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Lab Resource: Genetically-Modified Single Cell Line

Generation of a dual edited human induced pluripotent stem cell Myl7-GFP reporter line with inducible CRISPRi/dCas9

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A B S T R A C T

Temporal regulation of CRISPRi activity is critical for genetic screens. Here, we present an inducible CRISPRi platform enabling selection of iPSC-derived cardiomyocytes and reversible gene knockdown. We targeted a doxycycline-inducible dCas9-KRAB-mCherry cassette into the AAVS1 locus in an MYL7-mGFP reporter iPSC line. A clone with bi-allelic integration displayed minimally leaky CRISPRi activity and strong expression upon addition of doxycycline in iPSCs, iPSC-derived cardiomyocytes, and multilineage differentiated cells. The CRISPRi activity was validated by targeting the MYOCD gene in iPSC cardiomyocytes. In summary, we developed a robust inducible CRISPRi platform to interrogate gene function in human iPSC-derived cardiomyocytes and other cells.

Resource Table

Unique stem cell line identifier	SCVIi038-A
Alternative name(s) of stem cell line	AICS-0052-003-iKRABdCas9
Institution	Stanford University
Contact information of the reported cell line distributor	Ioannis Karakikes
Type of cell line	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 30–34 Sex: Male Ethnicity: Asian Limited clinical information: EKG
Cell Source	Skin fibroblasts
Method of reprogramming	Non-integrating, episomal
Clonality	Clonal
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	Matrigel-coated feeder-free culture, mTeSR1 media
Type of Genetic Modification	Transgene generation
Associated disease	N/A
Gene/locus	AAVS1 (OMIM 102699)/ 19q13
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	RNP
All genetic material introduced into the cells	pAAVS1-NDi-CRISPRi (Gen1) Plasmid #73497

(*continued on next column*)

Resource Table (*continued*)

Analysis of the nuclease-targeted allele status	PCR for WT allele and confirmation of integration by junction PCR and Sanger sequencing
Method of the off-target nuclease activity surveillance	In silico prediction and targeted PCR with Sanger sequencing
Name of transgene	CRISPRi/dCas9-KRAB
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	Positive (neomycin)
Inducible/constitutive system details	TET-On
Date archived/stock date	08/20/20
Cell line repository/bank	https://hpscreg.eu/user/cellline/edit/SCVIi038-A
Ethical/GMO work approvals	N/A
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pAAVS1-NDi-CRISPRi (Gen1) was a gift from Bruce Conklin (Addgene plasmid # 73497; https://n2t.net/addgene:73497; RRID: Addgene_73497) Dox-inducible CRISPR interference (CRISPRi) knock-in construct into the AAVS1 locus with mCherry marker.

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1. Resource utility

The dual edited Myl7-GFP/dCas9 iPSC line can be used for genetic screens in differentiated cardiomyocytes, and other iPSC-derivatives, providing a platform for systematic interrogation of normal and disease states in early fetal heart development.

