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Pooled CRISPR-activation screening coupled with single-cell RNA-seq in mouse embryonic stem cells

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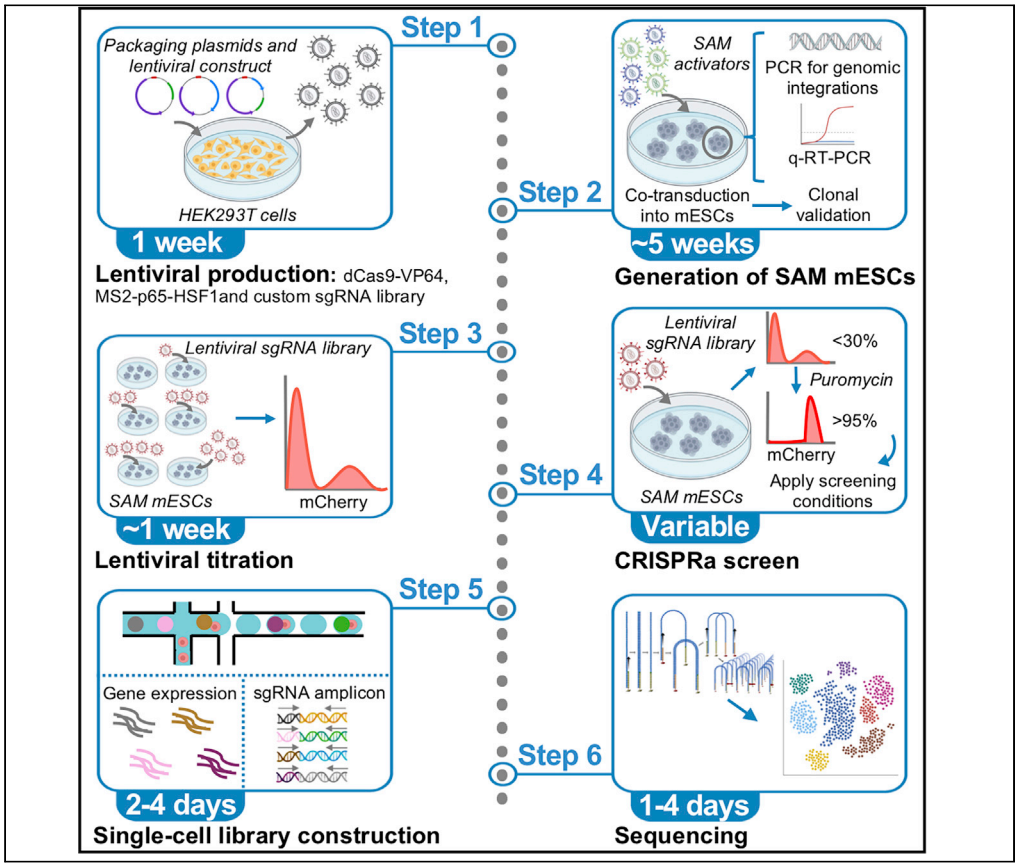

CRISPR/Cas9 screens are a powerful approach to identify key regulators of biological processes. By combining pooled CRISPR/Cas9 screening with a single-cell RNA-sequencing readout, individual perturbations can be assessed in parallel both comprehensively and at scale. Importantly, this allows gene function and regulation to be interrogated at a cellular level in an unbiased manner. Here, we present a protocol to perform pooled CRISPR-activation screens in mouse embryonic stem cells using 10× Genomics scRNA-seq as a readout.

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Highlights

Protocol for CRISPRa screens with single-cell readout to interrogate gene function

Detailed description of CRISPRa screening procedures in mouse embryonic stem cells

Detailed steps on how to construct derived single-cell sgRNA amplicon libraries

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Pooled CRISPR-activation screening coupled with single-cell RNA-seq in mouse embryonic stem cells

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SUMMARY

CRISPR/Cas9 screens are a powerful approach to identify key regulators of biological processes. By combining pooled CRISPR/Cas9 screening with single-cell RNA-sequencing readout, individual perturbations can be assessed in parallel both comprehensively and at scale. Importantly, this allows gene function and regulation to be interrogated at a cellular level in an unbiased manner. Here, we present a protocol to perform pooled CRISPR-activation screens in mouse embryonic stem cells using 10× Genomics scRNA-seq as a readout.

For complete information on the generation and use of this protocol, please refer to Alda-Catalinas et al. (2020).

BEFORE YOU BEGIN

Prior steps: candidate selection and custom sgRNA library construction

Timing: 6–9 weeks

The CRISPR-activation (CRISPRa) method used in this protocol is "synergistic activation mediator" or SAM (Konermann et al., 2015), a robust and potent method for transcriptional upregulation of endogenous targets. In this protocol, we describe the steps from lentiviral production of a custom CRISPRa library and subsequent transduction into mouse embryonic stem cells (mESCs) to generation and sequencing of single-cell RNA-sequencing (scRNA-seq) libraries from the perturbed cells. Previous steps not described here include selecting gene candidates to screen and the design, cloning, amplification, and quality control of the custom sgRNA library. Candidate selection is dependent on the biological question under investigation. The number of candidates that can be screened for depends on biological and technical considerations and project budget, but typically ranges from 100–500 genes. We recommend including 3–5 sgRNAs per target in the custom sgRNA library, along with 5%–10% of non-targeting sgRNAs out of the total size of the library. Careful consideration of sgRNA library size is particularly important for screens based on scRNA-seq read-outs, given the high costs of single-cell processing and sequencing. One of these considerations is the number of cells required to be sequenced per sgRNA, which highly depends on screening conditions and downstream transcriptional response expected to be captured. For instance, in Alda-Catalinas et al., 2020, we performed pilot studies and power analyses to estimate that approximately 400

sequenced cells were required to detect a ZGA-like transcriptional signature upon CRISPRa of a positive hit in mESCs. After knowing the coverage of cells/sgRNA that is required to detect the expected transcriptional response in the 10× Genomics scRNA-seq libraries, one can decide on the number of genes to screen for based on project budget. Not only 10× Genomics scRNA-seq library preparation but also sequencing costs should be considered, with the recommendation to sequence at a coverage of 30,000 read pairs per cell for gene expression libraries and at 5,000 read pairs per cell for sgRNA amplicon libraries (further details given in the sequencing chapter of this protocol). Following on the example described in Alda-Catalinas et al., 2020, for a sgRNA library size of 475 sgRNAs (covering 230 target genes, each with 2 sgRNAs), we performed 3 replicates, loading cells from each replicate across 8 lanes of a Chromium chip, each with 20,000 cells; after sequencing at the recommended coverage, this resulted in a dataset of 341,103 cells, out of which 203,894 cells passed all quality control parameters, resulting in an average coverage of 437 cells/sgRNA.

For a detailed description of the steps that need to be performed from CRISPRa sgRNA design to quality control via next generation sequencing (NGS) of the custom sgRNA library generated, we recommend following the protocol described by Joung et al., 2017, with the following modifications:

- Step 1Aiii or 1Bii from Joung et al., 2017: Design and synthesize the custom oligo library with the following flanking sequences around the sgRNA protospacers:
  a. 5′ flanking: 5′-TATCTTGTGGAAAGGACGAAACACCG-3′
  b. 3′ flanking: 5′-GTTTAAGAGCTAGGCCAACATGAGGATCACCCATG-3′

So that each oligo has the form: 5′-TATCTTGTGGAAAGGACGAAACACCG-sgRNA protospacer-GTTTAAGAGCTAGGCCAACATGAGGATCACCCATG-3′.

These flanking sequences allow for subsequent oligo library amplification and sgRNA library cloning into the plasmid backbone CROP-sgRNA-MS2 (Addgene #153457, Alda-Catalinas et al., 2020), which enables both CRISPRa via the SAM system (Konermann et al., 2015) and read-out of sgRNA protospacer sequences in 10× Genomics 3′ scRNA-seq libraries.

- Step 3 from Joung et al., 2017: Perform the PCR amplification of the pooled oligo library with the following primers (Table S1):
  a. Pooled_PCR_F: 5′-GTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAACACC-3′
  b. Pooled_PCR_R: 5′-ATTTAAACTTGCTAGGCCCTGCAGACATGGGTGATCCTCATGTTGGCCTAGCTCTTAAAC-3′

PCR amplification with these primers will result in a pooled oligo library that can be cloned into the plasmid backbone CROP-sgRNA-MS2 (Addgene #153457, Alda-Catalinas et al., 2020).

- Step 8 from Joung et al., 2017: Use the library plasmid backbone CROP-sgRNA-MS2 (Addgene #153457, Alda-Catalinas et al., 2020), which enables both CRISPRa via the SAM system (Konermann et al., 2015) and read-out of sgRNA protospacer sequences in 10× Genomics 3′ scRNA-seq libraries. Note this library backbone contains a 1,050-bp filler sequence between BsmBI restriction sites.

Cell culture of mouse embryonic stem cells and HEK293T lentiviral packaging cells

Timing: 1–2 weeks

1. Prepare culture media with the following components:
   a. Prepare serum/LIF media for mESC culture: DMEM (Gibco, 11995-040), 15% fetal bovine serum (Thermo Fisher Scientific, 10439024), 1 U/mL penicillin - 1 mg/mL streptomycin (Gibco, 15140-122), 0.1 mM nonessential amino acids (Gibco, 11140-050), 2 mM GlutaMAX (Gibco, 35050-061), 50 μM β-mercaptoethanol (Gibco, 31350-010), and 10^3 U/mL LIF (Stem Cell Institute, Cambridge).
   b. Prepare mESC media without LIF for lentiviral production in HEK293T cells: DMEM (Gibco, 11995-040), 15% fetal bovine serum (Thermo Fisher Scientific, 10439024), 1 U/mL penicillin - 1 mg/mL streptomycin (Gibco, 15140-122), 0.1 mM nonessential amino acids (Gibco, 11140-050), 2 mM GlutaMAX (Gibco, 35050-061) and 50 μM β-mercaptoethanol (Gibco, 31350-010).
   c. Prepare D10 media for maintenance of HEK293T cells: DMEM (Gibco, 11995-040), 15% fetal bovine serum (Thermo Fisher Scientific, 10439024) and 1 U/mL penicillin - 1 mg/mL streptomycin (Gibco, 15140-122).

Media should be filter sterilized, stored at 4°C for up to 2 weeks and pre-warmed to 37°C before use.

Note: for mESC culture, use only stem cell qualified FBS.

Note: the lentiviral transductions into mESCs in this protocol were optimized in feeder-free, serum/LIF conditions. Other culture conditions, such as serum-free 2i (±LIF) media may be used, but lentiviral transductions will likely require further optimization.

2. Prepare gelatin plates for mESCs:
   a. Coat tissue-culture treated plates or wells with 0.1% gelatin (Sigma, ES-006-B), for a minimum of 15 min at 37°C. The choice of plate size or well should be based on number of cells to be thawed:
      i. approximately 0.4 million cells per 6-well.
      ii. approximately 2.5 million cells per 100 mm plate.
      iii. approximately 5 million cells per 150 mm plate.

3. Thaw mESCs:

Note: this protocol was optimized using E14 mESCs (RRID: CVCL_C320). Other mESC lines may be used depending on the biological question, however, this may require additional optimization steps.

   a. Pre-warm culture media to 37°C.
   b. Obtain gelatin-coated plates or wells, aspirate gelatin and replace with pre-warmed culture media:
      i. 2 mL per 6-well.
      ii. 10 mL per 100 mm plate.
      iii. 20 mL per 150 mm plate
   c. Add 9 mL of pre-warmed culture media to a 15 mL tube.
   d. Thaw mESCs in cryovial by placing in a water bath at 37°C for 2–3 min, swirling gently.
   e. Transfer cells to a 15 mL tube containing pre-warmed media and spin down 300 × *g* for 3 min at room temperature (20°C–25°C).
   f. Aspirate supernatant, resuspend cell pellet in appropriate culture media volume and transfer cell suspension to prepared gelatin-coated cell culture plates or wells.
   g. Move plate containing cells to a 37°C, 5% CO_2 incubator.

4. Routine maintenance of mESCs:

Note: it is recommended to replace media daily.

Note: it is recommended to passage cells every 2–3 days; to avoid cell differentiation and loss of pluripotency, colonies should not be allowed to touch.

Note: it is recommended to maintain cells in 6-well or in 100 mm tissue-culture treated plates and expand them to 150 mm plates prior to performing the screen.

Note: approximate mESC seeding density conditions and culture media volumes:

i. approximately 0.4 million cells per 6-well in 2 mL.
ii. approximately 2.5 million cells per 100mm plate in 10 mL.
iii. approximately 5 million cells per 150mm plate in 20 mL.

a. Pre-warm media and coat gelatin plates as described in steps 1 and 2.
b. Aspirate media from cells and wash twice with PBS (Gibco, 14190144).
c. Aspirate PBS and add the following volumes of trypsin-EDTA (Gibco, 25200056):
   i. 0.5 mL per 6-well.
   ii. 1 mL per 100mm plate.
   iii. 3 mL per 150 mm plate.

