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Lab Resource: Genetically-Modified Single Cell Line

Introduction of a Geminin mScarlet Reporter into H2B-mTurq2 hiPSCs for Live-cell Imaging of Proliferation and Cell Cycling

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ABSTRACT

We previously generated a doxycycline-inducible H2B-mTurq2 reporter in hiPSCs to track cells and study cell division and apoptosis. To improve visualization of cycling cells, we introduced a ubiquitously transcribed mScarletI-Geminin (GMMN) (1–110) into the previously untargeted second *AAVS1* allele. Fusion to the *N*-terminal part of GMNN provided tightly controlled mScarletI expression during the cell cycle. mScarletI fluorescence increased gradually from the S-phase through the M-phase of the cell cycle and was lost at the metaphase-anaphase transition. The resulting hiPSC reporter line generated, which we named *ProLiving*, is a valuable tool to study cell division and cell cycle characteristics in living hiPSC-derived cells.

Resource Table:

Unique stem cell line identifier	LUMCi041-A-3 Synonym of CRMi003-A-3 Subclone of CRMi003-A
Alternative name(s) of stem cell line	*ProLiving*
Institution	Leiden University Medical Center
Contact information of the reported cell line distributor	Christiaan Arendzen, c.h.arendzen@lumc.nl
Type of cell line	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: Fetal tissue (umbilical cord blood) Sex: male No disease known
Cell Source	Cord Blood CD34 + derived NCRM-1 with a PAX3-Venus targeted allele
Method of reprogramming	Episomal reprogramming with *SOX2, KLF4, MYC, Oct4, Lin28* and SV40 large T antigen.
Clonality	Clonal, fluorescence-activated cell sorting (FACS)
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	N/A
Cell culture system used	Tissue culture treated multiwell plates (Greiner), hESC qualified Matrigel (Corning ref#354277), StemFlex medium (ThermoFisher Scientific Cat#A3349401)
Type of Genetic Modification	Targeted insertion of an all-in-one cassette (containing rtTA3G, H2B-mTurquoise2, puromycin *N*-acetyl-transferase)
Associated disease	N/A
Gene/locus	*AAVS1/PPP1R12C*
Method of modification/site-specific nuclease used	CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Electroporation
All genetic material introduced into the cells	CRISPR/Cas9 plasmid, *AAVS1* guide RNA plasmid, AAVS1-GMNN(1–110)-mScarletI targeting plasmid, BCL-XL expression plasmid
Analysis of the nuclease-targeted allele status	5′ PCR amplification and Sanger sequencing of the targeted region
Method of the off-target nuclease activity surveillance	N/A
Name of transgene	GMNN(1–110)-mScarletI
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	

(continued on next page)

