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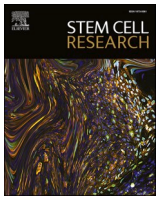

Human induced pluripotent stem cells for live cell cycle monitoring and endogenous gene activation

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ABSTRACT

The fluorescence ubiquitination cell cycle inhibitor (FUCCI) has been introduced to monitor cell cycle activity in living cells, including human induced pluripotent stem cells (hiPSC) and derived cell types. We have recently developed hiPSC with stable expression of dCas9VPR for endogenous gene activation and a Citrine-tagged ACTN2 cell line to monitor sarcomere development and function in muscle cells. Here, we present dual and triple transgenic hiPSC lines developed by genomic integration of FUCCI with and without dCas9VPR into the ROSA26 and AAVS1 loci, respectively, in the previously introduced ACTN2-Citrine line. Functionality of the transgenes was demonstrated in the novel hiPSC line, which we introduce as Myo-CCER and CraCCER.

1. Resource table

Unique stem cell line identifier	RUCDRi002-A-73 (CraCCER) RUCDRi002-A-74 (Myo-CCER)
	https://hpscreg.eu/cell-line/RUCDRi002-A-73https://hpscreg.eu/cell-line/RUCDRi002-A-74
Alternative name(s) of stem cell line	Myo-CCER CraCCER
Institution	Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Germany
Contact information of the reported cell line distributor	Eric Schoger
	eric.schoger@med.uni-goettingen.de
	Laura C. Zelarayán
	laura.zelarayan@med.uni-goettingen.de

(*continued on next column*)

(*continued*)

Type of cell line	Human induced pluripotent stem cell (hiPSC)
Origin	human
Additional origin info	Sex: male
Cell Source	Human CD34 + umbilical cord blood cell RUCDRi002-A-3 (Haertter et al. under revision)
Method of reprogramming	Non-integrating, episomal
Clonality	Monoclonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Previously reported by Baghbaderani et al. Stem Cell Rep. 2015 and confirmed by Whole Genome Sequencing conducted by Institute of Human Genetics, University Medical Center Göttingen

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(*continued*)

The cell culture system used	Feeder-free and serum-free culture conditions with Matrigel (growth factor reduced, BD Biosciences) and StemMACS iPS-Brew XF medium (Miltenyi Biotec)
Type of the Genetic Modification	Targeted integration of transgenes into human ROSA26 and human AAVS1 loci
Associated disease	N/A
Gene/locus	*ROSA26*, 3p25.4; *AAVS1*, 19q13.3
Method of modification / user-customisable nuclease (UCN) used, the resource used for design optimisation	CRISPR/Cas9 IDT DNA
User-customisable nuclease (UCN) delivery method	Nucleofection with RNP
All double-stranded DNA genetic material molecules introduced into the cells	Plasmids: pROSA26-CAG-FUCCI4.1; pAAVS1-CAG-dCas9VPR (Fig. 1A)
Analysis of the nuclease-targeted allele status	PCR & Sanger sequencing of the transgene at the targeted locus (Fig. 1B and C)
Method of the off-target nuclease activity prediction and surveillance	PCR & Sanger sequencing of the top 5 Off-targets (Supplementary Figure. 1A)
Descriptive name of the transgene	FUCCI4.1 and dCas9VPR
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	-
Inducible/constitutive expression system details	Constitutive expression under the ubiquitous CAG promoter
Date archived/stock creation date	18th March 2024
Cell line repository/bank	These cell lines are stored in the Central Bio-bank of the University Medical Center Göttingen: https://hpscreg.eu/cell-line/RUCDRi002-A-73 and https://hpscreg.eu/cell-line/RUCDRi002-A-74
Ethical/GMO work approvals	Reference number: 10/9/15
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

2. Resource utility

For (cardiac) muscle regeneration, it is essential to understand the regulatory mechanism governing cell proliferation to identify pro-proliferative factors and inhibitors of aberrant proliferation. To comprehend the endogenous mechanisms of cell cycle induction, it is therefore necessary to gain further insight into gene networks that control cell cycle activity. This is particularly important for adult cardiac myocytes, which have a limited regenerative capacity. This cell line combines a fluorescent cell cycle indicator (FUCCI) (Sakaue-Sawano et al., 2008, Murganti et al., 2022, Kasamoto et al., 2023), re-engineered to be used in combination with a CRISPR/dCas9-based system for endogenous gene activation (CRISPRa) (Schoger et al. Stem Cell Res. 2021) in a hiPSC line expressing a fluorescently tagged sarcomere component (ACTN2-Citrine) for live-cell fluorescence imaging of cell cycle progression in myocytes (RUCDRi002-A-3). The presented cell lines (RUCDRi002-A-73 and RUCDRi002-A-74) can be obtained for non-commercial use by third parties using appropriate material transfer agreements (MTA). See (Table 1).

