Chapter 6

High-Throughput Screening of a Luciferase Reporter of Gene Silencing on the Inactive X Chromosome

Alissa Keegan, Kathrin Plath, and Robert Damoiseaux

Abstract

Assays of luciferase gene activity are a sensitive and quantitative reporter system suited to high-throughput screening. We adapted a luciferase assay to a screening strategy for identifying factors that reactivate epigenetically silenced genes. This epigenetic luciferase reporter is subject to endogenous gene silencing mechanisms on the inactive X chromosome (Xi) in primary mouse cells and thus captures the multilayered nature of chromatin silencing in development. Here, we describe the optimization of an Xi-linked luciferase reactivation assay in 384-well format and adaptation of the assay for high-throughput siRNA and chemical screening. Xi-luciferase reactivation screening has applications in stem cell biology and cancer therapy. We have used the approach described here to identify chromatin-modifying proteins and to identify drug combinations that enhance the gene reactivation activity of the DNA demethylating drug 5-aza-2′-deoxycytidine.

Key words X chromosome inactivation, Chromatin, High-throughput screening, DNA methylation, Luciferase

1 Introduction

In the field of cancer biology there is a growing appreciation that tumorigenesis is frequently driven by epigenetic events such as tumor suppressor gene silencing by DNA methylation [1, 2]. Development of therapies targeting epigenetic pathways is hampered by the difficulty of monitoring treatment efficacy. Drugs such as DNA methylation inhibitors and histone deacetylase inhibitors are believed to reprogram the oncogenic cell fate through gene expression changes [2, 3]. Conventional assays of cytotoxicity such as those applied to chemotherapeutics are not ideal to measure epigenetic activity of drug candidates [3]. Changes in gene expression secondary to epigenetic reprogramming may take weeks to take effect and can be difficult to reproduce ex vivo [3]. Therefore, accurate reporter systems are needed to develop epigenetically acting drug regimens. As an example, Cui and coauthors described

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such a reporter gene system that demonstrated clinically relevant behavior in response to pharmacologic treatment [4]. They targeted a fluorescent reporter downstream of an aberrantly methylated tumor suppressor gene, *SFRP1*, in a colon cancer cell line and used *SFRP1-GFP* activation to monitor the effect of the DNA demethylating drug 5-aza-2′-deoxycytidine (5-aza-2′-dC or decitabine) [4]. They found that more prolonged 5-aza-2′-dC exposure promoted *SFRP1-GFP* expression, which may help explain why many patients with acute myeloid leukemia and myelodysplastic syndrome demonstrate a delayed response to 5-aza-2′-dC treatment [4, 5]. The reporter approach described here is of similar principle but makes modifications to the reporter gene that are more compatible with high-throughput screening and that have the potential to lead to more diverse, biologically relevant findings. First, we use a luciferase reporter that replaces fluorescence imaging with a rapid, enzymatic assay that gives quantitative readout of reporter activity. Second, rather than targeting the reporter to an aberrantly methylated gene, we have used a targeting approach that leads to endogenous reporter gene silencing in mouse development through the process of X chromosome inactivation (XCI). By using XCI as the model system of gene silencing and chromatin change, we increase the potential applications beyond cancer therapy to stem cell biology, as we describe below.

We adapted the model system of XCI to siRNA and chemical screening. XCI is one of the most studied examples of gene silencing in mammals and affects an entire chromosome. It is a mechanism of dosage compensation for X-linked genes between mammalian sexes that involves chromosome-wide transcriptional silencing beginning in early female embryonic development [6]. Initiation of XCI occurs with sequential events including upregulation and spread of the long noncoding RNA *Xist*, loss of RNA Polymerase II, gain of repressive histone methylation marks such as histone H3K27me3 and H3K9me2, histone deacetylation, and gain of DNA methylation at CpG islands [7]. The choice of which of the two X chromosomes is inactivated is random, however, if *Xist* is deleted off one X chromosome in female mice, that X chromosome will remain active and the wildtype X chromosome will undergo inactivation in 100% of cells [8]. Once established, the inactive X chromosome (Xi) is remarkably robust as it is maintained for the lifetime of the female [6]. Complete X chromosome reactivation (XCR) occurs in the female germline to transmit genetic information from both X chromosomes [6]. In tissue culture, complete XCR occurs from somatic mouse cells that undergo successful transcription-factor induced reprogramming to the induced pluripotent stem cell (iPSC) state [9]. XCR occurs as one of the final events in the course iPSC reprogramming [10]. XCR is a faithful marker of complete reprogramming to the iPSC state and has immediate applications in stem cell biology. For instance,

