin

This protocol is similar to the "Yeast light activation assay" previously described.<sup>26</sup> Yeast strain BS017 transformed with a human rhodopsin mutant gene in the pRS316 pTDH3 vector was incubated overnight in 5 mL SD-URA media (BioShop Canada) at 30 °C, while shaking. To rescue P23H rhodopsin function, 20  $\mu$ mol/L 9-*cis* retinal in ethanol (Sigma-Aldrich) was added to the overnight culture media. The following morning, cells were diluted to OD<sub>600</sub> 0.05 in fresh 5 mL SD-URA media containing 5  $\mu$ mol/L 9-*cis* retinal, in a LightSafe 50 mL centrifuge tube (Sigma-Aldrich), and incubated for 2 h protected from light at 30 °C, while shaking. Cultures were then exposed to light using a Fiber-Lite MI-150 high intensity illuminator (Dolan-Jenner) set to full intensity for 15 minutes at room temperature. After 100  $\mu$ L samples were taken for analysis, cultures were placed back in the 30 °C shaking incubator. An additional 5  $\mu$ mol/L 9-*cis* retinal after each light bleach was added to cultures in order to rescue the signaling function of P23H rhodopsin.

#### Flow Cytometry Analysis of Rhodopsin Expressing Yeast Strains

Yeast strain BS017 transformed with a plasmid were cultured in SD-URA media, while yeast strains stably expressing rhodopsin (BS023, BS024, BS025, BS026) and GFP-tagged rhodopsin (BS027, BS028, BS032, BS033) were cultured in SD-Complete media. Cells were treated with the protein synthesis inhibitor cycloheximide (BioShop Canada), to a final concentration of 10 µg/mL. The mCherry signal, indicative of G protein activation, of at least 6000 cells was measured for each sample with a Miltenyi Biotec MACSQuant VYB flow cytometer. In independent experiments, the GFP signal of at least 6000 cells was used to quantify rhodopsin expression levels, measured with an Invitrogen Attune NxT flow cytometer. The mean fluorescence was determined using FlowJo.

#### **Compound Library Screening by 96-Well Fluorescence Plate-Based Assay**

The LOPAC1280 library (Sigma-Aldrich) and 3000 compounds from the Ontario Institute for Cancer Research (OICR) peptidomimetic library "OICR-L110" (ChemDiv) were selected for screening (4280 total compounds). Overnight cultures of yeast strains BS025 (EM rhodopsin) and BS026 (P23H EM rhodopsin) were diluted to an initial OD<sub>600</sub> 0.3 with SD-Complete media, and 500  $\mu$ L of BS025 or BS026 dilution was dispensed to column 1 or 2-12, respectively, of a 96-well deep-well block (89237-526, VWR). 720 nL of dimethyl sulfoxide (DMSO) vehicle or small molecule compound was dispensed with an Echo 550 (Labcyte) into columns 1 and 12, or 2-11, respectively, of a V bottom 96-well plate (3363, Corning). 120  $\mu$ L SD-Complete media (BioShop Canada) was added to each well and mixed with a Bravo Automated Liquid Handling Platform (Agilent). 100  $\mu$ L from each well was then added to the corresponding well in the deepwell block containing yeast, for a total volume of 600  $\mu$ L per well and final concentration of 10  $\mu$ mol/L LOPAC1280 (0.1 % DMSO) or 5  $\mu$ mol/L peptidomimetic (0.5 % DMSO) compound, with yeast at a final  $OD_{600}$  0.25. Each well contained a 6 mm borosilicate glass bead (Sigma-Aldrich) to maintain yeast in suspension while culturing. Cultures were grown in the deep-well blocks for 6 h in at 30 °C, while shaking. 100 µL from each well was then transferred to a 96-well black, clear bottom plate (Costar 3603, Corning), and mCherry fluorescence (585 nm excitation/615 nm emission) and cell density (600 nm absorbance) were quantified using a Synergy Neo plate reader (BioTek Instruments). Potential hit compounds were tested as above in triplicate. False positives were identified by incubating compound with yeast strain BS017, which contains the mCherry reporter but does not express rhodopsin, then measuring fluorescence and cell density as above.

