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Fluorescence Reporter-Based Genome-Wide RNA Interference Screening to Identify Alternative Splicing Regulators

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Summary

Alternative splicing is a regulated process that leads to inclusion or exclusion of particular exons in a pre-mRNA transcript, resulting in multiple protein isoforms being encoded by a single gene. With more than 90% of human genes known to undergo alternative splicing, it represents a major source for biological diversity inside cells. Although in vitro splicing assays have revealed insights into the mechanisms regulating individual alternative splicing events, our global understanding of alternative splicing regulation is still evolving. In recent years, genome-wide RNA interference (RNAi) screening has transformed biological research by enabling genome-scale loss-of-function screens in cultured cells and model organisms. In addition to resulting in the identification of new cellular pathways and potential drug targets, these screens have also uncovered many previously unknown mechanisms regulating alternative splicing. Here, we describe a method for the identification of alternative splicing regulators using genome-wide RNAi screening, as well as assays for further validation of the identified candidates. With modifications, this method can also be adapted to study the splicing regulation of pre-mRNAs that contain two or more splice isoforms.

Keywords

Alternative splicing; Genome-wide; RNA interference; Flow cytometry; RBFOX2; Pre-mRNA

1. Introduction

RNA interference (RNAi) has allowed researchers to overcome challenges associated with classical genetic approaches and enabled them to perform high-throughput gene silencing (knockdown) experiments in cells and organisms. Combining the power of genetic screens with phenotypic assays, RNAi screening has made it possible for researchers to identify new genes and/or gene networks involved in regulating critical cellular processes. RNAi is now widely used in high-throughput screens in both basic and applied biology, and has allowed researchers to address key questions underlying a wide variety of biological processes, including signal transduction, cell viability, cell or organelle morphology, protein localization and/or function, drug resistance, and alternative splicing (1–6).

A number of genome-wide RNAi libraries have been developed by academic and commercial entities, with newer libraries emerging as our understanding of effective strategies to design and deliver RNAi reagents improves (7). Readers unfamiliar with RNAi screening strategies are referred to past reviews on assay development and optimization, high-throughput cell-based pooled format RNAi screens (1, 2, 8), arrayed format RNAi screens (1, 9), and *in vivo* screening (10). So far, hundreds of large-scale, cell-based and *in vivo* RNAi screens have been carried out in *Drosophila melanogaster*, mouse and human cells. Furthermore, numerous databases are available that support the browsing and analysis of results from these large-scale RNAi screens (11).

The RNAi screen described below is based on a previous publication from our group in which we sought to gain insights into the mechanism-of-action of the splicing regulator RBFOX2 by performing a genome-wide loss-of-function short hairpin RNA (shRNA) screen to identify factors that, in addition to the RBFOX2 itself, are required for splicing repression (6). Our screening strategy (Figure 1) was based upon an experimental system developed by Wang et al. (12) for the identification of exonic splicing silencers (ESSs) from a random sequence pool. This system uses a three-exon mini-gene construct that serves as a reporter for exon silencing (see inset to Figure 1). Exons 1 and 3 of this construct form a complete mRNA encoding green fluorescent protein (GFP), and exon 2 contains a cloning site into which an oligonucleotide can be inserted. Exon 2 is normally included to form an mRNA that does not encode functional GFP. However, insertion of an ESS sequence (in our case, the binding site for RBFOX2) into exon 2 can cause skipping of this exon, producing an mRNA encoding functional GFP. The mini-gene is constructed in an expression vector designed for use with Flp-In™-293 cells, which contain a single Flp recombination target (FRT) integration site. Integration of the mini-gene at a single genomic site is mediated by the Flp recombinase, which is encoded by a plasmid. The cell line containing the stably integrated splicing reporter is first sorted by fluorescence activated cell sorting (FACS) to obtain a population of cells that is 100% GFP-positive (GFP/Flp-In-293 cells). These cells are then used to perform a genome-wide shRNA screen. Briefly, the GFP/Flp-In-293 cells are stably transduced with an shRNA library; we used The RNAi Consortium (TRC)-Hs1.0 lentiviral human shRNA library comprising ~85,000 shRNAs, which we divided into 22 pools (~5000 shRNAs/pool) to facilitate high-throughput screening. The stably transduced cells from each pool are then FACS sorted to isolate the population of cells in which GFP expression has been significantly diminished and/or lost (GFP-negative), which is the expected result for the loss of splicing repressor function. For each pool, the GFP-negative population of cells is expanded, and the FACS sorting was repeated in order to minimize the number of false-positives. The shRNAs in the purified GFP-negative population of cells are identified by sequence analysis. Positive candidates are validated by stably transducing the GFP/Flp-In-293 cells with an individual shRNA directed against the candidate gene, and performing FACS analysis as well as other assays using reporter and endogenous target genes.

