Video Article

siRNA Screening to Identify Ubiquitin and Ubiquitin-like System Regulators of Biological Pathways in Cultured Mammalian Cells

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Abstract

Post-translational modification of proteins with ubiquitin and ubiquitin-like molecules (UBLs) is emerging as a dynamic cellular signaling network that regulates diverse biological pathways including the hypoxia response, proteostasis, the DNA damage response and transcription. To better understand how UBLs regulate pathways relevant to human disease, we have compiled a human siRNA “ubiquitome” library consisting of 1,186 siRNA duplex pools targeting all known and predicted components of UBL system pathways. This library can be screened against a range of cell lines expressing reporters of diverse biological pathways to determine which UBL components act as positive or negative regulators of the pathway in question. Here, we describe a protocol utilizing this library to identify ubiquitome-regulators of the HIF1A-mediated cellular response to hypoxia using a transcription-based luciferase reporter. An initial assay development stage is performed to establish suitable screening parameters of the cell line before performing the screen in three stages: primary, secondary and tertiary/deconvolution screening. The use of targeted over whole genome siRNA libraries is becoming increasingly popular as it offers the advantage of reporting only on members of the pathway with which the investigators are most interested. Despite inherent limitations of siRNA screening, in particular false-positives caused by siRNA off-target effects, the identification of genuine novel regulators of the pathways in question outweigh these shortcomings, which can be overcome by performing a series of carefully undertaken control experiments.

Video Link

The video component of this article can be found at <http://www.jove.com/video/51572/>

Introduction

Modification of proteins with ubiquitin and ubiquitin-like molecules (UBLs) represents an expansive biochemical system that regulates diverse biological pathways and stress responses. The covalent attachment of UBLs to their target proteins can have various outcomes regulating the stability, localization, function or interconnectivity of the substrate¹. The enzymatic steps underlying UBL modification were first established for ubiquitin, and now serve as a paradigm for modification with most UBLs, including SUMO, NEDD8, ISG15 and FAT10. For modification to occur, the carboxylate group of the UBL diglycine motif is first activated by an E1 activating enzyme to form a high-energy thiol that is transferred to the active-site cysteine of an E2 conjugating enzyme. The E2 then interacts with a substrate-bound E3 ligase to mediate transfer of the UBL onto (usually) a target lysine residue creating a branched chain (isopeptide) linkage². Successive rounds of modification can occur to build isopeptide chains onto the substrate, which for ubiquitin can occur through any of its seven lysines, or through its N-terminal methionine to create linear ubiquitin chains. These modifications form discrete topologies with diverse purposes such as creating new interaction motifs and targeting proteins for degradation prior to UBL removal by specialist proteases. In the case of ubiquitin there are two E1 enzymes, 30-40 E2 conjugating enzymes, at least 600 E3 ligases and approximately 100 deubiquitylating enzymes (DUBs). While the pathways are less expansive for the other 10 or so UBLs, the overall ubiquitome complexity affords huge diversity in the biological outcome of a particular UBL modification. However, while major advances in UBL biology have been made, the precise cellular roles of the majority of these ubiquitome components remain unknown.

The use of short interfering ribonucleic acid (siRNAs) has emerged as a powerful tool in reverse genetics due to the ability of siRNAs to specifically target cellular mRNAs for destruction, allowing the role of individual genes to be examined in different biological contexts³. Whole genome screens have been used to identify and validate new regulators of many cellular processes, and have created a wealth of useful data accessible to the wider scientific community. However, while whole genome screens have proven extremely useful, targeted screens are becoming increasingly popular as they are cheaper, faster, involve less data management and report only on members of the genome in which the investigator is most interested. Therefore, to better understand which cellular processes UBL family components are involved in, we have compiled a human siRNA library targeting all known and predicted components of the ubiquitome. This includes the UBLs, E1 activating enzymes, E2 conjugating enzymes, E3 ligases, ubiquitin-binding domain (UBD)-containing proteins and DUBs. This library can be

used to screen against a wide range of reporter cell lines of distinct biological problems, thus allowing the unbiased identification of novel UBL components governing these pathways.