#### **Data Analysis**

mCherry fluorescence of column 1 (DMSO, EM rhodopsin, strain BS025) of each plate served as High Control, while mCherry fluorescence column 12 (DMSO, P23H EM rhodopsin, strain BS026) served as Low Control. Assay results were normalized on a per-plate basis using mCherry fluorescence measurements, with the following equation:

% Rescue = 
$$100 \times \frac{\text{Test Well} - \text{Mean Low Control}}{\text{Mean High Control} - \text{Mean Low Control}}$$

Wells with cell density >3 standard deviations (SD) below the mean Low Control cell density were eliminated from further characterization. Potential hit compounds were identified using a cutoff of three times the SD of the mean Low Control % Rescue, on a per plate basis. Plate Z'factor was calculated using the following equation,<sup>31</sup> where SD<sub>H</sub> and SD<sub>L</sub> are the standard deviations of mCherry fluorescence for the plate High Control and Low Control respectively:

Plate Z'-Factor = 
$$1 - \frac{3(SD_H - SD_L)}{Mean High Control - Mean Low Control}$$

Signal-to-background ratio (S/B) of mCherry fluorescence was calculated as the ratio between the mean High Control and mean Low Control in the experiment. During assay development, the Low Control for S/B was either the yeast strain BS017 expressing P23H EM rhodopsin from a plasmid, or the yeast strain BS026 expressing P23H EM rhodopsin from the yeast genome.

#### Dose-Response Analysis and Light Activation Test with Compound L251-0001

Yeast strain BS026 (P23H EM rhodopsin) was incubated overnight in SD-Complete media. Yeast cells were diluted to OD<sub>600</sub> 0.05 into 600 µL fresh media, containing up to 320 µmol/L of peptidomimetic compound L251-0001 in DMSO. (Named by MolPort Cat#, also MolPort-010-860-710. Original library identifier OICR0005849A01). At the highest compound concentrations, the cultures contained up to 0.3 % DMSO. Cultures were then incubated for 6 h in at 30 °C, while shaking. Pharmacological rescue with 9-*cis* retinal was performed as described above, with the additional step of adding 50 µmol/L L251-0001 after diluting overnight cultures to OD<sub>600</sub> 0.05 in 5 mL fresh SD-Complete media, and utilizing yeast strains BS023 (WT rhodopsin) and BS024 (P23H rhodopsin) instead of expressing rhodopsin from a plasmid. Following light activation, the mCherry signal of at least 6000 cells was measured for each sample with a Miltenyi Biotec MACSQuant VYB. The mean mCherry fluorescence was determined using FlowJo.

## **Results and Discussion**

#### **Rescue of Plasmid Expressed P23H Rhodopsin**

We first sought to determine if incubation with 9-*cis* retinal, a well-characterized pharmacological chaperone of rhodopsin, would rescue the light-dependent activation of P23H rhodopsin. Previous studies have shown that incubating cells with retinal during P23H rhodopsin biosynthesis is crucial to stabilizing the protein.<sup>4-5, 12</sup> Thus, yeast strains were incubated with 20 µmol/L 9-*cis* retinal overnight, then 5 µmol/L 9-*cis* retinal after refreshing the media. To rescue P23H rhodopsin, 5 µmol/L 9-*cis* retinal was also added after each time the samples were exposed to light, a step which is required to activate rhodopsin. This treatment rescued approximately 20 % of P23H rhodopsin's signaling activity in response to light (**Figure 1**), where the expression of the fluorescent reporter protein mCherry served to indicate rescued G protein activation.

However, this repetitive addition of light-sensitive retinal followed by exposing cultures to light was not conducive to a compound screening assay. Thus, we sought to determine if the function of constitutively active rhodopsin could be rescued by incubating with 9-*cis* retinal. The previously characterized constitutively active synthetic mutant was generated by site-directed mutagenesis,<sup>32</sup> with or without the pathogenic P23H mutation (**Figure 2A**). We have shown that expressing E113Q M257Y<sup>32</sup> (herein referred to as "EM") rhodopsin in yeast constitutively activates the downstream G protein signaling pathway, leading to robust mCherry fluorescence without needing to activate rhodopsin with light.<sup>26</sup> We hypothesized that by incubating P23H EM rhodopsin with 9-*cis* retinal a rescued phenotype could be observed, without laborious light-activation procedures. However, despite incubation with high concentrations of 9-*cis* retinal, no downstream signaling pathway activation was observed (**Figure 2B**). Increasing the length of incubation to 16 h did not improve the signal (data not shown). Retinal also did not display the

expected inverse agonism of EM rhodopsin,<sup>32</sup> as G protein signaling in yeast remained high even in the presence of 1 mmol/L retinal, which suggests that retinal binding differs in live yeast versus protein purified from mammalian cells.