2. Resource details

Inducible expression of dCas9 without silencing is crucial to

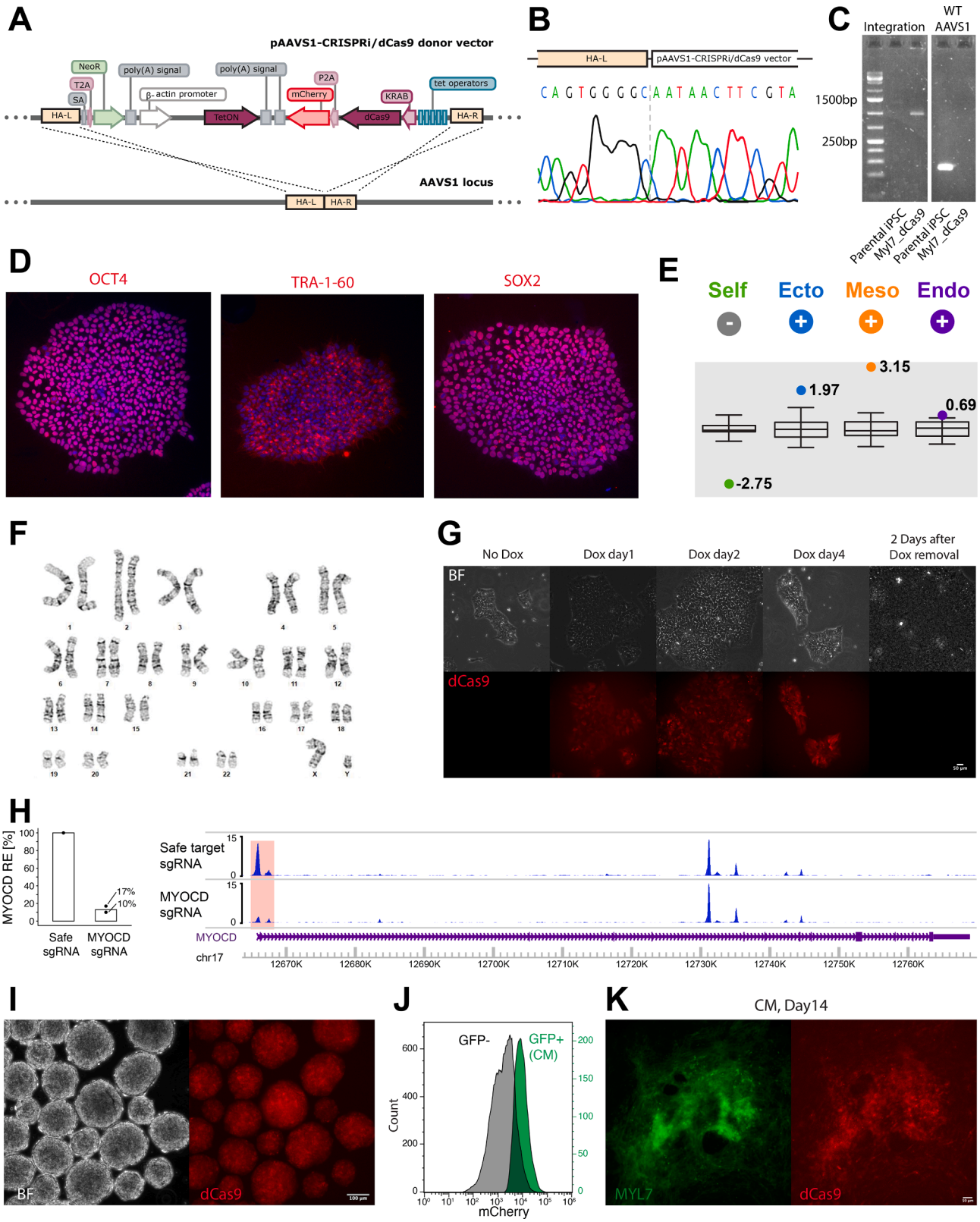

Fig. 1. Generation and characterization of inducible CRISPRi iPSCs.

successfully implementing CRISPR interference (CRISPRi) systems in functional genomics (Mandegar et al., 2016). Here, we describe the development of a robust inducible CRISPRi system in human induced pluripotent stem cells (CRISRPi-iPSCs) that displays efficient gene repression activity in undifferentiated iPSCs, and importantly, in post-differentiated cells, such as cardiomyocytes. This iPSC line is also endogenously tagged at the MYL7 locus with mEGFP, allowing for purification of iPSC-derived cardiomyocytes as well as live-cell microscopy of sarcomere structure and dynamics. The line has minimally leaky CRISPRi activity and strong, stable, homogenous induction of dCas9-KRAB expression upon addition of doxycycline both in iPSCs and in differentiated cells, allowing targeted but reversible gene knockdown.

