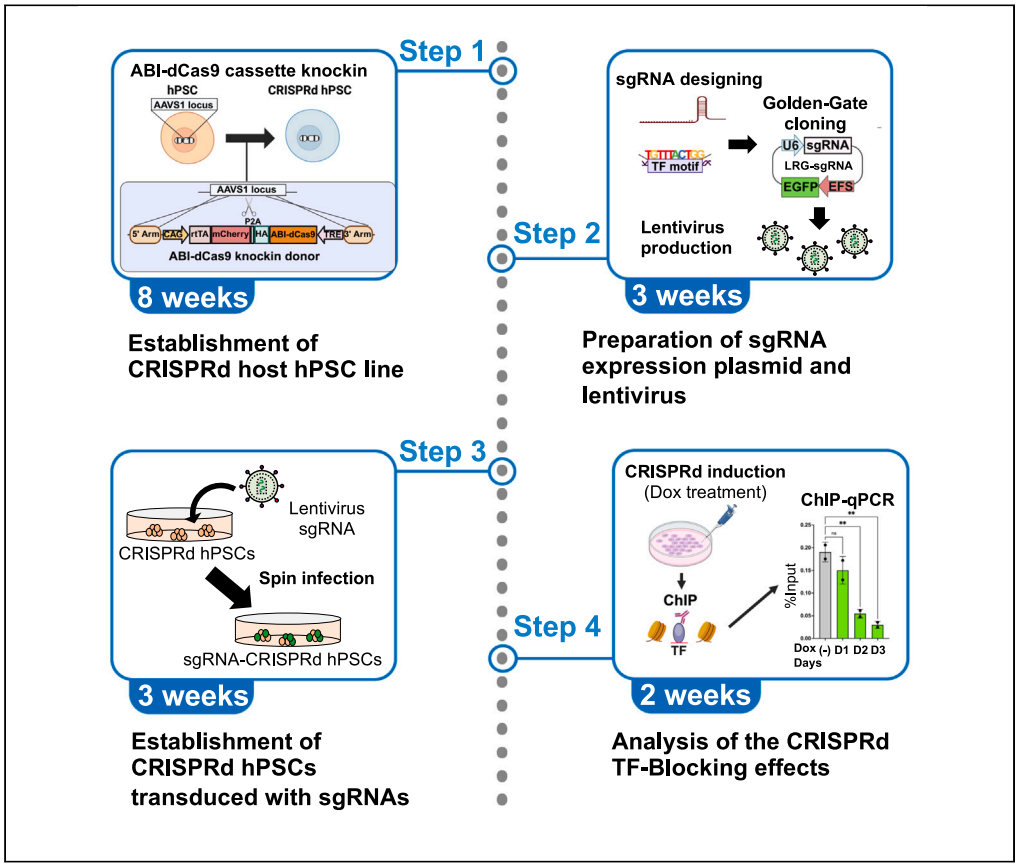
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for establishing inducible CRISPRd system for blocking transcription factor-binding sites in human pluripotent stem cells



Transcription factor (TF) gene knockout or knockdown experiments provide comprehensive downstream effects on gene regulation. However, distinguishing primary direct effects from secondary effects remains challenging. To assess the direct effect of TF binding events, we present a protocol for establishing a doxycycline (Dox)-inducible CRISPRd system in human pluripotent stem cells (hPSCs). We describe the steps for establishing CRISPRd host hPSCs, designing and preparing single-guide RNA (sgRNA) expression lentivirus vectors, generating CRISPRd hPSCs transduced with sgRNAs, and analyzing CRISPRd TF-block effects by chromatin immunoprecipitation (ChIP)-qPCR.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

Satoshi Matsui, Joseph R. Shiley, Morgan Buckley, Hee-Woong Lim, Yueh-Chiang Hu, Christopher N. Mayhew, Makiko Iwafuchi

matsui.satoshi.3p8@osaka-u.ac.jp (S.M.)
christopher.mayhew@cchmc.org (C.N.M.)
makiko.iwafuchi@cchmc.org (M.I.)

Highlights

Dox-inducible CRISPRd system to assess the direct effect of TF binding events

Protocol for generating CRISPRd hPSCs transduced with sgRNAs

FOXA-CRISPRd system blocks FOXA binding 2 days after Dox induction

Matsui et al., STAR Protocols 5, 103233
September 20, 2024
https://doi.org/10.1016/j.xpro.2024.103233

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocol for establishing inducible CRISPRd system for blocking transcription factor-binding sites in human pluripotent stem cells

Satoshi Matsui,[1,3,4,*] Joseph R. Shiley,[1,3] Morgan Buckley,[1,3] Hee-Woong Lim,[2] Yueh-Chiang Hu,[1] Christopher N. Mayhew,[1,4,*] and Makiko Iwafuchi[1,4,5,*]

[1]Division of Developmental Biology, Center for Stem Cell & Organoid Medicine, Cincinnati Children's Hospital Medical Center, Cincinnati, Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, OH 45229, USA

[2]Department of Biomedical Informatics, Cincinnati Children's Hospital Medical Center, Cincinnati, Department of Pediatrics, College of Medicine, University of Cincinnati, Cincinnati, OH 45229, USA

[3]These authors contributed equally

[4]Technical contact

[5]Lead contact

*Correspondence: matsui.satoshi.3p8@osaka-u.ac.jp (S.M.), christopher.mayhew@cchmc.org (C.N.M.), makiko.iwafuchi@cchmc.org (M.I.)
https://doi.org/10.1016/j.xpro.2024.103233

SUMMARY

Transcription factor (TF) gene knockout or knockdown experiments provide comprehensive downstream effects on gene regulation. However, distinguishing primary direct effects from secondary effects remains challenging. To assess the direct effect of TF binding events, we present a protocol for establishing a doxycycline (Dox)-inducible CRISPRd system in human pluripotent stem cells (hPSCs). We describe the steps for establishing CRISPRd host hPSCs, designing and preparing single-guide RNA (sgRNA) expression lentivirus vectors, generating CRISPRd hPSCs transduced with sgRNAs, and analyzing CRISPRd TF-block effects by chromatin immunoprecipitation (ChIP)-qPCR.

For complete details on the use and execution of this protocol, please refer to Matsui et al.[1]

BEFORE YOU BEGIN

In the CRISPRd system, deactivated Cas9 (dCas9) is recruited to a single-guide RNA (sgRNA)-targeted TF-binding site and sterically blocks the TF binding events upon Dox treatment.[2] dCas9 can outcompete TFs for binding as the dCas9 dissociation rate is significantly slower (hours) than the dissociation rate of TFs (seconds). Using this approach, we successfully blocked endogenous FOXA and PRDM1 binding sites in hPSC-derived foregut and validated a direct effect of FOXA and PRDM1 binding events in recruiting co-factors.[1] In this protocol, we describe the detailed steps to generate the Dox-inducible CRISPRd hPSC lines targeting a FOXA binding site, including the establishment of host hPSCs integrated Dox-inducible dCas9 expression cassettes, design and cloning of a sgRNA into a lentivirus plasmid, transduction of the lentivirus into the host hPSCs, and validation of CRISPRd efficiency by performing ChIP-qPCR. This protocol is broadly applicable for blocking endogenous TF sites in various cell types.

Institutional permissions

This protocol requires institutional approval for handling biohazardous agents. We received permission from the CCHMC Institutional Biosafety Committee for the use of human derived materials and recombinant viral vectors.

Source of CRISPR/Cas9 plasmid

To complete this protocol, plasmids for the expression of Streptococcus pyogenes-derived Cas9 (SpCas9) and sgRNA, as well as a plasmid that serves as the donor template to introduce the CRISPRd cassette into the AAVS1 safe harbor locus are required. These can be generated in-house or are available on Addgene (e.g., PX458-AAVS1; Plasmid #113194).

1. Obtain or generate plasmid for SpCas9 and sgRNA co-expression to target the AAVS1 locus.
   a. For CRISPR/Cas9-mediated targeting of the human AAVS1 safe harbor locus, the gRNA spacer sequence 5′-GGGGCCACTAGGGACAGGAT-3′ was utilized.[3]
   b. Oligonucleotides sgRNA_T2_Forward and sgRNA_T2_Reverse containing this gRNA spacer sequence and the overhangs were ligated and subcloned into pX459M2-HF, a modified pX459 plasmid (a kind gift from Dr. Feng Zhang)[4] containing a high-fidelity eSpCas9(1.1) and optimized sgRNA scaffold.[5,6]
   c. Multiple protocols describing the oligonucleotide annealing and Golden-Gate cloning have been described (e.g., Sahu et al., 2023, step16).[7]

   Note: A comparable SpCas9/sgRNA expression plasmid expressing the same AAVS1-specific gRNA spacer sequence with the original sgRNA scaffold is available at Addgene (#113194).

   Note: Many other polycistronic plasmids, combinations of plasmids, and ribonucleoprotein complexes can be used to introduce SpCas9 and AAVS1-specific sgRNA to cells.[8,9]

   ⚠ CRITICAL: Use Sanger sequencing to confirm the genomic AAVS1 sequence in the targeted cell line matches the gRNA sequence. Primer pair 1 (Figure 1A) was used to Sanger sequence the genomic AAVS1 sequence immediately flanking the gRNA target site. These primer sequences can be found in the key resources table.

2. Generate the donor plasmid for targeting the CRISPRd cassette to AAVS1.
   a. The targeting plasmid backbone used in this study (pAAVS1-NDi-CRISPRi-Gen1, Addgene #73497) was a kind gift of Dr. Bruce Conklin.[10]
   b. The CRISPRd-2A-mCherry fragment was generated from pHR-PGK-ABI-dCas9-P2A-mCherry (Addgene #121513), a kind gift from Dr. Stanley Qi.[11]
      i. Primers AgeI_ABI-dCas9 and AflII_ABI-dCas9 were used to generate a PCR amplicon from pHR-PGK-ABI-dCas9-P2A-mCherry containing the ABI-dCas9-P2A-mCherry elements flanked by 5′-AgeI and 3′-AflII restriction sites to facilitate subcloning.
   c. The pAAVS1-NDi-CRISPRi (Gen1) plasmid and AgeI-CRISPRd-2A-mCherry-AflII PCR fragment were digested with AgeI and AflII. The 8567 bp and 5952 bp fragments, respectively, were gel purified and ligated using T4 DNA ligase to generate donor plasmid pAAVS1-NDi-CRISPRd (Addgene #216531).
   d. The resulting plasmid consists of the following functional elements:
      i. Left and right homology arms for targeting the AAVS1 safe harbor locus.
      ii. Gene trap-mediated expression of neomycin resistance gene (NeoR) from the endogenous PPP1R12C promoter.
      iii. Constitutive CAG promoter mediated expression of the reverse tetracycline transactivator (rtTA).
      iv. Tet-On promoter for inducible co-expression of catalytically inactive SpCas9 fused to ABI, which can be used for abscisic acid-dependent recruitment of PYL-fused proteins, and mCherry.[11]

   Note: The methods described in this protocol are broadly applicable for targeting other transgenes to the AAVS1 locus of human hPSCs.

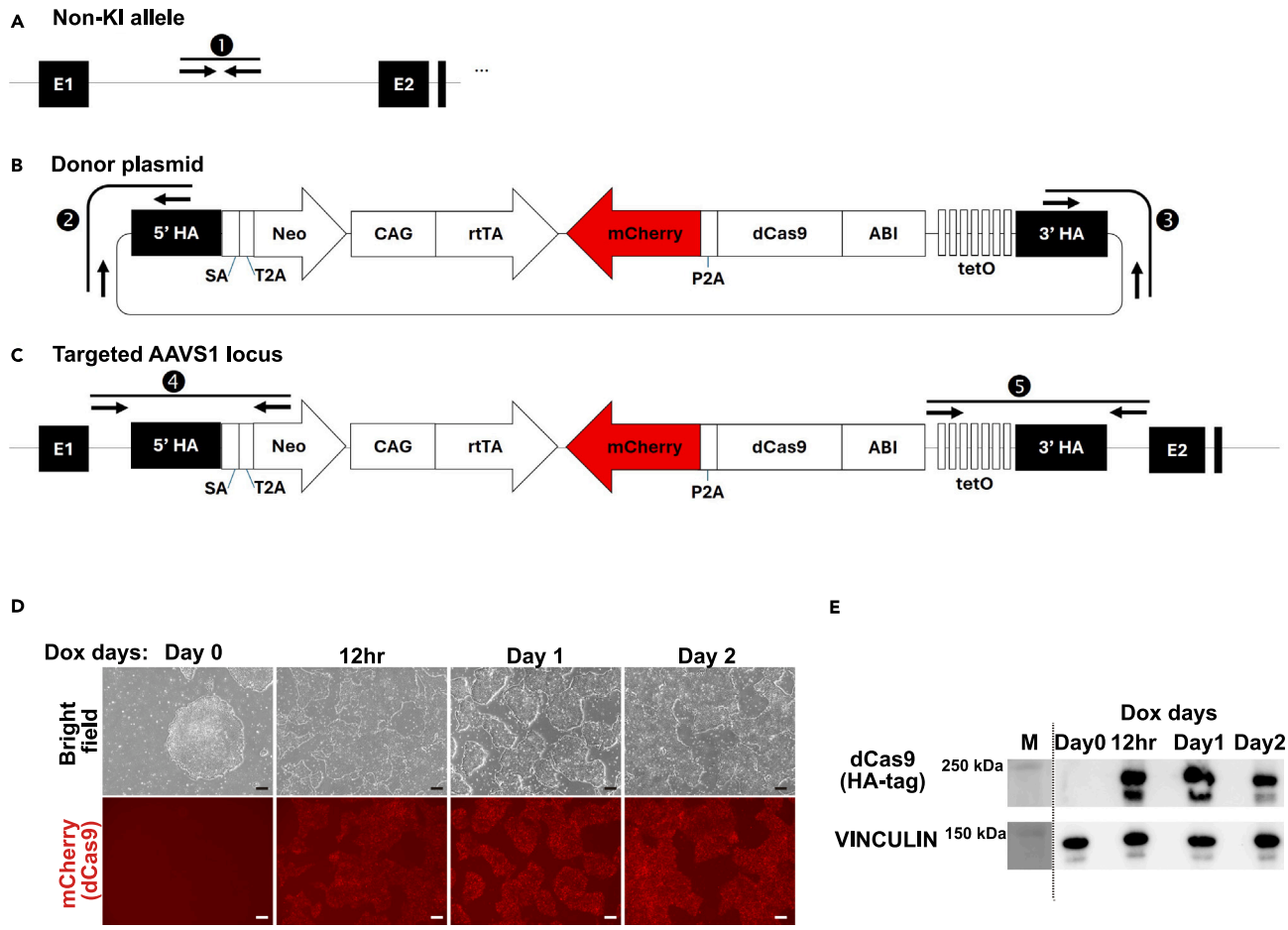


Figure 1. Overview of the genotyping strategy of the ABI-dCas9 cassette at the AAVS1 locus and verification of dCas9 expression in the CRISPRd host hPSCs

(A) Schematic of the non-KI allele. Primer pair 1 specifically amplifies the AAVS1 alleles that are untargeted or with indels only.

(B) Schematic of the pAAVS1-NDi-CRISPRd donor plasmid. Primer pairs 2 and 3 detect specifically amplified plasmid sequences that are not integrated *via* homologous recombination into the AAVS1 locus.

(C) Schematic of CRISPRd cassette correctly targeted to the AAVS1 locus. Primer pairs 4 and 5 specifically amplify the 5′- and 3′-junctions, respectively. Approximate primer locations are shown in the table at Step 12.

(D) mCherry marker expression to validate dCas9 induction upon Dox treatment. Scale bars = 300 μm.

(E) Western blot analysis to validate dCas9 induction upon Dox treatment along with internal control, VINCULIN. HA-tag conjugated dCas9 was detected with HA-tag antibody. dCas9 expression was rapidly induced after 12 h of 2 μM Dox treatment.

⚠ CRITICAL: High-quality, endotoxin-free plasmid DNA is required for hPSC transfection.

hPSC maintenance culture

Timing: 2 weeks

In this section, we describe the protocol for culturing hPSCs for daily maintenance. We use iPSC72_3 human induced pluripotent stem cells (iPSCs; RRID: CVCL_A1BW) and culture them in mTeSR1 on Matrigel- or Cultrex SCQ-coated plates. We routinely passage hPSCs using Gentle Cell Dissociation Reagent (GCDR). The volumes of cell culture materials for different sizes of culture plates are listed below.

The volumes of culture materials per single well

Culture scale	mTeSR1 culture volume	Coating solution volume	GCDR volume	mTeSR1 volume for GCDR passage
96-well plate	100 μL	50 μL	50 μL	100 μL
24-well plate	500 μL	250 μL	250 μL	250 μL
12-well plate	1 mL	0.5 mL	500 μL	500 μL
6-well plate	2 mL	1 mL	1 mL	1 mL

3. Preparation of a Matrigel- or Cultrex SCQ-coated 6-well plate.
   a. Dilute the desired amounts of Matrigel or Cultrex SCQ (final concentration: 40 μg/mL) in cold DMEM/F12.

   Note: The dilution factor of Matrigel and Cultrex depends on the lot number. Please refer to the certificates of analyses here (Matrigel: https://www.corning.com/worldwide/en/products/life-sciences/resource-library.html?productNumber=354277&lotNumber=; Cultrex SCQ: https://www.bio-techne.com/p/cell-culture/cultrex-stem-cell-qualified-reduced-growth-factor-basement-membrane-extract_3434-010-02#product-datasheets-anchor) for specific instructions.

   b. Add 1 mL of the coating solution to each well of a 6-well plate.
   c. Coat it for 30 min to 1 h at 37°C in the 5% CO_2 incubator before plating hPSCs.

   Alternatives: You can coat a plate at 20°C–25°C for a minimum 1 h or at 4°C for 16–24 h. If you coat a plate a day before plating, seal the plate with parafilm and store at 4°C. The shelf life of the coated plate is 2 weeks at 4°C.

4. Thawing cryopreserved hPSC vial.
   a. Warm up mTeSR1 media and the coated plate at 20°C–25°C for 15–30 min.

   ⚠ CRITICAL: If using a water bath, do not allow mTeSR1 to dwell at 37°C for more than 15 min.

   b. Rapidly thaw a cryopreserved hPSC vial in a 37°C water bath.
   c. Carefully transfer the hPSC suspension to a 15 mL conical tube.
   d. Add 5 mL of mTeSR1 dropwise to the tube and gently mix.
   e. Centrifuge hPSC suspension at 300 × *g* for 3 min at 20°C–25°C.
   f. Aspirate the supernatant and detach the hPSC pellet by tapping the tube.
   g. Aspirate the coating solution from the plate.
   h. Add 2 mL of fresh mTeSR1 to the hPSC pellet and gently mix by pipetting up and down 2 to 3 times.

   ⚠ CRITICAL: Keep hPSC clumps as big as possible.

   i. Transfer the hPSC suspension to the coated plate.
   j. Place the plate in a 37°C and 5% CO_2 incubator and move it side to side to evenly distribute hPSC clumps across it.
   k. Starting the next day, perform daily media changes with mTeSR1.