The following protocol describes how to perform a rigorous targeted siRNA ubiquitome screen to identify novel regulators of the HIF1A-dependent response to hypoxia. Under normal oxygen tension, HIF1A is subject to prolyl hydroxylation that causes it to be recognized and targeted for degradation by the Von Hippel Lindau (VHL) E3 ligase complex⁴. Hypoxia inhibits prolyl hydroxylation leading to the stabilization of HIF1A and its subsequent binding to hypoxia response elements (HREs) to drive gene expression. Here, we describe a screen using U2OS osteosarcoma cells stably expressing firefly luciferase under the control of three tandem copies of the Hypoxia Response Element (U2OS-HRE cells)⁵. This protocol can be adapted for any biological pathway if a robust read-out of reporter activity is achievable and can be coupled with appropriate positive and negative controls.

Protocol

1. Assay Development Stage

Note: prior to initiating the siRNA screen, an assay development stage is critical to set out important parameters for screening with the reporter cell line. It is essential to invest significant effort at this stage as this will underpin the future success of the screen.

1. To characterize the hypoxia-responsiveness of U2OS-HRE reporter cell line, grow 2 x 75 cm² flasks of U2OS-HRE cells to 80-90% confluence in Dulbeccos Modified Eagle Medium (DMEM) supplemented with 10% FBS.
2. Aspirate the media from one flask of cells, wash twice with 10 ml PBS and detach cells by adding 2 ml 0.05% trypsin-EDTA solution and incubating at 37 °C for 5 min. Resuspend cells in 8 ml DMEM 10% FBS and pipette up and down to create a homogenous cell suspension.
3. Pipette 20 µl of the suspension to a cell counting chamber, in duplicate, and insert the chamber into an automated cell counter. Click "Display Image" on the cell counter software and ensure the cells are in focus. Click "Count" and make a note of the cell density. Repeat this for the other duplicate and calculate the average cell density. Dilute the cells with DMEM 10% FBS to a concentration of 60,000 cells/ml.
4. Using a multichannel pipette, add 100 µl (6,000 cells) of the diluted cells to the same three columns (e.g. A5-H5, A6-H6 and A7-H7) of 5 sterile white-walled 96-well assay plates, and transfer to a humidified 37 °C incubator at 5% CO₂ overnight. Note: these plates will be used to determine the hypoxia-dependent luciferase output for a range of hypoxia exposures: 0 hr, 2 hr, 6 hr, 10 hr and 24 hr.
5. At the end of the following day, note the time and add the 24 hr hypoxia plate to the hypoxia workstation set to 1% oxygen. The following morning, calculate the time 10 hr, 6 hr and 2 hr before the 24 hr plate is due to be removed from the hypoxia workstation. At these times, add the 10 hr, 6 hr and 2 hr plates to the hypoxia workstation.
6. Prepare 30 ml of 2x combined luciferase lysis/assay buffer (50 mM Tris Phosphate pH 7.8; 16 mM MgCl₂; 2 mM DTT; 2% Triton-X-100; 30% glycerol; 1 mM ATP; 1% BSA; 0.25 mM luciferin and 8 µM Na₄P₂O₇). Note: add the components in the order listed and allow the BSA at least 30 min to dissolve before the addition of luciferin and Na₄P₂O₇.
7. Remove all plates from the hypoxia workstation at the appropriate time. Add 100 µl of 2x luciferase lysis/assay buffer to the appropriate wells of the assay plate and cover with clear film. Shake the plates on a plate shaker for 10 min at 500 rpm to thoroughly lyse the cells.
8. Transfer the plate stack to an automated 96 well plate luminometer rack. Under the "Protocols" menu, select "abs 595" and highlight all wells to be read in the on-screen plate map under the "well selection" menu. Click "run" to measure luminescence of each well then calculate the average reading of each plate. Calculate the hypoxia-dependent fold-increase of reporter activity by dividing the average reading of each hypoxia plate by the average reading of the normoxia (0 hr hypoxia) control plate. Note: it is essential to observe a robust hypoxia-dependent luciferase response to be suitable for screening. A five-ten fold increase in reporter activity is considered excellent.
9. Create a master control plate that contains four high control (HIF1A siRNA) replicates (wells A1-D1), four low control (FIH1 siRNA) replicates (wells A12-D12), four buffer only control replicates (wells E1-H1) and four non-target siRNA control replicates (E12-H12), where all siRNA pools are at a concentration of 200 nM. Note: High and low controls are established based on known pathway regulators that increase and decrease the hypoxia response respectively. Alternatively, assay development using a subset of the library may be used to identify suitable controls.
10. Using a multichannel pipette, transfer 10 µl of each control siRNA to a sterile white walled assay plate. Prepare transfection mix of 0.1 µl transfection reagent in 10 µl reduced-serum medium (1:100) per well, and transfer 10 µl of transfection mix to each control well. Pipette up and down briefly to mix, and leave the plate to rest for 20-60 min to allow transfection complex formation.
11. Prepare a cell suspension of 75,000 cells/ml with the second flask of U2OS-HRE cells. Using a multichannel pipette, add 80 µl (6,000 cells) of the cell suspension to each transfection mix. Transfer the plate to a humidified 37 °C incubator with 5% CO₂ for 24 hr, then transfer to a hypoxia workstation for a further 24 hr and perform luciferase assays as described in steps 1.6-1.8.
12. Calculate the Z-factor for the high and low controls using the formula $Z = 1 - [3 \times (\text{standard deviation of high control} + \text{standard deviation of low control}) / (\text{mean of high control} - \text{mean of low control})]$. Note: a value of between 0.5-1 indicates an excellent assay and should be strived for.