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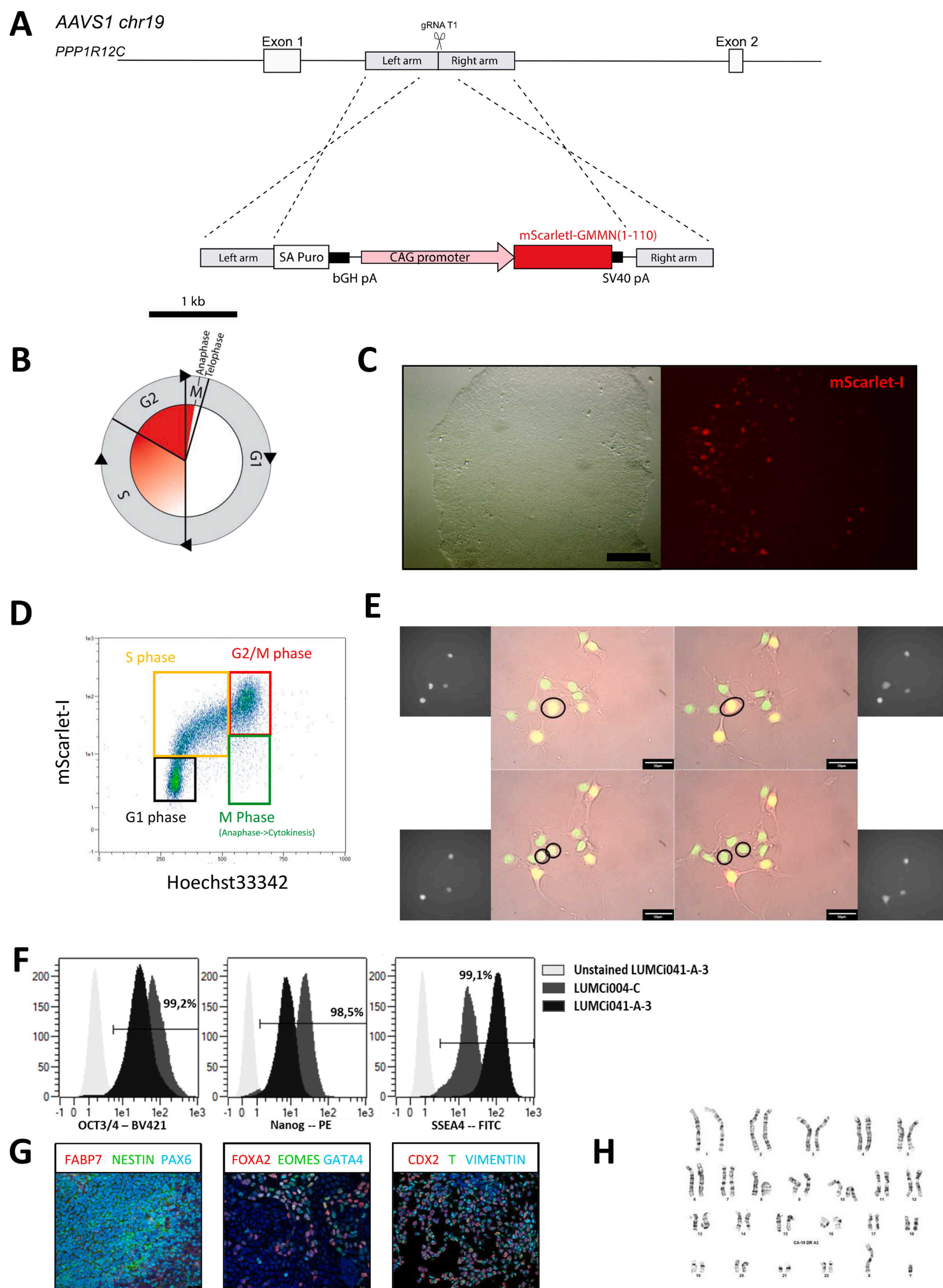

Fig. 1. Characterization and functional validation of LUMCi041-A-3.

Table 1
Characterization and validation.

Classification (optional *italicized*)	Test	Result	Data
Morphology	Photography	Normal	Fig. 1C
Pluripotency status evidence for the described cell line	Quantitative analysis *(Flow cytometry)*	OCT3/4: 99.2 % Nanog:98.5 % SSEA4:99.1 %	Fig. 1F
	Qualitative analysis *(Imaging living cells)*		Fig. 1G
Karyotype	G-banding	Normal, 46, XY	Fig. 1H
Genotyping for the desired genomic alteration/ allelic status of the gene of interest	Genomic DNA PCR specific for homology directed insertion (3′ donor arm)	Specific Integration into one of the AAVS1 alleles was verified by PCR screening	Fig. S1B
Verification of the absence of random plasmid integration events	PCR/Southern	Not done	
Parental and modified cell line genetic identity evidence	STR analysis	24 markers analysed. Profile matches with parental line (LUMCi041-A-2)	Results available on hPSCreg.eu and submitted in archive with journal
Mutagenesis/ genetic modification outcome analysis	Sequencing (genomic DNA PCR)	Heterozygous *AAVS1* allele A: DOX inducible H2B-mTurquoise2 *AAVS1* allele B: CAG-mScarletI-GMNN(1–110)	
	PCR-based analyses	Detection of correctly-inserted CAG-mScarletI-GMNN(1–110)	Fig. S1B
	Southern Blot or WGS; western blotting (for knock-outs, KOs)	Not done	
Off-target nuclease analysis-	PCR across top 5/10 predicted top likely off-target sites, whole genome/exome sequencing	1 insertion found in SNORA25-AL603632.1 which was not present in parental line.	Fig. S1E
Specific pathogen-free status	Mycoplasma *[mandatory]*	Mycoplasma testing using Lonza MycoAlert, Mycoplasma detection kit, negative	Fig. S1D
Multilineage differentiation potential	Directed Trilineage differentiation analysed with immunofluorescence.	Positive immunostaining for germ layer specific markers: Ectoderm (PAX6, NESTIN, FABP7), Endoderm (FOXA2, EOMES, GATA-4), Mesoderm (T, CDX2, Vimentin)	Fig. 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	Not done	
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping	Not done	
	HLA tissue typing	Not done	

(*continued*)

