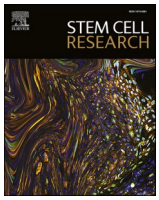
Stem Cell Research 77 (2024) 103378

Contents lists available at ScienceDirect

Stem Cell Research

journal homepage: www.elsevier.com/locate/scr



Generation of homozygous and heterozygous *REEP1* knockout induced pluripotent stem cell lines by CRISPR/Cas9 gene editing

M. Korneck [a,b,c], A. Leonhardt [b], L. Schöls [a,b], S. Hauser [a,b,*]

[a] Hertie Institute for Clinical Brain Research, University of Tübingen, Tübingen, Germany
[b] German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany
[c] Graduate School of Cellular and Molecular Neuroscience, University of Tübingen, Tübingen, Germany

A B S T R A C T

REEP1 is a transmembrane protein in the endoplasmic reticulum (ER) membrane that is involved in shaping and remodeling of the ER. Mutations in *REEP1* cause SPG31, an autosomal dominant form of hereditary spastic paraplegia (HSP). Here we show the generation of a homozygous and a heterozygous *REEP1* knockout induced pluripotent stem cell line suitable for *in vitro* disease modelling using the CRISPR/Cas9 editing system.

Resource Table:

Unique stem cell lines identifier	HIHCNi008-A-3 HIHCNi008-A-4
Alternative name(s) of stem cell lines	iPSC-REEP1_homKOiPSC-REEP1_hetKO
Institution	Hertie Institute for Clinical Brain Research, University of Tübingen, and German Center for Neurodegenerative Diseases (DZNE), Tübingen, Germany
Contact information of the reported cell line distributor	Stefan Hauser Stefan.Hauser@dzne.de
Type of cell lines	iPSC
Origin	Human
Additional origin info *(applicable for human ESC or iPSC)*	Age: 74 Sex: Male Ethnicity: Caucasian
Cell Source	Fibroblasts
Method of reprogramming	Non-integrating episomal plasmids
Clonality	Clonal (single cell seeding and manual colony picking)
Evidence of the reprogramming transgene loss (including genomic copy if applicable)	RT-PCR
Cell culture system used	Matrigel, Essential-8 medium
Type of Genetic Modification	CRISPR/Cas9-mediated gene knockout
Associated disease	Spastic Paraplegia 31, autosomal dominant (SPG31), OMIM #610250
Gene/locus	*REEP1*, 2p11.2
Method of modification/site-specific nuclease used	Site-specific nuclease (SSN) CRISPR/Cas9
Site-specific nuclease (SSN) delivery method	Nucleofection
All genetic material introduced into the cells	Cas9, crRNA1, crRNA2
Analysis of the nuclease-targeted allele status	Sequencing of the targeted and untargeted allele
Method of the off-target nuclease activity surveillance	PCR/sequencing of eight potential exonic off-target sites
Name of transgene	N/A
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	N/A
Inducible/constitutive system details	N/A
Date archived/stock date	N/A
Cell line repository/bank	https://hpscreg.eu/cell-line/HIHCNi008-A-3 https://hpscreg.eu/cell-line/HIHCNi008-A-4
Ethical/GMO work approvals	Institutional Review Board of the Medical Faculty, University of Tübingen, Approval Number: 598/2011BO1
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	N/A

1. Resource utility

REEP1 is involved in ER remodeling and trafficking and is thought to play an important role in axon maintenance. When mutated, it causes hereditary spastic paraplegia type 31 (SPG31). *REEP1* KO-iPSCs provide a valuable cell source to model HSP in a disease-relevant *in vitro* system.

* Corresponding author.
E-mail address: Stefan.Hauser@dzne.de (S. Hauser).

https://doi.org/10.1016/j.scr.2024.103378
Received 4 December 2023; Received in revised form 28 February 2024; Accepted 4 March 2024
Available online 5 March 2024
1873-5061/© 2024 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC license (http://creativecommons.org/licenses/by-nc/4.0/).

2. Resource details

REEP1 is a transmembrane protein located in the ER membrane and interacts with atlastin-1 and spastin. (Park et al., 2010). It plays a role in various cellular transport and packaging processes associated with the ER, such as mitochondrial trafficking or lipid droplet formation, and is highly expressed in the disease-relevant cell type, cortical neurons (Hauser et al., 2020). Mutations in the *REEP1* gene are associated with hereditary spastic paraplegia type 31 (SPG31), an autosomal dominant form of HSP whose disease mechanism is thought to be haploinsufficiency of REEP1 (Beetz et al., 2008).

