Journal of Visualized Experiments

# Enforced Activation of Enhancer RNAs in situ Through the dCas9 Synergistic Activation Mediator System

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE61460R2 |
| **Full Title:** | Enforced Activation of Enhancer RNAs in situ Through the dCas9 Synergistic Activation Mediator System |
| **Section/Category:** | JoVE Genetics |
| **Keywords:** | Enhancer RNA, eRNA, Enhancer, CRISPR activation (CRISPRa), dCas9, SAM, synthetic epigenetic activator, Gene regulation, transcriptional activation system. |
| **Corresponding Author:** | Wenbo Li<br><br>UNITED STATES |
| **Corresponding Author's Institution:** | |
| **Corresponding Author E-Mail:** | Wenbo.Li@uth.tmc.edu |
| **Order of Authors:** | Zian Liao |
| | Joo-Hyung Lee |
| | Wenbo Li |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Houston, Texas, USA |

Cover letter

**Wenbo Li, Ph.D.**
**Assistant Professor**
**Biochemistry & Molecular Biology**
**McGovern Medical School**
**6431 Fannin Street, Houston, 77030, TX.**
**Tel: 713-500-6103**
**Email: Wenbo.li@uth.tmc.edu**

Dear Stephanie and Vineeta,

We again thank you for the invitation to submit a paper on generating CRISPR activation of enhancer RNAs for functional studies. We thank you for your efforts in the process of reviewing this paper.

We have fully read the comments from the two Reviewers and the editorial team. We have made all required changes as requested, and included thorough discussion on technical details. I am pleased to submit the fully revised manuscript by Liao and Lee et al. entitled *"**Enforced Activation of Enhancer RNAs in situ Through the dCas9 Synergistic Activation Mediator System**"* (JoVE61460) for your further consideration as an *Article* to *JOVE*.

As you can see this revised manuscript consists of a main text, a rebuttal letter to Reviewer's comments, 6 figures, one supplementary table and one material table. We hope that you will enjoy reading this manuscript, and will find it now fully suitable for publication as an *Article* to *JOVE*.

Thank you for your consideration.

Best regards,

Wenbo Li
Assistant Professor
CPRIT Scholar

**TITLE:**

# Enforced Activation of Enhancer RNAs In Situ Through the dCas9 Synergistic Activation Mediator System

**AUTHORS AND AFFILIATIONS:**

Zian Liao[1,2,*], Joo-Hyung Lee[1,*], Joanna Krakowiak[1], Ruoyu Wang[1,2], Wenbo Li [1,2]

[1]Department of Biochemistry and Molecular Biology, McGovern Medical School, University of Texas Health Science Center, Houston, TX, USA.

[2]Graduate School of Biomedical Sciences, University of Texas MD Anderson Cancer Center and UTHealth, Houston, TX, USA.

*These authors contributed equally.

Corresponding author:

Wenbo Li (Wenbo.Li@uth.tmc.edu)

Email addresses of co-authors:

Zian Liao (Zian.Liao@uth.tmc.edu)

Joo-Hyung Lee (Joo.Hyung.Lee@uth.tmc.edu)

Joanna Krakowiak (Joanna.Krakowiak@uth.tmc.edu)

Ruoyu Wang (Ruoyu.Wang@uth.tmc.edu)

**KEYWORDS:**

enhancer RNA, eRNA, enhancer, CRISPR activation, CRISPRa, dCas9, SAM, synthetic epigenetic activator, gene regulation, transcriptional activation system

**SUMMARY:**

Enhancer RNAs (eRNAs) are non-coding RNAs produced from active enhancers. An optimal approach to study eRNA functions is to manipulate their levels in the native chromatin regions. Here we introduce a robust system for eRNA studies by using CRISPR-dCas9-fused transcriptional activators to induce the expression of eRNAs of interest.

**ABSTRACT:**

Enhancers are pivotal genomic elements scattered through the mammalian genome and dictate tissue-specific gene expression programs. Increasing evidence has shown that enhancers not only provide DNA binding motifs for transcription factors (TFs) but also generate non-coding RNAs that are referred to as eRNAs. Studies have demonstrated that eRNA transcripts can play significant roles in gene regulation in both physiology and disease. Commonly used methods to investigate the function of eRNAs are constrained to “loss-of-function” approaches by knockdown of eRNAs, or by chemical inhibition of the enhancer transcription. There has not been a robust method to conduct “gain-of-function” studies of eRNAs to mimic specific disease conditions such as human cancer, where eRNAs are often over-expressed. Here, we introduce a method for precisely and robustly activating eRNAs for functional interrogation of their roles by

applying the dCas9 mediated Synergistic Activation Mediators (SAM) system. We present the entire workflow of eRNA activation, from the selection of eRNAs, the design of gRNAs to the validation of eRNA activation by RT-qPCR. This method represents a unique approach to study the roles of a particular eRNA in gene regulation and disease development. In addition, this system can be employed for unbiased CRISPR screening to identify phenotype-driving eRNA targets in the context of a specific disease.

**INTRODUCTION:**

The human genome contains a constellation of regulatory elements[1-3]. Among these, enhancers emerge to be one of the most critical categories[4-6]. Enhancers play essential roles in regulating development, and are responsible for generating spatial-temporal gene expression programs to determine cell identity[5-7]. Conventionally, enhancers are only considered to be DNA elements that provide binding motifs for transcription factors (TFs), which then control target gene expression[6,8]. However, a series of studies found that many active enhancers also transcribe non-coding enhancer RNAs (i.e., eRNAs)[4,9,10].

The level of eRNA transcription was found to correlate with the activity of an enhancer[4,10]. Active enhancers produce more eRNA transcripts and show higher levels of epigenome markers associated with active transcription, such as H3K27ac and H3K4me1[9,11,12]. Some studies have demonstrated that eRNA transcripts can play important roles in transcriptional activation of target genes[10,12]. A large number of eRNAs were identified to be deregulated in human cancers[13-16], many of which exhibited high cancer type specificity and clinical relevance. These findings bring opportunities that the elucidation of eRNAs that can drive/promote tumorigenesis may offer novel targets for therapeutic intervention[13,15].

Current methods to study eRNA functions are almost exclusively based on knockdown strategies that used small interference RNAs (siRNA), short hairpin RNAs (shRNAs), or antisense oligonucleotides (ASOs, of which locked nucleic acids (LNAs) are the commonly used type in research)[10,12,17]. However, human diseases such as cancer predominantly show over-expression of eRNAs as compared to their adjacent normal tissue[15], demanding tools to "overexpress" eRNAs to mimic their disease-relevant expression patterns for functional studies. To achieve this, a plasmid-based ectopic overexpression system is not optimal because the exact transcription start and termination sites of eRNAs remain largely unclear. In addition, a plasmid expression system may alter the location of eRNAs, causing potential artifacts of their functions[18]. Here we provide a detailed protocol to facilitate the functional characterization of eRNAs by enforcing their "overexpression" in the native genomic locus of their production (i.e., in situ), which is based on the CRISPR/dCas9-Synergistic Activation Mediators System (SAM).

The SAM system was initially developed for activating coding genes and long intergenic non-coding RNAs (lincRNAs) associated with BRAF inhibitor resistance in melanoma cells[19]. Unlike other CRISPR activation (CRISPRa) technologies, the SAM system consists of a combination of transcription activators to confer robust transcriptional activation of target regions. These activators include: an enzymatically dead Cas9 (dCas9) fused with VP64 (i.e., dCas9-VP64); a

guide RNA containing two MS2 RNA aptamers, and an MS2-p65-HSF1 fusion activator protein. The presence of the MS2 aptamers in the gRNA can recruit the MS2-p65-HSF1 fusion protein to the vicinity of dCas9/gRNA binding sites. Among these, VP64 is an engineered tetramer of the herpes simplex VP16 transcriptional activator domain, which has been shown to strongly activate gene transcription by recruiting general transcription factors[20-22]. The MS2-p65-HSF1 fusion protein consists of three parts. The first part, the MS2-N55K, is a mutant form of MS2 binding protein that has a stronger affinity[23]; the other two parts of this fusion protein are the transactivation domain of p65 and heat shock factor 1 (HSF1), both of which are transcription factors that possess strong transactivation domains and can induce robust transcription programs[24,25]. Therefore, the SAM system essentially created a highly potent activator complex to activate transcription of designated coding genes and lincRNAs[19].

**PROTOCOL:**

The entire workflow of this protocol is shown in **Figure 1.**

**1. Enhancer RNA (eRNA) selection**

1.1. Identify a putative enhancer region of interests by using binding peaks of chromatin immunoprecipitation sequencing (ChIP-Seq) data, i.e., of histone modifications (e.g., H3K4me1 and H3K27ac), or of transcription coactivators (e.g., p300).

1.2. Identify the eRNA of interest by intersecting the ChIP-Seq peak with RNA-seq signals (e.g., from total RNA-seq or from nascent RNA-seq such as Global Run-On Sequencing (GRO-Seq).

NOTE: The region chosen for designing gRNAs should be usually limited to the "enhancer core" region, where TFs or coactivators (e.g., p300) showed clear ChIP-seq peaks (**Figure 2A**). The dCas9 and its fusion coactivators will be recruited by a gRNA to this region to mimic the native binding of coactivators (**Figure 2A**). If specific datasets such as Cap Analysis of Gene Expression (CAGE) [4] or GRO-cap[26] are available, they can be used to precisely determine the "enhancer core", the region between two transcription start sites of the eRNAs transcribed to opposite directions[4,10,26].

**2. gRNA design**

2.1. Use common CRISPR gRNA design tools such as CRISPOR[27] to select gRNAs with low potential off targeting (http://crispor.tefor.net/).

NOTE: Other tools like Benchling[28], or CHOPCHOP[29] can also be used as additional options for gRNA design.

2.2. Paste the enhancer core DNA sequence into the **Step 1** column in the CRISPOR website, then click the dropdown button to choose the corresponding genome (e.g., human) in the **Step 2** column. Click the dropdown button to set the Protospacer Adjacent Motif (PAM) sequence as "NGG" in the **Step 3** column and then click "**Submit**" button to generate guide sequences with a length of 20 bp.

2.3. Choose guides with highest specificity scores in CRISPOR tool, i.e., low off-target potential, then add “CACCG” to the 5’ end, and “C” to the 3’ end, respectively.

NOTE: Select the guides with highest specificity scores (>85 in CRISPOR is preferred).

2.4. Order oligonucleotides for each sense and antisense sequence from commercial sources.

NOTE**:** Additional instructions on CRISPR/Cas9 gRNA design can be found in other studies[30,31]. The overhangs in step 2.2 will make the gRNA compatible with the SAM gRNA backbone (Addgene #61427), which uses the BsmBI restriction enzyme.

**3. Clone gRNAs into a lentiviral construct**

3.1. To anneal oligos mix 1 µL of each paired oligo at 100 µM, 1 µL of 10x T4 ligation buffer, 0.5 µL of T4 DNA Ligase (400,000 units/mL) and 6.5 µL of $H_2O$ to reach a total volume of 10 µL. Incubate at 37 °C for 30 min, then 95 °C for 5 min, and ramp down to 25 °C at 5 °C/min. Dilute to 100 µL using $H_2O$.

3.2. Digest the gRNA backbone by mixing 2 µL of 10x restriction enzyme (RE) Buffer, 300 ng of lenti_gRNA(MS2)_zeo backbone plasmid (Addgene #61427) in 1 µL, 1 µL of BsmBI enzyme, and 16 µL of $H_2O$ to reach a total volume of 20 µL. Incubate at 55 °C for 15 min.

3.3. Mix ligation components with 20 µL digestion product by adding 2.5 µL of 10x T4 ligation buffer, 1 µL of diluted annealing product and 1.5 µL of T4 DNA ligase (400,000 units/mL) into a 25 µL system. Incubate at room temperatures for 30 min.

3.4. Transform 2 µL of the ligation mix from step 3.3 into Stbl3 competent *E.coli* cells. Plate them on an ampicillin LB-agar plate and incubate overnight at 37 °C.

3.5. Pick and inoculate a single bacteria colony and extract plasmid. Send it for Sanger sequencing to confirm that the gRNA sequence is correctly inserted.

NOTE: Sequences for the primers and gRNAs are available in **Supplementary Table 1.** Stbl3 chemically competent *E.coli* cells are recommended to be used here because they have a higher plasmid DNA yield and higher plasmid stability when generating instability-prone lentivirus plasmids[32].