   ⚠ CRITICAL: Examine cultures daily using a stereomicroscope and manually remove differentiated colonies by scraping them with a sterile glass pipette.

5. Passaging hPSCs.
   a. Warm mTeSR1 and a coated plate at 20°C–25°C for 15–30 min.

b. Aspirate the coating solution from the coated plate and add 2 mL of fresh mTeSR1 to each well of the coated 6-well plate.
c. Aspirate the culture media from the 1 mL of hPSC culture plate and add GCDR to each well.
d. Incubate at 20°C–25°C for 3.5–6 min.

⚠ CRITICAL: Optimal GCDR incubation time depends on colony sizes (e.g., 5 min for 70%–80% confluent hPSCs; 4 min for 50–60% confluent hPSCs). See STEMCELL Technologies manual "Maintenance of Human Stem Cells in mTeSR1" (https://cdn.stemcell.com/media/files/manual/10000005505-Maintenance_of_Human_Pluripotent_Stem_Cells_mTeSR1.pdf").

e. Aspirate GCDR and add 1 mL of mTeSR1 to each well.
f. Lift the colonies into the mTeSR1 with a sterile cell scraper.
g. Dissociate the hPSC clumps by slowly pipetting up and down with a P1000 pipette under a stereomicroscope.

⚠ CRITICAL: The optimal size of the hPSC clumps after trituration should uniformly be 50–150 μm. Do not break clumps into single cells. While serological pipets can be used for trituration, we have found that using a p1000 micropipette in conjunction with a stereoscope to assess clump size after each trituration provides the best control of clump size.

h. Transfer hPSC clumps to the fresh mTeSR1-filled coated plate using a P200 pipette.

Note: When replating triturated cell clumps as described here, the optimal quantity of clumps to transfer is dependent on a number of variables, including GCDR incubation time, triturated clump size, desired density, forecasted recovery time, source confluency, and source density. With GCDR, passaging ratios typically range from 1:20 (for plates at 40–50% confluency) to 1:60 (for plates at ~90% confluency) depending on these variables (see STEMCELL Technologies manual "Maintenance of Human Stem Cells in mTeSR1" https://cdn.stemcell.com/media/files/manual/10000005505-Maintenance_of_Human_Pluripotent_Stem_Cells_mTeSR1.pdf).

i. Place the plate into the 37°C and 5% CO_2 incubator and move the plate side to side.
j. After 72 h of incubation, perform daily media changes with mTeSR1.

Note: Monitor daily and remove differentiated colonies with a glass pipette under a stereomicroscope.

6. Cryopreservation of hPSCs.
   a. Thaw mFreSR at 4°C for 12–16 h.
   b. Warm DMEM/F12 at 20°C–25°C for 15 min.
   c. Aspirate the culture media from the hPSC culture plate.
   d. Rinse a culture well (6-well plate) with 2 mL of DMEM/F12.
   e. Add 1 mL of GCDR and incubate at 20°C–25°C for 3.5–5 min.

⚠ CRITICAL: Optimal GCDR incubation time depends on the colony size. GCDR incubation should be for less time than typically used to passage the cells (e.g., 5–6 min for 70%–80% confluent hPSCs).

   f. Aspirate GCDR and gently wash with 1 mL of DMEM/F12.
   g. Aspirate DMEM/F12 and add 1 mL of ice-cold mFreSR.

Note: If the hPSC culture is at low density, use 1 mL of mFreSR for 2 wells (500 μL per each well) and combine them into 1 vial.

h. Gently detach colonies with a sterile cell scraper.
i. Slowly transfer clumps to a 1.2 mL cryovial.

⚠ CRITICAL: hPSC clumps should not be dissociated. Keep clumps as large as possible.

j. Place the cryovial into an isopropanol freezing container and store at −80°C for 16–24 h.
k. Transfer vials to liquid nitrogen the next day.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FOXA2 (5 μg of antibody for 50 μg of chromatin in a ChIP reaction)	Millipore	Cat# 07-633; RRID: AB_390153
PRDM1 (0.05 μg of antibody for 37.5 μg of chromatin in a ChIP reaction)	Cell Signaling Technology	Cat# 9115S; RRID: AB_2169699
RING1B/RNF2 (1.5 μg of antibody for 18.75 μg of chromatin in a ChIP reaction)	Bethyl	Cat# A302-869A; RRID: AB_10632773
H2AK119ub1 (1.3 μg of antibody for 12 μg of chromatin in a ChIP reaction)	Cell Signaling Technology	Cat# 8240S; RRID: AB_10891618
HA-tag (0.13 μg of antibody for 37.5 μg of chromatin in a ChIP reaction)	Cell Signaling Technology	Cat# 3724; RRID: AB_1549585
HA.11-tag (1:1,500 dilution for western blotting)	BioLegend	Cat# 901514; RRID: AB_2565336
VINCULIN (1:2,500 dilution for western blotting)	Sigma-Aldrich	Cat#V9131; RRID: AB_477629
Mouse TrueBlot ULTRA: anti-mouse Ig HRP (1:2,000 dilution for western blotting)	Rockland	Cat# 18-8817-33; RRID: AB_2610851
Bacterial and virus strains		
NEB stable competent *E. coli*	NEB	Cat# C3040H
Chemicals, peptides, and recombinant proteins		
mTeSR1	STEMCELL Technologies	Cat# 07174
DMEM/F-12, HEPES	Thermo Fisher Scientific	Cat# 11330032
DMEM, high glucose	Thermo Fisher Scientific	Cat# 11965118
IMDM, GlutaMAX supplement	Thermo Fisher Scientific	Cat# 31980097
Ham's F-12 nutrient mix, GlutaMAX supplement	Thermo Fisher Scientific	Cat# 31765092
Opti-MEM	Thermo Fisher Scientific	Cat# 31985062
FBS	Thermo Fisher Scientific	Cat# 10437-028
KnockOut Serum replacement	Thermo Fisher Scientific	Cat# 10828028
DPBS (no calcium, no magnesium)	Thermo Fisher Scientific	Cat# 14190144
Chemically defined lipid concentrate	Thermo Fisher Scientific	Cat# 11905031
Poly(vinyl alcohol)	Sigma-Aldrich	Cat# 363170
1-Thioglycerol	Sigma-Aldrich	Cat# M6145
Insulin solution from bovine pancreas	Sigma-Aldrich	Cat# I0516-5ML
Transferrin	Sigma-Aldrich	Cat# T3309-100MG
ActivinA	Shenandoah Cell Guidance Systems	Cat# 800-0; Cat# GFH6
CHIR99021	Sigma-Aldrich	Cat# SML1046-25MG
PI-103	Tocris Bioscience	Cat# 2930-1
LDN193189	Sigma-Aldrich	Cat# SML0559-5MG
A83-01	Sigma-Aldrich	Cat# SML0788-5MG
ATRA	Sigma-Aldrich	Cat# R2625-100MG
bFGF	Thermo Fisher Scientific	Cat# PHG0261

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
BMP4	R&D Systems	Cat# 314-BP-050
hESC-qualified Matrigel	Corning	Cat# 354277
Cultrex stem cell qualified reduced growth factor basement Membrane extract	Bio-Techne	Cat# 3434-010-02
Gentle Cell Dissociation reagent	STEMCELL Technologies	Cat# 07174
ReLeSR	STEMCELL Technologies	Cat# 100-0483
Accutase	STEMCELL Technologies; Sigma-Aldrich	Cat# 07922; Cat# A6964-500ML
TrypLE Express Enzyme (1X)	Thermo Fisher Scientific	Cat# 12605010
mFreSR	STEMCELL Technologies	Cat# 5854
Y27632	STEMCELL Technologies	Cat# 72304
CloneR	STEMCELL Technologies	Cat# 5888
Doxycycline	Sigma-Aldrich	Cat# D9891-5G
G418 sulfate (50 mg/mL)	Thermo Fisher Scientific	Cat# 10131035
Polybrene infection/transfection reagent	Sigma-Aldrich	Cat# TR-1003-G
Polyethylenimine branched	Sigma-Aldrich	Cat# 408727-100ML
TransIT-LT1	Mirus Bio	Cat# MIR 2300
Tris-HCl, pH 8.0 (1 M)	Santa Cruz Biotechnology	Cat# sc-301953
Triton X-100 (100%)	Acros Organics	Cat# 327371000
Proteinase K (20 mg/mL)	Thermo Fisher Scientific	Cat# EO0491
Phusion polymerase	NEB	Cat# M0530S
KOD Xtreme Hot Start DNA polymerase	Millipore	Cat# 71975-3
rSAP	NEB	Cat# M0371S
AflII	NEB	Cat# R0520S
AgeI-HF	NEB	Cat# R3552S
FastDigest Esp3I	Thermo Fisher Scientific	Cat# FD0454
Esp3I	NEB	Cat# R0734S
T4 DNA ligase	NEB	Cat# M0202L
T4 polynucleotide kinase	NEB	Cat# M0201L
Adenosine 5′-triphosphate (ATP)	NEB	Cat# P0756S
NEB 10-beta/stable outgrowth medium	NEB	Cat# B9035S
PFA	Fisher Scientific	Cat# F79-500
UltraPure glycine	Thermo Fisher Scientific	Cat# 15527013
RNaseA	Thermo Fisher Scientific	Cat# EN0531
Protease K	Roche	Cat# 03115828001
cOmplete protease inhibitor cocktail	Roche	Cat# 11836170001
Critical commercial assays		
GeneJET Gel Extraction Kit	Thermo Fisher Scientific	Cat# K0691
GeneJET Plasmid Miniprep Kit	Thermo Fisher Scientific	Cat# K0502
ZymoPURE II Plasmid Midiprep Kit	Zymo Research	Cat# D4201
ZymoPURE II Plasmid Maxiprep Kit	Zymo Research	Cat# D4202
Endo-free plasmid maxi kit	QIAGEN	Cat# 12362
TaqMan genotyping master mix	Thermo Fisher Scientific	Cat# 4371355
TaqMan copy number assay, NeoR	Thermo Fisher Scientific	Cat# 4400291
TaqMan copy number reference assay, human RNase P	Thermo Fisher Scientific	Cat# 4403328
Lenti-X concentrator	Takara Bio	Cat# 631231
Aurum total RNA lysis solution	Bio-Rad	Cat#7326802
Aurum Total RNA Mini Kit	Bio-Rad	Cat#7326820
iScript cDNA Synthesis Kit	Bio-Rad	Cat#1708890
Dynabeads protein G for immunoprecipitation	Thermo Fisher Scientific	Cat# 10004D
QuantiFluor ONE dsDNA system	Promega	Cat# E4870
PowerUp SYBR Green master mix	Thermo Fisher Scientific	Cat# A25742
Deposited data		
Raw gel image data in Mendeley data	This study	

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental models: Cell lines		
hPSC (72_3)	CCHMC Pluripotent Stem Cell Facility	RRID: CVCL_A1BW
Dox-inducible CRISPRd, host hPSC (72_3)	Matsui et al.[1]	N/A
HEK293T	ATCC	Cat# CRL-11268; RRID: CVCL_1926
Oligonucleotides		
AflII _ABI-dCas9_Forward: 5′-CATCGACTTAAG GCCACCATGACGCGTGTGCCTTT-3′	IDT	N/A
AgeI_ ABI-dCas9_Reverse: 5′-TATACCGGTTTA CTTGTACAGCTCGTCCATGCCGCCG-3′	IDT	N/A
sgRNA_T2_Forward: 5′-CACCGGGGCCACTA GGGACAGGAT-3′	IDT	N/A
sgRNA_T2_Reverse: 5′-AAACATCCTGTCCC TAGTGGCCCC-3′	IDT	N/A
Primers for detecting untargeted AAVS1 (primer pair 1): Fwd: 5′-TGTCACCAGATAAG GAATCTGCCTAAC-3′: Rev: 5′-TAATGTGGC TCTGGTTCTGGGTAC-3′	N/A	N/A
Primers for detecting random targeting plasmid integration (primer pair 2): Fwd: 5′-GAGTGGAG GAAGACGGAACC-3′: Rev: 5′-GAATAAGGGCG ACACGGAAATGT-3′	N/A	N/A
Primers for detecting random targeting plasmid integration (primer pair 3): Fwd: 5′- CCACCTCT GACTTGAGCGTC-3′: Rev: 5′- GGGTTTGAGTC CTTGGCAAG-3′	N/A	N/A
Primers for detecting correct targeted AAVS1 integration (primer pair 4): Fwd: 5′-TCGACTTC CCCTCTTCCGATG-3′: Rev: 5′-ATGATGGATA CTTTCTCGGCAGGAG-3′	Fwd: Oceguera-Yanez et al.[3] Rev: N/A	N/A
Primers for detecting correct targeted AAVS1 integration (primer pair 5): Fwd: 5′-CAGTTGG AGGAGAATCCACCCAAAAG-3′: Rev: 5′-GAAT TGCTCCAGGCGATCTGAC-3′	N/A	N/A
gRNAs for CRISPRd	IDT	Table in step16
LRG-gRNA sequencing Primer: 5′-GAGGGCC TATTTCCCATGATT-3′	IDT	N/A
Primers for ChIP-qPCR and RT-qPCR	IDT	Table S1
Recombinant DNA		
pX459M2-HF-AAVS1	Ran et al.[4] Chen et al.[5]	N/A
pAAVS1-NDi-CRISPRd (ABI-dCas9-mCherry)	Matsui et al.[1]	Cat# 216531; RRID_Addgene_216531
pAAVS1-NDi-CRISPRi (Gen 1)	Mandegar et al.[10]	Cat# 73497; RRID_Addgene_73497
pHR-PGK-ABI-dCas9-P2A-mcherry	Wang et al.[11]	Cat# 121513; RRID_Addgene_121513
LRG (Lenti_sgRNA_EFS_GFP)	Shi et al.[12]	Cat# 65656; RRID_Addgene_65656
LRG-sgRNA.FOXA-ZEB2_CRISPRd_v2	Matsui et al.[1]	Cat# 216173; RRID_Addgene216173
psPAX2	Laboratory of Didier Trono	Cat# 12260; RRID_Addgene_12260
pMD2.G	Laboratory of Didier Trono	Cat# 12259; RRID_Addgene_12259
Software and algorithms		
homer v4.11	Heinz et al.[13]	http://homer.ucsd.edu/homer/
CRISPOR	Concordet et al.[14]	http://crispor.gi.ucsc.edu/
GraphPad Prism v9.3.1 (350)	Dotmatics	https://www.graphpad.com/features
Other		
BZ-X810	Keyence	N/A
QuantStudio5	Thermo Fisher Scientific	N/A
Veriti 96-well fast thermal cycler	Thermo Fisher Scientific	Cat# 4375305
MiniAmp thermal cycler	Thermo Fisher Scientific	Cat# A37834
Quantus Fluorometer	Promega	Cat# E6150
S220 focused-ultrasonicator	Covaris	Cat# 500217

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
milliTUBE 1 mL AFA fiber	Covaris	Cat# 520135
Nuclease-free 1.5 mL microcentrifuge tubes	Eppendorf	Cat# 022363212
5PRIME Phase Lock GelTM-Light	Quantabio	Cat# 2302820
DynaMag-2 magnet	Thermo Fisher Scientific	Cat# 12321D

MATERIALS AND EQUIPMENT

Cell culture reagents

mTeSR1 medium		
Reagent	Final concentration	Amount
mTeSR1 basal medium	N/A	400 mL
mTeSR1 5× supplement	1×	100 mL
Total	**N/A**	**500 mL**
Store at −80°C for up to 6 months or 4°C for up to 2 weeks.		

Note: Make aliquots for storage (e.g., 40 mL each).

Matrigel solution for coating a plate		
Reagent	Final concentration	Amount
DMEM/F12	1×	12.5 mL
Matrigel hESC-Qualified Matrix	1×	Variable
Total	**N/A**	**12.5 mL**
Store coated plates at 4°C, wrapped in parafilm, for up to 2 weeks.		

Alternatives: Cultrex Stem Cell Qualified Reduced Growth Factor Basement Membrane Extract (Bio-Techne, #3434-005-02) is also a suitable coating for hPSC culture. The final concentration of Cultrex working solution should be 40 μg/mL. When diluted with DMEM/F12, Cultrex can be used to coat a plate under the same condition as Matrigel coating.

mTeSR1 + 100 μg/mL G418		
Reagent	Final concentration	Amount
mTeSR1 medium	N/A	12 mL
G418 sulfate (50 mg/mL)	100 μg/mL	24 μL
Total	**N/A**	**12 mL**
Make fresh for each use.		

mTeSR1 + CloneR supplement		
Reagent	Final concentration	Amount
mTeSR1 medium	1×	9 mL
10× CloneR supplement	1×	1 mL
Total	**N/A**	**10 mL**
Make fresh for each use.		

2 mM Doxycycline (Dox)		
Reagent	Final concentration	Amount
Doxycycline hyclate (M.W.: 512.94)	2 mM	5 mg
DMSO	1×	4.87 mL
Total	**N/A**	**4.87 mL**
It can be stored at −80°C for at least 1 year.		

mTeSR1 + 10 μM Y27632		
Reagent	Final concentration	Amount
Y27632 (10 mM)	10 μM	10 μL
mTeSR1	1×	10 mL
Total	**N/A**	**10 mL**
Make fresh for each use.		

Lentivirus Transduction media		
Reagent	Final concentration	Amount
Polybrene (10 mg/mL)	8 μg/mL	4 μL
mTeSR1 + 10 μM Y27632	1×	5 mL
Total	**N/A**	**5 mL**
Make fresh for each use.		

CDM2		
Reagent	Final concentration	Amount
IMDM, GlutaMAX Supplement	49%	250 mL
F12, GlutaMAX Supplement	49%	250 mL
PVA solution (10%)	0.98 mg/mL (0.098%)	5 mL
1-thioglycerol (11.5 M)	441.7 μM	19.6 μL
Chemically defined lipid concentrate	0.98%	5 mL
Insulin (10 mg/mL)	0.69 μg/mL	35 μL
Transferrin (30 mg/mL)	14.7 μg/mL	250 μL
Total	**N/A**	**510.3 mL**
Store at −80°C for up to 6 months or 4°C for up to 2 months.		

Note: Pass through the 0.22 μm filter unit and make aliquots for storage.

CDM3		
Reagent	Final concentration	Amount
IMDM, GlutaMAX Supplement	43.7%	225 mL
F12, GlutaMAX Supplement	43.7%	225 mL
KnockOut Serum Replacement	9.7%	50 mL
PVA solution (10%)	0.97 mg/mL (0.097%)	5 mL
Penicillin-Streptomycin (10,000 U/mL)	0.97%	5 mL
Chemically defined lipid concentrate	0.97%	5 mL
Total	**N/A**	**515 mL**
Store at −80°C for up to 6 months or 4°C for up to 2 months.		

Note: Pass through the 0.22 μm filter unit and make aliquots for storage.

10% FBS-supplemented DMEM		
Reagent	Final concentration	Amount
DMEM	1×	45 mL
FBS	10%	5 mL
Total	**N/A**	**50 mL**
Store at 4°C for up to 3 months.		