Note: Be sure to cover the entire plate by carefully swirling it.

d. Incubate cells in trypsin-EDTA for 2–5 min in a 37°C, 5% CO_2 incubator until colonies dissociate and detach. Gently tap the sides of the plates to detach most of the cells from the surface.
e. Add the following volumes of serum/LIF culture media and gently pipet up and down to create a single-cell suspension:
   i. 2.5 mL per 6-well.
   ii. 9 mL per 100mm plate.
   iii. 15 mL per 150mm plate.
f. Split cells at a ratio of 1:5 to 1:8 into a new gelatinized tissue-culture plate with pre-warmed media.
g. Culture mESCs for at least 1–2 weeks (or 4–5 passages) before proceeding with this protocol.

5. Freezing down mESCs:

Note: freeze cells at a density of 4–5 million cells/mL.

a. Prepare a 2× freezing media containing 20% (v/v) tissue-culture grade dimethylsulfoxide (DMSO; Sigma-Aldrich D2650), 50% (v/v) fetal bovine serum (Thermo Fisher Scientific, 10439024) and 30% (v/v) serum/LIF media. Filter, sterilize and store at 4 C for up to 2 weeks.
b. Label cryovials with cell type, date, and number of cells.
c. Trypsinize cells following steps 4a–4e.
d. Transfer cell suspension to a 15 mL tube, count cells and aliquot if necessary.
e. Spin down tube(s) 300 × *g* for 3 min at room temperature (20°C–25°C).
f. Aspirate supernatant(s), resuspend cell pellet(s) in 250–500 μL of serum/LIF media and transfer to a pre-labelled cryovial.
g. Add the same volume of pre-chilled 2× freezing media.
h. Immediately slow freeze in a −70°C freezer (i.e., using a Mr. Frosty cryocontainer; Nalgene) and approximately 24 h later, transfer to liquid nitrogen for longer cryopreservation.

6. Thaw HEK293T cells (ATCC CRL-3216):
   a. Pre-warm D10 culture media to 37°C.
   b. Add 9 mL of pre-warmed culture media to a 15 mL tube.

c. Thaw HEK293T cells (ATCC CRL-3216) in cryovial by placing in a water bath at 37°C for 2–3 min, swirling gently.
d. Transfer cells to 15 mL tube containing pre-warmed media and spin down 300 × *g* for 3 min at room temperature (20°C–25°C).
e. Aspirate supernatant, resuspend cell pellet in appropriate D10 media volume and transfer cell suspension to cell culture flask or plate.

Note: Recommended seeding density is approximately 30,000 cells/cm^2 (i.e.,: 6 million cells in a T175 flask with 30–40 mL of D10 media).

f. Move plate containing cells to a 37°C, 5% CO_2 incubator.

7. Routine maintenance of HEK293T cells:

Note: it is recommended to replace media every 2–3 days.

Note: it is recommended to passage cells every 2–3 days or when they reach 70% confluency.

Note: recommended seeding density for HEK293T maintenance is approximately 30,000 cells/cm^2. We recommend maintaining and growing cells in T175 flasks, seeding approximately 6 million cells in 30–40 mL of D10 media. The following volumes for routine maintenance of HEK293T cells refer to T175 flasks.

a. Pre-warm D10 media to 37°C.
b. Aspirate media from cells and wash twice with PBS (Gibco, 14190144).
c. Aspirate PBS and add 5 mL trypsin-EDTA (Gibco, 25200056).
d. Incubate cells in trypsin-EDTA for 2–5 min in a 37°C, 5% CO_2 incubator until cells detach. Gently tap the sides of the plates to detach most of the cells from the surface.
e. Add 12 mL of pre-warmed D10 media and gently pipet up and down to create a single-cell suspension.
f. Split cells at a ratio of 1:3 to 1:5 by transferring into a new cell culture flask.
g. Culture HEK293T cells for 1–2 passages before proceeding with this protocol.

8. Freezing down HEK293T cells:

Note: freeze cells at a density of approximately 10 million cells/mL.

a. Prepare a 2× freezing media containing 20% (v/v) tissue-culture grade dimethylsulfoxide (DMSO; Sigma-Aldrich D2650), 50% (v/v) fetal bovine serum (Thermo Fisher Scientific, 10439024) and 30% (v/v) D10 media. Filter sterilize and stored at 4°C for up to 2 weeks.
b. Label cryovials with cell type, date, and number of cells.
c. Trypsinize cells following steps 7a–7e.
d. Transfer cell suspension to a 15 mL tube, count cells and aliquot if necessary.
e. Spin down tube(s) 300 × *g* for 3 min at room temperature (20°C–25°C).
f. Aspirate supernatant(s), resuspend cell pellet(s) in 250–500 μL of D10 media and transfer to a pre-labelled cryovial.
g. Add the same volume of pre-chilled 2× freezing media.
h. Immediately slow freeze in a −70°C freezer (i.e., using a Mr. Frosty cryocontainer; Nalgene) and approximately 24 h later, transfer to liquid nitrogen for longer cryopreservation.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Murine LIF	Wellcome – MRC Cambridge Stem Cell Institute	https://www.stemcells.cam.ac.uk/research/facilities/tissueculture
TransIT transfection reagent	Mirus Bio	Cat# MIR2700
Polybrene	Millipore	Cat# TR-1003-G
Lenti-X Concentrator	Takara	Cat# 631231
DNaseI	Thermo Fisher Scientific	Cat# EN0521
Blasticidin	InvivoGen	Cat# ant-bl-1
Hygromycin	InvivoGen	ant-hg-1
Puromycin	InvivoGen	ant-pr-1
2× KAPA HiFi Master Mix	Kapa Biosystems	Cat# KR0389
Brilliant III SYBR master mix	Agilent Technologies	Cat# 600882
Critical commercial assays		
AllPrep DNA/RNA Mini Kit	Qiagen	Cat# 80204
RevertAid First-Strand cDNA synthesis kit	Thermo Fisher Scientific	Cat# K1622
Chromium Next GEM Single Cell 3′ GEM, Library & Gel Bead Kit v3.1	10× Genomics	Cat# PN-1000121 or Cat#PN-1000128 for 16 or 4 reactions, respectively
Chromium Next GEM Chip G Single Cell Kit	10× Genomics	Cat# PN-1000120
Chromium Next GEM Chip G Single Cell Kit	10× Genomics	Cat# PN-1000127
Single Index Kit T Set A	10× Genomics	Cat# PN-1000213
AMPure XP beads	Beckman Coulter	Cat# A63881
SPRIselect Reagent Kit	Beckman Coulter	Cat# B23318
Nextera XT Index Kit	Illumina	Cat# FC-131-2001, FC-131-2002, FC-131-2003, FC-131-2004 or FC-131-1001
Agilent Bioanalyzer High Sensitivity chip	Agilent	Cat# 5067-4626
Deposited data		
Raw and quantified sequencing data: bulk RNA-seq data of E14 and SAM mouse ESCs	Alda-Catalinas et al., 2020	GEO: GSE135509 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135509)
Raw and quantified sequencing data: 10× Genomics CRISPRa screen dataset	Alda-Catalinas et al., 2020	GEO: GSE135621 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135621)
Experimental models: cell lines		
Mouse: E14 embryonic stem cells	Hooper et al., 1987	RRID: CVCL_C320 https://discovery.lifemapsc.com/stem-cell-differentiation/in-vitro-cells/inner-cell-mass-mus-musculus-e14-university-of-edinburgh
Human: HEK293T	ATCC	ATCC CRL-3216
Oligonucleotides		
Genomic PCR primers for dCas9-VP64 and MS2-p65-HSF1	Alda-Catalinas et al, 2020	Table S1
q-RT-PCR primers	This paper	Table S1
Primers for sgRNA amplicon libraries	Alda-Catalinas et al, 2020	Table S1
Primers for PCR amplification of a pooled sgRNA library to be cloned in CROP-sgRNA-MS2 lentiviral backbone	This paper	Table S1
Recombinant DNA		
pMD2.G	Didier Trono	Addgene plasmid #12259
psPAX2	Didier Trono	Addgene plasmid #12260
Lenti dCas9-VP64_Blast	Konermann et al., 2015	Addgene plasmid #61425
Lenti MS2-p65-HSF1_Hygro	Konermann et al., 2015	Addgene plasmid #61426

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Lenti CROP-sgRNA-MS2	Alda-Catalinas et al., 2020	Addgene plasmid #153457
Software and algorithms		
CellRanger v2.1	(Zheng et al., 2017)	Github: https://github.com/10XGenomics/cellranger
Scripts to analyse CROP-seq screens	Hill et al., 2018	Github: https://github.com/shendurelab/single-cell-ko-screens
Scripts for quality control and analysis of CRISPRa CROP-seq	Alda-Catalinas et al., 2020	Github: https://github.com/gtca/crispra_zga
Other		
0.45 μm PES filter	Sartorius	Cat# 16533

STEP-BY-STEP METHOD DETAILS

Lentiviral production of SAM activators and custom sgRNA library

Timing: 1 week

Three lentiviruses are produced in this step: (1) dCas9-VP64_Blast, (2) MS2-p65-HSF1_Hygro and (3) the custom CRISPRa sgRNA library. HEK293T cells are used for lentiviral packaging by co-transfecting the plasmid of interest with 2nd generation lentiviral packaging plasmids. These packaging plasmids are pMD2.G (Addgene #12259), which expresses a VSV-G envelope, and psPAX2 (Addgene #12260), which contains Gag, Pol, Rev, and Tat inserts. When these three lentiviral productions are complete, dCas9-VP64_Blast and MS2-p65-HSF1_Hygro lentiviruses will be first simultaneously transduced into mESCs in the next step to generate a constitutive cell line that enables CRISPRa (this cell line will be referred to as SAM mESCs). The custom sgRNA library will be then transduced into SAM mESCs at a low multiplicity-of-infection (MOI) to introduce the custom CRISPRa perturbations.

Note: safety considerations need to be evaluated when working with lentiviruses and it may require additional lab biosafety procedures.

1. Prepare HEK293T cells (ATCC CRL-3216) for transfection. Three transfections will be performed here: (1) dCas9-VP64_Blast, (2) MS2-p65-HSF1_Hygro and (3) the custom CRISPRa sgRNA library. Seed 3.5 million HEK293T cells per transfection in 100 mm tissue culture plates with 10 mL of pre-warmed D10 media, 16–20 h before transfection.

⚠ CRITICAL: use low-passage (<20 passages) HEK293T cells for optimal lentiviral production.

Note: we recommend starting with one transfection per plasmid/library in a 100 mm plate and scale it up later if necessary.

2. Plasmid co-transfection for lentiviral packaging:
   a. 16–20 h after seeding HEK293T cells as described in previous step, cells should have reached 80%–90% confluency.
   b. Prepare a single-tube reaction mix for each transfection.
      i. Obtain three sterile 1.5 mL tubes. Label 1–3.
      ii. To each tube, add 1.5 mL of opti-MEM (Gibco, 31985).
      iii. To each tube containing opti-MEM, add plasmid/library of interest together with lentiviral packaging plasmids as follows:
         - Tube 1: 3.5 μg of pMD2.G (Addgene #12259), 6.5 μg of psPAX2 (Addgene #12260) and 10 μg of lenti dCas9-VP64_Blast (Addgene #61425).

- Tube 2: 3.5 μg of pMD2.G (Addgene #12259), 6.5 μg of psPAX2 (Addgene #12260) and 10 μg of lenti MS2-p65-HSF1_Hygro (Addgene #61426).
- Tube 3: 3.5 μg of pMD2.G (Addgene #12259), 6.5 μg of psPAX2 (Addgene #12260) and 10 μg of the custom sgRNA library cloned into CROP-sgRNA-MS2 (Addgene #153457).

iv. Vortex briefly.
v. Add 60 μL of transIT-293 transfection reagent (Mirus Bio, 2700) to each tube.
vi. Vortex briefly.
vii. Incubate 30 min at room-temperature.

c. During the 30 min incubation time, aspirate the D10 media from the three plates of HEK293T cells and add 8.5 mL per plate of pre-warmed mESC media without LIF: DMEM (Gibco, 11995-040), 15% fetal bovine serum (Thermo Fisher Scientific, 10439024), 1 U/mL penicillin - 1 mg/mL streptomycin (Gibco, 15140-122), 0.1 mM nonessential amino acids (Gibco, 11140-050), 2 mM GlutaMAX (Gibco, 35050-061) and 50 μM β-mercaptoethanol (Gibco, 31350-010).
d. Add each transfection mix from step 2bvii drop-wise to one of the 100 mm plates of HEK293T cells prepared in step 2c.

3. Return plates to incubator and culture at 37°C in 5% CO_2 for 48 h.
4. For each plate, collect 10 mL of lentiviral supernatant with a 20 mL syringe and filter into a 15 mL tube through a 0.45 μm PES filter (Sartorius, 16533). Prepare aliquots of 1 mL of the clarified supernatant for (1) dCas9-VP64_Blast (Addgene #61425) and (2) MS2-p65-HSF1_Hygro (Addgene #61426).

⚠ CRITICAL: use only cellulose acetate or polyethersulfone (PES) (low protein binding) filters. Do not use nitrocellulose filters as nitrocellulose binds surface proteins on the lentiviral envelope and destroys the virus. As an alternative to filtering the lentiviral supernatant, centrifuge at 500 × *g* for 10 min and discard pellet.