2. Primary Screen

Note: once these basic conditions from the assay development stage are in place, the primary screen can be performed in triplicate in 96 well-plate format using the following protocol.

1. Grow 7 x 75 cm² flasks of U2OS-HRE cells to 80-90% confluence in DMEM supplemented with 10% v/v fetal bovine serum (FCS).
Note: the following steps 2.2-2.13 should be carried out on Day 1.
2. Initiate replicate 1 by preparing a master control plate containing 200 µl of each control as described in step 1.9 and thawing out a dilution series of the siRNA ubiquitome library for a minimum of 30 min. Note: each well contains a pool of 4 siRNAs assembled in 17 x 96-well plates with columns 1 and 12 left empty for controls.
3. In a laminar flow hood, label (or bar-code) 17 sterile white walled assay plates with lids from numbers 1-17, corresponding to each plate of the siRNA library series.

4. Centrifuge the master control plate and the siRNA ubiquitome library briefly (1 min at 2,000 x g) to ensure all siRNAs accumulate at the bottom of the well.
5. Use an automated liquid dispenser to robotically "stamp" 10 µl of each control from the master control plate onto the 17 assay plates, using the same 96-well tip stack for each plate.
6. Using a fresh 96-well tip stack for each of the 17 library plates, transfer 10 µl of each ubiquitome siRNA to its corresponding assay plate using an automated liquid dispenser.
7. Prepare 40 ml transfection reagent for the 17 assay plates using the ratio established in 1.10. Note: this includes an additional 20 ml to account for the "dead volume" in the cell dispenser. The "dead volume" refers to the volume of liquid continually present within the tubing network in the cell dispenser.
8. Use an automated cell dispenser to transfer 10 µl of transfection reagent to each well in the 17 assay plates. Briefly shake the assay plates on a shaker (1 min at 500 rpm) to allow thorough mixing of siRNA and transfection reagent, then leave still at room temperature for 20-60 min to allow siRNA:transfection reagent complex formation.
9. Wash two 75 cm² flasks of U2OS-HRE cells twice with 10 ml PBS and detach with 2 ml trypsin-EDTA at 37 °C for 5 min. Add 8 ml DMEM 10% FBS to each flask and pipette up and down several times to create a homogenous cell suspension. Combine cells from both flasks and transfer to a sterile 50 ml plastic tube.
10. Calculate cell concentration by pipetting 20 µl cells in duplicate to a cell counting chamber and calculate the average cell concentration from two readings of an automated cell counter as described in step 1.3.
11. Prepare 155 ml of cell suspension by diluting with DMEM 10% FBS to a concentration of 75,000 cells per ml in a sterile plastic container. Note: this volume is sufficient for 17 plates and includes an additional 25 ml for the dead volume in the cell dispenser.
12. Add a sterile magnetic stirrer to the cell suspension and place on a stirrer set up inside the laminar flow hood to limit cell clumping. Equilibrate the automated cell dispenser with the cell suspension and set it to dispense 80 µl of cells per well (6,000 cells).
13. Place each of the 17 plates, in turn, onto the automated cell dispenser and dispense cells. Record the time and stack plates in groups of 5 then place stacks in a humid 37 °C incubator at 5% CO₂ for 24 hr. Note: the final concentration of siRNA pool will be 20 nM in 100 µl total volume.
Note: the following steps 2.14-2.15 should be carried out on Day 2.
14. Initiate the second replicate on day 2, following the procedure outlined in Day 1 for replicate 1 (2.2-2.13).