**4. gRNA efficiency test**

NOTE: Although it may not be necessary for every gRNA, it is recommended that researchers examine the quality of gRNA by performing Surveyor assay (i.e., mismatch cleavage assay) to detect indels or mutations that can only be efficiently generated by good quality gRNAs[33,34]. Other methods such as Tracking of Indels by Decomposition (TIDE) can also be used to determine

gRNA efficiency[30,35]. Surveyor nuclease is a member of a family of mismatch-specific endonucleases that can cut double-strand DNA with mismatches (**Figure 3A**). The quality of gRNAs can be revealed by the efficacy of producing smaller DNA species. Practically, surveyor cutting efficacy can also be affected by the transfection efficiency of gRNAs and Cas9.

4.1. Transfect gRNA together with the pSpCas9(BB)-2A-Puro (Addgene #62988) plasmid that expresses the Cas9 protein into 293T cells using a lipid-based transfection reagent. Use 1.2 µg/mL for each plasmid per well in a 6 well plate. Continue to culture the cells for 3 days after transfection. Harvest and extract genomic DNA according to the manufacturer's protocol[36].

4.2. Use polymerase chain reaction (PCR) to amplify the targeted enhancer region from genomic DNA. Use PCR conditions shown in **Supplementary Table 2**. Use primers in **Supplementary Table 1** for an example enhancer, NET1e. Denature the PCR products by incubating at 95 °C for 10 min, and re-hybridize them by ramping down from 95 °C to 25 °C at the speed of -0.3 °C/s to allow the single-strand DNA (ssDNA) with and without gRNA-induced indels or mutations to anneal to each other.

4.3. Digest the hybridized DNA by the Surveyor nuclease (**Figure 3A**) following the manufacturer's protocol[37]. Mix 400 ng of hybridized DNA from step 4.2, 1 µL of Surveyor nuclease, 1 µL of Surveyor enhancer and 5 µL of 0.15 M $MgCl_2$ in a 50 µL system. Incubate at 42 °C for 60 min. Analyze the DNA on an agarose gel. Use negative control, such as cells with Cas9 only but without targeting gRNAs, in the assay and gel electrophoresis (**Figure 3B**).

**5. Lentivirus generation**

5.1. Add psPAX2, pMD2.G, and a target plasmid (e.g., lenti_dCas9-VP64_Blast, Addgene #61425; or lenti_MS2-p65-HSF1_Hygro, Addgene #61426) at the ratio of 3 µg : 1 µg : 4 µg in a 1.5 mL polypropylene tube. Mix them with 500 µL of Opti-MEM and incubate for 5 min at room temperature.

5.2. In a separate tube, put 10 µL of the lipid-based transfection reagent in 500 µL of Opti-MEM, and incubate for 5 min at room temperature.

5.3. Combine the products from steps 5.1 and 5.2 and incubate for 20 min at room temperature.

5.4. Plate 293T cells one day before the transfection and let them reach a confluence of ~30% in a 10 cm dish at the time of transfection. Add 4 mL of regular medium (DMEM with 10% FBS), then add the complex from the step 5.3 dropwise to cells. Add DMEM with 10% FBS to make the final volume up to 6 mL. Incubate overnight in a cell incubator at 37 °C with 5% $CO_2$.

5.5. The next day, change the medium to 10 mL of new DMEM with 10% FBS. Harvest the medium 24 h after the medium change. Use a syringe filter (e.g., 0.45 µm) to filter the virus-containing medium and then proceed to step 6 or store the virus in -80 °C.

NOTE: Lentivirus operations require a BioSafety Level II cabinet. Caution needs to be taken to safely handle the virus-associated experiments; and if any container has direct contact with the viral medium, it needs to be bleached for more than 20 min before disposal as biohazard.

**6. Cell culture**

6.1. Maintain cells in a $CO_2$ cell culture incubator at 37 °C with 5% $CO_2$.

6.2. Culture MCF7 and 293T cells in Dulbecco's Modified Eagle Medium (DMEM) medium with 10% FBS.

6.3. Grow cells in 10 cm dishes and split at a 1:3 to 1:5 ratio when confluent.

**7. Cell infection and selection**

7.1. Seed the target cells (e.g., MCF7) directly in viral medium mixture containing 0.5 mL of lenti_dCas9-VP64_Blast (Addgene #61425) and 0.5 mL lenti_MS2-p65-HSF1_Hygro (Addgene #61426). Add 8 μg/mL Hexadimethrine bromide to increase the efficiency of infection. Use a well of uninfected cells as a negative control for examining the efficacy of antibiotic selection.

NOTE: The amount of viral medium is recommended as follows: 6 mL viral medium for 10 cm dish, 1 mL for a well of a 6 well plate, and 0.5 mL for a well of a 12 well plate.

7.2. At 24 h post-infection, add to the cells fresh medium containing Blasticidin (5 μg/mL for MCF7 cells) and Hygromycin (200 μg/mL for MCF7 cells).

7.3. Keep cells in antibiotic selection medium until the negative control cells die out.

NOTE: Time may vary for different cell lines to become stable. For MCF7, it usually takes 5-7 days for non-infected cells to completely die out. A killing curve using a range of antibiotic concentrations should be tested for a new cell line prior to the experiments. It is acceptable to use a cell mixture for the next steps, but an alternative is to pick single-cell colonies that are homogeneous in terms of expression of the two effector proteins. The stable line obtained is referred to as the SAM-effector parental line (e.g., MCF7 SAM-effector line) in this paper. It is recommended that a shared SAM-effector parental cell line be used for infection by different targeting or non-targeting gRNAs, especially if their effects will be compared.

7.4. Use western blotting to determine whether the cells stably express the two effector proteins (an example is shown in **Figure 4A**). Use reverse transcription of total RNAs followed by quantitative PCR (RT-qPCR) as an alternative method to examine the expression levels of the two mRNAs (dCas9-VP64 and MS2-p65-HSF1, **Figure 4B**).

NOTE: Primers for examining the mRNA levels of the two dCas9 effectors are available in **Supplementary Table 1**.

7.5. Generate lentivirus of gRNAs constructed in step 3.5 and infect the stable SAM cell line dually expressing dCas9-VP64 and MS2-p65-HSF1 with individual gRNA lentivirus. Add Zeocin (100 µg/mL for MCF7 cells) to the medium 24 h post gRNA viral transduction. Generate a negative control that expresses non-targeting gRNA (NT-gRNA) in the parental SAM cell line.

NOTE: Ensure that the selection drug is specific for the construct of interest.

**8. RNA extraction and quantitative RT-PCR to examine eRNA levels**

8.1. Extract total RNAs from SAM cell lines expressing either NT-gRNA or targeting gRNAs using an RNA extraction kit[38], or other phenol chloroform based method. Use cells of ~80% confluency in one well of a six-well plate for RNA extraction.

NOTE: No significant difference was observed in our practice for eRNA detection when RNAs are extracted either by commercial binding columns or by phenol chloroform reagents.

8.2. Make complementary DNA (cDNA) from the purified RNA by reverse transcription reaction with random hexamer, following the manufacturer's protocol[39]. Use conditions in **Supplementary Table 2**.

NOTE: Because the majority of eRNAs are non-polyadenylated[1,2,4,9,10], random hexamer is routinely used for cDNA generation.

8.3. Design primers for RT-qPCR measuring target eRNAs using a reputable primer designing tool (e.g., Primer 3). Test the amplification linearity of the primer pairs by examining if the primers will linearly amplify serial diluted cDNAs and show expected qPCR cycle differences. Use conditions in **Supplementary Table 2**.

NOTE: The primers for RT-qPCR should target the highly transcribed region in the nascent RNA-seq, and the linearity test of primers should include a broad range of cDNA dilutions to ensure that all possibly encounterable eRNA levels are tested. An example of linearity test is shown in **Figure 5A**.

8.4. Perform RT-qPCR and analyze the eRNA expression levels in control cells (SAM cell line with NT-gRNA) and in SAM cell line with eRNA-targeting gRNAs (e.g., NET1e gRNA#1) (e.g., **Figure 6A**). Primers for *NET1e* RNA are shown in **Supplementary Table 1**. Use conditions in **Supplementary Table 2.**

**9. dCas9 ChIP and qPCR**

NOTE: This step is an optional experiment to validate the binding of dCas9/SAM-gRNA complex to the target enhancer by the specific gRNAs. While it is encouraged that users perform this step, it is not necessary to test every single gRNA. Refer to an example shown in **Figure 5B.** Refer to

primers listed in **Supplementary Table 1**.

9.1. Cross-link cells

9.1.1. Remove the medium from cells and add 1% formaldehyde dissolved in phosphate buffered saline (PBS). Leave for 10 min.

9.1.2. Add 2.5 M glycine at 1:20 volume to quench the cross-linking and wash cells twice with ice-cold PBS. Add 700 µL of ice-cold PBS and scrape the cells to a 1.5 mL tube.

9.1.3. Centrifuge at 2,000 *x g* for 5 mins at 4 °C. Proceed to step 9.2, or snap freeze and store at -80 °C.

9.2. Sonicate

9.2.1. Make fresh LB1, LB2 and LB3 buffers. LB1: 50 mM Hepes-KOH (pH 7.5), 140 mM NaCl, 1 mM EDTA, 10% glycerol, 0.5% NP-40, 0.25% Triton-X 100 and 1x Protease inhibitor. LB2: 10 mM Tris-HCl (pH 8.0), 200 mM NaCl, 1 mM EDTA, 0.5 mM EGTA and 1x Protease inhibitor. LB3: 10 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine and 1x Protease inhibitor. Supplement 1x Protease inhibitor freshly to the buffer before the experiment.

9.2.2. Add 1 mL of buffer LB1 to cell pellets, pipette well, rotate at 4 °C for 10 min, and spin at 2,000 *x g* for 5 mins at 4 °C. Pour off the supernatant, add 1 mL of LB2 buffer, rotate at 4 °C for 10 min, and spin at 2,000 *x g* for 5 min at 4 °C. Pour off the supernatant and remove the excess of the LB2 buffer. Add 300 µL of buffer LB3.

9.2.3. Sonicate and fragment the chromatin DNA to an average size of ~200-400 bp using a proper sonicator system. After the sonication, add 30 µL of 10% Triton-X 100 and mix well. Test the proper sonication time if a new cell line is used.

9.2.4. Centrifuge at 14,000 *x g* to remove the pellet. Transfer the supernatant to the new tube and add 630 µL of LB3 and 70 µL of Triton X-100 to a total volume of 1 mL. Add 2 µg of the Cas9 antibody to the supernatant and rotate at 4 °C overnight.

9.3. Immuno-complex capture and reverse cross-linking

9.3.1. Prepare RIPA buffer (50 mM HEPES pH 7.6, 1 mM EDTA, 0.7% Na-deoxycholate, 1% NP-40, 0.5 M LiCl) and elution buffer (1% SDS and 0.1 M $NaHCO_3$).

9.3.2. Wash Protein G with 1% BSA in PBS 3x and LB3 buffer once.

9.3.3. Add 30 µL of Protein G beads to the sample and incubate at 4 °C for 4 h.

9.3.4. Wash the beads-immuno-complex in 500 µL of RIPA buffer 6x. Do not let beads dry out.

9.3.5. Wash the beads-immuno-complex once with TE buffer; remove the buffer.

9.3.6. Add 200 µL of elution buffer to the beads-immuno-complex and vortex; then put it in a temperature-adjustable heated shaker set to 65 °C with 600 rpm shaking for 6-16 h to reverse crosslink.

9.4. DNA extraction

9.4.1. Remove tubes from the shaker, briefly spin down the beads and put them on a magnetic stand. Transfer 200 µL of the supernatant to a fresh tube and add 200 µL of TE buffer.

9.4.2. Add 1 µL of RNase A (1 mg/mL) to the tube and incubate at 37 °C for 1 h.

9.4.3. After 1 h of incubation, add 2 µL of Proteinase K (20 mg/mL) to the sample and incubate it at 65 °C for 2 h.

9.4.4. Centrifuge briefly and add 400 µL of phenol-chloroform-isoamyl alcohol mixture. Mix well, then centrifuge for 5 min at 10,000 *x g.*

9.4.5. Move 400 µL of the upper layer to a 1.7 mL tube containing 16 µL of 5 M NaCl and 1 µL glycogen (20 µg/µL) and mix well.