Cell lysis buffer		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	50 mM	250 μL
Triton X-100 (100%)	0.5%	25 μL
Proteinase K (20 mg/mL)	200 μg/mL	50 μL
Nuclease-free water	N/A	4675 μL
Total	**N/A**	**5 mL**

Note: Cell lysis buffer without Proteinase K can be prepared in batches and stored at 4°C for up to 1 month. Add proteinase K before each use and keep on ice.

Solution for transfection

PEI stock solution (1:100)		
Reagent	Final concentration	Amount
PEI	10 μg/mL	250 μL
Nuclease-free water	N/A	to 25 mL
Total	**N/A**	**25 mL**
It can be stored at −80°C for at least 1 year.		

PEI working solution (1:1000)		
Reagent	Final concentration	Amount
PEI stock solution	1 μg/mL	5 mL
Nuclease-free water	N/A	to 50 mL
Total	**N/A**	**50 mL**
It can be stored at −80°C for at least 1 year. After thawing, store at 4°C for up to 2 months. Avoid freezing and thawing.		

Note: Adjust pH to 7.0–8.0 by adding 6N HCl. Pass through 0.22 μm filter.

Solution for cell lysis and chromatin sonication

Lysis Buffer1 (need >500 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	10 mM	25 μL
NaCl (5 M)	10 mM	5 μL
IGEPAL (100%)	0.5%	13 μL
10× Complete Protease Inhibitor EDTA free	1×	250 μL
Nuclease-free water	N/A	2208 μL
Total	**N/A**	**2500 μL**
Prepare fresh and keep on ice.		

Note: Dissolve IGEPAL at 20°C–25°C by rotating and then place on ice.

Lysis Buffer2 (need >750 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	50 mM	200 μL
EDTA (0.5 M)	10 mM	80 μL
SDS (10%)	0.32%	128 μL
10× Complete Protease Inhibitor EDTA free	1×	400 μL
Nuclease-free water	N/A	3192 μL
Total	**N/A**	**4000 μL**
Prepare fresh and keep at 20°C–25°C.		

IP Dilution Buffer (need >450 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	20 mM	50 μL
NaCl (5 M)	150 mM	75 μL
EDTA (0.5 M)	2 mM	10 μL
Triton X-100 (20%)	1.0%	125 μL
10× Complete Protease Inhibitor EDTA free	1×	250 μL
Nuclease-free water	N/A	1990 μL
Total	**N/A**	**2500 μL**
Prepare fresh and keep on ice.		

Sonication Buffer (need 20–200 μL per sample)		
Reagent	Final concentration	Amount
Lysis Buffer2	0.625×	625 μL
IP Dilution Buffer	0.375×	375 μL
Total	**N/A**	**1000 μL**
Prepare fresh and keep on ice.		

Solution for ChIP

PBS-T (need 1.1 mL per sample)		
Reagent	Final concentration	Amount
1× PBS	1×	5489 μL
Tween-20 (10%)	0.02%	11 μL
Total	**N/A**	**5500 μL**
Prepare fresh and keep on ice.		

Sonication Buffer (need 50–100 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	40 mM	20 μL
NaCl (5 M)	56 mM	5.6 μL
EDTA (0.5 M)	7 mM	7.0 μL
Triton X-100 (20%)	0.40%	10 μL
SDS (10%)	0.20%	10 μL
10× Complete Protease Inhibitor EDTA free	1×	50 μL
Nuclease-free water	N/A	397 μL
Total	**N/A**	**500 μL**
Prepare fresh and keep on ice.		

IP Dilution Buffer with CPI (for ChIP reaction, need 25–50 μL per sample) and without CPI (for washing beads-antibody conjugate, need 1.0 mL per sample)			
Reagent	Final concentration	Amount (with CPI)	Amount (w.o. CPI)
Tris-HCl, pH 8.0 (1 M)	20 mM	5 μL	100 μL
NaCl (5 M)	150 mM	8 μL	150 μL
EDTA (0.5 M)	2 mM	1.0 μL	20 μL
Triton X-100 (20%)	1.0%	13 μL	250 μL
10× Complete Protease Inhibitor EDTA free (CPI)	1×	25 μL	-
Nuclease-free water	N/A	199 μL	4480 μL
Total	**N/A**	**250 μL**	**5000 μL**
Prepare fresh and keep on ice.			

FA Lysis Buffer (need 500 μL per sample)		
Reagent	Final concentration	Amount
HEPES-KOH, pH 7.5 (1 M)	50 mM	125 μL
NaCl (5 M)	150 mM	75 μL
EDTA (0.5 M)	2 mM	10.0 μL
Triton X-100 (20%)	1.0%	125 μL
Sodium Deoxycholate (10%)	0.10%	25 μL
Nuclease-free water	N/A	2140 μL
Total	**N/A**	**2500 μL**
Prepare fresh and keep on ice.		

NaCl Buffer (need 500 μL per sample)		
Reagent	Final concentration	Amount
HEPES-KOH, pH 7.5 (1 M)	50 mM	125 μL
NaCl (5 M)	500 mM	250 μL
EDTA (0.5 M)	2 mM	10 μL
Triton X-100 (20%)	1.0%	125 μL
Sodium Deoxycholate (10%)	0.10%	25 μL
Nuclease-free water	N/A	1965 μL
Total	**N/A**	**2500 μL**
Prepare fresh and keep on ice.		

LiCl Buffer (need 500 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	100 mM	250 μL
LiCl (5 M)	500 mM	250 μL
IGEPAL (100%)	1.0%	25 μL
Sodium Deoxycholate (10%)	1.0%	250 μL
Nuclease-free water	N/A	1725 μL
Total	**N/A**	**2500 μL**
Prepare fresh and keep on ice.		

Note: Dissolve IGEPAL at 20°C–25°C by rotating and then place on ice.

10 mM Tris-HCl, pH 8.0 (need 500 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	10 mM	25 μL
Nuclease-free water	N/A	2475 μL
Total	**N/A**	**2500 μL**
It can be stored at 4°C for at least 1 year.		

TES (need 180 μL per sample)		
Reagent	Final concentration	Amount
Tris-HCl, pH 8.0 (1 M)	50 mM	250 μL
EDTA (0.5 M)	10 mM	100 μL
SDS (10%)	1.0%	500 μL
Nuclease-free water	N/A	4150 μL
Total	**N/A**	**5000 μL**
It can be stored at 20°C–25°C for 1 year.		

STEP-BY-STEP METHOD DETAILS

Establishment of CRISPRd hPSC host line

Timing: 8–10 weeks

The following steps describe the CRISPR/Cas-mediated generation of a human hPSC line harboring integration of a cassette for Dox-inducible expression of ABI-dCas9-P2A-mCherry in the AAVS1 safe harbor locus. Once the identity and quality of targeted clones are authenticated and functionally validated, these cells can then be used in subsequent steps for sgRNA-lentivirus transduction and CRISPRd-mediated inhibition of TF binding to DNA (step 23).

Prepare hPSCs for transfection

Timing: 1 week

In this section, we describe the process for plating hPSCs to be used for reverse transfection.

1. Coat a 6-well plate with Matrigel or Cultrex as described in the hPSC maintenance culture.
2. Passage hPSCs into 6-wells of a 6-well plate.
3. Continue daily media changes until cells reach ~75% confluence.
4. Proceed with reverse transfection.

Note: A minimum of 4 × 10^6 cells are required for reverse transfection.

⚠ CRITICAL: Confirm that the hPSCs used for gene editing have been robustly quality controlled and authenticated before targeting. At a minimum, we recommend that cells are documented to be mycoplasma-free, have a normal karyotype, have the capacity to differentiate into ectoderm, endoderm, and mesoderm lineages, and have had their identity authenticated (e.g., by short tandem repeat [STR] analysis).[15]

CRISPRd hPSC line generation: reverse transfection

Timing: 1 h

5. Prepare reverse transfection reagents for reverse transfection.
   a. Prepare a hESC-qualified Matrigel-coated 6-well plate as described in the hPSC Maintenance Culture.
   b. Prepare mTeSR1 + CloneR supplement and warm up at 20°C–25°C.
   c. Aspirate Matrigel from the plate and add 500 μL of mTeSR1 + CloneR supplement to 2 of the wells, respectively.
   d. Incubate the plate in an incubator during the remainder of the reverse transfection protocol to allow the media and plate to equilibrate for at least 15 min.
   e. Prepare TransIT-LT1:DNA complexes.

Note: In two sterile 1.5-mL Eppendorf tubes labeled "Transfected" and "Untransfected", prepare the following transfection mixes:

TransIT-LT1:DNA complexes

Reagent	Transfected	Untransfected
pX459M2-HF-AAVS1	2 μg	-
pAAVS1-NDi-CRISPRd	2 μg	-

(*Continued on next page*)

Continued

Reagent	Transfected	Untransfected
TransIT-LT1 Reagent	12 12 µL	12 µL
Opti-MEM	13–425 µL	413 µL
Total	**425 µL**	**425 µL**

f. Mix gently by pipetting and incubate transfection complexes for 10–15 min at 20°C–25°C.

Note: Maximum volume of combined DNA should not exceed 20 µL. We prepare our plasmid DNA to a concentration of 1 µg/µL in 10 mM Tris-HCl, pH 8.5.

⚠ CRITICAL: Incubate TransIT-LT1:DNA complexes for no longer than 15 min.

6. Reverse transfect hPSCs.
   a. Remove the plate containing hPSCs to be transfected from the incubator.
   b. Aspirate media and add 1 mL of 37°C DMEM/F12 to each well.
   c. Aspirate and add 1 mL of 37°C Accutase per well and return the plate immediately to the incubator.

⚠ CRITICAL: All reagents used for cell detachment should be at 37°C when added to cells.

   d. Starting after 3 min, check cell dissociation by gently tapping the dish and inspecting the cells under an inverted microscope.

Note: If cells have not detached, return the plate to the incubator.

   e. Incubate up to a total of 10 min or until ~80% of colonies have lifted from the surface.

⚠ CRITICAL: Cells should detach within 7–8 min. In our experience, if extended incubation or excessive trituration is required, the viability of the transfected cells will be diminished.

   f. Add 1 mL of 37°C mTeSR1 to each well.
   g. Gently collect and combine the contents of all wells into a single 15 mL conical tube.
   h. Use a 5 mL serological pipette to gently triturate the cells to complete dissociation into single cells.
   i. Determine viable cell count and transfer 4 × 10^6 cells into a separate 15 mL conical tube.
   j. Centrifuge at 300 × *g* for 3 min at 20°C–25°C.
   k. During centrifugation, remove the plate set up in step 5 from the incubator, label one well as 'Transfected' and one well as 'Untransfected', and add the TransIT-LT1:DNA complexes prepared in step 5 to each respective well.
   l. After centrifugation, carefully aspirate the supernatant and gently resuspend the cell pellet in 2 mL of 37°C mTeSR1 + CloneR supplement.
   m. Add 1 mL of resuspended cells (2 × 10^6 cells) to each well of the plate containing transfection complexes.
   n. Return the plate to the incubator and gently move the plate back and forth to evenly distribute the cells across the plate.

CRISPRd hPSC line generation: selection, excision, and expansion of targeted clones

Timing: 2–3 weeks

This step describes the selection of targeted clones with G418 and the isolation of discrete clones.

Note: It is possible that the optimal G418 selection concentration and duration will differ for each hPSC line targeted. Therefore, it is advisable to generate a kill curve to identify the lowest concentration of G418 that kills untransfected cells in a defined time period. We have found that G418-supplemented mTeSR1 at a concentration of 100 μg/mL G418 can effectively eliminate untransfected cells after approximately a duration of 6 days.

7. Plate transfected hPSCs for G418 selection.
   a. Starting 24 h post-transfection, perform daily media changes with mTeSR1 + CloneR supplement to allow cells to recover from transfection.

   Note: Recovery following reverse transfection typically takes 2–4 days post reverse transfection. Cell density should reach ~90% before proceeding.

   b. Repeat steps 6a-h to generate a single-cell suspension of transfected hPSCs using Accutase.
   c. Remove a small aliquot from each tube to determine the viable cell count.
   d. Centrifuge remaining cells at 300 × *g* for 3 min at 20°C–25°C.
   e. Aspirate supernatant from each conical tube and resuspend cells at 2×10^5 cells/mL in mTeSR1 + CloneR supplement.
   f. Plate 1 mL per well (2×10^5 cells) of transfected cells into 5 wells of a hESC-qualified Matrigel-coated plate. Plate 1 mL (2×10^5 cells) of untransfected cells into the remaining well.

   Note: The number of wells plated with transfected cells can be scaled up as needed to generate additional clones.

   g. Return the plate to the incubator and gently move the plate back and forth to evenly distribute the cells across the plate.
8. Selection of targeted cells with G418 (see troubleshooting, problem 1).
   a. Starting the next day, aspirate spent media and feed daily with 2mL/well of mTeSR1 + 100 μg/mL G418 for a total of 6 days.

   Note: At the point at which G418 selection is discontinued, multiple small colonies should be visible in wells plated with transfected cells.

   ⚠ CRITICAL: All untransfected cells should be killed before discontinuing G418 treatment and expanding targeted clones. Note that it may be necessary to prolong G418 exposure beyond 6 days and up to 10 days.

   b. Continue daily media changes with 2 mL of mTeSR1 without G418 until colonies become large enough to manually excise as described in step 9f below (until colonies exhibit a typical, developed size of approximately 500 μm diameter).

   Note: The number of days for surviving cells to reach the density at which they can be excised can vary. Generally, resistant clones will recover within 7 days after the exposure to G418 is terminated.

9. Excision of G418 resistant clones (see troubleshooting, problem 2).
   a. Prepare a hESC-qualified Matrigel-coated 48-well plate in advance.
   b. Aspirate hESC-qualified Matrigel from each well of the 48-well dish and add 250 μL of mTeSR1.
   c. Aspirate spent media from one well of the 6-well dish containing clones and add 1 mL of 20°C–25°C GCDR.
   d. Incubate cells at 20°C–25°C for 3 min.

Note: We have found that short exposure to GCDR prior to excision significantly increases clone recovery.

Note: Expose cells to GCDR one well at a time rather than all at once.

Alternatives: because the pAAVS1-CRISPRd cassette contains a doxycycline-inducible mCherry reporter, G418-resistant cells putatively targeted with the CRISPRd cassette can also be isolated by fluorescence activated cell sorting following exposure to 100 μg/mL doxycycline for approximately 3 days. Clonal lines can then be generated following plating mCherry-positive cells.

e. Aspirate GCDR and gently add 2 mL of 20°C–25°C mTeSR1 to the side of the well to avoid disrupting colonies.
f. Identify a single colony that is isolated from other colonies.
g. Use a sterile P200 pipette to gently scrape the isolated colony while pulling media into the pipette tip.
h. Collect all of the fragments of the isolated colony in the P200 pipette tip and transfer the fragments to a single well of the 48-well dish by depositing the entirety of the contents of the P200 pipette tip.

Note: To avoid cross-contamination between isolated colonies, ensure each isolated and excised colony is deposited into a different well of the 48-well dish.

i. Using the same pipette tip, gently triturate the clumps to break them into uniform pieces of 50 to 150 μm each.
j. Discard the pipette tip and repeat for all clones to be collected.

Note: While excising clones, it may be necessary to replace the mTeSR1 in the well in which you are working. Frequently replacing the mTeSR1 can prevent cross-contamination due to cell debris accumulation and accommodate the loss of media during the colony isolation process.

⚠ CRITICAL: We aim to excise 48 discrete colonies selected with G418 per experiment for genotyping. If no G418-resistant colonies form, confirm whether the transfection was successful and/or optimize selection conditions (see troubleshooting, problem 1). If too many cells survive G418 selection and isolated clones cannot be excised, the chances of recovering mixed clones consisting of multiple genotypes will be increased (see troubleshooting, problem 2).

k. Move the 48-well plate containing excised clones to the incubator.
l. Continue daily media changes with mTeSR1 until clones are ready for passaging (typically within 6–7 days).

10. Clone expansion for maintenance and genotyping.
    a. Prepare two hESC-qualified Matrigel-coated 24-well plates in advance. Wells should be labeled 1–48.
    b. Aspirate Matrigel, add 500 μL of mTeSR1 to each well, and place dishes in the incubator.
    c. Prepare 48 sterile, nuclease-free 1.5 mL microcentrifuge tubes labeled 1 to 48.
    d. Aspirate spent media from each well of the 48-well dish containing excised clones and add 125 μL of ReleSR (20°C–25°C) to each well.

Note: We recommend processing a maximum of 24 wells at a time.

e. Aspirate nearly all the ReleSR within 1 min, leaving a small film covering the cells in each well.
f. Return the plate to the incubator and incubate for 5 min.

g. Add 125 μL of mTeSR1 per well.
h. Gently tap the side of the plate to release cell clumps into the media.
i. Retrieve the 24-well plates from the incubator.
j. Using a P200 pipette, gently triturate cell clumps until they are 50–150 μm in diameter.
k. Transfer ~25% of the triturated contents of each well of the 48-well plate to one well of the 24-well plate.
l. Transfer the remaining ~75% of the material in each well to the corresponding microcentrifuge tube.
m. Repeat for all 48 clones.
n. Use the cells collected in the microcentrifuge tubes for genotyping.
o. Return the 24-well plates to the incubator and continue with daily media changes.

Alternatives: Instead of transferring dissociated cells into microfuge tubes in step 10L, cells can be transferred into a v-bottom 96-well plate for pelleting cells. A multichannel pipette can then be used for subsequent cell washing, lysis, and PCR steps.

Note: depending on the brand of 96-well plate used, the volume of DPBS used for cell washing in step 11c below may need to be reduced.

Note: Once expanded to 24-well plates, excised clones can either be cryopreserved or cultured until the genotyping has been completed. Optimally, the genotyping can be completed within 6–7 days, prior to the clones requiring additional passaging.

CRISPRd hPSC line generation: clone genotyping

Timing: 1–2 weeks

In this section, we provide an overview of the genotyping methods we use to identify correctly targeted clones.

11. Preparation of crude genomic DNA lysate.

Note: There are many methods to extract genomic DNA. We have found that the simplest method is to lyse the cells in a buffer containing detergent and proteinase K and to directly use the crude lysate for PCR analysis.

a. Pellet cells by centrifugation at 1000 × *g* for 3 min at 20°C–25°C.
b. Carefully aspirate the supernatant to avoid loss of pelleted cells.
c. Add 250 μL of Dulbecco's phosphate buffered saline (DPBS) to each tube and resuspend cells by brief vortexing.
d. Pellet cells by centrifugation at 1000 × *g* for 3 min at 20°C–25°C.
e. Carefully aspirate the supernatant to avoid loss of pelleted cells.
f. Add 100 μL of Cell lysis buffer to each tube and briefly vortex to disperse the cell pellet.
g. Incubate at 55°C for a minimum of 2 h.

Pause point: This incubation can be left for up to 24 h if necessary.

h. Heat-inactivate proteinase K by incubating tubes at 95°C for 10 min.

Note: For long-term storage, crude lysates can be frozen at −20°C.