15. After 24 hr of transfection, transfer plates from replicate 1 in a sterile environment to a hypoxia workstation set at 1% oxygen and leave for 24 hr to induce the HRE reporter.
Note: the following steps 2.16-2.20 should be carried out on Day 3.
16. Initiate the third replicate, following the procedure outlined in Day 1 for replicate 1 (2.2-2.13).
17. After 24 hr of transfection, transfer plates from replicate 2 in a sterile environment to a hypoxia workstation set at 1% oxygen and leave for 24 hr to induce the HRE reporter.
18. Two hours before replicate 1 plates are to be removed from the hypoxia workstation, prepare 200 ml of 2x luciferase lysis/assay buffer as described in 1.6. Note: this volume includes an additional 20 ml to ensure the buffer reservoir remains well covered.
19. Remove replicate 1 assay plates from the hypoxia workstation after 24 hr hypoxia exposure and using an automated liquid dispenser, add 100 µl of 2x luciferase lysis/assay buffer to each assay plate and cover with clear film.
20. Shake the plates on a cell shaker for 10 min at 500 rpm to thoroughly lyse the cells. Transfer each plate in turn to a luminometer plate reader and record luminescence as described in step 1.8.
Note: the following steps 2.21-2.22 should be carried out on Day 4.
21. After 24 hr of transfection, transfer plates from replicate 3 in a sterile environment to a hypoxia workstation set at 1% oxygen and leave for 24 hr to induce the HRE reporter.
22. Read the replicate 2 plates by following steps 2.18-2.20 used for replicate 1.
Note: the following step 2.23 should be carried out on Day 5.
23. Read the replicate 3 plates by following step 2.18-2.20 used for replicate 1.
24. Compile all 3 replicates of the primary screen, and calculate the Z factor for each plate using the formula given in 1.12. Note: if any plate has a Z factor of less than 0.5, consider repeating this plate to improve the data quality.
25. Calculate the coefficient of variance (CV) for the high and the low controls by using the following formula: 100 x standard deviation of control/average of control. Note: CV should be as low as possible, it is reasonable to expect 10-15% variation. If variation is significantly higher, consider repeating the plate.
26. Calculate percent activation of each siRNA per plate by using the following formula: [(mean of high control – siRNA score) / (mean of high control – mean of low control)] x 100. In addition, calculate the non-target (NT)-fold of each siRNA by using the formula [Sample data/mean of NT]. For both percent activation and NT-fold, calculate the average of the three replicates and compile the values.
27. Using the raw data, calculate the Spearman's Rank Correlation Coefficient (SRCC) to determine how closely the three replicate plates correlate using the formula:
$$r = 1 - \left(\frac{6 \sum d^2}{n(n^2 - 1)} \right)$$

where r = SRCC; d = difference between the two numbers in each pair of ranks and n = number of pairs of data. Note: a good correlation between plate replicates will give values close to 1. If one replicate shows low SRCC against the other 2 plates, investigate further and consider repeating that plate.
28. Decide which siRNAs are of sufficient interest for secondary screening (up to 80). Employ user-defined cut offs (e.g. selecting all siRNAs showing less than 5% or over 95% percent activation, or less than 0.5 NT-fold or over 1.5 NT-fold) or apply bioinformatics or knowledge-based reasoning to search for clusters of hits falling within the same UBL pathway. Note: normally, a combination of these approaches determines which siRNA are taken into secondary screening.