#### Genomic Expression of Rhodopsin Improves Downstream Signaling

As plasmid-based expression is more variable than the expression of genomically integrated genes in yeast,<sup>33</sup> we postulated that genomic integration of rhodopsin could improve a rescue phenotype. Following successful integration of EM rhodopsin into the yeast genome, mCherry positive cells increased from 42 % to 87 % of the total (Figure 3A), and increased the signal-tobackground ratio (S/B) from 19 to 22 versus plasmid-based expression of EM rhodopsin (Figure **3B**). As mCherry expression is dependent on functional rhodopsin being expressed, these results suggested that expression of EM rhodopsin was improved by integrating the gene into the yeast genome. Relative protein expression was investigated by generating additional strains with GFPtagged rhodopsin variants integrated into the yeast genome. Quantified by flow cytometry, expression of P23H rhodopsin was 44 % lower than WT rhodopsin, while P23H EM expression was 34 % lower than EM rhodopsin (Suppl. Fig. S2). P23H rhodopsin variants integrated into the yeast genome exhibited poor membrane localization (Suppl. Fig. S3), similar to plasmidexpressed P23H rhodopsin as reported previously.<sup>26</sup> In addition, genomic integration lead to GFP accumulation in the vacuole, indicating significant overexpression, which we did not previously observe with plasmid-expressed rhodopsin.

Although the mCherry S/B ratio was improved by using genome-integrated rhodopsin, the mCherry signal from the P23H EM strain was not improved by incubation with up to 20  $\mu$ mol/L 9-*cis* retinal for either 6 h or 16 h (data not shown). Combined with the results from

plasmid-expressed EM rhodopsin, this suggests that in yeast, 9-*cis* retinal does not act as a pharmacological chaperone of rhodopsin when it also contains the EM mutations.

#### **Development of 96-Well Fluorescence Plate Reader Assay**

In instances where a positive control compound cannot be found for a compound screening assay, an artificial rescue phenotype (i.e. EM rhodopsin) can be used as a positive control instead.<sup>34</sup> The high S/B ratio between strains expressing EM rhodopsin versus P23H EM rhodopsin provided a foundation to develop an unbiased phenotypic screening assay, requiring only that G protein signaling is rescued by a compound, which is predicated on correct folding and trafficking of rhodopsin to the plasma membrane. A general goal for pharmacological chaperone screens is to discover compounds that do not compete for the native ligand binding pocket,<sup>19</sup> thus the inability of retinal to bind EM rhodopsin is not expected to negatively impact screening applications of our assay.

Up to this point, all fluorescence measurements were conducted with flow cytometrybased methods, which can be difficult to adapt to a compound screen due to lower throughput. We therefore investigated the use of a fluorescence plate reader-based assay, which could measure fluorescence in up to 96 wells at once. When using flow cytometry, mCherry expression can be detected just one hour after rhodopsin has been activated by light, and measurement at six hours gives a robust S/B,<sup>26</sup> which is comparable to other fluorescent reporters of yeast mating pathway activation.<sup>35</sup> Based on this, we reasoned that six hours would be sufficient time to detect rescued rhodopsin function during a compound screen.

However, fluorescence plate reader measurements are typically less sensitive than flow cytometry, and the fluorescence signal is influenced by cell density. Thus, various initial cell

densities were tested to ensure the S/B remained high, balanced with the desire to have a high compound-to-cell ratio during a six hour incubation. An initial cell density of  $OD_{600}$  0.25 followed by culturing the yeast for six hours lead to a S/B of >12 in these initial tests, while maintaining a relatively low number of cells in the well (**Suppl. Fig. S4**). Increasing the cell density to  $OD_{600}$  0.5 or 1.0 only modestly improved the S/B, so an initial  $OD_{600}$  0.25 was deemed optimal and sufficient. Anticipating concentrations as high as 0.5 % DMSO in the media during the compound screen, we measured S/B levels at increasing cell densities in 0.5 % DMSO media (**Suppl. Fig. S4**). The presence of 0.5 % DMSO did not significantly affect mCherry expression or yeast growth, consistent with previous observations when expressing human GPCRs in yeast.<sup>27</sup> Although the S/B ratio of the fluorescence plate reader-based assay was approximately half that of flow cytometry-based measurements, the sensitivity of the plate reader-based assay appeared to be sufficient to detect a rescued signaling function of P23H EM rhodopsin.