Pause point: This step completes lentiviral production for (1) dCas9-VP64_Blast (Addgene #61425) and (2) MS2-p65-HSF1_Hygro (Addgene #61426). For these, store aliquoted supernatant at −70°C for long-term or proceed immediately with one aliquot of each to step 6. For the clarified supernatant of the custom sgRNA library (3), proceed immediately to step 5 for lentiviral concentration.

5. Concentrate the lentiviral preparation of custom sgRNA library cloned into CROP-sgRNA-MS2 (Addgene #153457):
   a. Combine 1 volume of Lenti-X Concentrator (Takara, 631231) with 3 volumes of lentiviral supernatant (i.e., add 3.3 mL of Lenti-X Concentrator to 10 mL of lentiviral supernatant).
   b. Mix by inversion.
   c. Incubate at 4°C for 4 h to 16 h.

Note: manufacturer recommends incubation from 30 min to overnight, however, the shortest time we have tested is 4 h.

   d. Centrifuge mix at 1,500 × *g* for 45 min at 4°C. The resulting white pellet contains the concentrated lentivirus.
   e. Remove supernatant and resuspend pellet with fresh serum/LIF media to 1/10 to 1/20 of the original volume of clarified lentiviral supernatant (500 μL to 1 mL). Prepare 100 μL aliquots of the concentrated lentivirus and store at −70°C.

Pause point: Store aliquots of the custom sgRNA library lentivirus at −70°C until step 19.

Generation and quality control of SAM mESCs

Timing: 4–5 weeks

A clonal mESC line constitutively expressing dCas9-VP64_Blast and MS2-p65-HSF1_Hygro is generated in this step via transduction of the lentiviral particles prepared in steps 1–4. This cell line will be used to perform CRISPRa via the SAM system (Konermann et al., 2015) after lentiviral transduction of individual sgRNAs or a sgRNA library cloned into the backbone CROP-sgRNA-MS2 (Addgene #153457).

6. Titer dCas9-VP64_Blast and MS2-p65-HSF1_Hygro lentiviral preparations in mESCs. Construct a lentiviral dilution curve for each lentivirus:
   a. Trypsinize a confluent plate of mESC and count the cell suspension.
   b. In preparation for seeding 120,000 cells per well in 400 µl of serum/LIF culture media in 12 gelatinized wells of a 24-well plate (6 wells to be used for dCas9-VP64_Blast titration and 6 for MS2-p65-HSF1_Hygro titration), count 1.56 million cells (13× 120,000 cells, i.e., counting for an extra well to account for pipetting errors) and spin down 300 × *g* for 3 min at room temperature (20°C–25°C); discard supernatant and resuspend cell pellet in 5.2 mL of serum/LIF culture media (13× 400 µl).
   c. Add polybrene (Millipore, TR-1003-G) to the 5.2 mL of cell suspension to a final concentration of 16 µg/mL.
   d. Add 400 µl of cell suspension + polybrene per well to 12 gelatinized wells of a 24-well plate.
   e. If dCas9-VP64_Blast and/or MS2-p65-HSF1_Hygro lentiviral preparations from step 4 had been stored at −70°C, thaw 1 aliquot of each on ice.
   f. Add 10^3 U/mL LIF (Stem Cell Institute, Cambridge) to each lentiviral preparation.
   g. Add lentivirus and/or additional serum/LIF culture media to individual wells of the 24-well plate of mESCs prepared in step 6d, as follows:
      i. dCas9-VP64_Blast lentivirus:
         - Well 1: 400 µl lentivirus
         - Well 2: 200 µl lentivirus + 200 µl serum/LIF media
         - Well 3: 100 µl lentivirus + 300 µl serum/LIF media
         - Well 4: 50 µl lentivirus + 350 µl serum/LIF media
         - Well 5: 25 µl lentivirus + 375 µl serum/LIF media
         - Well 6: 400 µl serum/LIF media. This non-transduced well will be used as a +antibiotic (blasticidin) selection or kill control.
      ii. MS2-p65-HSF1_Hygro lentivirus:
         - Well 1: 400 µl lentivirus
         - Well 2: 200 µl lentivirus + 200 µl serum/LIF media
         - Well 3: 100 µl lentivirus + 300 µl serum/LIF media
         - Well 4: 50 µl lentivirus + 350 µl serum/LIF media
         - Well 5: 25 µl lentivirus + 375 µl serum/LIF media
         - Well 6: 400 µl serum/LIF media. This non-transduced well will be used as a +antibiotic (hygromycin) selection or kill control.

   Note: the final volume in each well is 800 µl, making the final concentration of polybrene 8 µg/mL.

   h. Mix each well thoroughly by pipetting up and down and return plate to incubator (37°C in 5% CO_2).
   i. After 24 h, remove media from each well, wash twice with PBS and add 800 µl of fresh serum/LIF culture media. Troubleshooting 1
   j. Approximately 24 h later (approximately 48 h after transduction), trypsinize and split each well of cells into 2 gelatinized wells of a 24-well plate. For each condition of cells transduced with dCas9-VP64_Blast, culture one well of cells in 800 µl of serum/LIF media and the other well in 800 µl of serum/LIF media containing 20 µg/mL blasticidin (InvivoGen, ant-bl-1). Similarly, for each condition of cells transduced with MS2-p65-HSF1_Hygro, culture one well of cells in 800 µl of serum/LIF media and the other well in 800 µl serum/LIF media containing 200 µg/mL hygromycin (InvivoGen, ant-hg-1). Add also 20 µg/mL blasticidin to one well of

non-transduced cells and 200 μg/mL hygromycin to another well of non-transduced cells; these will be used as non-transduced +antibiotic selection controls. Two wells will remain as non-transduced no-antibiotic controls in serum/LIF media.

k. For the following 6–7 days, expand cells from individual wells when they reach 80% confluency. To calculate viral titer, this expansion should be done without discarding any cells, by transferring all cells to a gelatinized 6-well plate for the first passage and subsequently to gelatinized 100 mm plates for the later passages.

l. The transduced cells have been selected for dCas9-VP64 or MS2-p65-HSF1 expression when there are no viable cells in the respective non-transduced +antibiotic control condition (likely to occur 6–7 days after addition of blasticidin/hygromycin). When selection is complete, the viral titer can be calculated. Trypsinize, count and record the total number of cells in each well/plate. Troubleshooting 2

m. For each lentivirus and for each transduction condition (as indicated in step 6 g), calculate MOI as the number of cells in the transduced +antibiotic condition divided by the number of cells in the transduced no-antibiotic condition. For example, if the number of cells in condition 3 (100 μl lentivirus + 300 μl serum/LIF media) of dCas9-VP64_Blast titration is 10 million in the +blasticidin condition and 20 million in the no-blasticidin condition, the resulting MOI is 0.5.

7. Transduce dCas9-VP64_Blast and MS2-p65-HSF1_Hygro lentiviruses into mESCs at a MOI of 0.5–0.7:

a. Calculate the volume of dCas9-VP64_Blast lentivirus and MS2-p65-HSF1_Hygro lentivirus to transduce 2.5 million cells at a MOI of 0.5–0.7 each, based on calculations in step 6 m.

Note: for instance, if for the titration experiment described in step 6 with 120,000 mESCs, 50 μl of dCas9-VP64_Blast lentivirus resulted in a MOI of 0.5–0.7, and 100 μl of MS2-p65-HSF1 lentivirus resulted in a MOI of 0.5–0.7, scale up these volumes for 2.5 million cells. For dCas9-VP64_Blast this would result in (2.5×10^6 cells × 50 μl) / 1.2×10^5 cells in titration experiment = 1,042 μl; for MS2-p65-HSF1_Hygro, (2.5×10^6 cells × 100 μl) / 1.2×10^5 cells in titration experiment = 2,083 μl.

b. Thaw aliquots of the lentiviral preparations dCas9-VP64_Blast and MS2-p65-HSF1_Hygro from step 4 on ice.

c. Combine the required volume of dCas9-VP64_Blast lentivirus with the required volume of MS2-p65-HSF1_Hygro lentivirus according to calculations in step 7a, and add 10^3 U/mL LIF (Stem Cell Institute, Cambridge) to the combined lentiviral mix. If lentiviral preparations were successful, the total combined volume should not exceed 15 mL. If required, top up to 15 mL with serum/LIF culture media.

d. Add 8 μg/mL polybrene (Millpore, TR-1003-G) to the 15 mL of combined lentivirus and mix gently by pipetting.

e. Trypsinize a confluent plate of low-passage mESC (<10 passages) and count the cell suspension. Transfer a total of 2.5 million cells to a new tube and spin down 300 × *g* for 3 min at room temperature (20°C–25°C). Discard the supernatant and resuspend cell pellet in the ~15 mL mix from step 7d containing the combined lentiviral preparation + LIF + polybrene.

⚠ CRITICAL: we recommend using low-passage mESCs (<10 passages) for clonal cell line generation, especially if differentiation protocols are to be applied during screening.

f. Seed cells + lentiviral mix in a 100 mm gelatinized tissue culture plate and return plate to incubator (37°C in 5% CO_2).

⚠ CRITICAL: when seeding cells, ensure an even distribution throughout the plate.

g. Seed an additional 2.5 million mESCs in another 100 mm gelatinized tissue culture plate to be used as a non-transduced +antibiotic selection control. Add 8 μg/mL polybrene (Millpore, TR-1003-G), mix gently and return plate to incubator (37°C in 5% CO_2).
h. After 24 h, remove media for both plates, wash twice with PBS and add 10 mL of fresh serum/LIF culture media per plate.

8. Select cells for dCas9-VP64 and MS2-p65-HSF1 expression:
   a. Approximately 24 h after refreshing serum/LIF culture media (approximately 48 h after transduction), replace media with serum/LIF media containing 20 μg/mL blasticidin (InvivoGen, ant-bl-1) and 200 μg/mL hygromycin (InvivoGen, ant-hg-1), both to the plate of cells transduced with dCas9-VP64_Blast/MS2-p65-HSF1_Hygro and to the non-transduced +antibiotic selection control plate.
   b. Grow cells in blasticidin+hygromycin selection media for 10–14 days, until individual colonies (derived from single transduced cells) are observed in the plate of transduced cells and there are no viable cells in the non-transduced +antibiotic selection control plate.

Note: 20 μg/mL blasticidin and 200 μg/mL hygromycin should kill all non-transduced cells within a week, the extra few days will allow resistant cells to form large independent colonies. Troubleshooting 2 and 3

9. Manual picking of resistant colonies and clonal expansion:
   a. Use a bright-field microscope inside a tissue culture hood.
   b. Prepare a 96-well U-shaped-bottom plate with 25 μl of Trypsin-EDTA (Thermo Fisher Scientific, 25200056) in 30 wells. Additionally, coat all the wells of a 24-well plate with 0.1% gelatin.
   c. Remove media from plate of transduced and selected cells from step 8b and wash once with PBS.
   d. Add 10 mL of PBS and inspect plate under a microscope with a 4× magnification.
   e. To pick a desired colony, circle the colony with a 20 μL pipette tip and, with volume set of 15 μL, scrape the colony to dislodge it and then aspirate.

⚠ CRITICAL: Select colonies that are spaced well enough apart to avoid contamination from surrounding colonies.

⚠ CRITICAL: Select colonies that appear rounded or oval, with a phase contrast bright edge and often a dark necrotic center. Do not pick differentiated colonies that appear flat and surrounded by fibroblast-like cells.

   f. Transfer the picked colony to a single well of a 96-well U-shaped-bottom plate containing 25 μl of trypsin-EDTA and pipette up and down to help dissociate the colony.
   g. Repeat steps 9e–9f for 5–10 colonies or the maximum number of colonies that you can pick in approximately 5 min.
   h. Add 175 μl of serum/LIF culture media to colonies in 96-well plate.

⚠ CRITICAL: do not leave colonies in trypsin-EDTA for longer than 5 min before neutralizing with serum/LIF media. If necessary, do fewer colonies at the time and check their dissociation in trypsin-EDTA under a microscope. Always check that colonies are dissociated before adding serum/LIF media.

   i. Repeat steps 9e–9 h until 20–30 colonies have been picked.
   j. Transfer each dissociated colony in the 96-well plate to a well in a gelatinized 24-well plate and add 600 μl of serum/LIF media to each well. Return plate to incubator and culture cells (37°C and 5% CO_2).

k. 24–72 h later (at 70%–80% confluency), trypsinize and transfer clones to a gelatinized 6-well plate with 2 mL of serum/LIF media.

10. When clones in the 6-well plate reach 70%–80% confluency, harvest cell pellets for genomic DNA and RNA extraction, and cryopreserve clonal cell lines:
    a. Trypsinize each well containing a clone and divide sample 1:1:1 into three 2 mL tubes. Also trypsinize a confluent plate of non-transduced mESCs.
    b. Use one of the tubes from each clone for genomic DNA/RNA extraction. To do that, spin down 300 × *g* for 3 min at 4°C, remove supernatant and immediately snap freeze pellet in dry ice or liquid nitrogen. In parallel, follow the same steps for non-transduced mESCs to be used as a negative control for downstream analysis of genomic DNA and RNA.
    c. Use another tube from each clone for cryopreservation, following step 5 of the "before you begin" section.
    d. Use the last tube from each clone for routine mESC maintenance by seeding cells in an individual gelatinized well from a 6-well plate in serum/LIF culture media. These cells can be used later for further cryopreservation or for further genomic DNA/RNA harvesting.