3. Resource details

A modified FUCCI expression construct (Sakaue-Sawano et al., 2008) under CAG promoter control was integrated into the ROSA26 locus on human chromosome 3 by CRISPR/Cas9 homology directed repair in the TC1133-ACTN2-Citrine (RUCDRi002-A-3) hiPSC line (Myocyte Cell Cycle Reporter = Myo-CCER). Subsequently, we integrated a CAG-driven dCas9VPR expression cassette into the AAVS1 locus resulting in a triple transgenic hiPSC line (CRISPRa Cell Cycle Reporter = CraCCER). The targeted loci and integrated transgene expression cassettes for both hiPSC lines are schematically summarized in Fig. 1A. Successful integration into both loci (heterozygous: FUCCI, homozygous: dCas9VPR) was confirmed by PCRs amplifying the interfaces of the inserted constructs and the endogenous loci or the wild-type loci in Myo-CCER and CraCCER hiPSC clones, respectively, (Fig. 1B) followed by Sanger sequencing (Fig. 1C). SNP-based karyotyping validated genomic integrity of Myo-CCER and CraCCER cells as compared to the parental reference line (Fig. 1D). We evaluated off-target editing/integrations and checked the top 5 predicted off-target sites (Pliatsika and Rigoutsos, 2015) by PCR and Sanger sequencing without detecting unintended editing events in both Myo-CCER and CraCCER cells, respectively (Supplementary Fig. 1A). Stem cell properties were maintained as evaluated by immunofluorescence microscopy (Fig. 1E) and flow cytometry with over 93 % and 94 % OCT4 positive and 92 % and 95 % TRA-1-60 positive cells, for both Myo-CCER and CraCCER cells, respectively (Supplementary Fig. 1B). Expression of the cell cycle sensor was confirmed by fluorescence microscopy and faithfully indicated distinct cell cycle phases (G1: mCherry+, S: mCherry+/ECFP+, G2: ECFP+) exemplified in Myo-CCER hiPSC (Fig. 1F). Expression of dCas9VPR in CraCCER cells was confirmed by immunoblotting. We also validated endogenous gene activation by enhancing expression of *KLF15* as previously demonstrated (Schoger et al. 2021 Stem Cell Res.) upon gRNA delivery compared to controls (NT gRNA=non-targeted gRNA) confirming the capacity of these cells to be used for CRISPRa applications (Fig. 1G). Differentiation into the three germ layers was confirmed for both cell lines, Myo-CCER and CraCCER, by directed differentiation into endodermal, mesodermal and ectodermal lineages followed by immunofluorescence microscopy (FOXA2, ACTN2, and PAX6, respectively). Additionally, Citrine-tagged ACTN2 was detected in differentiated hiPSC-cardiomyocytes (Fig. 1H). Absence of mycoplasma contamination (Supplementary Fig. 1C) and random plasmid integration (Supplementary Fig. 1D) was checked by PCRs in both, Myo-CCER and CraCCER, hiPSC lines. In summary, we developed transgenic cell cycle indicator cell lines (FUCCI), which can be used in combination with CRISPR/dCas9-based single or multiplexed endogenous gene expression control (dCas9VPR) in a fully characterized hiPSC line and differentiated cells including bona fide myocytes (ACTN2-Citrine positive cells).

4. Material & methods

4.1. Cell lines and culture

Human induced pluripotent stem cells were cultured on Matrigel (Corning) coated plates or flasks in StemMACS Brew (Milteny Biotech) as described previously (Tiburcy et al., 2017). Cells were dissociated with Versene (Gibco) and replated in StemMACS Brew supplemented with 5 μmol/L ROCK inhibitor Y-27632 (Stemgent). Cells were checked for mycoplasma (Supplementary Fig. 1C) using the VenorGeM Advance kit (Minerva Biolabs).

