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Lab Resource: Genetically-Modified Multiple Cell Lines

Establishment of two homozygous CRISPR interference (CRISPRi) knock-in human induced pluripotent stem cell (hiPSC) lines for titratable endogenous gene repression

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A B S T R A C T

Using nuclease-deficient dead (d)Cas9 without enzymatic activity fused to transcriptional inhibitors (CRISPRi) allows for transcriptional interference and results in a powerful tool for the elucidation of developmental, homeostatic and disease mechanisms. We inserted dCas9KRAB (CRISPRi) cassette into the *AAVS1* locus of hiPSC lines, which resulted in homozygous knock-in with an otherwise unaltered genome. Expression of dCas9KRAB protein, pluripotency and the ability to differentiate into all three embryonic germ layers were validated. Furthermore, functional cardiomyocyte generation was tested. The hiPSC-CRISPRi cell lines offer a valuable tool for studying endogenous transcriptional repression with single and multiplexed possibilities in all human cell types.

1. Resource table

Unique stem cell line identifier	RUCDRi002-A-16 (CRISPRi), RUCDRi002-A-17 (CRISPRi2) https://hpscreg.eu/cell-line/RUCDRi002-A-16 https://hpscreg.eu/cell-line/RUCDRi002-A-17
Alternative name of stem cell line	TC-1133-CRISPRi, TC-1133-CRISPRi2
Institution	Institute of Pharmacology and Toxicology, University Medical Center Goettingen, Germany
Contact information of distributor	Laura C. Zelarayán, laura.zelarayan@med.uni-goettingen.de
Type of cell line	Human induced pluripotent stem cell (hiPSC)
Origin	Human induced pluripotent stem cell (hiPSC) (LhiPSC-GR1.1), Accession: CVCL_RL65 (https://commonfund.nih.gov/stemcells/lines)
Additional origin info	Age: N/A Sex: male Ethnicity if known: N/A
Cell Source	Umbilical Cord Blood Cell (CD34 +)

(*continued on next column*)

(*continued*)

Method of reprogramming	Non-integrating, episomal
Associated disease	No disease was diagnosed
Type of Genetic Modification	Transgene generation *(overall structure, resistance, reported),*
Associated disease	*N.A*
Gene/locus	Targeted transgenesis in AAVS1. Integration: CRISPR/dCas9KRAB/tdTomato, 19q13.3
Method of modification/site-specific nuclease used	Site-specific nuclease (SSN) CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	RNP
All genetic material introduced into the cells	HDR donor vectors: 1.) *pAAVS1-CAG-dCas9KRAB-T2A-tdTomato-EF1alpha-puro* and 2.) *pAAVS1-CAG-dCas9KRAB-T2A-tdTomato-WPRE*
Analysis of the nuclease-targeted allele status	Sequencing of the targeted allele and PCR for the untargeted allele
Method of the off-target nuclease activity surveillance	Targeted PCR/sequencing
Name of transgene	CRISPR/dCas9KRAB/tdTomato(/WPRE) EF1alpha-Puromycin (CRISPRi line)

(*continued on next page*)

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(*continued*)

Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	
Date archived/stock date	30th September 2020
Cell line repository/bank	The cell lines are stored in the Central Biobank of the University Medical Center Göttingen.
Ethical/GMO work approvals	Reference number: 10/9/15
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	Lenti-dCas9-KRAB-blast was a kind gift from Gary Hon's Lab (Addgene #89567).

1.1. Resource utility

Transcriptional interference by CRISPR/dCas9 system presents several advantages over genome editing strategy for loss-of-function studies including: dynamic, reversible and titratable transcriptional repression, along with reduced off-target effects (Gilbert et al., 2014). The generated hiPSC-CRISPRi lines are suitable for titrating targeted single and multiple gene repression along with cell tracing for basic and translational studies in iPSCs and differentiated cell types.