9.4.6. Add 800 µL of 100% ethanol and leave overnight at -20 °C.

9.4.7. Next morning, centrifuge the tubes at 4 °C at 14,000 *x g* for 15 min.

9.4.8. Remove the supernatant and wash the pellet with 1 mL of 70% ethanol. Spin down at 14,000 *x g* for 10 min.

9.4.9. Remove ethanol, air dry pellet, and resuspend in 50 µL of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA).

9.5. Perform ChIP-qPCR to examine the recruitment of the SAM complex to the targeting site by a pair of enhancer core-targeting primer. Use a primer pair targeting an irrelevant region as a negative control.

**10. Cell growth assay and other functional tests of eRNA over-activation**

10.1. Trypsinize SAM cell lines expressing the non-targeting gRNA or eRNA-targeting gRNAs and plate ~3,000 cells per well in a 96 well plate.

10.2. Measure cell growth by using a live-cell imager or other methods (e.g., cell counting, or

water-soluble tetrazolium salt-1 assay).

10.3. Use the half-maximal inhibitory concentration (IC50) to test the cellular responses to specific cancer drugs in cells with or without *NET1e* over-expression by SAM [15].

NOTE: Results of cell growth assays and drug sensitivity tests of breast cancer cells are presented after overexpression of an eRNA transcribed adjacent to *NET1* gene, which was referred to as *NET1e*[15]. Other assays can be conducted at the cellular or organismal levels based on the need of each specific project.

**REPRESENTATIVE RESULTS:**

**Figure 1** illustrates the overall workflow of this protocol. Our focus was on a representative eRNA, *NET1e*[15], which is over-expressed in breast cancer, for which SAM system was used to activate and study it's biological role in regulating gene expression, cell proliferation and cancer drug response. For this *NET1* enhancer, several p300 ChIP-Seq peaks, flanked by transcribed eRNA transcripts (**Figure 2A,B**) were marked. Based on strong GRO-Seq signals, one of the p300 ChIP-Seq peaks was selected as the enhancer core (where bi-direction eRNA transcripts originate, **Figure 2B**). *NET1* enhancer region was also marked by dense ChIP-Seq peaks of H3K4me1 and H3K27ac. Multiple gRNAs were designed within this enhancer core p300 peak, with two of the gRNAs denoted by arrows in the inlet (**Figure 2B**). A chromatin loop between *NET1* enhancer core and *NET1* promoter can be detected in the data of Chromatin Interaction Analysis by Paired-End Tag Sequencing (ChIA-PET, black arch, **Figure 2B**), suggesting the direct regulatory relationship between the *NET1e* and the *NET1* gene.

The quality of designed gRNA can be tested by two different methods i.e., Surveyor assay (**Figure 3**) or ChIP-qPCR (**Figure 5**)**.** In **Figure 3A**, a schematic is used to illustrate the procedure of surveyor assay used to examine the quality of gRNAs. In surveyor assay, a PCR product spanning the gRNA recognition site was amplified from 60 ng genomic DNA, which was subjected to denaturing and rehybridization, and subsequently surveyor cleavage. In the control cells transfected with Cas9 plasmid only (pSpCas9(BB)-2A-Puro, Addgene #62988), only one band of 1,205 bp (indicated by the blue asterisk, Lane 1 in **Figure 3B**) was detected. By contrast, the assay using genomic DNAs from cells transfected with *NET1e* gRNA#1 together with Cas9 generated two cleavage products of 777 bp and 428 bp (denoted by orange asterisks), respectively (lane 2, **Figure 3B**). Similarly, shown in lane 3, the co-transfection of *NET1e* gRNA#2 with Cas9 caused Surveyor digestion of the 1,205 bp PCR product into two digested products of 905 bp and 300 bp (marked by red asterisks), respectively (**Figure 3B**). The sizes of these cleavage products matched the expected sizes of digestion due to the in-dels or mutations generated by the gRNAs, indicating that gRNAs and Cas9 can cut our targeted enhancer DNA in cells.

MCF7 parental SAM cell line was successfully generated, which expresses the two effector proteins as shown both by western blotting (**Figure 4A**) and RT-qPCR (**Figure 4B**). After infecting this parental line with NT-gRNAs or specific gRNAs targeting *NET1* enhancer, ChIP-qPCR was conducted to test if the gRNA can recruit dCas9-VP64 (one of the effectors) to our target site. Prior to ChIP-qPCR, the amplification linearity of ChIP-qPCR primers needs to be tested by a PCR-

cycle versus diluted-cDNA/ChIP-DNA standard curve**,** as shown in **Figure 5A**. For each pre-diluted standard shown in the x axis of **Figure 5A**, four folds of serial dilution were conducted to generate them from sonicated genomic DNAs. A standard curve was plotted between delta cycle threshold (ΔCT) values (y axis) against the relative quantity ($log_2$) of each diluted standard to the initial amount of genomic DNA (**Figure 5A**). This specific primer set conferred an R square of 0.994, and we generally recommend primer sets with an R square higher than 0.95. We then conducted ChIP by using a ChIP-quality antibody targeting Cas9 and found that the dCas9-VP64 protein was recruited by our *NET1e* gRNA to the target *NET1* enhancer region, but not to another irrelevant genomic region (**Figure 5B**).

RT-qPCR was used to examine the expression levels of the targeted NET1e RNA after SAM activation by two different gRNAs in MCF7 SAM cells. **Figure 6A** shows that in cells with engineered SAM system targeting *NET1* enhancer, >30-fold of *NET1e* upregulation was successfully achieved with two different gRNAs. Because *NET1e* was overexpressed in breast tumors than adjacent normal tissue based on our analysis of the Cancer Genome Atlas (TCGA) datasets[15], and because its overexpression correlated with poor survival and altered response to a set of cancer drugs[15], we tested if the enforced overexpression of *NET1e* by SAM can directly alter cell proliferation or cellular response to cancer drugs. We conducted cell growth assay and measured cell confluence by a live cell imager. The confluences were measured every 6 h and normalized to that at 0 h, which showed that SAM-enforced overexpression of *NET1e* accelerated cell growth (**Figure 6B**). We selected the BEZ235 and the Obatoclax to examine cell response to cancer drugs because *NET1e* expression is positively correlated with IC50 of these two drugs based on the analysis from Cancer Therapeutics Response Portal (CTRP) and Genomics of Drug Sensitivity in Cancer (GDSC). The result suggested that *NET1e* overexpression directly conferred resistance to specific cancer drugs (**Figure 6C**)**.**

**FIGURE AND TABLE LEGENDS:**

**Figure 1: A work-flow chart demonstrating procedures used to generate the SAM system for enforced activation of eRNAs of interest.**

**Figure 2: Epigenetic features of *NET1 enhancer* and its neighborhood in MCF7 cells.** (**A**) A diagram showing the structure of the "Enhancer core" to denote the DNA region bound by transcription factors and cofactors, which is used for gRNA design to anchor SAM system in step 2 of the protocol. (**B**) A snapshot of genome browser tracks of ChIP-Seq, GRO-Seq, and ChIA-PET of indicated factors at *NET1* and *NET1e* loci in MCF7 cells. GRO-Seq is stranded (Red: Watson strand, Orange: Crick strand). The zoom-in inlet to the right demonstrates the specific "enhancer core" used for designing two gRNAs (red lines and pointed by arrows). ChIA-PET track indicates a potential regulatory relationship between *NET1e* and *NET1* gene, with the black and grey arch denoting chromatin loops. Figure 2B is modified from Zhang, Z. et al.[15]. The MCF7 ChIA-PET dataset is from ENCODE data portal[1].

**Figure 3: Surveyor nuclease digestion assay to test gRNA quality.** (**A**) The workflow of the Surveyor assay. The red dot on the DNA indicates in-dels or mutations generated by CRISPR/Cas9

to the target region. The forward primer was designed to be ~300-400 bp from the cutting site and the reverse primer ~800-900 bp from the cutting site, allowing easy discerning of their cutting in gel electrophoresis. (**B**) An agarose gel picture of Surveyor digestion assay. Blue asterisk indicates uncleaved DNA from PCR, while orange and red asterisks indicated cleaved products by Surveyor.

**Figure 4: Examination of dCas9-VP64 and MS2-p65-HSF1 expression in stable MCF7 SAM cell line.** (**A**) Western blots with a Cas9 antibody in parental MCF7 cells (MCF7 WT), and in the MCF7 cell line expressing dCas9-VP64 and MS2-p65-HSF1 proteins (MCF7 SAM). GAPDH was used as a loading control. (**B**) RT-qPCR results show the mRNA levels of dCas9-VP64 and MS2-p65-HSF1 in MCF7 WT and MCF7 SAM, respectively. The values were normalized to GAPDH. Error bars represent mean ± SD; N=3. P value: Student's t-test.

**Figure 5: ChIP-qPCR to validate dCas9-VP64 recruitment to *NET1* enhancer.** (**A**) Amplification linearity test of the primer set used for the *NET1 enhancer* ChIP-qPCR. (**B**) ChIP-qPCR based on Cas9 antibody using primers specific for the *NET1* enhancer region, and an irrelevant genomic region as a negative control (hg19, chr14:35,025,431-35,025,595). Error bars represent mean ± SD; N=3. P value: Student's t-test.

**Figure 6**: ***NET1e* expression level, cell growth assay, and responses to anti-cancer drugs in MCF7 *NET1e*-SAM cells**. (**A**) RT-qPCR result showing the expression levels of *NET1e,* or its neighboring gene*, NET1,* in the SAM cell lines expressing non-targeting gRNA (NT-gRNA), *NET1e* gRNA#1 o *NET1e* gRNA#2. The values are normalized to GAPDH. Error bars represent mean ± SD; N=3. P value: Student's t-test. (**B**) Normalized cell confluence of MCF7 SAM cells expressing gRNAs as indicated. (**C**) The IC50 of two drugs (Obatoclax and BEZ235) in SAM cell lines expressing *NET1e* gRNA#1 or NT-gRNA. The relative cell amount was measured with live cell imager. Error bars represent mean ± SD. Student's t-test was used to calculate the p values. This figure is modified from Zhang et al[15].

**Supplementary Table 1: List of primer sequences for gRNAs, Surveyor assay, RT-qPCR, and ChIP qPCR.**

**Supplementary Table 2: PCR and RT-qPCR experimental setup and reaction conditions.**

**DISCUSSION:**

Based on our data, we conclude that the SAM system is suitable for studying the role of eRNAs in regulating cellular phenotypes, e.g., cell growth or drug resistance. However, careful gRNA designing is required for robust eRNA activation, due to the following reasons. First of all, the transcription start site (TSS) of eRNA in each specific cell lines/types remains less clearly annotated. Due to this, epigenomic information (e.g., ChIP-Seq of H3K27ac, of transcription factors, or of p300), transcriptional activity depicted by GRO-Seq (or additional CAGE[4] or GRO-cap[26] datasets if available) need to be utilized to deduce the transcription starting sites so that gRNAs can be designed in a way to avoid the potential impediment of normal enhancer transcription by the dCas9/SAM complex. Second, gRNAs provided by current design tools may

show differential eRNA activation potency in SAM systems, the basis of which remains mechanistically unclear. It can be due to different binding dynamics of distinct gRNA sequences[30], or due to the relative location of the gRNA/dCas9 binding site versus the other critical regulatory DNA elements near the enhancer core region. To ensure robust eRNA activation and to reduce off-targeting, we recommend readers to design multiple gRNAs (n>3) and test their actual activation efficacy by RT-qPCR. If a consistent result is generated by multiple gRNAs, it is considered conclusive. Furthermore, the process of generating stable lines also varies based on the choice of cell lines, which may require different selection time, length, and antibiotic concentrations. Finally, we found it important to ensure the quality of primer sets for examining eRNAs in RT-qPCR, as their abundance is sometimes one or two magnitudes lower than common mRNAs. The primers' performance in qPCR and their linear range of amplification should be carefully examined. It is also important and necessary to remove the trace of genomic DNAs in RNA samples when the levels of eRNAs are examined, because these DNAs may confound RT-qPCR results.

We largely followed the original method developed by the group of Feng Zhang et al.[19]. The modifications here is to target the dCas9-SAM complex to enhancers and to activate eRNAs instead of lincRNAs or mRNAs.