3. Secondary Screen

Note: a confirmatory secondary screen is carried out based on a maximum of 80 siRNAs of interest from the primary screen. This number can be conveniently carried out with each replicate plated on a single 96 well plate with full controls as per the primary screen (see 2.2). It is very useful

to confirm that the regulators identified in the primary screen reproducibly elicit the same phenotype, and this will assist in refining the triage decisions on which hits should be carried through to the final tertiary/deconvolution screen.

1. Grow 1 x 75 cm² flask of U2OS-HRE cells to 80-90% confluence in DMEM with 10% FBS.
2. Note the plate number and location of the primary screen hits, and plan their new location on the secondary screen cherry picked master plate, leaving columns 1 and 12 free for controls. Prepare the control master plate as in 1.9 but with a total volume of each control of 50 μ l.
3. Thaw a second dilution series of the ubiquitome library (set aside for cherry-picking individual siRNA pools) for a minimum of 30 min. Centrifuge the plates briefly (1 min at 2,000 x g) then add 50 μ l of the secondary screen siRNAs to their new plate locations on the cherry picked master plate.
4. Carry out the secondary screen using the same basic protocol outlined for the primary screen with the following modifications: (a) run all three replicates on the same day in one assay plate per replicate and (b) adjust volumes of reagents accordingly.

4. Tertiary/Deconvolution Screen

Note: tertiary or deconvolution screening is performed on a maximum of 20 siRNAs from the secondary screen. This step is to examine the effect of knockdown using each individual siRNA from the original pool of four. Normally, at least two individual siRNA duplexes from each pool should illicit the same phenotype to have a reasonable degree of confidence that the observed phenotype is not due to siRNA off-target effects. To further increase confidence at this stage, additional individual siRNA duplexes targeting the gene in question can be designed and tested for their ability to elicit the given phenotype. Results from these experiments may then be used to calculate the H score, where $H = 0.6$ or over (*i.e.* where at least 3 out of 5 individual siRNAs elicit the phenotype) is considered acceptable⁶.

1. Grow one 75 cm² flask of U2OS-HRE cells to 80-90% confluence in DMEM with 10% FBS.
2. Create a plate map for the tertiary screen by planning the location of the four individual siRNA duplexes of each hit on the tertiary screen master plate, leaving columns 1 and 12 free for controls. Prepare the control master plate as in 1.9 but with a total volume of each control of 50 μ l.
3. Thaw the deconvolution/individual siRNA plates and add 50 μ l of the individual siRNAs (200 nM) into each well of the tertiary screen master plate according to the plate map (4.2) to allow for three x 10 μ l replicates and a comfortable 20 μ l excess.
4. Carry out the tertiary screen using the same basic protocol outlined for the primary screen with the following modifications: (a) run all three replicates on the same day in one assay plate per replicate and (b) adjust volumes of reagents accordingly

Note: Ideally the threshold for individual siRNA duplexes should be set at the same stringency cut-off as for the pool. However, it may be acceptable to relax the threshold by 10-20% for individual siRNAs, especially where at least one other duplex falls within the set threshold. It is worthwhile bearing in mind that the individual effect of siRNAs may be less than that of the pool, and conversely, in some cases individual siRNAs may show a stronger effect on the cellular phenotype in isolation than when it exists in the pool (even when concentration is accounted for).

Representative Results

Prior to screening, the hypoxia-responsiveness of U2OS-HRE cells is established. U2OS-HRE cells express a reporter construct consisting of firefly luciferase fused downstream of three tandem copies of the hypoxia response element, which is bound by the HIF1A/HIF1B heterodimer upon exposure to hypoxia (**Figure 1A**). Cells are placed in a hypoxia workstation for a range of times to establish which hypoxia exposure produces the most effective response for screening. U2OS cells express low levels of HIF2A, therefore luciferase readings are largely dependent on hypoxia-mediated HIF1A stabilization⁷. Exposure of U2OS-HRE cells to hypoxia for 0 hr, 2 hr, 4 hr, 6 hr and 24 hr results in a hypoxia-dependent induction of luciferase activity (**Figure 1B**). Exposure of U2OS-HRE cells to hypoxia for 24 hr results in a ~10 fold induction of activity (**Figure 1B**), therefore this represents an excellent response for siRNA screening.