2. Resource details

Mammalian cell transcription can be modified by dCas9KRAB with high levels of precision without genetically altering the target sequence (Mandegar et al., 2016; Qi et al., 2013). We previously generated a CRISPR/dCas9-hiPSC-based system for enhancing endogenous gene expression (Schoger et al., 2020). Here we expanded the toolbox by generating hiPSC lines with a CRISPR-based system for titrating transcriptional repression. The CRISPR/Cas9-based genome editing approach was used to target the *AAVS1* human genomic locus of LhiPSC-GR1.1 (TC-1133) cells. A cassette containing dCas9KRAB fused to Krueppel-associated box (KRAB) repression domain along with either a previously described tdTomato (CRISPRi line) followed by a puromycin resistance under the control of the *EF1a* promoter or a tdTomato including the posttranscriptional regulatory element of the woodchuck hepatitis virus (WPRE) to boost the efficiency of transgene expression (Zufferey et al., 1999) (CRISPRi2) under the control of the *CAG* promoter was inserted (Fig. 1A).

Cells were selected by tdTomato expression and genotyped by PCR (Fig. 1B and Supplementary Fig. 1A, primer binding shown in Fig. 1A, **black** primers amplified only the wild-type (WT) allele; **green** primers amplified the inserted construct). Subsequently, two positive clones per line (CRISPRi #1 and #9; CRISPRi2 #5 and #7) were expanded, analysed and cryopreserved. DNA sequencing data corroborated both, correct and homozygous knock-in transgene integration in the *AAVS1* locus in all lines (Fig. 1C). Clones that were positive for an intact WT locus were used as electroporated control cells. The top five predicted genome-wide off-target sites were analyzed by PCR and sequenced. Control electroporated and non-electroporated (reference) lines were used for comparison. The analyzed sequences showed no editing event (Supplementary Fig. 1A). All lines tested negative for mycoplasma. SNP-based karyotyping demonstrated genomic integrity of CRISPRi/i2 as well as of control cells as compared to the reference line (Schoger et al., 2020) (Fig. 1D and Table 1). Cell growth and morphology were comparable to controls (Fig. 1Ei). Western blot and confocal microscopy corroborated expression of dCas9KRAB and tdTomato in CRISPRi/i2, respectively, as compared to control cells. Enhanced dCas9KRAB expression was confirmed in the CRISPRi2 line (Fig. 1Eii and E*iv*, n = 2 different passages). Pluripotency was assessed by immunofluorescence and Western blot to analyse the expression of stemness markers OCT4 and TRA1-60 (Fig. 1Eiii and *iv*), and by flow cytometry analysis which showed 97.46% OCT4 (CRISPRi) or 96.22% (CRISPRi2) and 92.14% TRA1-60 (CRISPRi) or 84.37% (CRISPRi2) positive cells (Fig. 1Ev and Table 1). Spontaneous differentiation capacity into all three germ layers was tested by formation of embryoid bodies (EBs) and directed differentiation in the CRISPRi/i2 and control lines. Immunofluorescence analysis confirmed expression of α-1-Feto-protein (AFP), β-III-Tubulin and α-Smooth Muscle Actin (ACTA2), supporting endodermal, ectodermal and mesodermal fate, respectively (Fig. 1F). Analysis of transcript levels showed expression of Paired Box 3 (*PAX3*) and Microtubule-Associated Protein 2 (*MAP2*) indicating ectodermal differentiation; T-box transcription factor T (*TBXT*) indicating mesodermal fate and α-Feto-Protein (*AFP*) indicative of endodermal differentiation (Supplementary Fig. 1B and Table 1). Furthermore, we investigated the suitability of the CRISPRi/i2 lines for generating cardiomyocytes by directed 2D differentiation which resulted in spontaneously beating cells (data not shown, available by the authors) with robust cardiac Troponin T (TNNT2) cardiomyocyte marker expression (Supplementary Fig. 1C). Finally, we validated the functionality of the CRISPRi/i2 lines by ascertaining the repression of *KLF15* expression. A combination of three (g)RNAs, which were designed to hybridize to the correct is 5́-upstream sequence of the *KLF15* transcriptional start site (TSS), was able to significantly repress transcription of *KLF15* in CRISPRi and stronger in CRISPRi2 line compared to their respective parental lines transduced with non-targeting (NT) gRNAs. Control cells did not show transcriptional repression independent of the transfected gRNA (Fig. 1G).