Unique stem cell line identifier	LUMCi041-A-3 Synonym of CRMi003-A-3 Subclone of CRMi003-A
Inducible/constitutive system details	Doxycycline-inducible expression of H2B-mTurquoise2 via CAG promoter driven consitutive expression of rtTA3G. Constitutive expression of GMNN(1–110)-mScarletI via the CAG promoter. *PAX3* promoter regulated Venus expression (*PAX3* knockin)
Date archived/stock date	N/A
Cell line repository/bank	https://hpscreg.eu/cell-line/CRMi003-A-3
Ethical/GMO work approvals	See parental line CRMi003-A at hpscreg.eu/cell-line/LUMCi041-A-2LUMC GMO permit: IG06-02
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	pCas9_GFP vector (Addgene #44719) *AAVS1* guide vector: (Addgene #41818) BCL-XL vector: Li et al, Nucl. Acids Res. 2018

1. Resource utility

We introduced a mScarletI-Geminin(GMNN)(1–110) cell cycle reporter into the *AAVS1* locus of a *PAX3-Venus* hiPSC line already containing a doxycycline-inducible H2B-mTurquoise2 (LUMC041-A-2). The hiPSC reporter line (LUMC041-A-3) generated, which we subsequently refer to as *ProLivIng*, allows for tracking of living cells as well as analysis of specific cell cycle stages in real time.

2. Resource details

Geminin (GMNN) is a nuclear factor that safeguards DNA replication by inhibiting unwanted reinitiation of replication on an origin already activated through binding to CDT1, an essential component of the replication licensing system (Esperanza et al., 2018). Expression of GMNN is tightly controlled during the cell cycle as GMNN is ubiquitinated and degraded in the G1-phase of the cell cycle after which it gradually accumulates again from early S-phase until the end of the metaphase. The destruction box, together with the nuclear localisation sequences (NLS), is located in the first 110 amino acids of the protein. As a result GMNN1-110 that is *N*-terminally fused to a fluorescent protein like citrine enables visualization of cell cycle progression in live cells and is a component of the widely used fluorescent ubiquitination-based cell cycle indicator (FUCCI) system (Sakaue-Sawano et al, 2008). To improve tracking and visualization of different stages of the cell cycle in cycling LUMC041-A-2-derived cells (Arendzen et al, 2021), we additionally introduced a GMNN(1–110) fusion gene into the untargeted *AAVS1* allele of LUMC041-A-2 using CRISPR/Cas9 (Fig. 1A). As GMNN (1–110) fusion partner, we opted for mScarlet-I, which is a bright monomeric red fluorescent protein designed for cellular imaging (Bindels et al, 2017) and whose excitation and emission spectrum can be easily separated from those of mTurquoise2 and Venus (https://www.fpbase.org/). Expression of mScarlet-I should enable identifation of different cell cycle stages in living cells (Fig. 1B). After electroporation, hiPSC colonies with nuclear mScarlet-I expression were identified (Fig. 1C), passaged, and further purified using fluorescence activated cell sorting (FACS) (Fig. S1A). Correct homologous recombination events were identified by *AAVS1 locus* specific amplification of mScarlet-I-GMNN(1–110) (Fig. S1B). We determined the cell cycle stage specificity of mScarlet-I expression in multiple clones using flow cytometry (Fig. 1D & Fig. S1C). All but one clone yielded similar cell cycle distributions, with a gradual increase in mScarlet-I expression from the S- through the M stages, as reported before (Fig. S1C). Clone A3 was selected for further experiments. First, we investigated whether it could be used to determine specific cell cycle stages using live cell

