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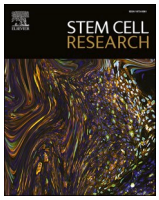

Lab Resource: Single Cell Line

Genome engineering of a neuronal specific, optogenetic, induced pluripotent stem cell line

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A B S T R A C T

Control of neuronal activity by optogenetic tools is increasingly explored in disease modelling and optogenetics and holds great promise for regenerative therapy. To investigate neuronal connectivity with other excitable cells we established an optogenetic induced pluripotent stem cell line. The Syn*f*Chrimson line harbors a stably integrated, fast, red light-activatable channel (f-Chrimson), under the control of *synapsin* promotor in the *AAVS1* locus. Multielectrode array analysis showed that Syn*f*Chrimson derived neurons are light-activatable. The specificity of the Syn*f*Chrimson function in neurons was validated by cardiomyocyte differentiations which do not respond to light stimulations.

1. Resource Table

Unique stem cell line identifier	RUCDRi002-A-71 https://hpscreg.eu/cell-line/RUCDRi002-A-71
Alternative name(s) of stem cell line	Syn*f*Chrimson
Institution	Institute of Pharmacology and Toxicology, University Medical Center Göttingen, Germany
Contact information of the reported cell line distributor	Maria-Patapia Zafeiriou patapia.zafeiriou@med.uni-goettingen.de Kea Aline Schmoll keaaline.schmoll@med.uni-goettingen.de
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
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. 2015 (https://doi.org/10.1016/j.stemcr.2015.08.015) and confirmed by Whole Genome Sequencing conducted by

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Unique stem cell line identifier	RUCDRi002-A-71 https://hpscreg.eu/cell-line/RUCDRi002-A-71
	Institute of Human Genetics, University Medical Center Göttingen / Repairon GmbH
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 transgene to a specific locus
Associated disease	N/A
Gene/locus	*AAVS1*, 19q13.3
Method of modification / user-customisable nuclease (UCN) used, the resource used for design optimisation	CRISPR/Cas9 IDT, Benchling, CRISPOR
User-customisable nuclease (UCN) delivery method	Nucleofection with RNP
All double-stranded DNA genetic material molecules introduced into the cells	Plasmid: pAAVS1-SYN*f*Chrimson (Fig. 1B)