In summary, homozygous hiPSC-CRISPRi/i2 lines are pluripotent and can differentiate into all germ cell derivates as well as showed its potential for interference with endogenous gene transcription according to the dCas9KRAB expression levels. This represents an invaluable tool for *in vitro* human development studies and disease modelling.

3. Materials and methods

3.1. Cell lines and culture

The LiPSC-GR1.1 cell line and derivates (Lonza, Walkersville, USA) were cultured in Matrigel (Corning) coated flasks under standard conditions (37 °C, 5% CO_2; BD Biosciences) and daily media changes with StemMACS iPS-Brew XF (Milteny Biotec). Cells were passaged with Versene 1:5000 (Gibco) and resuspended in StemMACS iPS-Brew XF (Milteny Biotec) supplemented with 5 μmol/L ROCK inhibitor Y27632 (Stemgent).

3.2. Molecular cloning of donor plasmid

From a previously published dCas9VPR construct (Schoger et al., 2020), the VPR transactivator domain was exchanged with the KRAB domain (ZNF10 KRAB domain) from Lenti-dCas9-KRAB-blast (a gift from Gary Hon's Lab) by InFusion HD cloning (TAKARA) using NheI and MluI (both Thermo Fisher Scientific) sites. The dCas9KRAB-2A-tdTomato construct was cloned into a previously described AAVS1-donor construct (Schoger et al., 2020) using FseI (NEB) and SmiI (Thermo Fisher Scientific) sites via InFusion HD cloning. For CRISPRi2, the bGH-polyA-EF1a-Puro cassette was removed by PacI and KspAI (both Thermo Fisher Scientific) and the WPRE site was amplified from a pGIPZ construct (Horizon Discovery) and integrated. Primers are listed in Table 2.

3.3. Gene targeting and clonal selection

Alt-R S.p. HiFi Cas9, tracrRNA and crRNA were used for targeting the AAVS1 locus (5′ GGGGCCACUAGGGACAGGAUGUUUUAGAGCUAUGCU 3′) (IDT Integrated DNA Technologies) together with pAAVS1-CAG-dCas9KRAB-2A-tdTomato-EF1alpha-Puro or pAAVS1-CAG-dCas9KRAB-2A-tdTomato-WPRE template constructs were electroporated into hLiPSC-GR1.1 with Neon Transfection System (Thermo Fisher Scientific) according to manufacturer's protocol with 2 pulses à 1000 V for 30 ms.

