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Lab Resource: Genetically-Modified Single Cell Line

Establishment of a second generation homozygous CRISPRa human induced pluripotent stem cell (hiPSC) line for enhanced levels of endogenous gene activation

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A B S T R A C T

CRISPR/Cas9 technology based on nuclease inactive dCas9 and fused to the heterotrimeric VPR transcriptional activator is a powerful tool to enhance endogenous transcription by targeting defined genomic loci. We generated homozygous human induced pluripotent stem cell (hiPSC) lines carrying dCas9 fused to VPR along with a WPRE element at the *AAVS1* locus (CRISPRa2). We demonstrated pluripotency, genomic integrity and differentiation potential into all three germ layers. CRISPRa2 cells showed increased transgene expression and higher transcriptional induction in hiPSC-derived cardiomyocytes compared to a previously described CRISPRa line. Both lines allow studying endogenous transcriptional modulation with lower and higher transcript abundance.

1. Resource table

Unique stem cell line identifier	RUCDRi002-A-15 (CRISPRa2) https://hpscreg.eu/cell-line/RUCDRi002-A-15
Alternative name of stem cell line	TC-1133-CRISPRa2
Institution	Institute of Pharmacology and Toxicology, University Medical Center Goettingen, Germany
Contact information of distributor	Laura C. Zelarayán, laura.zelarayan@med.uni-goettingen.de Eric Schoger, eric.schoger@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/dCas9VPR/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 vector *pAAVS1-CAG-dCas9VPR-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/dCas9VPR/tdTomato/WPRE
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A

(*continued on next page*)

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(*continued*)

Date archived/stock date	30th September 2020
Cell line repository/bank	Our cell line is 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)	N/A

2. Resource utility

The generated hiPSC-CRISPRa2 line was modified to allow for higher levels of dCas9VPR transgene expression as compared to previous described hiPSC-CRISPRa system. This modification results in increased transcriptional induction expanding the titrability of gene enhancement an can be applied to hiPSC-derived cells.

3. Resource details

Several systems, based on modified nuclease inactive dCas9, have been developed. These systems allow transcriptional modulation with high levels of precision without altering the genetic information (Mandegar et al., 2016; Qi et al., 2013). We previously generated a hiPSC-CRISPRa-based system for enhancing single and multiplexed endogenous gene expression (Schoger et al., 2020a). In order to expand CRISPR/Cas9 functions for transcriptional activation, we generated hiPSC-CRISPRa lines with increased activation efficiency.

A cassette containing dCas9 fused to heterotrimeric VPR transactivator consisting of VP64, p65, and RTA domains along with a tdTomato including the posttranscriptional regulatory element of woodchuck hepatitis virus (WPRE), to boost the transgene expression (Zufferey et al., 1999), under the control of the *CAG* promoter was inserted in the *AAVS1* human genomic locus of LhiPSC-GR1.1 (TC-1133) cells (Fig. 1A). Cells were selected by tdTomato expression and genotyped by PCR (Fig. 1B, primer binding shown in Fig. 1A, **black** primers amplified only the wild-type (WT) fragment; **green** primers amplified the inserted construct-*AAVS1* locus interface). Subsequently, two positive clones (CRISPRa2 #8 and #11) 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 carried an unmodified WT locus were used as electroporated control (ctrl) cells. The top five predicted off-targets were analyzed by PCR and subsequent Sanger sequencing (Supplementary Figure 1A). Control electroporated and non-electroporated (Schoger et al., 2020a) 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 CRISPRa2 as well as of control cells as compared to the reference line (Schoger et al., 2020a) (Fig. 1D and Table 1). Cell growth and morphology were comparable to controls (Fig. 1Ei). Confocal microscopy (Fig. 1Eii) and Western blot (Fig. 1Eiv) corroborated expression of tdTomato and dCas9 in CRISPRa2, respectively. CRISPRa2 showed higher transgene expression compared to previously described CRISPRa hiPSCs (Schoger et al., 2020a) (n = 3 different passages). Pluripotency was assessed by immunofluorescence to analyze the expression of stemness marker OCT4 and TRA1-60 (Fig. 1Eiii). Flow cytometry analysis confirmed 90% OCT4 and 99% TRA1-60 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 of CRISPRa2 lines. Immunofluorescence analysis confirmed expression of α-1-Feto-protein (AFP), β-III-Tubulin and α-Smooth Muscle Actin (ACTA2), further supporting endodermal,