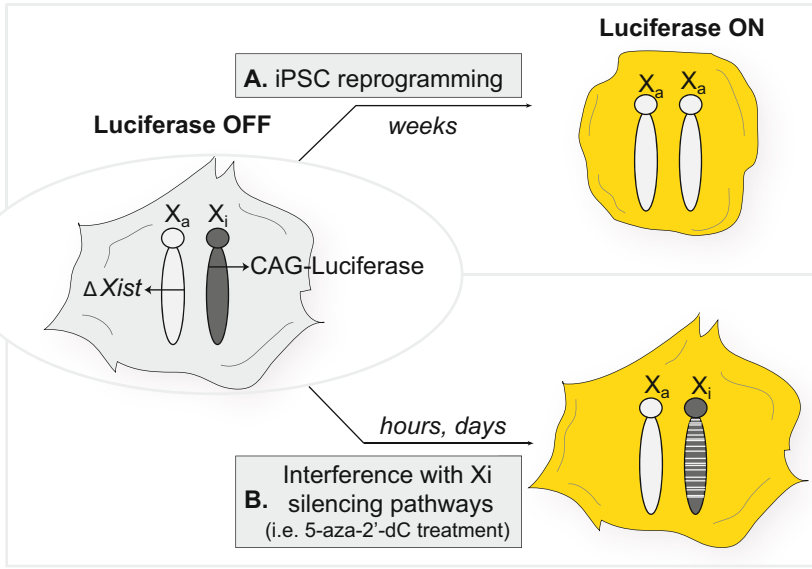

Fig. 1 Applications of X chromosome reactivation screening. Primary mouse embryonic fibroblasts (MEFs) bearing a luciferase transgene on the inactive X chromosome (Xi) do not have luciferase signal at baseline. (**a**) Transcription factor-induced reprogramming to iPSCs culminates in complete reactivation of the Xi to an active X chromosome (Xa) with concomitant luciferase (yellow) upregulation. (**b**) Interference with individual repressive chromatin pathways present on the Xi in MEFs leads to upregulation of some Xi-linked genes including the luciferase reporter in a percentage of treated cells

screens can be designed to increase the efficiency of iPSC reprogramming by monitoring for earlier and higher rates of XCR in response to protein overexpression, gene knockdown, or chemical treatment (Fig. 1).

Monitoring of XCR is also relevant to cancer biology. Partial XCR can be elicited by targeting the pathways of DNA methylation and histone deacetylation with the same epigenetic drugs used in cancer treatment such as 5-aza-2′-dC and trichostatin A [3, 11]. Partial XCR means that for a treated cell population, only a small percentage of cells reactivate the expression of inactive X chromosome (Xi)-linked gene being assayed. Partial XCR likely reflects that multiple layers of chromatin silencing with some redundancy maintain the Xi and that the threshold for reactivation differs across cells in a population and across loci on the Xi. Nonetheless, the XCR effect of various chemical and gene knockdown treatments is reproducible [11, 12]. A benefit to studying epigenetic drug targets in the XCI model system is that the activity of treatments can be detected by XCR activity in as few as 1–3 days [12]. The short timecourse of the reporter assay in primary cells avoids epigenetic drift and secondary cellular changes that may indirectly affect chromatin silencing. As we show here, XCR is highly amenable to high-throughput screening. Cells bearing a luciferase reporter

gene on the Xi do not have detectable background reactivation [12]. The luciferase reporter is also sensitive and detects low rates of partial XCR [12]. Furthermore, the Xi-luciferase reporter is suitable to combination drug screening since it undergoes in vivo silencing due to XCI, which involves multiple repressive chromatin pathways, and thus reflects the multilayered nature of repressive chromatin changes. Here, we describe how to adapt an XCR assay to 384-well format with high-throughput siRNA and chemical screening. We detail the important considerations when scaling the experiment to a genome-wide mouse siRNA library. Finally we touch on approaches to normalize screening data and identify and validate hits. Using the methods described here, we were able to identify knockdown of a chromatin silencing factor *Atf7ip* as an XCI maintenance factor and to identify a combination drug treatment that enhances the DNA demethylating ability of 5-aza-2′-dC [12].