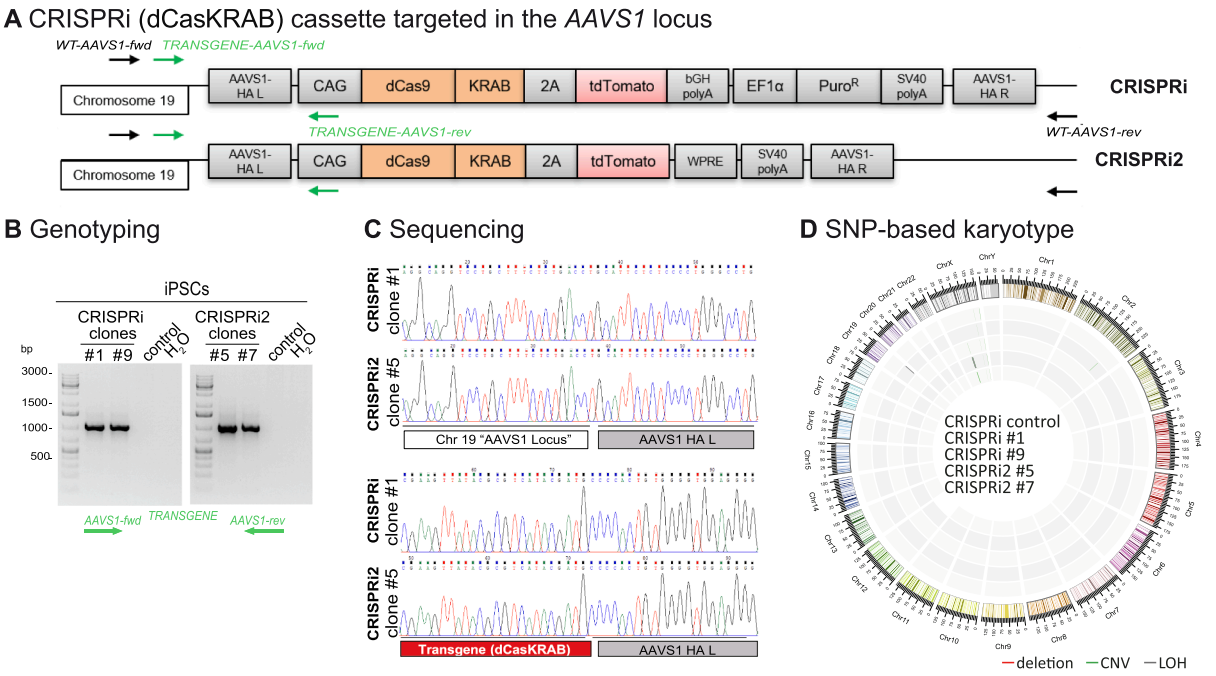
A CRISPRi (dCasKRAB) cassette targeted in the AAVS1 locus
WT-AAVS1-fwd
TRANSGENE-AAVS1-fwd
Chromosome 19
AAVS1-HA L
CAG
dCas9
KRAB
2A
tdTomato
bGH polyA
EF1α
PuroR
SV40 polyA
AAVS1-HA R
CRISPRi
TRANSGENE-AAVS1-rev
WT-AAVS1-rev
WPRE
CRISPRi2
B Genotyping
iPSCs
CRISPRi clones
CRISPRi2 clones
#1 #9
#5 #7
control
H2O
bp
3000-
1500-
1000-
500-
AAVS1-fwd
TRANSGENE
AAVS1-rev
C Sequencing
CRISPRi clone #1
CRISPRi2 clone #5
Chr 19 "AAVS1 Locus"
AAVS1 HA L
Transgene (dCasKRAB)
D SNP-based karyotype
CRISPRi control
CRISPRi #1
CRISPRi #9
CRISPRi2 #5
CRISPRi2 #7
deletion
CNV
LOH
E Stemness & transgene expression
control
CRISPRi #1
CRISPRi2 #5
brightfield
tdTomato
TRA1-60 OCT4 Hoechst
100 µm
controls
ctr#1 ctr#3
kDa
140-
50-
40-
35-
Cas9
OCT4
GAPDH
IgG control - CRISPRi
CRISPRi #1
IgG control - CRISPRi2
CRISPRi2 #5
TRA1-60 APC
OCT4 FITC
F Germ layer differentiation
AFP Hoechst
βIII-Tubulin Hoechst
ACTA2 Hoechst
Immunofluorescence
Control
CRISPRi
CRISPRi2
G Transcriptional repression
KLF15
gRNA3
gRNA2
gRNA 1
TSS
-197 bp
-175 bp
-44 bp
relative expression
control gRNAs
KLF15 gRNAs
**
control
CRISPRi
CRISPRi2

Fig. 1.