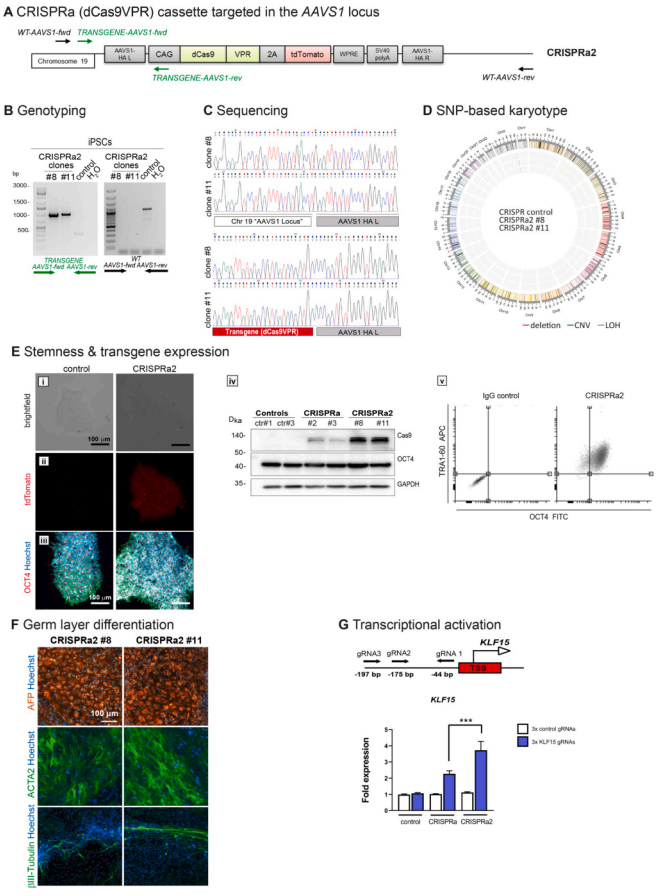

Fig. 1.

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 CRISPRa2 lines for generating cardiomyocytes by directed monolayer differentiation which resulted in spontaneously beating cells (data available by the authors) with robust α -Actinin 2 (ACTN2) and cardiac Troponin T (TNNT2) cardiomyocyte marker expression (Supplementary Fig. 1C). Finally, we validated the functionality of the CRISPRa2 cell lines by ascertaining the activation of *KLF15* expression. Three gRNAs were designed to the 5′ upstream region of *KLF15,* cloned into the corresponding vector for packaging into lentiviral particles and transduced side-by-side into CRISPRa (Schoger et al., 2020a), CRISPRa2, and control iPSC cardiomyocytes. *KLF15* transcriptional activation was observed in CRISPRa and CRISPRa2, compared to their respective parental lines transduced with a non-targeting (NT) gRNA. Importantly, gene activation was significantly more potent in CRISPRa2 than the induction achieved by the CRISPRa line (Schoger et al., 2020a). Control cells did not show activation independent of the gRNAs transduced (Fig. 1G).

In summary, homozygous hiPSC-CRISPRa2 lines are pluripotent and can differentiate into all germ cell derivates. As proof-of-concept for the control of endogenous gene transcription targeting of the *KLF15* locus was demonstrated with higher transcript abundance compared to the previous hiPSC-CRISPRa version (Schoger et al., 2020a). Controlling gene transcription in iPSC derivatives offers attractive possibilities for

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 = 90% TRA1-60 = 99%	Fig. 1 panel E
Genotype	SNP-based human microarray	Normal (Copy number events were reported if larger than 3.5×10^5 bps and 1×10^6 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, but 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. 1B 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	

mechanistic studies and target validation.