12. Genotyping for clone verification.

Note: We use a combination of PCR genotyping and Sanger sequencing to genotype clones. As depicted in Figures 1A–1C, the PCR strategy involves: i) detecting transgene knock-in (KI) to the correct genomic locus using pairs of internal and external primers that flank the 5′- and 3′-junctions between the transgene and the AAVS1 genomic sequence outside the homology arms (primer pairs 4 and 5), ii) detecting the presence of a non-KI AAVS1 allele, to facilitate identification of heterozygous and homozygous transgene knock-in (primer pair 1), and iii) detecting random integration of donor plasmid using primer pairs that amplify 5′ and 3′ sides of the plasmid backbone sequences outside of the targeted cassette (primer pairs 2 and 3).

a. Prepare PCR reaction mix.

Reagent	Final concentration	Volume for 1× (uL)
Forward primer (100 μM)	1 μM	0.2 μL
Reverse primer (100 μM)	1 μM	0.2 μL
5× Phusion HF Buffer	1×	4.0 μL
dNTP (10 μM)	0.2 μM	0.4 μL
Phusion polymerase	N/A	0.1 μL
DMSO (100%)	3.0%	0.6 μL
Genomic DNA in cell lysis buffer	N/A	1.0 μL
Nuclease-free water	N/A	13.5 μL
Total	**N/A**	**20 μL**

Note: We routinely use Phusion polymerase for genotyping PCR reactions. Using different polymerases will require optimization of PCR conditions, including changes in temperatures and lengths of time for each step according to the manufacturer's instructions.

Note: include a negative control reaction with no added genomic DNA (i.e., 1.0 μL of cell lysis buffer) to ensure that PCR reagents are not contaminated with genomic DNA.

Alternatives: A 2× Phusion High-Fidelity PCR Master Mix is commercially available (NEB; M0531S) and can be used for convenience.

b. Run PCR reaction.

Steps	Temperature	Time	Cycles
Initial denaturation	98°C	5 min	1
Denature	98°C	15 s	35 cycles
Anneal	Primer pair dependent	15 s	
Extension	72°C	15–30 s/kb	
Final extension	72°C	7 min	1
Hold	4°C	infinite	

Note: The donor plasmid (pAAVS1-NDi-CRISPRd) contains the GC-rich CAG promoter, making PCR and sequencing of this region challenging.

Note: The recommended annealing temperatures and amplicon size for each primer pair are listed below.

Primer pair	Tm	Size (bp)
1	67°C	171
2	65°C	219
3	64°C	230
4	66°C	1619
5	66°C	1345

Note: See key resources table for sequences of each primer pair used for genotyping.

c. Run PCR products in an agarose gel containing 0.5 μg/mL ethidium bromide at 100 V for 1 h. Include a 100-base-pair ladder.

13. Genotyping workflow and interpretation of results.
    a. Identify clones containing a targeted integration of the CRISPRd transgene.
        i. Subject lysates from all 48 clones to separate PCR reactions using primer pairs 4 and 5 (Figures 1A–1C).
        ii. Identify clones with amplicons for both primer pairs.

Note: This will putatively identify clones in which at least one AAVS1 allele has undergone integration of the CRISPRd cassette.

b. For clones containing evidence of targeted integration using primer pairs 4 and 5, perform PCR using primer pair 1 to determine zygosity.

Note: Include a positive control reaction containing genomic DNA extracted from untargeted cells.

Note: For each clone that is positive using primer pairs 4 and 5, there are 2 possible genotypes: (1) If an amplicon is generated using primer pair 1, the putative genotype is a mono-allelic targeted integration of the transgene. (2) If an amplicon is not generated using primer pair 1, the putative genotype is a bi-allelic targeted integration of the transgene.

Note: The absence of an amplicon for using primer pair 1 does not exclude the possibility of targeted integration at one AAVS1 allele and a large deletion in the second AAVS1 allele that disrupts primer binding.

c. Subject genomic DNA from putative mono- and bi-allelic targeted clones to PCR using primer pairs 2 and 3 to confirm the absence of random genomic integration.

Note: Include a positive control reaction containing donor plasmid DNA.

Note: Amplicon generation for either primer pair indicates the random genomic integration of the targeting plasmid. Clones with random integration should not be used.

Note: For detection of random integration, the use of 2 primer pairs that anneal to discrete regions of the targeting plasmid backbone is necessary.

Note: See the table below for a summary of the interpretation of PCR genotyping results and putative genotypes.

	Primer pair				
Putative genotype	1	2*	3*	4	5
Unmodified or indels only	+	-	-	-	-
Unmodified + random integration	+	+	+	-	-
Heterozygous	+	-	-	+	+
Heterozygous + random integration	+	+	+	+	+
Homozygous	-	-	-	+	+
Homozygous + random integration	-	+	+	+	+

Note: *A positive result for either primer pair 2 or 3, or both confirms random integration.

⚠ CRITICAL: Sanger sequencing of genomic DNA across the targeted AAVS1 region is essential to confirm PCR genotyping. This includes sequencing to confirm the unmodified or indels in the non-KI AAVS1 alleles.

⚠ CRITICAL: It is important to analyze off-target gene editing associated with wild-type SpCas9-activity. RNA design sites such as CRISPOR (http://crispor.tefor.net/)[16] provide a list of the most likely predicted off-target loci and primers that can be used to amplify across each locus. We recommend amplifying each of the top 5 predicted off-target loci and sequencing PCR amplicons to confirm the lack of off-target editing in selected clones.

⚠ CRITICAL: To support the PCR-based random integration genotyping approach we describe above and to confirm correct AAVS1 targeting, we also recommend performing a transgene copy number assay (e.g., using Applied Biosystems TaqMan copy number assays on NeoR, following the manufacturer's recommended protocol).

⚠ CRITICAL: Identified clones should be robustly quality controlled before use in CRISPRd experiments. At a minimum, we recommend cells to be mycoplasma-free, have a normal karyotype, express markers of the undifferentiated state (e.g., by immunofluorescence and flow cytometric analysis), have the capacity to differentiate into ectoderm, endoderm, and mesoderm lineages, and have had their identity authenticated (e.g., by STR analysis).[15]

⚠ CRITICAL: When working with newly generated lines, before proceeding with sgRNA transduction, we also advise validating the expression of the ABI-dCas9 transgene (Figures 1D and 1E). This requires optimizing Dox concentration and timing, assessing the inducibility and reversibility of ABI-dCas9 expression.

Design and cloning of a sgRNA to a lentivirus plasmid

Timing: 1–2 weeks

In this section, we provide protocols for designing and generating a sgRNA expression lentivirus plasmid, targeting a FOXA binding motif at the FOXA-PRDM1 co-binding site in the *ZEB2* locus, by Golden-Gate cloning (Figure 2A).

14. Generate transcription factor (TF)-motif *.bed file.
    a. Perform a *de novo* motif search within the TF ChIP-seq or CUT&RUN peaks using Homer.[13]

```
>> findMotifsGenome.pl <peak file> <genome> <output folder>
```

where,
i. <peak file> is a BED-format ChIP-seq or CUT&RUN peak file from Homer or MACS.
ii. <genome> is the genome version in Homer. hg38 for this particular experiment.
iii. <output folder> is an arbitrary user-defined folder.

    b. Obtain Homer motifs for FOXA and PRDM1.
        i. Open <output folder>/homerResults.html in a genome browser to see the list of discovered *de novo* motifs.
        ii. Identify FOXA and PRDM1 motifs from the list and save the corresponding motif files (*.motif).
    c. Perform motif scan within the ChIP-seq or CUT&RUN peaks.

```
>> annotatePeaks.pl <peak file> <genome> -m <motif file> -mbed motif.bed -noann -nogene
```

where,
i. <peak file> is the same peak file from 14-a.

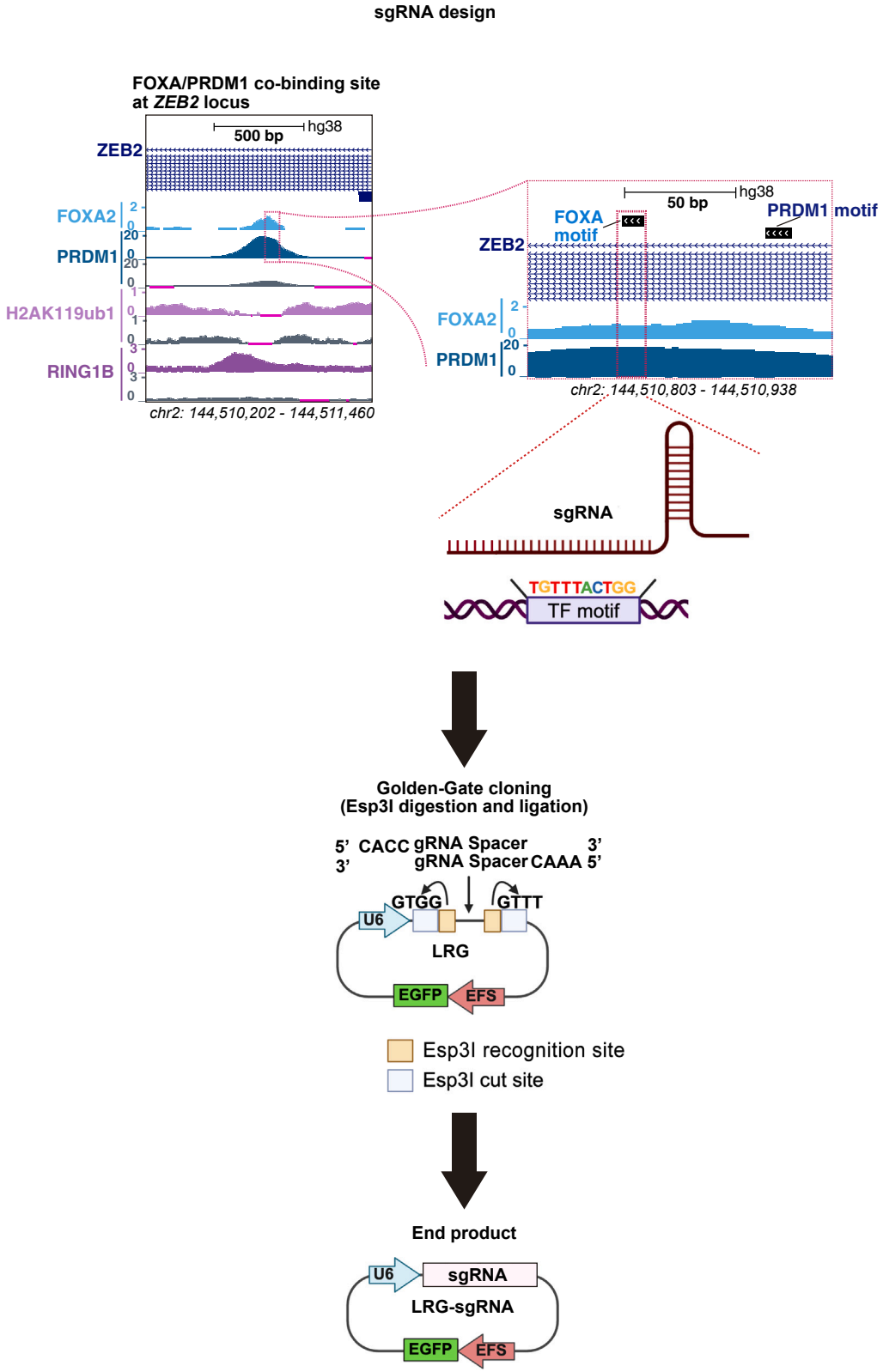
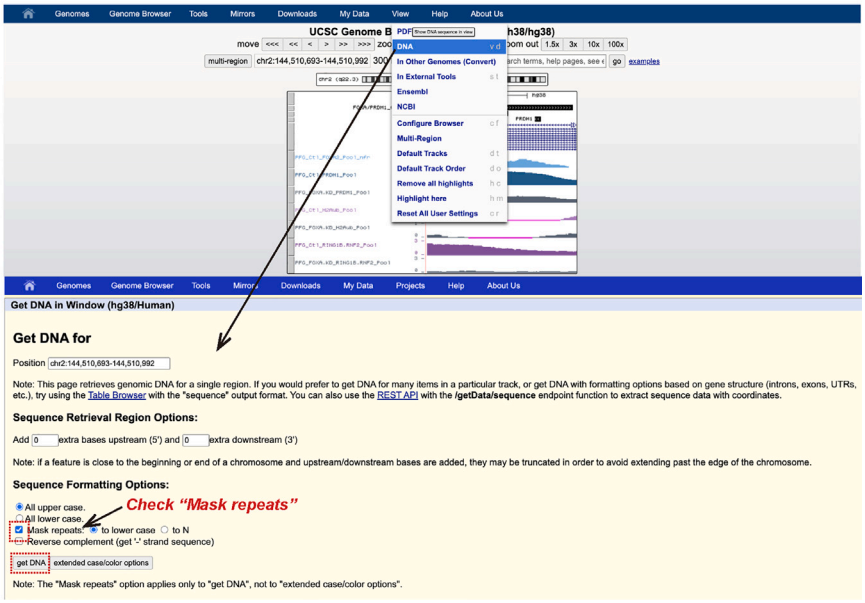
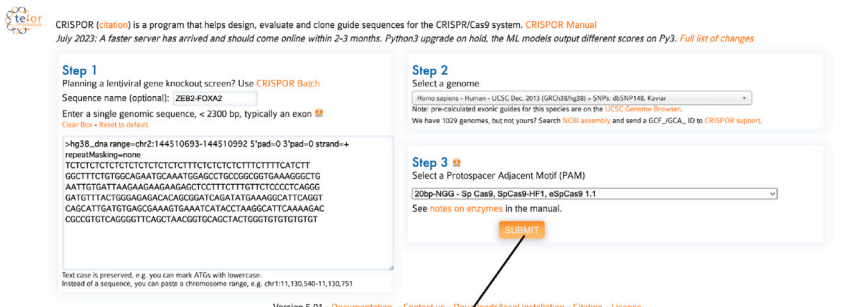


Figure 2. Schematic workflow of FOXA-CRISPRd sgRNA design and Golden-Gate cloning to the LRG lentiviral plasmid

A


Genomes
Genome Browser
Tools
Mirrors
Downloads
My Data
View
Help
About Us
UCSC Genome B
h38/hg38)
move
zoom out
1.5x
3x
10x
100x
multi-region
chr2:144,510,693-144,510,992
go
examples
PDF
DNA
In Other Genomes (Convert)
In External Tools
Ensembl
NCBI
Configure Browser
Multi-Region
Default Tracks
Default Track Order
Remove all highlights
Highlight here
Reset All User Settings
Genomes
Genome Browser
Tools
Mirrors
Downloads
My Data
Projects
Help
About Us
Get DNA in Window (hg38/Human)
Get DNA for
Position
chr2:144,510,693-144,510,992
Note: This page retrieves genomic DNA for a single region. If you would prefer to get DNA for many items in a particular track, or get DNA with formatting options based on gene structure (introns, exons, UTRs, etc.), try using the Table Browser with the "sequence" output format. You can also use the REST API with the /getData/sequence endpoint function to extract sequence data with coordinates.
Sequence Retrieval Region Options:
Add
extra bases upstream (5') and
extra downstream (3')
Note: if a feature is close to the beginning or end of a chromosome and upstream/downstream bases are added, they may be truncated in order to avoid extending past the edge of the chromosome.
Sequence Formatting Options:
All upper case.
All lower case.
Mask repeats:
to lower case
to N
Reverse complement (get '-' strand sequence)
get DNA
extended case/color options
Note: The "Mask repeats" option applies only to "get DNA", not to "extended case/color options".
Check "Mask repeats"

B


CRISPOR (citation) is a program that helps design, evaluate and clone guide sequences for the CRISPR/Cas9 system. CRISPOR Manual
July 2023: A faster server has arrived and should come online within 2-3 months. Python3 upgrade on hold, the ML models output different scores on Py3. Full list of changes
Step 1
Planning a lentiviral gene knockout screen? Use CRISPOR Batch
Sequence name (optional):
ZEB2-FOXA2
Enter a single genomic sequence, < 2300 bp, typically an exon
Clear Box • Reset to default
>hg38_dna range=chr2:144510693-144510992 5'pad=0 3'pad=0 strand=+ repeatMasking=none
TCTCTCTCTCTCTCTCTCTCTTTCTCTCTCTCTTTCTTTTCATCTT
GGCTTTCTGTGGCAGAATGCAAATGGAGCCTGCCGGCGGTGAAAGGGCTG
AATTGTGATTAAGAAGAAGAAGAGCTCCTTTCTTTGTTCTCCCCTCAGGG
GATGTTTACTGGGAAGAGACACAGCGGATCAGATATGAAAGGCATTCAGGT
CAGCATTGATGTGAGCGAAAGTGAAATCATACCTAAGGCATTCAAAAGAC
CGCCGTGTCAGGGGTTCAGCTAACGGTGCAGCTACTGGGTGTGTGTGTGT
Text case is preserved, e.g. you can mark ATGs with lowercase.
Instead of a sequence, you can paste a chromosome range, e.g. chr1:11,130,540-11,130,751
Step 2
Select a genome
Homo sapiens - Human - UCSC Dec. 2013 (GRCh38/hg38) + SNPs: dbSNP148, Kaviar
Note: pre-calculated exonic guides for this species are on the UCSC Genome Browser.
We have 1029 genomes, but not yours? Search NCBI assembly and send a GCF_/GCA_ ID to CRISPOR support.
Step 3
Select a Protospacer Adjacent Motif (PAM)
20bp-NGG - Sp Cas9, SpCas9-HF1, eSpCas9 1.1
See notes on enzymes in the manual.
SUBMIT
Version 5.01 - Documentation - Contact us - Downloads/local installation - Citation - License

C

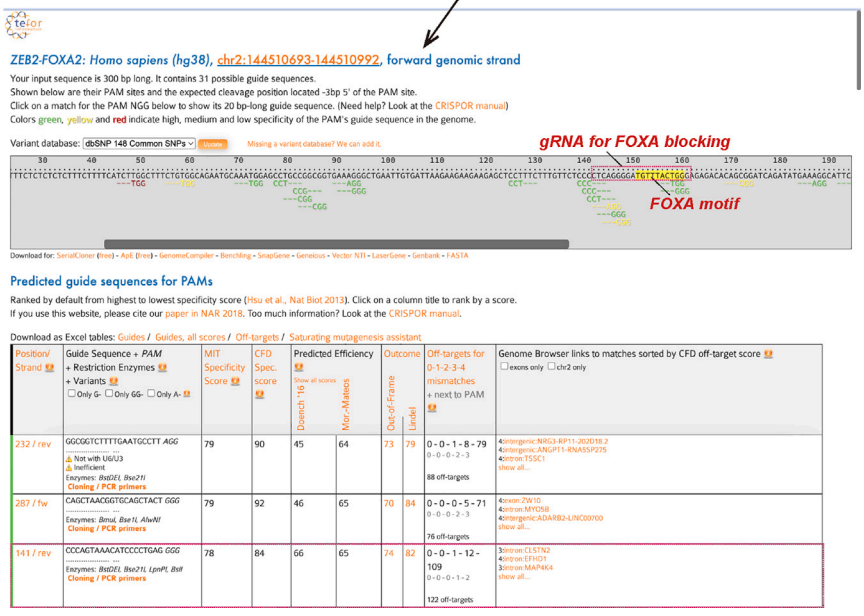

ZEB2-FOXA2: Homo sapiens (hg38), chr2:144510693-144510992, forward genomic strand
Your input sequence is 300 bp long. It contains 31 possible guide sequences.
Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site.
Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the CRISPOR manual)
Colors green, yellow and red indicate high, medium and low specificity of the PAM's guide sequence in the genome.
Variant database: dbSNP 148 Common SNPs
Missing a variant database? We can add it.
gRNA for FOXA blocking
FOXA motif
Download for: SerialCloner (free) - ApE (free) - GenomeCompiler - Benchling - SnapGene - Geneious - Vector NTI - LaserGene - Genbank - FASTA
Predicted guide sequences for PAMs
Ranked by default from highest to lowest specificity score (Hsu et al., Nat Biot 2013). Click on a column title to rank by a score.
If you use this website, please cite our paper in NAR 2018. Too much information? Look at the CRISPOR manual.
Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant
Position/ Strand
Guide Sequence + PAM + Restriction Enzymes + Variants
MIT Specificity Score
CFD Spec. score
Predicted Efficiency
Doench '16
Mor.-Mateos
Outcome
Out-of-Frame
Linkel
Off-targets for 0-1-2-3-4 mismatches + next to PAM
Genome Browser links to matches sorted by CFD off-target score
232 / rev
GGCGGTCTTTTGAATGCCTT AGG
Not with U6/U3
Inefficient
Enzymes: BstDEI, Bse21I
Cloning / PCR primers
79
90
45
64
73
79
0-0-1-8-79
0-0-0-2-3
88 off-targets
4intergenic:NRG3-RP11-202D18.2
4intergenic:ANGPT1-RNA5SP275
4intron:TSSC1
show all...
287 / fw
CAGCTAACGGTGCAGCTACT GGG
Enzymes: BmuI, BseYI, AflwNI
Cloning / PCR primers
79
92
46
65
70
84
0-0-0-5-71
0-0-0-2-3
76 off-targets
4exon:ZW10
4intron:MYO5B
4intergenic:ADARB2-LINC00700
show all...
141 / rev
CCCAGTAAACATCCCCTGAG GGG
Enzymes: BstDEI, Bse21I, LpnPI, BslI
Cloning / PCR primers
78
84
66
65
74
82
0-0-1-12-109
0-0-0-1-2
122 off-targets
3intron:CLSTN2
4intron:EFHD1
3intron:MAPK4
show all...

