Video Article

High-throughput Screening for Chemical Modulators of Post-transcriptionally Regulated Genes

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Abstract

Both transcriptional and post-transcriptional regulation have a profound impact on genes expression. However, commonly adopted cell-based screening assays focus on transcriptional regulation, being essentially aimed at the identification of promoter-targeting molecules. As a result, post-transcriptional mechanisms are largely uncovered by gene expression targeted drug development. Here we describe a cell-based assay aimed at investigating the role of the 3' untranslated region (3' UTR) in the modulation of the fate of its mRNA, and at identifying compounds able to modify it. The assay is based on the use of a luciferase reporter construct containing the 3' UTR of a gene of interest stably integrated into a disease-relevant cell line. The protocol is divided into two parts, with the initial focus on the primary screening aimed at the identification of molecules affecting luciferase activity after 24 hr of treatment. The second part of the protocol describes the counter-screening necessary to discriminate compounds modulating luciferase activity specifically through the 3' UTR. In addition to the detailed protocol and representative results, we provide important considerations about the assay development and the validation of the hit(s) on the endogenous target. The described cell-based reporter gene assay will allow scientists to identify molecules modulating protein levels via post-transcriptional mechanisms dependent on a 3' UTR.

Video Link

The video component of this article can be found at https://www.jove.com/video/52568/

Introduction

For a long period transcriptional regulation of gene expression was thought to play a major if not exclusive role in controlling protein production. Accumulating evidence, however, indicates that post-transcriptional regulation contributes as much as, if not more than, transcriptional regulation to determine cellular protein abundance[1,2]. Post-transcriptional control of gene expression is much more complex and elaborate than was at first thought. In fact, all the various stages of post-transcriptional control have emerged to be regulated, including mRNA processing, localization, turnover, translation[3] as well as the newly described reversible RNA methylation[4]. From a number of post-transcriptional regulation processes potentially affected, the assay described below focuses on those involving the mRNA 3' untranslated region (3' UTR). 3' UTR broadly affects mRNA fate mainly via specific interaction of RNA-binding proteins and non-coding RNAs to its regulatory sequences and/or secondary structures[5]. Therefore, small molecules that alter these interactions or interfere with upstream signal transduction pathways will shift the balance that regulates protein abundance. This therapeutic approach is of particular importance for human pathologies for which evidence exists showing that disease-relevant genes are subjected to post-transcriptional regulation, which thus represents a potential target for pharmacological intervention. Therefore, systems allowing for screening of mRNA levels' modulators through interference with post-transcriptional control mechanisms in a high-throughput format can become valuable tools in the identification of potential novel treatments.

The described cell-based reporter gene assay allows for the identification of compounds able to modulate mRNA fate via mechanisms dependent on its 3' UTR. It consists of several principal components (**Figure 1**) and requires few preliminary steps to validate the feasibility of the assay. First of all, the reporter construct is designed to include the 3' UTR from a gene of interest. The 3' UTR is fused to the end of a reporter gene such as firefly luciferase (**Figure 1**). Any changes in post-transcriptional control mechanisms exerted through the inserted 3' UTR will alter the stability and/or translation efficiency of this chimeric transcript, resulting in varied luciferase levels. Therefore, changes in luciferase activity serve as indirect measure of affected post-transcriptional regulation of the gene of interest. The second component of the system is a control reporter construct, an identical expression plasmid that expresses the same reporter gene but does not contain any 3' UTR. The two reporter plasmids can be designed in the lab using conventional molecular biology protocols or purchased from commercial sources.

