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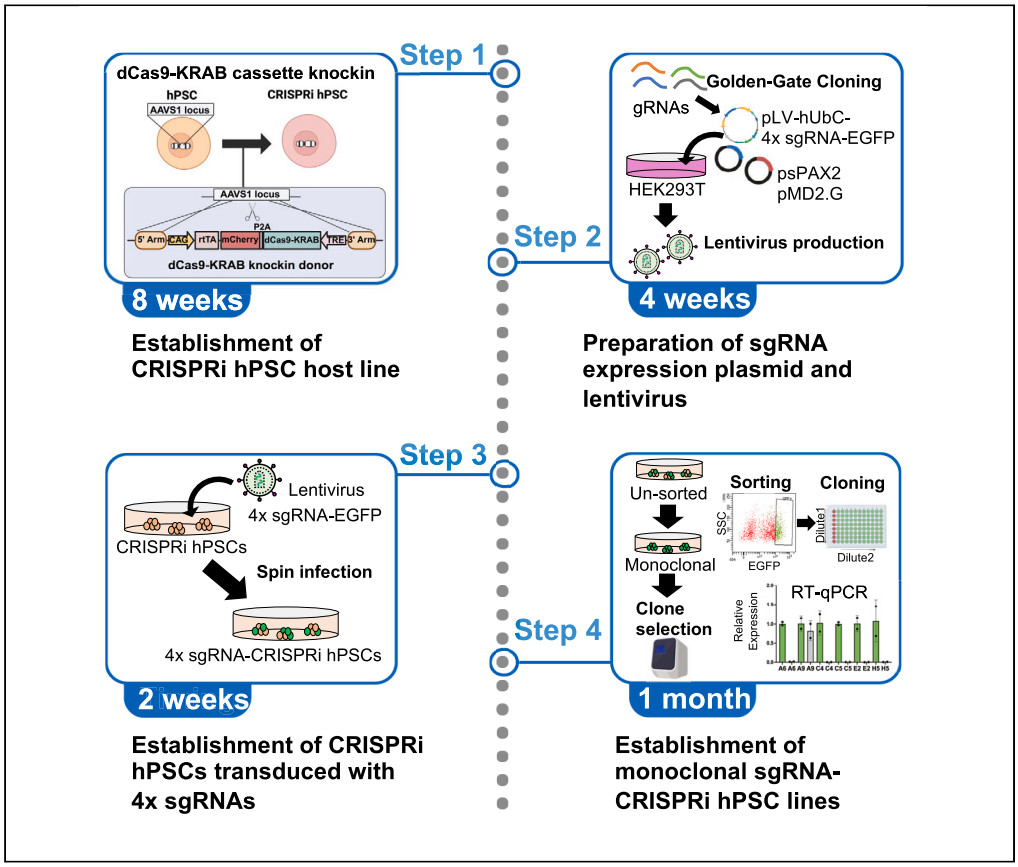
Protocol

Protocol for establishing inducible CRISPR interference system for multiple-gene silencing in human pluripotent stem cells



Inducible loss-of-function strategies are crucial for understanding gene function. However, creating inducible, multiple-gene knockout models is challenging and time-consuming. Here, we present a protocol for establishing a doxycycline-inducible CRISPR interference (CRISPRi) system to concurrently silence multiple genes in human induced pluripotent stem cells (hPSCs). We describe the steps for establishing host CRISPRi hPSCs, designing and cloning single-guide RNAs (sgRNAs) into a lentivirus plasmid, and establishing monoclonal CRISPRi hPSC lines transduced with sgRNAs. We also detail the procedures for selecting effective CRISPRi clones.

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

Satoshi Matsui, Joseph R. Shiley, Marissa Granitto, ..., Yueh-Chiang Hu, Christopher N. Mayhew, Makiko Iwafuchi

christopher.mayhew@cchmc.org (C.N.M.)
makiko.iwafuchi@cchmc.org (M.I.)

Highlights

Dox-inducible CRISPRi system to silence multiple genes concurrently

Instructions for generating CRISPRi hPSCs transduced with four sgRNAs

FOXA1/A2/A3-CRISPRi system represses expression of all three *FOXA* genes by 95%

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Protocol for establishing inducible CRISPR interference system for multiple-gene silencing in human pluripotent stem cells

Satoshi Matsui,[1,2] Joseph R. Shiley,[1,2] Marissa Granitto,[1,2] Katie Ludwig,[1] Morgan Buckley,[1] Sandra Koigi,[1] Gerardo Mirizio,[1] Yueh-Chiang Hu,[1] Christopher N. Mayhew,[1,3,*] and Makiko Iwafuchi[1,3,4,*]

[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]These authors contributed equally

[3]Technical contact

[4]Lead contact

*Correspondence: christopher.mayhew@cchmc.org (C.N.M.), makiko.iwafuchi@cchmc.org (M.I.)
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SUMMARY

Inducible loss-of-function strategies are crucial for understanding gene function. However, creating inducible, multiple-gene knockout models is challenging and time-consuming. Here, we present a protocol for establishing a doxycycline-inducible CRISPR interference (CRISPRi) system to concurrently silence multiple genes in human induced pluripotent stem cells (hPSCs). We describe the steps for establishing host CRISPRi hPSCs, designing and cloning single-guide RNAs (sgRNAs) into a lentivirus plasmid, and establishing monoclonal CRISPRi hPSC lines transduced with sgRNAs. We also detail the procedures for selecting effective CRISPRi clones.

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

BEFORE YOU BEGIN

This protocol describes the detailed steps for generating a doxycycline (Dox)-inducible CRISPRi system in hPSCs to silence multiple genes. The system employs a deactivated Cas9 (dCas9) fused with a Krüppel associated box (KRAB) repression domain, which silences genes or cis-regulatory elements targeted by single-guide RNAs (sgRNAs) through the deposition of H3K9me3 repressive epigenetic marks.[2,3] Using this approach, we have achieved over 95% knockdown efficiency of the functionally redundant FOXA family of transcription factor (TF) genes (*FOXA1*, *FOXA2*, and *FOXA3*) in hPSC-derived endoderm cells.[1] This protocol outlines detailed steps for generating Dox-inducible *FOXA1/A2/A3*-triple CRISPRi hPSC lines, including establishing host hPSCs with a Dox-inducible dCas9-KRAB expression cassette, designing sgRNAs, performing Golden-Gate cloning of multiple sgRNAs into a single lentivirus plasmid, transducing the lentivirus into the host hPSCs, generating monoclonal CRISPRi lines, and validating knockdown efficiency. This protocol is broadly applicable for silencing genes or cis-regulatory regions 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.

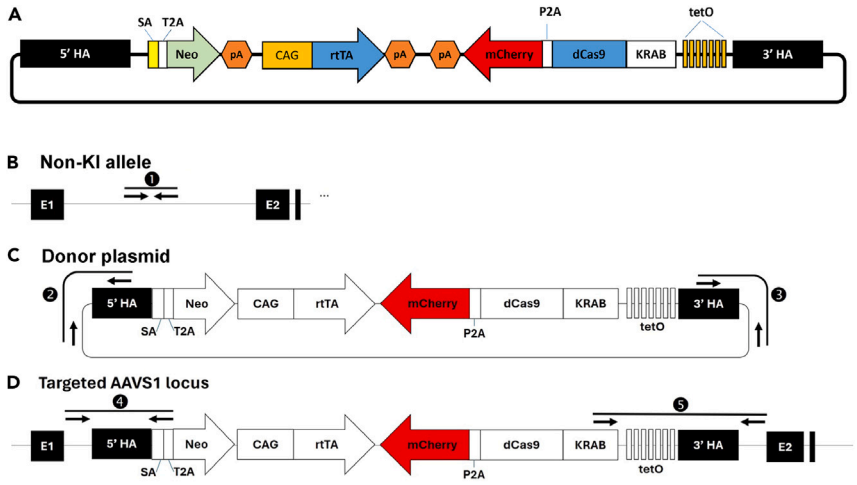

Figure 1. Overview of targeting vector and genotyping strategy of the dCas9-KRAB cassette at the AAVS1 locus

(A) The map of the targeting vector (Addgene #73497).