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry			
	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers (Flow cytometry)	anti-OCT3/4 BV421 anti-Nanog PE anti-SSEA4 FITC	1:25 1:5 1:25	BD Biosciences Cat#565644 RRID: AB_2739320 BD Biosciences Cat#560483 RRID: AB_1645522 Miltenyi Cat#130–098-371 RRID: AB_2653517
Differentiation Markers Ectoderm Endoderm Mesoderm	Rb anti-PAX6 (D3A9V) Alexa 647 conj.Rb anti-FABP7 (D8N3N) Alexa 555 conj.Ms anti-Nestin (10C2) Alexa 488 conj.Rb anti-FOXA2 (D56D6) Alexa 555 conj.Rb anti-Eomes (D8D1R) Alexa 488 conj. Rb anti-GATA4 (D3A3M) Alexa 647 conj.Rb anti-CDX 2 (D11D10) Alexa 555 conj.Rb anti-Brachyury (D2Z3J) Alexa 488 conj.Rb anti-Vimentin (D21H3) Alexa 647 conj.	1:200 1:100 1:200 1:500 1:100 1:200 1:500 1:200 1:400	Cell signalling Technology Cat#60433 RRID: AB_2797599 Cell signalling Technology Cat#13347 RRID: AB_2572210 Cell signalling Technology Cat#33475 RRID: AB_2799037 Cell signalling Technology Cat#3143 RRID: AB_2104879 Cell signalling Technology Cat#81493 RRID: AB_2799974 Cell signalling Technology Cat#36966 RRID: AB_2799108 Cell signalling Technology Cat#12306 RRID: AB_2797879 Cell signalling Technology Cat#81694 RRID: AB_2799983 Cell signalling Technology Cat#9856 RRID: AB_10834530
Secondary antibodies Differentation markers			
Other staining	DAPI Hoechst33342	1 µg/ml 1 µg/ml	ThermoFisher Cat#D3571 RRID: AB_2307445 ThermoFisher Cat#62249 RRID: AB_2651133
Site-specific nuclease			
Nuclease information	Nuclease type/nomenclature	spCas9 (Addgene #44719)	
Delivery method	Transfection	Vectors delivered using electroporation. The Neon electroporation system (ThermoFisher) was used.	
Selection/enrichment strategy	Manual selection under fluorescence microscope and by FACS	mScarlet-I positive cells were selected by FACS	
Primers and Oligonucleotides used in this study			
	Target	**Forward/Reverse primer (5′-3′)**	
e.g. Genotyping (desired allele/transgene presence detection)	AAVS1 - pUCM (5′ flank) *AAVS1*	#1047: 5′-GACCTGCCTGGAGAAGGAT-3′ AAVS1DOXrev: 5′-AGTCACCCAGGAGTCATTTG-3′	
e.g. Targeted mutation analysis/sequencing	Sequencing data from both alleles	Sanger sequencing of the allele before (Arendzen et al. 2021) and after targeting.	
gRNA oligonucleotide/crRNA sequence		gRNA[T2]: 5′-GGGGCCACTAGGGACAGGAT-3′	
Genomic target sequence(s)	Including *PAM* and other sequences likely to affect UCN activity	GGGGCCACTAGGGACAGGAT*TGG*	
Top off-target mutagenesis predicted site sequencing (for CRISPR/Cas9)	SNORA24-AL603632.1 DAPK2-RP11-111E14.1 RNU6-J15P-RNA5SP106 RP11-93L14.1-CTNNA3 CPNE5	5′-CTAACTATGAAGCCTTGAGTAAC-3′/5′-GAGCAGTATAATGAATGCAC-3′ 5′-CACAGAGACATGTCTCTTTGTG-3′/5′-GACAATGGCACATCTGATCCAG-3′ 5′-GAGATGAAGACTCCAATTTAG-3′/5′-CACTAATGATGTTCACTCAACAG-3′ 5′-CACTCACACACCTCACCAAGAC-3′/5′-GTTCTTGGAGAGCCATTTATTTC-3′ 5′-GATGTGTATAAGAGACAGGGCTGGTCCCTGAAGACATC-3′/5′-CGTGTGCTCTTCCGATCTCAGGGCTCTACTCACATAG-3′	
ODNs/plasmids/RNA templates used as templates for HDR-mediated site-directed mutagenesis. Backbone modifications in utilized ODNS have to be noted using standard nomenclature.	plasmid	pUCM AAVS1-mScarletI-GMNN(1–110)	

imaging. After DOX treatment, which induces H2B-mTurq2, expression of mScarlet-I was found to be the highest during mitosis with a rapid degradation of mScarlet-I in anaphase recapitualting the flow cytometry results (Fig. 1E). Prior to registration of the Proliferation Live Imaging (*ProLivIng)* hiPSC line at hPSCreg.eu, we confirmed pluripotency on the basis of OCT4, Nanog and SSEA4 expression compared to unstained cells and another benchmark hiPSC line (Fig. 1F) in undifferentiated cultures and the expression of PAX6/Nestin (ectoderm), FOXA2/GATA4 (endoderm) and CDX2/T (mesoderm) upon directed differentiation into the three germ layers (Fig. 1G). In addition, we demonstrated the absence of mycoplasma (Fig. S1D) and a normal 46, XY karyotype (Fig. 1H). To test for potential CRISPR/Cas9 mediated off-targets we PCR amplified and Sanger sequenced five genomic regions that were predicted to contain gRNA target sequences, one of which is a validated AAVS1-T2 gRNA off-

target sequence. We found one 1 nucleotide deletion in one of the 5 potential off-target sequences, namely in a large 800 kb intergenic region of *SNORA24-AL603632.1* Fig. S1E). Because this deletion occurred in a gene desert, the deleted nucleotide is very poorly conserved and the generated iPSC line has not demonstrated abnormal differentiation and cell cycling, this cell line is perfectly suited for study of cell division and cell cycle characteristics in living hiPSC-derived cells.