2 Materials

1. Mouse embronic fibroblast (MEF) medium, 1×: Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, nonessential amino acids, L-glutamine, penicillin–streptomycin, and β-mercaptoethanol.
2. PLB lysis buffer (Promega).
3. Luciferase assay reagent I (LAR) (Promega).
4. GloMax microplate luminometer (Promega).
5. Genome-wide mouse siRNA library provided as Silencer Mouse Druggable Genome siRNA library V3 and Silencer Mouse Genome siRNA V3 Extension Set (Ambion, Life Technologies).
6. Multidrop 384 reagent dispenser (Thermo Scientific).
7. BenchCel 4× plate handler with Vcode bar code print and apply station, and Vprep pipetting system with a 96 LT head (all Velocity11, Agilent Technologies).
8. Matrix 384-well tissue culture plates (Thermo Scientific).
9. PlateLoc thermal microplate sealer (Agilent Technologies).
10. Dnmt1 siRNA (Ambion, ID # AM161526).
11. 5-Aza-2′-deoxycytidine (5-aza-2′-dC) (Sigma-Aldrich). Resuspend in DMSO and bring to 100 mM, then freeze aliquots in −80 °C. Thaw aliquot at room temperature just before adding to tissue culture media.

12. Microsource, Biomol enzyme inhibitor and bioactive lipid libraries, Prestwick chemical library, and NIH clinical collections. Stored at 10 mM in DMSO.
13. 2× MEF medium: Dulbecco's modified Eagle medium supplemented with 20% fetal bovine serum, 2× nonessential amino acids, 2× L-glutamine, 2× penicillin–streptomycin, and 1× β-mercaptoethanol.
14. 5× siRNA buffer (Dharmacon, GE Healthcare).
15. Tissue-culture grade phosphate buffer saline (PBS) (Life Technologies).
16. Trypsin–EDTA 0.25%, phenol red (Life Technologies).
17. Opti-MEM reduced serum medium (Life Technologies).
18. Lipofectamine RNAiMAX transfection reagent (Life Technologies).
19. ELx405 select deep well washer (Bio-Tek Instruments).
20. ONE-Glo luciferase assay reagent (Promega).
21. FlexStation II benchtop multimode microplate reader (Molecular Devices).
22. Biomek FXP laboratory automation workstation (Beckman Coulter).

3 Methods

3.1 Preparing Xi-Luciferase Reporter Cells

MEFs were isolated from Xi-luciferase female transgenic embryos bearing a CAG promoter-driven luciferase transgene in the *Hprt* locus on the X chromosome as well as deletion of *Xist* on the other X chromosome [12]. The deletion of *Xist*, a noncoding RNA required for XCI, ensures that the luciferase-bearing transgene is silenced in all cells [8, 12]. Xi-luciferase female double transgenic embryos ($Xi^{CAG\text{-}Luciferase}Xa^{\Delta Xist}$) are derived at an expected 1:4 Mendelian ratio from a cross of male luciferase reporter mice ($X^{CAG\text{-}Luciferase}Y$) and female mice heterozygous for *Xist* knockout ($X^{\Delta Xist}X^{wt}$). This cross also produces littermate females lacking the *Xist* deletion ($X^{CAG\text{-}Luciferase}X^{wt}$) that express luciferase at baseline due to random XCI. Therefore, genotyping and careful tissue culture technique are necessary to avoid contamination with luciferase-expressing littermate cells.

1. Isolate MEFs by standard methods from embryos (d13 or d14 d.p.c.) in 1× MEF media in 15 cm^2 plates (*see* **Note 1**) [13].
2. Upon reaching 80–90% confluence, sample MEFs for *Hprt*, *Xist*, and *Zfy* (to confirm absence of Y chromosome) genotyping by PCR, and freeze the remainder of cells in 1× MEF media with 20% DMSO.