4.2. Molecular cloning

The FUCCI expression cassette was PCR amplified using ES-FUCCI, a gift from Pierre Neveu (Addgene plasmid #62451; https://n2t.net/addgene:62451; RRID: Addgene_62451), as a template. The Citrine fluorescent protein was replaced by PCR amplification of ECFP from pcDNA3.1-CMV-CFP;UBC-Cre25nt, which was a gift from Jacco van Rheenen (Addgene plasmid #65727; https://n2t.net/addgene:65727; RRID: Addgene_65727). The CDT1-mCherry and GMNN-ECFP sequences were

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Morphology in bright-field microscopy showed typical hiPSC features	Fig. 1F
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Immunocytochemistry: OCT4, TRA-1-60	Fig. 1E
	Quantitative analysis (Flow Cytometry)	Pluripotency is tested regularly by flow cytometry: >92 % of positive cells for TRA-1-60 and OCT4 expression.	Supplementary Fig. 1B
Karyotype	Digital Karyotyping	46XY, no difference from parental line (TC1133)	Fig. 1D
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	The generated hiPSC lines showed a heterozygous insertion of the *FUCCI* transgene in the ROSA26 locus and a homozygous integration of dCas9VPR in the AAVS1 locus	Fig. 1B
	Evaluation of the − (homo-/hetero-/hemi-) zygous status of introduced genomic alteration (s) by Sanger sequencing	Sanger sequencing of PCR products confirmed heterozygous integration of the *FUCCI* transgene in the ROSA26 locus and a homozygous integration of dCas9VPR in the AAVS1 locus	Fig. 1C
	Transgene-specific PCR (when applicable)	PCR primers targeting transgene specific features were used and confirmed transgene integration. Functionality of transgenes was confirmed	Fig. 1B, 1C, 1F and G
Verification of the absence of random plasmid integration events	PCR	Backbone plasmid PCR showed no evidence of random integration of FUCCI4.1 or dCas9VPR	Supplementary Fig. 1D
Parental and modified cell line genetic identity evidence	STR analysis	DNA Profiling 16 loci (D8S1179, D21S11, D7S820, CSF1PO, D3S1358, TH01, D13S317, D16S539, D2S1338, AMEL, D5S818, FGA, D19S433, vWA, TPOX and D18S51) were tested with AmpFLSTR Identifiler Plus PCR Amplification Kit; 100 % matched	Submitted to journal archive
Mutagenesis / genetic modification outcome analysis	Genomic DNA PCR product with subsequent Sanger sequencing	PCRs amplified the expected region. Transgene and integration amplicons were Sanger sequenced and confirmed integration.	Fig. 1B and C
	PCR-based analyses	N/A	N/A
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	N/A	N/A
Off-target nuclease activity analysis	PCR across top 5 predicted top likely off-target sites, Sanger sequencing	Top 5 off-target PCR amplicons showed correct base pair size, subsequent Sanger sequencing showed no mutations (comparison to parental RUCDRI002-A cell line).	Supplementary Fig. 1A
Specific pathogen-free status	Mycoplasma	Mycoplasma test was negative. (Venor GeM Advance, Minerva Biolabs)	Supplementary Fig. 1C
Multilineage differentiation potential	Directed differentiation	The hiPSC lines were differentiated into 1.) endoderm, 2.) mesoderm (cardiomyocytes), 3.) ectoderm and evaluated by 1. FOXA2, 2. ACTN2, 3. PAX6 (immuno-) fluorescence microscopy	Fig. 1H
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype − additional histocompatibility info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

then cloned to be expressed in tandem divided by a T2A self-cleaving peptide sequence and a GSG linker (FUCCI4.1). The pROSA26 vector, derived from pAAVS1 (Schoger et al. 2021 Stem Cell Res.) by exchanging the AAVS1 homology regions for PCR amplified human ROSA26 homology regions using the following primers: human ROSA26 HR-R fwd: TAAGCAGGTACCATCGGCTCTGGCGCGGG, human ROSA26 HR-R rev: TGCTTAACGCGTTAGCCCCTTAAGCCTAGGCA and human ROSA26 HR-L fwd: TAAGCATGTACAACTTGGTCCCTGGGCGTTG, human ROSA26 HR-L rev: TGCTTAATGCATGAACGTGAACTAGGGAG-GAAT as well as MluI and KpnI or Bsp1407I and NsiI restriction sites (Thermo Fisher Scientific), was used to clone pROSA26-CAG-FUCCI-4.1. The pAAVS1-CAG-dCas9VPR vector was generated as previously described (Schoger et al. 2021 Stem Cell Res.) by omitting the tdTomato reporter cassette.