4. Materials and methods

4.1. Cell lines and culture

Induced pluripotent stem cells (LiPSC-GR1.1 and derivates (Lonza, Wakersville, USA) were cultured on Matrigel (Corning) coated flasks in StemMACS iPS-Brew XF (Milteny Biotec) with daily media changes. Versene 1:5000 (Gibco) was used for passaging and cells were resuspended in StemMACS iPS-Brew XF with 5 μmol/L ROCK inhibitor Y27632 (Stemgent).

4.2. Molecular cloning of donor plasmid

The original pAAVS1-CAG-dCas9VPR-T2A-tdTomato sequence was reported before (Qi et al., 2013). The CRISPRa2 construct was derived by removal of the bGH-polyA-EF1a-Puro cassette with PacI and KspAI (both Thermo Fisher Scientific) and the WPRE site was amplified from a pGIPZ construct (Horizon Discovery). Primers are listed in Table 2.

4.3. Gene targeting and clonal selection

Alt-R S.p. HiFi Cas9, tracrRNA and crRNA were used to target the AAVS1 safe harbor locus (5′ GGGGCCACUAGGGACAGGAUGUUUUAGAGCUAUGCU 3′) (IDT Integrated DNA Technologies) and electroporated together with the HDR donor plasmid into LiPSC-GR1.1 with a Neon Transfection System (Thermo Fisher Scientific).

4.4. Karyotyping

SNP-based human microarray using genomic DNA (QIAamp DNA Mini kit (Qiagen)) was performed with 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) with the CNVpartition 3.2.0 algorithm and default settings. Copy number events were reported if larger than 3.5×10^5 bps and 1×10^6 bps for loss of heterozygosity.

4.5. Genotyping and sequence analysis

RedExtract-N-Amp Tissue PCR Kit (Sigma Aldrich) was used for extraction and genotyping of iPSC clones. PCR products were Sanger-sequenced at Seqlab Goettingen. Primers are listed in Table 2.

4.6. RNA isolation and qPCR analysis

Total RNA was extracted with TRIzol reagent (Thermo Fisher Scientific). Complementary DNA was synthesized with Random Hexamers, dNTPs and M-MLV reverse transcriptase (all Promega). Takyon ROX SYBR 2x Master Mix dTTP Blue (Eurogentec) was used and reactions were performed on a 7900 HT Real Time Cycler (Applied Biosystems). Relative gene expression levels were determined based on standard curves comparisons and normalization to *TBP* expression. Primers are listed in Table 2. RT-PCR reactions were performed with 30 cycles for *PAX3, MAP2, TBXT, AFP* and dCas9VPR and with 32 cycles for *AFP* and *TBP*.

4.7. Cell authentication/ STR analysis

PCR-single-locus-technology was used for cell line authentication. 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.

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

Directed germ line differentiation into ectodermal and endodermal cells was performed using the STEMdiff Trilineage Differentiation Kit (StemCell Technologies). Mesodermal cells were differentiated in basal medium (RPMI 1640 + GlutaMAX, 2% B27 supplement 200 μmol/L L-ascaorbic acid, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin) supplemented with 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); subseqeuntly, cardiomyocytes

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
	Goat anti-mouse IgG-Alexa Fluor®488	1:250	Thermo Fisher Scientific, Cat# A11029, RRID: AB_138404
	Donkey anti-rabbit IgG Alexa Fluor 555	1:200	Thermo Fisher Scientific Cat# A-31572, RRID: AB_162543
	Donkey anti-mouse IgG Alexa Fluor 488	1:200	Thermo Fisher Scientific 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_2617138
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	*dCas9VPR-fwd*	GCCTCGCACCAACACCAAC	
	dCas9VPR-rev	GCCTCTTCCTTCTGGGGAATCAC	
	KLF15-fwd	TGCGCCAAGTTCAGCCGC	
	KLF15-rev	GCGTGGCCTGGGACAATAGG	
DNA genotyping & sequencing	*WT-AAVS1-fwd*	CGGAACTCTGCCCTCTAACG	
	WT-AAVS1-rev	ATCCTCTCTGGCTCCATCGT	
	TRANSGENE-AAVS1-fwd	CCGGACCACTTTGAGCTCTA	
	TRANSGENE-AAVS1-rev	GGCTATGAACTAATGACCCCG	
Cloning primers	*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	