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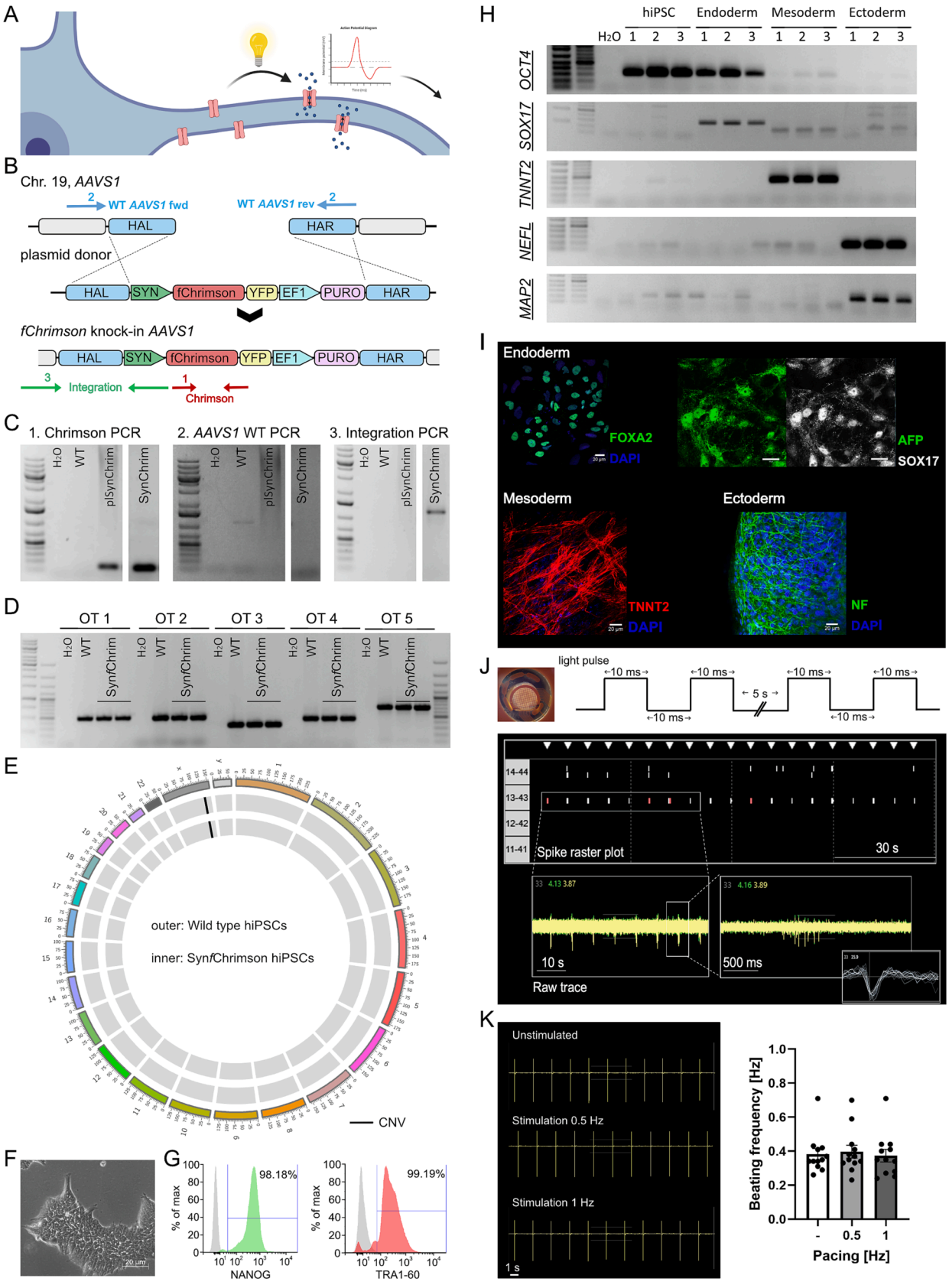

Fig. 1. Generation and characterization of Synfchrimson.

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Morphology in bright-field microscopy showed typical hiPSC features	Fig. 1 Panel F
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	N/A	N/A
	Quantitative analysis (Flow Cytometry)	Pluripotency is tested regularly by flow cytometry: >98 % of positive cells for NANOG, TRA1-60 and OCT3/4 expression.	Fig. 1G, Supplementary Fig. 1C
Karyotype	Digital Karyotyping	46XY, no difference from parental line (TC-1133)	Fig. 1E
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR across the edited site	The generated hiPSC line showed a homozygous insertion of the *SYNfChrimson* transgene in the AAVS1 locus	Fig. 1C, Supplementary Fig. 1A
	Evaluation of the - (homo-/hetero-/hemi-) zygous status of introduced genomic alteration (s) by Sanger sequencing	The generated hiPSC line showed a homozygous insertion of the *SYNfChrimson* transgene in the AAVS1 locus	Supplementary Fig. 1A
	Transgene-specific PCR (when applicable)	Chrimson PCR, validated by Sanger sequencing	Fig. 1C
Verification of the absence of random plasmid integration events	PCR	Backbone PCR showed no evidence for random integration of Syn*f*Chrimson	Supplementary Fig. 1E
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 the archive
Mutagenesis / genetic modification outcome analysis	Genomic DNA PCR product with subsequent Sanger sequencing	The *AAVS1* WT PCR with gDNA showed no band, while transgene and integration PCR were positive. Transgene and integration amplicons were Sanger sequenced and showed homozygous integration.	Fig. 1C, Supplementary Fig. 1A
	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 basepair size, subsequent Sanger sequencing showed no mutations (comparison to parental RUCDRI002-A RUCDRI002-A cell line).	Fig. 1D, Supplementary Fig. 1B
Specific pathogen-free status	Mycoplasma	Mycoplasma testing was negative. (Venor GeM Advance, Minerva Biolabs)	Supplementary Fig. 1D
Multilineage differentiation potential	Directed differentiation	The hiPSC line was differentiated into -ectoderm lineage, more specifically to Bio-Engineered Neuronal Organoids (BENOs) (Zafeiriou, 2020). -mesoderm lineage by differentiation to cardiomyocytes and Engineered Heart Muscle (EHM) (Schneider, et al., 2023). -endoderm lineage by directed differentiation (Ghorbani-Dalini, 3 Biotech 2020).Immunohistochemistry and marker expression via RT-PCR confirmed differentiation in the respective germlayers.	Fig. 1H/I
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