4.3. Cell line generation and clonal expansion

Nucleofections were performed with the Amaxa 4D Nucleofector and P3 Primary Cell 4D-Nucleofector X Kit L (Lonza), Alt-R S.p. HiFi Cas9, Alt-R CRISPR/Cas9 tracrRNA (IDTDNA), and 2 μg of plasmid DNA in subsequent reactions. The Alt-R CRISPR/Cas9 crRNAs (IDTDNA) used for the integration of transgenes are summarized in Table 2. Nucleofected hiPSC were singularized for clonal expansion.

4.4. Digital karyotyping

SNP-based human microarray using genomic DNA was performed. The gDNA was sent to the Life & Brain GmbH for Illumina Bead Array analysis or digital karyotyping and the result was analyzed by Genome Studio v2.0 (Illumina). Copy number variations higher than $3.5*10^5$ bp and loss of heterozygosity higher than $1*10^6$ bp were considered significant.

4.5. Genotyping and sanger sequencing

DNA was isolated with the NucleoSpin Tissue Kit (Macherey Nagel). PCRs were performed with CloneAmp HiFi PCR Premix (Takara Bio) and primers are listed in Table 2. Sanger sequencing was performed by Microsynth Seqlab GmbH. Possible random integration of donor plasmid DNA was checked by PCR targeting the F1 origin of replication of the transfer plasmids (Supplementary Fig. 1D).

4.6. RNA isolation and RT-qPCR analysis

RNA was isolated from transfected hiPSC using the NucleoSpin RNA Kit (Macherey Nagel) according to manufacturer's protocol. For cDNA synthesis, 1 μg of total RNA was transcribed using MMLV-Transverse transcriptase (Promega), dNTP (Promega), and Oligo dT (20xT) (Eurofins genomics). Takyon ROX SYBR 2X MasterMix dTTP blue (Eurogentec) was used to perform qPCRs on an Applied Biosystems 7900HT Fast Real-Time PCR System (Applied Biosystems).

4.7. Cell line 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 checked with the AmpFLSTR Identifiler Plus PCR Amplification Kit.