Pause point: Store cell pellets from step 10b at −70°C long-term or proceed immediately to step 11. Cryopreserved clonal lines (step 10c) can be stored long-term in liquid nitrogen.

11. Extract genomic DNA and RNA for each clone and for non-transduced mESCs. We recommend using the AllPrep DNA/RNA Mini Kit (Qiagen, 80204), following manufacturer's instructions, for simultaneous purification of genomic DNA and RNA for each clone. Measure DNA and RNA concentration using a nanodrop or similar.

Pause point: RNA can be stored long-term at −70°C. For DNA purifications, store at −20°C or proceed immediately to step 12.

12. Perform a PCR on the genomic DNA from each clone to identify those containing dCas9-VP64_Blast and MS2-p65-HSF1_Hygro integrations:
    a. Samples to run:
        i. Genomic DNA from harvested clones (20–30 samples).
        ii. Genomic DNA from non-transduced mESCs (negative control).
        iii. Lenti dCas9-VP64_Blast plasmid DNA (positive control).
        iv. Lenti MS2-p65-HSF1_Hygro plasmid DNA (positive control).
    b. Set up 2 PCR reactions for each clone, the negative control and the corresponding positive control. The first PCR reaction will test for integration of dCas9-VP64_Blast and the second for integration of MS2-p65-HSF1_Hygro.

Note: We use DreamTaq DNA Polymerase (Thermo Fisher Scientific, EP0702). Alternative DNA polymerases for standard PCR applications on genomic DNA can be suitable following manufacturer's instructions but may require optimization.

PCR for dCas9-VP64_Blast:

Reagent	Final concentration / amount	Amount
10× DreamTaq Buffer	1×	2.5 µl
dNTP Mix (10 mM each)	0.2 mM	0.5 µl
dCas9-VP64_F primer (10 µM)*	0.4 µM	1 µl
dCas9-VP64_R primer (10 µM)**	0.4 µM	1 µl
Template DNA (genomic DNA from clones, or genomic DNA from non-transduced mESCs, or lenti dCas9-VP64_Blast plasmid DNA)	0.2–1 µg for genomic DNA samples; 0.5–1 ng for plasmid DNA	Variable
DreamTaq DNA Polymerase	0.625 U	0.125 µl

(*Continued on next page*)

Continued

PCR for dCas9-VP64_Blast:

Reagent	Final concentration / amount	Amount
Nuclease-free water	n/a	Up to 25 µl
Total	n/a	**25 µl**

*sequence of forward primer (Table S1): 5′-CATCGAGCAGATCAGCGAGT-3′
**sequence of reverse primer (Table S1): 5′-CGATCCGTGTCTCGTACAGG-3′

PCR for MS2-p65-HSF1_Hygro

Reagent	Final concentration / amount	Amount
10× DreamTaq Buffer	1×	2.5 µl
dNTP Mix (10 mM each)	0.2 mM	0.5 µl
MS2-p65-HSF1_F primer (10 µM)*	0.4 µM	1 µl
MS2-p65-HSF1_R primer (10 µM)**	0.4 µM	1 µl
Template DNA (genomic DNA from clones, or genomic DNA from non-transduced mESCs, or lenti MS2-p65-HSF1_Hygro plasmid DNA)	0.2–1 µg for genomic DNA samples; 0.5–1 ng for plasmid DNA	Variable
Nuclease-free water	n/a	Up to 25 µl
Total	n/a	**25 µl**

*sequence of forward primer (Table S1): 5′-AAGCCTGAACTCACCGCTAC-3′
**sequence of reverse primer (Table S1): 5′-TTGGGAATCCCCGAACATGG-3′

c. Run PCRs with the following conditions:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	1 min	1
Denaturation	95°C	30 s	35
Annealing	58°C	1 min	
Extension	72°C	1 min	
Final Extension	72°C	5 min	1
Hold	4°C	Forever	

Note: if alternative DNA polymerases are used, follow manufacturer's instructions for cycling conditions.

d. Agarose gel analysis: run PCR products in a 1% agarose gel with HyperLadder IV (Biolane, BIO-33029). The expected band size for a clone expressing dCas9-VP64_Blast is 275 bp and for MS2-p65-HSF1_Hygro is 672 bp. No bands should be observed in the PCR product from genomic DNA of non-trasduced mESCs (Figure 1).

13. Verify dCas9-VP64 and MS2-p65-HSF1 mRNA expression by q-RT-PCR in clones containing both dCas9-VP64_Blast and MS2-p65-HSF1_Hygro genomic integrations, as identified in step 12d, using the RNA extracted in step 11. Include non-transduced mESCs as a negative control.
    a. Treat 2 µg of RNA from each sample with DNaseI (Thermo Fisher Scientific, EN0521), following manufacturer's instructions, to remove any contaminating genomic DNA.
    b. Perform cDNA synthesis from 0.5 µg DNaseI-treated total RNA from each sample using RevertAid First-Strand cDNA synthesis kit (Thermo Fisher Scientific, K1622), following manufacturer's instructions with the following specifications:
        i. Template RNA: 0.5 µg of DNaseI-treated total RNA.

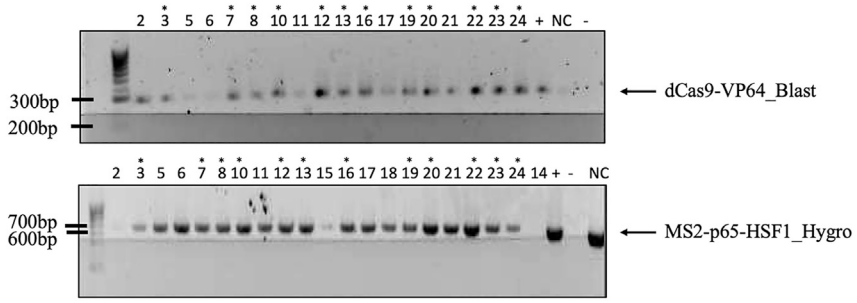

Figure 1. Screening for genomic integrations of dCas9-VP64_Blast and MS2-p65-HSF1 by PCR and agarose gel electrophoresis (step 12d)

Agarose gel electrophoresis (1% agarose) showing the products resulting from PCR on genomic DNA from selected clones using the primers and conditions described in step 12, designed to amplify a region within dCas9-VP64_Blast integration (top panel) and a region within MS2-p65-HSF1_Hygro integration (bottom panel). The ladder used is HyperLadder IV (Biolane, BIO-33029); the numbers reflect different clones; the PCR positive controls (+) were plasmid DNA of dCas9-VP64_Blast (top panel) or plasmid DNA of MS2-p65-HSF1_Hygro (bottom panel); the PCR negative controls (-) were genomic DNA from the parental line (non-transduced) E14 mESCs; non-clonal (NC) shows the PCR product from the respective polyclonal line. The expected band size for a clone containing dCas9-VP64_Blast integrations is 275 bp and for MS2-p65-HSF1_Hygro is 672 bp. Asterisks above numbers indicate that those clones show clear bands of the right size to be selected for both dCas9-VP64_Blast and MS2-p65-HSF1_Hygro integrations. See also Table S1 for primer sequences.

ii. Primer: random hexamer.

c. Dilute the cDNA from each sample by adding 180 µl of nuclease-free water to the 20 µl of cDNA obtained in step 13b, to a total volume of 200 µl.

d. Perform a q-RT-PCR using Brilliant III SYBR master mix (Agilent Technologies, 600882), following manufacturer's instructions. Set up four reactions for each cDNA with the following primers, in technical triplicates (Table S1 for primer sequences):

i. *Gapdh* (house-keeping gene control):
- Forward primer: 5′-GGTGGTGAAGCAGGCATCT-3′
- Reverse primer: 5′-CGGCATCGAAGGTGGAAGA-3′

ii. *CycloB1* (house-keeping gene control):
- Forward primer: 5′-GACAGACAGCCGGGACAAGC-3′
- Reverse primer: 5′-GGGGATTGACAGGACCCACA-3′

iii. dCas9-VP64:
- Forward primer: 5′-AACCTATGCCCACCTGTTCG-3′
- Reverse primer: 5′-AGGATTGTCTTGCCGGACTG-3′

iv. MS2-p65-HSF1:
- Forward primer: 5′-CTGGGAGAGGGCTCCTACTT-3′
- Reverse primer: 5′-TCATGGTTGGGCCAGGATTC-3′

e. Quantify relative levels of dCas9-VP64 and MS2-p65-HSF1 by the comparative CT method with normalization to average *Gapdh* and *CycloB1* levels. Clones with high expression of dCas9-VP64 and MS2-p65-HSF1 should have a fold change of >1000 in relative expression to non-transduced mESCs (Figure 2). These are referred to as SAM mESC clonal lines.

14. Although not strictly necessary, we highly recommend performing RNA-seq on 2–3 clones selected as positive for genomic DNA integrations and mRNA expression of both dCas9-VP64 and MS2-p65-HSF1, as well as non-transduced mESCs as a control. This experiment will help ensuring that the selected SAM mESC clonal line to be used for screening is similar to the parental line and global gene expression has not been altered as a result of dCas9-VP64_Blast and MS2-p65-HSF1_Hygro lentiviral transduction and antibiotic selection.

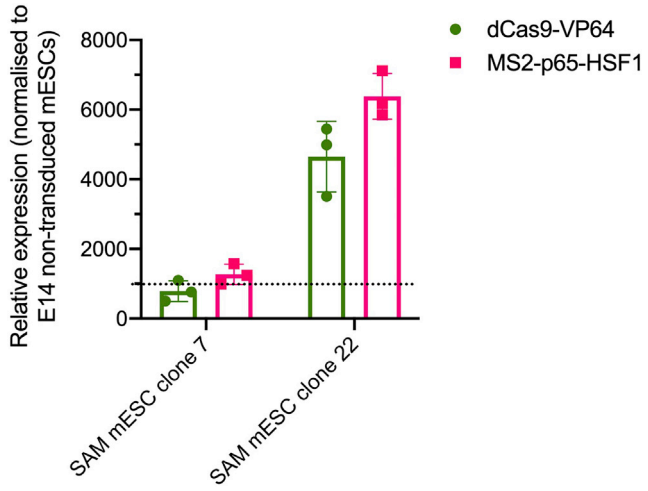

Figure 2. dCas9-VP64 and MS2-p65-HSF1 expression of two SAM mESC clones compared to the parental line E14, analyzed by q-RT-PCR (step 13e)

Analysis of dCas9-VP64 (green) and MS2-p65-HSF1 (pink) relative mRNA levels by q-RT-PCR in two different SAM mESC clones against its parental (non-transduced) cell line E14. Each clone was analyzed in three experimental replicates and the q-RT-PCR performed in two technical replicates. Data are shown as relative expression to the parental line E14 mESCs, after normalization of each transgene's CT values to average CT values of the house-keeping genes *Gapdh* and *CycloB1*. Each dot represents the value for each experimental replicate, and bars represent mean plus and minus standard deviation. The dotted horizontal line at y=1000 indicates the minimum relative expression of both dCas9-VP64 and MS2-p65-HSF1 that a clone should have to be selected for further characterization and/or use in a screen. In this example, clone 22 was selected over clone 7. See also Table S1 for primer sequences.

a. Construct RNA-seq libraries using the TruSeq Stranded Total RNA Library Prep Human/Mouse/Rat Kit (Illumina, 20020596), starting with 1 μg of DNaseI-treated RNA and following manufacturer's instructions.
b. Sequence at a coverage of 10–20 million reads per sample, 50 bp single-end reads.
c. Verify dCas9-VP64 and MS2-p65-HSF1 expression in the positive clones, compared to non-transduced mESCs (Figure 3).
d. Perform differential gene expression analysis using DESeq2 and EdgeR for each clone, normalized to non-transduced mESCs.
e. Select a clone with none or few differentially expressed genes compared to non-transduced mESCs (Figure 3). This clone will be used to perform the screen (referred to as SAM mESCs).