Table 1

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1 panel E
Phenotype	Immunocytochemistry	Assess staining of pluripotency markers OCT4, TRA1-60	Fig. 1 panel E
	Flow cytometry	Assess antigen levels & cell surface markers OCT4 = 97.5% (CRISPRi), = 96.2% (CRISPRi2) TRA1-60 = 92.1% (CRISPRi), = 84.4% (CRISPRi2)	Fig. 1 panel E
Genotype	SNP-based human microarray	Normal (Copy number events were reported if larger than 3.5 $\times$ 10^5 bps and 1 $\times$ 10^6 bps for loss of heterozygosity)	Fig. 1 panel D and data available with authors
Identity	Microsatellite PCR (mPCR)	not performed	
	STR analysis	16 loci were tested with AmpFLSTR Identifiler Plus PCR Amplification Kit; 100% matched	Submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	Homozygous, insertion	Fig. 1 panel B and C (Heterozygous insertion, data available with the authors)
	Southern Blot OR WGS	not performed	
Microbiology and virology	Mycoplasma	MycoALERT PLUS Mycoplasma Detection Kit (Lonza) Ratios < 1 were considered mycoplasma free. Tested by bioluminescence Result: negative	not shown, available with author
Differentiation potential	Embryoid body formation OR Teratoma formation OR Scorecard OR Directed differentiation	Embryoid body formation, expression of AFP, α-SMA and βIII-Tubulin Tri-lineage directed differentiation, expression of PAX3, MAP2, TBXT, AFP	Fig. 1 panel F and Supplementary Fig. 1 panel B and C
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	
	HLA tissue typing	N/A	

3.4. Karyotyping

SNP-based human microarray was performed with genomic DNA (QIAamp DNA Mini kit (Qiagen)) using the Infinium Global Screening Array-24 v3.0 BeadChip and the iScan array scanner (Illumina). Digital karyotypes were analyzed in GenomeStudio v2.0 software (Illumina) using the CNVpartition 3.2.0 algorithm with default settings. Copy number events were reported if larger than 3.5 $\times$ 10^5 bps and 1 $\times$ 10^6 bps for loss of heterozygosity.

3.5. Genotyping and sequence analysis

Genomic DNA was isolated and genotyping was performed with RedExtract-N-Amp Tissue PCR Kit (Sigma Aldrich). PCR products were sequenced at Seqlab Goettingen. Primers are listed in Table 2.

3.6. RNA isolation and qPCR analysis

RNA was isolated extracted with TRIzol (Thermo Fisher Scientific) following manufacturer's protocol. Reverse transcription was performed with Random Hexamers dNTPs and M−MLV reverse transcriptase (all Promega). RT-PCR and quantitative PCR were performed using Takyon ROX SYBR 2x Master Mix dTTP Blue (Eurogentec) with 1/5 diluted cDNA input on a 7900 HT Real Time Cycler (Applied Biosystems). Gene expression levels were determined based on standard curve comparison and normalization to *TBP* expression. Primers are listed in Table 2. RT-PCRs were performed with 30 cycles for PAX3, MAP2, TBXT and dCas9KRAB and with 32 cycles for AFP and TATA-binding protein (TBP).

3.7. Cell authentication/ STR analysis

Sample identification analysis was performed by PCR-single-locus-technology. 16 independent PCR-systems D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51 were investigated with AmpFLSTR Identifiler Plus PCR Amplification Kit.