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

(C) Schematic of the pAAVS1-Ndi-CRISPRi donor plasmid. Primer pairs 2 and 3 specifically amplify plasmid sequences that are not integrated *via* homologous recombination into the AAVS1 locus.

(D) Schematic of the CRISPRi cassette correctly integrated into 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 13.

Source of CRISPR/Cas plasmids

To complete this protocol, plasmids for the expression of Streptococcus pyogenes-derived Cas9 (SpCas9) and single-guide RNA (sgRNA), as well as a plasmid that serves as the donor template to introduce the CRISPRi 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 following guide-RNA (gRNA) spacer sequence 5′-GGGGCCACTAGGGACAGGAT-3′ was utilized.[4]
   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)[5] containing a high-fidelity eSpCas9(1.1) optimized sgRNA scaffold.[6,7]
   c. Multiple protocols describing oligonucleotide annealing and Golden-Gate cloning have been described (e.g., Sahu et al., 2023; step 15).[8]

   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.[9–11]

   ⚠ CRITICAL: Use Sanger sequencing to confirm the genomic AAVS1 sequence in the targeted cell line matches the gRNA sequence. Primer pair 1 (Figure 1B) 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. Obtain the targeting plasmid (AAVS1-NDi-CRISPRi) for knock-in of the CRISPRi cassette to the AAVS1 locus.

a. The targeting plasmid (pAAVS1-NDi-CRISPRi-Gen1, Addgene #73497) used in this study was a kind gift of Dr. Bruce Conklin (Figure 1A).[2]
b. The 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 the Kox1 KRAB transcriptional repression domain and mCherry.[12]

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

⚠ 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 using a 6-well plate. We use human induced pluripotent stem cells 72_3 (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 of 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 it at 4°C. The shelf life of the coated plate is 2 weeks at 4°C.

4. Thawing cryopreserved hPSCs.
   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 2–3 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 hPSC culture plate and add 1 mL of 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 an appropriate portion of hPSC clumps (see Note below) 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" for detailed instructions).

i. Place the plate into a 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 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/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
Bacterial and virus strains		
NEB 5-alpha competent *E. coli*	NEB	Cat# C2987H
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
Opti-MEM	Thermo Fisher Scientific	Cat# 31985062
IMDM, GlutaMAX supplement	Thermo Fisher Scientific	Cat# 31980097
Ham's F-12 nutrient mix, GlutaMAX supplement	Thermo Fisher Scientific	Cat# 31765092
FBS	Thermo Fisher Scientific	Cat# 10437-028

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
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
Activin A	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
BMP4	R&D Systems	Cat# 314-BP-050
Forskolin	Sigma-Aldrich	Cat# F3917-10MG
Corning Matrigel hESC-qualified matrix	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
Accutase	STEMCELL Technologies Sigma-Aldrich	Cat# 07922 Cat# A6964-500ML
ReLeSR	STEMCELL Technologies	Cat# 100-0483
TrypLE Express enzyme (1×)	Thermo Fisher Scientific	Cat# 12605010
mFreSR	STEMCELL Technologies	Cat# 5854
CloneR	STEMCELL Technologies	Cat# 05888
Y27632	STEMCELL Technologies	Cat# 72304
7-AAD	Thermo Fisher Scientific	Cat# 00-6993-50
Doxycycline	Sigma-Aldrich	Cat# D9891-5G
Polybrene infection/transfection reagent	Sigma-Aldrich	Cat# TR-1003-G
Polyethylenimine branched	Sigma-Aldrich	Cat# 408727-100ML
G418 sulfate (50 mg/mL)	Thermo Fisher Scientific	Cat# 10131035
Plasmocin	InvivoGen	Cat# ant-mpp
Penicillin-Streptomycin	Thermo Fisher Scientific	Cat# 15140122
Proteinase K, recombinant, PCR grade	Thermo Fisher Scientific	Cat# EO0491
TransIT-LT1	Mirus Bio	Cat# MIR 2300
Tris-HCl, pH 8.0	Santa Cruz Biotechnology	Cat# sc-301953
Triton X-100 (100%)	Acros Organics	Cat# 327371000
Phusion polymerase	NEB	Cat# M0530S
AgeI-HF	NEB	Cat# R3552S
NotI-HF	NEB	Cat# R3189S
BbsI-HF	NEB	Cat# R3539L
FastDigest Esp3I	Thermo Fisher Scientific	Cat# FD0454
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
SOC outgrowth medium	NEB	Cat# B9020S
N,N-Dimethylformamide	Fisher Scientific	Cat# D119-500
Isopropyl-β-D-thiogalactoside	Roche	Cat# 10724815001
X-Gal	GoldBio	Cat# X4281C
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

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Endo-free plasmid maxi kit	QIAGEN	Cat# 12362
Taqman copy number assay, NeoR	Thermo Fisher Scientific	Cat# 4400291
Taqman copy number reference assay, human RNAseP	Thermo Fisher Scientific	Cat# 4403328
TaqMan genotyping master mix	Thermo Fisher Scientific	Cat#4371355
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
PowerUp SYBR Green master mix	Thermo Fisher Scientific	Cat#A25742
Experimental models: Cell lines		
hPSC (72_3)	CCHMC Pluripotent Stem Cell Facility	RRID: CVCL_A1BW
Dox-inducible CRISPRi, host hPSC (72_3)	Matsui et al.[1]	N/A
HEK293T	ATCC	Cat# CRL-11268; RRID:CVCL_1926
Oligonucleotides		
sgRNA_T2_Forward: 5′-CACCGGGGCCACTAGGGACAGGAT-3′	IDT	N/A
sgRNA_T2_Reverse: 5′-AAACATCCTGTCCCTAGTGGCCCC-3′	IDT	N/A
Primers for detecting untargeted AAVS1 (primer pair 1) Fwd: 5′-CCCCTTACCTCTCTAGTCTGTGC-3′ Rev: 5′-CTCAGGTTCTGGGAGAGGGTAG-3′	Oceguera-Yanez F et al.[4]	N/A
Primers for detecting random targeting plasmid integration (primer pair 2) Fwd: 5′-GAGTGGAGGAAGACGGAACC-3′ Rev: 5′-CAGTGTTATCACTCATGGTTATGGC-3′	N/A	N/A
Primers for detecting random targeting plasmid integration (primer pair 3) Fwd: 5′-GGGTTTGAGTCCTTGGCAAG-3′ Rev: 5′-CCACCTCTGACTTGAGCGTC-3′	N/A	N/A
Primers for detecting correct targeted AAVS1 integration (primer pair 4) Fwd: 5′-TCGACTTCCCCTCTTCCGATG-3′ Rev: 5′-GAGCCTAGGGCCGGGATTCTC-3′	Oceguera-Yanez F et al.[4]	N/A
Primers for detecting correct targeted AAVS1 integration (primer pair 5) Fwd: 5′-CAGTTGGAGGAGAATCCACCCA AAAG-3′ Rev: 5′-CAGGCAGTTAGTGACTTAGCATCC-3′	N/A	N/A
CRISPRi gRNAs	IDT	Table in step 14
M13R Primer 5′-TCACACAGGAAACAGCTATGAC-3′	IDT	N/A
RT-qPCR primers	IDT	Table S1
Recombinant DNA		
pAAVS1-NDi-CRISPRi (Gen 1)	Mandegar et al.[2]	Cat# 73497; RRID_Addgene_73497
pX459M2-HF-AAVS1	Ran et al.[5] Chen et al.[6]	N/A
pmU6-gRNA	Kabadi et al.[13]	Cat# 53187; RRID_Addgene_53187
phU6-gRNA	Kabadi et al.[13]	Cat# 53188; RRID_Addgene_53188
phH1-gRNA	Kabadi et al.[13]	Cat# 53186; RRID_Addgene_53186
ph7SK-gRNA	Kabadi et al.[13]	Cat# 53189; RRID_Addgene_53189
pLV GG hUbC-EGFP	Matsui et al.[1]	Cat# 216161; RRID_Addgene_216161
pLV-hUbC-EGFP-sgRNA.FOXA1/A2/A3_CRISPRi	Matsui et al.[1]	Cat# 216166; RRID_Addgene_216166
psPAX2	Laboratory of Didier Trono	Cat# 12260; RRID_Addgene_12260
pMD2.G	Laboratory of Didier Trono	Cat# 12259; RRID_Addgene_12259
Software and algorithms		
CRISPOR	Concordet et al.[14]	http://crispor.gi.ucsc.edu/
GraphPad Prism v9.3.1 (350)	Dotmatics	https://www.graphpad.com/features
Other		
MoFlo XDP	Beckman Coulter	N/A
BD FACSAria II	BD Biosciences	N/A
BZ-X810	Keyence	N/A
QuantStudio3	Thermo Fisher Scientific	N/A
Veriti 96-Well Fast Thermal Cycler	Thermo Fisher Scientific	Cat# 4375305
MiniAmp Thermal Cycler	Thermo Fisher Scientific	Cat# A37834
Nuclease-free 1.5 mL microcentrifuge tubes	Eppendorf	Cat# 022363212

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 is 40 μg/mL. After dilution with DMEM/F12, Cultrex can be used to coat plates using the same process 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**
Store at −80°C for at least 1 year.		

mTeSR1 + 10 μM Y27632		
Reagent	Final concentration	Amount
mTeSR1	1×	10 mL

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Continued		
Reagent	Final concentration	Amount
Y27632 (10 mM)	10 μM	10 μL
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.		