Optional: before embarking on a screen, we highly recommend performing pilot studies by testing CRISPRa with individual sgRNAs targeting 2–5 genes of interest, as well as non-targeting controls, in 2–3 selected SAM mESC clones. After doing these experiments, the screen can be performed in the clone that shows the most robust and highest CRISPRa efficiencies. To do that, 1) design and clone individual sgRNAs into the lentiviral backbone CROP-sgRNA-MS2 (Addgene #153457), following the steps 69–80 described in Joung et al., 2017; 2) generate sgRNA lentiviruses, following steps 1–5 of this protocol; 3) titer sgRNA lentiviruses in a clone of SAM mESCs, following steps 15–27 of this protocol; 4) transduce and select sgRNA lentiviruses targeting genes of interest as well as non-targeting controls at a MOI<0.3 into the different SAM mESC clones that are to be tested, following steps 28–33 of this protocol; 5) harvest RNA 7–10 days after sgRNA transduction, following steps 10b and 11 of this protocol; 6) perform q-RT-PCR with custom primers and/or RNA-seq, following steps 13 and 14 of this protocol, to compare levels of target gene activation across the different SAM mESC clones, normalized to respective non-targeting sgRNA samples. Here, we provide some positive

control sgRNA sequences that induce high and robust target gene activation in SAM mESCs, as well as non-targeting control sgRNAs:

Target gene	sgRNA protospacer	Mean log_2 fold change expression to non-targeting controls in 10× Genomics scRNA-seq screen data described in Alda-Catalinas et al., 2020
Dppa3	GAACTGGCTGGGATTGCGCA	1.83
Plac8	ATTTGGTAAGAGATGGCTTT	1.24
Carhsp1	TCCAGCCGCTGCCGAGTCCC	0.94
E2f5	GGCAGTCGGGTTCCCTCAGG	0.76
Lin28a	GTCAGAGACCAGAGCAGTGG	0.64
Stat3	TAAGGAATGGCCAGCTGGCT	0.63
Ncoa3	GGAGGTGAAGAGGACGTTCT	0.60
Smarca5	GCGTAGGTAGCTGTCCTGGT	0.50
Dppa2	ACACAGGAGGACCCTCCCTC	0.50
Ezh2	TTCGGAGCGAGCTCCAGCCC	0.46
Non-targeting control	GCTTTCACGGAGGTTCGACG	N.A
Non-targeting control	ATGTTGCAGTTCGGCTCGAT	N.A

Optional: if special conditions are to be applied during the screen, such as differentiation protocols or drug treatments, it is recommended that these conditions are tested across different SAM mESC clones in comparison to parental mESCs, to verify that the selected clones behave as expected.

Lentiviral titration of custom sgRNA library in SAM mESCs

Timing: 1–2 weeks

After a clone of SAM mESCs is selected for the screen, the functional titer of the lentiviral custom sgRNA library needs to be calculated in the selected clone so that the screen is then performed at a low MOI.

15. If required, thaw the selected clone of SAM mESCs, passage at least twice and culture in serum/LIF media containing 20 μg/mL blasticidin (InvivoGen, ant-bl-1) and 200 μg/mL hygromycin (InvivoGen, ant-hg-1) for 5 days before proceeding to the next step, to ensure expression of dCas9-VP64 and MS2-p65-HSF1.
16. Trypsinize a confluent plate of SAM mESCs and count the cell suspension. In preparation for seeding 120,000 cells per well in 780 μl of serum/LIF culture media in 8 gelatinized wells of a 24-well plate, count 1.08 million cells (9× 120,000 cells, i.e., counting for an extra well to account for pipetting errors) and spin down 300 × *g* for 3 min at room temperature (20°C–25°C). Discard supernatant and resuspend cell pellet in 7.02 mL of serum/LIF culture media (9× 780 μl).
17. Add polybrene (Millpore, TR-1003-G) to the 7.02 mL of cell suspension to a final concentration of 8.2 μl/mL.
18. Add 780 μl of cell suspension + polybrene per well to 8 gelatinized wells of a 24-well plate.
19. Thaw 1 aliquot of the lentiviral custom sgRNA library from step 5e on ice.
20. Add custom sgRNA library lentivirus and/or additional serum/LIF culture media to individual wells of the 24-well plate of SAM mESCs prepared in step 18, as follows:
    a. Well 1: 20 μl lentivirus
    b. Well 2: 10 μl lentivirus + 10 μl serum/LIF media
    c. Well 3: 5 μl lentivirus + 15 μl serum/LIF media

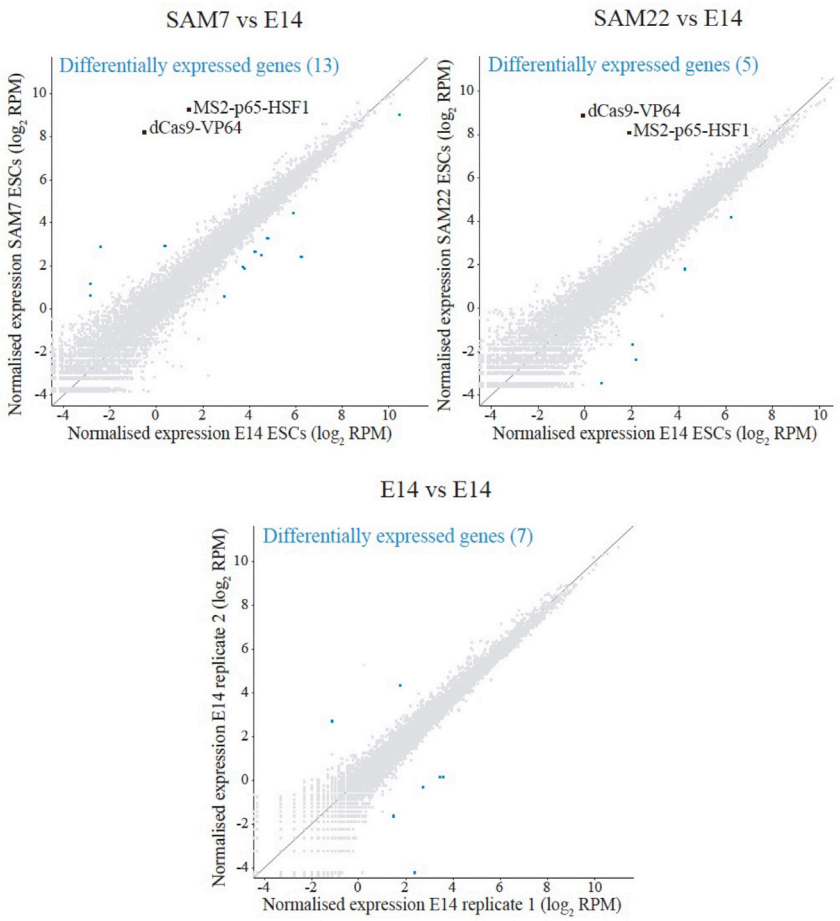

Figure 3. Transcriptome of two SAM mESC clones compared to the parental line E14 (step 14)

Scatterplots showing normalized gene expression in log_2 reads per million (RPM) of two different SAM mESC clones against its parental (non-transduced) cell line E14 (top panels), as well as scatterplot between two replicates of non-transduced E14 (bottom panel), analyzed by RNA-sequencing, highlighting dCas9-VP64 and MS2-p65-HSF1 transcripts in black and differentially expressed endogenous genes (DEGs) in blue. The transcriptome analysis was done with two replicates of each SAM mESC clone and three replicates of E14. DEGs were determined using DESeq2 (FDR<5%), EdgeR (FDR<5%) and intensity difference filter (FDR<5%), with the high-confidence DEGs defined as the intersection between the three statistical tests and a log_2 fold change >2. In this example, clone 22 was selected over clone 7 due to having less DEGs and, by q-RT-PCR, showing higher relative expression levels of dCas9-VP64 and MS2-p65-HSF1 (see also Figure 2).

d. Well 4: 2.5 μl lentivirus + 17.5 μl serum/LIF media
e. Well 5: 1.25 μl lentivirus + 18.75 μl serum/LIF media
f. Well 6: 0.625 μl lentivirus + 19.375 μl serum/LIF media
g. Well 7: 20 μl serum/LIF media. This non-transduced well will be used as a +antibiotic (puromycin) selection or kill control.
h. Well 8: 20 μl serum/LIF media. This well will be used as a non-transduced no-antibiotic control.

Note: the final volume in each well is 800 μl, making the final concentration of polybrene 8 μl/mL.

21. Mix each well thoroughly by pipetting up and down and return plate to incubator (37°C in 5% CO_2).

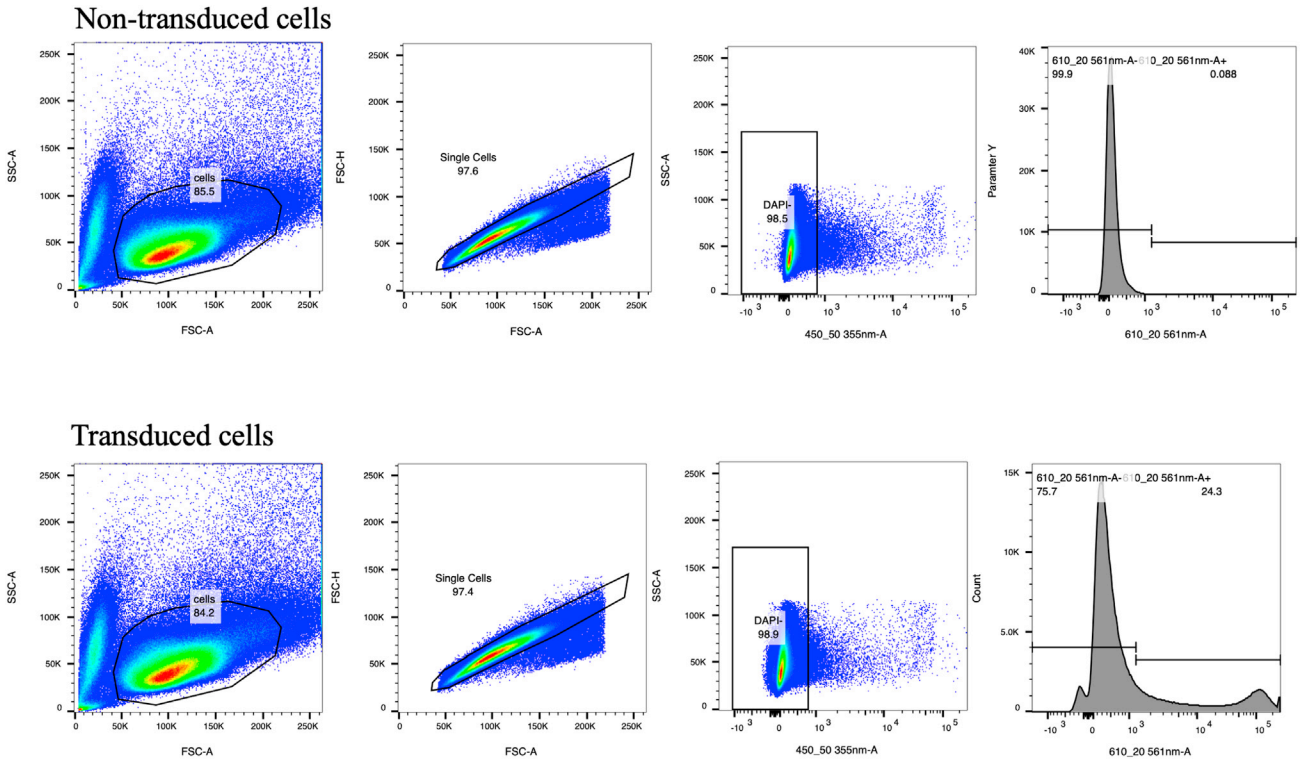

Figure 4. Flow cytometry gating strategy (steps 25, 27, 31 g, and 32 b)

Flow cytometry scatterplots and histograms showing a representative gating strategy for steps 25, 27, 31 g, and 32 b. After gating on single cells, DAPI$^-$ cells are selected (viable cells) and, within those, mCherry fluorescence is analyzed. mCherry$^+$ gate should be set based on a control of non-transduced cells.

22. After 24 h, remove media from each well, wash twice with PBS and add 800 µl of fresh serum/LIF culture media. Troubleshooting 1
23. Approximately 24 h later (approximately 48 h after transduction), trypsinize and expand cells from each individual well to a gelatinized well from a 6-well plate with 2 mL of serum/LIF media per well.
24. Approximately 24 h later (approximately 72 h after transduction), prepare cells for mCherry flow cytometry analysis:
    a. Prepare ice-cold PBS containing 0.1% FBS.
    b. Trypsinize cells.
    c. Re-plate half of the cells per condition in a gelatinized well of a 6-well plate. These cells will be used to test antibiotic selection.
    d. With the rest of the cells, count approximately 0.5 million cells per condition and spin them down at 300 × *g* for 3 min at room temperature (20°C–25°C). Resuspend pellet in 500 µl ice-cold PBS containing 0.1% FBS.
    e. Pass cells through a 40 µm cell strainer (Falcon, 352340) into a 5 mL round bottom tube for flow cytometry analysis.
    f. Add 1 µg/mL of DAPI and incubate for 15 min at room temperature (20°C–25°C).
25. Perform flow cytometry analysis gating on live cells expressing the lentiviral custom sgRNA library (DAPI$^-$/mCherry$^+$). Set the mCherry$^+$ gate using the non-transduced no-antibiotic control condition, calculate the percentage of mCherry$^+$ cells and determine the MOI of each transduced condition (Figures 4 and 5). For the purpose of this protocol, we calculate MOI of the custom sgRNA library as the percentage of mCherry$^+$ cells at this step, i.e., 10% mCherry$^+$ cells translates into an MOI of 0.1.