(*continued*)

Unique stem cell line identifier	RUCDRi002-A-71 https://hpscreg.eu/cell-line/RUCDRi002-A-71
Analysis of the nuclease-targeted allele status	Sanger sequencing of the transgene within the targeted locus (Supplementary Fig. 1A)
Method of the off-target nuclease activity prediction and surveillance	Sanger sequencing of the top 5 Off-targets (Fig. 1D, Supplementary Fig. 1B)
Descriptive name of the transgene	SYN*f*Chrimson
Eukaryotic selective agent resistance cassettes (including inducible, gene/cell type-specific)	Puromycin resistance
Inducible/constitutive expression system details	Constitutive expression under the neuron-specific *synapsin* (SYN)-promoter
Date archived/stock creation date	20th November 2023
Cell line repository/bank	https://hpscreg.eu/cell-line/RUCDRi002-A-71
Ethical/GMO work approvals	Reference number: 10/9/15
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

2. Resource utility

Optogenetic control of electrically excitable cells is a powerful technique that allows cell stimulation in a high spatiotemporal resolution. Optogenetic stimulation of neurons can be achieved by light stimulation of rhodopsin channels expressed on the membrane of the cells leading to a depolarization and thus action potential propagation (Fig. 1**A**). Table 1.

3. Resource Details

To generate a neuronal specific optogenetic cell line, we inserted the Syn*f*Chrimson into the safe harbour *AAVS1* on Chr.19 (Schoger et al., 2020) by CRISPR/Cas9-mediated homology directed repair (HDR). The integrated Syn*f*Chrimson sequence comprised of a red-shifted variant of channel rhodopsin f-Chrimson under the human synapsin (SYN)-promoter and a C-Terminal YFP tag (Fig. 1**B**) (Mager, 2018). The correctly modified iPSC-clone was identified by genotyping utilizing primers against different regions of the construct as indicated in Fig. 1**B** and Sanger sequencing **(Supplementary** Fig. 1**A).** As negative and positive controls, the original iPSC line was used (WT) as well as the donor