The method described here is a general screening approach that can be used to identify splicing repressors and/or co-repressors. In principle, this screening strategy could also be applied to identify alternative splicing regulators regulating complex alternative splicing events such as the splicing regulation of pre-mRNAs that contain two or more splice

isoforms with appropriate modifications to the reporter construct such as the one described by Moore et al. (see ref. 5).

2. Materials

Prepare all solutions using ultrapure double distilled water (ddH₂O). Store all commercially obtained reagents according to the manufacturer's instructions.

2.1. Cell Lines and Culture Conditions

1. Cell lines: Flp-In™-293 cells (Thermo Fisher Scientific) and 293T cells (American Tissue Culture Collection) (*see Note*¹).
2. Cell culture medium: DMEM high glucose medium (Invitrogen) containing 10% fetal bovine serum (FBS) (Invitrogen)/Penicillin-Streptomycin (Invitrogen). Mix well and store at 4°C. Prior to starting the cell culture experiment, warm the media in a 37°C water bath for about 15 min.

2.2. Lentivirus Preparation, Transduction and Determination of Multiplicity of Infection

1. 10 cm tissue culture plates.
2. TRC Lentiviral Human Genome shRNA Library (GE Dharmacon) divided into 22 pools, and corresponding positive (RBFOX2) and negative (non-silencing, also called non-targeting) control shRNAs.
3. Lentiviral packaging plasmids pMD2.G (Addgene plasmid #12259) and psPAX2 (Addgene plasmid #12260).
4. Effectene Transfection Reagent kit (QIAGEN), which includes Effectene reagent, Enhancer and EC buffer.
5. 0.45 µm filters (Millipore).
6. Polybrene (100 µg/µL) (*see Note*²).
7. Puromycin (5 mg/mL).
8. Phosphate buffered saline (PBS; 10×): 25.6 g Na₂HPO₄·7H₂O, 80 g NaCl, 2 g KCl, 2 g KH₂PO₄, ddH₂O to 1 liter. Autoclave prior to use. Store at room temperature.
9. Crystal violet staining solution: 40% methanol, 10% acetic acid, 0.01% (w/v) crystal violet in ddH₂O. Store at room temperature.

¹293T cells are the preferred cell line for virus preparation due to their high transfection efficiency and their ability to support high expression of virally-encoded proteins. Use of early passage 293T cells will ensure high titer virus is obtained.

²Polybrene improves overall transduction efficiency by enhancing receptor-independent virus absorption through the cell membrane. Pilot experiments should be done to determine the optimum of polybrene required by cells for maximum transduction efficiency. Use of more than optimum concentration of polybrene will result in unwanted toxicity to the cells.

2.3. Preparation of Stable Cell Lines Carrying the GFP Reporter Construct

1. pcDNA5/FRT vector (Thermo Fisher Scientific) containing the GFP reporter with an RBFOX2-binding site inserted into exon 2 (see ref. 12).
2. pOG44 Flp-recombinase expression vector (Thermo Fisher Scientific).
3. Hygromycin B (50 mg/mL) (AG Scientific Incorporation).
4. Cloning cylinders.

2.4. Flow Cytometry Sorting and Analysis

1. Flow cytometer and analyzer, such as a BD FACSCalibur flow cytometer (BD Biosciences).
2. FACS tubes.
3. Collection media: DMEM + 20% FBS/Penicillin-Streptomycin.
4. Trypsin-EDTA (0.25%, Invitrogen).