To establish suitable high and low controls, siRNAs to HIF1A and FIH1 are used to reverse transfect U2OS-HRE cells. Cells are transfected with the controls for 24 hr and exposed to hypoxia for a further 24 hr before luciferase assays are performed. HIF1A siRNA diminishes the hypoxia signal to around 10% whereas the FIH1 siRNA enhances the hypoxia signal to around 170% relative to non-target siRNA (**Figure 2**). These controls result in an average Z factor of 0.8, which is an excellent score for screening. The screening parameters have now been successfully established for a robust screen.

The primary screen is carried out in 96-well plate format using the workflow demonstrated in **Figure 3**. Control and library siRNAs are stamped onto assay plates (**Figure 3A**), and U2OS-HRE cells are reverse transfected then incubated at 37 °C for 24 hr. Assay plates are then exposed to hypoxia for 24 hr (**Figure 3B**) before luciferase assays are performed and the data analyzed (**Figure 3C**). Quality control analysis of the screen includes determining the Z factor for each plate, where a Z factor above 0.5 is desirable^{8,9}. The Z factor is calculated for each plate of the triplicate primary screen using the formula in **Figure 4A**. The Z factors for each plate are visualized using a scatter plot, where it can be seen each plate has a Z factor greater than 0.5 (**Figure 4B**). Calculating the Z factor is important as it reports on how good a separation window exists between controls and can highlight potential problems with individual screen assay plates. The CV of the high and low controls are also calculated, and the averages are 5.95% $\pm$ 0.4 and 8.04% $\pm$ 1.2 respectively. The primary screen results in a distribution of siRNA duplexes forming a continuum between the low and high controls. Typically, there will be approximately equal numbers of positive and negative regulators, with the majority of siRNAs having no effect on the reporter (**Figure 5**). Regulators of interest are taken through to secondary then tertiary screening to establish a list of high-confidence regulators for follow-up analysis and validation.

Figure 1. Hypoxia exposure induces U20S-HRE luciferase reporter. (A) Schematic representing the hypoxia reporter construct in U20S-HRE cells used for siRNA screening. Three tandem copies of the hypoxia response element (HRE) are bound by the HIF1A/HIF1B heterodimer to drive the expression of firefly luciferase. (B) Increasing exposure of U20S-HRE cells to 2 hr, 6 hr, 10 hr and 24 hr to hypoxia results in increasing amounts of reporter luciferase activity. 24 hr hypoxia exposure results in ~10 fold increase in luciferase activity compared to 0 hr control (normoxia). Scale bars represent the standard error of the mean.

Figure 2. Establishing high and low siRNA controls for screening. U2OS-HRE cells reverse transfected with siRNA to HIF1A (low control) and FIH1 (high control) decrease and increase the response to 24 hr hypoxia exposure respectively. These controls give a Z factor of 0.8, which is considered an excellent score for screening. The effect of non-target (NT) siRNA is shown for comparison. Error bars represent the standard errors of the mean.

Figure 3. Screening workflow to identify regulators of the HIF1A hypoxia response. (A) Arrangement of 96 well plates for siRNA screening. Control siRNAs are stamped onto the outside columns as indicated and the ubiquitome siRNA library is stamped onto the internal columns, spread over 17 plates. U2OS-HRE cells are reverse-transfected and incubated at normoxia for 24 hr. (B) Stacks of assay plates are transferred in a sterile environment to a hypoxia workstation set at 1% oxygen for 24 hr. (C) Plates are removed from the hypoxia workstation and luciferase activity assays are performed on all assay plates. Luminescence is recorded on a luminometer and data analysis performed to establish the reporter activity of cells transfected with each library siRNA.