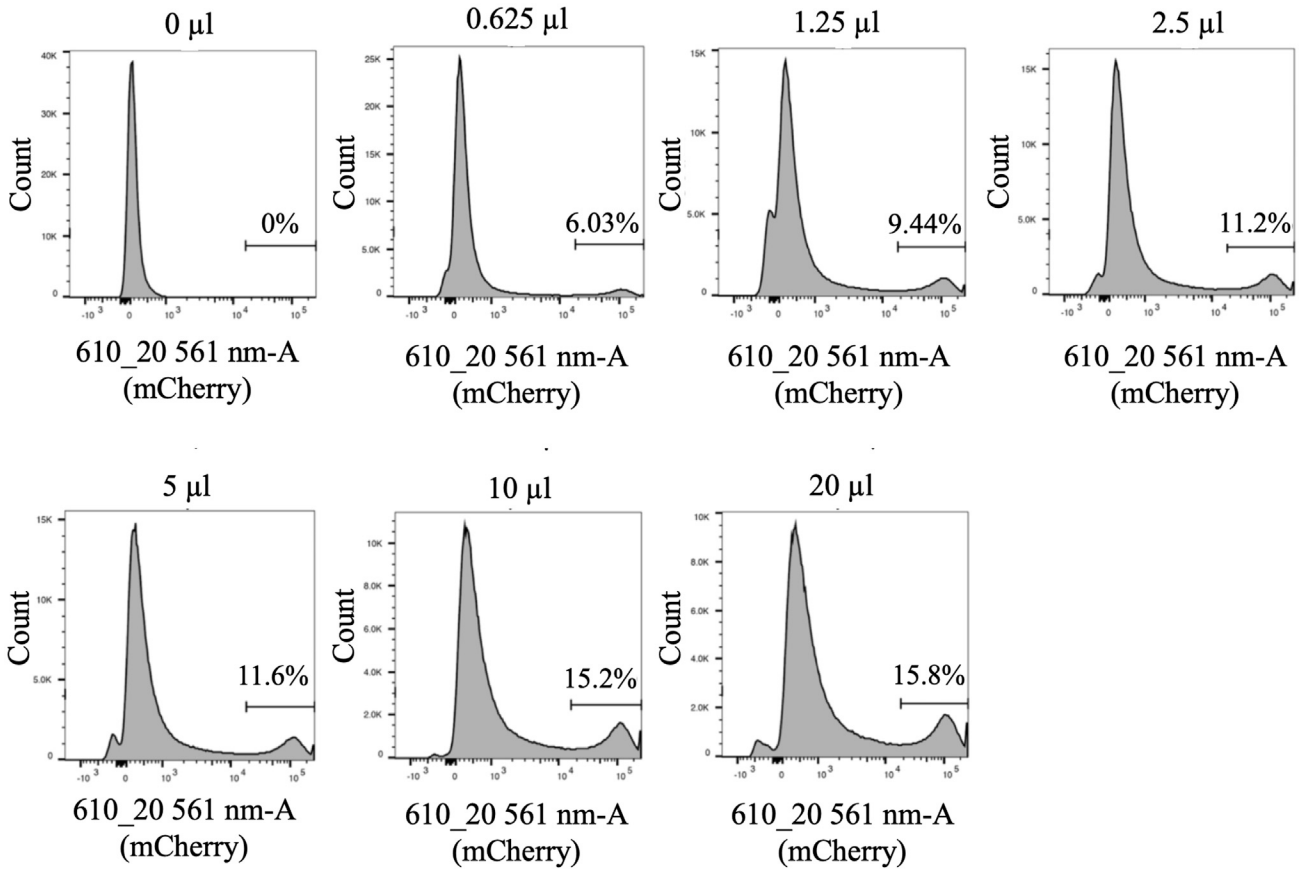

Figure 5. mCherry histograms for lentiviral custom sgRNA library titration, analyzed by flow cytometry (step 25)

Flow cytometry histograms showing expression of mCherry in SAM mESCs transduced with different volumes of a custom sgRNA library cloned into CROP-sgRNA-MS2 backbone, analyzed three days after transduction and prior to puromycin selection; percentages of cells transduced (expressing mCherry) are shown.

26. To verify selection of transduced cells, 4–5 h after step 24c (or once cells have settled down and attached to the plate), replace media with serum/LIF media containing 1 µg/mL puromycin (InvivoGen, ant-pr-1) to the wells with transduced cells and to the non-transduced +antibiotic control condition. Do not add puromycin to the non-transduced no-antibiotic control well.
27. The transduced cells have been selected for expression of the custom sgRNA library when there are no viable cells in the non-transduced +antibiotic control condition (likely to occur 3–4 days after addition of puromycin). To verify selection, repeat the flow cytometry analysis following steps 24–25. At this point, all transduced conditions should have 95%–100% mCherry^{+} cells (Figure 6). Troubleshooting 4

Lentiviral transduction and screening

Timing: 2 weeks to several weeks depending on screening conditions

After titrating the lentiviral custom sgRNA library, the library is transduced into SAM mESCs at a MOI<0.3 and the screen is carried out in basal conditions (serum/LIF media) or applying specific screening conditions. These conditions should be optimized on a case-by-case basis. Here, we describe the steps to perform CRISPRa screens in basal conditions:

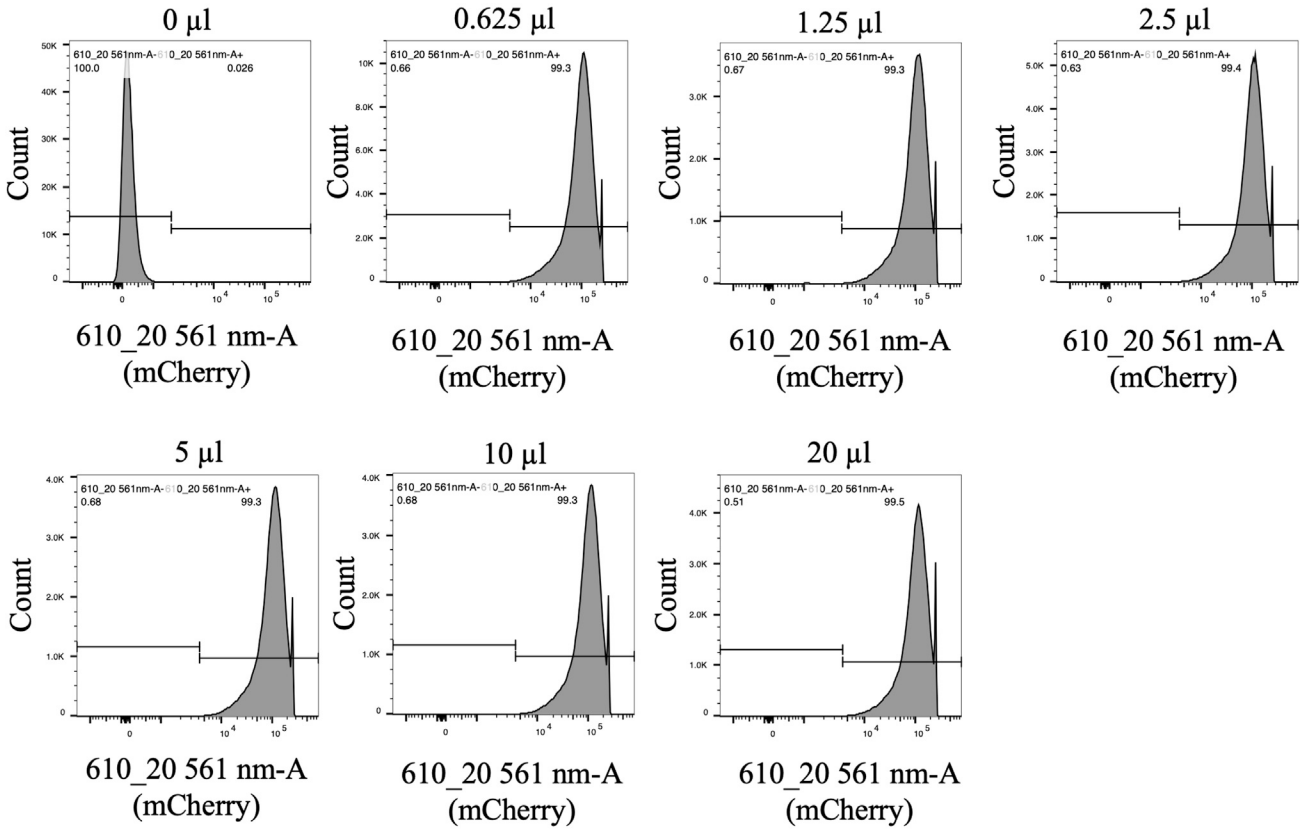

Figure 6. mCherry histograms for lentiviral custom sgRNA library titration and puromycin selection, analyzed by flow cytometry (step 27)

Flow cytometry histograms showing expression of mCherry in SAM mESCs transduced with different volumes of a custom sgRNA lentiviral library cloned into CROP-sgRNA-MS2 backbone, and selected for 4 days with 1 µL/mL puromycin; percentages of cells transduced and selected (expressing mCherry) are shown.

28. If required, thaw SAM mESCs, passage at least twice and culture in serum/LIF media containing 20 µg/mL blasticidin (InvivoGen, ant-bl-1) and 200 µg/mL hygromycin (InvivoGen, ant-hg-1) for 5 days prior to commencing screening to ensure expression of dCas9-VP64 and MS2-p65-HSF1.
29. Calculate the number of cells needed for transduction to maintain a representation of 1000 cells per sgRNA at a MOI<0.3. For example: for a library of 500 sgRNAs, a total of 500,000 cells are needed to maintain library representation throughout the screen; considering a MOI of 0.1 (i.e., 10% mCherry$^+$ cells after transduction and prior to puromycin selection), 5 million cells need to be transduced so that, after antibiotic selection, 500,000 cells remain.

Note: while a final representation of 500 cells per sgRNA might suffice, an additional 0.4–1 million cells are required for the flow cytometry analyses described in steps 31 and 32b; therefore, we recommend, if possible, to aim for a representation of 1000 cells per sgRNA.

30. Transduce the lentiviral custom sgRNA library at a MOI<0.3 into the number of SAM mESCs calculated in step 29. For a medium-scale screen (library of approximately 500 sgRNAs), we recommend using a 150 mm gelatinized cell culture plate with a total of 20 mL of serum/LIF culture media.
    a. Calculate the volume of lentivirus required to transduce the required number of cells at a MOI<0.3 (i.e., <30% mCherry$^+$ cells after transduction and prior to puromycin selection), based on calculations performed in step 25.

Note: for instance, if for the titration experiment described in steps 15–25 with 120,000 mESCs, 10 μl of lentivirus resulted in a MOI of 0.15 (i.e., 15% mCherry$^+$ cells), and the number of cells calculated for transduction in step 29 was 5 million, the amount of lentivirus to use in this step would be (5 × 10^6 cells × 10 μl) / 1.2 × 10^5 cells in titration experiment = 416.67 μl.

b. Thaw the required number of aliquots of the lentiviral preparation of custom sgRNA library cloned into CROP-sgRNA-MS2, from step 5e, on ice.
c. Trypsinize a confluent plate of SAM mESCs, count the cell suspension and obtain the number of cells calculated in step 29. Spin down 300 × *g* for 3 min at room temperature (20°C–25°C), discard supernatant and resuspend pellet in 1 mL of serum/LIF media.

⚠ CRITICAL: we recommend using low-passage SAM mESCs (<10 passages), especially if differentiation protocols are to be applied during screening.

d. To the resuspended cell pellet, add the volume of lentivirus calculated in step 30a. Mix thoroughly by pipetting up and down.
e. Top up with serum/LIF media to the appropriate final volume to be used in the tissue culture plate (see step 4 of "before you begin" section for appropriate final volumes in different cell culture plates).
f. Add 8 μg/mL polybrene (Millpore, TR-1003-G) and mix gently by pipetting.
g. Seed cells resuspended in the lentiviral mix + polybrene in the appropriate gelatinized cell culture plate. Return plate to incubator (37°C in 5% CO_2).
h. Plate two additional 100 mm plates with 2.5 million SAM mESCs to be used as 1) non-transduced +antibiotic selection control and 2) non-transduced no-antibiotic control. Add 8 μg/mL polybrene (Millpore, TR-1003-G) and mix gently.
i. 24 h after transduction, remove media, wash twice with PBS and add fresh serum/LIF culture media.

31. After 48 h (72 h after transduction), verify low MOI. Prepare both the transduced cells and the non-transduced no-antibiotic control for mCherry flow cytometry analysis:
    a. Prepare ice-cold PBS containing 0.1% FBS.
    b. Trypsinize cells.
    c. Count 0.2–0.5 million cells to be used for flow cytometry analysis and re-plate the rest of the cells in an appropriate gelatinized cell culture plate in serum/LIF media.
    d. With the cells to be used for flow cytometry analysis, spin them down at 300 × *g* for 3 min at room temperature (20°C–25°C). Resuspend pellet in 200–500 μl ice-cold PBS containing 0.1% FBS.
    e. Pass cells through a 40 μm cell strainer (Falcon, 352340) into a 5 mL round bottom tube for flow cytometry analysis.
    f. Add 1 μg/mL of DAPI and incubate for 15 min at room temperature (20°C–25°C).
    g. Perform flow cytometry analysis gating on live cells expressing the lentiviral custom sgRNA library (DAPI$^-$/mCherry$^+$). Set the mCherry$^+$ gate using the non-transduced control (Figure 4). Verify MOI<0.3 based on the percentage of mCherry$^+$ cells.