To construct this line, the dCas9-KRAB construct (Addgene #73497) (Mandegar et al., 2016) was integrated at the AAVS1 'genomic safe harbor' locus (Fig. 1A-B) of the WTC11-MYL7eGFP iPSC line (AICS-0052 cl.3) by CRISPR/Cas9-mediated genome editing. The bi-allelic insertion of the transgene in the AAVS1 locus was verified by PCR amplification of the 5′ integration junction (1 kb) (Fig. 1C). Pluripotency was verified by immunostaining for OCT3/4, SOX2, and TRA1-60 (Fig. 1D), and the trilineage potential was confirmed by Scorecard assay at passage 42 (Fig. 1E; A15876, Thermofisher). The cells showed normal karyotype at passage 42 (Fig. 1F). To assess the CRISPRi activation and reversibility, the iPSCs were treated with 1ug/ml Doxycycline. We observed a robust mCherry signal at 24hr after induction. The mCherry signal persisted for four days and was undetectable two days after Doxycycline withdrawal, indicting tunable and reversible expression of the CRISPRi (Fig. 1G). We validated the CRISPRi activity by targeting the MYOCD locus (Table 1, Table 2) in iPSC-derived cardiomyocytes and performing qPCR analysis for relative expression and ATACseq on FACS purified GFP+ cells. Upon adding doxycycline from day three of differentiation, we observed a significant reduction in MYOCD relative expression (about 85%) and accessibility of the MYOCD locus at day 15 of post-differentiation, indicating that the CRISPRi activity is preserved in differentiated cardiomyocytes (Fig. 1H, left and right, respectively). We also observed homogeneous and robust expression of mCherry upon adding doxycycline during tri-lineage Embryoid bodies (EB) mediated differentiation of the CRISPRi-iPSCs, suggesting that dCas9-KRAB expression is not silenced in multiple differentiated cell lineages (Fig. 1I, Day 4 EBs). To verify that the construct was minimally silenced during differentiation and in differentiated cardiomyocytes, we use flow cytometry to sort out GFP+ and GFP- cells at day 15 of cardiomyocyte differentiation. We observed that the vast majority of the GFP+ population were also mCherry+ (Fig. 1J) and verified by fluorescence microscopy (Fig. 1K).

3. Materials and methods

3.1. Generation and maintenance of the iPSC line

The iPSCs were cultured in mTeSR1 (STEMCELL Technologies) on Matrigel (BD Biosciences) coated plates at 37 °C and 5%CO2/5%O2. For transgene insertion, 250,000 iPSCs were nucleofected (1200 V, 20 ms, 1 pulse) with 60 pmoles sgRNA (Synthego) targeting the AAVS1 locus, 20 pmoles SpCas9 nuclease (Synthego), and 1 μg CRISPRi plasmid using the Neon Transfection System (ThermoFisher Scientific) per the manufacturer's instructions. When cells reached 75% confluency, they were dissociated by DPBS-EDTA at 37 °C for 7–10 min and replated in mTeSR1 containing 5 μM Y-27632 (Selleckchem). For selection, the iPSCs were grown in the presence of 50 μg/ml G418 for five days, followed by single-cell seeding. The reversible expression of the dCas9 transgene was confirmed by the addition of 1 μg/ml Doxycycline Hyclate (Calbiochem) for four days and then removed for two days.

3.2. PCR and sequencing

Genomic DNA was extracted using Quick Extract solution (Lucigen)

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Typical primed pluripotent human stem cell morphology	Fig. 1G
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Positive for pluripotency markers: OCT4, TRA1-60, SOX2	Fig. 1D
Karyotype	Karyotype (Wicell)	Normal male karyotype (46, XY), no clonal abnormalities detected Resolution: 425–450 bands	Fig. 1F
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site or targeted allele-specific PCR	Homozygous integration of at the AAVS1 locus	Fig. 1B,C
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR/Southern	N/A	N/A
Parental and modified cell line genetic identity evidence	Verification of the mGFP-tag in the MYL7 locus of the parental line	PCR	N/A
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR or RT-PCR product)	Verified presence of integration in single edited allele and absence of integration in wild-type allele. Mono-allelic transgene insertion.	Fig. 1B
	PCR-based analyses	Detection of correctly targeted construct	Fig. 1C (Sample Lane 2)
	ATACseq	Verified knockdown of the targeted accessible peak in comparison to a Safe targeting guide	Fig. 1H
Off-target nuclease analysis-	PCR across top predicted likely off-target sites	PCR of predicted off-target site; Sanger sequencing	No off-target effect observed
Specific pathogen-free status	Mycoplasma testing by MycoAlert Detection Kit; passage 35	Negative	N/A
Multilineage differentiation potential	Embryoid body spontaneous differentiation; RNA isolation RNeasy kit (Qiagen); Taqman Scorecard (ThermoFisher Scientific)	Tri-lineage differentiation potential	Fig. 1I
Donor screening (OPTIONAL)	HIV1+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