2.5. Genomic DNA Isolation and Identification of Candidate shRNAs by DNA Sequencing

1. Cell lysis buffer: 0.5% (v/w) SDS, 200 µg/mL of protease K, 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 10 mM EDTA (pH 8.0). Store at room temperature.
2. Phenol:chloroform:isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM EDTA. Store at 4°C.
3. Chloroform. Store at room temperature.
4. Sodium acetate (3 M): Dissolve 408.1 g of sodium acetate•3H₂O (MW 136) in 800 mL of ddH₂O. Adjust the pH to 5.2 with glacial acetic acid. Store at room temperature.
5. Ethanol (100% and 70%).
6. TE buffer (1×): 10 mM Tris, 1 mM EDTA (pH 8.0).
7. Taq PCR buffer (10×): 100 mM KCl, 100 mM (NH₄)₂SO₄, 200 mM Tris-HCl, pH 8.75, 20 mM MgSO₄, 1% Triton X-100, 0.1% BSA. Store at -20°C. Alternatively, it can be purchased.
8. Taq DNA polymerase
9. dNTPs (final concentration 10 mM each A, C, G, T).
10. Primers: Primer1 For-TRC (10 µM), TACGATACAAGGCTGTTAGAGAG; Rev-TRC (10 µM), CGAACCGCAAGGAACCTTC, Sequencing primer (MF22; 5 µM), AAACCCAGGGCTGCCTTGAAAAAG.
11. Dimethyl sulfoxide (DMSO).
12. DNase and RNase free agarose for gel electrophoresis.
13. QIAquick Gel Extraction Kit (QIAGEN).

14. pGEM®-T Easy Vector Systems kit (Promega), which contains the pGEM®-T Easy Vector, control insert DNA, 2× Rapid Ligation Buffer and T4 DNA Ligase.
15. DH5α competent cells. Store at −80°C.
16. 2× LB broth: Dissolve 20 g of peptone, 10 g of yeast extract, and 5 g of NaCl in 1 L of ddH₂O. Autoclave prior to use. Store at room temperature.
17. LB Amp plates: Add 15 g of agar to 1 L of 2× LB broth and autoclave for 25 min. Cool down and add ampicillin (100 µg/mL). Pour into 10 cm dishes, let solidify and store at 4°C.
18. Isopropyl-β-D-thiogalactopyranoside (IPTG; 1M).
19. 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (BCIG or X-gal; 50 mg/mL).

3. Methods

Carry out all cell culture experiments in an ultraviolet-sterilized vacuum hood at room temperature unless otherwise specified. Incubate cells in a 5% CO₂ incubator at 37°C.

3.1. shRNA Lentivirus Preparation

1. On day 1, plate 2×10⁵ 293T cells in each of 24 individual 10 cm tissue culture plates; use one plate for each of the 22 shRNA pools, one plate for the positive (RBFOX2) shRNA control, and one plate for the negative (non-silencing) control shRNA. Shake the plates well to make sure the cells are evenly spread. Incubate at 37°C for 16 h.
2. On day 2, aspirate old medium and add 10 mL of pre-warmed fresh medium onto the cells. Incubate the cells at 37°C until the transfection mixture is added. Prepare the transfection mixture by mixing 5 µg of pooled shRNA plasmids (or positive and negative control shRNA plasmids), 2.5 µg of pMD2.G (VSV-G envelope expressing plasmid) and 5 µg of psPAX2 (lentiviral packaging plasmid) in 300 µL of EC buffer. Add 32 µL of Enhancer, mix well by brief vortexing, and let it sit at room temperature for 5 min. Add 80 µL of Effectene, vortex and let it sit at room temperature for another 20 min. Dispense 0.5 mL of fresh medium to the transfection mixture and, while holding the plate still, gently dispense the entire mixture evenly on top of the cells.
3. On day 3, aspirate all of the medium and add 10 mL of pre-warmed fresh medium. Incubate at 37°C for 48 h.
4. On day 5, collect the supernatant with a syringe and dispense it through a 0.45 µm filter to remove cell debris. Aliquot the supernatant (1 mL aliquots) into microcentrifuge tubes and store at −80°C (*see Note*³).

³It is important to note that freeze-thawing will lead to drop in virus titer. Therefore, we recommend avoiding multiple freeze-thaw cycles.