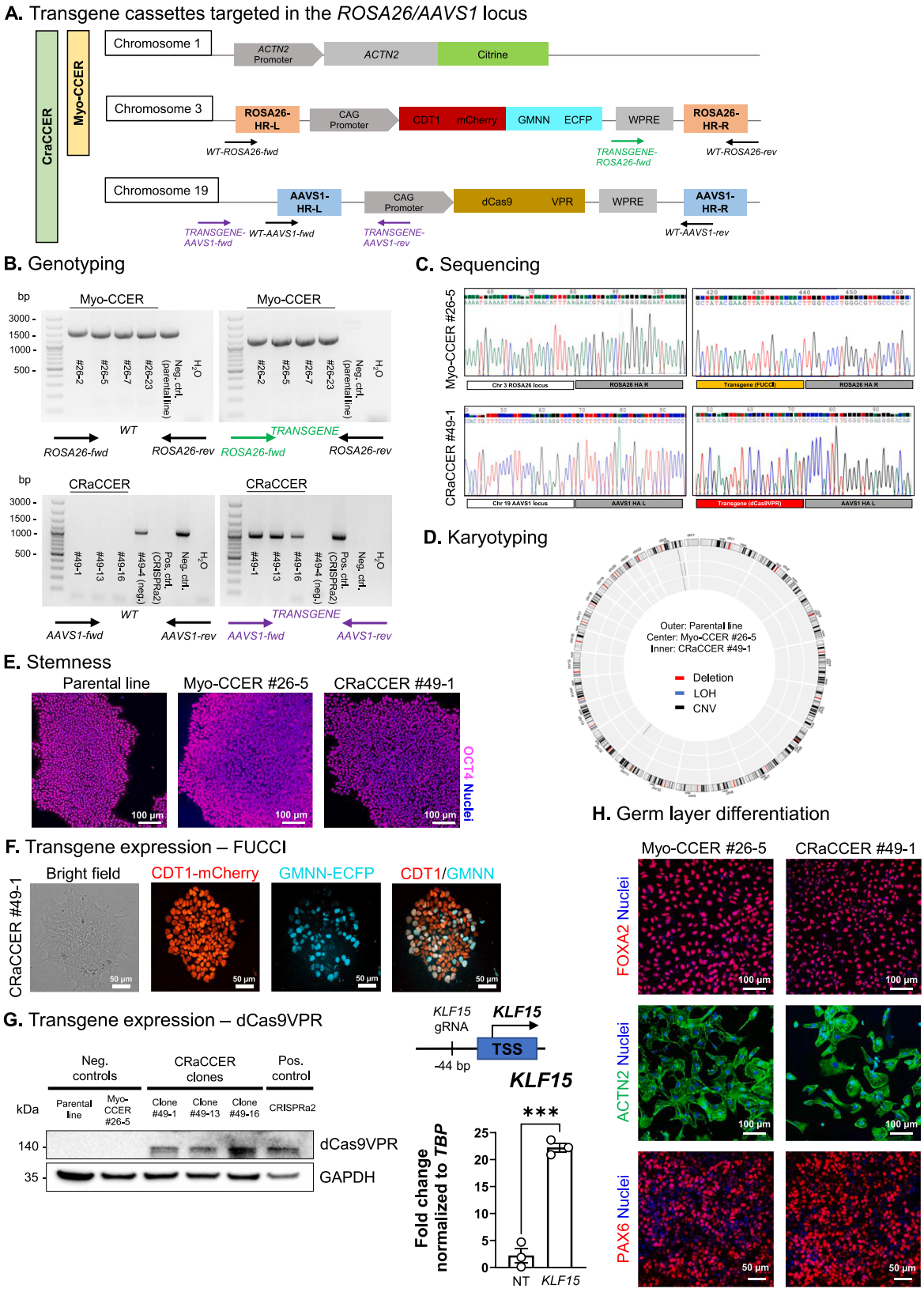

Fig. 1. Generation and characterization of Myo-CCER and CraCCER hiPSC lines.

Table 2
Reagents details.