The selection of an appropriate cell line is critical for the assay construction. Which cell line is the optimal model depends on numerous factors ranging from clinical requirements like maximal resemblance to pathology to just practical issues like availability, transfectability, and growth characteristics. Importantly, prior to proceed one should make sure that fusion of 3' UTR to the reporter gene has an expected effect on the level of the chimeric transcript. The absence of significant difference in luciferase signal from the 3' UTR-bearing and control reporter constructs

transiently transfected in the selected cells would indicate that the 3' UTR is not functional in this cellular model, prompting for search of alternative ones. For the high-throughput format, the 3' UTR-bearing and control luciferase reporter constructs should be stably integrated in the selected cell line. Using a stably integrated over transiently transfected cell line is preferable for several reasons. This will broaden the choice of a cellular model including hard-to-transfect cell lines, decrease the variability in the data arising from fluctuations in transfection efficiency, subside the costs. Moreover, upon transient transfection cells are very often overloaded with a DNA plasmid leading to the saturation of the system. In these settings a further increase in the reporter expression might be challenging, resulting in decreased sensitivity towards potential upregulating compounds.

Finally, when all the assay components are set up, it is critical before moving to the high-throughput format to determine the feasibility of the assay, *i.e.*, if luciferase activity can be indeed up- and down-regulated specifically via the inserted 3' UTR. The best controls would be small molecules reported to modulate the stability or translatability of mRNA through the 3' UTR of interest. If having these is not possible, surrogate controls imitating the desired effect are accepted. These could be miRNAs or RNA-binding proteins known to exert their effects via binding to the 3' UTR of interest. Evaluation of such controls before the primary screening not only indicates the functionality of the developed assay, but also allows for estimation of its dynamic range, sensitivity and performance in the high-throughput format.

Using the described protocol we screened a 2,000-compound library[6]. Neuroblastoma, the most common extracranial solid tumor of infancy, was used as a biological model and the 3' UTR of the *MYCN* oncogene, whose amplification strongly predicts adverse outcome of neuroblastoma, as a target gene[7]. **Figure 2** outlines the experimental layout. The screening was divided in three runs with the batch size of 27 plates screened in one run. The batch of 27 plates included Spectrum Collection library plates n.1-n.8+n.25 (first run), n.9-n.16+n.25 (second run) and n.16-n.24+n.25 (third run), each plate screened in triplicate. Thus, one library plate (n.25) was assayed within all three runs making possible inter-run comparison. The protocol below describes a single run of 9 library plates tested in triplicate.

Protocol

NOTE: The throughput of such assay systems depends on the available HTS lab equipment. This protocol is facilitated by a Tecan Freedom EVO 200 robot, which performs liquid handling in 96-well format. Miniaturization to 384-well format is also possible. The robotic liquid handling system is positioned under a laminar flow hood in order to maintain aseptic conditions during all experimental steps. If no liquid-handling automation is available, the protocol can be readily adapted to low-throughput format.

Primary Screening

1. Day 1: Prepare and Seed Cells

NOTE: Seed cells to yield 80% confluence at the time of assaying luciferase activity, *i.e.*, 48 hr after seeding.

1. Resuspend trypsinized CHP134-mycn3UTR neuroblastoma cells to a concentration of 2×10^5 cells/ml in RPMI containing 10% FBS and 1% L-glutamine. For 27 plates prepare at least 250 ml of cell suspension taking into account liquid handling, troughs dead volumes and repeated pipetting steps. Pour the cell suspension into sterile reservoir and place it on the robot desk.
2. Place 9 barcoded white flat-bottom 96-well plates and a box of 200 µl sterile pipet tips on the robot desk. Mix the cell suspension by pipetting before each dispensation step to avoid cells settling under gravity. Dispense 75 µl of cells into each well of 9 plates to get a final density of 1.5×10^4 cells per well.
3. Cover the plates with the lids and leave them outside of the incubator for 30 min to allow the cells to sediment to the bottom of the well. This will minimize edge effects.
4. Repeat steps 1.2 and 1.3 twice in order to prepare a total number of 27 plates.
5. Place the plates at 37 °C and 5% CO_2 and incubate for 24 hr.

2. Day 2: Treat the Cells with Library Compounds

NOTE: The Spectrum Collection small molecule library consists of 2,000 compounds arranged in 8 rows and 10 columns in 25 96-well plates at the concentration of 10 mM in DMSO. The wells on the first and last columns are left empty for controls. Compound screening assays are typically performed at 1–10 µM compound concentration[8]. The screening protocol described here fixes 2 µM as the working concentration and 24 hr as the assay end point.