A

$$Z \text{ factor} = 1 - \frac{3 (\sigma_p + \sigma_n)}{(\mu_p - \mu_n)}$$

B

Figure 4. Quality control is performed by calculating the Z factor of each plate. (A) The Z factor for each plate is calculated using the formula given, where s represents the standard deviation, m represents the mean, p the positive control and n the negative control. (B) Scatter plot displaying the calculated Z factor of each plate from the three replicates of the ubiquitome screen. A cut-off of 0.5 is applied, and in this case all plates display a Z factor > 0.5 and therefore pass the quality control.

Figure 5. Chart showing the distribution of the siRNA library. Chart displaying the frequency of siRNAs from the triplicate ubiquitome siRNA screen that result in the corresponding percent activation. Low and high controls are set to 0% and 100% respectively. The siRNA library is distributed evenly between the high and low control, whereby the majority of siRNAs have no effect on the cellular phenotype. A small proportion of siRNAs are observed to increase and decrease the response, and therefore represent the potential novel regulators of the pathway.

Discussion

The use of genome-wide siRNA screens in mammalian cells has proven to be extremely valuable in identifying novel regulators of distinct biological pathways. Here, we have described the use of a targeted ubiquitome siRNA screen to identify regulators of the HIF1A-mediated cellular response to hypoxia. Targeted screens are becoming increasingly attractive as they are generally cheaper, quicker, easier to manage and report only on the pathway components in which the investigators are interested^{7, 10, 11}.

It is critical to put a major effort into the assay development stage to ensure the basic cell reporter is suitable for screening, and that reproducibility and low-variability can be achieved. It is also important to select robust positive and negative controls to create a window of separation within which the library regulators will fall. In addition, these act as an internal control in each assay plate of the screen allowing for the rapid identification of any problems with individual plates.

One common problem in high throughput screening is the occurrence of plate edge effects, which can be caused by a local difference in humidity at the outside wells of the plate relative to the internal wells. Having the appropriate controls in place and creating a heat map of the plate output can help to identify this problem. We have found that edge effects can be overcome by using moist tissue paper at the base of the plates, and placing a stiff, transparent plastic bag over the stack of plates to make the microenvironment of each plate inside the incubator more even.

A limitation of siRNA screening is the presence of false-positives and false negatives. False negatives can occur due to a number of factors including insufficient knockdown of the corresponding mRNA, or the ability of residual mRNA to produce enough active protein to efficiently perform its function. False-positives are more problematic, as they can cause an investigator to invest significant time following up a hit that may not actually govern the pathway in question. False-positives can be generated through a number of mechanisms including via siRNA off-target effects. This is where the siRNA knocks down not only the mRNA in question but also other, unidentified targets that are the true pathway regulators^{12, 13}. A number of approaches are commonly used and accepted as confirmation that the observed phenotype is a true result of knocking down the target gene in question¹⁴. For example, the problem of off-target effects can be overcome in part by tertiary screening, whereby the likelihood of more than one individual siRNA from the starting pool of four having the same off-target effect is decreased. In addition, successfully performing rescue experiments with siRNA-resistant cDNAs corresponding to the hit will also rule out off-target effects. It should be noted that this approach in itself is not without caveats, for example exogenously overexpressed proteins will not necessarily act identically to the endogenous counterpart due to altered stoichiometry of the relevant complexes or perturbed post-translational modifications *etc.* An alternative approach to hit validation therefore is to generate or acquire knockout or knock-in cell lines corresponding to the hits, and determine if these cell lines display the same phenotype as the siRNA knockdown. Thus, limitations of siRNA screening can be overcome with careful experimental follow-up. Furthermore, the unbiased identification of genuine modifiers of the pathways under scrutiny far outweighs the problems associated with potentially identifying false positives.

In summary, siRNA screening with a targeted "ubiquitome" siRNA library is a powerful method to identify novel members of ubiquitin and ubiquitin-like modifiers regulating distinct biological pathways. The protocol described here can be applied to any biological problem if there is a robust read-out of reporter activity. The final list of high confidence hits from the screen represents the starting point from where to establish the mechanistic basis of how each hit governs the pathway in question. Ultimately, this will add to the growing knowledge of how ubiquitin and ubiquitin-like modifiers regulate distinct biological pathways.

Disclosures

The authors have nothing to disclose.

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