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency marker	anti-Nanog PE, human	1:50	Miltenyi Biotec, Clone: REA314, CAT #130–117-377, RRID:AB_2751383
	anti-TRA-1–60 anti-human, Vio, 488 REAfinity™	1:50	Miltenyi Biotec, Clone REA157, CAT#130–106-872, RRID:AB_2654228
	anti-Oct3/4 anti-human APC 647, REAfinity™	1:50	Miltenyi Biotec, Clone: REA622, CAT# 130–123-257, RRID:AB_2819457
Isotype control for pluripotency marker	REA Control (I)-PE, human	1:50	Miltenyi Biotec, Lot: 5190226237, CAT#130–104-613, RRID:AB_2661678
	REA Control Antibody (S) human igG1, FITC 488, REAfinity™	1:50	Miltenyi Biotec, Clone: REA293, CAT #130–113-437, RRID:AB_2733689
	REA Control Antibody (I) human igG1, APC, REAfinity™	1:50	Miltenyi Biotec, Clone: REA293, #130–120-709, RRID:AB_2784399
Differentiation Markers	(FOXA2) Anti-HNF-3β (RY-7)	1:20	Santa Cruz #sc-101060, RRID:AB_1124660
	Alpha-1-Fetoprotein (AFP)	1:500	Dako #A0008, RRID:AB_2650473
	SOX17	1:20.000	R&D Systems, #AF1924, RRID:AB_355060
	TNNT2 (cTNT)	1:400	Abcam #ab8295, RRID:AB_306445
	Anti-Neurofilament (NF)	1:20000	Biolegend CAT#822601, RRID:AB_2564859
Secondary antibodies	Goat anti-Chicken IgY (H + L) Alexa Fluor488	1:400	Thermo Fisher Scientific #A-11039, RRID: AB_2534096
	Goat anti-Mouse IgG1 Cross-Adsorbed Alexa Fluor 633	1:400	Thermo Fisher Scientific #A-21126, RRID: AB_2535768
	Goat anti-Rabbit IgG (H + L) Highly Cross-Adsorbed Alexa Fluor 546	1:400	Thermo Fisher Scientific #A-11035, RRID: AB_2534093
	Alexa fluor 633 goat anti-rabbit IgG	1:400	Invitrogen #A-21070, RRID:AB_2535731
	Alexa fluor 546 rabbit anti-goat IgG (H + L)	1:400	Thermo Fisher Scientific #A-21085, RRID: AB_2535742
Nuclear stain	Hoechst33342	1:10000	Thermo Fisher Scientific, Cat #11534886
Site-specific nuclease			
Nuclease information	Nuclease type/version	Alt-R® S.p. HiFi Cas9 Nuclease V3, IDT DNA	
Delivery method	Electroporation	Amaxa 4D Nucleofector, Lonza, Program CA-137	
Selection/enrichment strategy	Selection cassette	Puromycin selection cassette	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
RT-qPCR	*OCT4*	Fwd: GGGGGTTCTATTTGGGAAGGTATTCAGCCAAACG Rev: CCACACTCGGACCACATCCTTCTCGAGCC 370 bp, Fig. 1 Panel H	
	SOX17	Fwd: ATACGCCAGTGACGACCAGA Rev: TTCCACGACTTGCCCAGCAT 300 bp, Fig. 1 Panel H	
	TNNT2	Fwd: TGAGGGAGAGCAGAGACCAT Rev: ACGAGCTCCTCCTCCTCTTT 377 bp, Fig. 1 Panel H	
	NEFL	Fwd: GCGCTATGCAGGACACGAT Rev: GTCTCCTCGCCTTCCAAGAG 160 bp, Fig. 1 Panel H	
	MAP2	Fwd: GCTAAGTCCGTGAGCGGTG Rev: TGTGTCGTGTTCTCAAAGGGT 217 bp, Fig. 1 Panel H	
Genotyping (desired allele/transgene presence detection)	Integration pSyn-*f*Chrimson	Fwd: CGGAACTCTGCCCTCTAACG Rev: ATGCGCAATTTGGGGAATGG 1176 bp, Fig. 1 Panel C	
	Chrimson PCR	Fwd: TGCTGAAGCTGAGCCCT Rev: TGGATCAGGATGTGCTC 124 bp, Fig. 1 Panel C	
	AAVS1 Wildtype PCR	Fwd: CGGAACTCTGCCCTCTAACG Rev: ATCCTCTCTGGCTCCATCGT 1106 bp,Fig. 1 Panel C	
Random integration-detecting PCRs	plasmid backbone PCR	Fwd: AACATACGAGCCGGAAGCATAAAGTGTAAAG Rev: GCCTATGGAAAAACGCCAGCAACG 364 bp, Supplementary Figure E	
gRNA oligonucleotide/crRNA sequence	Alt-R® CRISPR- Cas9 crRNA Alt-R® CRISPR- Cas9 tracrRNA	GGGGCCACTAGGGACAGGAT_TGG, IDT DNA [1] #1072532, IDT DNA	
Genomic target sequence(s)	Including PAM and other sequences likely to affect UCN activity	GRCh38: 19q13.3	
Bioinformatic gRNA on– and -off-target binding prediction tool used, specific sequence/outputs link(s)	IDT, CRISPOR, etc.	**http://crispor.tefor.net/crispor.py?batchId=VSQbIKzSjN4Y5GDnTz3J** https://cm.jefferson.edu/Off-Spotter/	
Primers for top off-target mutagenesis predicted site sequencing	OT1 448 bp OT2 492 bp OT3 344 bp	Fwd: TGAAGAAACAACCCGTTTCC Rev: TTCCCAGGAAACGATGAGAC Fwd: CCCTTGCTGAAGATCACACA Rev: CGTATGTTGCCCCCTACACT Fwd: GGCACAGAAGCATGAAGTGA Rev: CCTCCAGGTGCTGCTTACTC	