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	**Dilution**	**Company Cat # and RRID**
Pluripotency Markers	Mouse anti-OCT3/4, mouse anti-TRA-1–60, rabbit anti-NANOG, mouse anti-SOX2	1:200	Santa Cruz Cat #SC-5279, Millipore Cat #MAB4360, Santa Cruz Cat #SC-33759, Cell Signaling Cat #4900S
Differentiation markers	N/A	N/A	N/A
Secondary antibodies	Goat Anti-Mouse IgG Alexa fluor 594, Goat Anti-Rabbit IgG Alexa fluor 488	1:800, 1:400	Invitrogen Cat #A11032, Invitrogen Cat #A11070
Nuclear stain	DAPI	1 drop	Invitrogen Cat #R37606
Site-specific nuclease			
Nuclease information	SpCas9	Synthego	
Delivery method	Nucleofection	Neon Transfection System (ThermoFisher)	
Selection/ enrichment strategy	50 μg/ml G418		

Primers and Oligonucleotides used in this study		
	Target	**Forward/Reverse primer (5′-3′)**
AAVS1 gRNA sequence	AAVS1 locus gRNA	TCCCTAGTGGCCCCACTGTG
Junction PCR	Integration at AAVS1 locus	Fw: TTGAGCTCTACTGGCTTCTGCGCRv: GCCCTGTGGGAGGAAGAGAAGAGG (1 kb amplicon)
WT allele	AAVS1 locus	Fw: CGGTTAATGTGGCTCTGGTTRv: AGGATCCTCTCTGGCTCCAT (250 bp amplicon)
Off-target locus	Chr22: 48335634–48335655	Fw: GGAGAGGAGAAGAGGATACAGAC Rv: TCCAGAAGCCTGCAGGCTGA
MYOCD qPCR primer	MYOCD	idtdna.com, PrimeTime primers, Assay ID: Hs.PT.58.23073756
ACTB qPCR primer	ACTB	Fw: TTCTACAATGAGCTGCGTGTG Rv: GGGGTGTTGAAGGTCTCAAA
sgRNA sequences	Safe guide gRNA	GGAAATTGCTTCTGGTTTAT
	MYOCD gRNA	GAGGTTGGCCAGGAGCAGCG

and PCR-amplified with GoTaq HotStart polymerase (Promega). Integration of the pAAVS1-CRISPRi vector at the AAVS1 locus was confirmed with vector-specific (within SA site) and AAVS1 locus-specific primers that amplified the 5′ integration junction (1 kb product). A second primer set (within HA-L and HA-R) did not amplify the WT AAVS1 junction spanning the cut site, indicating biallelic insertion. PCR cycling condition: 95 °C 2 min; 95 °C 15sec, 60 °C 15sec, 72 °C 1 min (40 cycles); 72 °C 1 min.

3.3. Immunostaining

The cells were fixed with 4% PFA for 10 min at 37 °C and then washed 3 times for 5 min with DPBS. The cells were then permeabilized in DPBS with 0.1% Triton for 10 min at room temperature, followed by blocking for 1 hr at room temperature with DPBS/0.1% Triton X/1% BSA. Cells were incubated with primary antibodies at 4 °C overnight. The cells were then washed 3 times for 5 min each with DPBS and incubated with a secondary antibody for 1 hr at room temperature. After washing 3 times for 5 min each, a drop of NucBlue was added to counterstain the DNA.

3.4. sgRNA transduction to iPSCs

Individual gRNAs were cloned by synthesizing the complementary sense and antisense strand oligos separately, annealing them, then ligating them into the recipient lentiviral plasmid, pMCB619 (Addgene 171011). Lentivirus was generated by transfection of the lentiviral vector (1000 ng) and packaging plasmids pCMV-dR8.91 (900 ng) and pCMV-VSV-G (100 ng) with 12 μL of polyethyleneimine (PEI, 1 mg/mL; Polysciences 24765–1) into HEK293T cells that had been grown to 60–80% confluence in 6-well plates. Total volume of media was 2 mL per transfection. About 48 hr after transfection, the cell medium containing virus was harvested in 0.5 mL aliquots, and filtered through a 0.45 um filter. The harvested virus was concentrated 10-fold using Lenti-X Concentrator (Takara Bio), following manufacturer's recommendations, and resuspended in PBS. iPSCs were kept growing in log-phase, plated in 6-well plates, then transduced with virus at 60–80% confluence. 48 hr after transduction, cells were selected with puromycin at 0.4 ug/mL until cells were at least 95% BFP+.