Cell sorting media		
Reagent	Final concentration	Amount
10× CloneR supplement	1×	2 mL
Plasmocin	1×	40 μL
Penicillin-Streptomycin (10,000 U/mL)	1×	200 μL
mTeSR1	1×	18 mL
Total	**N/A**	**20 mL**
Make fresh for each use.		

CDM2 media		
Reagent	Final concentration	Amount
IMDM, GlutaMAX Supplement	49%	250 mL
F12, GlutaMAX Supplement	49%	250 mL
10% PVA solution	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 media		
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
10% PVA solution	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.

Mesendoderm induction media (with Dox)		
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**
Make fresh for each use.		

Definitive endoderm induction media (with Dox)		
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**
Make fresh for each use.		

Foregut induction media (with Dox)		
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**
Make fresh for each use.		

Liver bud progenitor induction media (with Dox)		
Reagent	Final concentration	Amount
CDM3	1×	12 mL
ActivinA (10 μg/mL)	10 ng/mL	12 μL
BMP4 (30 μg/mL)	30 ng/uL	12 μL
Forskolin (1 mM)	1 μM	12 μL
Doxycycline (2 mM)	2 μM	12 μL
Total	**N/A**	**12 mL**
Make fresh for each use.		

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 2–3 months.		
Solution for preparation of sgRNA expression lentivirus (Steps 16–20)		

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**
Store 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**
Store 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 to 8.0 by adding 6N HCl. Pass through 0.22 μm filter.

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	to 5 mL
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.

STEP-BY-STEP METHOD DETAILS

Establishment of CRISPRi 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 KRAB-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-lentiviral transduction and CRISPRi-mediated knockdown of target genes (step 22).

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 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]

CRISPRi hPSC line generation: Reverse transfection

Timing: 1 h

5. Prepare 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:

Reagent	Transfected	Untransfected
pX459M2-HF-AAVS1	2 ug	-
pAAVS1-Ndi-CRISPRi (Addgene #73497)	2 ug	-
TransIT-LT1 Reagent	12 μL	12 μL
Opti-MEM	to 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 wash.
   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. Gently move the plate back and forth to evenly distribute the cells across the plate. Return the plate to the incubator.

CRISPRi 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. 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 2 mL/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 the 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-CRISPRi cassette contains a doxycycline-inducible mCherry reporter, G418-resistant cells putatively targeted with the CRISPRi 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 50–150 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 mTeSR1 to each well and place dishes in the incubator.
    c. Prepare 48 sterile, nuclease-free 1.5 mL microcentrifuge tubes labeled 1–48.
    d. Aspirate spent media from each well of the 48-well dish containing excised clones and add 125 μL of ReleSR at 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/well of mTeSR1.
    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 10 L, 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.

CRISPRi 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 Figure 1B to D, 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 as indicated below.

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 the changes in temperatures and lengths of time for each step according to the manufacturer's instructions.

Note: Include a control reaction with no added genomic DNA (i.e., 1.0 μL of cell lysis buffer) to ensure 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 as indicated below.

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-CRISPRi, Gen1) 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	536
2	65.5°C	579
3	65.5°C	230
4	72°C	1248
5	68°C	1446

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 an appropriate 100-base-pair ladder.

13. Genotyping workflow and interpretation of results.
    a. Identify clones containing a targeted integration of the CRISPRi transgene.
        i. Subject lysates from all 48 clones to separate PCR reactions using primer pairs 4 and 5.
        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 CRISPRi 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 a 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 (pAAVS1-Ndi-CRISPRi, Gen1).

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.

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

⚠ 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. gRNA design sites such as CRISPOR (http://crispor.tefor.net/)[14] 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 CRISPRi 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 flowcytometric analysis), have the capacity to differentiate into ectoderm, endoderm, and mesoderm lineages, and have had their identity authenticated (e.g., by STR analysis).[15]

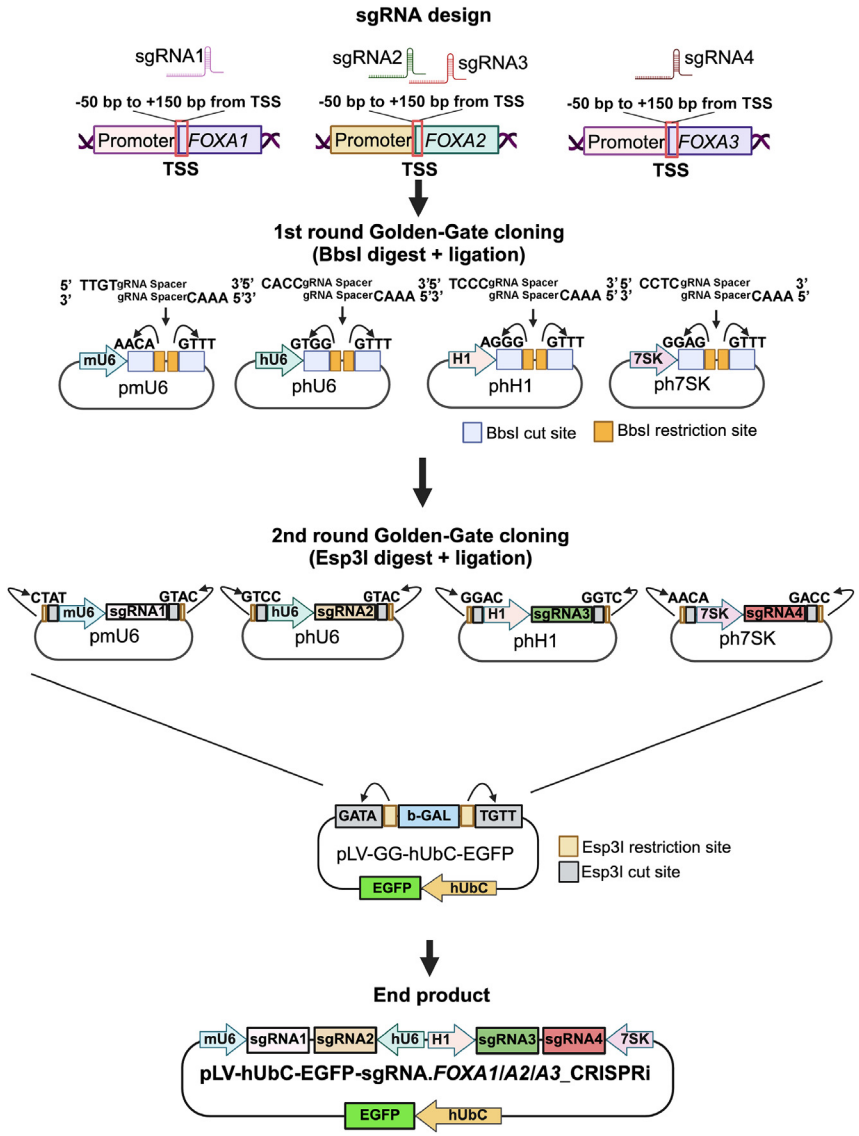

Figure 2. Schematic workflow of the 2-step Golden-Gate cloning for integrating four sgRNA sequences into a lentivirus plasmid

⚠ CRITICAL: When working with newly generated lines, before proceeding with sgRNA transduction, we also advise validating the expression of the dCas9-KRAB transgene. This requires optimizing Dox concentration and timing, assessing the inducibility and reversibility of dCas9-KRAB expression.[2]

Design and cloning of four sgRNAs to a lentivirus plasmid

Timing: 2–3 weeks

In this section, we provide a step-by-step protocol for the design and generation of a sgRNA expression lentivirus plasmid by Golden-Gate cloning of four gRNA spacers (Figure 2). This modified protocol, based on the referenced study,[13,16] demonstrates the high efficiency of integrating four sgRNAs into the pLV-GG-hUbC-EGFP lentivirus plasmid.