3.2. Determining the Multiplicity of Infection for Lentiviral shRNA Pools

1. Plate 1×10^4 293T cells in each well of a 6-well plate and incubate at 37°C for ~16 h. Use one plate for each of the 22 pools, plus two more for the positive and negative control shRNAs.
2. Thaw the virus supernatant, and make a series of six 10-fold serial dilutions in DMEM media containing 10% FBS/Penicillin-Streptomycin. Mix 100 μL of diluted virus with 900 μL of fresh medium. Add polybrene to a final concentration of 10 $\mu\text{g/mL}$. Gently dispense the virus mixture on top of the 293T cells and incubate at 37°C for 24 h.
3. Aspirate the media containing virus and add 10 mL of fresh medium. Incubate at 37°C for 24 h.
4. Add 1.5 $\mu\text{g/mL}$ of puromycin to each plate and incubate at 37°C until colonies begin to form (usually about 7–10 days). Change the media containing puromycin every 2 days.
5. Wash colonies with $1 \times \text{PBS}$ and stain with crystal violet staining solution at room temperature for 20 min. Wash the colonies multiple times with ddH_2O until the water runs colorless. Air-dry the plate and count the colonies. Calculate the multiplicity of infection for the lentiviral supernatants using the following formula:

$$\text{MOI (particle forming units (pfu) / mL)} = \text{colony number} \times \text{dilution factor} \times 10$$

3.3. Preparation of Stable Cell Lines Carrying the GFP Reporter Construct

1. Plate 2×10^6 Flp-InTM-293 cells in a 10 cm plate. Incubate the cells at 37°C for 16 h.
2. Transfect the cells with 2 μg of pcDNA5/FRT-based reporter plasmid and 1 μg of pOG44 plasmid using the Effectene Transfection Reagent kit. Incubate at 37°C for 24 h.
3. Aspirate all of the medium and add 10 mL of pre-warmed fresh medium.
4. Add hygromycin B (150 $\mu\text{g/mL}$) (*see Note 4*) to enrich cells containing the stably integrated reporter construct. Incubate the cells at 37°C for ~2 weeks to allow for individual colonies to form. Change the medium containing hygromycin every 4 days. It takes about 8–10 days to wipe out cells that do not carry a stable integration of the construct.
5. Isolate individual colonies (8–10) into 6-well plates using cloning cylinders according to manufacturer's instructions.

⁴It is essential to empirically determine the concentration of hygromycin required for killing the Flp-InTM-293 cells. Use of sub-optimal concentrations of hygromycin will result in colonies growing on the plate that do not contain stably integrated reporter construct.

6. Expand the colonies for 6–8 days in order to obtain enough cells for FACS sorting.
7. Sort the cells using a flow cytometer. Use the parental Flp-InTM-293 cells and GFP reporter plasmid-transfected cells as controls to set the gates for the analysis. First, gate for the live cell population in the forward versus side-scatter plot. Next, gate for the GFP-positive cells in the GFP channel: set the gate so that >90% of the cells appear to be GFP-positive in the GFP reporter plasmid-transfected cells and 100% cells appear GFP-negative in the parental Flp-InTM-293 cells. Sort all the colonies based on these gates and collect the GFP-positive cells in collection media containing DMEM and 20% FBS/Penicillin-Streptomycin.
8. Plate these cells in 10 cm plates containing DMEM and 10% FBS/Penicillin-Streptomycin. Select the colony that shows maximum mean fluorescence intensity of GFP signal for further experiments.

3.4. shRNA Library Transduction and Selection

1. Plate 2×10^6 GFP/Flp-In-293 cells in 24 individual 10 cm plates, one for each shRNA pool and two for the positive (RBFOX2) and negative (non-silencing) control shRNAs. Incubate at 37°C for 12–16 h.
2. Transduce the cells with the lentiviral shRNA pools and control shRNAs in a total volume of 10 mL DMEM media containing 10% FBS/Penicillin-Streptomycin and polybrene (10 µg/mL) to achieve a multiplicity of infection (MOI) of 0.2.
3. Change media after 24 h and add puromycin (1.5 µg/mL) to select the cells carrying shRNA. Change media containing puromycin after every two days. Usually it takes about 3–4 days to completely wipe out cells that do not contain an integrated shRNA.