3.5. Validation of CRISPRi-mediated knockdown

Inducible dCas9-mCherry expression was validated by induction of 1ug/ml Doxycycline Hyclate (Calbiochem) followed by fluorescence microscopy capturing mCherry signal from the construct (Fig. 1G). Similarly, mCherry was captured by flow cytometry (Fig. 1J). Knockdown validation was performed by transducing iPSCs using a lentivirus expressing the sgRNAs for MYOCD and "safe" target (Negative control gRNAs that target non-functional, non-genic regions). The transduced iPSCs were then differentiated the into cardiomyocytes and inducing the cells with 1ug/ml Dox on days 3–15 of differentiation. The cells were harvested on day 15, sorted for GFP+ signal (MYL7 positive cells), and assayed for accessibility and mRNA expression (Corces et al., 2017).

3.6. Induction of CRISPRi in iPSC-derived cardiomyocytes

The iPSCs were differentiated to cardiomyocytes as previously described (Feyen et al., 2021). Briefly, at about 70% cell confluency, the culture media was changed to 3 mL RPMI with B27 supplement (without Insulin) with 6 μM CHIR90021. On days 1 and 2, 2 and 1 mL RPMI with B27 supplement (without Insulin) were added, receptively. On day 3 of differentiation, the media was changed to RPMI/B27 -Insulin with 3 μM IWP2. Media was refreshed every other day. After Day 9, the media was changed to RPMI/B27 with Insulin and refreshed every other day. On day 3 to 15, Dox was added to the differentiated cells, and then the cells were FACS sorted.

3.7. qPCR assay

Total RNA was harvested from day15 cardiomyocytes after four days of Glucose starvation (RPMI -Glucose supplemented with B27 +insulin, ThermoFisher 11879020, Gibco 17504044). Total RNA amounts from two biological repeats were then measured by Nanodrop (Thermo Scientific, NanoDrop 2000) and 5 ng total RNA was RT and quantitively assayed (Luna Universal One-Step RT-qPCR Kit, New England Biolabs, E3005S) using MYOCD and ACTB primers (see below) on a QuantStudio 3 qPCR machine (Applied Biosystems™). Ct values were extracted using Design & Analysis software (2.6) and transformed to Relative expression (RE) values following standard Comparative Ct analysis calculations.

3.8. Spontaneous differentiation

Embryoid bodies (EB) were formed using the Aggrewell-400 protocol according to the manufacturer's protocol (STEMCELL Technologies). Briefly, the iPSCs were dissociated with Gentle Cell Dissociation Reagent (100–0485, STEMCELL Technologies) and seeded into the Aggrewell 400 24-well plate pre-coated with the anti-adherence rinsing solution

(07010, STEMCELL Technologies) at a density of 1.2e6 cells/well in Aggrewell EB Formation Medium (05893, STEMCELL Technologies). After 24hr, half of the media was replaced with fresh Aggrewell EB Formation Medium. 48 hr after seeding, we harvested EBs and moved them to an ultra-low attachment 6-well plate (CLS3471-24EA, Corning) in TeSR™-E6 media (05946, STEMCELL Technologies). We maintained EBs in culture for 6 days, replacing media every other day.

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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2022.102754.

References

Mandegar, M.A., et al., 2016. CRISPR Interference Efficiently Induces Specific and Reversible Gene Silencing in Human iPSCs. Cell Stem Cell 18 (4), 541–553.
Corces, M.R., et al., 2017. An improved ATAC-seq protocol reduces background and enables interrogation of frozen tissues. Nat. Meth. 14 (10), 959–962.
Feyen, D.A.M., et al., 2021. Unfolded Protein Response as a Compensatory Mechanism and Potential Therapeutic Target in PLN R14del Cardiomyopathy. Circulation 144 (5), 382–392.