1. Thaw 9 compound library plates at room temperature.
2. Prepare two troughs with sterile PBS and PBS-0.5% DMSO solutions and place them on the robot desk. For each library plate, prepare and label accordingly one dilution plate — a polypropylene U-bottom 96-well plate with a working volume of at least 400 µl. Fill each well of columns 2 to 11 of dilution plates with 398 µl of sterile PBS using the liquid handler's fixed tips. Fill columns 1 and 12 with 50 µl of sterile PBS with 0.5% DMSO in order to reproduce the vehicle concentration in the control wells (0.02%).
3. Place two library plates, the correspondingly labeled dilution plates, two boxes of 50 µl sterile pipet tips and six cells plates on the robot desk. Uncover cells plates.
4. Pipet 2 µl of the 10 mM stock solution from one of two library plates in the corresponding dilution plate and mix well. This will result in an intermediate compounds concentration of 50 µM. Using the same set of tips, dispense 3 µl of the 50 µM dilution in 3 barcoded white 96-well plates with cells. This dilution scheme results in 2 µM working concentration of each tested compound.
   NOTE: To avoid unnecessary manipulations with the tips, use a full set of 96 tips from the beginning, even though the library plates contain only 80 compounds. This is possible since the first and the last columns of the library plates are empty.
5. Replace the pipet tips and repeat Step 2.4 for the second library plate.
6. Cover the cells plates and return them to the incubator for 24 hr.

7. Repeat Steps 2.3-2.6 two more times for the remaining library plates.

3. Day 3: Perform Luciferase Assay

NOTE: Before performing the luciferase assay, it is possible to multiplex it with a cell viability assay based on reduction of resazurin in order to obtain a cell-viability index from each well[9]. Multiplexing luciferase and viability assay assays results in a reduction of luciferase signal that, however, can be ignored if the cells provide high enough level of luciferase activity. Luciferase activity can be detected by a variety of commercial and homemade[10,11] luciferase assay reagents with stable luminescent signal.

1. Equilibrate the reconstituted luciferase reagent of choice to room temperature. Make sure the volume is enough for all the assayed plates. For 27 plates 170 ml of luciferase reagent will be required taking into account liquid handling, troughs dead volume and repeated pipetting steps. Pour the luciferase reagent into a reservoir and place it on the robot desk.
2. Remove 9 plates with cells from the incubator, place them on the robot desk, uncover and wait until equilibrated to room temperature. Add 50 µl of the luciferase reagent per well plate by plate. Between the plates, introduce a delay equal to the luminescence reading time of one plate. This will assure that all the plates are measured within equal time interval from the reagent addition.
3. After a 30 min incubation, place the first plate in the luminometer and measure luminescence after a brief shaking step. Repeat for all plates. Record the barcode of each plate and associate it to the corresponding data file to avoid mistakes arising from a large number of handled plates.
4. Repeat Steps 3.2-3.3 twice for the remaining 18 plates with cells.
5. Collect the remaining luciferase reagent and store it at -20 °C.

4. Day 4: Data Analysis

1. Process experimental data using normalization of all samples to the average of on-plate vehicle-treated controls. For each library compound, calculate the mean value $\bar{x}$ and SD over triplicates.
2. Select up-regulating and down-regulating hits by setting a threshold of $\bar{x} \pm k \times SD$, where the mean value $\bar{x}$ and SD are computed over all assay processed values, and k is a defined constant.
3. Select an arbitrary threshold cut-off < 20% of vehicle-treated controls when a reduction of luciferase signal is most probably associated with compound toxicity. Discarding the hits below this threshold will significantly reduce a number of false-positive hits in the counter-screening.
   NOTE: Instead of control-based normalization, experimental data can be processed applying Z score or its robust analog B score[12]. In addition, alternative statistical approaches for hit selection are available[12].

5. Counter-screening

NOTE: Compounds identified in the primary screen (labeled 'hits') are confirmed and evaluated for the specificity by a counter-screening.