Alternatives: For a single or dual sgRNA cloning, please refer https://weissman.wi.mit.edu/crispr/ or https://www.jostlab.org/_files/ugd/1b15a0_67b14b03e176400793390fcfa9914842.pdf.[17]

14. Design gRNA spacer sequences.
    a. Select the top 1 or 2 scored gRNA sequences for each target gene from a list of the human next-generation, genome-scale CRISPRi gRNA library (Supplementary File 3, the "hCRISPRi-v2.1" tab, in the reference paper).[16]

Note: The reference study designed human and mouse whole gene CRISPRi gRNA libraries based on a comprehensive algorithm that incorporates chromatin, position, and sequence features to accurately predict highly effective gRNA for CRISPRi.[16]

⚠ CRITICAL: If your gene of interest has multiple transcription start sites (TSSs), confirm whether a selected gRNA is located within −50 and +150 bp upstream and downstream of the active TSS of your cell of interest. If not, please use the alternative design option below. An active TSS can be determined by RNA-seq and chromatin accessibility data in your cell of interest on a genome browser (e.g., USCS genome browser: https://genome.ucsc.edu/).

Alternatives: Design gRNA sequence using CRISPOR (http://crispor.tefor.net/).[14] CRISPOR automatically designs gRNA sequences and provides potential off-target information. Design gRNA sequence within −50 and +150 bp of the upstream or the downstream of the active TSS of your cell of interest.

    b. Order DNA oligos with specific overhang sequences for cloning into a BbsI site of the donor plasmids, as indicated in the table below.

Promoter/Donor plasmid	Oligo name	Overhang	gRNA Protospacer sequences	Overhang
pmU6 (Addgene #53187)	gRNA-FOXA3-Rank1-sense	5′-TTGTTTG	AGCGCTCTGGATCTCTCAG	
	gRNA-FOXA3-Rank1-as	5′-AAAC	CTGAGAGATCCAGAGCGCT	CAA-3′
phU6 (Addgene #53188)	gRNA-FOXA1-Rank1-sense	5′-CACCG	GCAGCCCGCTCACTTCCCG	
	gRNA-FOXA1-Rank1-as	5′-AAAC	CGGGAAGTGAGCGGGCTGC	C-3′
phH1 (Addgene #53186)	gRNA-FOXA2-Rank3-sense	5′-TCCCA	GTGCCGAGCTGCCCCGAGG	
	gRNA-FOXA2-Rank3-as	5′-AAAC	CCTCGGGGCAGCTCGGCAC	T-3′
ph7SK (Addgene #53189)	gRNA-FOXA2-Rank2-sense	5′-CCTCG	AAAGACGAGCGCTTACCTC	
	gRNA-FOXA2-Rank2-as	5′-AAAC	GAGGTAAGCGCTCGTCTTT	C-3′

⚠ CRITICAL: All sequences in the CRISPRi gRNA library are prepended with a 5′ G to enable robust transcription from the U6 promoter, whether or not this base was present in the genomic sequence.[16] Since we use the H1 promoter (5′ A for robust transcription), the 5′ G should be removed. We include the 5′ G for mU6/hU6/7SK promoters and 5′ A for H1 promoter in the overhang sequences in the gRNA design table.

15. Golden-Gate cloning of four sgRNAs into a host lentivirus plasmid.
    a. 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

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Reagent	Final concentration	Amount
Nuclease-free water	N/A	24.45 mL
Total	**N/A**	**25 mL**

Note: Store at 20°C–25°C for up to 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 inactivate 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).

b. 1st round Golden-Gate cloning of four individual sgRNAs into four separate donor plasmids: pmU6 (Addgene #53187), phU6 (Addgene #53188), phH1 (Addgene #53186), and ph7SK (Addgene #53189).

i. Prepare Golden-Gate reaction mix as indicated below.

Reagent	Final concentration	Amount
Donor plasmid (100 ng/uL)	46 fmol (molar ratio 1)	1 μL
Annealed, phosphorylated oligo DNA (0.1 μM)	100 fmol (molar ratio 2)	1 μL
BbsI-HF (20 U/uL)	1 U/uL	1 μL
T4 DNA Ligase (400 U/uL)	2 U/uL	1 μL

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Reagent	Final concentration	Amount
10× DNA Ligase Reaction Buffer	1×	2 μL
Nuclease-free water	N/A	14 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: 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	10–20 cycles
	16°C	10 min	
	37°C	30 min	1
	75°C	15 min	1
Hold	4°C	infinite	

c. Transformation of ligated DNA into competent *E. coli*.
   i. Thaw 50 μL of NEB 5-alpha Competent *E. coli* on ice.
   ii. Add 5 μL of ligated DNA and mix cells and DNA by pipetting or tapping.
   iii. Incubate on ice for 30 min.
   iv. Heat shock in a 42°C water bath for 30 s.
   v. Place on ice for 2 min.
   vi. Add 500 μL of super optimal broth with catabolite repression (SOC) media.
   vii. Place cells at 37°C for 1 h with shaking.
   viii. Spread 50–100 μL of the transformed competent cells on a Kanamycin-containing LB plate and incubate at 37°C for 12–18 h.

d. Colony isolation, small-scale plasmid purification, and plasmid verification
   i. Pick 2 single colonies and transfer each colony into a separate bacteria culture tube containing 2.0 mL of Kanamycin-containing LB medium.
   ii. Incubate bacterial culture at 37°C for 16 h in a shaking incubator.
   iii. Centrifuge bacterial culture at 3000 × *g* for 10 min at 4°C.
   iv. Purify plasmid using a mini-prep kit.
   v. Measure concentration by Nanodrop.
   vi. Digest purified plasmid with BbsI at 37°C for 1 h as indicated below.

Reagent	Final concentration	Amount
Mini-prep plasmid	100 ng	Variable
BbsI-HF (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 on agarose gel with 0.5 μg/mL ethidium bromide at 100V for 1 h.

Note: If gRNA is successfully integrated, the BbsI restriction sites will be removed in the donor plasmids.

viii. Confirm the integrated gRNA spacer sequence using Sanger sequencing with M13R Primer (5′-TCACACAGGAAACAGCTATGAC- 3′).

e. 2nd round Golden-Gate cloning of four sgRNAs into a host lentivirus plasmid (Addgene #216161) (see troubleshooting, problem 3).

i. Prepare 2nd round of Golden-Gate reaction mix as indicated below.

Reagent	Final amount	Amount
pLV-GG-hUbC-EGFP (100 ng/uL) (Addgene #216161)	200 ng	2 μL
phU6+sgRNA (100 ng/uL)	200 ng	2 μL
pmU6+sgRNA (100 ng/uL)	200 ng	2 μL
phH1+sgRNA (100 ng/uL)	200 ng	2 μL
ph7SK + sgRNA (100 ng/uL)	200 ng	2 μL
Esp3I FastDigest	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**

⚠ CRITICAL: The enzymatic activity of Esp3I FastDigest dramatically decreases after 1 year of storage.

⚠ 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	10 min	30 cycles
	16°C	15 min	
	37°C	30 min	1
	80°C	5 min	1
Hold	4°C	infinite	

Note: Increasing the cycle number improves ligation efficiency.

iii. Chill ligated DNA on ice for 5–10 min.

f. Transformation of ligated DNA into Stable competent *E. Coli*.

i. Prepare IPTG/X-Gal solution as indicated below.