32. Select cells with puromycin for expression of the custom sgRNA library:
    a. 4–5 h after step 31c (or once cells have settled down and attached to the plate) and after verifying cells were transduced at a MOI<0.3, replace media with serum/LIF media containing 1 μg/mL puromycin (InvivoGen, ant-pr-1) to the transduced cells and to non-transduced +-antibiotic control condition. Do not add puromycin to the non-transduced no-antibiotic control.
    b. The transduced cells have been selected for expression of the custom sgRNA library when there are no viable cells in the non-transduced +antibiotic control condition (likely to occur 3–4 days after addition of puromycin). To verify selection, repeat the flow cytometry analysis

following step 31. At this point, 95%–100% of cells from the transduced condition should be mCherry^{+}. Troubleshooting 4

33. After selection is complete, proceed to step 34. Alternatively, depending on screening conditions, cells can be cultured for longer, subjected to differentiation protocols, drug treatments, or similar.

Note: We have tested efficient target gene activation by CRISPRa SAM as early as day 6 post-sgRNA transduction and up to 15 days post-sgRNA transduction. However, the optimal time point to harvest cells in the screen needs to be determined in pilot studies using sgRNAs targeting genes of interest with known biological function, by testing the optimal time point of target gene activation and expected downstream transcriptional changes.

Harvest of perturbed cells and sample preparation for 10× Genomics scRNA-seq

Timing: 1–1.5h

Cells are harvested and prepared to run in the 10× Genomics Chromium Controller. It is critical to ensure a clean and highly viable single-cell suspension for optimal formation of single-cell droplets or GEMs.

34. Prepare ice-cold PBS containing 0.1% FBS, and cool-down a centrifuge with 15 and/or 50 mL tube buckets to 4°C.
35. Remove media and wash cells twice with room temperature (20°C–25°C) PBS.
36. Trypsinize cells very briefly (2–3 min) and then add serum/LIF media.
    a. For a 100 mm plate, use 1 mL of trypsin and 3 mL of serum/LIF media.
    b. For a 150 mm plate, use 3 mL of trypsin 9 mL of serum/LIF media.

⚠ CRITICAL: do not trypsinize cells for any longer than necessary as this can affect viability.

37. Gently mix cells by pipetting up and down to obtain a single-cell suspension. Check cells are properly detached and dissociated under a microscope.

⚠ CRITICAL: clumps of cells will lead to blockages in the 10× Genomics Chromium Controller run, therefore, it is critical to ensure colonies are well dissociated.

38. Further inactivate trypsin with extra serum/LIF media and gently mix by pipetting up and down.
    a. For a 100 mm plate, use 6 mL of serum/LIF media to obtain a final single-cell suspension of 10 mL.
    b. For a 150 mm plate, use 13 mL of serum/LIF media to obtain a final single-cell suspension of 25 mL.
39. Transfer cell suspension to a 15 or 50 mL tube and spin down 300 × *g* for 3 min at 4°C.
40. Resuspend cell pellet in 1 mL of ice-cold PBS containing 0.1% FBS.
41. Pass cell suspension through a 40 μm cell strainer (Falcon, 352340) into a new 15 mL tube.
42. Repeat step 41.

⚠ CRITICAL: steps 40 and 41 are critical to ensure a clean single-cell suspension.

43. Keep cells on ice and count cell suspension using a viability dye. Make appropriate dilutions for counting if needed.

Note: we recommend counting cells using an automated cell counter with a viability read-out.

⚠ CRITICAL: aim for very accurate cell counting. We recommend counting three different aliquots of each cell suspension/dilution, with two counts for each aliquot.

⚠ CRITICAL: do not proceed with cell suspensions that show <90% viability as this will result in poor scRNA-seq results. Troubleshooting 5

44. Dilute counted cell suspension in PBS containing 0.1% FBS, or concentrate cells by centrifugation (300 × *g* for 3 min at 4°C) to obtain a cell suspension of 700–1200 cells /mL, in ice-cold PBS containing 0.1% FBS. Keep cells on ice. Immediately proceed to step 45.

⚠ CRITICAL: to maintain >90% viability, it is critical to work fast during sample counting and dilution and to not exceed 1.5h from cell trypsinization (step 36) to loading in the 10× Genomics Chromium Controller (step 45). It is also critical to keep cell suspensions on ice.

Construction of 10× Genomics scRNA-seq libraries and sgRNA amplicon libraries

Timing: 2–4 days

3′ scRNA-seq 10× Genomics libraries are constructed following manufacturer's instructions. Additionally, a custom PCR enrichment is performed to amplify sgRNA-containing fragments and construct sgRNA amplicon libraries. Targeted amplification of sgRNAs is based on the strategy described in Hill et al., 2018, which improves the sgRNA-cell assignment rate.

Note in Alda-Catalinas et al., 2020, 10× Genomics single cell 3′ v2 kits were used to build the scRNA-seq libraries. However, these kits are no longer available and so the latest version of 3′ kits (v3.1 at the time of publication) need to be used (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry).

45. Run the single-cell suspension generated in step 44 in the 10× Genomics Chromium Controller using 3′ v3.1 kits and protocol (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry), following manufacturer's instructions.

⚠ CRITICAL: the number of single cells that need to be sequenced and, consequently, the number of cells to load per lane in the 10× Genomics Chromium chip and total number of lanes/chips to run, depends on screening conditions and downstream transcriptional response to be captured. In Alda-Catalinas et al., 2020, we generated 10× Genomics scRNA-seq libraries for on average 437 cells per sgRNA. This number was calculated using pilot studies. We recommend performing similar pilot studies for the biological process of interest, or to use prior knowledge, to determine the number of cells that need to be sequenced per sgRNA to have sufficient coverage of the expected transcriptional response. This is crucial to determine the total number of perturbed cells that need to be loaded across one or multiple 10× Genomics Chromium chips.

46. Follow the manufacturer' s protocol (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry) up to step 2.4a (cDNA quality control and quantification).
47. Following cDNA quantification, for each 10× Genomics sample/lane, set aside 10 ng of the full-length cDNA obtained after step 2.3m of the manufacturer's protocol. These 10 ng cDNA samples will be used to construct sgRNA amplicon libraries, as described in step 49 of this protocol.

48. Continue to build the 3′ gene expression libraries with 10 μL of the full-length cDNA , following steps 3.1–3.7 of the manufacturer' s protocol (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry).

49. Construct a sgRNA amplicon library for each 10× Genomics sample/lane, using the 10 ng full-length cDNA sample(s) saved in step 47 of this protocol. These libraries are built using three rounds of semi-nested PCR amplification, monitored by KAPA SYBR.

Note: the PCR reactions described below to build sgRNA amplicon libraries are compatible with full-length cDNA generated using 3′ scRNA-seq v2/v3/v3.1 10× Genomics kits, but might not be compatible with future versions of 3′ scRNA-seq kits if the chemistry is modified by 10× Genomics.

a. Prepare a master-mix for PCR1 on ice using the following reagents and volumes. Prepare a PCR reaction for each cDNA sample in a 96-well or 384-well plate:

sgRNA amplicon PCR1

Reagent	Final concentration / amount	Amount
Amp_sgRNA_1F primer (10 μM)*	1.5 μM	1.5 μl
Amp_sgRNA_1R primer (10 μM)**	1.5 μM	1.5 μl
2× KAPA HiFi Master Mix (Kapa Biosystems, KR0389)	1×	5 μl
Full-length cDNA from 10× Genomics protocol step 2.3m	10 ng	Variable
Nuclease-free water	n/a	Up to 10 μl
Total	n/a	**10 μl**

*sequence of forward primer (Table S1): TTTCCCATGATTCCTTCATATTTGC (primes an outer part of the U6 promoter).
**sequence of reverse primer (Table S1): CTACACGACGCTCTTCCGATCT (partial TruSeq Read 1 primer).

b. Run PCR in a q-RT-PCR-compatible instrument, with the following conditions:

PCR1 Cycling Conditions

Steps	Temperature	Time	Cycles
Initial Denaturation/ Enzyme activation	95°C	5 min	1
Denaturation	95°C	30 s	Variable (monitor amplification in the screen of the q-RT-PCR instrument; stop reaction before reaching amplification plateau to avoid PCR overcycling; in our experience, at 10–12 cycles) (Figure 7).
Annealing/Extension/Data acquisition	65°C	45 s	

c. Purify the resulting reactions using 1× AMPure XP beads (Beckman Coulter, A63881):

i. Obtain AMPure XP beads from 4°C storage and warm to room temperature (20°C–25°C) for 30 min.
ii. If required, recover the PCR products from the q-RT-PCR plate into PCR tube strips or into a 96-well plate.
iii. To each PCR product, add 10 μl of nuclease-free water.
iv. Vortex to resuspend the AMPure XP beads.
v. Add 20 μl AMPure XP beads (1×) to each sample and mix by pipetting up and down 10 times.
vi. Incubate 15 min at room temperature (20°C–25°C) to allow DNA to bind the beads.
vii. Place PCR tube strip or 96-well plate on an appropriate magnetic stand until the solution clears.
viii. Remove and discard supernatant(s).
ix. Add 100 μl of 80% ethanol to each pellet. Wait 30 s.
x. Remove the ethanol.

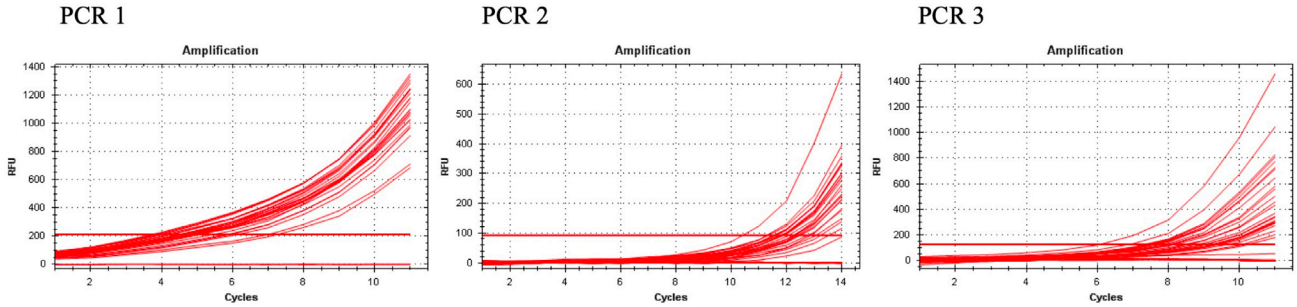

Figure 7. qPCR monitoring of sgRNA amplicon libraries (steps 49b, 49f, 49j)

Relative fluorescent units (RFU, y axis) vs number of qPCR cycles (x axis) in each of the three semi-nested PCRs on barcoded sgRNAs using 10× Genomics full-length cDNA of SAM mESCs transduced with a custom CROP-sgRNA-MS2 lentiviral library. Each line represents a different 10× Genomics cDNA sample obtained from the same cell suspension. qPCRs were performed with KAPA SYBR in a CFX384 Touch Real-Time PCR Detection System machine. Following amplification at real-time on the instrument screen, the reactions were stopped before reaching a steady state of amplification. See also Table S1 for primer sequences.

xi. Repeat ethanol wash (steps ix and x) for a total of 2 washes.

xii. Centrifuge PCR tube strip or 96-well plate briefly and place back on appropriate magnetic stand.

xiii. Remove any remaining ethanol from each sample and air dry for 1–2 min.