1. Cherry pick the selected hits from the aliquots stored in single 2D barcoded tubes and create new "hit plates", saving the corresponding layout. Do not forget to leave free positions for controls.
   NOTE: In the absence of an efficient cherry-picking system, one might test both the control and 3' UTR-bearing cells in parallel in the primary screening. In this case, specific selection of compounds with effects dependent only on the introduced 3' UTR will be possible after the primary screening eliminating the need of counter-screening. However, the parallel screening in two cell lines will double the assay costs.
2. Following the protocol for the primary screening (section "Day 1: Prepare and Seed Cells"), for each "hit plate" prepare three 96-well plates of each CHP134-mycn3UTR and CHP134-CTRL stable cells.
3. Following the protocol for the primary screening (section "Day 2: Treat the Cells with Library Compounds"), use each hit plate to treat three CHP134-mycn3UTR and three CHP134-CTRL plates at 2 µM working concentration.
   NOTE: While the counter-screen described here is performed in triplicates at the single dose of 2 µM, it might be beneficial to substitute the standard replicates and test the hits in 3 concentrations as 10-fold dilutions ranging from 2 µM to 20 nM. Applying this approach would not only allow to confirm the data of the primary screen, but also to obtain preliminary dose-response data on the primary hits, minimizing false-positive rates. In this case, a different analysis pipeline should be applied to process the experimental data and confirm the hits.
4. Following the protocol for the primary screening (section "Day 3: Perform Luciferase Assay"), measure luciferase activity in all CHP134-mycn3UTR and CHP134-CTRL plates.
5. Following the protocol for the primary screening (section "Day 4: Data Analysis"), normalize experimental data from treated wells to the average of on-plate vehicle-treated controls and calculate the mean and SD over replicates. For each compound tested, compute the fold change of luciferase activity in CHP134-mycn3UTR vs. CHP134-CTRL cells. Select arbitrary fold change cut-offs of >1.5 and significance p-values of <0.01.
   NOTE: In the described assay settings the determinant parameter for hit specificity is a significant fold change of luciferase activity in CHP134-mycn3UTR vs. CHP134-CTRL cells. If needed, one might also assess compound toxicity by performing concurrently a cell viability assay on the selected compounds. This might be especially beneficial if the primary hits are counter-screened in a dose response analysis. For a single dose, our experience indicates a strong correlation of reduced luciferase activity with decreased cell viability[6]. Therefore, decreased luciferase activity in target cells as well as control cells can be considered as an indicator of compound toxicity at the tested concentration.

Representative Results

Using the described approach, we screened a 2,000-compound library for potential modulators of post-transcriptional control mechanisms exerted through the 3' UTR of the *MYCN* gene. **Figure 3** depicts the results of the primary screening exemplified by a single library plate. Luciferase signal displayed as percentage of vehicle-treated controls was obtained by measuring luciferase activity in triplicate plates of

CHP134-mycn3UTR cells treated with compounds of a single library plate. As expected, the majority of compounds had no effect on luciferase activity as indicated by values close to the 100% baseline. The hits (clear squares) were selected setting a threshold of $\bar{x} \pm 2$ SD, where the mean value $\bar{x}$ and SD are computed over all assay values. Compounds reducing luciferase activity below 20% (the one marked with asterisk) should be removed from consideration as the detected inhibition most probably results from pronounced cellular toxicity of the compound.

Figure 4 illustrates few representative examples of data obtained in the counter-screening of about 100 compounds. As can be judged from normalized luciferase signal in CHP134-CTRL cells, compound W at 2 µM concentration has no effect on cell viability, while compound X displays some cytotoxic activity. Both compounds W and X, however, cause MYCN 3' UTR-specific up-regulation of luciferase activity, as indicated by fold change difference and t-test. Compound Y at 2 µM concentration seems to compromise viability of CHP134 cells; in terms of specificity there is no difference in luciferase activity in CHP134-mycn3UTR and CHP134–CTRL cells. Finally, the increase in luciferase activity detected in the primary screening for compound Z is not truly dependent on MYCN 3' UTR, since an equivalent up-regulation is observed in CHP134-CTRL cells. We did not detect any hit resulting in MYCN 3' UTR-specific down-regulation of luciferase activity.