#### **Pilot Screen of 4280 Compounds**

To investigate the feasibility of our phenotypic screening strategy, a pilot screen of 4280 compounds was performed. The yeast strain containing P23H EM rhodopsin integrated into the genome was incubated with compounds from chemical libraries, where observed mCherry fluorescence would indicate successful rescue of rhodopsin's signaling function. The LOPAC1280 library was chosen based on its well characterized suite of chemicals, with  $\approx$ 50 % of compounds targeting human GPCRs.<sup>36</sup> A peptidomimetic compound library compiled by OICR was also selected based on previous reports of peptidomimetics functioning as pharmacological chaperones of GPCRs.<sup>24</sup> The 4280 compounds were screened over the course of three days, utilizing 96-well deep-well blocks to culture the yeast in the presence of compound. mCherry signal was measured using a fluorescence plate reader, and % Rescue was normalized

to the "High Control" in rows of the same plate (EM strain). The scatterplot of the normalized results of the pilot screen is shown in **Figure 4**. An average Z'-factor of  $0.65\pm0.11$  (SD) for controls in the complete library screen was achieved, indicating a robust screening assay.<sup>31</sup> A cutoff for determining potential hits was set at a % Rescue 3SD above the plate Low Control. Forty-eight compounds were found above this cutoff; however, they were each very close to the noise of the assay.

These 48 compounds and one additional compound of interest (13-*cis*-retinoic acid) were tested again for their ability to rescue P23H EM rhodopsin function in our plate reader-based assay, this time in triplicate. Four peptidomimetic compounds had one replicate above 3% rescue (**Suppl. Fig. S5**). The peptidomimetic "L251-0001" had previously shown the highest signal in the initial pilot screen and thus was selected for additional study. The 49 compounds were also screened to determine any residual fluorescence of the compounds themselves, which identified three false-positives from the original 48 compounds of interest (**Suppl. Fig. S6**).

#### **Characterization of L251-0001**

To better determine if compound L251-0001 could rescue rhodopsin's function, a dose-response assay was performed using the P23H EM strain, followed by measurement of mCherry fluorescence by flow cytometry (**Suppl. Fig. S7**). L251-0001 failed to increase the mCherry signal, showing no difference versus DMSO alone. The compound precipitated at (40, 80, 160, and 320) µmol/L, indicating a limit to an effective working concentration. With the failure of pharmacologically rescuing constitutively active rhodopsin with the compound, we instead attempted to further improve the rescue of the light-dependent activation of rhodopsin. Strains containing genomically integrated rhodopsin variants, without the constitutively active "EM"

mutations, were incubated with 50  $\mu$ mol/L L251-0001 and then 5  $\mu$ mol/L 9-*cis* retinal after each light bleach, similar to the protocol used in Figure 1. Unfortunately, this protocol did not rescue the light-dependent function either (data not shown).

#### **Future Refinement and Applications**

Although the pilot screen we performed did not discover a compound which rescues the function of P23H rhodopsin, these yeast-based methods were simple to perform, robust, and maintained a relatively low cost (\$10 to \$15 per 96-well plate). However, a larger high-throughput screening campaign, which typically involves 10 to 100 fold the number of compounds screened here, will require refinement of these methods. Miniaturizing the assay to 384-well plates may be possible,<sup>37</sup> facilitated by replacing the fluorescent reporter gene with an auxotrophic marker to enable a growth-based assay, although the lack of aeration can pose a challenge.<sup>30</sup> Plate-based assays also enable the monitoring of yeast growth and reporter gene expression during culturing, which was not explored here. It may be advantageous to incubate yeast for up to 48 h with compound and monitor phenotype, a strategy used to identify promising compounds in other yeast-based assays.<sup>28-30, 37</sup>

There is also the yeast cell wall to consider, which mammalian cells lack, which may impede the diffusion of compounds into the cell. The cell wall has a size exclusion of  $\approx 4500$ Da,<sup>38</sup> but the libraries screened here were under this size cut-off (OICR average 418 Da, LOPAC1280 average 337 Da). It remains possible that compounds which naturally diffuse into mammalian cells may bind the cell wall, limiting their diffusion. Evaluating the efficacy of the recently published non-retinal pharmacological chaperones of rhodopsin would help to reveal such differences between yeast and mammalian cell phenotypes.<sup>13-15</sup> Constitutively active GPCRs have been used in compound screens to discover antagonists or inverse agonists of GPCRs,<sup>39</sup> and pharmacological chaperones often act as an antagonist or inverse agonist of the GPCR they rescue. Thus, rather than screening for the rescued signaling function of P23H EM rhodopsin, the EM rhodopsin yeast strain could instead be used to discover novel antagonists and inverse agonists of rhodopsin, by screening for reduced constitutive activity. Such a screen would extend applications of the yeast-based screening assay described here, and hit compounds may additionally have applications as novel pharmacological chaperones of rhodopsin.