Figure 3. The workflow for designing a gRNA spacer using CRISPOR

(A) The UCSC genome browser website to obtain a target DNA sequence.

(B) The top page of the CRISPOR website shows the instructions for designing gRNA spacers.

(C) The result page of the CRISPOR website displays available gRNA spacers. The red dashed box indicates a gRNA candidate, and the yellow highlight represents a FOXA2 binding motif.

ii. <genome> is the same genome from 14-a (hg38).
iii. <motif file> is Homer motif files (*.motif) for FOXA and PRDM1 from 14-b.

Note: The output file, motif.bed contains all the coordinates of the corresponding motifs within the peak regions, which can be loaded into a genome browser for inspection and gRNA design.

Alternatives: We can obtain TF-motif *.bed file from JASPAR database (https://jaspar.elixir.no/) (e.g., FOXA2: https://jaspar.elixir.no/matrix/MA0047.4/).[16]

15. Design a gRNA spacer sequence.
    a. Load the motif.bed file into a genome browser to visualize the location of TF binding motifs and ChIP-seq or CUT&RUN signal on the genome using the UCSC genome browser (https://genome.ucsc.edu/) or IGV.[14]
    b. Identify a target genomic region of interest bound by the TFs of interest (e.g., FOXA and PRMD1) and containing the TF-binding motifs (Figure 2B) and obtain a 150- to 300-bp genomic sequence around the target genomic region from the UCSC genome browser (Figure 3A).

⚠ CRITICAL: To target a TF co-binding site and individually block each TF-binding motif , the TF motifs should be spaced at least 40 to 50 bp apart. This spacing ensures that the CRISPRd system can effectively target and block each TF-binding motif without interfering with others.

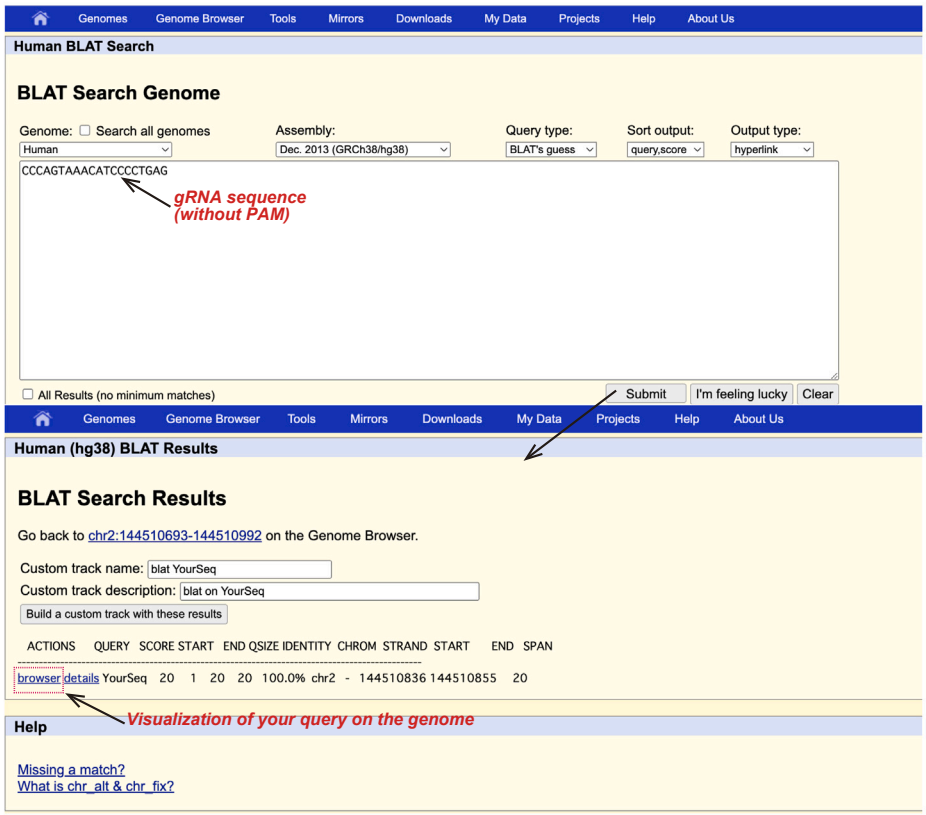
    c. Open CRISPOR (http://crispor.gi.ucsc.edu/)[17] and enter the obtained DNA sequence (Figure 3B).
    d. Select SpCas9 (20 bp-NGG) and the optimal reference genome, which matches your ChIP-seq or CUT&RUN data (e.g., hg38).
    e. Assess potential gRNA spacer sequences that cover the target TF binding motif with optimal MIT Specificity scores (>66), Doench '16 scores (>50), and Mor.-Mateos scores (>40) (Figure 3C).

⚠ CRITICAL: The gRNA spacer sequence should cover the target TF binding motif, ideally the entire motif. However, covering a major portion of the motif (e.g., 11 bp out of 14 bp) can still block the binding.[2]

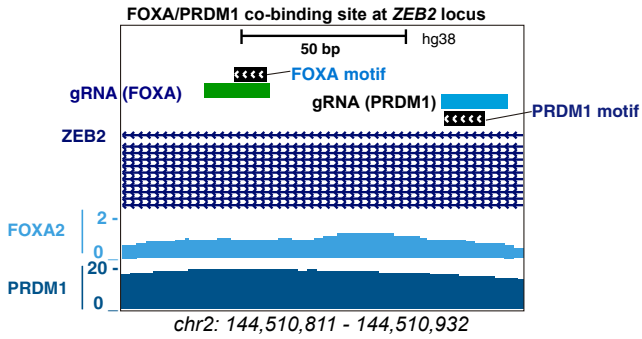
    f. Confirm the gRNA target location in the UCSC genome browser (Figure 4A).
        i. Open BLAT in the Tool tab.
        ii. Enter the designed gRNA spacer sequence.
        iii. Check "All Results"
        iv. Click "submit."
        v. Click "browser."
        vi. The result is shown in Figure 4B.
16. Golden-Gate cloning of a gRNA spacer sequence into a host lentivirus plasmid (see troubleshooting, problem 3).

Alternatives: To clone multiple sgRNAs into a host lentivirus plasmid, you can apply a 2-step Golden-Gate cloning protocol.[18,19] .

A


Genomes
Genome Browser
Tools
Mirrors
Downloads
My Data
Projects
Help
About Us
Human BLAT Search
BLAT Search Genome
Genome: Search all genomes
Human
Assembly:
Dec. 2013 (GRCh38/hg38)
Query type:
BLAT's guess
Sort output:
query,score
Output type:
hyperlink
CCCAGTAAACATCCCCTGAG
gRNA sequence
(without PAM)
All Results (no minimum matches)
Submit
I'm feeling lucky
Clear
Human (hg38) BLAT Results
BLAT Search Results
Go back to chr2:144510693-144510992 on the Genome Browser.
Custom track name: blat YourSeq
Custom track description: blat on YourSeq
Build a custom track with these results
ACTIONS QUERY SCORE START END QSIZE IDENTITY CHROM STRAND START END SPAN
browser details YourSeq 20 1 20 20 100.0% chr2 - 144510836 144510855 20
Visualization of your query on the genome
Help
Missing a match?
What is chr_alt & chr_fix?

B


FOXA/PRDM1 co-binding site at ZEB2 locus
50 bp
hg38
FOXA motif
gRNA (FOXA)
gRNA (PRDM1)
PRDM1 motif
ZEB2
FOXA2
2
0
PRDM1
20
0
chr2: 144,510,811 - 144,510,932

C

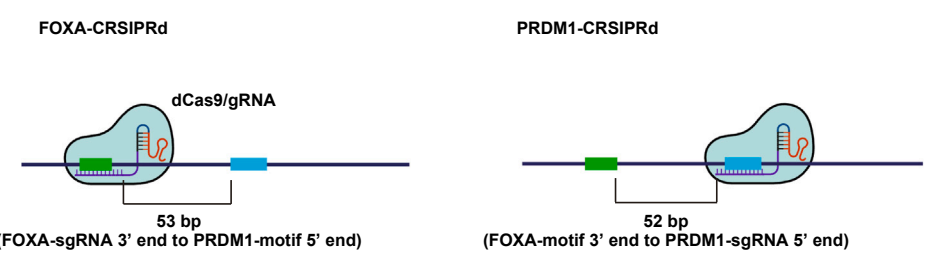

FOXA-CRSIPRd
PRDM1-CRSIPRd
dCas9/gRNA
53 bp
(FOXA-sgRNA 3' end to PRDM1-motif 5' end)
52 bp
(FOXA-motif 3' end to PRDM1-sgRNA 5' end)

Figure 4. Confirmation of the genomic location of a gRNA spacer sequence

(A) Utilize the BLAT Search in the UCSC genome browser website to verify the specificity of the gRNA sequence within the genome. The gRNA sequence aligns uniquely with the target genomic locus at chr2: 144510836–144510855 (*ZEB2* locus).

(B) Visualization of a gRNA target location on the UCSC genome browser. Each gRNA targeting the FOXA2 motif and PRDM1 motif, respectively, in the ZEB2 locus, covering each TF binding motif completely.

(C) Schematic representation of the CRISPRd system targeting the FOXA binding motif and PRDM1 binding motif on the *ZEB2* locus.

a. Design gRNA oligo DNA with specific overhang sequences for cloning into a Esp3I site, as indicated below.

Host plasmid	gRNA	Overhang sequence	gRNA Protospacer sequences (targeting a FOXA binding motif in the *ZEB2* locus)	Overhang sequence
LRG (Addgene #65656)	gRNA- FOXA.bs-ZEB2.locus-sense	5′-CACC**G**	CCCAGTAAACATCCCCTGAG	
	gRNA- FOXA.bs-ZEB2.locus-antisense	5′-AAAC	CTCAGGGGATGTTTACTGGG	C-3′

Note: We added an extra 5′ guanine (G) to the gRNA spacer sequence as the overhang sequence to enable robust transcription from the U6 promoter.[1]

b. Anneal sense and antisense oligo DNA.

i. Prepare the Annealing buffer as indicated below.

Annealing buffer		
Reagent	Final concentration	Amount
5 M NaCl	50 mM	250 μL
1 M Tris-HCl (pH 8.0)	10 mM	250 μL
0.5 M EDTA (pH 8.0)	1 mM	50 μL
Nuclease-free water	N/A	24.45 mL
Total	**N/A**	**25 mL**

Note: Store at 20°C–25°C for 1 year.

ii. Prepare an annealing reaction mix as indicated below.

Reagent	Final concentration	Amount
Sense oligo DNA (100 μM)	1.0 μM	0.5 μL
Antisense oligo DNA (100 μM)	1.0 μM	0.5 μL
Annealing buffer	N/A	49.0 μL
Total	**N/A**	**50.0 μL**

iii. Anneal oligo DNA by thermal cycler as indicated below.

Steps	Temperature	Time	Cycles
Denaturation	95°C	5 min	1
Annealing	95°C (−1°C/cycle)	1 min	70 cycles
Hold	4°C	infinite	

Note: The final concentration of annealed oligo is 1.0 μM,

iv. Prepare a phosphorylation mix as indicated below.

Reagent	Final concentration	Amount
Annealed oligo DNA	1.0 μM	50.0 μL
10× T4 PNK Reaction Buffer	1×	5.0 μL
ATP (10 mM)	1 mM	5.0 μL
T4 PNK (10 U/uL)	0.2 U/uL	1.0 μL
Total	**N/A**	**61.0 μL**

Note: The final concentration of annealed oligo is 0.82 μM.

v. Incubate at 37°C for 30 min.
vi. Heat inactivates at 65°C for 20 min.
vii. Dilute annealed, phosphorylated oligo DNA to 0.1 μM (100 fmol/uL) with TE (pH 8.0) for the next step (e.g., 6.1 μL of 0.82 μM phosphorylated oligo DNA + 43.9 μL of TE).

c. Golden-Gate cloning.
i. Prepare the Golden-Gate reaction mix as indicated below.

Reagent	Final concentration	Amount
LRG plasmid (100 ng/μL) (Addgene #65656)	46 fmol (molar ratio 1)	2 μL
Annealed oligo DNA (0.1 μM)	100 fmol (molar ratio 2)	1 μL
FastDigest Esp3I	N/A	1 μL
T4 DNA ligase (400 U/uL)	2 U/uL	1 μL
10× DNA Ligase Reaction Buffer	1×	2 μL
Nuclease-free water	N/A	to 20 μL
Total	**N/A**	**20 μL**

Note: We use a molar ratio of 1:2 host plasmid to insert for reaction. You can calculate the molar ratio by NEBioCalculator (https://nebiocalculator.neb.com/#!/ligation).

⚠ CRITICAL: Isoschizomers, BsmBI, is not applicable for the Golden-Gate reaction below due to its different optimal reaction temperature (55°C).

⚠ CRITICAL: The T4 ligase buffer contains ATP, which degrades quickly. We recommend aliquoting the buffer and discarding the aliquots after 2 or 3 freeze-and-thaw cycles to maintain optimal ligation.

Alternatives: T7 DNA ligase can be used, as it ligates only sticky ends, reducing background and improving specificity in the ligation reaction.

ii. Incubate the Golden-Gate mix as indicated below.

Steps	Temperature	Time	Cycles
Golden gate assembly	37°C	5 min	20 cycles
	16°C	10 min	
	37°C	30 min	1
	75°C	15 min	1
Hold	4°C	infinite	

Note: Increasing the cycle number improves ligation efficiency.

d. Transformation of ligated DNA into competent *E. coli*.
i. Thaw 50 μL of NEB Stable Competent *E. coli* on ice.
ii. Add 5 μL of ligated plasmid to the competent cells and mix by pipetting or tapping.
iii. Incubate on ice for 30 min.
iv. Heat shock the competent cell mixture in a 42°C water bath for 30 s.
v. Place cells on ice for 2 min.
vi. Add 950 μL of NEB 10-beta/Stable Outgrowth Medium to the mixture.
vii. Place the mixture at 37°C for 1 h with shaking.
viii. Spread 50 to 100 μL of the transformed competent cells on an Ampicillin-containing LB plate and incubate at 30°C for 24 h.

⚠ CRITICAL: For better clone stability of the lentivirus plasmid, incubate plates at 30°C.

e. Colony isolation, small-scale plasmid purification, and plasmid verification.
   i. Pick 4 single colonies and transfer each colony into a separate bacteria culture tube containing 2 mL of Ampicillin-containing LB medium.
   ii. Incubate bacterial culture at 30°C for 16–20 h in a shaking incubator.

⚠ CRITICAL: For better clone stability, pick colonies from fresh transformants (plates with bacteria transformed no more than 3 days ago) and incubate in liquid culture at 30°C.

   iii. Centrifuge at 3000 × *g* for 10 min at 4°C.
   iv. Discard LB media and purify plasmid DNA using a mini-prep kit (e.g., GeneJET Plasmid Miniprep Kit: Thermo Fisher Scientific).
   v. Measure concentration by Nanodrop.
   vi. Prepare a restriction enzyme reaction mix as indicated below, and incubate at 37°C for 2 h.

Reagent	Final concentration	Amount
Mini-prep plasmid	100 ng	Variable
Esp3I (20 U/uL)	0.4 U/uL	0.5 μL
10× CutSmart Buffer	1×	1 μL
Nuclease-free water	N/A	to 10 μL
Total	**N/A**	**10 μL**

   vii. Run digested plasmid in agarose gel with 0.5 μg/mL ethidium bromide at 100 V for 1 h.

Note: If sgRNA is successfully integrated, the Esp3I restriction sites will be removed from the host plasmids. The expected band sizes of the empty host plasmid are 7413 bp and 1885 bp.

   viii. Confirm the integrated gRNA spacer sequence using Sanger sequencing with LRG-gRNA sequencing Primer (5′-GAGGGCCTATTTCCCATGATT-3′).

f. Amplify successfully cloned plasmids on a midi or maxi-culture scale.
   i. Transform and incubate successfully cloned plasmid into Stable competent *E. coli* cells as described in step 16d.
   ii. Pick ~3 single colonies and transfer each colony into a separate bacteria culture tube containing 2 mL of ampicillin-containing LB media.
   iii. Incubate at 30°C for 16–20 h in a shaking incubator.
   iv. Dilute one of the starter bacteria cultures in 1:1000 in ampicillin-containing LB media (e.g., transfer 50 μL of starter bacteria culture to 50 mL media).
   v. Incubate at 30°C for 18–24 h in a shaking incubator.
   vi. Centrifuge at 3000 × *g* for 10 min at 4°C.
   vii. Discard LB media and purify plasmid DNA using a midi- or maxi-prep kit.
   viii. Measure concentration by Nanodrop.
   ix. Validate the plasmid sample by restriction enzyme digestion as described in steps 16e-vi to vii.

Preparation of sgRNA expression lentivirus

Timing: 9 days

In this section, we describe lentivirus packaging for the sgRNA expression plasmid in HEK293T cells. Our protocol uses a LentiX concentrator (Takara Bio) for easy and fast virus purification, capable of concentrating the virus 60–70 times.