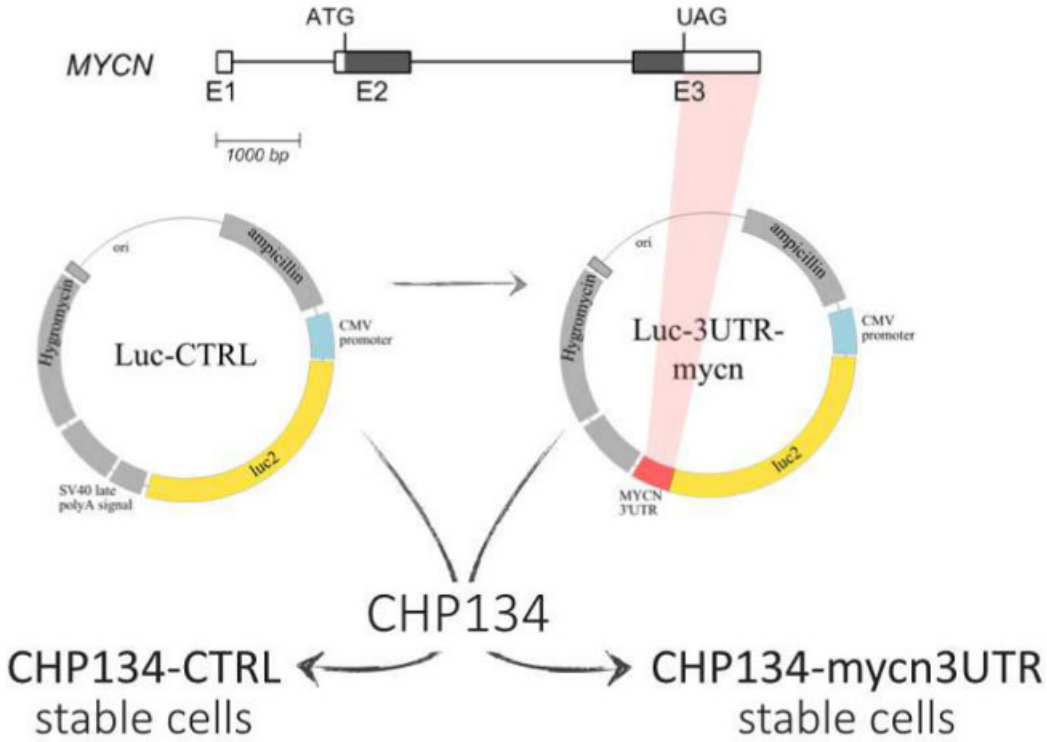

Figure 1: Components of the cell-based reporter gene assay. The upper panel displays schematic diagram of the *MYCN* gene. The MYCN transcript (NM_005378.4) is drawn to scale. Boxes represent the exons with shaded regions corresponding to the CDS, lines represent introns. The reporter constructs Luc-CTRL and Luc-3UTR-mycn are schematically represented below. CMV, cytomegalovirus; SV40, simian virus 40. The CHP134 neuroblastoma cell line was chosen to produce stably transfected cells using the aforementioned plasmids. Please click here to view a larger version of this figure.