Here we introduce a highly effective method to activate the transcription of targeted enhancers at its native chromosomal sites (i.e., in situ*)*. This method complements conventional approaches to study eRNAs in gene regulation that often emphasize on the knockdown of eRNAs by shRNAs, siRNAs, or ASOs[10,17]. Indeed, it has been largely infeasible to apply strategies to activate the transcription of eRNAs for functional interrogation until the advent of CRISPR mediated epigenetic tools. This is because ectopic expression of an eRNA driven by a heterologous promoter may not be optimal in two aspects: 1) the full-length transcript of eRNAs from their transcriptional start to end sites (particularly the ends) are difficult to be determined or are possible of mixed species in a cell; 2) the ectopic expression may alter the location of eRNAs, or may not contain proper structural features of the RNAs due to the lack of genomic/epigenomic contexts. CRISPR-dCas9 based epigenetic activation of eRNAs overcomes the above problems and provides an ideal method to "over-express" eRNAs in situ. Importantly, the enhanced eRNA expression recapitulates the disease-relevant enhancer over-activation commonly observed in human cancers[13-16], permitting subsequent studies of the pathological consequence.

Here we used NT-gRNA as a control for eRNA-targeting gRNAs to deduce the effects of the eRNA over-activation. However, there are other controls that can be employed. For example, cells with eRNA-targeting gRNAs with or without one of the two effector proteins may also be considered to deduce the genes affected by the effector proteins. Additional genome-wide tools such as ChIP-seq may be utilized to examine the global binding patterns of dCas9 effectors together with a targeting gRNA to further test the specificity of the SAM activator. Regardless, we consider that a robust conclusion should always be based on at least two separate gRNAs generating consistent results. Currently, little knowledge is available in terms of how the enforced binding of dCas9 effectors may impede the normal binding of transcription factors or cofactors on enhancers, which should be taken into consideration when users are designing and/or interpreting their

experiments. This should be an important research question for future work to better take advantage of this robust tool. While our mainly focus was on the oncogenic functions of eRNAs in this paper, the SAM system can be applied to examine eRNA functions in other biological or pathological settings[40-42]. One limit of this system is that it is not rapidly inducible, nor is it reversible, which may elicit indirect effects on cell growth that is not directly caused by eRNA activation. Therefore, one of the future directions to improve this protocol is to make it inducible and reversible. This will permit our manipulation of enhancers and eRNAs with higher temporal or spatial precision.

**ACKNOWLEDGMENTS:**

This work is supported by grants to W.L (Cancer Prevention and Research Institute of Texas, CPRIT RR160083 and RP180734; NCI K22CA204468; NIGMS R21GM132778; The University of Texas UT Stars Award; and the Welch foundation AU-2000-20190330) and a post-doctoral fellowship to J.L (UTHealth Innovation for Cancer Prevention Research Training Program Post-doctoral Fellowship, CPRIT RP160015). We acknowledge the original publicataion[15] where some of our current figures were adopted from (with modifications), which follows the Creative Commons license (https://creativecommons.org/licenses/by/4.0/).

**DISCLAIMERS**:

The content is solely the responsibility of the authors and does not necessarily represent the official views of the Cancer Prevention and Research Institute of Texas.

**REFERENCES:**

1. Consortium, E. P. An integrated encyclopedia of DNA elements in the human genome. *Nature*. **489** (7414), 57-74 (2012).
2. Djebali, S. et al. Landscape of transcription in human cells. *Nature*. **489** (7414), 101-108 (2012).
3. Kundaje, A. et al. Integrative analysis of 111 reference human epigenomes. *Nature*. **518** (7539), 317-330 (2015).
4. Andersson, R. et al. An atlas of active enhancers across human cell types and tissues. *Nature*. **507** (7493), 455-461 (2014).
5. Heinz, S., Romanoski, C. E., Benner, C., Glass, C. K. The selection and function of cell type-specific enhancers. *Nature Reviews Molecular Cell Biology*. **16** (3), 144-154 (2015).
6. Ong, C. T., Corces, V. G. Enhancer function: new insights into the regulation of tissue-specific gene expression. *Nature Reviews Genetics*. **12** (4), 283-293 (2011).
7. Hnisz, D. et al. Super-enhancers in the control of cell identity and disease. *Cell*. **155** (4), 934-947 (2013).
8. Grossman, S. R. et al. Systematic dissection of genomic features determining transcription factor binding and enhancer function. *Proceedings of the National Academy of Sciences of the United States of America*. **114** (7), 1291-1300 (2017).
9. Kim, T. K. et al. Widespread transcription at neuronal activity-regulated enhancers. *Nature*. **465** (7295), 182-187 (2010).
10. Li, W., Notani, D., Rosenfeld, M. G., Enhancers as non-coding RNA transcription units: recent insights and future perspectives. *Nature Reviews Genetics*. **17** (4), 207-223 (2016).

11. Creyghton, M. P. et al. Histone H3K27ac separates active from poised enhancers and predicts developmental state. *Proceedings of the National Academy of Sciences of the United States of America*. **107** (50), 21931-21936 (2010).
12. Li, W. et al. Functional roles of enhancer RNAs for oestrogen-dependent transcriptional activation. *Nature*. **498** (7455), 516-520 (2013).
13. Lee, J. H., Xiong, F., Li, W. Enhancer RNAs in cancer: regulation, mechanisms and therapeutic potential. *RNA Biology*. 1-10 (2020).
14. Sur, I., Taipale, J. The role of enhancers in cancer. *Nature Reviews Cancer*. **16** (8), 483-493 (2016).
15. Zhang, Z. et al. Transcriptional landscape and clinical utility of enhancer RNAs for eRNA-targeted therapy in cancer. *Nature Communications*. **10** (1), 4562 (2019).
16. Chen, H. et al. A Pan-Cancer Analysis of Enhancer Expression in Nearly 9000 Patient Samples. *Cell*. **173** (2), 386-399 (2018).
17. Kopp, F., Mendell, J. T. Functional Classification and Experimental Dissection of Long Noncoding RNAs. *Cell*. **172** (3), 393-407 (2018).
18. Lubelsky, Y., Ulitsky, I. Sequences enriched in Alu repeats drive nuclear localization of long RNAs in human cells. *Nature*. **555** (7694), 107-111 (2018).
19. Konermann, S. et al. Genome-scale transcriptional activation by an engineered CRISPR-Cas9 complex. *Nature*. **517** (7536), 583-588 (2015).
20. Beerli, R. R., Segal, D. J., Dreier, B., Barbas, C. R. Toward controlling gene expression at will: specific regulation of the erbB-2/HER-2 promoter by using polydactyl zinc finger proteins constructed from modular building blocks. *Proceedings of The National Academy of Sciences of the United States of America*. **95** (25), 14628-14633 (1995).
21. Gilbert, L. A. et al. CRISPR-mediated modular RNA-guided regulation of transcription in eukaryotes. *Cell*. **154** (2), 442-451 (2013).
22. Hirai, H., Tani, T., Kikyo, N., Structure and functions of powerful transactivators: VP16, MyoD and FoxA. *International Journal of Developmental Biology*. **54** (11-12), 1589-1596 (2010).
23. Lim, F., Spingola, M., Peabody, D. S., Altering the RNA binding specificity of a translational repressor. *Journal of Biological Chemistry*. **269** (12), 9006-9010 (1994).
24. Schmitz, M. L., Baeuerle, P. A., The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO Journal*. **10** (12), 3805-3817 (1991).
25. Vihervaara, A., Sistonen, L., HSF1 at a glance. *Journal of Cell Science*. **127** (Pt 2), 261-266 (2014).
26. Core, L. J. et al. Analysis of nascent RNA identifies a unified architecture of initiation regions at mammalian promoters and enhancers. *Nature Genetics*. **46** (12):1311-1320 (2014).
27. Haeussler, M. et al. Evaluation of off-target and on-target scoring algorithms and integration into the guide RNA selection tool CRISPOR. *Genome Biology*. **17** (1), 148 (2016).
28. Benchling [Biology Software]. Retrieved from https://benchling.com (2020).
29. Labun, K. et al. CHOPCHOP v3: expanding the CRISPR web toolbox beyond genome editing. *Nucleic Acids Research.* **47** (W1), W171-W174 (2019).
30. Hanna, R. E., Doench, J. G., Design and analysis of CRISPR-Cas experiments. *Nature Biotechnology*. (2020).
31. Nageshwaran, S. et al. CRISPR Guide RNA Cloning for Mammalian Systems. *Journal of Visualized Experiments*. **140**, e57998 (2018).

661 32. Al-Allaf, F. A., Tolmachov, O. E., Zambetti, L. P., Tchetchelnitski, V., Mehmet, H.
662 Remarkable stability of an instability-prone lentiviral vector plasmid in Escherichia coli Stbl3. *3*
663 *Biotech*. **3** (1), 61-70 (2013).
664 33. Qiu, P. et al. Mutation detection using Surveyor nuclease. *Biotechniques*. **36** (4), 702-707
665 (2004).
666 34. Ran, F. A. et al. Genome engineering using the CRISPR-Cas9 system. *Nature Protocols*. **8**
667 (11), 2281-2308 (2013).
668 35. Brinkman, E. K., Chen, T., Amendola, M., van Steensel, B. Easy quantitative assessment of
669 genome editing by sequence trace decomposition. *Nucleic Acids Research*. **42** (22), e168 (2014).
670 36. ZYMO. Quick-DNA Miniprep Kit. Retrieved from
671 https://files.zymoresearch.com/protocols/_d3024_d3025_quick-dna_miniprep_kit.pdf (2020).
672 37. IDT. SURVEYOR Mutation Detection Kit. Retrieved from
673 https://sfvideo.blob.core.windows.net/sitefinity/docs/default-source/user-guide-
674 manual/surveyor-kit-for-gel-electrophoresis-user-guide.pdf?sfvrsn=a9123407_6 (2020).
675 38. ZYMO, Quick-RNA Miniprep Kit. Retrieved from
676 https://files.zymoresearch.com/protocols/_r1054_r1055_quick_rna_miniprep_kit.pdf (2020).
677 39. Thermo Fisher Scientific. SuperScript IV Reverse Transcriptase Product Manual.
678 Retrieved from https://assets.thermofisher.com/TFS-
679 Assets/LSG/manuals/SSIV_Reverse_Transcriptase_UG.pdf (2020).
680 40. Ounzain, S. et al. Functional importance of cardiac enhancer associated noncoding RNAs
681 in heart development and disease. *Journal of Molecular and Cellular Cardiology*. **76**, 55-70 (2014).
682 41. McCleland, M. L. et al. CCAT1 is an enhancer-templated RNA that predicts BET sensitivity
683 in colorectal cancer. *Journal of Clinical Investigation*. **126**(2), 639-652 (2016)
684 42. Miao, Y. et al. Enhancer-associated long non-coding RNA LEENE regulates endothelial
685 nitric oxide synthase and endothelial function. *Nature Communications*. **9**(1), 292 (2018).
686

