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Lab Resource: Multiple Cell Lines

Generation of heterozygous and homozygous hESC H9 sublines carrying inactivating mutations in *RB1*

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A B S T R A C T

Inactivation of the tumor suppressor gene *RB1* is causal for development of retinoblastoma, a tumor of the neural retina arising in children under the age of five. In addition, secondary *RB1* mutations are found in many other tumor types. To investigate retinoblastoma formation in vitro, stem cells with inactivated *RB1* can be differentiated into neural retina. To enable such studies, two sublines of hESC line H9 carrying mutations in *RB1* exon 3 in heterozygous or homozygous state were generated and characterized. Homozygous mutation led to loss of RB1 protein expression.

Resource table

Unique stem cell lines identifier	WAe009-A-12 WAe009-A-13
Alternative names of stem cell lines	C7 (homozygous deletion, WAe009-A-12) G12LS (heterozygous deletion, WAe009-A-13)
Institution	University Hospital Essen, University Duisburg-Essen, Essen, Germany
Contact information of distributor	Dr. Laura Steenpass, laura.steenpass@uni-due.de Dr. Deniz Kanber, deniz.kanber@uni-due.de
Type of cell lines	ESC
Origin	Human
Cell Source	Human ESC line H9 purchased from WiCell
Clonality	Clonal
Method of reprogramming	N/A
Multiline rationale	clones selected for deletion in heterozygous and homozygous state
Gene modification	YES
Type of modification	Indels in *RB1* exon 3
Associated disease	Retinoblastoma
Gene/locus	*RB1*, chromosome 13q14.2
Method of modification	CRISPR/Cas9 nuclease
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	C7 12.05.2018 G12LS 12.05.2018
Cell line repository/bank	N/A
Ethical approval	Approval obtained from the Robert-Koch Institute, Berlin, Germany (Az.3.04.02/0101) and from the local Ethical Review Board University Duisburg-Essen (16–7215-BO)

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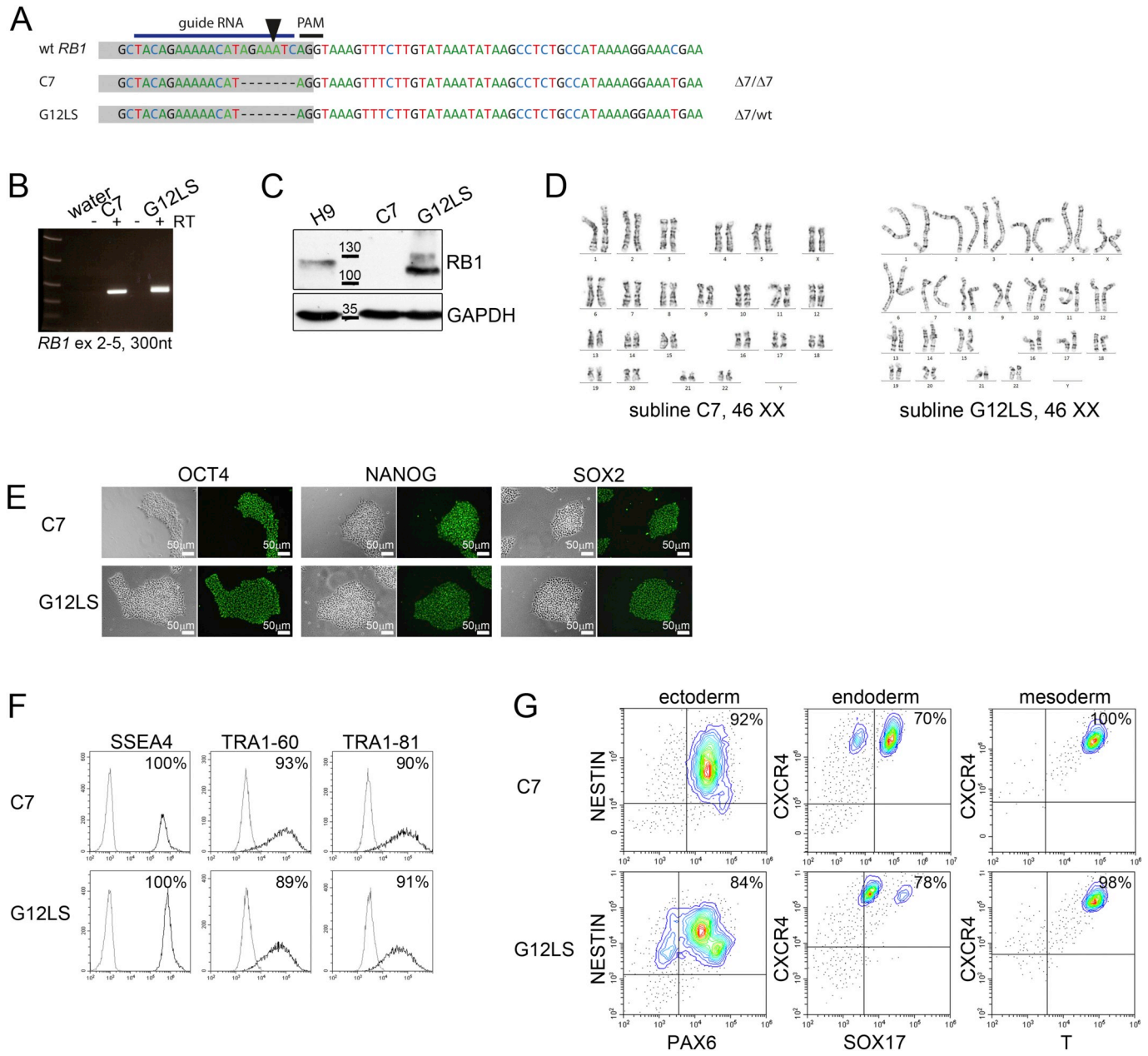