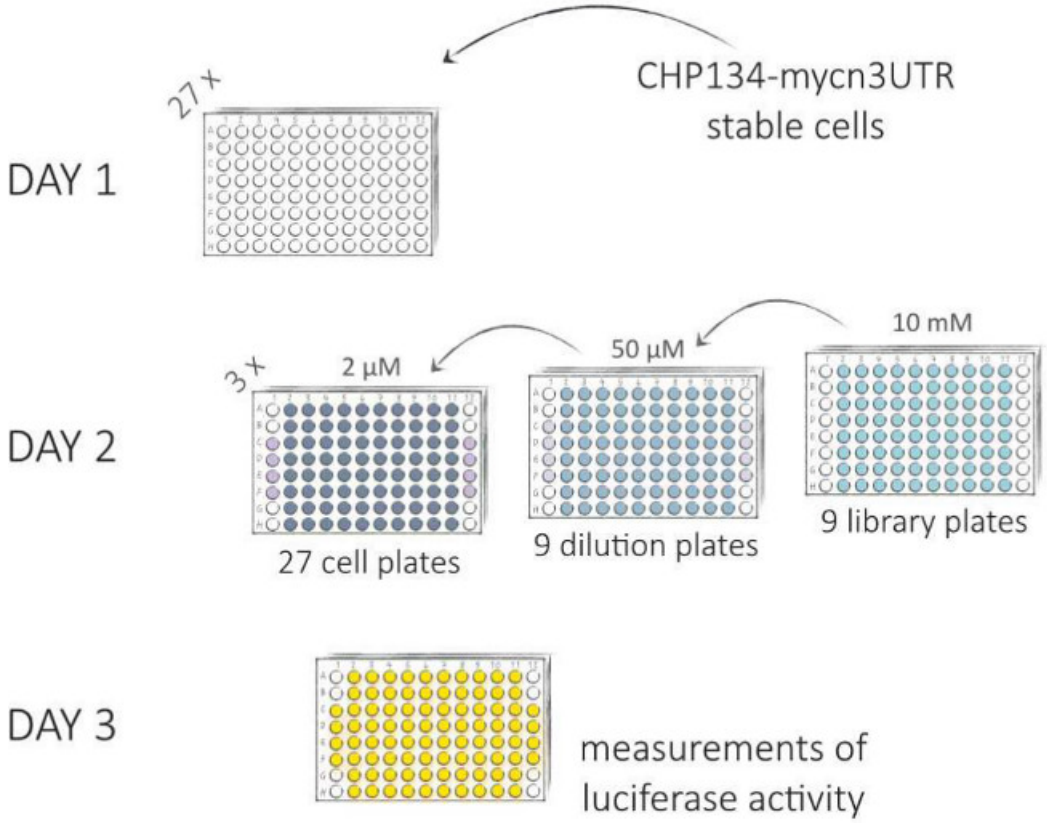

Figure 2: Outline of the primary screening. Each single compound library plate was used to treat triplicate plates of CHP134-mycn3UTR cells. Luciferase activity was measured after 24 hr of treatment.