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Table 2 (*continued*)

Antibodies and stains used for immunocytochemistry/flow-cytometry		
	Antibody	Dilution Company Cat # and RRID
	OT4 446 bp OT5 705 bp	Fwd: TTTTCCCAGGAAACGATGAG Rev: GCTCCCAGCTCTCCCTAAGT Fwd: ATCAGCAGGGCCACTAGAGA Rev: AGCAAAGCTCCTCAAACCAA [1] Fig. 1 Panel D
ODNs/plasmids/RNA molecules used as templates for HDR-mediated site-directed mutagenesis.	Original plasmid and modifications Backbone modifications in utilized ODNS have to be noted using standard nomenclature.	pCAG_AAVS1 modified pAAVS1 integration vector (System Biosciences, catalogue #GE602A-1)

plasmid (*plSynfChrimson*), respectively (Fig. 1**C**). The top five suspected off-targets identified by Off-spotter (Thomas Jefferson University) were amplified by PCR and sequenced with no evidence for off-target CRISPR editing (Fig. 1**D, Supplementary** Fig. 1**B**) (Schoger et al., 2020). Digital karyotyping showed no copy number variations introduced by editing (Fig. 1**E**). Brightfield microscopy revealed a normal stem cell morphology (Fig. 1**F**), while pluripotency marker analysis showed more than 99 % positive cells for NANOG and TRA 1–60 (Fig. 1**G, Supplementary** Fig. 1**C**). The potential of the iPSC line to differentiate into all three germ layers was demonstrated by RT-PCR analysis (Fig. 1**H**) and immunofluorescence (Fig. 1**I**). Following directed differentiation towards endoderm, we found positive expression for SOX17, FOXA2 and AFP. Directed differentiation towards the mesodermal lineage showed positive expression for cardiomyocyte marker TNNT2 (cTNT). Finally, the Syn*f*Chrimson iPSC line was utilized in our previously established BioEngineered Neuronal Organoid (BENO) model (Zafeiriou, 2020) and clearly resulted in successful neuronal generation as shown by neurofilament expression. To validate that light stimulation leads to neuronal depolarization, we generated BENOs and plated them on a multielectrode array (MEA) system (Maestro, Axion biosystems). Local field potentials were recorded while paired light pulses (10 ms width and 10 ms interpulse interval) were delivered every 5 s. Fig. 1**J s**hows representative spike raster plot showing bursts (red) or single neuronal spikes (white) in all electrodes of a d60 BENO. The light pulses are indicated with a white arrowhead. The inserts show the raw traces of one electrode in low (left panel) and high temporal magnification (right panel). To validate that only neurons are stimulated by light we plated Syn*f*Chrimson cardiomyocytes on the same multiwell array (Fig. 1**K).** Cardiomyocytes exhibited a beating frequency of 0.4 Hz, which was not affected by light stimulations at a frequency of 0.5 or 1 Hz. This result provide proof that the Syn*f*Chrimson expression is only in neurons and thus allows neuronal specific stimulation. A specific use of the Syn*f*Chrimson line in a tissue model context has been recently reported in Schneider et al. (Schneider, et al., 2023).