Classification	Antibody	Dilution	Company # and RRID
Immunofluorescence	Rabbit anti-OCT4	1:50	Abcam, Cat# 19857, RRID: AB_445175
	Mouse anti-TRA-1–60	1:50	Thermo Fisher Scientific, Cat# MA1-023, RRID: AB_2536699
	PAX6-APC	1:50	Miltenyi, Cat# 130–107–829, RRID: AB_2653168
	Mouse anti-FOXA2	1:50	Santa Cruz Biotechnology, Cat# sc-101060, RRID: AB_1124660
	Goat anti-rabbit IgG-Alexa Fluor®633	1:400	Thermo Fisher Scientific, Cat# A-21070, RRID: AB_2535731
	Goat anti-mouse IgG-Alexa Fluor®633	1:400	Thermo Fisher Scientific, Cat# A-21052, RRID: AB_2535719
Immunoblotting	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
	Rabbit anti-mouse-HRP	1:10,000	Dako, Cat# P0260, RRID: AB_2636929
	Goat anti-rabbit-HRP	1:5,000	Dako, Cat# P0448, RRID: AB_2617138
	Primer and oligonucleotides	**Sequence (5′ − 3′)**	
crRNA	Alt-R CRISPR-Cas9 crRNA ROSA26	CUAGGCUUAAGGGGCUAACUGUUUUAGAGCUAUGCU	
	Alt-R CRISPR-Cas9 crRNA AAVS1	GGGGCCACUAGGGACAGGAUGUUUUAGAGCUAUGCU	
Transgene integration	ROSA26 fwd	GTTGGAGTAAAGCTCCTGTCA	
	ROSA26 rev	AGATGTTGCCTAACCTCAGATC	
	AAVS1 fwd	CGGAACTCTGCCCTCTAACG	
	AAVS1 rev	ATCCTCTCTGGCTCCATCGT	
	Transgene ROSA26 fwd	AGACGAGTCGGATCTCCCTT	
	Transgene AAVS1 fwd	CCGGACCACTTTGAGCTCTA	
	Transgene AAVS1 rev	GGCTATGAACTAATGACCCCG	
Random integration check	F1 ORI fwd	CCGAGATAGGGTTGAGTGTTGTTCCAG	
	F1 ORI rev	CCCTGTAGCGGCGCATTAAGCG	
qPCR	KLF15 fwd	AGCAAGGACTTGGATGCCTG	
	KLF15 rev	AGGGCAGGTTCAAGTTGGAG	
gRNA (CRISPRa)	KLF15 gRNA fwd	GCGCCGCGAAGGCTCGCAGG	
	KLF15 gRNA rev	CCTGCGAGCCTTCGCGGCGC	
	NT gRNA fwd	GTCCAGCGGATAGAATGGCG	
	NT gRNA rev	CGCCATTCTATCCGCTGGAC	
Off-target PCR	ROSA26 Off-target 1 fwd	GAGGGTGACATTCAGCTGGT	
	ROSA26 Off-target 1 rev	GTCTGGGACACCTGTGCATA	
	ROSA26 Off-target 2 fwd	TAATACTGACACCTCCGCCA	
	ROSA26 Off-target 2 rev	CAATTCAGAATCACCCGCTG	
	ROSA26 Off-target 3 fwd	CCTCAATCCACTGTCAAAGCA	
	ROSA26 Off-target 3 rev	TCCTATACAAGGTGCACTGG	
	ROSA26 Off-target 4 fwd	AATGCCTGTAGGTTGACCTG	
	ROSA26 Off-target 4 rev	GCATGCCTCTAGGCTTATCC	
	ROSA26 Off-target 5 fwd	GGAGTGCTTTCACCAGAAGAT	
	ROSA26 Off-target 5 rev	TATTCCTTAGAGCCAGATGGG	
	AAVS1 Off-target 1 fwd	TGAAGAAACAACCCGTTTCC	
	AAVS1 Off-target 1 rev	TTCCCAGGAAACGATGAGAC	
	AAVS1 Off-target 2 fwd	CCCTTGCTGAAGATCACACA	
	AAVS1 Off-target 2 rev	CGTATGTTGCCCCCTACACT	
	AAVS1 Off-target 3 fwd	GGCACAGAAGCATGAAGTGA	
	AAVS1 Off-target 3 rev	CCTCCAGGTGCTGCTTACTC	
	AAVS1 Off-target 4 fwd	TTTTCCCAGGAAACGATGAG	
	AAVS1 Off-target 4 rev	GCTCCCAGCTCTCCCTAAGT	
	AAVS1 Off-target 5 fwd	ATCAGCAGGGCCACTAGAGA	
	AAVS1 Off-target 5 rev	AGCAAAGCTCCTCAAACCAA	

4.8. Germ line and cardiomyocyte differentiation

Directed germline differentiation towards ectoderm was performed using the STEMdiff Neural Induction Medium (StemCell Technologies) according to manufacturer's protocol. For endoderm differentiation, the protocol described by Qiu et al. was utilized, and modified using B27 minus insulin instead of B27 minus vitamin A (Qiu et al., 2021). For mesoderm induction, base medium (RPMI 1640 + GlutaMAX, 2 % B27 minus insulin, 200 μmol/L L-ascorbic acid, 1 mmol/L Na-pyruvate, 100 U/mL penicillin, 100 μg/mL streptomycin) was used 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-2 (Peprotech) for 3 days. For the subsequent cardiomyocyte differentiation over 9 days, the base medium was supplemented with 5 μmol/L IWP4 (ReproCELL) and B27 supplement was used instead of B27 minus insulin (Tiburcy et al., 2017).

4.9. Immunocytochemistry

Cells were fixed with 4 % formaldehyde for 15 min at room temperature, followed by permeabilization with (0.2 % BSA, 0.3 % Triton X-100 in PBS), blocking (5 % BSA, 0.1 % Triton X-100 in PBS) and incubation with primary antibodies (Table 2) diluted in (2 % BSA, 0.1 % Triton X-100 in PBS) for 16 h at 4 °C. Cells were washed with PBS and incubated with secondary antibodies for 2 h at room temperature. Images were taken with a CQ1 System (Yokogawa).