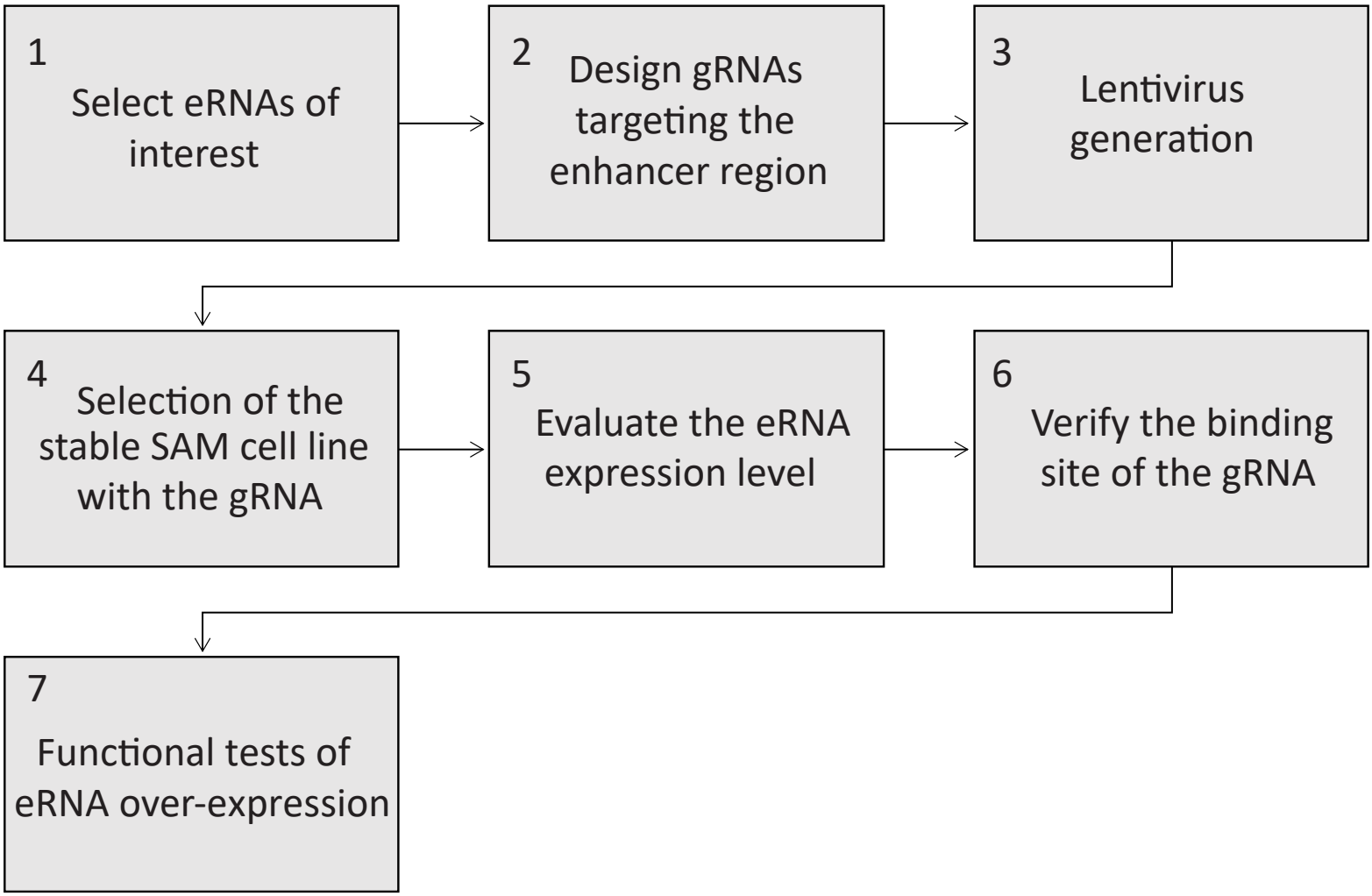



**Figure 1**

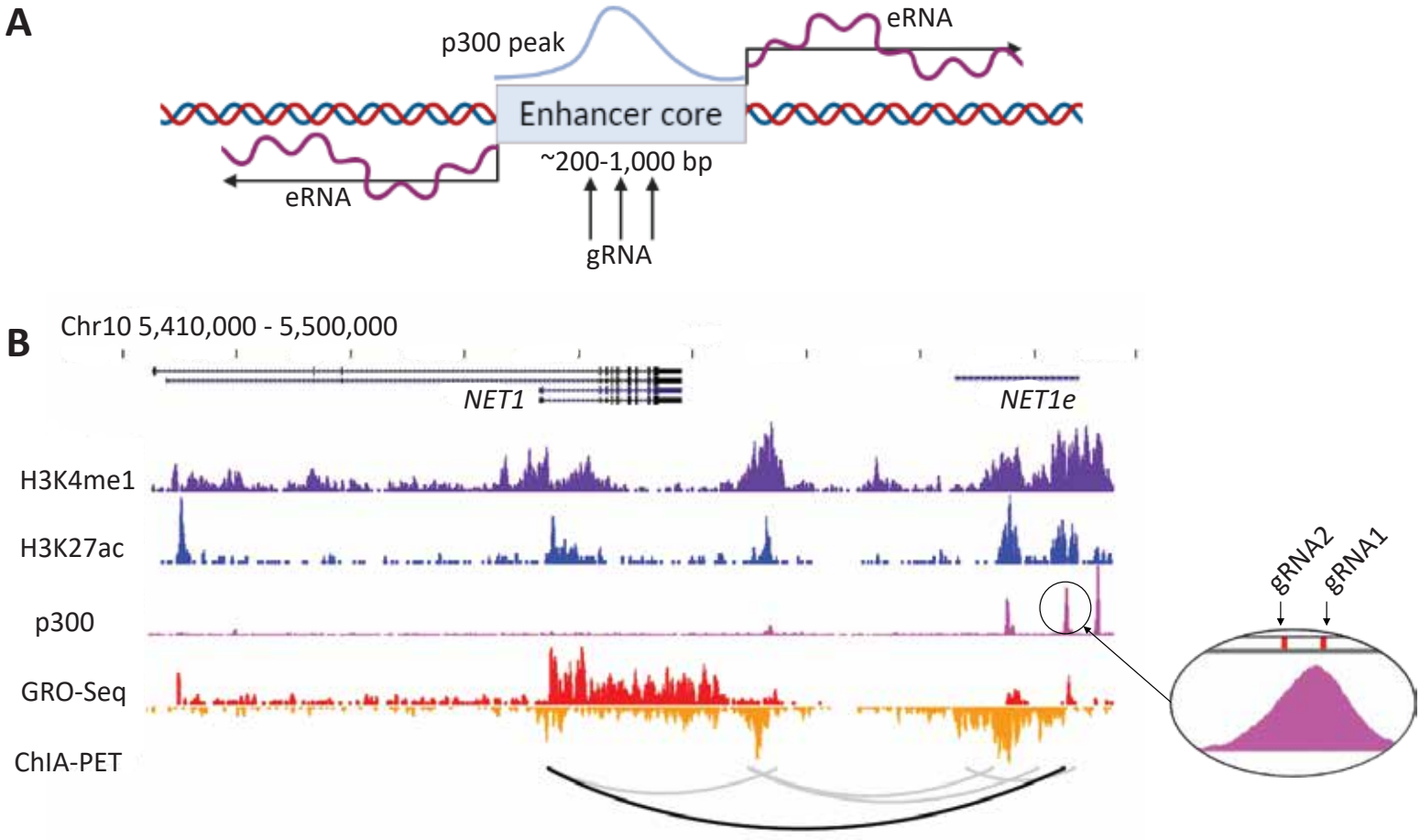
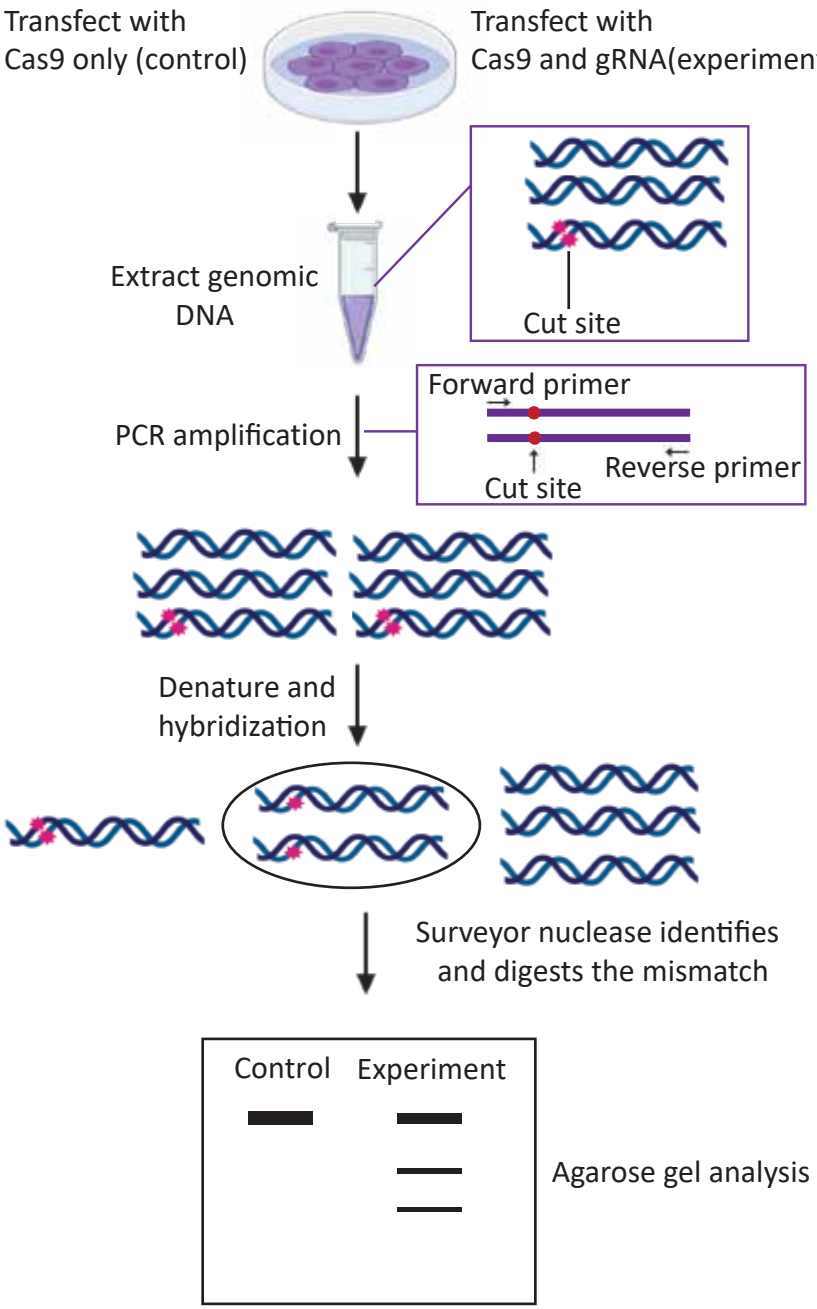