3.8. Directed germ layer, embryoid body (EB) and cardiomyocytes differentiation

Ectoderm and endoderm commitment was tested with STEMdiff Trilineage Differentiation Kit (StemCell Technologies) according to supplyer's protocol for 7 days. Mesoderm differentiation was performed in basal medium (RPMI 1640 + GlutaMAX, 2% B27, 200 μmol/L L-ascorbic acid, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin) and 9 ng/mL Activin A (Bio-Techne), 1 μmol/L CHIR99021 (Merck Chemicals GmbH), 5 ng/ml BMP4 (Bio-Techne) and 5 ng/mL FGF (Preprotech) for 3 days followed by cardiac differentiation in basal medium supplemented with 5 μmol/L IWP4 (ReproCELL Europe Ltd.) for 9 days as described before (Tiburcy et al., 2017, Circulation, doi:10.1161/CIRCULATIONAHA.116.024145). Cardiomyocytes were metabolically selected for 5 days (RPMI 1640, no glucose, 2.2 mmol/L Na-lactate, 100 μmol/L β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin). For EB formation, 5 $\times$ 10^4 iPSCs were combined with 2.5 $\times$ 10^4 mouse embryonic fibroblasts on a 96-well plate in hES medium (DMEM-F12, 15% KnockOut Serum Replacement, 1 $\times$ MEM Non-Essential Amino Acids (Thermo Fisher Scientific), 50 μmol/L β-mercaptoethanol (Serva Electrophoresis) and 2 μM Thiazovivin (Merck Millipore)). Plates were centrifuged for 5 min at 250 $\times$ g and co-cultures were grown in suspension. At day 2, medium was changed to differentiation medium (IMDM with Glutamax, 20% Fetal Bovine Serum (Thermo Fisher Scientific), 1 $\times$ MEM Non-Essential Amino Acids Solution and 450 μmol/L 1-Thioglycerol (Sigma-Aldrich)) for further 6 days. Medium was changed every other day. At day 8, EBs were plated onto 0.1% gelatin-coated coverslips and cultured for up to one month in differentiation medium with medium changes every other day.

Table 2

Antibodies used for immunocytochemistry/flow-cytometry/immunoblotting

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:50	Abcam, Cat# 19857, RRID: AB_445175
	Mouse anti-TRA1-60-DyLight 650	1:50	Thermo Fisher Scientific, Cat# MA1-023-D650, RRID: AB_2536702
Differentiation Markers	Mouse anti- ACTA2	1:3,000	Sigma-Aldrich, Cat# A2547, RRID: AB_476701
	Rabbit anti-AFP	1:100	Agilent, Cat# A0008, RRID: AB_2650473
	Mouse anti-β-III-Tubulin	1:2,000	Covance, Cat# MMS-435P, RRID: AB_2313773
	Rabbit anti-TNNT2	1:200	Abcam, Cat# ab45932, RRID: AB_956386
Secondary antibodies	Goat anti-rabbit IgG-Alexa Fluor®488	1:250	Thermo Fisher Scientific, Cat# A11008, RRID: AB_143165
	Donkey anti-rabbit IgG	1:200	Thermo Fisher Scientific
	Alexa Fluor 555		Cat# A-31572, RRID: AB_162543
	Donkey anti-mouse IgG	1:200	Thermo Fisher Scientific
	Alexa Fluor 488		Cat# A-21202, RRID: AB_141607
Primary antibodies for immunoblots	Rabbit anti-Cas9	1:2,000	Diagenode, Cat# C15310258, RRID: AB_2715516
	Mouse anti-GAPDH	1:10,000	Proteintech, Cat# 60004–1-Ig, RRID: AB_2107436
Secondary antibodies for immunoblots	Rabbit anti-mouse-HRP	1:10,000	Dako, Cat# P0260, RRID: AB_2636929
	Goat anti-rabbit-HRP	1:5,000	Dako, Cat# P0448, RRID: RRID: AB_2617138T
Control antibodies	Normal rabbit IgG	1:50	EMD Millipore, Cat# 12–370, RRID: AB_145841
	Normal mouse IgG	1:20	Santa Cruz, Cat# sc-2025, RRID: AB_737182
	Rabbit anti-mouse IgG-Alexa Fluor®633	1:250	Thermo Fisher Scientific, Cat# A-21052, RRID: AB_2535719