⚠ CRITICAL: Avoid using growth-retarded HEK293T cells. This can cause low transfection efficiency. Ideally, cells within 4 passages post-thawing should be used.

17. Thawing HEK293T cells (Day 0; Passage 0 post-thaw).
    a. Thaw a HEK293T cryovial in a 37°C water bath.
    b. Remove the vial from the bath as soon as it is thawed and spray with 70% Ethanol.
    c. Transfer the cell suspension to a 15 mL conical tube containing 9 mL of the basal media (10% FBS-supplemented DMEM).
    d. Centrifuge at 300 × *g* for 5 min at 20°C–25°C.
    e. Aspirate supernatant and resuspend cell pellets with 1 mL of the basal media.
    f. Transfer the cell suspension into a 10 cm dish containing 10 mL of basal media, slide the dish forward and backward, and then left to right to ensure equal distribution of the cells.
    g. Place the culture dish in a 37°C 5% CO_2 incubator.

Note: Once cells reach sub-confluency (70%–80%), proceed to passage.

18. Passaging HEK293T cells (Day 3; Passage 1).
    a. Aspirate the culture media from the sub-confluent culture dish and wash the dish with 5 mL of 1× PBS, twice.
    b. Add 2.5 mL of TryPLE Express.
    c. Incubate at 37°C or 20°C–25°C for 1–5 min.
    d. Add 2.5 mL of the basal media and pipet up and down (about 5 times) to dissociate cells into single cells.
    e. Transfer the cell suspension to a 15-mL conical tube.
    f. Centrifuge at 300 × *g* for 5 min at 20°C–25°C.
    g. Aspirate supernatant and resuspend the cell pellet with 1 mL of the basal media.
    h. Transfer the desired amount of the cell suspension into a 10 cm dish containing 10 mL of fresh basal media (1:6 to 1:10 split), slide the dish forward and backward, and then from left to right to ensure equal distribution of the cells.
    i. Place the culture dish in a 37°C 5% CO_2 incubator.
19. Passaging HEK293T for virus packaging (Day 5; Passage 2).

⚠ CRITICAL: The basal media (10% FBS-supplemented DMEM) should not contain any antibiotics.

    a. Follow the passaging protocol 18-a to 18-g.
    b. Transfer 1 × 10^6 cells into a 10 cm dish containing 10 mL of fresh basal media without antibiotics, slide the dish forward and backward, and then from left to right to ensure equal distribution of the cells.
    c. Place the culture dish in a 37°C 5% CO_2 incubator, slide the dish again, and gently close the incubator door.
20. Transfection of lentivirus, packaging, and envelope plasmids into HEK293T cells (Day 6; 24 h post-plating)

⚠ CRITICAL: The confluency of HEK293T cells should be 30–40%. Do not use sub-confluent cells.

    a. Prepare transfection mix as indicated below (see troubleshooting, problem 4).

Reagent	Amount
LRG-sgRNA.FOXA-ZEB2_CRISPRd_v2 (Addgene #216173)	5.0 μg
psPAX2 (Addgene #12260)	3.4 μg
pMD2.G (Addgene #12259)	1.6 μg
Opti-MEM	500 μL

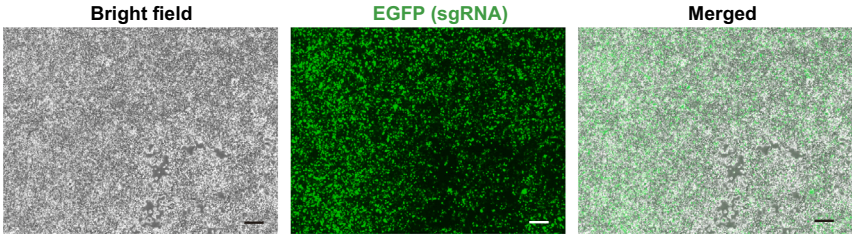


Figure 5. EGFP marker expression from a sgRNA-lentivirus plasmid in HEK293T cell

Transfection efficiency of the sgRNA lentivirus plasmid is assessed by monitoring EGFP expression. Scale bars = 300 μm.

⚠ CRITICAL: Using a low concentration of plasmid may reduce transfection efficiency. Concentrate plasmids if a concentration is lower than 0.5 μg/μL.

b. Add 15 μL (DNA [ug]: PEI [uL] = 1:1.5) of PEI working solution (1 μg/mL) to 500 μL of the transfection mix.
c. Immediately vortex for 15 s.
d. Incubate the transfection mix for 10 min at 20°C–25°C.
e. Add 600 μL of DMEM (without FBS and antibiotics) to the transfection mix.
f. Add the entire amount of the transfection mix (1.1 mL) to the HEK293T cells in a 10 cm dish dropwise. Distribute drops over the entire dish by gently rocking the dish.
g. Place the dish into the 37°C 5% CO_2 incubator.

21. Replace with fresh 10% FBS-supplemented DMEM (Day 7: 14–18 h post-transfection).

⚠ CRITICAL: 24 h post-transfection, lentivirus will start to be produced. Handle all procedures as Risk Group Level 2.

Note: If the culture media becomes yellow before 60 h post-medium change, collect the lentivirus supernatant (keep it at 4°C), feed cells with new media, and pool the two supernatants for concentration at step 22.

22. Collect and concentrate lentivirus supernatant (Day 9; ~60 h post-medium change).
    a. Confirm transfection efficiency by EGFP expression using a fluorescence microscope. Virus-producing cells can be identified by morphological changes (i.e., rounder shape compared to non-transfected cells).

    Note: A transfection efficiency of more than 80–90% results in a higher titer of virus (Figure 5).

    b. Collect lentivirus supernatant into a 15- or 50-mL conical tube.
    c. Centrifuge at 700 × *g* for 10 min at 4°C to remove cell debris.
    d. Filter the supernatant on a 0.45 μm PES syringe filter.

    ⚠ CRITICAL: Do not use a Nitrocellulose filter, as it can degrade the virus.

    e. Add 1/3 volume of Lenti-X Concentrator (e.g., 3 mL for 9 mL supernatant) and mix by gentle inversion.
    f. Incubate the mixture at 4°C for 1 h.
    g. Centrifuge mixture at 1500 × *g* in a swing bucket at 4°C for 45 min.
    h. Carefully remove supernatant, taking care not to disturb the off-white pellet. Any residual supernatant can be removed with a pipette tip.
    i. Gently pipette to resuspend the pellet in ~100 μL of plain DMEM without FBS and antibiotics (1/100th of the original volume).

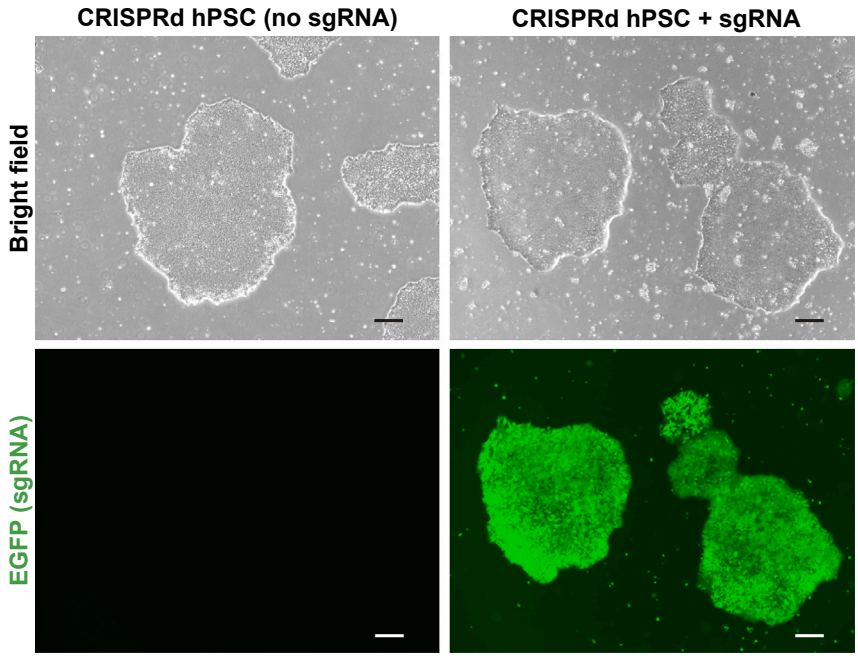


Figure 6. EGFP marker expression in CRISPRd hPSCs transduced with sgRNA lentivirus

Successful lentivirus transduction is assessed by monitoring EGFP expression. The left panels show sgRNA-untransduced controls. Scale bars = 300 μm.

j. Make single-use aliquots (10–20 μL each), snap freeze on dry ice, and store at −80°C.

Alternatives: Lentivirus can also be purified and concentrated by ultracentrifugation, which can achieve a higher level of virus concentration.

sgRNA-lentivirus transduction into the CRISPRd host hPSCs

Timing: 1–2 weeks

In this section, we describe the stepwise protocol for sgRNA-lentivirus transduction into CRISPRd host hPSC line by spin infection,[20] which enables high transduction efficiency in hPSCs (>80%) (Figure 6).

23. Lentivirus transduction (see troubleshooting, problem 5).

⚠ CRITICAL: Follow your institute's guidelines for lentivirus handling.

a. Bring reagents (mTeSR1, DMEM/F12, Accutase, and Matrigel- or Cultrex-coated 12-well plates) to 20°C–25°C.
b. Prepare mTeSR1 + 10 μM Y27632 and Transduction media (see materials and equipment).
c. Aspirate media and briefly rinse cells with DMEM/F12.
d. Aspirate DMEM/F12 and add Accutase (800 μL per one well of a 6-well plate).
e. Place cells into a 37°C 5% CO_2 incubator and incubate for 10–15 min.
f. Add mTeSR1 (1 mL per one well of a 6-well plate).
g. Dissociate cells into single cells by pipetting using a P1000 pipet.
h. Transfer the cell suspension into a 15 mL conical tube.

i. Take 10 μL of cell suspension and mix it with 10 μL of trypan blue for cell counting.
j. Count the cell number.
k. Transfer a desired number of cells (4.0 × 10^5 cells per sample to be transduced) to a 15 mL conical tube.
l. Centrifuge at 350 × *g* for 5 min at 20°C–25°C.
m. Aspirate the supernatant and resuspend the pellet with transduction media (500 μL per one transduction sample).
n. Transfer 500 μL of cell suspension into a 1.5-mL tube.
o. Thaw a lentivirus aliquot.

⚠ CRITICAL: Avoid repeating the freeze-thaw processes of a lentivirus aliquot. One freeze-thaw cycle may result in a 20–30% loss of viral titer. Thaw a virus aliquot up to 90% at 37°C water bath or in your hand to minimize the exposure time to −20°C ice crystals. Also, ensure the thawing temperature is lower than 40°C, as the virus may become nonviable at temperatures exceeding 40°C.

p. Add 10–15 μL of lentivirus (e.g., 60-times concentration by Lenti-X Concentrator).

Alternatives: If you use highly concentrated lentivirus by ultracentrifugation (e.g., 250-times concentration), 1 μL of virus is sufficient.

q. Centrifuge at 3200 × *g* for 30 min at 20°C–25°C.
r. Remove the supernatant using a P1000 pipet.
s. Resuspend the pellet with 1 mL of mTeSR1 + 10 μM Y27632.
t. Aspirate Matrigel- or Cultrex-coating solution from a 12-well plate.
u. Plate cells on Matrigel- or Cultrex-coated 12 well plates (4.0 × 10^5 cells per well).
v. Place cells into a 37°C 5% CO_2 incubator.
w. Starting the next day, perform daily media changes with mTeSR1.

⚠ CRITICAL: When transduced cells reach 50–70% confluency, transfer the cells to a 6-well-plate scale using the GCDR passage method (as described above). From 12-well to 6-well passages, the dissociated colonies by GCDR tend to be smaller compared to conventional 6-well to 6-well passages. Therefore, incubate the colonies in a 12-well plate with GCDR for 3 min, instead of 5 min.

Validating CRISPRd efficiency by ChIP-qPCR assay

Timing: 1–2 weeks

In this section, we describe the protocol for validating FOXA-CRISPRd efficiency during hPSC to foregut (FG) differentiation by performing chromatin immunoprecipitation (ChIP)-qPCR assay.

24. Differentiate FOXA-CRISPRd hPSCs into foregut cells with Dox treatment.

Note: For a TF ChIP-qPCR assay, prepare one full 6-well plate per replicate to obtain 1.3–1.6 × 10^7 foregut cells.

a. Plate FOXA-CRIPSRd hPSCs for differentiation (Day 0).
   i. Coat a 6-well plate with Matrigel or Cultrex, warm-up materials, dissociate cells, and count cell numbers as described in step 23a–j.

Note: Prepare mTeSR1 + 10 μM Y27632 during the Accutase dissociation step.

ii. Transfer a desired number of cells to a 50-mL conical tube.

Note: For one replicate, plate 7.2–9.0 × 10^6 cells in total (1.2–1.5 × 10^6 cells per one well of a 6-well plate). 70% confluent hPSCs in one well of a 6-well plate contain 2.5–3.5 × 10^6 cells.

iii. Centrifuge at 350 × *g* for 5 min at 20°C–25°C.
iv. Aspirate supernatant and resuspend cells with mTeSR1 + 10 μM Y27632.
v. Aspirate coating solution from a 6-well plate.
vi. Transfer cell suspensions into each well of the coated 6-well plate (1.2–1.5 x 10^6 cells per well).
vii. Place the plate into a 37°C 5% CO_2 incubator.

b. Differentiate FOXA-CRISRPd hPSCs into mesendoderm (ME) with Dox treatment (Day 1).

Note: Dox-negative condition or control-sgRNA should be prepared as a control.

i. Warm up DMEM/F12 and CDM2 media at 20°C–25°C.
ii. Prepare ME induction media as indicated below.

ME induction media

Reagent	Final concentration	Amount
CDM2	1×	12 mL
ActivinA (100 μg/mL)	100 ng/mL	12 μL
CHIR99021 (2 mM)	2 μM	12 μL
PI-103 (50 μM)	50 nM	12 μL
Doxycycline (2 mM)	2 μM	12 μL
Total	**N/A**	**12 mL**

Note: For dCas9/CRISPRd induction, add Dox.

iii. Rinse cells with DMEM/F12 (1 mL per well of a 6-well plate).
iv. Add fresh ME induction media to cells (2 mL per well of a 6-well plate).
v. Place the plate into a 37°C 5% CO_2 incubator.

c. Differentiate induction into definitive endoderm (DE) with Dox treatment (Day 2).
i. Warm up DMEM/F12 and CDM2 at 20°C–25°C.
ii. Prepare DE induction media as indicated below.

DE induction media

Reagent	Final concentration	Amount
CDM2	1×	12 mL
ActivinA (100 μg/mL)	100 ng/mL	12 μL
LDN193189 (250 μM)	250 nM	12 μL
Doxycycline (2 mM)	2 μM	12 μL
Total	**N/A**	**12 mL**

Note: For dCas9/CRISPRd induction, add Dox.

iii. Rinse cells with DMEM/F12 (1 mL per well of a 6-well plate).
iv. Add fresh DE induction media to cells (2 mL per well for a 6-well plate).
v. Place the plate into a 37°C 5% CO_2 incubator.

d. Differentiation induction into foregut (FG) with Dox treatment (Day 3).
i. Warm up DMEM/F12 and CDM3 at 20°C–25°C.

ii. Prepare DE induction media as indicated below.

FG induction media		
Reagent	Final concentration	Amount
CDM3	1×	12 mL
A83-01 (1 mM)	1 μM	12 μL
ATRA (2 mM)	2 μM	12 μL
bFGF (10 μg/mL)	10 ng/uL	12 μL
BMP4 (30 μg/mL)	30 ng/mL	12 μL
Doxycycline (2 mM)	2 μM	12 μL
Total	N/A	12 mL

Note: For dCas9/CRISPRd induction, add Dox.

⚠ CRITICAL: ATRA is a photosensitive small molecule. Turning off the light in a culture hood while making FG induction media to avoid photodegradation. Once ATRA's color (yellow) turns clear, stop using it.

iii. Rinse cells with DMEM/F12 (1 mL per well of a 6-well plate).
iv. Add fresh FG induction media to cells (2 mL per well of a 6-well plate).
v. Place the plate into a 37°C 5% CO_2 incubator.

25. Crosslink foregut cells and sonicate chromatin for ChIP-qPCR assay (Day 4).

In this section, we describe the protocol for crosslinking and sonicating chromatin (200–300 ng/uL) for ChIP-qPCR experiments.

a. Warm up Accutase, DMEM/F12, and 10% FBS supplemented DMEM/F12 at 20°C–25°C.
b. Dissociate cells and count cell numbers as described in step 23a–j.
c. Transfer a desired number of cells to a 15-mL conical tube.

Note: When quenching the Accutase reaction, use FBS, BSA, or KOSR-containing basal media.

Note: For a TF ChIP-qPCR experiment, 1.2–2.0 × 10^7 cells are required.

d. Centrifuge at 350 × *g* for 5 min at 20°C–25°C.
e. Aspirate supernatant and resuspend cells with 10 mL of 1% Formaldehyde in 1× PBS as indicated below.

Note: The maximum number of cells for crosslinking in 10 mL of 1% Formaldehyde is 3.0 × 10^7 cells. For crosslinking > 3.0 × 10^7 cells, divide the cells into two tubes.

1% Formaldehyde in 1× PBS		
Reagent	Final concentration	Amount
Formaldehyde (37%)	1%	1081 μL
1× PBS	1×	38919 μL
Total	N/A	40 mL

Note: Prepare fresh and keep at 20°C–25°C.

⚠ CRITICAL: Follow your institute's guidelines for Formaldehyde handling.

f. Incubate and crosslink cells for exactly 10 min at 20°C–25°C.

g. Quench the crosslink reaction by adding 500 μL of 2.5 M glycine (final 0.125 M, 1:20 dilution) as indicated below.

2.5 M Glycine		
Reagent	Final concentration	Amount
Ultrapure Glycine	2.5 M	0.38 g
Nuclease-free water	1×	2 mL
Total	N/A	2 mL

Note: Prepare fresh and keep at 20°C–25°C.

h. Incubate cells for 5 min at 20°C–25°C.
i. Centrifuge at 600 × *g* for 5 min at 4°C.
j. Discard the supernatant and wash the pellet with 5 mL of ice-cold 1× PBS.
k. Centrifuge at 600 × *g* for 5 min at 4°C.
l. Repeat wash steps 25j and k.
m. Aspirate the supernatant and snap freeze the pellet on dry ice.
n. Store crosslinked pellet at −80°C.

Note: Crosslinked pellets can be stored for over 1 year at −80°C.

26. Sonicate chromatin

Note: This protocol is optimized for sonication of 1.0–3.0 × 10^7 crosslinked cells.