3.5 FACS Sorting

1. On day 10 post infection, aspirate the media from the plates.
2. Rinse the cells with 1× PBS. Add 1 ml 0.25% trypsin to each plate and incubate at room temperature for ~2 min with occasional agitation. Visually inspect the plates to ensure complete detachment of the cells.
3. Add 1 ml of 1× PBS with 10% FBS to neutralize the trypsin and dissociate the cells into a single cell suspension by repeated pipetting. Collect the cells in FACS tubes and store at 4°C.
4. Sort the cells using a FACS sorter and analyzer. Use the parental Flp-InTM-293 cells and non-silencing shRNA-infected GFP/Flp-In-293 cells as controls to set the gates for FACS sorting. First, gate for the live cell population in the forward versus side-scatter plot. Next, gate for the GFP-positive cells in the GFP channel: set the gate so that >90% of the cells appear to be GFP-positive in the non-silencing shRNA control and 100% cells appear GFP-negative in the parental

Flp-In™-293 cells. Sort all the 22 pools based on these gates and collect the GFP-negative cells in collection media containing DMEM and 20% FBS/ Penicillin-Streptomycin.

5. Collect the sorted GFP-negative cells from individual pools separately and plate them on a 10 cm dish in media containing DMEM with 10% FBS/Penicillin-Streptomycin and puromycin (1.5 µg/mL). Incubate at 37°C for 4 days, changing the media containing puromycin every 2 days.
6. On day 17 post-infection, repeat steps 1–5 and proceed to the next section with the collected GFP-negative cells.

3.5. Genomic DNA Isolation and shRNA Identification

1. Pellet down the cells at 5,000 rpm for 5 min, collect the GFP-negative cells and resuspend them in 500 µL of cell lysis buffer. Incubate the cell lysate at 55°C overnight.
2. Add an equal volume of phenol:chloroform:isoamyl alcohol. Mix and centrifuge at 10,000 rpm for 15 min. Transfer the aqueous phase into a new 1.5 mL microcentrifuge tube and extract again with an equal volume of chloroform.
3. Precipitate the DNA by adding 0.1 volume of 3 M sodium acetate and 2 volumes of 100% ethanol. Mix well by vortexing and leave at –80°C for at least 1 h. Spin in a tabletop centrifuge at top speed at 4°C for 30 min, and wash the pellet with 1 mL of 70% ethanol. Pour off the ethanol and invert the microfuge tube onto paper towel to drain the residual ethanol. Air-dry the pellet at room temperature overnight, dissolve it in 100 µL of TE buffer, and measure the DNA concentration (*see Note* ⁵).
4. To amplify the lentiviral shRNA, set up a PCR reaction containing the following components: ~100 ng genomic DNA, 2.5 µL 10× Taq buffer, 1 µL 10 mM dNTPs, 1 µL For-TRC primer, 1 µL Rev-TRC primer, 1 µL DMSO, 0.5 µL Taq DNA polymerase, 18 µL ddH₂O.
5. Program a PCR machine with the following cycling program and run the samples:
 - Step 1 94°C for 2 min
 - Step 2 94°C for 30 s
 - Step 3 55°C for 45 s
 - Step 4 72°C for 1 min
 - Step 5 Go to Step 2 for 34 additional cycles
 - Step 6 72°C for 5 min

⁵We use a NanoDrop spectrophotometer (Thermo Scientific) to accurately measure DNA concentrations in solutions of precipitated DNA and PCR reactions.

Step 7 4°C indefinitely

6. Run the PCR product on a 1% agarose gel containing 10 µl ethidium bromide (10 mg/ml stock). A ~700 bp PCR product should be observed. Elute the product from the gel using a QIAquick Gel Extraction Kit.
7. Ligate the eluted PCR product into the TA cloning vector (pGEM®-T) by setting up a ligation reaction as follows: 3 µL of PCR product, 1 µL of vector, 5 µL of 2× Rapid Ligation buffer, and 1 µL of T4 DNA ligase. Incubate the ligation reaction at 16°C overnight.
8. The next day, transform the ligation reaction into DH5α competent cells. Plate the transformation mix onto LB Amp plates onto which 10 µL of IPTG and 50 µL of X-gal have been spread evenly.
9. Incubate the plates at 37°C for ~16 h until the blue and white colonies can be clearly distinguished (*see Note 6*).
10. Aliquot 25 µL of ddH₂O into a series of PCR tubes, one for each colony to be picked (*see Note 7*). Pick a single white colony from the LB Amp plate using a pipette tip, place the tip in the PCR tube and mix well by pipetting. Remove 5 µL from each tube and dispense into a fresh PCR tube, and store the remaining 20 µL at 4°C.
11. Prepare a PCR master mix (by multiplying the following recipe by the number of colonies to be screened) and add 15 µL to each tube prepared for PCR in step 10: 2 µL of 10× Taq Buffer, 1 µL dNTPs, 0.5 µL For-TRC primer, 0.5 µL Rev-TRC primer, 0.25 µL Taq DNA polymerase, 10.75 µL ddH₂O.
12. Program a PCR machine with the following cycling program and run the samples:
 - Step 1 95°C for 2 min
 - Step 2 94°C for 1 min
 - Step 3 55°C for 1 min
 - Step 4 72°C for 3 min
 - Step 5 Go to Step 2 for 34 cycles
 - Step 6 72°C for 5 min
 - Step 7 4°C indefinitely
13. To make sure the PCR reaction worked, load 5 µL of the reaction mixture on a 1% agarose gel. Again, a ~700bp PCR product should be observed.