3. If MEFs are identified from the litter as Xi-luciferase MEFs on the basis of carrying both the luciferase allele and the *Xist* deletion, thaw the Xi reporter MEFs in 15 cm^2 plates and passage two more times at a 1:6 split. With the first passage, a sample of cells should be tested to confirm absence of luciferase activity at baseline (*see* **Note 2**). Plate a suspension of 60,000 cells in a 12-well plate well for 48–72 h, then lyse the adherent cells in 200 μL of PLB buffer for 20 min on an orbital shaker. Clear the lysate with a short centrifugation step of 12,000 × *g* for 30 s then assay 20 μL of cleared lysate with 50 μL of LARI reagent on a luminometer. At the second split, MEFs derived from different Xi-luciferase reporter embryos should be pooled together to ensure homogeneity in the batches frozen for the screen. Freeze each 15 cm^2 plate of cells to one vial. Thaw one vial to two 15 cm^2 plates and count cells 24 h later to estimate yield from the frozen batches in preparation for screening (*see* **Note 3**).
4. In addition to thawing cells 24 h prior to screening, autoclave one tissue-culture grade 500 mL bottle with a magnetic stir bar inside per each set of 30 library plates that will be screened. This glassware will be used to sterilely dispense a homogenous suspension of cells to 384-well plates.

3.2 siRNA Library Preparation

We chose to use a genome-wide mouse siRNA library provided as 0.25 nmol dried oligonucleotide in 153 384-well plates. Two columns were left empty on each plate for positive siRNA control and no siRNA control. The library was resuspended by centrifuging each plate for 2 min at 1000 × *g*, adding 50 μL of RNAse free water with a Multidrop reagent dispenser in an RNAse-free biological safety cabinet, resealing, vortexing, and centrifuging again at 1000 × *g* for 2 min. Four sample plates were randomly chosen to confirm RNA concentration between 64 and 75 ng/μL. Copies of the library were transferred by BenchCel 4× plate handler with Vcode bar code application, and Vprep pipettor with a 96 LT head. 2 μL of siRNA suspension from source plates were moved into white opaque 384-well tissue culture plates, sealed with plate sealer, and then frozen in −80 °C.

3.3 Screen Controls

Loss of DNA methylation by deletion of or interference with the maintenance methyltransferase *Dnmt1* is known to elicit XCR. Therefore we tested combinations of *Dnmt1* knockdown and 5-aza-2′-dC, a DNMT1-inhibiting chemical, treatment to produce luciferase signal in 384-well format [11, 14]. We chose a *Dnmt1* siRNA that reduced *Dnmt1* RNA levels to <10% of control levels as measured by RT-qPCR from MEFs treated in the 12-well format [12]. We then moved to optimizing the assay in 384-well format and found that *Dnmt1* knockdown in the presence of low

concentration 5-aza-2′-dC (0.2 μM), reliably produced luciferase reactivation [15]. Addition of 5-aza-2′-dC was likely necessary to reduce DNA methylation to a lower level such that knockdown of *Dnmt1* boosted Xi-luciferase reactivation signal to a threshold detectable in the 384-well assay. The low concentration of 5-aza-2′dC without *Dnmt1* knockdown did not produce background signal [15]. Therefore, we added siRNA against *Dnmt1* to all 16 wells of the last column of each siRNA screening plate as a positive control. We also added 5-aza-2′-dC to a final concentration of 0.2 μM to each screening well including positive and negative controls and library samples. The negative control, occupying the 16 wells in the penultimate column of each screening plate, contained all the reagents with exception of siRNA. For the chemical screen, we used high concentration 5-aza-2′-dC (10 μM) in one row of each plate as a positive control.