Fig. 1. Generation and characterization of RB1 modified H9 sublines C7 (WAe009-A-12) and G12LS (WAe009-A-13).

Table 1
Summary of lines.

hESC line names	Abbreviation in figures	Gender	Age	Ethnicity	Genotype of locus	Disease
H9_RB1ex3_C7 (WAe009-A-12)	C7	female	blastocyst	NA	RB1 −/−	Retinoblastoma
H9_RB1ex3_G12LS (WAe009-A-13)	G12LS	female	blastocyst	NA	RB1 +/−	Retinoblastoma

Resource utility

With 3D organoid models, modelling development of retinoblastoma in human neural retina cells in vitro has become feasible. Retinoblastoma is caused by inactivation of the tumor suppressor *RB1*. To generate appropriate starting cells for such experiments, the *RB1* gene was inactivated by insertion of indels into exon 3 using CRISPR/Cas9.

Resource details

H9 hESCs were modified in *RB1* exon 3 by insertion of random indel mutations using the CRISPR/Cas9 nuclease system (Fig. 1A, positions of guide RNA and PAM sequence are shown above the sequence alignment, grey box indicates 3′-end of *RB1* exon 3). 288 clonal cell lines were screened using GeneScan fragment length analysis. In total 88 clones showed modifications and eight sublines with heterozygous, two with compound heterozygous and one with homozygous mutations were

Table 2
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Fig. 1E
Phenotype	Qualitative analysis	Immunocytochemistry for OCT4, NANOG, SOX2	Fig. 1E
	Quantitative analysis	FACS analysis TRA1–60: C7 93%, G12LS 89% TRA1–81: C7 90%, G12LS 91% SSEA4: C7, G12LS 100%	Fig. 1F
Genotype	Karyotype (G-banding) and resolution	46 XX, Resolution 450 (C7) and 550 (G12LS)	Fig. 1D
Identity	Microsatellite PCR (mPCR) OR	N/A	N/A
	STR analysis	Promega Powerplex 16 HS	submitted in archive with journal
Mutation analysis (IF APPLICABLE)	Sequencing	heterozygous and homozygous indels in *RB1* exon 3	Fig. 1A
	Southern Blot OR WGS	N/A	N/A
Microbiology and virology	Mycoplasma	Mycoplasma testing by PCR; Negative	Supplementary file
Differentiation potential	Directed differentiation	Ectoderm (PAX6/NESTIN): C7 92%, G12LS 84% Endoderm (SOX17, CXCR4): C7 70%, G12LS 78% Mesoderm (T, CXCR4): C7 100%, G12LS 98%	Fig. 1G
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info (OPTIONAL)	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