Reagent	Final concentration	Amount
IPTG (100 mM)	25 mM	40 μL
X-Gal (20 mg/mL)	15.4 mg/mL	120 μL
Total	**N/A**	**160 μL**

ii. Spread 100 μL IPTG/X-Gal mix on an ampicillin-containing LB plate.
iii. Dry IPTG/X-Gal coated plate at 20°C–25°C for 20–30 min.
iv. Thaw 50 μL of NEB Stable competent *E. coli* on ice.
v. Add 5 μL of ligation mix and mix cells and DNA by pipetting or tapping.
vi. Incubate on ice for 30 min.
vii. Heat shock the competent cell mixture in a 42°C water bath for 30 s.
viii. Place cells on ice for 2 min.
ix. Add 950 μL of NEB 10-beta/Stable Outgrowth Medium to the mixture.
x. Place the mixture at 30°C for 1 h with shaking.
xi. Spread 50–100 μL of the transformed competent cells on the IPTG/X-Gal/Ampicillin-containing LB plate.
xii. Incubate at 30°C for 24 h.

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

g. Colony isolation, small-scale plasmid purification, and plasmid verification.
i. Pick 4–8 white, single colonies and transfer each colony into a separate bacteria culture tube containing 2 mL of Ampicillin-containing LB medium.
ii. Incubate at 30°C for 16–20 h in a shaking incubator.

⚠ CRITICAL: For better clone stability, isolate 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 3100 × *g* for 10 min at 4°C.
iv. Discard LB media and purify plasmid DNA using a mini-prep kit.
v. Measure concentration by Nanodrop.
vi. Prepare 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
NotI-HF (20 U/uL)	0.4 U/uL	0.5 μL
AgeI-HF (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 on agarose gel with 0.5 μg/mL ethidium bromide at 100 V for 1 h.

Note: If four sgRNAs are successfully integrated, the expected band sizes are 7589 bp and 3820 bp. The expected band sizes of the empty host plasmid are 7589 bp and 2863 bp.

h. Amplify successfully cloned plasmids on a midi or maxi-culture scale.
i. Transform and incubate successfully cloned plasmid into NEB Stable competent *E. coli* as indicated in step f.
ii. Pick ~ 3 single colonies and transfer each colony into a separate bacteria culture tube containing 2.5 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. Check insert by restriction enzyme digestion as described in step 15-g (vi-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 (TakaraBio) 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.

16. 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 it 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.

17. 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 (Thermo Fisher Scientific, #12605010).
    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.
18. 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 17-a to 17-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.

19. 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
pLV-hUbC-EGFP-sgRNA.FOXA1/A2/A3_CRISRPi (Addgene #216166)	5.0 ug
psPAX2 (Addgene #12260)	3.4 ug
pMD2.G (Addgene #12259)	1.6 ug
Opti-MEM	500 μL

⚠ 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.

20. 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 21.

21. Collect and concentrate lentivirus supernatant (Day 9; ~60 h post-medium change).
a. Confirm transfection efficiency by observing 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 3).

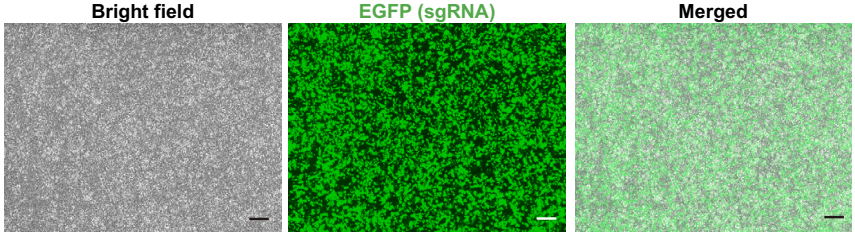

Figure 3. EGFP marker expression from sgRNA-lentivirus plasmids in HEK293T cell
Images were acquired just before the lentivirus purification step (Day 9). This level of transfection efficiency is sufficient to yield enough lentivirus for spin-infection. Scale bars = 300 μm.

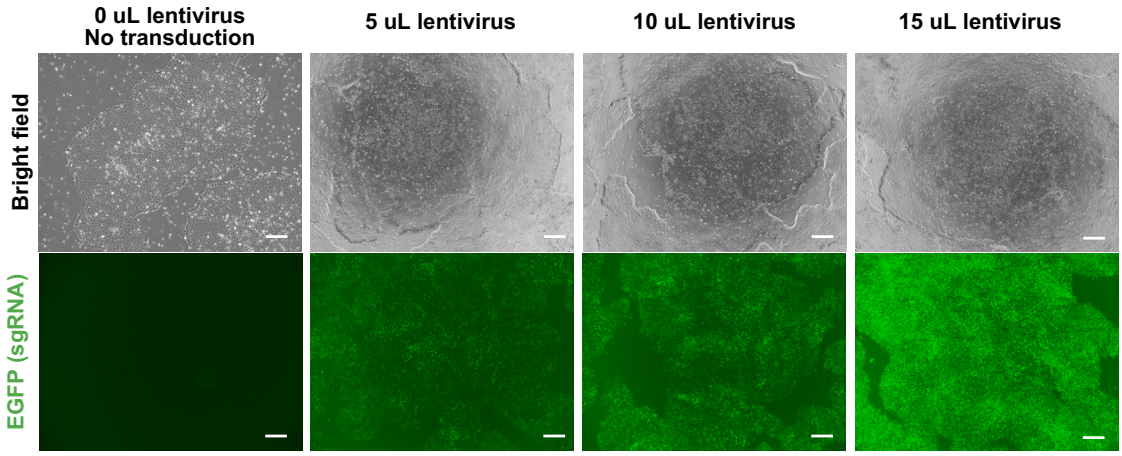

Figure 4. EGFP marker expression from sgRNA-lentivirus transduced hPSCs

Successful lentivirus transduction is monitored by EGFP expression. The percent of transduced cells increased with the amount of lentivirus transduced. Scale bars = 300 μm.

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 (This step may be omitted).

⚠ 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 30 min to 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 ($1/100^{th}$ of the original volume).
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 CRISPRi host hPSCs

Timing: 1–2 weeks

In this section, we describe the stepwise protocol for sgRNA-lentivirus transduction into the CRISPRi host hPSC line by spin infection,[18] which enables high transduction efficiency in hPSCs (>80%) (Figure 4).

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

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

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 (STEMCELL Technologies, #72302) and Transduction media (see Material).
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 (e.g., 4 times) 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 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 non-viable 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 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.

Establishment of monoclonal CRISPRi hPSC lines transduced with sgRNAs

Timing: 5–6 weeks

This section describes the protocol for establishing monoclonal CRISPRi hPSC lines transduced with sgRNAs. We sort cells expressing high levels of sgRNA-EGFP using a cell sorter and subsequently perform single-cell cloning through limiting dilution. From one 96-well plate, we expect to obtain 5 to 11 clones.

Alternatives: We experienced inconsistent and lower CRISPRi knockdown efficiency without monoclonal selection. However, the monoclonal selection process can introduce clonal effects unrelated to the CRISPRi system, such as those related to cell differentiation. If this is a concern, you can freshly transduce sgRNA-lentivirus with a consistent MOI each time and omit the monoclonal selection process.

23. Sorting sgRNA-EGFP-positive cells.

Note: Prepare sgRNA-EGFP-untransduced hPSCs as a negative control.

a. Bring reagents (mTeSR1, DMEM/F12, Accutase, and Cultrex or Matrigel-coated 96 and 6 well plates) to 20°C–25°C.
b. Prepare Cell sorting media as indicated in the Material section.
c. Aliquot 5 mL of Cell sorting media into a 15 mL conical tube for cell collection.
d. Aliquot 1.5 mL of Cell sorting media into a 15 mL conical tube and add 7-AAD (1:2000) for live dead cell staining.
e. Aspirate culture media from a cell culture plate and briefly rinse cells with DMEM/F12.
f. Aspirate DMEM/F12 and add 800 μL of Accutase (per one well of a 6-well plate).
g. Place cells into a 37°C 5% CO_2 incubator and incubate for 12 min.
h. Add 1 mL of mTeSR1 per one well of a 6-well plate.
i. Dissociate cells into single cells by pipetting using a P1000 pipette.
j. Transfer the cell suspension to a 15 mL conical tube.
k. Count cell number.
l. Transfer a desired number of cells to a 15 mL conical tube as indicated below.