3.4 siRNA Knockdown Assay Optimization

The optimization of siRNA knockdown in 384-well format is assessed by reporter activity with positive control knockdown. As with knockdown of *Dnmt1* (*see* Subheading 3.3), this siRNA should be validated for target gene knockdown from a larger assay format such that RNA yield is sufficient for RT-qPCR. Once a positive control such as the *Dnmt1* knockdown and 5-aza-2′-dC (0.2 μM) treatment is established (*see* Subheading 3.3), the assay can be optimized to 384-well format by comparing the signal distribution between positive and negative control samples. We recommend optimizing the assay to maximize the *Z*-factor, which is a coefficient reflective of assay dynamic range and data signal variations (Fig. 2) [16]. A feasible assay for screening has a *Z*-factor > 0, and an excellent assay has a *Z*-factor higher than 0.5. Pilot experiments should sequentially test experimental variables for raising the *Z*-factor of the assay (*see* **Note 4**). The experimental variables in our optimization are summarized in Table 1 in the order that we tested them (*see* **Note 5**). Since our final assay *Z*-factor was 0.11, which is predictive of generating false positives and false negatives, we screened the siRNA library in duplicate to add statistical power.

3.5 siRNA Screening of Xi-Reporter MEFs

1. One day prior to transfection thaw and plate the Xi-luciferase reporter MEFs. The number of vials of cells should be sufficient to distribute 2000 cells to each 384-well well 24 h later, accounting for 20% extra cells (*see* **Note 6**). We recommend screening one 30-plate batch from the library on the first day of screening then increasing to two 30-plate batches on subsequent days. The determination of the number of vials of cells to thaw should be made from a previous test of cell yield from the frozen batches (refer to Subheading 3.1).

$$\text{Z-factor} = 1 - \frac{3\,(\text{StDev}_{\text{sample}}) + 3\,(\text{StDev}_{\text{control}})}{\left|\text{Mean}_{\text{sample}} - \text{Mean}_{\text{control}}\right|}$$

Adapted from Zhang et al., 1999

Fig. 2 *Z*-factor quality coefficient for screening assay

Table 1
Experimental variables for screening optimization

Experimental variable	Optimal condition	Other condition(s) assayed
Cell Number	2000 cells	750–2500 cells
5-aza-2′-dC concentration	0.2 μM	0.05–0.15 μM
Incubation time after knockdown	72 h	48 or 60 h
Luciferase plate reader	Acquest (Molecular Devices)	Wallac 1420 Victor2 (Perkin Elmer) or Tecan
384-well plate	Matrix (Thermo Scientific)	Greiner (Sigma-Aldrich)
Luciferase assay system	ONE-Glo (Promega)	Bright-Glo (Promega)
Luciferase reagent volume	20 μL	10–30 μL
Transfection reagent type	RNAimax (Life Technologies)	DharmaFECT (Dharmacon)
Transfection reagent volume	0.03 μL	0.02–0.15 μL
Luciferase assay incubation time	20 min	45 min
Culture media aspiration step	Incude	Exclude

2. Prepare 2× MEF media. This 2× MEF media will be diluted in each sample by reduced-serum media in the transfection step.
3. Thaw the 30 siRNA screening plates per batch, centrifuge for 1 min at 1000 × *g*, wipe down with RNAse reducing solution in a Biological Safety Cabinet, peel off cover, and stack screening plates.
4. Dilute *Dnmt1* siRNA in 5× siRNA Buffer to distribute 2 μL solution containing 1 pmol siRNA to the 16 wells of the positive control column.
5. Trypsinize MEFs thawed onto 15 cm^2 plates 24 h prior by washing in PBS then treating for 5 min with 2.5 mL of Trypsin-EDTA 0.25% in a humidified 37 °C incubator. Resuspend trypsinized cells in 18 mL of MEF media and pool into 50 mL tissue-grade conical tubes. Centrifuge the cells at

300 × *g* for 5 min, then carefully aspirate off supernatant, and store cell pellet on ice.