⚠ CRITICAL: The efficiency of sonication can vary greatly with cell type, crosslinking conditions, and cell number. Optimize the sonication conditions to achieve the vast majority of DNA fragments at 300–400 bp (for histone ChIP) and 400–500 bp (for transcription factor ChIP), with a faint trail of larger DNA fragments extending above.

a. Resuspend a crosslinked cell pellet in 500 μL of Lysis Buffer1.
b. Incubate the cell suspension on ice for 10 min.
c. Transfer the cell lysate to a 1.5-mL tube and centrifuge at 650 × *g* for 5 min at 4°C.
d. Remove the supernatant and resuspend the pellet with 625 μL of Lysis Buffer2.
e. Incubate the cell lysate on ice for 10 min.
f. Dilute the lysate with 375 μL of IP Dilution Buffer, as indicated below (Total volume is 1 mL).
g. Transfer the whole lysate slowly to a milliTUBE 1 mL ATA Fiber tube (#520135, Covaris). Add an additional 20–200 μL Sonication Buffer (indicated below) up to the top using surface tension. Remove air bubbles on the lysate surface by blowing air using a P200 pipette, then quickly place the cap from above and screw it on.

⚠ CRITICAL: Ensure that there are no air bubbles in the tube, as they can reduce sonication efficiency.

h. Sonicate the cell lysate by Covaris S220, as indicated below.

Sonication conditions of Covaris S220	
Parameter	Condition
Temperature	5°C–9°C.
Peak incident power	150 Watt
Duty factor	10.0%
Cycle/Burst	200 counts
Duration	210 s

Note: Extend sonication time to 330 s for histone ChIP.

i. Transfer the sonicated cell lysate to a pre-chilled 1.5-mL tube on ice.
j. Centrifuge at 12000 × *g* for 10 min at 4°C to remove insoluble debris.
k. Transfer the supernatant into a new pre-chilled 1.5-mL tube on ice and mix well.
l. Transfer 30 μL of the sample into a separate 1.5-mL tube to purify DNA and confirm sonication efficiency (store at −20°C or proceed to step 28).
m. Make aliquots of the remaining sample (e.g., 200 μL × 5), snap freeze aliquots on dry ice, and store at −80°C (for ChIP step 27).

27. Chromatin immunoprecipitation (ChIP).

Note: A ChIP condition (amount of antibody and chromatin) should be optimized for each antibody. Test a range of amounts for chromatin (e.g., 25 μg, 50 μg, and 75 μg) and/or antibody (e.g., 2.5 μg, 5 μg, and 10 μg).

⚠ CRITICAL: Perform all ChIP steps on ice until the chromatin elution step.

a. Prepare Dynabeads-Antibody conjugate and immunoprecipitation reaction (ChIP Day 1).
   i. Resuspend Dynabeads Protein A or G for Immunoprecipitation (Thermo Fisher Scientific) by vortexing for more than 30 s.

⚠ CRITICAL: Binding specificities and affinities of Protein A and G to an antibody differ between antibody host species and antibody subclass. Select Protein A or G depending on the antibody of interest.

   ii. Transfer 1/5 of the ChIP reaction volume of Dynabeads to a 1.5-mL low-binding tube for each ChIP reaction (e.g., 25 μL of beads for a 125 μL ChIP reaction).

⚠ CRITICAL: Antibody binding efficiency may decrease if Dynabeads are resuspended in more than 5 times their original volume.

   iii. Place the sample tube on a magnetic stand, wait for the solution to clear, and remove the supernatant.
   iv. Remove the tube from the magnetic stand, resuspend beads with 500 μL of PBS-T by inverting the tube, and briefly spin down the tube.
   v. Repeat the wash steps 27a-iii and iv.
   vi. Place the tube on the magnetic stand and remove the supernatant.
   vii. Resuspend each bead aliquot with twice the original bead volume of PBS-T (e.g., 50 μL for 25 μL of beads).
   viii. Add a desired amount of antibody and mix gently by pipetting.
   ix. Transfer the beads-antibody conjugate to a low-binding 0.6 mL tube.
   x. Incubate on a rotating rack in a cold room for 2–6 h.
   xi. While incubating the beads-antibody conjugate, start thawing chromatin on ice, and prepare Sonication buffer and IP dilution buffer.
   xii. Dilute sonicated chromatin in Sonication Buffer with pre-chilled IP Dilution Buffer at a volume ratio of 4:1 (e.g., 160 μL of chromatin in Sonication buffer and 40 μL of IP Dilution Buffer). Keep on ice until beads-antibody conjugate is ready.

Example of chromatin dilution for ChIP

Reagent	Final concentration	Amount
Sonicated chromatin in Sonication Buffer (0.2 μg/μL)	25 μg	125 μL
Sonication Buffer	N/A	35 μL

(*Continued on next page*)

Continued

Reagent	Final concentration	Amount
IP Dilution Buffer with CPI	N/A	40 μL
Total	**N/A**	**200 μL**

xiii. Ensure to aliquot ~10% amount of the sonicated chromatin (e.g., 2.5 μg) into an extra tube to serve as an input sample and store at −20°C until proceeding to step 28 for reverse chromatin crosslinks.
xiv. After 2–6 h of incubation of the beads-antibody conjugate, briefly spin the mixture and transfer to a low-binding 1.5-mL tube.
xv. Place the tube on a magnetic stand, wait for the solution to clear, and remove the supernatant.
xvi. Remove the tube from the magnetic stand, resuspend beads with 500 μL of IP Dilution Buffer (without CPI) by inverting the tube, and briefly spin down the tube.
xvii. Repeat the wash steps 27a-xv and xvi.
xviii. Place the tube on the magnetic stand and remove the supernatant.
xix. Resuspend the beads-antibody conjugate with the diluted chromatin mixture from step 27a-xii.
xx. Transfer the sample to a new low-binding 0.6-mL tube.
xxi. Incubate on a rotating rack in a cold room for 12–16 h.

b. Wash beads and elute chromatin (ChIP Day 2).
i. Prepare Day-2 ChIP buffers.
ii. Briefly spin the sample tube and transfer the sample to a low-binding 1.5-mL tube.
iii. Place the tube on a magnetic stand, wait for the solution to clear, and remove the supernatant.
iv. Remove the tube from the magnetic stand, resuspend beads with 500 μL of pre-chilled FA lysis buffer by inverting the tube, and spin down briefly.
v. Repeat the wash steps 27b-iii and iv with pre-chilled 500 μL of NaCl buffer.
vi. Repeat the wash steps 27b-iii and iv with pre-chilled 500 μL of LiCl buffer.
vii. Repeat the wash steps 27b-iii and iv with pre-chilled with 500 μL of 10 mM Tris-HCl (pH 8.0).
viii. Place the tube on a magnetic stand, wait for the solution to clear, and remove the supernatant.
ix. Resuspend the beads with 60 μL of TES to elute chromatin.
x. Incubate the sample at 65°C for 15 min with shaking by ThermoMixer F1.5 (Eppendorf).
xi. Place the tube on the magnetic stand, wait for the solution to clear, and transfer the supernatant to a new 1.5-mL tube (no low binding).
xii. Repeat elution steps (steps 27b-viii–xi) two more times by resuspending beads in 60 μL of TES and combine the three elutes (total 180 μL).

28. Reverse chromatin crosslinks and purify DNA.
a. Reverse chromatin crosslinks.
i. Bring up the volume of sonicated chromatin (From step 26L) or ChIP samples (from step 27b-xii) to 180 μL with TE buffer.
ii. Add 7.2 μL of 5 M NaCl.
iii. Transfer samples into a 0.2-mL PCR tube.
iv. Incubate at 65°C for 4–8 h (for sonicated chromatin) or 2–8 h (for ChIP samples) in a thermal cycler with a 65°C heated lid.
v. Add 0.9 μL of 10 mg/mL RNase, DNase free (final concentration 50 ng/uL).
vi. Incubate at 37°C for 30–60 min.
vii. Add 1.9 μL of 20 mg/mL Protease K (final concentration 0.2 mg/mL).
viii. Incubate at 37°C for 2–16 h.

b. Phenol/Chloroform DNA purification.

⚠ CRITICAL: Since phenol and chloroform are toxic reagents, handle these reagents under a chemical hood.

i. Centrifuge a Phase-Lock-Gel (PLG) tube (Quantabio) or MaXtract High-Density tube (QIAGEN) at 16000 × *g* for 30 s.
ii. Transfer the reverse-crosslinked chromatin (about 180 μL) to the pre-spun PLG tube.
iii. Add 180 μL of Phenol/Chloroform/Isoamyl alcohol into the PLG tube.
iv. Mix immediately by inverting the tube more than 15 times (Do not vortex).
v. Centrifuge at 16000 × *g* for 5 min at 20°C–25°C.
vi. Add 180 μL of chloroform into the PLG tube.
vii. Mix immediately by inverting a tube more than 15 times (Do not vortex).
viii. Centrifuge at 16000 × *g* for 5 min at 20°C–25°C.
ix. Carefully transfer the aqueous layer to a fresh 1.5-mL tube.
x. Add 0.9 μL of 20 mg/mL glycogen and 360 μL of 100% ethanol, and mix well by inverting.
xi. Incubate at −20°C for 30 min to 16 h.
xii. Centrifuge at 16000 × *g* for 10 min at 4°C and remove supernatant.
xiii. Wash the pellet with 500 μL of 80% ethanol.
xiv. Centrifuge at 16000 × *g* for 5 min at 4°C, remove the supernatant.
xv. Air dry the pellet until it becomes transparent (uncap the tube and cover it with a Kim wipe).
xvi. Resuspend the pellet with 20 μL (for ChIP samples) or 30 μL (for sonicated chromatin) of TE buffer and leave it for 30 min at 20°C–25°C or for 16 h at 4°C to dissolve completely.
xvii. Measure the DNA concentration using a Quantus (Promega) or Qubit Fluorometer (Thermo Fisher Scientific) (for ChIP samples) or a Nanodrop (for sonicated chromatin to validate the sonication efficiency).
xviii. Store at −20°C (for ChIP samples, proceed to step 29; for sonicated chromatin, proceed to step 28c)

c. Validate the sonication efficiency using reverse-crosslinked sonicated chromatin samples.
i. Prepare 1.3% agarose gel in 1× TAE Buffer without ethidium bromide.

Note: A thinner gel provides a clear image.

ii. Mix 0.5 μg of purified DNA with 6× Orange G loading dye.

Note: Orange G dye migrates approximately at the 50-bp position, while Bromophenol Blue dye migrates at the 300-bp position and may cover the DNA smear of interest (200–500 bp).

iii. Run on the agarose gel in 1× TAE Buffer at 100 V for 1–2 h.
iv. Stain the gel with 0.5 μg/mL ethidium bromide in 1× TAE Buffer for 15 min at 20°C–25°C.
v. Wash gel for 5 min with water, twice.
vi. Image the gel.

29. ChIP-qPCR assay to validate CRISPRd efficiency and its effect.
a. Design ChIP-qPCR primers for the CRISPRd target site (FOXA binding site/motif) to confirm FOXA-CRISPRd efficiency and for the binding site(s) of FOXA's co-factors (TFs and chromatin modifiers) to analyze their recruitment in FOXA-CRISPRd cells.
i. Visualize the ChIP-seq signal for target factors (e.g., FOXA and FOXA's co-factors) in the UCSC genome browser or IGV to identify a genomic region of interest (Figure 2B).
ii. Use the UCSC genome browser to obtain the DNA sequence of the target genomic site. Select a region of about 300–500 bp around the peak summit containing the TF binding motif.
iii. Access Primer-BLAST at https://www.ncbi.nlm.nih.gov/tools/primer-blast/ (Figure 7A).
iv. Paste the DNA sequence of interest into the "Enter accession, or FASTA sequence" box.

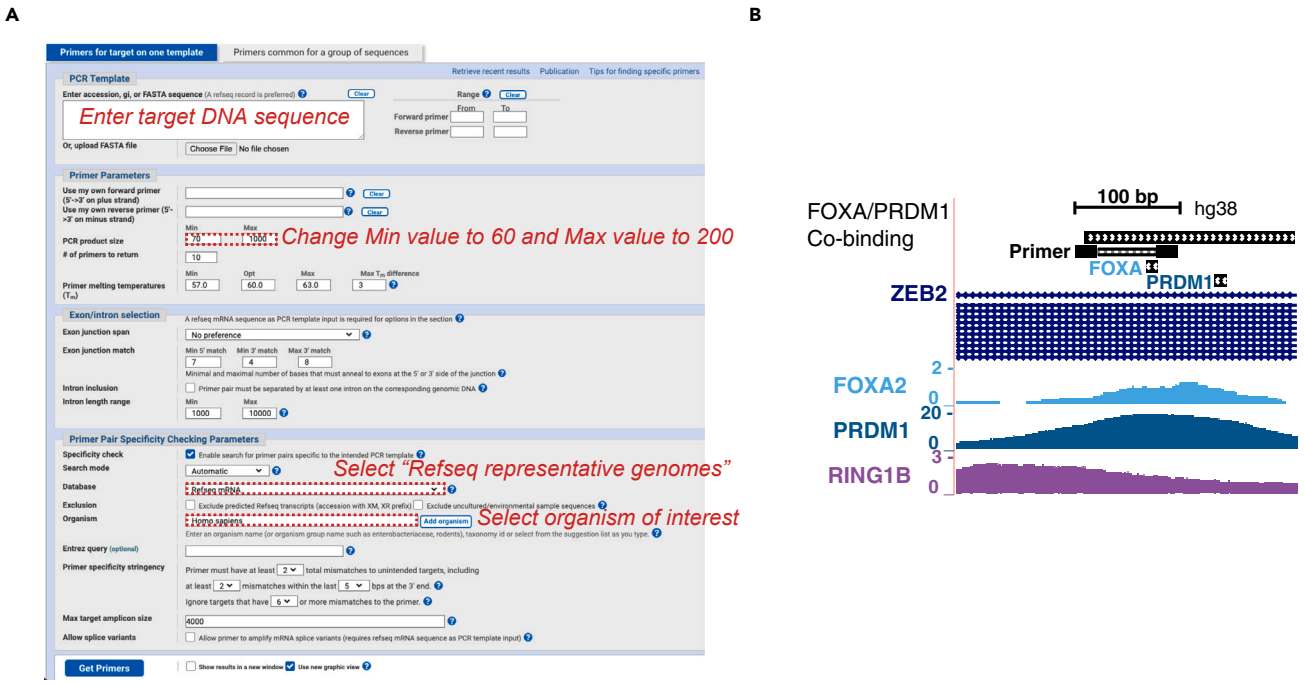


Figure 7. Overview of designing ChIP-qPCR primers

(A) Utilize the Primer-BLAST website to design primers. Adjust parameters as indicated in the red dotted boxes.

(B) The UCSC genome browser window displaying a FOXA ChIP-seq peak and the location of the designed primers at the *ZEB2* locus. The PCR amplicon includes a FOXA2 binding motif.

v. Adjust the PCR product size range from 60 to 200 bp to ensure amplification efficiency.
vi. Choose "Refseq representative genomes" as the database to search against.
vii. Specify the organisms of interest (e.g., Homo sapiens).
viii. Click "Get Primers" to generate primer pairs for the target region.

Note: Ideally, select a primer set that includes the target factor's binding motif (Figure 7B) and design a primer set around the peak summit for optimal ChIP-qPCR results.

b. ChIP-qPCR (see troubleshooting, problem 6).
   i. Dilute reverse cross-linked Input DNA 10 times with 1× TE buffer (pH 8.0).
   ii. Measure the concentration of the diluted input DNA using a fluorometer like Quantus (Promega) or Qubit (Thermo Fisher Scientific) to generate a standard curve for qPCR quantification.
   iii. Further dilute the Input DNA with Nuclease-free water, if needed, to achieve a final concentration ranging from 0.05 to 0.5 ng/uL. This dilution ensures that the input DNA falls within the linear range of the qPCR assay.
   iv. Dilute the ChIP DNA with Nuclease-free water, if needed, to achieve a final concentration ranging from 0.05 to 0.5 ng/uL (typically 1:2 to 1:10 dilution).
   v. Prepare 5-fold serial dilutions of the Input DNA to a standard curve for quantification. For example, 4 ng/uL, 0.8 ng/uL, and 0.16 ng/uL using Nuclease-free water.
   vi. Prepare a qPCR reaction mix as indicated below. See Table S1 for primer sequences.

Reagent	Final concentration in 10 μL qPCR reaction	Amount
PowerUP SYBR Green PCR Mix	1×	5 μL
Forward + Reverse Primer Mix (10 μM each)	0.5 μM each	0.5 μL
Nuclease-free water	N/A	2.5 μL
Total	**N/A**	**8 μL**

vii. Apply 8 μL of qPCR reaction mix into each well of a 96- or 384-well plate.
viii. Apply 2 μL of ChIP DNA, input DNA, and the serial dilutions of the input DNA (for a standard curve) to their respective wells in the PCR plate.
ix. Centrifuge the PCR plate at 800 × *g* for 1 min.
x. Run qPCR using a real-time PCR instrument with the following parameters.

Steps	Temperature	Time	Cycles
UDG activation	50°C	2 min	1
Polymerase activation	95°C	2 min	1
Denaturation	95°C	15 s	45 cycles
Annealing/Extension	60°C	1 min	
Melting curve stage	95°C	15 s	1
	60°C	1 min	1
	95°C	15 s	1

xi. After the qPCR run, perform a melting curve analysis to validate the specificity of the PCR products. Look for a single, specific peak for each reaction, indicating the amplification of the target sequence.
xii. Use the standard curve generated from the serial dilution of the input DNA to quantify the amount of DNA in the ChIP samples and Input samples. Back-calculate the amount of DNA in the original sample (before dilution) using the dilution factor, and calculate the percentage of input (%input) for each ChIP sample.

EXPECTED OUTCOMES

In the FOXA-CRISPRd system, dCas9 protein expression is induced about 12 h after Dox treatment and maintained with continued Dox treatment (Figures 1D, 1E, 8A, and 8B). ChIP-qPCR results indicate that recruitment of dCas9 to the FOXA binding motif in the *ZEB2* locus and subsequent blocking of FOXA2 binding occur 2 days after Dox treatment (Figure 8C). The efficiency of blocking FOXA binding was approximately 65%–70%. Notably, the FOXA-CRISPRd system did not affect endogenous FOXA expression (Figure 8D), ruling out the possibility that the reduction of FOXA binding at the target site was due to reduced FOXA protein level. Furthermore, blocking FOXA binding impaired the recruitment of the co-binding TF PRDM1 and the PRC1 core subunit RING1B, leading to reduced deposition of the PRC1 mark H2AK119ub1 (Figure 8E). Importantly, the RT-qPCR result indicated that the blocking FOXA binding also derepressed the *ZEB2* expression at 2 days after Dox treatment (Figure 8F). Collectively, analyses using the FOXA-CRISPRd system led to the conclusion that FOXA binding is involved in the recruitment of PRDM1 and PRC1 to repress target gene *ZEB2* expression.

QUANTIFICATION AND STATISTICAL ANALYSIS

Details of statistics tests, sample sizes, biological replicates, and error bars are indicated in the figure legends. For ChIP-qPCR, we prepared 2 technical replicates and used standard deviation (SD) as an error bar. For RT-qPCR, we prepared 3 technical replicates and used the standard error of the mean (SEM) as an error bar. Data were derived from independent experimental repeats for the FOXA-CRISPRd line. All statistical tests for RT-qPCR and ChIP-qPCR were performed by GraphPad Prism9. The two-tailed unpaired t-test was performed to assess the differences between the two groups. Ordinary one-way ANOVA was used to assess the difference among more than two groups.