4. Materials and methods

4.1. Cell Culture and Nucleofection of hiPSCs

The RUCDRi002-A iPSC line was cultured on Matrigel (growth factor reduced, BD Biosciences) with StemMACS iPS-Brew XF (Miltenyi Biotec) in a defined, serum free environment in 37 °C with a 5 % CO_2 atmosphere. Cells were passaged every 3–4 days with Versene (Thermo Fisher Scientific) and 10 μM ROCKi. $2*10^6$ Cells were electroporated with the 4D Amaxa Nucleofector system (Lonza, program CA-137). We used the ALT-R CRISPR/Cas9 Ribonucleoprotein (RNP, IDT DNA) system, consistent of a preassembled crRNA and tracrRNA (1:1) with a Alt-R® S. p. HiFi Cas9 Nuclease V3 (1:3) in nucleofector solution. This was electroporated with the donor plasmid *plSYNfChrimson*. After 2 days under 0.3 μM puromycin selection, colonies of around 1 mm^2 size were picked and cultured in a 48-well-plate.

4.2. Genotyping, pluripotency and validation of cell line

Genomic DNA (gDNA) was isolated (Macherey Nagel, Nucleo Spin Tissue) and amplified by PCR (Sigma redExtract, 35 cycles) targeting three different regions to identify (1) f-Chrimson, (2) its integration, or (3) its absence (WT PCR) and validated by Sanger sequencing. The top 5 off-targets were also amplified and sequenced. For digital karyotyping gDNA was sent to the Life & Brain for Illumina Bead Array analysis. Digital karyotype was analysed 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. Morphology of the cells showed typical hiPSC features and pluripotency. Briefly, $2*10^6$ Cells were fixed (4 % formaldehyde) for 15–20 min at RT, and washed three times in PBS-/-. Following antibody staining (45 min at 4 °C) against TRA1-60, NANOG, OCT3/4 and their corresponding isotype controls (Miltenyi Biotec). Cells were washed with PBS-/- and analysed using an LSR 2 flow cytometer (BD Biosciences) and FACSDiva Software. Mycoplasma contamination was tested regularly by Venor GeM Advance (Minerva Biolabs) (**Supplementary** Fig. 1). STR analysis was performed by Eurofins genomics.

Cells were differentiated into all three germ layers: (1) neuroectoderm was achieved by BENO (Zafeiriou, 2020) differentiation, followed by PCR-validated by PCR (NucleoSpin RNA, Macherey Nagel and DreamTaq, Thermo Fisher) for NEFL and MAP2 expression and by wholemount immunofluorescence as described earlier (Zafeiriou, 2020); (2) for proof of mesoderm induction, cardiomyocytes and Engineered Human Myocardium (EHM) were generated, validated by PCR and immunohistochemistry (IHC) for TNNT2 (Schneider, et al., 2023); (3) endoderm differentiation (Ghorbani-Dalini, 3 Biotech 2020), followed by PCR and IHC validation of SOX17 and AFP expression, respectively. Cells for IHC were differentiated on coverslips and fixed on day 9 with 4 % formaldehyde. After 1 h blocking at RT, cells were stained by primary antibodies overnight at 4 °C. The following day, cells were washed with PBS-/- for 30 min and finally stained with secondary antibodies (2 h) (cf. Table 2) and nuclear stain (Hoechst33342) for 10 min, prior visualization.

4.3. Validation of function by Multielectrode array (MEA)

Syn*f*Chrimson derived BENOs and cardiomyocytes were plated on a MEA multiwell plate (Lumos MEA 48, Axion BioSystems), after coating the 16-electrodes by a droplet of Matrigel. Light stimulations (590 nm) were performed by the Lumos Optical Stimulation System by (Axion BioSystems).

CRediT authorship contribution statement

Kea Aline Schmoll: . **Thomas Mager:** Resources, Methodology. **Timothy Tse:** Investigation. **Ahmed Alameldeen:** Investigation. **Wolfram-Hubertus Zimmermann:** Writing – review & editing, Resources. **Maria-Patapia Zafeiriou:** .

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Maria Patapia Zafeiriou reports financial support was provided by German Research Foundation. Wolfram Hubertus Zimmermann reports a relationship with Myriamed GmBH that includes: equity or stocks. Maria Patapia Zafeiriou and Wolfram Hubertus Zimmermann has patent #WO2018228948A1 licensed to Maria Patapia Zafeiriou and Wolfram Hubertus Zimmermann. No 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..

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103317.

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