6. Dilute a stock of 5-aza-2′-dC from 100 mM in DMSO to 100 μM in 2× MEF media.
7. Mix a transfection solution of Opti-MEM reduced serum media at room temperature and Lipofectamine RNAimax transfection mix in a ratio of 20 μL of Opti-MEM to 0.03 μL RNAimax per each well, again accounting for 20% extra solution. Incubate the transfection solution for 20 min prior to adding to siRNA.
8. Using a Multidrop, distribute 20 μL of transfection mix to each well of the plates including positive controls and the no siRNA column. Ensure transfection mix incubates with siRNAs from 30 min to 1 h prior to addition of cells.
9. Assuming 20 μL of cell suspension is administered to each well, depending on the number of 384 plates being screened, transfer the appropriate volume of 2× MEF media warmed to 37 °C into the 500 mL tissue culture bottle with stir bar. Resuspend the cell pellet with 10 mL of this 2× MEF media and transfer to the 500 mL bottle, carefully triturating to homogenize the cells and agitating on a stir plate. Dilute 5-aza-2′-dC to 0.04 μM in this cell solution. Deliver 20 μL of the cell and 5-aza-2′-dC solution to the transfection mix while maintaining gentle stirring to avoid cell clumping (*see* **Note 7**).
10. Incubate cells in siRNA knockdown mixture with 5-aza-2′-dC for 72 h in a humidified 37 °C incubator at 5% CO_2.
11. In batches of 14 plates, prior to measuring luciferase activity, aspirate off 20 μL of media using a well washer (*see* **Note 8**).
12. Using a Multidrop distribute 20 μL of One-Glo luciferase assay reagent and incubate for 20 min.
13. Measure luciferase activity with microplate reader. Perform visual inspection of plate heat map for presence and absence of high luciferase signal in positive and negative control wells, respectively. Take note of any plates that have a higher density of high luciferase values as they make represent technical errors.

3.6 Chemical Screening of Xi-Reporter MEFs

1. Analogously to the siRNA screening protocol, 1 day prior to treatments, thaw approximately two vials of cells in 4 15 cm^2 plates, or enough cells to distribute 2000 cells to each 384-well 24 h later, accounting for 20% extra.
2. Prepare 50 μL of cell suspension with 2000 cells per well in 1× MEF media with 0.2 μM 5-aza-2′-dC, accounting for 20% extra. As before, prepare cell suspension in a 500 mL bottle with magnetic stir bar while distributing 50 μL of solution per well by Multidrop.

3. Prepare a positive control mixture for a row of wells on each plate by mixing 50 μL of cell suspension with 2000 cells per well in 1× MEF media with high concentration 5-aza-2′-dC (10.0 μM). Distribute 50 μL of positive control mixture by multichannel pipette.
4. Add 0.5 μL of screening chemical in DMSO by liquid handling system to rows excluding the positive control row.
5. Incubate cells in chemical treatment mixture with 5-aza-2′-dC for 72 h in a humidified 37 °C incubator at 5% CO_2.
6. As with siRNA screening approach, measure luciferase activity with microplate reader with visual inspection of plate signal heat map.

3.7 Data Analysis

3.7.1 Data Normalization

High-throughput screening with siRNAs, as opposed to chemicals, is subject to unique sources of variability partly due the biology of siRNA with off-target effects and partly due to the required addition of a transfection step [17]. Systematic error can be seen within a batch as incubation times vary across plates or with regard to plate layout as siRNA screens are more susceptible to edge effects [17]. The first level of quality assurance should be in real-time with visualization of plate luciferase values as they are collected. Many plate readers, such as the FlexStation microplate reader, display a heat map with real-time data collection, which facilitates identification of errors so that plates can be tagged for closer analysis later and so that technical errors can be immediately corrected. Data normalization can be achieved by comparing the sample to positive or negative controls or by comparing to the individual plate distribution as part of the *z*-score (*see* **Note 9**) [17]. *See* Birmingham et al. [17] for an in-depth discussion of data normalization strategies.

3.7.2 Hit Selection

The identification of hits from siRNA library screens is complicated by a high rate of false positives [17]. The main feature that enhances selection of biologically active hits from siRNA screens is the redundancy included in the siRNA library design [17]. For instance, the genome-wide mouse Silencer siRNA library includes three unique siRNAs against each gene. We increased redundancy by screening this library in duplicate because of the mediocre *Z*-factor of our optimized assay. We pooled data such that six data points represented each gene. Then we applied the Redundant siRNA Activity (RSA) analysis which ranks all siRNAs by normalized value (robust *z*-score in this case) then analyzes siRNAs according to their gene targets [18]. Genes are assigned *p*-values on the basis of whether siRNAs against that individual gene cluster higher in the ranking than would be expected by chance [18]. Validation of the method of analysis can be confirmed by identification

of the gene target of the positive control siRNA from the library by RSA analysis. For instance, our top RSA hit with the p value of 3.2×10^{-6} was *Dnmt1* [15].