⚠ CRITICAL: Do not air dry for longer than 2 min; an over-dried pellet can result in decreased elution efficiency.

xiv. Remove PCR tube strip or 96-well plate from the magnet.

xv. Add 21 µl of nuclease-free water to each sample and mix by pipetting up and down 10 times.

xvi. Incubate 10 min at room temperature (20°C–25°C).

xvii. Place the PCR tube strip or 96-well plate on the magnet until the solution clears.

xviii. For each sample, transfer 20 µl of the supernatant containing the purified PCR product to a new PCR tube strip or well of 96-well.

d. Prepare 1:25 dilutions for each purified PCR1 product by adding 1 µl of sample to 24 µl of nuclease-free water. The remaining PCR1 sample can be stored long-term at −20°C.

e. Prepare a master-mix for PCR2 on ice using the following reagents and volumes. Prepare a PCR reaction for each sample in a 96-well or 384-well plate using as input 1 µl of the 1:25 dilution of purified PCR1 product from step 49d:

sgRNA amplicon PCR2

Reagent	Final concentration / amount	Amount
Amp_sgRNA_2F primer (10 µM)*	1.5 µM	1.5 µl
Amp_sgRNA_2R primer (10 µM)**	1.5 µM	1.5 µl
2× KAPA HiFi Master Mix (Kapa Biosystems, KR0389)	1×	5 µl
1:25 dilution of purified PCR1 product from step 49d	n/a	1 µl
Nuclease-free water	n/a	1 µl
Total	n/a	**10 µl**

*sequence of forward primer (Table S1): GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCTTGTGGAAAGGACGAAACAC (primes on the U6 promoter adjacent to the sgRNA protospacer sequence and adds a standard Nextera Read 2 primer).
**sequence of reverse primer (Table S1): AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC (adds the remainder of TruSeq Read 1 and the adapter P5).

f. Run PCR in a q-RT-PCR-compatible instrument, with the following conditions:

PCR2 cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation/ Enzyme activation	95°C	5 min	1
Denaturation	95°C	30 s	Variable (monitor amplification in the screen of the q-RT-PCR instrument; stop reaction before reaching amplification plateau to avoid PCR overcycling; in our experience, at 12–15 cycles) (Figure 7).
Annealing/Extension/Data acquisition	65°C	45 s	

g. Purify the resulting reactions using 1× AMPure XP beads (Beckman Coulter, A63881), following step 49c.

h. Prepare 1:25 dilutions for each purified PCR2 product by adding 1 μl of sample to 24 μl of nuclease-free water. The remaining PCR2 sample can be stored long-term at −20°C.

i. Prepare a master-mix for PCR3 on ice using the following reagents and volumes. Prepare a PCR reaction for each sample in a 96-well or 384-well plate using as input 1 μl of the 1:25 dilution of purified PCR2 product from step 49 h:

sgRNA amplicon PCR3

Reagent	Final concentration / amount	Amount
Nextera XT P7 index (10 μM)*. Use a separate index for each sample.	1.5 μM	1.5 μl
Amp_sgRNA_2R primer (10 μM)**	1.5 μM	1.5 μl
2× KAPA HiFi Master Mix (Kapa Biosystems, KR0389)	1×	5 μl
1:25 dilution of purified PCR2 product from step 49h	n/a	1 μl
Nuclease-free water	n/a	1 μl
Total	n/a	**10 μl**

*sequence of forward primer: Nextera XT P7 index in the form of: CAAGCAGAAGACGGCATACGAGAT-[8 bp Index]-GTCTCGTGGGCTCGG. These adaptors can be obtained from the Illumina kits "Nextera XT Index Kit" (Illumina, FC-131-2001, FC-131-2002, FC-131-2003, FC-131-2004 or FC-131-1001).

**sequence of reverse primer (Table S1): AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTC (same reverse primer from PCR2).

⚠ CRITICAL: to pool sgRNA amplicon libraries from different samples for multiplexed sequencing, follow Illumina's index adapters pooling guide (https://support.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/experiment-design/index-adapters-pooling-guide-1000000041074-10.pdf). If sgRNA amplicon libraries are going to be sequenced together with 3′ scRNA-seq gene expression libraries, make sure appropriate index sets are used to ensure no index overlap in the multiplexed pool.

j. Run PCR in a q-RT-PCR-compatible instrument, with the following conditions:

PCR3 cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation/ Enzyme activation	95°C	5 min	1
Denaturation	95°C	30 s	Variable (monitor amplification in the screen of the q-RT-PCR instrument; stop reaction before reaching amplification plateau to avoid PCR overcycling; in our experience, at 10–12 cycles) (Figure 7).
Annealing/Extension/Data acquisition	72°C	45 s	

k. Purify the resulting reactions using 1× AMPure XP beads (Beckman Coulter, A63881), following step 49c. These are the final sgRNA amplicon libraries.

l. Run 1 μL of each of the resulting sgRNA amplicon libraries at 1:10 dilution on an Agilent Bioanalyzer High Sensitivity chip (Agilent, #5067-4626), following manufacturer's instructions.

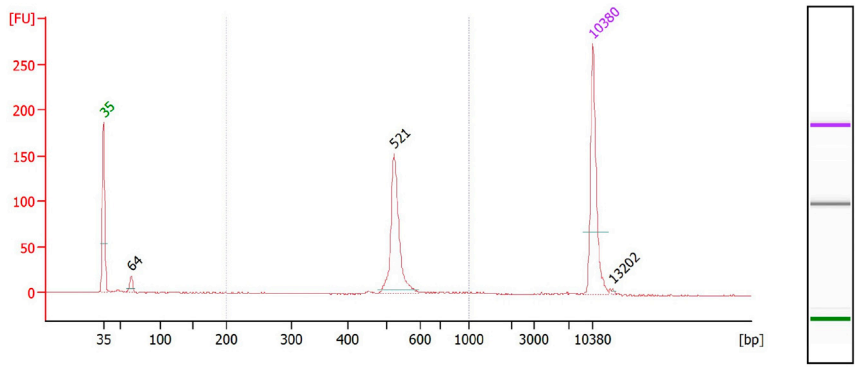

Figure 8. Bioanalyzer traces of final sgRNA amplicon libraries (step 49l)

Bioanalyzer electropherogram showing fluorescence units (FU, y axis) vs fragment size in base pairs (bp, x axis) of a representative sgRNA amplicon library of SAM mESCs transduced with a custom CROP-sgRNA-MS2 lentiviral library. Numbers above electropherogram peaks indicate the size in bp of detected fragments, including a lower molecular weight marker of 35 bp (green) and a higher molecular weight marker of 10,380 bp (purple). sgRNA amplicon libraries derived from cells transduced with CROP-sgRNA-MS2 constructs generate amplicons of approximately 520 bp.

The expected size of the sgRNA amplicon is approximately 520 bp (Figure 8). Troubleshooting 6

Sequencing

Timing: 1–4 days

The resulting 10× Genomics 3′ scRNA-seq gene expression libraries and sgRNA amplicon libraries are Illumina paired-end constructs which contain P5 and P7 adaptors, an 8 bp sample index, a 16 bp 10× Genomics cell barcode and a 12 bp UMI. The 3′ gene expression libraries contain TruSeq Read 1 and TruSeq Read 2 adapters as primers sites for paired-end sequencing, whereas the sgRNA amplicon libraries contain TruSeq Read 1 and Nextera Read 2 as primer sites. TruSeq Read 1 is used to sequence the 10× Genomics cell barcode and UMI using 28 cycles, both in the 3′ gene expression and sgRNA amplicon libraries. TruSeq Read 2 is used to read the 3′ end of transcripts using 91 sequencing cycles in the 3′ gene expression libraries, whereas Nextera Read 2 is used to read the sgRNA protospacer in the sgRNA amplicon libraries. The i7 read is used to sequence the sample index in both library types.

We recommend following 10× Genomics guidance to sequence 3′ gene expression libraries on an Illumina sequencer (https://support.10xgenomics.com/single-cell-gene-expression/library-prep/doc/user-guide-chromium-single-cell-3-reagent-kits-user-guide-v31-chemistry) with a minimum sequencing depth of 20,000 read pairs per cell (30,000 read pairs per cell is preferable) and 28 cycles for Read 1, 8 cycles for i7 index, 0 cycles for i5 index and 91 cycles for Read 2.

sgRNA amplicon libraries can be pooled together with 3′ gene expression libraries for sequencing, providing no overlapping sample indexes were used (see step 49i of this protocol). Sequencing coverage for sgRNA amplicon libraries should be a minimum of 5,000 read pairs per cell. If sgRNA amplicon libraries are sequenced separately, use appropriate percentage of PhiX as these libraries have low complexity. The same sequencing parameters are used for sgRNA amplicon libraries: 28 cycles for Read 1, 8 cycles for i7 index, 0 cycles for i5 index and 91 cycles for Read 2.

EXPECTED OUTCOMES

An example of a sequencing dataset obtained as a final outcome of a pooled CRISPRa screen in mESCs, analyzed by 10× Genomics scRNA-seq and described in Alda-Catalinas et al., 2020, can be found in GEO: GSE135621 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135621).

QUANTIFICATION AND STATISTICAL ANALYSIS

The complex scRNA-seq dataset generated as an output of this protocol requires extensive computational analysis. An example is detailed in Alda-Catalinas et al., 2020. Documented scripts for quality control, assignment of sgRNAs to cells and downstream analysis of this type of datasets can be found in https://github.com/gtca/crispra_zga (Alda-Catalinas et al., 2020). Other resources can be found in https://github.com/shendurelab/single-cell-ko-screens (Hill et al., 2018).

LIMITATIONS

A key consideration for this protocol to be successful is an optimal sgRNA design that allows target gene activation of the screening candidates. While in Alda-Catalinas et al., 2020 we designed the sgRNA library based on Konermann et al., 2015, we now recommend using the CRISPRa sgRNA design strategy described in Sanson et al., 2018, or the associated online design tool (https://portals.broadinstitute.org/gppx/crispick/public) for optimal sgRNA activity. We also recommend including 3–5 sgRNAs per target gene. However, despite all these considerations, a limitation of this protocol is that some candidate genes might not be successfully CRISPR-activated by any of their sgRNAs included in the custom sgRNA library and, therefore, their function cannot be interrogated.

Furthermore, although pooled CRISPR screening read-out by scRNA-seq allows for comprehensive and high throughput interrogation of candidate genes, the nature of single-cell sequencing might prevent detection of transcriptomic changes induced by weak regulators which might otherwise be detected by bulk RNA-seq (Alda-Catalinas et al., 2020). Similarly, if the transcriptional changes expected to be induced by screen hits involve lowly expressed genes, their associated transcripts might not be detected by scRNA-seq. Given these limitations, performing the pilot studies recommended in this protocol is critical for an optimal experimental design.

Lastly, we have only tested this protocol in undifferentiated and untreated SAM mESCs. Although it might be possible to perform screens in mESCs under differentiation conditions or under different drug treatments, we have not studied how SAM mESCs respond to these conditions.

TROUBLESHOOTING

Problem 1

There is considerable cell death in all conditions of the titration experiment (i.e., cells are floating in the media) 24 h after transduction, before starting antibiotic selection; this is likely due to very high lentiviral concentrations.

Potential solution

Repeat lentiviral titration starting with higher (less concentrated) lentiviral dilutions.

Problem 2

All cells in the non-transduced +antibiotic control condition either die within 1–3 days after addition of blasticidin/hygromycin or there is cell survival after 7–8 days of treatment with blasticidin/hygromycin; this is likely due to wrong antibiotic concentration.

Potential solution

In our hands, 20 μg/mL blasticidin and 200 μg/mL hygromycin are the optimal antibiotic concentrations for E14 mESCs, however, it is possible to optimize it on a case-by-case basis by performing a kill

curve for the antibiotic of interest. To construct a kill curve, titrate different concentrations of the antibiotic on non-transduced mESCs and choose the lowest concentration that kills all cells after 4–7 days.

Problem 3

No or very few resistant colonies are observed; this is likely due to low lentiviral titer.

Potential solution

Concentrate dCas9-VP64_Blast and MS2-p65-HSF1_Hygro lentiviruses obtained in step 4 by following step 5 (Lenti-X Concentrator; Takara, 631231). An alternative method for lentiviral concentration is ultracentrifugation at 88,000 × *g* for 2 h at 4°C.

Problem 4

Transduced cells are not selected (i.e., <95% $mCherry^+$ cells) after 4 days of puromycin treatment and/or there is incomplete cell death in the non-transduced +antibiotic control condition; this is likely due to wrong puromycin concentration.

Potential solution

In our hands, 1 μg/mL puromycin is the optimal concentration for E14 mESCs, however, it is possible to optimize it on a case-by-case basis by performing a puromycin kill curve. To construct a kill curve, titrate different concentrations of the antibiotic on non-transduced mESCs and choose the lowest concentration that kills all cells after 3–4 days.

Problem 5

The described 10× Genomics sample preparation procedure in steps 34–43 consistently results in <90% cell viability.

Potential solution

The described protocol should ensure high cell viabilities in mESC samples, however, if this is not the case, potential solutions include FACS-sorting on $DAPI^-$ cells or processing the sample with a dead cell removal kit (i.e., Miltenyi Biotec, 130-090-101).

Problem 6

The Bioanalyzer traces are flat and/or no ~520 bp peak is observed; this could be due to low concentration of libraries and/or poor amplification.

Potential solution

Run the undiluted libraries in Bioanalyzer; if a ~520 sharp peak is still not observed, go back to step 49a and repeat sgRNA amplicon library preparation with increased number PCR of cycles.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wolf Reik (wolf.reik@babraham.ac.uk).

Materials availability

CROP-sgRNA-MS2 plasmid has been deposited to Addgene (CROP-sgRNA-MS2, 153457).

Data and code availability

An example of a CRISPRa scRNA-seq dataset in mESCs (Alda-Catalinas et al., 2020) is accessible through GEO Series accession number (GSE135622; https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE135622). Code generated in Alda-Catalinas et al., 2020, which allows quality

control and analysis of CRISPRa scRNA-seq datasets is available in Github: https://github.com/gtca/crispra_zga

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2021.100426.

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AUTHOR CONTRIBUTIONS

C.A.-C designed, implemented, and wrote the protocol. C.A.-C., M.A.E.-M., and W.R. conceived, designed, and supervised the study. C.A.-C., M.A.E.-M., and W.R. reviewed and edited the protocol.

DECLARATION OF INTERESTS

W.R. is a consultant and shareholder of Cambridge Epigenetix. All other authors declare no competing interests.

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