selected for further analysis. Of these, sublines G12LS (heterozygous) and C7 (homozygous) were chosen for detailed characterization (Table 1). Sequencing analysis revealed the same seven basepair deletion in subline G12LS on one allele and in subline C7 on both alleles (Fig. 1A). Expression analysis on mRNA level by amplification of *RB1* exons 2 to 5, showed fragments of about 400 bp in both sublines (Fig. 1B). In subline C7 sequencing of this fragment confirmed deletion of seven basepairs on transcript level (NM_000321.2(RB1_v001):c.374_380del; www.mutalyzer.nl). In subline G12LS the fragment corresponded to the regular *RB1* transcript and a transcript carrying the seven basepair deletion in exon 3. In both sublines this resulted in a frameshift on the deletion allele and a premature stop codon in exon 4 (NM_000321.2(RB1_i001):p.(Glu125Valfs*9); www.mutalyzer.nl). Accordingly, RB1 protein was undetectable in subline C7 but still present in subline G12LS by western blot (Fig. 1C, full blot is shown in Supplementary file). Karyotype analysis nine passages after transfection (total passage 43) showed a normal karyotype of 46, XX in both sublines and descent from hESC line H9 was proven by STR analysis (Fig. 1D, STR file). Pluripotency was demonstrated qualitatively by expression of nuclear pluripotency markers OCT4, NANOG and SOX2 by immunofluorescence and quantitatively by FACS analysis of surface markers TRA1–60, TRA1–81 and SSEA4 (Fig. 1E: phase contrast and fluorescence images are shown, scale bar is 50 µm; Fig. 1F: FACS plots: X-axis: fluorescence intensity, Y-axis: counts, grey: isotype control, black: sample; percentage of positive cells is indicated). Efficient differentiation into all three germ layers was observed in both lines: In subline C7 92% of cells were double positive for ectoderm markers (PAX6, NESTIN), 70% for endoderm markers (SOX17, CXCR4) and 100% for mesoderm markers (T, CXCR4). In subline G12LS 84% of cells were double positive for ectoderm, 78% for endoderm and 98% for mesoderm marker gene expression (Fig. 1G, Table 2).

Materials and methods

Cell culture and nucleofection

H9 hESCs were obtained from WiCell (Thomson et al., 1998; www.wicell.org). Cells and derived sublines were cultured feeder-independent in StemMACS iPS-Brew XF, human (Miltenyi Biotec) on plastic coated with Matrigel (Corning) under 5% CO2 in a 37 °C humidified incubator. Medium was changed daily. Cells were passaged every 5 to 6 days using Cell Dissociation Buffer (Stemcell Technologies) at a ratio of 1/6. 8×10^5 cells were transfected with 5 µg of pX461nuc_RB1ex3_guideB using Human Stem Cell Nucleofector kit 2 (Lonza) and program B-016 in a Nucleofector II device. Cells were grown to confluency on Matrigel and then seeded at low density of 200 cells/cm^2 in StemMACS iPS-Brew XF, human supplemented with 10uM ROCK inhibitor Y-27632 for 24 h. Single colonies were isolated manually 7 days after transfection, without selection.

RT-PCR and western blot

RNA was isolated using the RNeasy mini kit (Qiagen). 500 ng of RNA were first treated with RNase-free DNaseI RQ1 (Promega) and reverse transcribed into cDNA using GeneAmp RNA PCR Core kit (Thermo). PCR (details in Supplementary file) was performed amplifying *RB1* exons 2 to 5, obtained fragments were sequenced in house.

Protein lysates were generated in lysis buffer (500 mM NaCl, 20 mM Tris pH 8, 1 mM EDTA, 0.5% NP-40) and used for standard western blotting and semi-dry blotting procedures (BioRad). Primary antibodies were incubated at 4 °C over night. Signals were detected by horseradish peroxidase coupled secondary antibody on film.

Karyotype analysis

Karyotype analysis was performed as described (Stanurova et al., 2016). At least eleven metaphases and three karyograms were analysed per clone.

Immunofluorescence

In brief, cells were grown on Matrigel-coated cover slips, fixed at room temperature with 4% paraformaldehyde for 15 min, permeabilized with 0.3% Triton-X-100 for 5 min, blocked in PBS/5% BSA for 60 min at room temperature and incubated with primary antibodies on 4 °C over night. Secondary antibody staining was performed for 60 min at room temperature. Antibodies were diluted in PBS/1.25% BSA (Table 3). Images were taken with a Zeiss Axiovert A1 microscope with ZENblue software.

FACS analysis

Surface marker staining: single cell suspension was prepared with Accutase and incubated with antibodies and isotype control immunoglobulins for 30 min at room temperature in PBS with 10% serum. Intracellular marker staining: Single cells were fixed in suspension with 4% paraformaldehyde at room temperature for 20 min, permeabilized in 0.1% Triton-X-100 in PBS for 15 min at room temperature and incubated with antibodies/isotype controls for 60 min at room

Table 3
Reagents details.