3.7.3 Hit Validation

The next step in data analysis is to generate a list of top-scoring hits for subsequent validation. For a high quality screen with a Z-factor ≥ 0.5 one may arbitrarily chose a cutoff by considering a technically feasible number of siRNAs to validate. Alternatively, one may hand-annotate gene function or apply gene ontogeny analysis to decide on a cutoff. In the case of the Xi-luciferase screen, the 50 top genes by RSA analysis (with the lowest RSA p-value) included genes with known chromatin functions but also genes that were more likely to represent false positives such as olfactory receptor genes and transmembrane channel subunits. Thus, we chose a cutoff of the top 54 genes but omitted those genes that were unlikely to have a role in Xi chromatin maintenance (*see* **Note 9**). For the validation assays, we recommend ordering resynthesized active siRNAs against the gene hits in order to ensure that the library annotation is accurate. To complete the validation of active siRNAs against gene hits, it is important to rule out a luciferase-specific effect. We retested active siRNAs with Xi-H2B-Citrine reporter MEFs that were synonymous to the Xi-luciferase reporter MEFs but carry a fluorescent reporter in the place of luciferase [12]. In summary, data analysis includes choosing an appropriate method to normalize data, applying statistical techniques to rank genes, determining a cutoff for hits, and validating the hit siRNA sequences in an assay with a different reporter gene readout. For chemical screening, many of these same considerations in data analysis and hit confirmation apply [19]. We performed both chemical and siRNA screening with the Xi-luciferase assay, as described here, and identified a protein target common to an siRNA and a chemical hit [15]. This overlap suggested identification of a biologically relevant pathway and thus we prioritized validation of these hits. We found the two screening approaches to be highly complimentary; the siRNA hit helped identify the intracellular target of the chemical compound and the identification of chemical compound allowed us to translate our observations to a disease model in which the chemical was used as a drug [15]. We believe that redundancy is a major characteristic of screening design that ensures identification of high confidence hits. Redundancy can be accomplished by using a library with many reagents directed against common pathways, by testing those reagents in replicates, and by screening with multiple modalities such as with siRNA and chemical libraries.

4 Notes

1. We found that MEFs derived from four individual Xi-luciferase embryos were sufficient to screen 300 384-well plates.
2. Measuring luciferase activity in addition to checking genotypes is an important control to avoid contaminating Xi-reporter cell stocks with luciferase-expressing littermate cells.
3. In our experience using 2000 cells per 384-well screening assay, we could screen 30 library plates/day by thawing two vials of cells to 4 15 cm^2 plates 24 h prior.
4. The methods describe a screen using empirically determined "optimal variables" as listed in Table 1. With each screen, we recommend optimizing to variables such as these using the *Z*-factor as shown in Fig. 2 to measure assay quality.
5. We recommend using at least half of a 384-well plate per variable to better assess the distribution of signal with each experimental condition. While early phases of optimization can include manually pipetted reagents, later optimization trials should attempt to replicate actual screening conditions including the use of manifold liquid handlers and other automated steps.
6. Whenever making large volumes of solutions to be aliquoted by Multidrop or multichannel pipette, calculate for 20% of extra solution considering losses in manifold tubing or reservoir.
7. Gentle agitation of the cell suspension is an important step for homogenous distribution of healthy cells. Avoid media foaming.
8. We found this step of removing media from adherent MEF cultures to have a large effect on increasing luminescence signal. Removing the supernatant increases the concentration of luciferase in the cell lysate in the next step.
9. An important caveat to using individual plate distribution for normalization is that the screening library has a relatively equal distribution of hits across plates due to random organization of genes in the library. We chose to normalize to a robust *z*-score which takes into account plate median and median absolute deviation and is therefore less sensitive to outliers than *z*-score [17].
10. Hand annotation should be performed with caution because it introduces bias and limits further exploration of unexpected and interesting gene pathways.

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