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## Figures



**Figure 1.** Flow cytometry-based assay to measure light-dependent activation of rhodopsin in yeast. Partial rescue of P23H rhodopsin activation in response to light was observed, following incubation with 20  $\mu$ mol/L 9-*cis* retinal overnight, followed by 5  $\mu$ mol/L 9-*cis* retinal after each light bleach (P23H Rescue Light). The initial mCherry fluorescence of each sample (zero hour time point) was subtracted from each, to compare the relative change in fluorescence. Data points represent averages of four individual colonies, each from a 5 mL culture. Error bars represent SEM, \*P <0.05 as determined by a Two-way ANOVA.



**Figure 2.** (**A**) Yeast strain schematic illustrating mCherry expression in response to rhodopsin activation. Wild-type (WT) rhodopsin requires both light and retinal to activate the downstream pathway. The synthetic E113Q M257Y (EM) mutations in rhodopsin create a constitutively active receptor, leading to mCherry expression at a high level, without light or retinal. The addition of the pathogenic P23H mutation (P23H EM) abolishes constitutive mCherry expression. (**B**) Yeast transformed with a plasmid containing human rhodopsin E113Q M257Y (EM) with or without the P23H mutation was incubated for 6 h with the indicated concentration of 9-*cis* retinal. The expected increase in mCherry fluorescence, indicative of rescued signaling function, was not observed. Data points represent averages of three individual colonies, each from a 600 µL culture. Error bars represent standard deviation.



**Figure 3.** (**A**) Representative flow cytometry results from yeast strains expressing EM rhodopsin. Integrating rhodopsin into the genome of the reporter yeast strain (EM Strain) significantly increased the percentage of mCherry positive yeast cells, versus plasmid based expression. (**B**) The signal to background ratio (S/B) between EM and P23H EM rhodopsin expressing strains was slightly improved with genome integrated versus plasmid-based expression of rhodopsin. Data points represent averages of three individual colonies, each from a 5 mL culture. Error bars represent standard deviation.



**Figure 4.** Compound screening to discover pharmacological chaperones of P23H EM rhodopsin. 4280 compounds from LOPAC1280 and the OICR peptidomimetic libraries were screened in singlicate, at 10  $\mu$ mol/L and 5  $\mu$ mol/L respectively. Data are shown normalized to the High Control, a strain expressing constitutively active EM rhodopsin. A cutoff value of 3 times the standard deviation of the mean plate Low Control (P23H EM + DMSO only), was used to determine hits. The screen had an average plate Z'-score of 0.65±0.11 with an average plate S/B of 14.7±4.7.

## Supplemental Material Screening of Chemical Libraries Using a Yeast Model of Retinal Disease

Benjamin M. Scott<sup>1,2,3</sup>, Leanne E. Wybenga-Groot<sup>4</sup>, C. Jane McGlade<sup>5,6</sup>, Elise Heon<sup>7</sup>, Sergio G. Peisajovich<sup>1</sup>, Belinda S.W. Chang<sup>1,8,9\*</sup>

<sup>1</sup>Department of Cell and Systems Biology, University of Toronto, Toronto, ON, Canada <sup>2</sup>Department of Chemistry and Biochemistry, University of Maryland, College Park, MD, USA <sup>3</sup>Biosystems and Biomaterials Division, National Institute of Standards and Technology, Gaithersburg, MD, USA <sup>4</sup>SPARC BioCentre, The Hospital for Sick Children, Toronto, ON, Canada <sup>5</sup>Department of Medical Biophysics, University of Toronto, Toronto, ON, Canada

<sup>6</sup>Program in Cell Biology, The Hospital for Sick Children, Toronto, ON, Canada

<sup>7</sup>Department of Ophthalmology and Vision Science, The Hospital for Sick Children, Toronto, ON, Canada

<sup>8</sup>Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, ON, Canada

<sup>9</sup>Centre for the Analysis of Genome Evolution and Function, University of Toronto, ON, Canada