3. Materials and methods

3.1. Cell culture

hiPSCs were cultured using StemFlex medium (ThermoFisher) on hESC qualified Matrigel (Corning)-coated plates in a humified incubator at 37 °C and 5 % CO_2. Once a week, cells were passaged mechanicaly using gentle cell dissociation reagent (GCDR) (Stemcell Technologies).

3.2. Donor plasmid generation

The mClover fragment in pmClover-GMNN1-110 (Addgene #83915) was exchanged with a mScarlet-I fragment using the *Nco*I and *BsrgI* restriction sites. Subsequently, mScarlet-I-GMNN1-110 (*Pme*I-*Nco*I) was swapped with mTurq-H2B in pUCM-AAVS1-TO-H2B/mTurquoise2 using the *Hinc*II restriction sites to generate pUCM-AAVS1-TO-mScarletI-GMNN1-110.

3.3. Reporter insertion and clone selection

Plasmids (pUCM-AAVS1-TO-GMNN-mScarletI (200 ng) and pCas9_GFP (Addgene #44719) (225 ng), pgRNA_AAVS1-T2 (Addgene #41818) (75 ng) and pEF1_BCL-XL (50 ng) (Li et al, 2018)) were delivered into LUMCi041-A-2 by nucleofection (Neon system 10 µl, ThermoFisher Scientific)(conditions: 1000 v, 50 ms, 2 pulses). Immediately after electroporation cells were seeded onto Matrigel-coated plates in StemFlex medium supplemented with 10 µM Fasudil (LC Laboratories). Single mScarlet-I positive cells sorted by FACS (Aria II sorter (BD Bioscience)) two weeks after electroporation, were seeded into Matrigel (Corning)-coated 96-well plates containing StemFlex and Fasudil. Twelve clones were selected for characterization. Genomic DNA was isolated and 5′ homologous recombination specific flanks were amplified using a forward AAVS1 primer and a donor specific reverse primer (AAVS1-HRrev) with Phusion High-Fidelity PCR mix with GC Buffer (ThermoFisher). For off-target analysis, the same gDNA and polymerase where used. Overview of primers in Table 2.

3.4. Reporter validation

Single cells were harvested following GCDR incubation at 37 °C for 8 min. Harvested cells were treated with the Fix and Perm kit (ThermoFisher) and DNA stained with Hoechst33342 (ThermoFisher) according to manufacturer's instructions. Levels of mScarlet-I and Hoechst33342 were measured by flowcytometry (Milteny MaCSQuant VYB).

3.5. Trilineage differentiation

To determine functional pluripotency, reporter hiPSC clone A3 was differentiatied into *meso*-, endo- and ectoderm derivatives using the Trilineage differentiation kit (Stemcell Technologies) on Matrigel coated glass coverslips according the manufacturer's recommendations. Cells were fixed with 2 % PFA at RT for 20 min, permabilized with 0.05 % Triton-X100/1x PBS and incubated in PBS-4 %Normal Swine Serum containing the conjugated antibodies (Table 1) at 4 °C O/N. Nuclei were stained using DAPI. Coverslips were mounted in Prolong Gold (ThermoFisher) and images were captured on a SP8 confocol microscope (Leica).

3.6. Expression of pluripotency markers

hiPSCs were harvested as single cells by incubating with GCDR for 8 min at 37 °C and treated with the Fix and Perm kit. Cells were incubated for 60 min at RT with permeabilization medium containing conjugated antibodies for OCT3/4, Nanog and SSEA4 (Table 1). Samples were analysed on a MaCSQuant VYB Flowcytometer (Miltenyi).

3.7. Live cell imaging

Cells were passaged as single cells 24 h prior to the start of imaging. 1-5x10^3 cells were seeded onto a Matrigel coated 12-well plate in Stemflex containing 10 µM Fasudil. After 24 h, cells were imaged with a AF6000 imaging system (Leica) every 30 min for a period of 24 h.

3.8. Genome analysis

Karyotype was determined by G-banding by the Laboratory of Diagnostic Genome Analysis (LDGA), Leiden University Medical Center.

3.9. Mycoplasma testing

MycoAlert(Lonza) kit was used for detection of mycoplasma.

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

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2023.103031.

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