LIMITATIONS

A gRNA spacer sequence should be designed to cover a target TF binding motif. According to the original manuscript, a gRNA covering a major portion of a target motif (e.g., 11 bp out of 14 bp) successfully blocks the TF binding.[2] In our current CRISPRd system, the PAM sequence needs to be located close to the target TF motif. However, given that TFs typically bind to thousands of target sites, finding TF motifs near the PAM sequence should not be difficult. The footprint of dCas9 has

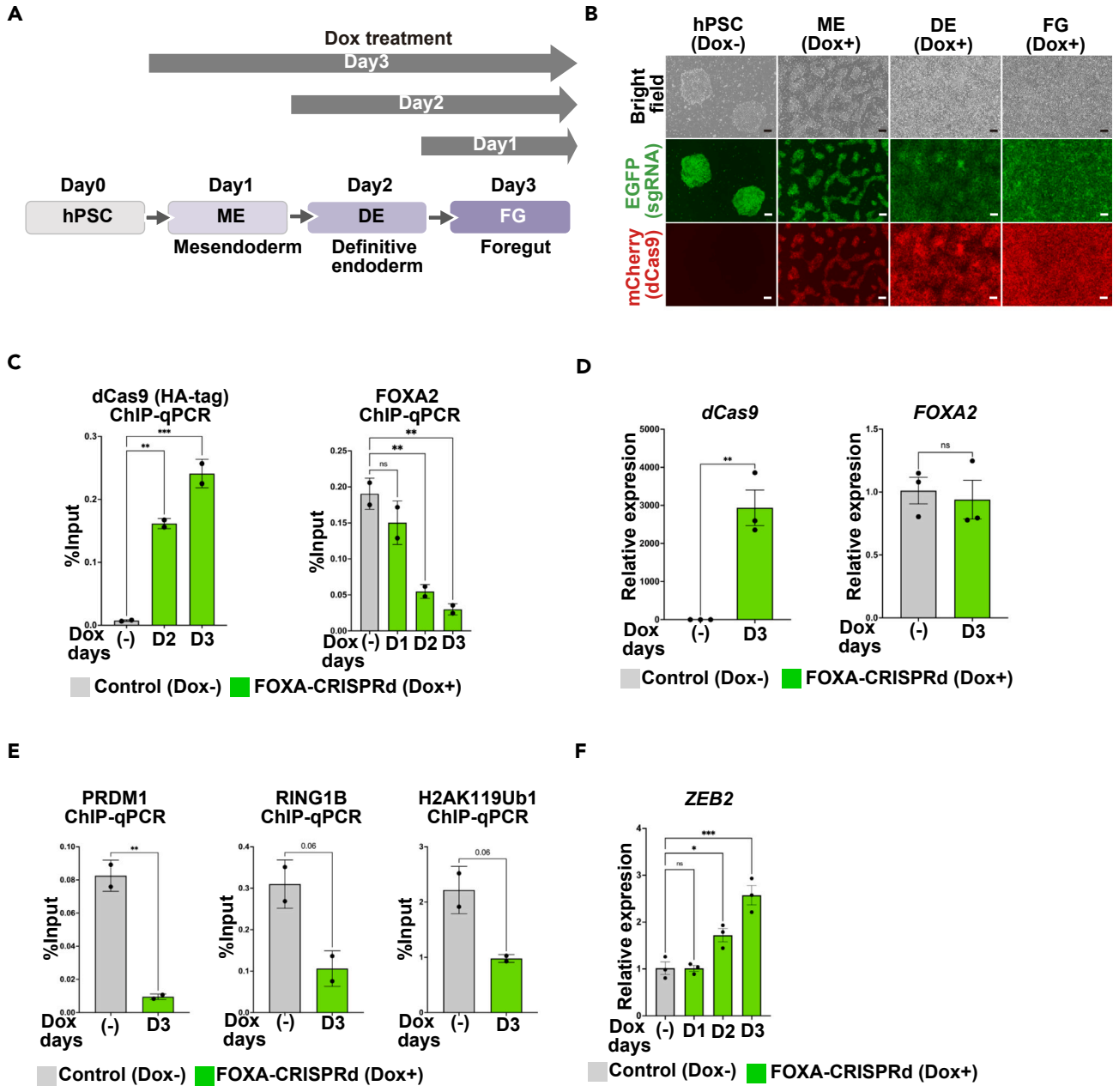


Figure 8. Results of FOXA-CRISPRd blocking experiments in human foregut cells

(A) Schematic overview of hPSC to foregut differentiation and timing of Dox treatment.

(B) sgRNA and dCas9 expression are assessed by monitoring EGFP and mCherry expression, respectively. Scale bars = 300 μm.

(C) dCas9 (HA-tag) and FOXA2 ChIP-qPCR enrichment at the *ZEB2* locus in control (Dox-negative) and FOXA-CRISPRd (Dox-treated) foregut cells.[1] $n = 2$ replicates, mean ± SD, $^{**}p < 0.01$, $^{***}p < 0.001$, and ns, not significant, based on one-way ANOVA and Dunnett's test.

(D) RT-qPCR analysis of *dCas9* and *FOXA2* gene expression in FOXA-CRISPRd foregut cells. $n = 3$ replicates, mean ± SEM, $^{**}p < 0.01$, and ns, not significant, based on a two-tailed unpaired t-test.

(E) PRDM1, RING1B, and H2AK119Ub1 ChIP-qPCR enrichment at the *ZEB2* locus in control (Dox-negative) and FOXA-CRISPRd (Dox-treated) foregut cells. $n = 2$ replicates, mean ± SD, $^{**}p < 0.01$, based on two-tailed unpaired t test.

(F) RT-qPCR analysis of *ZEB2* gene expression in control (Dox-negative) and FOXA-CRISPRd (Dox-treated) foregut cells.[1] $n = 3$ replicates, mean ± SEM, $^{*}p < 0.05$, $^{***}p < 0.001$, and ns, not significant, based on one-way ANOVA and Dunnett's test.

been defined as +14 bp and −8 bp from 5′ to 3′ ends of a 23-bp gRNA spacer sequence (45 bp total).[21] To block individual transcription factors exclusively at multiple TF co-binding sites, the TF motifs should be spaced at least 40 to 50 bp apart. If the distance between two TF binding motifs is shorter, a CRISPRd targeting one TF may also physically block the other TF binding site. Although dCas9 protein is detected 12 h after Dox treatment, at least 2 days of Dox treatment are required to effectively block a target TF binding. Since the sonication step randomly produces relatively long chromatin fragments (200–500 bp), the resolution of ChIP-qPCR results would be low. To improve the resolution, cleavage under targets & release using nuclease (CUT&RUN) can be used, which generates shorter chromatin fragments (< 200 bp) around the immunoprecipitation target protein.[22,23]

TROUBLESHOOTING

Problem 1

Poor recovery of drug-resistant cells following G418 selection. This could be caused by using inappropriate G418 concentration for the hPSC line used, low transfection efficiency, or low targeting efficiency (step-by-step method details steps 7 and 8).

Potential solution

Perform a kill curve experiment to identify the lowest concentration of G418 that kills all untransfected cells in a defined time period. Assess transfection efficiency by adding Dox (overnight) and monitor mCherry expression (if using pAAVS1-NDi-CRISPRd). The dCas9-KRAB expression kinetics in the Dox-on/off system differs between quantification methods (western blot vs. live mCherry fluorescence assays).[24] We recommend confirming dCas9-KRAB expression using a western blot assay. Targeting efficiency is occasionally low despite efficient transfection. In this case, recovery of targeted clones can be enhanced by scaling up the experiment.

Problem 2

Cell recovery following G418 selection is robust and discrete clones cannot be excised (step-by-step method details in step 9).

Potential solution

Subclone recovered lines to isolate a monoclonal population by plating cells at clonal density (i.e., 100–1000 cells/well of a 6-well plate) immediately following G418 selection.

Problem 3

Low efficiency or no DNA fragment integration through Golden-Gate cloning (step-by-step method details step 16).

Potential solution

The activity of the restriction enzyme and ligase is critical for cloning efficiency. Validate the enzyme activity by digesting the host plasmid. Particularly, Esp3I (FastDigest) does not work efficiently after the expiration date (after 1 year). Consider increasing the cycle numbers of the Golden-Gate reaction, possibly up to 30 cycles, as this may improve cloning efficiency. The T4 ligase buffer contains ATP, which degrades quickly. We recommend aliquoting the buffer and discarding the aliquots after 2 or 3 freeze-and-thaw cycles to maintain optimal ligation efficiency.

Problem 4

Low PEI-plasmid transfection efficiency for lentivirus production (step-by-step method details step 20).

Potential solution

Using a low concentration (less than 100 ng/uL) of lentivirus plasmid increases the volume of plasmid solution in a PEI transfection mix, thereby reducing transfection efficiency and resulting in a low virus yield. Concentrate the lentivirus plasmid to 500–1000 ng/uL for PEI transfection.

Problem 5

Low lentivirus transduction efficiency (step-by-step method details step 23).

Potential solution

Increase the amount of lentivirus used in transduction without affecting cell viability. Consider purifying the transduced cells by cell sorting using the EGFP marker expression.

Problem 6

No target TF blocking was observed in a CRISPRd system (step-by-step method details step 29b).

Potential solution

Ensure the expression of mCherry and EGFP marker genes, which represent dCas9 and sgRNA expression, respectively. If cells do not show high positivity (> 80% positive), increase the amount of Dox to enhance mCherry/dCas9 expression, and refer to Solution 3 to increase EGFP/sgRNA expression. To confirm dCas9 recruitment of a target site, perform dCas9-HA ChIP-qPCR (use a HA-tag antibody, i.e., Cell Signaling Technology, Cat# 3724). If significant dCas9 recruitment is not observed over a targeted site, consider redesigning gRNA or increasing the number of gRNAs around the target site.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Makiko Iwafuchi (makiko.iwafuchi@cchmc.org).

Technical contact

Technical questions on executing this protocol should be directed to and will be answered by the technical contact, Satoshi Matsui (matsui.satoshi.3p8@osaka-u.ac.jp), Christopher N. Mayhew (christopher.mayhew@cchmc.org) and Makiko Iwafuchi (makiko.iwafuchi@cchmc.org).

Materials availability

Newly generated materials in this study are available upon request. Plasmids generated in this study are available through Addgene.

Data and code availability

Further information and requests for code should be directed to and will be fulfilled by Hee-Woong Lim (heewoong.lim@cchmc.org).

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103233.

ACKNOWLEDGMENTS

We thank T. Takebe for sharing equipment at the Digestive Disease Research Core Center in CCHMC (Pluripotent Stem Cell Facility, Genome Edition Core, and DNA sequencing Core). This work was supported by the Japan Society for the Promotion of Science Foundation (Postdoctoral Fellowship to S.M.), the Uehara Memorial Foundation (Postdoctoral Fellowship to S.M.), the Cincinnati Children's Research Foundation (Trustee Award and Center for Pediatric Genomics Pilot Award to M.I.), and the National Institutes of Health (P30 DK078392 and R01GM143161 to M.I.).

AUTHOR CONTRIBUTIONS

Methodology, S.M., M.I., M.B., C.N.M., J.R.S., Y.-C.H., and H.-W.L.; investigation, S.M., J.R.S., and M.B.; writing – original draft, S.M., J.R.S., C.N.M., and M.I.; writing – review and editing, all authors; supervision, C.N.M. and M.I.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

1. Matsui, S., Granitto, M., Buckley, M., Ludwig, K., Koigi, S., Shiley, J., Zacharias, W.J., Mayhew, C.N., Lim, H.-W., and Iwafuchi, M. (2024). Pioneer and PRDM transcription factors coordinate bivalent epigenetic states to safeguard cell fate. Mol. Cell *84*, 476–489.e10. https://doi.org/10.1016/j.molcel.2023.12.007.

2. Shariati, S.A., Dominguez, A., Xie, S., Wernig, M., Qi, L.S., and Skotheim, J.M. (2019). Reversible Disruption of Specific Transcription Factor-DNA Interactions Using CRISPR/Cas9. Mol. Cell *74*, 622–633.e4. https://doi.org/10.1016/j.molcel.2019.04.011.

3. Oceguera-Yanez, F., Kim, S., Matsumoto, T., Tan, G.W., Xiang, L., Hatani, T., Kondo, T., Ikeya, M., Yoshida, Y., Inoue, H., and Woltjen, K. (2016). Engineering the AAVS1 locus for consistent and scalable transgene expression in human iPSCs and their differentiated derivatives. Methods *101*, 43–55. https://doi.org/10.1016/j.ymeth.2015.12.012.

4. Ran, F.A., Hsu, P.D., Wright, J., Agarwala, V., Scott, D.A., and Zhang, F. (2013). Genome engineering using the CRISPR-Cas9 system. Nat. Protoc. *8*, 2281–2308. https://doi.org/10.1038/nprot.2013.143.

5. Chen, B., Gilbert, L.A., Cimini, B.A., Schnitzbauer, J., Zhang, W., Li, G.W., Park, J., Blackburn, E.H., Weissman, J.S., Qi, L.S., and Huang, B. (2013). Dynamic imaging of genomic loci in living human cells by an optimized CRISPR/Cas system. Cell *155*, 1479–1491. https://doi.org/10.1016/j.cell.2013.12.001.

6. Slaymaker, I.M., Gao, L., Zetsche, B., Scott, D.A., Yan, W.X., and Zhang, F. (2016). Rationally engineered Cas9 nucleases with improved specificity. Science *351*, 84–88. https://doi.org/10.1126/science.aad5227.

7. Sahu, S., Sullivan, T., Southon, E., Caylor, D., Geh, J., and Sharan, S.K. (2023). Protocol for the saturation and multiplexing of genetic variants using CRISPR-Cas9. STAR Protoc. *4*, 102702. https://doi.org/10.1016/j.xpro.2023.102702.

8. Xu, H., Kita, K., Bang, U., Gee, P., and Hotta, A. (2021). Optimized electroporation of CRISPR-Cas9/gRNA ribonucleoprotein complex for selection-free homologous recombination in human pluripotent stem cells. STAR Protoc. *2*, 100965. https://doi.org/10.1016/j.xpro.2021.100965.

9. Caillaud, A., Lévêque, A., Thédrez, A., Girardeau, A., Canac, R., Bray, L., Baudic, M., Barc, J., Gaborit, N., Lamirault, G., et al. (2022). FACS-assisted CRISPR-Cas9 genome editing of human induced pluripotent stem cells. STAR Protoc. *3*, 101680. https://doi.org/10.1016/j.xpro.2022.101680.

10. Mandegar, M.A., Huebsch, N., Frolov, E.B., Shin, E., Truong, A., Olvera, M.P., Chan, A.H., Miyaoka, Y., Holmes, K., Spencer, C.I., et al. (2016). CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. Cell Stem Cell *18*, 541–553. https://doi.org/10.1016/j.stem.2016.01.022.

11. Wang, H., Xu, X., Nguyen, C.M., Liu, Y., Gao, Y., Lin, X., Daley, T., Kipniss, N.H., La Russa, M., and Qi, L.S.C. (2018). CRISPR-Mediated Programmable 3D Genome Positioning and Nuclear Organization. Cell *175*, 1405–1417.e14. https://doi.org/10.1016/j.cell.2018.09.013.

12. Shi, J., Wang, E., Milazzo, J.P., Wang, Z., Kinney, J.B., and Vakoc, C.R. (2015). Discovery of cancer drug targets by CRISPR-Cas9 screening of protein domains. Nat. Biotechnol. *33*, 661–667. https://doi.org/10.1038/nbt.3235.

13. Heinz, S., Benner, C., Spann, N., Bertolino, E., Lin, Y.C., Laslo, P., Cheng, J.X., Murre, C., Singh, H., and Glass, C.K. (2010). Simple combinations of lineage-determining transcription factors prime cis-regulatory elements required for macrophage and B cell identities. Mol Cell *38*, 576–589. https://doi.org/10.1016/j.molcel.2010.05.004.

14. Concordet, J.P., and Haeussler, M. (2018). CRISPOR: Intuitive guide selection for CRISPR/Cas9 genome editing experiments and screens. Nucleic Acids Res. *46*, W242–W245. https://doi.org/10.1093/nar/gky354.

15. Ludwig, T.E., Andrews, P.W., Barbaric, I., Benvenisty, N., Bhattacharyya, A., Crook, J.M., Daheron, L.M., Draper, J.S., Healy, L.E., Huch, M., et al. (2023). ISSCR standards for the use of human stem cells in basic research. Stem Cell Rep. *18*, 1744–1752. https://doi.org/10.1016/j.stemcr.2023.08.003.

16. Sandelin, A., Alkema, W., Engström, P., Wasserman, W.W., and Lenhard, B. (2004). JASPAR: an open-access database for eukaryotic transcription factor binding profiles. Nucleic Acids Res. *32*, D91–D94. https://doi.org/10.1093/nar/gkh012.

17. Thorvaldsdóttir, H., Robinson, J.T., and Mesirov, J.P. (2013). Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Brief. Bioinform. *14*, 178–192. https://doi.org/10.1093/bib/bbs017.

18. Kabadi, A.M., Ousterout, D.G., Hilton, I.B., and Gersbach, C.A. (2014). Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. Nucleic Acids Res. *42*, e147. https://doi.org/10.1093/nar/gku749.

19. Matsui, S., Shiley, J.R., Granitto, M., Ludwig, K., Buckley, M., Koigi, S., Mirizio, G., Hu, Y.-C., Mayhew, C.N., and Iwafuchi, M. (2024). Protocol for establishing inducible CRISPR interference system for multiple-gene silencing in human pluripotent stem cells. STAR Protoc *5*. https://doi.org/10.1016/j.xpro.2024.103221.

20. Kodaka, Y., Asakura, Y., and Asakura, A. (2017). Spin infection enables efficient gene delivery to muscle stem cells. Biotechniques *63*, 72–76. https://doi.org/10.2144/000114576.

21. Sapozhnikov, D.M., and Szyf, M. (2021). Unraveling the functional role of DNA demethylation at specific promoters by targeted steric blockage of DNA methyltransferase with CRISPR/dCas9. Nat. Commun. *12*, 5711. https://doi.org/10.1038/s41467-021-25991-9.

22. Skene, P.J., and Henikoff, S. (2017). An efficient targeted nuclease strategy for high-resolution mapping of DNA binding sites. Elife *6*, e21856. https://doi.org/10.7554/eLife.21856.

23. Skene, P.J., Henikoff, J.G., and Henikoff, S. (2018). Targeted in situ genome-wide profiling with high efficiency for low cell numbers. Nat. Protoc. *13*, 1006–1019. https://doi.org/10.1038/nprot.2018.015.

24. Han, J.L., Heinson, Y.W., Chua, C.J., Liu, W., and Entcheva, E. (2023). CRISPRi gene modulation and all-optical electrophysiology in post-differentiated human iPSC-cardiomyocytes. Commun. Biol. *6*, 1236. https://doi.org/10.1038/s42003-023-05627-y.