**Figure 2**

**A**




**B**

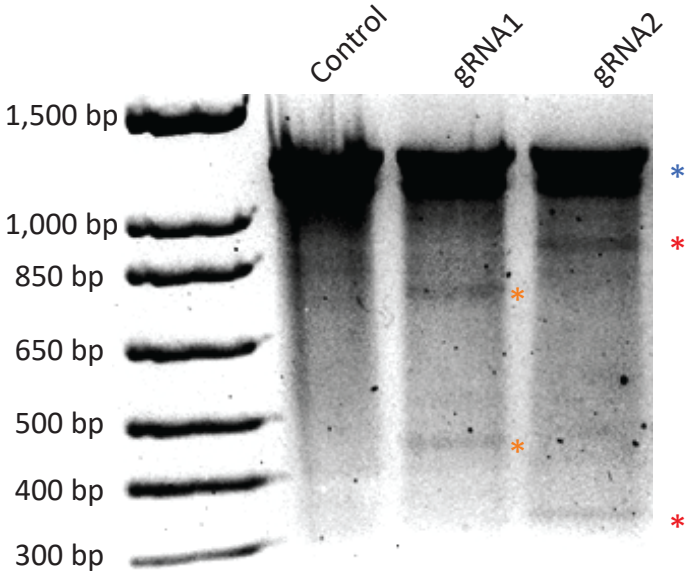



**Figure 3**

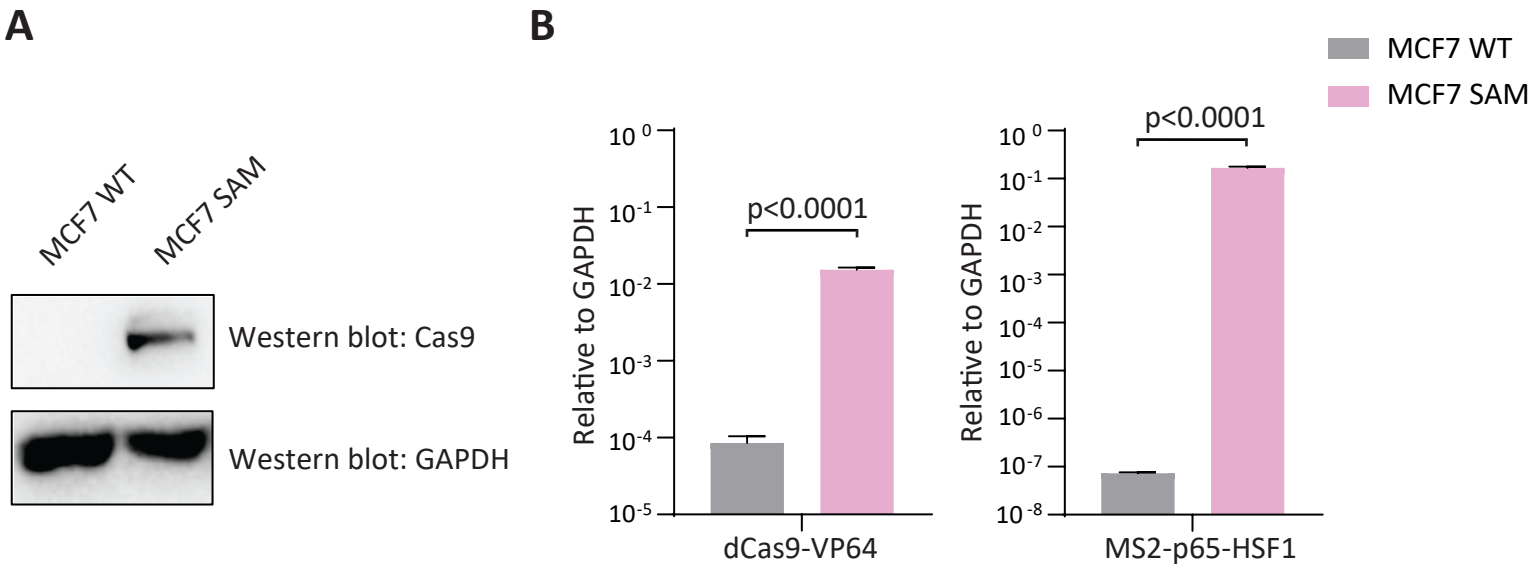



**Figure 4**

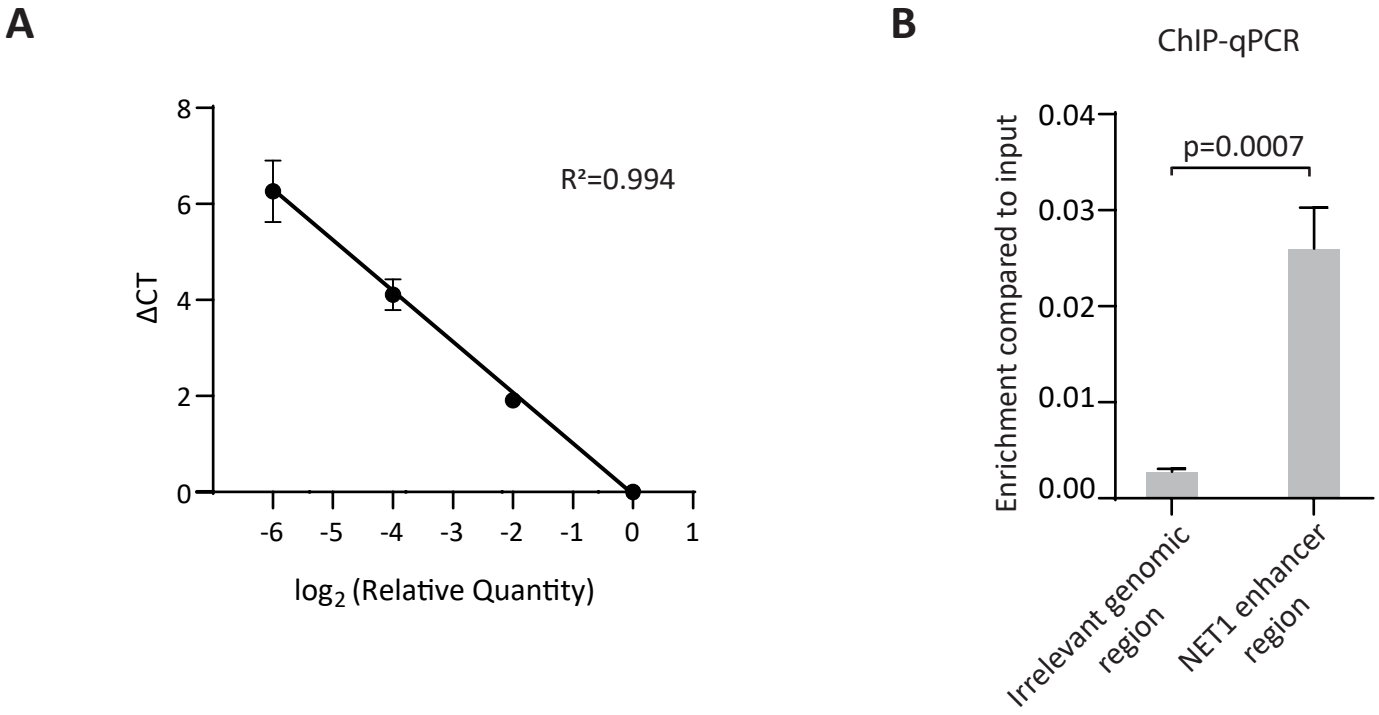



**Figure 5**

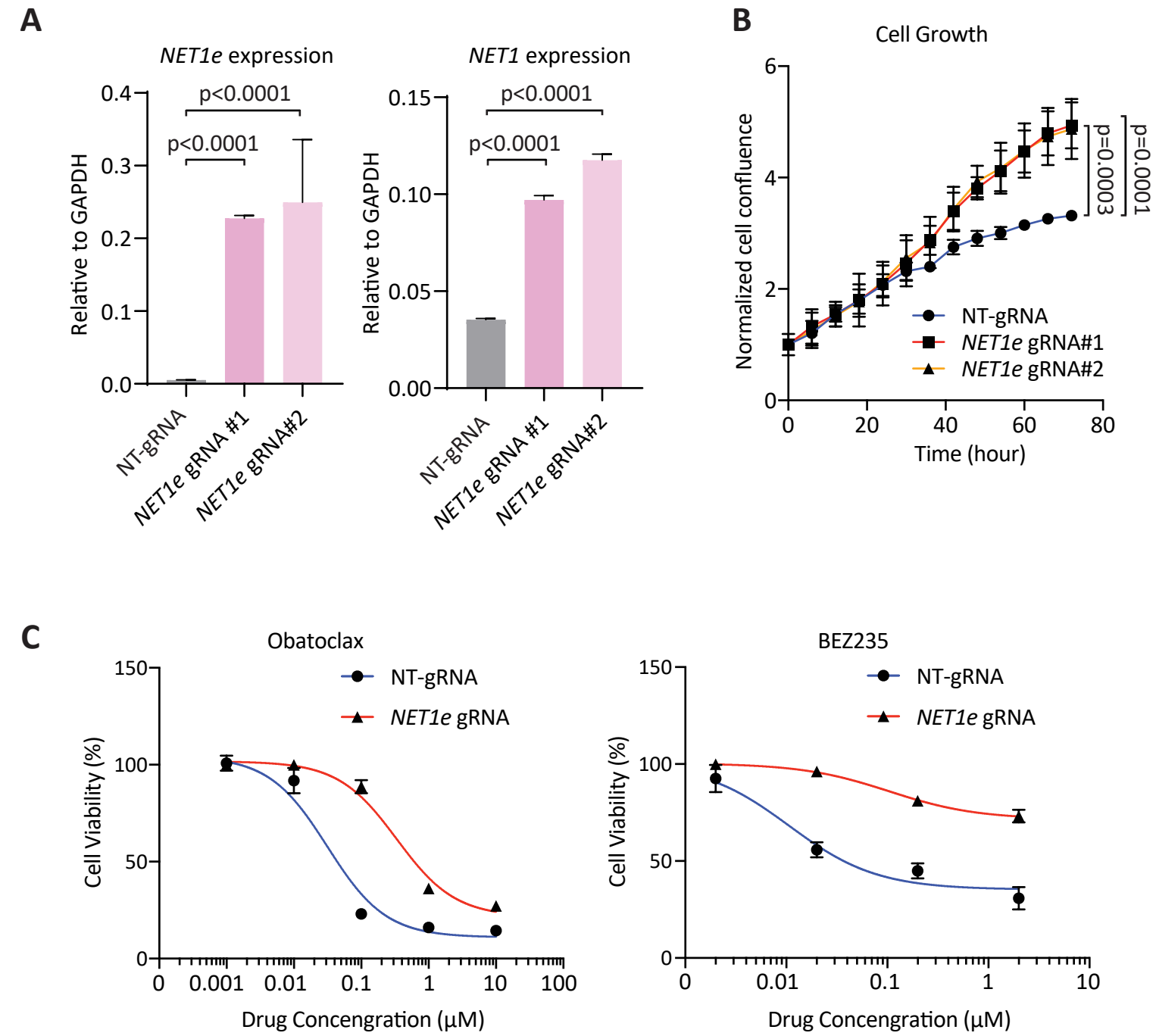



**Figure 6**

Table of materials



| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Blasticidin | Goldbio | B-800-100 | |
| BsmBI restriction enzyme | New England BioLabs Inc. | R0580S | |
| Cas9 mAb | Active Motif | 61757 | Lot: 10216001 |
| Deoxynucleotide (dNTP) Solution Mix | New England BioLabs Inc. | N0447S | |
| Dulbecco's Modified Eagle Medium | Corning | 10-013-CM | |
| Dynabeads Protein G | Thermo Fisher Scientific | 65002 | |
| EDTA | Thermo Fisher Scientific | BP118-500 | |
| EGTA | Sigma | E3889 | |
| Fetal Bovine Serum | GenDEPOT | F0900-050 | |
| Glycogen | Thermo Fisher Scientific | 10814010 | |
| Hepes-KOH | Thermo Fisher Scientific | BP310-100 | |
| Hexadimethrine Bromide | Sigma | H9268 | |
| Hygromycin B | Goldbio | H-270-25 | |
| IGEPAL CA630 | Sigma | D6750 | |
| IncuCyte live-cell imager | Essen BioScience | IncuCyte S3 Live-Cell Analysis System | |
| lenti_dCAS-VP64_Blast | Addgene | 61425 | |
| lenti_gRNA(MS2)_zeo backbone | Addgene | 61427 | |
| lenti_MS2-p65-HSF1_Hygro | Addgene | 61426 | |
| LiCL | Sigma | L9650 | |
| Lipofectamine 2000 | Thermo Fisher Scientific | 11668-500 | |
| NaCl | Sigma | S3014 | |
| Na-Deoxycholate | Sigma | D6750 | |

| | | |
|---|---|---|
| $NaHCO_3$ | Thermo Fisher Scientific | BP328-500 |
| N-lauroylsarcosine | Sigma | 97-78-9 |
| Opti-MEM Reduced Serum Medium | Thermo Fisher Scientific | 31985070 |
| PES syringe filter | BASIX | 13-1001-07 |
| Protease Inhibitor Cocktail Tablet | Roche Diagnostic | 11836145001 |
| pSpCas9(BB)-2A-Puro | Addgene | 62988 |
| Q5 High-Fidelity DNA Polymerase | New England BioLabs Inc. | M0491S |
| Q5 Reaction Buffer | New England BioLabs Inc. | B9027S |
| Quick-DNA Miniprep | ZYMO Research | D3025 |
| Quick-RNA Miniprep | ZYMO Research | R1054 |
| Restriction enzyme buffer | New England BioLabs Inc. | B7203S |
| RT-qPCR Detection System | Thermo Fisher Scientific | Quant Studio3 |
| SDS | Thermo Fisher Scientific | BP359-500 |
| Sonicator | Qsonica | Q800R2 |
| Sso Advanced Universal SYBR Green Supermix | Bio-Rad Laboratories | 1725274 |
| Stbl3 competent cell | Thermo Fisher Scientific | C7373-03 |
| Superscript IV reverse transcript | Thermo Fisher Scientific | 719000 |
| Surveyor Mutation Detection Kits | Integrated DNA Technologies | 706020 |
| T4 DNA Ligase | New England BioLabs Inc. | M0202S |
| T4 DNA Ligase Reaction Buffer | New England BioLabs Inc. | B0202S |

| | | |
|---|---|---|
| TE buffer | Thermo Fisher Scientific | 46009CM |
| Thermal cycler | Bio-Rad Laboratories | T100 |
| Thermomixer | Sigma | 5384000020 |
| Zeocin | Thermo Fisher Scientific | ant-zn-1p |

**Editorial comments:**

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
**RESPONSE**: We thank the Editors for the suggestions, and have now fully proofread the manuscript.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points.
**RESPONSE**: We have now formatted the manuscript as instructed.

3. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Lipofectamine, Thermomixer, etc.
**RESPONSE**: We have now removed these commercial languages. For example, we use “lipid-based transfection reagent” to replace Lipofectamine. We use “temperature-adjustable heated shaker” to replace “Thermomixer”.

4. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.”
**RESPONSE**: We have now used imperative tense throughout the revised manuscript, and have reformatted other text as “NOTE”.

5. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).
**RESPONSE**: We have revised accordingly.

6. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
**RESPONSE**: We have revised accordingly.

7. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
**RESPONSE**: We have revised accordingly.

8. Please ensure you answer the “how” question, i.e., how is the step performed?
**RESPONSE**: We have revised accordingly.

9. 1.1: How is this done? Please include all actions.
**RESPONSE**: We have revised accordingly, adding new details and used imperative tense. We would like to clarify that this protocol is aimed for users who have basic knowledge of genome biology and ChIP-Seq. It is out of the scope of this protocol to describe how to use a genome browser or how to look at ChIP-Seq data.

10. 2: Please include all the button clicks in the software to show how this is done.
**RESPONSE**: We have revised accordingly, adding new details and buttons to click. This is a rather straightforward website for users. We also cited other papers about gRNA design in the NOTE in section 2 (**Line 161**).

11. 7: Please include all the actions involved in each step.
**RESPONSE**: We have revised accordingly.

12. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
**RESPONSE**: We have highlighted about 2.75 pages.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
**RESPONSE**: As instructed, we obtained copyright permission from Nature Communications on Mar 10th 2020. We have uploaded this information as “***Copyright Permission.docx***” together with the re-submission. In addition, we clearly cited the original source of the results in the legend of Figure 6.

14. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
**RESPONSE**: We have revised the discussion section according to this guideline.

15. Please do not abbreviate the journal titles in the references section.

**RESPONSE**: We have revised accordingly.

---

**Reviewers' comments:**
**Reviewer #1:**
Manuscript Summary:

This method addresses an issue in the field - how to figure out what eRNAs are doing. In this work they report a system to induce eRNA expression using CRISPR-Cas9 fusions proteins directed to enhancers. In general, this seems a reasonable technique, and the authors provide some controls to show that the Cas9 fusion is directed to the location or interest, that it induces expression of the associated eRNA, and upregulates the expected c
**Response :** We thank this Reviewer for the overall positive assessment of this paper.

Major Concerns:

**Q1**: Some of the controls are inadequately described or of questionable execution (see below). One issue is that there are no controls for the effect of introducing the these Cas9-fusions on gene expression globally, nor is the specificity of action and binding assayed (other than using a single non-binding control region). These may be good tools, but use in practice would need to include much more extensive controls to ensure the specificity of their action.
**Response 1:** We thank the Reviewer for this cogent suggestion. Indeed, for common practices, non-targeting gRNAs (NT) were often co-transduced with dCas9-fusion proteins to serve as negative controls to reveal direct effects of dCas9-fusions and targeting RNAs. This has been the case for a few published papers (see the end of this letter, References [a,b]). We agree with the Reviewer that additional controls can be included to further study the effects of the dCas9 and/or fusion proteins, e.g. by using dCas9-SAM together with gRNAs targeting a region close to the targeted enhancer, but not in the central core; or dCas9 together with targeting gRNAs, or by using targeting gRNAs only. Additional genome-wide tools such as ChIP-seq may be utilized to examine the global binding patterns of dCas9-fusion together with a targeting gRNA. We also concur with the Reviewer that this current manuscript is to provide a general guideline for a useful tool. These additional investigations are better fit for separate scientific exploration. We have now added discussion of these in the revised manuscript (between **Lines 563-574)**. We consider that a robust conclusion should always be based on at least two separate gRNAs generating consistent results. We thank the Reviewer for this insightful question.

**Q2**: Also, as a general question, showing that the Cas9 protein is targeted to the correct spot and cuts at the correct spot in the Surveyor assay is fine, but isn't upregulation of the eRNA the only thing that matters? If a weak or no ChIP signal was observed, but the eRNA was up, wouldn't you go forward with experiment anyway? Doing qPCR on the eRNA is much easier, faster, and more sensitive than ChIP.
**Response 2:** We thank the Reviewer for this valuable comment. We provided additional experiments of Surveyor assay and ChIP-qPCR with the aim to help ensure the gRNA efficacy and

dCas9-fusion binding to the enhancer of interest. Practically speaking, as pointed out by this Reviewer, eRNA induction is a more direct evidence and easier readout for the purpose of the tool described in our paper. We have now added in the manuscript that Surveyor assays and ChIP may not be required for every single experiment for eRNA activation by dCas9/SAM, but an option (**new sections 4 and 9**), especially if multiple gRNAs were used and consistent results were generated. But we also consider that these two assays are helpful tests of the gRNA targeting. This is relevant to the Question 1 aforementioned, in which the Reviewer mentioned the possible gRNA off-targeting or non-specific gene regulation by dCas9 fusion proteins. For example, in a case that eRNA expression level is induced by dCas9/SAM together with a gRNA as compared to a non-targeting gRNA, however, if neither surveyor assay nor ChIP show positive results, caution needs to be taken to examine if gRNAs have off-targeting to some general activator genes, or if any overall cellular abnormality may account for the eRNA changes. We thank the Reviewer for this chance of discussion.

**Q3**: Figure 5A - The standard curve is odd. The details were not given, but it looks like perhaps 3 or 4-fold DNA dilutions were used? If so, a deltaCT of the most concentrated DNAs should be 2 cycles apart, or a 2^deltaCT of 4. Although there may be a difference of 4x between the highest and next highest concentration, I do not understand the units of ~4e-8 for the y-axis? Why is the value in the 10-8 range? Also, for this curve to be useful, the authors need to show that both the input and the ChIP samples fall within the range of this standard curve. It seems unlikely that they would, as the enrichment compare to input is ~0.025, which means that there is 40-fold less ChIP DNA than input. That would not allow both the input and ChIP to fit within the presented range of the standard curve.

**Response 3:** We apologize for the mistake/lack of clarity in making the plot. We sequentially diluted ChIP input genomic DNA from 0.5% down to 0.0078125% with a 4-fold differences by each dilution (0.5%, 0.125%, 0.03125%, 0.0078125%), and conducted qPCRs to assess the efficiency of the primer pair. Each diluted concentration should have ~2 cycles of higher PCR cycles than the previous dilution. We plotted the original plot as 2^-CT (the label in the original plot was incorrect). Now we have adjusted it to deltaCT values (ΔCT, the difference between CT values of serial diluted samples versus the undiluted DNA) for easier interpretation of the results. We have also added more description in the Figure legend of Figure 5.

As pointed out by the Reviewer, the input and the ChIP samples should have PCR cycles that fall in the range of the linearity test of the qPCR primers, which is a basic routine that we use in the lab. In this specific case, the input (cycle 25) and the sample (cycle 30) fall with the range of the linearity test (~24.5-30.5 cycles).

Minor Concerns:

**Q4**: The authors often referring to recent evidence of eRNAs…it's not that recent. What's more important is they seem to correlate with associate gene regulation.

**Response 4:** We thank the Reviewer for this suggestion, and we have now removed the usage of "recent" in our description.

**Q5**: Page 2 - Do eRNA producing enhancers have "higher levels of epigenomic markers" or "epigenomic marks associate with activated transcription". It's not the former, as not all epigenomic marks have been enumerated, so if it's the latter, which marks other than the one they point out?

**Response 5:** We thank the Reviewer for this important comment. Indeed, it is more appropriate/correct to use "epigenomic marks associated with active transcription" in this specific case. We have listed H3K27ac and H3K4me1 and transcriptional coactivators such as p300 as good marks (**Line84 and Line127**). However, an exhaustive list of epigenomic marks associated with eRNA-producing enhancers perhaps remains an active area of research investigation. We request the Reviewer to consider that this is beyond the score of this protocol paper.

**Q6**: Page 3, Line 2.1.2 - What is meant by a "high off-target score"? CRISPOR reports the gRNAs with the fewest off-target sites as the most specific and assigns them a low score - are the authors suggesting a high number of off-target binding sites is desirable?

**Response 6:** We apologize for this imprecise wording. Undoubtedly, we hope to identify gRNAs with minimal off-targeting. In CRISPOR, indeed as the Reviewer commented, it provides gRNAs with fewest off target sites and gives them a low score. Other tools, such as Benchling, give an overall off-target score for each guide RNA. In that case, a higher score (i.e. what we meant "off target score" in the original sentence) indicates an gRNA with fewer off-targeting. We have rephrased the sentence to clarify this point (**Line145** in the revised paper).

**Q7**: Figure 3B - The surveyor test shows very weak cleavage - and yet these were effective gRNAs? Does this mean that any discernible cleavage is a good predictor of activity? Is more cleavage better? This seems like a fair amount of effort for an unclearly described benefit.

**Response 7:** We thank the Reviewer for further discussion on this point (see Response 2 as well). The efficiency of DNA cut by the surveyor assay may be affected by several factors, such as the gRNA score, the transient transfection efficiency (and therefore the cells that bear both the gRNA and Cas9). Indeed, in our experiments in Figure 4B, the gRNA and the Cas9 were on two separate plasmids, which reduced the efficacy of co-expressing them in the same cells and contributed to the relatively lower rate of cleavage in Figure 3. As we have discussed in Response 2, Surveyor assay is used as an additional test of gRNA efficacy. In cases that the cutting was observed, regardless of the cleavage being full or partial, it proves that gRNAs do have specific targeting to desired sites. However, the Reviewer perhaps agrees that in cases that if a surveyor assay or ChIP did not show positive results, caution needs to be taken to examine if gRNAs have off-targeting, or if any overall cellular abnormality may account for eRNA changes. Exactly how strong a gRNA/dCas9-SAM complex needs to bind an enhancer to induce transcriptional changes is an interesting question that itself deserves further investigation. We thank the Reviewer for this chance for discussion to clarify our thoughts. We have made changes to Section 4 that suggest Surveyor assay or other similar assays as optional but helpful experiments (**Line191**).

**Q8**: Page 5, line 214 - We've found it is vital to filter lentivirus harvested from 293T cells before using in experiments. Otherwise 293T cells in the viral supernatant can contaminate the downstream cultures - even with dual selection. We use 0.2 micro filtering.

**Response 8:** We fully agree with this comment. It is a routine step to filter the lenti-viral medium in our lab as well. We have added the description of the filtering step in the revised manuscript (**Line234** in the revised manuscript). We thank the Reviewer for this comment.

**Q9**: Page 5, Line 232 - How much virus-containing medium do you add to cells? What scale do you recommend? There are details here that are important for someone wanting to do these experiments themselves.

**Response 9:** We often use 1ml of virus-containing medium for a well in a 6 well plate. We have now added the recommended viral medium amount to the revised manuscript (**Line272**). We thank the Reviewer for this comment.

**Q10**: Page 9, Line 375 - Cell number is measured by confluence? The graph reports normalized confluence - what does that mean? Normalized to the confluence on Day 1? What if the gRNA affects the ability of the cell to sense contact rather than divide? At a minimum, this seems a measure of the ability to reach high confluence, not cell growth. Were cell counts done at any point?

**Response 10:** We thank the Reviewer for the comment. We agree that the specific data we presented primarily measured cell confluence, which was used as a proxy for cell proliferation. Changes in cell attachment, cell shape or contact sensing may contribute to changes in confluence. We also often use other methods to measure cell growth including cell number counting. For this, we count cell numbers using membrane permeable dye Vybrant® Dye Cycle™ Green (Life Technologies, Cat#V35004) by Incucyte using its fluorescent imaging function. We additionally also count cells for specific samples using cell counters to confirm results. Additional work is now underway to examine tumor cell growth in animal models as well. We thank the Reviewer for this opportunity to discuss.

---

**Reviewer #2:**

Manuscript Summary:

The authors detail a new method for activating transcription of enhancer RNAs using a combination of deactivated Cas9 (dCas9) based approaches. eRNAs are important for regulating transcription of enhancer associated genes. However, there are a scarcity of approaches available to reduce or activate eRNA transcript levels without perturbing underlying chromatin features or transcription factor binding sites. Moreover, overexpression of eRNAs to replicate overexpression in many human cancers fails to accurately reproduce the cis expression profile of eRNAs and requires acurrate mapping of 3' and 5' ends of eRNAs, which are poorly defined. The research proposed here thus provides a useful set of tools for studying eRNA function. However, the current manuscript lacks a sufficient level of detail in places necessary for a methods paper, and this should be addressed prior to acceptance.