Sample name	Cell type	Fluorescent protein and dye information	Cell number
Isotype control	hPSC (no EGFP)	No color	1.0–2.0 × 10^6
7-AAD stain control	hPSC (no EGFP)	7-AAD	1.0–2.0 × 10^6
EGFP expression control	hPSC (sgRNA-EGFP)	EGFP	1.0–2.0 × 10^6
Sorting sample	hPSC (sgRNA-EGFP)	EGFP and 7-AAD	1.0–2.0 × 10^7

m. Centrifuge at 350 × *g* for 5 min at 20°C–25°C.
n. Resuspend a cell pellet with Cell sorting media as indicated below.

Sample name	Cell type	Cell sorting media volume
Isotype control	hPSC (no EGFP)	500 μL
7-AAD single control	hPSC (no EGFP)	500 μL
EGFP expression control	hPSC (sgRNA-EGFP)	500 μL
Sorting sample	hPSC (sgRNA-EGFP)	1–2 mL

o. Pass cell suspension through a 70-μm cell strainer.

⚠ CRITICAL: The use of a pass-through filter is crucial to prevent clogging of a cell sorter.

p. Remove doublets and sort the top 20% of the EGFP-high population using a cell sorter (Figure 5A).
q. Centrifuge sorted cells at 350 × *g* for 10 min at 20°C–25°C.
r. Resuspend the cell pellet with Cell sorting media to adjust concentration to 1.0 × 10^4 or 1.0 × 10^5 cells/mL.

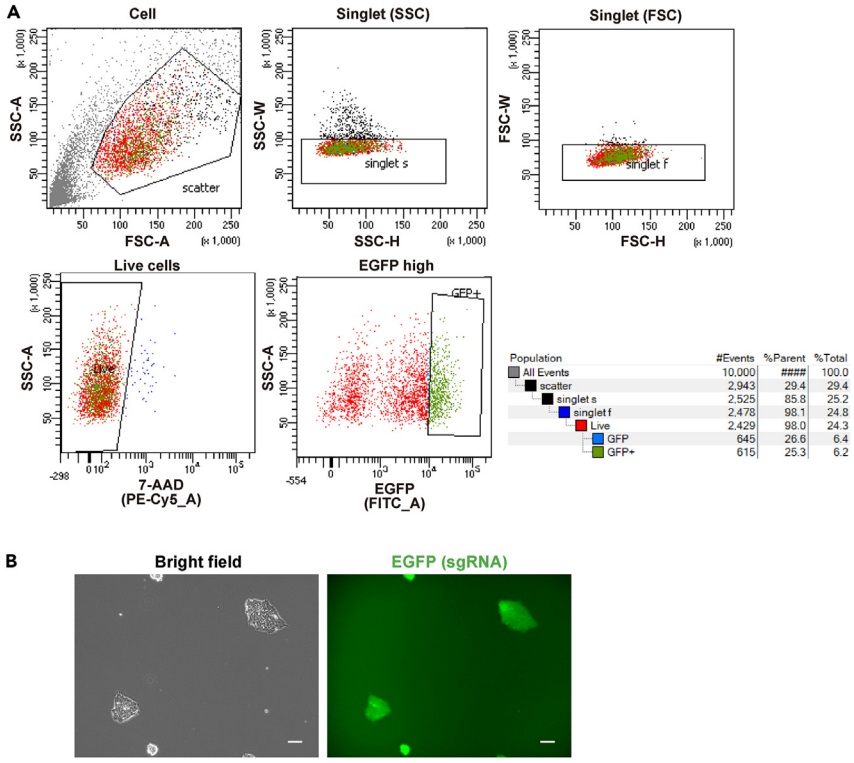

Figure 5. Cell sorting of sgRNA-transduced CRISPRi hPSCs

(A) Gating panels of cell sorting for sgRNA-EGFP expressing CRISPRi hPSCs.

(B) Images of sorted sgRNA-CRISPRi polyclonal hPSCs (Day 5). sgRNA expression was monitored by EGFP signal. Scale bars = 100 μm.

Note: Determine cell number based on a cell sorter's cell counter.

s. Transfer a desired number of cells to a new conical tube.

Note: For a limiting dilution, 4000 cells are required. For expanding polyclonal cells, 3000–4000 cells per one well of a 6-well plate are required (Figure 5B).

24. Single-cell cloning by a limiting dilution (Day 0).

Alternatives: Before establishing clonal lines, you can expand sorted polyclonal cells and generate single-cell suspension by Accutase dissociation (as described in step 22, a to j) for subsequent limiting dilution (Figure 6A).

a. Aspirate coating solution from a 96-well plate.
b. Add 100 μL of Cell sorting media to each well of a 96-well plate using an 8-channel pipette.
c. Resuspend 4000 sorted EGFP-positive cells in 100 μL of Cell sorting media, as shown in the table in the Materials and Equipment section.
d. Add the cell suspension to an A1 well (top right well of a 96-well plate) and mix well by pipetting.
e. Transfer 100 μL of the cell suspension from an A1 well to a B1 well.
f. Repeat the transfer of 100 μL of cell suspension from a B1 well to a C1 well and continue down to a H1 well (serial dilution).
g. Discard 100 μL of cell suspension in a H1 well.

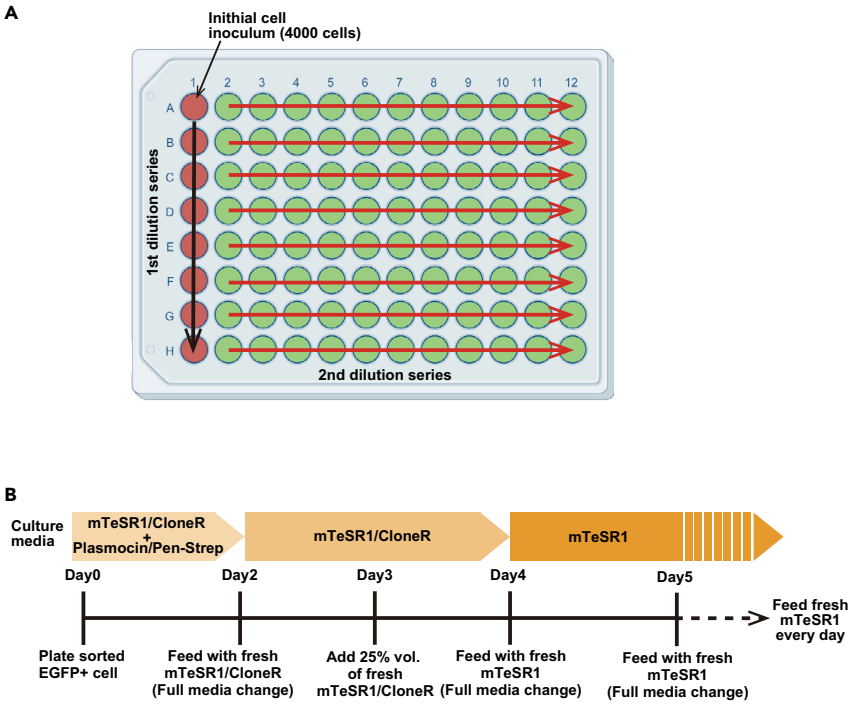

Figure 6. Summary of cell cloning and expansion strategy

(A) Schematic overview of limiting dilution for single-cell cloning.

(B) Cell culture timeline for sorted sgRNA-CRISPRi hPSCs. The schedule for medium change is the same for both polyclonal and monoclonal hPSCs.

h. Add 100 μL of additional Cell sorting media to each well of Colum 1.

Note: The total volume in each well of Column 1 is 200 μL.

i. Transfer 100 μL of cell suspension from Column 1 (A1 to H1) to Column 2 (A2 to H2) using an 8-channel pipette and mix well by gently pipetting.
j. Repeated the transfer of 100 μL of cell suspension using an 8-channel pipette across rows to A12 to H12
k. Discard 100 μL of cell suspension in A12 to H12.
l. Place the 96-well plate into a 37°C 5% CO_2 incubator.