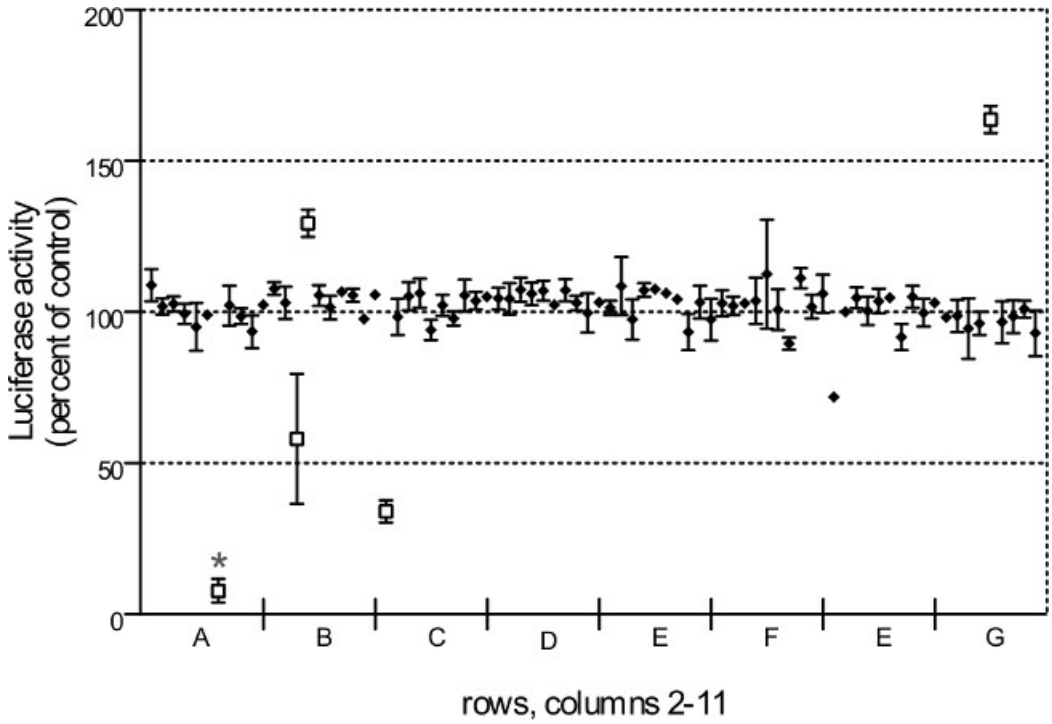

Figure 3: Plate-well scatter plot of normalized values representing a single 96-well plate from the primary screening. CHP134-mycn3UTR cells growing in 96-well plates were treated with library compounds at 2 μM concentration. Each point corresponds to luciferase activity detected for a single compound after 24 hr of treatment and normalized to the mean luciferase signal detected in vehicle-treated controls within the same plate. Data points are displayed following the compounds position within a 96-well plate (10 compounds in columns 2-10 from raw A to G). Mean over three replicates ± SD is shown. The hits are displayed as clear squares. A hit characterized by reduced luciferase activity below 20% is marked with asterisk.