Primers

	Target	Forward/Reverse primer (5'-3')
Differentiation marker	*PAX3-fwd*	AGAAGCCGAACCACCTTCAC
	PAX3-rev	GGGTTGGAAGGAATCGTGCT
	MAP2-fwd	CCACCTAGAATTAAGGATCA
	MAP2-rev	GGCTTACTTTGCTTCTCTGA
	TBXT-fwd	AATTGGTCCAGCCTTGGAAT
	TBXT-rev	CGTTGCTCACAGACCACA
	AFP-fwd	ACTCCAGTAAACCCTGGTGTTG
	AFP-rev	GAAATCTGCAATGACAGCCTCA
Normalizing gene	*TBP-fwd*	GCACAGGAGCCAAGAGTGAA
	TBP-rev	TTGTTGGTGGGTGAGCACAA
RT qPCR	*dCas9KRAB fwd*	TCAGTCAATTACGGGGCTCT
	dCas9KRAB rev	CCCTGGTGAAGTCCACAAAC
	KLF15-fwd	AGCAAGGACTTGGATGCCTG
	KLF15-rev	AGGGCAGGTTCAAGTTGGAG
DNA genotyping & sequencing	*WT-AAVS1-fwd*	CGGAACTCTGCCCTCTAACG
	WT-AAVS1-rev	ATCCTCTCTGGCTCCATCGT
	TRANSGENE-AAVS1-fwd	CCGGACCACTTTGAGCTCTA
	TRANSGENE-AAVS1-rev	GGCTATGAACTAATGACCCCG
Cloning primers	*InFusion MluI-KRAB fwd*	CGTCGACTTGACGCGTCTGCGGCCGCTGGATCCGAT
	InFusion NheI-KRAB rev	CTCTGCCCTCGCTAGCTCCACTGCCTGTACA AACTTTGCGT
	AAVS1-InFusion-fwd	GACCGGTTCTATTGGCTCTAGAGGATCGAA
	AAVS1-InFusion-rev	CCGGTCCAGCCATTTCGATTTACTTGTACA
	AAVS1-WPRE fwd	AGCAATCGATTTAATTAACAATTGAATCAACCTCTGGATTACAAAATTTG
	AAVS1-WPRE rev	CTTAATCGATGAATTAATTCCAGGCGGGGAG
	pGIPZ-MCS fwd	TCGAGGATATCATTTAAGGTACCTAAGCATTAATTAAGC
	pGIPZ-MCS rev	GGCCGCTTAATTAATGCTTAGGTACCTTAAATGATATCC
gRNA oligonucleotides	*KLF15-1-fwd*	CACCGCGCCGCGAAGGCTCGCAGG
	KLF15-1-rev	AAACCCTGCGAGCCTTCGCGGCGC
	KLF15-2-fwd	CACCGCGTGCGCGTCTGGCAGCTC
	KLF15-2-rev	AAACGAGCTGCCAGACGCGCACGC
	KLF15-3-fwd	CACCGGACCAGGCAGCGTGTTGGG
	KLF15-3-rev	AAACCCCAACACGCTGCCTGGTCC
	CT1-fwd	CACCGTCCAGCGGATAGAATGGCG
	CT1-rev	AAACCGCCATTCTATCCGCTGGAC
	CT2-fwd	CACCGGAGCGGTTTTGGATATTAG
	CT2-rev	AAACCTAATATCCAAAACCGCTCC
	CT3-fwd	CACCGTATGAGCGCGATGAAGGTG
	CT3-rev	AAACCACCTTCATCGCGCTCATAC
Off-target analysis	*Off-target-1-fwd*	TGAAGAAACAACCCGTTTCC
	Off-target-1-rev	TTCCCAGGAAACGATGAGAC
	Off-target-2-fwd	CCCTTGCTGAAGATCACACA
	Off-target-2-rev	CGTATGTTGCCCCCTACACT
	Off-target-3-fwd	GGCACAGAAGCATGAAGTGA
	Off-target-3-rev	CCTCCAGGTGCTGCTTACTC
	Off-target-4-fwd	TTTTCCCAGGAAACGATGAG
	Off-target-4-rev	GCTCCCAGCTCTCCCTAAGT
	Off-target-5-fwd	ATCAGCAGGGCCACTAGAGA
	Off-target-5-rev	AGCAAAGCTCCTCAAACCAA

3.9. Immunoblotting

Proteins were extracted and quantified using ROTI Quant (Carl Roth). Proteins were seperated on SDS-PAGE, transferred to ROTI PVDF membranes (Carl Roth), blocked in 5% milk and incubated with primary and secondary antibodies (Table 2). Membranes were analyzed using a ChemiDoc MP Imaging System (BioRad).