25. Media change schedule (Day 2 to Day 4) (Figure 6B).
    a. Media change (Day 2).
        i. Bring mTeSR1 to 20°C–25°C.
        ii. Prepare a 1:10 dilution of CloneR in mTeSR1 (e.g., 1.2 mL of CloneR in 10.8 mL of mTeSR1).
        iii. Aspirate culture media from the cell culture plate and add fresh CloneR-mTeSR1 media in each well (e.g., 100 μL per each well of a 96-well plate).
        iv. Place the 96-well plate into a 37°C 5% CO_2 incubator.
    b. Media change (Day 3).
        i. Bring mTeSR1 to 20°C–25°C.
        ii. Prepare a 1:10 dilution of CloneR in mTeSR1.
        iii. Add 25% volume of fresh CloneR-mTeSR1 media to each well of the 96-well plate (e.g., 25 μL per one well of the 96-well plate).
        iv. Place the 96-well plate into a 37°C 5% CO_2 incubator.
    c. Media change (Day 4).

i. Bring mTeSR1 to 20°C–25°C.
ii. Aspirate culture media from the cell culture plate and add fresh mTeSR1.
iii. Place well into a 37°C 5% CO_2 incubator.

⚠ CRITICAL: Once cells reach 60% confluence, passage cells into a larger scale well plate (e.g., 24-well pate). If you perform a GCDR passage, use the amounts listed below.

Well scale	GCDR volume	Incubation time	mTeSR1 volume	Trituration method
96-well plate	50 μL	1–2 min	100 μL	P200 pipette (set 100 μL)
24-well plate	250 μL	2–3 min	250 μL	P200 pipette (set 200 μL)
12-well plate	500 μL	3–4 min	500 μL	P1000 pipette (set 500 μL)
6-well plate	1 mL	4–5 min	1 mL	P1000 pipette (set 1000 μL)

iv. Expand single-colony-derived hPSCs into a 6-well plate scale.

Note: The normal hPSC culture protocol (e.g., GCDR passage and feeding with mTeSR1) is applicable during the clonal expansion steps.

Selection of effective CRISPRi clones

Timing: 3 days

This section describes the selection of effective CRISPRi clones using the RT-qPCR method. We present *FOXA1/2/3*-CRIPSRi hPSC as an example. Single-cell cloning allows for the selection of the most efficient CRISPRi lines and facilitates reproducible experiments, compared to polyclonal cell lines.

26. Plate monoclonal CRISPRi hPSC lines for CRISPRi induction (Day 0).

Note: We pick one clone (2 replicates) for Dox-negative control and all clones for Dox-treated CRISPRi samples (2 replicates).

a. Dissociate and plate monoclonal CRISPRi hPSCs as described in step 22 (a to j).
b. Transfer the desired number of cells to a conical tube.

Note: 3.0×10^5 cells (hPSC culture for 3–4 days) and 1.2×10^6 cells (endoderm differentiation) are required for one well of a 6-well plate.

c. Centrifuge at 350 × *g* for 5 min at 20°C–25°C.
d. Aspirate the supernatant and resuspend the cell pellet with mTeSR1 + 10 μM Y27632.
e. Aspirate coating solution from a culture plate.
f. Transfer cell suspensions into each well of the coated plate.
g. Place the plate into a 37°C 5% CO_2 incubator.

27. CRISPRi induction by Dox treatment during endoderm differentiation (Day 1–3)
a. Bring appropriate culture media and thaw 2 mM Dox to 20°C–25°C.
b. Prepare Dox-containing culture media.

Note: If you perform CRISPRi during hPSC differentiation, prepare Dox-containing media using the appropriate media. A detailed human endoderm induction protocol is described in Matsui et al.[1]

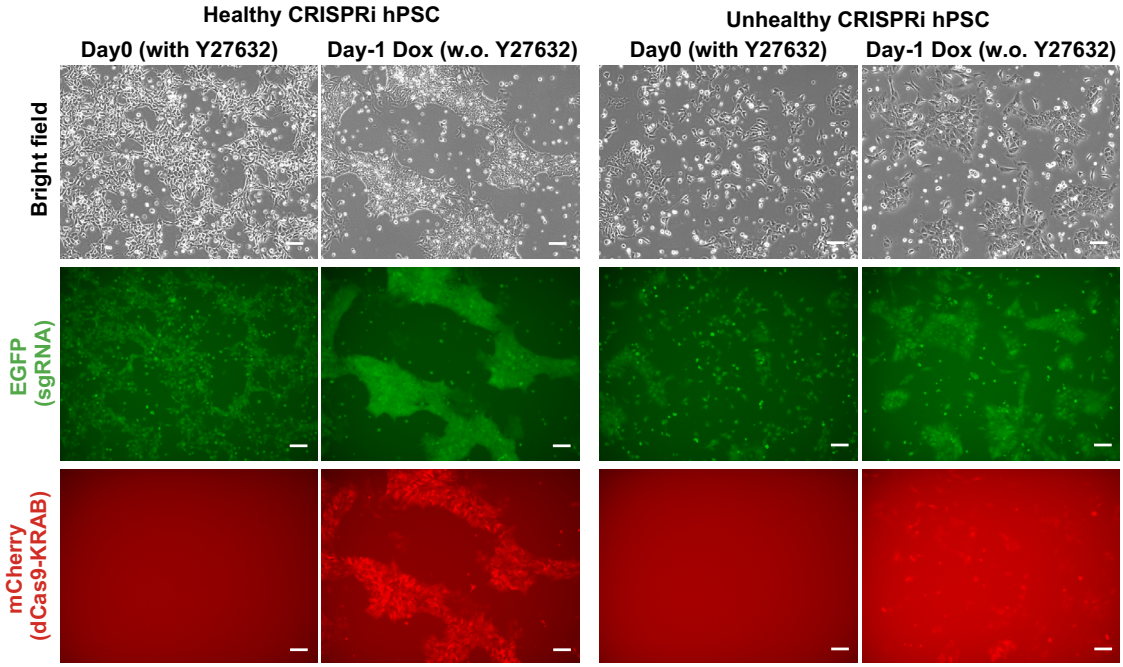

Figure 7. mCherry marker expression in healthy and unhealthy sgRNA-CRISPRi hPSCs

Left: Healthy state. Right: Unhealthy state. Unhealthy cells do not express mCherry 1 day after the Dox administration. Unhealthy cells do not form clusters, and their morphology is flattened on Day 0. Scale bars = 100 μm.

Note: The optimal concentration of Dox is 500 nM to 2 μM. Optimal concentration needs to be determined by titration.

c. Aspirate culture media from the culture plate and add fresh Dox-containing media.
d. Place the culture plate into the 37°C 5% CO_2 incubator.
e. Starting the next day, perform daily media changes with Dox-containing media to keep the Dox fresh.

Note: For evaluating CRISPRi knockdown efficiency, collect RNA samples from 1 to 4 days of Dox treatment along with Dox-negative controls and for performing RT-qPCR assays. Additionally, we recommend confirming target protein expression using a western blot assay.

Note: Confirm successful dCas9-KRAB expression by mCherry fluorescence imaging. mCherry expression can be observed within one day after Dox administration.

⚠ CRITICAL: If CRISPRi hPSCs appear unhealthy (e.g., extensive spontaneous differentiation), CRISPRi may not work effectively. Unhealthy hPSCs may not form colonies in the mTeSR1 + 10 μM Y27632 media (Figure 7).

28. Sample collection and evaluation of CRISPRi knockdown efficiency by RT-qPCR (Day 3) (see troubleshooting, problem 6).
    a. Lyse Dox-negative controls and Dox-treated CRISPRi samples and purify total RNA by an appropriate kit (e.g., Aurum Total RNA Mini Kit; Bio-Rad).
    b. Measure RNA concentration.
    c. Synthesis cDNA (e.g., iScript cDNA Synthesis Kit; BioRad) from 500 ng of total RNA.
    d. Dilute cDNA to 1:5 by adding DNase-free water (20 μL cDNA + 80 μL DNase-free water).
    e. Run RT-qPCR using primers for CRISPRi target genes.
    f. Analyze RT-qPCR data (Figure 8).