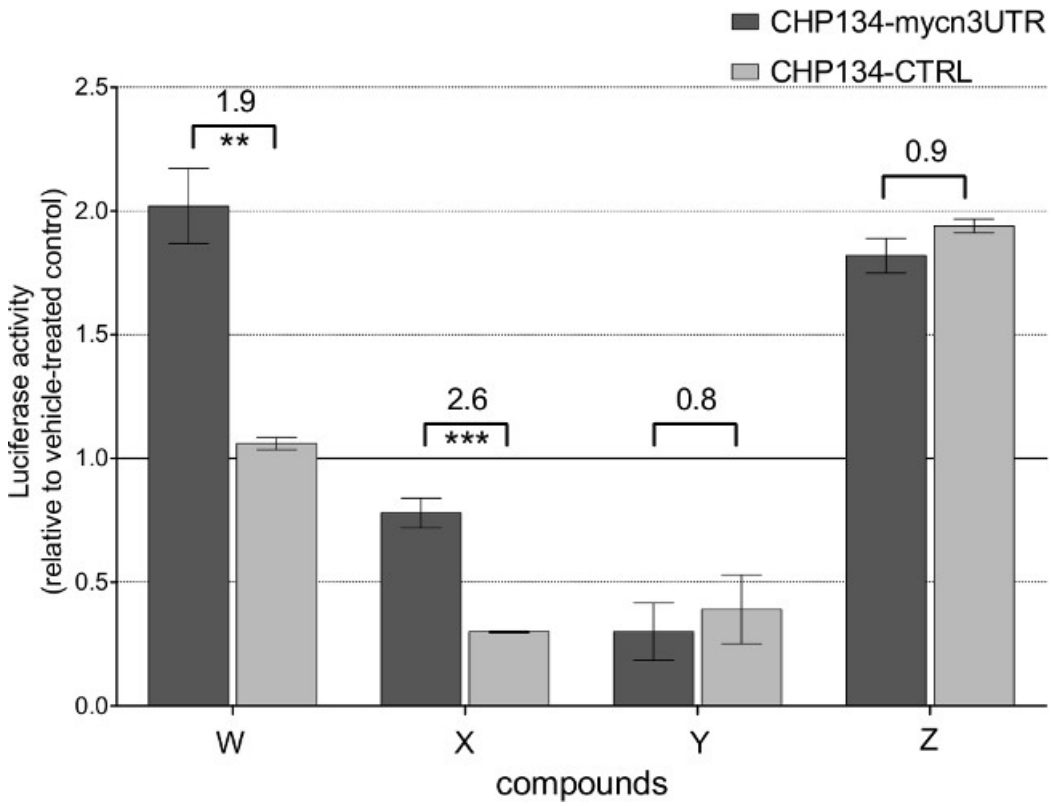

Figure 4: Representative examples of counter-screening. CHP134-mycn3UTR and CHP134-CTRL stable cells were treated in parallel with compounds pre-selected in the primary screening. The luciferase assay was performed after 24 hr of treatment at 2 µM concentration. The graph shows representative results for four selected compounds designated W, X, Y, and Z. Each bar corresponds to luciferase activity detected for a single compound and normalized to the mean luciferase signal of vehicle-treated controls in the indicated stable cells. Fold change difference and statistical significance in the two-tailed t-test are displayed, ** p <0.01, *** p <0.001.

Discussion

This protocol describes a cell-based reporter-gene assay aiming at the identification of modulators that target 3' UTR-dependent post-transcriptional processes. It encompasses the primary screening and the counter-screen and, if needed, can be accompanied by a cytotoxicity assay. The outcome of the primary screening is a number of valuable candidate compounds, whose reproducibility and specificity is further validated in the counter-screening.

The identification of primary hits is based on variations of luciferase activity upon treatment of cells expressing a reporter-3' UTR chimeric transcript. Retesting the hits in cells carrying the same reporter plasmid without the 3' UTR allows to discriminate between true hits (compounds modulating luciferase levels specifically via 3' UTR-dependent mechanisms) and false-positives (compounds changing luciferase activity via transcriptional responsiveness of a promoter). As evidenced by the counter-screening of primary hits, the assay demonstrates a considerable false-positive rate[6]. Several strategies could be employed to circumvent this limitation. Inhibiting transcription by drugs is an option to be considered for shorter treatments and mRNAs with short half-lives. Alternatively, one can substitute a viral promoter sensitive to external stimuli with the modified ribosomal protein promoter. The latter represents a constitutively active cellular promoter that was shown to be transcriptionally inert to a wide variety of agents and thus more suitable for post-transcriptional assessments[13]. Finally, use of bichromatic or dual-output reporters could help to unmask true post-transcriptional effects from a promoter-mediated activity already in the primary screening. Specifically, in a bidirectional promoter vector expressing two different reporter proteins, a 3' UTR is fused to one reporter, while the second reporter serves as an endogenous control; if the observed differences in expression are due to modulation of transcription, both reporters should be affected in the same way[14].

The described cell-based assay relies on a reporter readout that indirectly reflects post-transcriptional changes of endogenous gene expression. Therefore, the hits coming from the counter-screening should undergo further evaluation so that their anticipated effect could be recapitulated under physiological conditions. First, selected compounds should be assessed for their effect on the levels of the corresponding endogenous protein. Concurrent measurement of endogenous steady-state mRNA levels in combination with evaluation of its stability will finally rule out the possibility that observed effects are just a consequence of transcriptional changes. Then, dissecting a potential mechanism of action would provide further assurance of compound selectivity and specificity. Compounds modulating 3' UTR-dependent post-transcriptional processes should mainly result in affected transcript stability or translation efficiency. While mRNA stability can be monitored by measuring transcript levels after inhibition of transcription, changes in translation efficiency can be assessed by analysis of polysomal loading of the transcript of interest by sucrose density gradient centrifugation[15].

Overall, the developed cell-based reporter gene assay enables identification of molecules modulating protein levels via post-transcriptional mechanisms dependent on a 3' UTR. Variants of such assay can be proposed, involving a 5'UTR or both 5' and 3' UTR, different reporter genes

and different plasmid architectures. Importantly, this assay enables detection of both inhibitors and enhancers of gene expression, being the last especially relevant for genetic diseases. The identified compounds have strong therapeutic potential, as a number of pathologies have been linked to perturbed post-transcriptional processes[16–19]. Thus, implementation of such and similar assays aiming at post-transcriptional mechanisms in the drug-discovery process might provide new therapeutic approaches for diseases with no cure at the moment[20, 21].

Disclosures

The authors declare that they have no competing financial interests.

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