Antibodies			
	Antibody	Dilution	Company Cat # and RRID
Western blot			
pRB	Mouse anti-RB1	1:250	BD Biosciences Cat# 554136, RRID:AB_395259
GAPDH	Rabbit anti-GAPDH	1:5000	Cell Signal Technology Cat# 2118, RRID:AB_561053
Secondary antibody	Goat anti-mouse- HRP	1:1000	Thermo Fisher Scientific Cat# 32430, RRID:AB_1185566
	Goat anti-rabbit- HRP	1:1000	Thermo Fisher Scientific Cat# 32460, RRID:AB_1185567
Pluripotency markers			
StemLight Pluripotency antibody kit	Rabbit anti-Oct4 Rabbit anti-Nanog Rabbit anti-Sox2	1:200	Cell Signaling Technology Cat# 9656S, RRID:AB_10692662
FACS	Mouse anti-human SSEA4	5 µl/10^6 cells	BioLegend Cat# 330408 RRID: AB_1089200
FACS	Mouse IgG3κ, SSEA4 isotype	5 µl/10^6 cells	BioLegend Cat# 401321 RRID: AB_10683445
FACS	Mouse anti-human TRA1–60	10 µl/10^6 cells	BioLegend Cat# 330610 RRID: AB_2119065
FACS	Mouse IGMκ TRA1–60 isotype	10 µl/10^6 cells	BioLegend: Cat# 401611 RRID: not given
FACS	Mouse anti-human TRA-1-81	7.5 µl/10^6 cells	BioLegend: Cat# 330710 RRID: 2561742
FACS	Mouse IGMκ TRA1–81 isotype	7.5 µl/10^6 cells	BioLegend: Cat# 401617 RRID: not given
Differentiation markers			
FACS	Anti-human PAX6	10 µl/10^6 cells	Miltenyi Biotec Cat# 130–107-775 RRID: AB_2653167
FACS	REA(I) control, PAX6 istoype	10 µl/10^6 cells	Miltenyi Biotec Cat# 130–104-613 RRID: AB_2661678
FACS	Anti-human NESTIN	10 µl/10^6 cells	Invitrogen Cat#MA5–23650 RRID: AB_2608686
FACS	Mouse IgG1, NESTIN isotype control	10 µl/10^6 cells	Invitrogen Cat#MA5–18093 RRID: AB_2539476
FACS	Anti-human T	5 µl/10^6 cells	R&D Systems Cat# IC2085G RRID: not listed
FACS	Normal goat IgG, T isotype	5 µl/10^6 cells	R&D Systems Cat# IC108G RRID: AB_10890944
FACS	anti-human SOX17	2 µl/10^6 cells	Miltenyi Biotec Cat# 130–111-032 RRID: AB_2653493
FACS	REA(I) control, SOX17 isotype	2 µl/10^6 cells	Miltenyi Biotec Cat# 130–104-613 RRID: AB_2661678
FACS	Mouse anti-human CD184 (CXCR4)	5 µl/10^6 cells	Stemcell Technologies Cat# 60089AZ RRID: not listed
FACS	Mouse IgG2aκ CD184 isotype	5 µl/10^6 cells	Stemcell Technologies Cat# 60071AZ RRID: not listed

Primers		
	Target	Forward/Reverse primer (5′-3′)
Guide RNA exon 3	*RB1* exon 3	TACAGAAAAACATAGAAATC
Sequencing/GeneScan	*RB1* exon 3	TGCCATCAGAAGGATGTGTT/TTGGTCCAAGTTCTTTTTGTTTT product size wildtype: 394 nucleotides
RT-PCR	*RB1* exon 2–5	GTTTCATCTGTGGATGGAGTATT/GAACTGCTGGGTTGTGTCAA product size wildtype: 300 nucleotides

temperature in PBS with 10% serum. Cells were analysed on a CytoFLEX flow cytometer using CytExpert software (Beckman Coulter).

Germ layer in vitro differentiation

For directed monolayer differentiation the StemDiff Trilineage Kit (Stemcell Technologies) was used according to manufacturer's instructions.

STR and GeneScan analysis

For STR analysis, the Powerplex 16 HS system was used according to instructions (Promega). For fragment length analysis (GeneScan) exon 3 of *RB1* was amplified with one primer FAM-labelled. Fragments were analysed on an ABI Genetic Analyzer 3130XL and using the program GeneMarker (Softgenetics).

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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.2018.09.016.

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