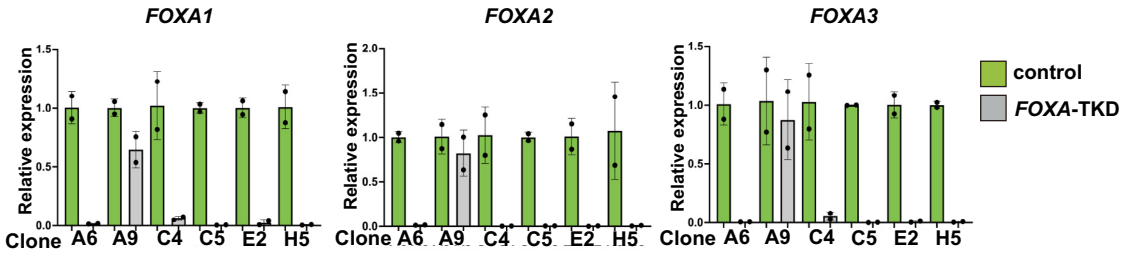

Figure 8. Screening knockdown efficiency for several *FOXA*1/2/3-CRISPRi clones by RT-qPCR

Evaluation of *FOXA*1/2/3 knockdown efficiency in *FOXA*1/2/3-CRISPRi foregut cells compared with control foregut cells (Dox-negative control) (n = 2, means ± SD). Five out of six clones showed more than 96–99% knockdown efficiency for all *FOXA* genes.

EXPECTED OUTCOMES

Using this protocol, we typically obtain 6 to 11 monoclonal CRISPRi hPSC lines from one 96-well plate. For the CRISPRi knockdown efficiency, *FOXA1/A2/A3*-CRISPRi repressed the expression of all three *FOXA* genes to 95% within 3 days of Dox treatment in endoderm differentiation (Figure 9).[1] We selected the three most effective CRISPRi clones for downstream experiments. Although CRISPRi knockdown efficiency depends on the gRNA spacer sequence design, using one or two gRNAs targeting one gene provides sufficient knockdown efficiency.

QUANTIFICATION AND STATISTICAL ANALYSIS

Detailed statistics tests, sample size, biological replicates, and error bars are provided in the figure legends. The data in Figure 9 were obtained from independent clones for *FOXA1/A2/A3*-CRISPRi clonal lines. All statistical tests for RT-qPCR and RNA-seq were performed by GraphPad Prism9. The two-tailed unpaired t-test was used to assess the differences between the two groups.

LIMITATIONS

This protocol is optimized for a hPSC line (iPSC72_3). Optimization may be required for other hPSC lines and cell types. The CRISPRi hPSCs were generated from WTC and WTB genetic backgrounds in

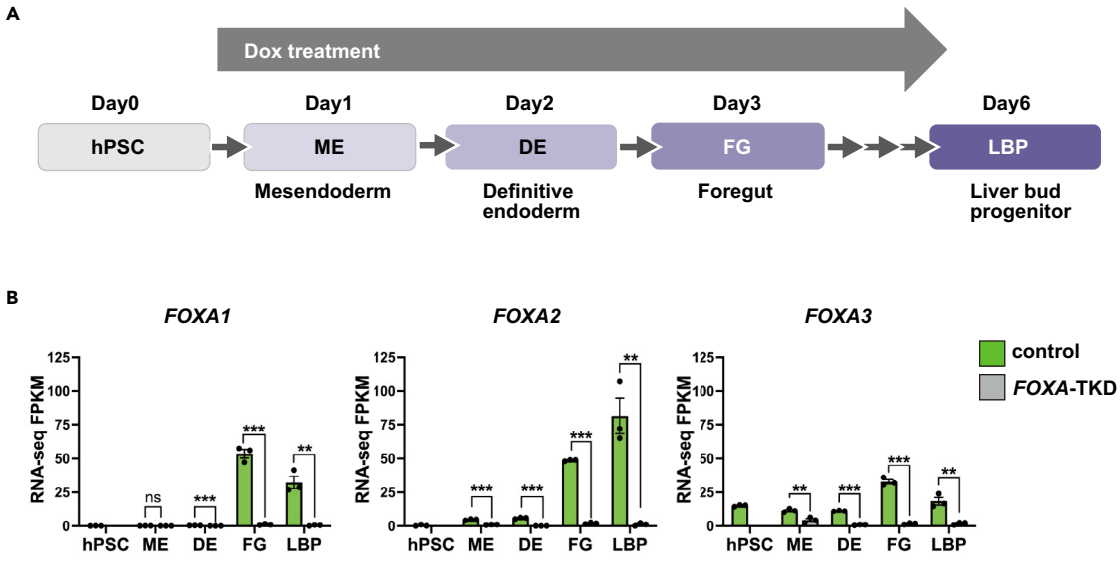

Figure 9. Examples of FOXA-CRISPRi knockdown experiments in human endoderm differentiation

(A) Schematic of stepwise endoderm differentiation and Dox administration to induce *FOXA*1/2/3-CRISPRi knockdown.

(B) Validation of *FOXA*1/2/3-CRISPRi knockdown efficiency by RNA-seq during hPSC to endoderm differentiation (n = 3 replicates from 3 independent *FOXA*-CRISPRi, Means ± SEM, $^{**}p < 0.01$, $^{***}p < 0.001$ and ns = not significant based on multiple unpaired t-tests).

the original paper.[2] The original paper also mentioned that Dox-mediated dCas9-KRAB expression is dramatically decreased after 15 days of differentiation for cardiomyocytes from hPSCs,[2] potentially due to silencing of the tetracycline response element. To analyze a specific gene function in terminally differentiated cells, you may have to use a constitutively expressed dCas9-KRAB system (e.g., CAG-dCas9-KRAB), transduce the Dox-inducible dCas9-KRAB cassette to terminally differentiated cells, or replace the Kox1-KRAB domain with a stronger repressor, ZIM3-KRAB domain.[19–21] The ZIM3-KRAB version of the CRISPRi system led to improved knockdown in post-differentiated cardiomyocytes derived from hPSCs.[21]

The CRISPRi system takes 1–2 days to silence the expression of endogenous genes at the mRNA level. Therefore, for more rapid protein loss-of-function experiments, another system like the Auxin-inducible Degron system might be more appropriate.[22]

TROUBLESHOOTING

Problem 1

Poor recovery of drug-resistant cells following G418 selection. This could be caused by using a suboptimal 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 (for 16–24 h) and monitor mCherry expression (if using pAAVS1-Ndi-CRISPRi). The dCas9-KRAB expression kinetics in the Dox-on/off system differs between quantification methods (western blot vs. live mCherry fluorescence assays).[21] We recommend confirming dCas9-KRAB expression using a western blot assay.

Targeting efficiency is occasionally low despite having an 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 too high and discrete clones cannot be excised (step-by-step method details step 9).

Potential solution

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

Problem 3

Low efficiency or no DNA fragment integration through Golden-Gate cloning (step-by-step method details step 15).

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) doesn't work after the expiration date (i.e., 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 19).

Potential solution

Using a low concentration (less than 500 ng/uL) of lentivirus plasmid increases the volume of plasmid solution in the PEI transfection mix, reducing the 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 22).

Potential solution

Increase the amount of lentivirus used in transduction without affecting cell viability.

Problem 6

CRISPRi knockdown efficiency is suboptimal based on RT-qPCR and western blot assays (step-by-step method details step 28).

Potential solution

Validate mCherry expression (representing dCas9-KRAB) and EGFP expression (representing sgRNA expression) in the CRISPRi cells using a fluorescence microscope. Depending on the genomic loci of sgRNA-EGFP-lentivirus insertion, sgRNA expression may be silenced. Select clones that resist silencing upon differentiation. Additional RT-qPCR and western blot analysis should be performed to confirm gene and protein expression levels. Unhealthy hPSCs (e.g., many spontaneous differentiations) negatively affect Dox-inducible dCas9-KRAB expression (Figure 7). If a CRISPRi target gene has multiple transcription start sites (TSSs), confirm that the designed gRNA targets the specific TSS used in a cell type of interest (see step 14).

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 contacts, 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 have been deposited to Addgene.

Data and code availability

This study did not generate/analyze datasets and code.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2024.103221.

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AUTHOR CONTRIBUTIONS

Methodology, M.I., C.N.M., J.R.S., M.G., S.M., K.L., G.M., and Y.-C.H.; investigation, M.G., J.R.S., S.M., M.B., K.L., S.K., and G.M.; 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.

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