3.10. Immunocytochemistry

Cells were fixed with ROTI Histofix 4% (Carl Roth), permeabilized (PBS with 0.2% BSA, 0.3% Triton X-100), blocked (PBS with 5% BSA, 0.1% Triton X-100) and incubated with primary and secondary antibodies (PBS with 2% BSA, 0.1% Triton X-100) (Table 2). Secondary antibodies alone served as signal controls. Nuclei were counterstained with 10 μg/ml Hoechst. Images were acquired with a Zeiss LSM710 confocal microscope and ZEN software (Zeiss).

3.11. Flow cytometry

Cells were fixed with ice-cold 70% ethanol, blocked, permeabilized and incubated with primary or suitable IgG antibodies (all in PBS with 5% FCS, 1% BSA and 0.5% Triton X-100) and, if applicable, secondary antibodies (Table 2). Nuclei were counterstained with 10 μg/ml Hoechst. Cells were counted with a LSRII Flow Cytometer and FACSDiva software (BD Biosciences).

3.12. CRISPRi-mediated repression

Oligonucleotides for *KLF15* 5′ TSS targeting gRNAs or non-targeted gRNA (NT) (both sequences listed in Table 2) were cloned into a triple gRNA expression construct (TRISPR) as described before (Schoger et al., 2019 Circ Res, doi: 10.1161/CIRCRESAHA.118.314522). The lentiviral transfer vector pGIPZ (Horizon Discovery) was modified by integration of a multiple cloning site (see table 2) using NotI and XhoI (both Thermo Fisher Scientific) sites. The gRNA expression cassette was cloned into the modified pGIPZ vector using KpnI and XhoI (both Thermo Fisher Scientific) sites. For lentiviral gRNA delivery, pGIPZ transfer vectors, pMD2.G and psPAX2 (a gift from Didier Trono's lab) were transfected with Turbofect (Thermo Fisher Scientific) into HEK293T (TAKARA) cells. Media (DMEM, 5% FCS and 100 U/mL penicillin, 100 μg/mL streptomycin (all Gibco)) was exchanged daily and lentiviral particles were harvested 48–96 h post-transfection. $5x10^5$ iPSC-CM were transduced with 1 mL lentiviral particle supernatant and cultured for 5 days. Quantitative PCRs were performed in replicates (n = 3–6) and gene repression was indicated as fold reduction compared to cells transfected with non-targeting (NT) gRNAs.

3.13. Off-target analysis

Genome-wide (hg38/GRCh38) mismatch based off-target prediction was performed using "Off-Spotter" (Pliatsika, V, and Rigoutsos, I, 2015, Biol. Direct 201510.1186/s13062-015-0035-z) "Off-Spotter: very fast and exhaustive enumeration of genomic lookalikes for designing CRISPR/Cas guide RNAs" Biol. Direct 10(1):4). For *AAVS1* gRNA, no off-target site was retrieved perfectly matching the employed gRNAs, only one off-target with two mismatches was predicted (Top 1) and additional off-targets with 3 or more mismatches were identified. The top 5 loci were selected, and flanking primers were designed for PCR amplification and Sanger sequencing. Off-target primers are listed in Table 2.

4. Statistics

Statistical analyses were performed with GraphPad Prism 8. Shapiro-Wilk test was employed to check for normal distribution and one-way ANOVA with Bonferroni correction was used for multiple group comparisons. Statistical significance was assumed if $p < 0.05$.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

STR analysis. Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2021.102473.

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