**Response :** We thank this Reviewer for the overall positive assessment of this paper. We will address the critiques and questions in the following paragraphs.

Major Concerns:

**Q11**: Line 100-105: The authors should explain in greater detail the specific role of each factor being recruited via the dCas9 recruitment system. For a methods paper such as this, the authors should not assume prior knowledge of the mechanisms of these factors and should therefore provide sufficient information to make the specific activation mechanism of each factor clear to the reader.

**Response 11:** We appreciate this insightful suggestion from the Reviewer. We have added more description of the role of each effector proteins to the introduction section to help the readers to understand the design of this system (**Lines111-119**).

**Q12**: Line 126. The authors should comment on the potential for disrupting normal enhancer function by targeting dCas9 complexes, analogous to CRISPRi approaches. This can be a major confounding effect when targeting enhancers using dCas9 based approaches and is one reason why CRISPRi approaches are currently more prevalent in the literature for studying enhancer function. Appropriate controls should be therefore considered (e.g ChIP-qPCR/ChIPseq for TFs, p300 or similar) at the earliest possible stage of the experimental design process. The authors should also make specific reference to the fact that this can be a problem.

**Response 12:** We thank the Reviewer for this question. The points made are well taken and we have now added some discussion on the potential impediment of normal binding of transcription factors or cofactors due to forced binding of dCas9 fusion proteins to enhancers (**Line571**) to the revised section of discussion. We ask the readers to be aware of the unknown biology of dCas9 based CRISPRa, including SAM.

Minor Concerns:

**Q13:** Line 168 (Step 3.4) Authors should explain the importance of using the Stbl3 line or similar here.

**Response 13:** We thank the Reviewer for this comment. We have now added the importance of Stbl3 in the manuscript (**Line186**).

**Q14**: Line 174 Authors should comment on other alternatives for checking efficiency of designed sgRNAs, e.g TIDE.

**Response 14:** Thank you for your suggestion, we have added the comment of alternative ways to check the gRNA efficiency in the note of section 4 of the manuscript (**Line194**).

**Q15:** Line 196: Negative controls are essential for this assay.

**Response 15:** Thank you for the comment. Indeed, here we used cells transfected with Cas9 plasmid only as a negative control, which shows no cutting of the target region in the Surveyor assay. We acknowledge that additional controls can be added to further validate the specificity and efficiency of the cutting. We have made this clear in section 4.3 (**Line213**).

**Q16**: Line 198: Authors should comment on appropriate Biosafety level and precautions for performing lentiviral transductions.

**Response 16:** We thank the Reviewer for this comment. We have now added the Biosafety level and precautions to **Line237** of the revised manuscript.

**Q17**: Lins 238: Authors should comment on need to optimise antibiotic concentration for different cell lines. How quickly should antibiotics be expected to kill non transduced cells? Are antibiotics applied immediately following transduction, or after a delay to allow genomic integration to occur?

**Response 17:** We thank the Reviewer for this comment. We have added comments about the concentration, timing and length of antibiotic selection in the discussion of the revised manuscript (**Line273**). The killing time using a range of antibiotics of non-infected cells is pre-determined for each cell line to be used. In our protocol, we start antibiotic selection about 24 hours after the infection.

**Q18**: Line 247: Antibody catalog and Lot number should be provided. Reference to Primer table should be included.

**Response 18:** Thank you for your suggestion, since JOVE requires that no commercial content shall be indicated in the manuscript, we choose to list this information in the table of materials as supplement files. We have also added the reference to the primer table in our revised text (**Line305**).

**Q19:** Line 263: Authors should include specific recommendations for kits used both for RNA purification and reverse transcription. eRNAs can be relatively low abundance, therefore it would be useful to include detail on recommended procedures for purification, for example specific kits versus phenol chloroform. This information would be of general interest. Similarly, do the authors experience differences in efficiency between different reverse transcriptases? Again, this information would be of broad interest to potential readers.

**Response 19:** We thank the Reviewer for mentioning this question. Although typical eRNA is of relatively lower abundance than typical mRNAs, we find no significant difference in detecting eRNAs in qPCR by using either RNA extraction kits (i.e. Qiagen or Zymo Mini RNA extraction kits) or by phenol chloroform based extraction method (e.g. TRIzol). Thus we did not specify the methods to extract RNAs in the manuscript. This seems to be supported by increasing amounts of literature that various RNA extraction methods are used that can successfully detect eRNAs. We respect this comment and have now added: "*No significant difference was observed in our practice for eRNA detection when RNAs are extracted either by commercial binding columns or by phenol chloroform reagents*" to the NOTE of section 8 **(Line 299**).

In terms of reverse transcriptases (RT enzymes), we routinely use RTIII or RTIV from ThermoFisher/Life Tech, but have not extensively tested all available RT enzymes, therefore we cannot provide a specific comment on the RT enzymes. The catalog numbers for our reagents are listed in the table of materials. We again thank this Reviewer for the opportunity to discuss this.

**Q20**: Line 274: Authors should provide more detail on assessing primer quality. "quality and amplification linearity of the primer pairs have to be examined": how do the authors do this?
**Response 20:** Thank you for your suggestion. We have now added this note in section 8.3 **Line292**.

**Q21:** Line 318: No mention is made of how much antibody to add, or which antibody should be used. Is antibody added directly to the supernatant and then recovered, or are beads conjugated to antibody before adding to the supernatant?
**Response 21:** Thank you for mentioning this question. We actually mentioned in our original manuscript that we used 2 µg of Cas9 antibody (**Line 315** in the original paper). The catalog number for this Cas9 antibody is Active Motif #61757. We directly added the antibody to the soluble portion of sonicated chromatin, and then recovered the immuno-complex by Protein G dynabeads. We included these information in the revised manuscript (**Line 344** of revised paper).

**Conclusion: We thank the editors and the two reviewers who provided many valuable comments and insight questions. We have now fully addressed these concerns and questions, and have revised the manuscript accordingly.**

**Additional References**:
a. Joung, J. et al., Genome-scale activation screen identifies a lncRNA locus regulating a gene neighbourhood. NATURE 548 343 (2017).
b. Bester, A. C. et al., An Integrated Genome-wide CRISPRa Approach to Functionalize lncRNAs in Drug Resistance. CELL 173 649 (2018).

Sup Table 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplementary Table 1.xlsx

| Name | Sequence | Function |
|---|---|---|
| NET1e gRNA#1-Fwd | CACCGGAGGAATGTGCAAACGGCCC | gRNA cloning |
| NET1e gRNA#1-Rev | AAACGGGCCGTTTGCACATTCCTCC | gRNA cloning |
| NET1e gRNA#2-Fwd | CACCGAGCCTACACCCTGGAGGTAC | gRNA cloning |
| NET1e gRNA#2-Rev | AAACGTACCTCCAGGGTGTAGGCTC | gRNA cloning |
| | | |
| NET1e-RT-qPCR-Fwd | CAGGCCTACCAGGATGGATA | RT-qPCR |
| NET1e-RT-qPCR-Rev | AGTTGACTTGGGTGGGACAG | RT-qPCR |
| NET1-RT-qPCR-Fwd | ACCTGGATGAAAAGCAGAGG | RT-qPCR |
| NET1-RT-qPCR-Rev | GGTAAGAGTGCCGTTCGTTC | RT-qPCR |
| GAPDH-Fwd | GTTTTTCTAGACGGCAGGTCA | RT-qPCR |
| GAPDH-Rev | AACATCATCCCTGCCTCTACT | RT-qPCR |
| dCas9-VP64-RT-qPCR-Fwd | GGCTTCGCCAACAGAAACTT | RT-qPCR |
| dCas9-VP64-RT-qPCR-Rev | CCTTCTGGGTGGTCTGGTTC | RT-qPCR |
| MS2-p65-HSF1-RT-qPCR-Fwd | CCAGGCCTACAAGGTGACAT | RT-qPCR |
| MS2-p65-HSF1-RT-qPCR-Rev | GCCTTCACGATGAGTTCACA | RT-qPCR |
| | | |
| NET1e-ChIP-qPCR-Fwd | GCCTACACCCTGGAGGTACA | ChIP-qPCR |
| NET1e-ChIP-qPCR-Rev | TTTCTGCAGGGAAGGTCATC | ChIP-qPCR |
| Negative control-ChIP-qPCR-Fwd | ATACTTACCTGGCAGGGGAG | ChIP-qPCR |
| Negative control-ChIP-qPCR-Rev | CAGGGGGAAAGCGCGAACGCA | ChIP-qPCR |
| | | |
| NET1e-gRNA-Surveyor-Fwd | TGAGTGGTCTCTACTCACCTTAGAAAC | Surveyor assay |
| NET1e-gRNA-Surveyor-Rev | AGATGAGGATGAGGGAATGGT | Surveyor assay |

| PCR reaction set up and conditions for Surveyor assay | | | | | |
|---|---|---|---|---|---|
| Amount | Reagent | | Temperature | Time | |
| 6 µl | 5X Q5 PCR buffer | | 98 °C | 2 min | |
| 0.6 µl | dNTP (10mM) | | 98 °C | 30 sec | |
| 0.3 µl | Q5 DNA Polymerase (2,000 U/ml) | → | 67 °C | 30 sec | |
| 100 ng | Genomic DNA | | 72 °C | 80 sec | X34 times |
| 1.5 µl | Forward primer (10 µM ) | | 72 °C | 2 min | |
| 1.5 µl | Reverse primer (10 µM ) | | 4 °C | infinite | |
| add to 30 µl | Water | | | | |

| cDNA synthesis reaction set up and conditions | | | | | | |
|---|---|---|---|---|---|---|
| Amount | Reagent | | Temperature | Time | | Amount |
| 1 µl | 50 µM random hexamers | | 65 °C | 5 min | | 4 µl |
| 1 µl | dNTP (10mM) | | put on ice | >1 min | | 1 µl |
| 800 ng | Template RNA(total RNA), max 11u | → | | | Add → | 1 µL |
| add to 13 µl | Water | | | | | 1 µl |

*NOTE: after the final step, dilute 1:10 with water and cDNA can be stored at -20°C

| qPCR reaction set up and conditions | | | | | |
|---|---|---|---|---|---|
| Amount | Reagent | | Temperature | Time | |
| 3 µl | cDNA template | | 95 °C | 5 min | |
| 2 µl | Forward primer and reverse primer (2.5 µM each ) | → | 95 °C | 15 sec | X 40 times |
| 5 µl | SYBR Green supeemix | | 60 °C | 40 sec | |

| Reagent | | Temperature | Time |
|---|---|---|---|
| 5× SSIV Buffer | | 23 °C | 10 min |
| 100 mM DTT | | 55 °C | 10 min |
| RNaseOUT™ Recombinant RNase Inhibitor | → | 80 °C | 10 min |
| SuperScript® IV Reverse Transcriptase (200 U/µL) | | | |

Journalpermissions <journalpermissions@springernature.com>

Tue 3/10, 9:18 AM

Lee, Joo Hyung

You forwarded this message on 3/10/2020 9:48 AM

****** EXTERNAL EMAIL ******

Dear Joo-Hyung,

Thank you for your recent Springer Nature permissions request. This work is licensed under the Creative Commons Attribution 4.0 International License, which permits unrestricted use, distribution, modification, and reproduction in any medium, provided you:

1) give appropriate acknowledgment to the original author(s) including the publication source,
2) provide a link to the Creative Commons license/include a notice of the CC license in legend and reference, and indicate if changes were made.

**You are not required to obtain permission to reuse this article, but you must follow the above two requirements.**

Images or other third party material included in the article are encompassed under the Creative Commons license, unless indicated otherwise in the credit line. If the material is not included under the Creative Commons license, users will need to obtain permission from the license holder to reproduce the material.

To view a copy of the Creative Commons license, please visit http://creativecommons.org/licenses/by/4.0/

Please let me know if you have any questions.

Best wishes,
Oda

**Oda Siqveland**
Rights Executive

**SpringerNature**
The Campus, 4 Crinan Street, London N1 9XW,
United Kingdom
T +44 (0) 207 014 6851

http://www.nature.com
http://www.springer.com
http://www.palgrave.com