were differentiated in basal medium and 5 μmol/L IWP4 (ReproCELL) as described before (https://doi.org/10.1161/CIRCULATIONAHA.116.024145). Cardiomyocytes were selected in RPMI, no glucose, 2.2 mmol/L Na-lactate, 100 μmol/L β-mercaptoethanol, 100 U/mL penicillin, 100 μg/mL streptomycin. EBs were generated by mixing $5{\cdot}10^4$ iPSC and $2.5{\cdot}10^4$ mouse embryonic fibroblasts in a 96-well plate in hES medium (DMEM-F12, 15% KnockOut Serum Replacement, 1x MEM Non-Essential Amino Acids (Thermo Fisher Scientific), 50 μmol/L β-mercaptoethanol (Serva Electrophoresis) and 2 μmol/L Thiazovivin (Merck Millipore)). Cells were pelleted for 5 min at 250 xg and EBs were grown in suspension. From day 2 onwards, EBs were cultured in differentiation medium (IMDM with GlutaMAX, 20% Fetal Bovine Serum, 1x MEM Non-Essential Amino Acids (Thermo Fisher Scientific), 450 μmol/L 1-Thioglycerol (Sigma Aldrich)) until day 6. EBs were plated onto 0.1% gelatin-coated coverslips at day 8 and cultured for up to one month in differentiation medium with media changes every other day.

4.9. Immunoblotting

Protein lysates were quantified with ROTI Quant (Carl Roth). Proteins were separated by SDS-PAGE and transferred onto ROTI PVDF membranes (Carl Roth). Membranes were blocked and incubated with primary and secondary HRP-coupled antibodies in 5% milk. Membranes were exposed to femtoLUCENT Plus-HRP (G-Biosciences) and images were taken with a ChemiDoc MP Imaging System (Bio-Rad). Antibodies are listed in Table 2.

4.10. Immunocytochemistry

Cells were fixed with ROTI Histofix (Carl Roth), permeabilized in PBS, 0.2% BSA, 0.3% Triton X-100 and blocked in PBS, 5% BSA, 0.3% Triton X-100 before incubation with antibodies diluted in PBS, 0.2% BSA, 0.3% Triton X-100. Nuclei were stained with 10 μg/ml Hoechst (Thermo Fisher Scientific). Antibodies are listed in Table 2.

4.11. Flow cytometry

Cells were fixed in ice-cold 70% ethanol and incubated with antbodies diluted in (PBS, 5% FCS, 1% BSA, 0.5% Triton X-100). Corresponding host organism IgGs served as control. Nuclei were stained with 10 μg/ml Hoechst (Thermo Fisher Scientific). Samples were analysed with a LSRII Flow Cytometer (BD Biosciences). Antibodies are listed in Table 2.

4.12. CRISPRa-mediated gene activation

KLF15 gRNAs targeted to the *KLF15* promoter region or non-targeted gRNAs (NT) encoding oligonucleotides were cloned into triple gRNA expression vectors (TRISPR) as described before (Schoger et al., 2020b). Guide RNA expression cassette was transferred into a modified pGIPZ (Horzion Discovery) construct for lentiviral particle generation in HEK293T cells transfected with pMD2.G, psPAX2 (both a gift from Didier Trono's Lab) and respective gRNA plasmid using TurboFect (Thermo Fisher Scientific). Lentiviral supernatants were used to transduce iPSC-cardiomyocytes and cells were cultured for 5 days post-transduction before proceeding with further analyses. Transcriptional changes are reported as fold-changes compared to corresponding cells transduced with NT gRNAs. Oligonucleotide sequences are listed in Table 2.

4.13. Off-target analysis

Mismatch-based off-target prediction was conducted for all gRNAs using "Off-Spotter" (https://doi.org/10.1186/s13062-015-0035-z). Top 5 predicted AAVS1 gRNA off-target sites (2–3 mismatches compared to on-target site sequence) were selected for examination of unintended edits. Off-target site primers are listed in Table 2.

4.14. Statistics

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

Acknowledgements

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