\*Correspondence: Belinda S. W. Chang, 25 Harbord St., Toronto ON Canada M5S 3G5 Tel: 416-978-3507 belinda.chang@utoronto.ca

| Strain | Descriptive Name      | Genotype  | Reference  |
|--------|-----------------------|---|------------|
| BS017  | Parent Strain         | W303 MATa far1Δ, mfa2::KanMX-pFUS1<br>mCherry, sst2::HygB, ste2::TRP1, gpa1::Gpa1-<br>Gαt (DCGLF) -LEU2, his3, ura3 | 1          |
| BS023  | WT Strain             | BS017 sst2::pTDH3 HuRh WT-HIS3  | This study |
| BS024  | P23H Strain           | BS017 sst2::pTDH3 HuRh P23H-HIS3  | This study |
| BS025  | EM Strain             | BS017 sst2::pTDH3 HuRh E113Q M257Y-<br>HIS3   | This study |
| BS026  | P23H EM Strain        | BS017 sst2::pTDH3 HuRh P23H E113Q<br>M257Y-HIS3   | This study |
| BS027  | WT GFP Strain         | BS017 sst2::pTDH3 HuRh WT GFP-HIS3  | This study |
| BS028  | P23H GFP Strain       | BS017 sst2::pTDH3 HuRh P23H P23H-HIS3   | This study |
| BS032  | EM GFP Strain         | BS017 sst2::pTDH3 HuRh E113Q M257Y<br>GFP-HIS3  | This study |
| BS033  | P23H EM GFP<br>Strain | BS017 sst2::pTDH3 HuRh P23H E113Q<br>M257Y GFP-HIS3   | This study |

**Table S1: Strain Genotypes** 

1. Scott, B. M.; Chen, S. K.; Bhattacharyya, N.; et al. Coupling of Human Rhodopsin to a Yeast Signaling Pathway Enables Characterization of Mutations Associated with Retinal Disease. *Genetics* **2019**, *211*, 597-615.



**Figure S1.** Plasmid Map of pBS603 containing a human rhodopsin gene. Shown here is the WT sequence, but plasmids were also generated with rhodopsin containing the P23H, E113Q M257Y, or P23H E113Q M257Y mutations. To characterize rhodopsin expression, GFP was inserted in-frame to the C-terminus of rhodopsin, via the BamHI site. Homology regions identical to the 800 base pairs on either side of the yeast SST2 ORF were inserted, to facilitate integration into the yeast genome by homologous recombination. The SST2 ORF had previously been deleted with the HygB selection marker, which was therefore no longer needed, serving as a landing pad for rhodopsin integration. Image generated using Benchling.



**Figure S2.** Rhodopsin expression was quantified by flow cytometry, where rhodopsin contains a C-terminal GFP tag and was integrated into the genome of the indicated strain. Data points represent averages of three individual colonies, each from a 3 mL culture. Error bars represent standard deviation.



**Figure S3.** Representative images of strains with GFP-tagged rhodopsin variants integrated into the yeast genome. Strains shown are BS027 (WT), BS028 (P23H), BS032 (EM), and BS033 (P23H EM). Scale bars are 5 µm.



**Figure S4.** The influence of cell density on mCherry fluorescence when measured using a fluorescence plate reader. The S/B ratio was calculated using EM (BS025) and P23H EM (BS026) rhodopsin-expressing yeast strains, with and without 0.5 % DMSO in the culture media. Data points represent averages of three individual colonies, each from a 600  $\mu$ L culture. Error bars represent standard deviation.



**Figure S5.** Testing compounds of interest in triplicate, following the pilot screen. % Rescue was determined by fluorescence plate reader-based assay. Four compounds had at least one replicate above the cutoff (3SD above the Low Control). Data points represent individual replicates, each from a 600 µL culture.



**Figure S6.** Determining false positives by incubating compounds with yeast strain BS017, which contains the mCherry reporter but does not express rhodopsin. % Rescue was then determined by fluorescence plate reader-based assay. AC-93253 iodide was significantly fluorescent, resulting in a strong but false % Rescue. Data points represent individual replicates, each from a 600  $\mu$ L culture.



**Figure S7.** Dose-Response Attempt with Compound L251-0001. Strain with genomic integration of P23H E113Q M257Y (EM) incubated with up to 320  $\mu$ mol/L of L251-0001 for 6 hours, before measuring mCherry fluorescence by flow cytometry. Data points represent averages of two individual colonies, each from a 600  $\mu$ L culture. Error bars represent standard deviation.