⁶Occasionally, there may be too many colonies growing on the plate due to high competency of DH5α cells. To avoid overcrowding, plate two or three 10-fold serial dilutions of the bacteria on different LB Amp plates containing X-gal and IPTG.

⁷The number of colonies to be picked for Sanger sequencing should be estimated based on the percentage of sorted cells collected after FACS. As a starting point, pick 10–20 colonies per pool. Sequencing of colonies from a plate should be stopped when the same shRNA is identified more than 3–4 times.

14. Dilute the PCR product by adding 80 μ L of ddH₂O to each tube and mix well by pipetting. Mix 2 μ L of the diluted PCR product with 2 μ L of 5 μ M MF22 sequencing primer and send for sequencing.
15. To identify shRNAs from the sequencing results, search for the sequence TTCAAAAA to find the beginning of the shRNA, TCTGAG to define the loop and CCGGTG to define the end within the sequencing reads. Then map the shRNA sequence onto the TRC shRNA library database (<https://www.broadinstitute.org/rnai/trc/lib>) to find the corresponding gene.

3.6. Validation of Candidate Genes

1. Prepare individual virus supernatants for each shRNA clone identified from the screen, as described above in *Section 3.1*.
2. Infect 2×10^5 Flp-InTM-293 cells with 0.5 mL of virus supernatant mixed with 10 mL of fresh medium and 10 μ g/mL of polybrene. Change media after 16 h and then add media containing 1.5 μ g/mL of puromycin. Select for 3–4 days.
3. After 10 days, perform FACS analysis in the candidate knockdown cells as described in *Section 3.5*. The analysis should be performed on 1×10^5 cells or more in order to obtain statistically significant results. FACS results obtained for candidate knockdowns should be compared against the control non-silencing shRNA knockdown cells from the same batch. Analyze the flow cytometry data using a software package such as FlowJo software (*see Note 8*).
4. Candidates should be validated using other assays, including PCR, to assess changes in isoform abundance of the reporter construct and of known endogenous target genes (6, 13).
5. It is critical to verify that the obtained results are not due to an off-target effect of the shRNA. To do this, select 2–3 unrelated shRNAs against the same target gene and test whether they confer similar loss of fluorescence signal by FACS and changes in isoform abundance of the reporter gene by PCR. Candidates that validate with multiple shRNAs can be considered a true candidate for follow-up studies. In addition, verify that the candidate shRNAs knock down their target genes with >60–70% knockdown efficiency using quantitative real-time RT-PCR (qRT-PCR) and/or immunoblot analyses (*see Note 9*).

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⁸Always use the parental Flp-InTM-293 cells and non-silencing shRNA-infected GFP/Flp-In-293 cells as controls when performing FACS sorting and/or analysis with the candidate shRNAs in order to avoid technical variations due to sorting on different days.

⁹There are several anticipated classes of “false-positives” that one should attempt to eliminate prior to further analysis. For example, shRNAs that result in a decreased level of the splicing repressor or lead to a general reduction of splicing would result in loss of GFP expression and thus score positively in the primary screen. To eliminate such candidates, analyze splicing repressor levels by immunoblotting and qRT-PCR analysis and eliminate those candidates that significantly decrease splicing repressor levels from further experiments.

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Figure 1.
Schematic of the genome-wide RNAi screening strategy.