4.10. Flow cytometry

Cells were fixed in ice-cold 70 % ethanol, permeabilized and blocked with Flow Buffer (5 % FCS, 1 % BSA, 0.5 % Triton X-100 in PBS). Antibodies (Table 2) were diluted in Flow Buffer and cells were incubated with the antibody mix for 1 h at room temperature. Nuclei were stained with Hoechst33342 (Thermo Fisher Scientific). Flow cytometry was performed with a LSRII Flow Cytometer and data analyzed FACSDiva software (BD Biosciences).

4.11. Immunoblotting

Cells were lysed in Lysis Buffer (350 mmol/L NaCl, 1 mmol/L $MgCl_2$, 20 mmol/L HEPES, 0.5 mmol/L EDTA, 0.1 mmol/L EGTA, 1 % Igepal CA-630, 20 % glycerol) and centrifuged for 20 min at 14,000 xg and

4 °C. 4x Loading Buffer (100 mmol/L TRIS-HCl, 5 % bromphenol blue, 8 % SDS, 10 % glycerol) was added and protein samples were denatured at 95 °C for 5 min. Electrophoresis was performed with Mini-PROTEAN TGX Precast Gels at 120 V for 1.5 h in Electrophoresis Buffer (25 mmol/L TRIS Base, 190 mmol/L glycine, 0.01 % SDS) and proteins transferred onto Nitrocellulose membranes in Transfer Buffer (25 mmol/L TRIS Base, 190 mmol/L glycine, 20 % methanol) at 100 V for 2 h. Membranes were blocked in 5 % non-fat dried milk diluted in TBST Buffer (20 mmol/L TRIS Base, 150 mmol/L NaCl, 0.1 % Tween) and incubated with primary antibodies for 16 h at 4 °C (Table 2). Membranes were washed with TBST and secondary antibodies (Table 2) were applied for 2 h at room temperature. Images were taken with a Gel Doc XR Gel Documentation System (Biorad).

4.12. CRISPR/dCas9VPR mediated gene activation

For CRISPR/dCas9VPR mediated gene activation, gRNAs were designed as previously described (Schoger et al. 2021 Stem Cell Res.). Cells were transfected with Lipofectamine Stem Reagent (Thermo Fisher Scientific) according to manufacturer's protocol. RNA was isolated two days post-transfection for transcript analyses by qPCR.

4.13. Off-target analysis

Possible off-target sites were identified using OFF-Spotter (Pliatsika and Rigoutsos, 2015) and PCR primers were designed flanking the identified genomic regions.

4.14. Statistics

GraphPad Prism 10.0 was used to perform statistical analyses. Gaussian normal distribution was confirmed by Shapiro-Wilk test. For two group comparisons, Student's *t*-test was used. Results were considered statistically significant if $p < 0.05$. Fig. 1G ***p-value < 0.001.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Eric Schoger reports financial support was provided by Deutsches Zentrum für Herz-und-Kreislauf-Forschung e.V. Eric Schoger reports financial support was provided by Deutsche Gesellschaft für Kardiologie – Herz- und Kreislaufforschung e.V. Norman Y. Liaw reports financial support was provided by Deutsches Zentrum für Herz-und-Kreislauf-Forschung e.V. Norman Y. Liaw reports financial support was provided by ADUMED Stiftung. Wolfram-H. Zimmermann reports financial support was provided by Deutsche Forschungsgemeinschaft. Wolfram-H. Zimmermann reports financial support was provided by Leducq Foundation. Wolfram-H. Zimmermann reports financial support was provided by Deutsches Zentrum für Herz-und-Kreislauf-Forschung e.V. Laura C. Zelarayan reports financial support was provided by Deutsche Forschungsgemeinschaft. Laura C. Zelarayan reports financial support was provided by Deutsches Zentrum für Herz-und-Kreislauf-Forschung e.V. Wolfram-H. Zimmermann reports a relationship with myriamed GmbH that includes: board membership and equity or stocks. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Rosa Kim: Writing – review & editing, Visualization, Validation, Methodology, Investigation. **Sebastian H. Nagel:** Writing – review & editing, Investigation. **Norman Y. Liaw:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Wolfram-H. Zimmermann:** Writing – review & editing, Resources, Funding acquisition, Conceptualization. **Laura C. Zelarayán:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Eric Schoger:** Writing – original draft, Validation, Supervision, Methodology, Investigation, Funding acquisition, Conceptualization.

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

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