The CRISPR/Cas9 system facilitates the study of cells bearing a disease-related knockout in conjugation with their isogenic control. This is a notable advantage especially in OMICS studies, as variations observed between lines can be more confidently attributed to the absence of the disease-relevant protein rather than to genomic differences between individuals. Here, we generated a homozygous and a heterozygous *REEP1* knockout iPSC line from a healthy control line using the CRISPR/Cas9 system to provide a tool to investigate the pathomechanism leading to SPG31. Fibroblasts from a healthy male donor were reprogrammed with OCT4, LIN28, SOX2, L-MYC and KLF4 using episomal plasmids. The resulting iPSCs (HIHCNi008-A, iPSC-CO2) were functionally, genetically, and morphologically verified and subsequently used for genetic modification (see Table 1). iPSC-CO2 were electroporated with RNP complexes carrying two different fluorescence-labeled crRNAs targeting 46 bp in exon 5 of the *REEP1* gene. After nucleofection, double-positive cells (containing both crRNAs) were sorted, seeded as single cells on Matrigel-coated plates and cultivated for 7–10 days. Colonies were then screened and the resulting clones were verified by Sanger sequencing to be c.329-391del p.Lys112Serfs*54 (Fig. 1B). One heterozygous and one homozygous clone were obtained containing one or two knockout alleles with 46 base pairs missing, resulting in a frame shift and a premature stop codon. Due to the small fragment size difference of 46 bp, only the KO band could be properly separated on the gel prior to sequencing, while the WT band still contained KO DNA fragments, resulting in a mixed sequence signal of the WT sequence in the heterozygous line. In addition, the knockout was validated on protein level via western blot. The knockout iPSCs (HIHCNi008-A, HIHCNi006-B) exhibited normal pluripotent stem cell morphology and were positively stained for the pluripotency markers alkaline phosphatase (ALP), TRA1-81, SOX2 and OCT4 (Fig. 1C). Furthermore, the integration of the reprogramming factors into the genome was excluded by PCR (supplemental file 1). At the transcriptional level, the expression of pluripotency related genes were comparable to those measured in human embryonic stem cells (hESCs). The levels of *OCT3/4, NANOG, SOX2, KLF4, C-MYC, TDGF1* and *DMNT3B* levels were assessed (Fig. 1E). In an embryoid body-based approach, trilineage differentiation capacity was verified by staining cells for early mesodermal (SMA), ectodermal (TUJ) and endodermal (FOXA2 and SOX17) markers (Fig. 1C). Short tandem repeat (STR) analysis verified cell identity (supplemental file) and a whole-genome short nucleotide polymorphism (SNP) array demonstrated genomic integrity of the knockout iPSCs (Fig. 1A).

Table 1
Characterization and validation.

Classification (optional *italicized*)	**Test**	**Result**	**Data**
Morphology	Photography	normal	Supplemental file
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Expression of pluripotency markers SOX2, OCT4 and TRA1-81	Fig. 1 panel C Fig. 1 panel D
	Quantitative analysis (RT-qPCR)	Alkaline phosphatase expression expression qRT-PCR for OCT4, NANOG, SOX2, KLF4, c-MYC, TDGF1 and DNMT3B	Figure 1 panel E
Karyotype	Whole genome SNP genotyping with InfiniumOmniExpressExome-8 BeadChip (Illumina) Spacing (kb): Mean: 3,03; Median: 1,36	46 XY No larger chromosomal aberrations or copy number variations upon CRISPR/Cas9 mediated genome editing	Fig. 1 panel A
Genotyping for the desired genomic alteration/allelic status of the gene of interest	PCR and sequencing across the edited site	homo-/heterozygous status verified Sanger sequencing (seq track); western blotting	Fig. 1 panel B Data not shown
	Transgene-specific PCR	N/A	N/A
Verification of the absence of random plasmid integration events	PCR	Non-integration of reprogramming plasmids verified	Supplemental file
Parental and modified cell line genetic identity evidence	STR analysis	5 sites, all matched D12S1648, D12S345, D6S1624, D6S265, D12S85, D12S1687, D12S1713	Supplemental file
Mutagenesis/genetic modification outcome analysis	Sequencing (genomic DNA PCR product)	c.329-391del p.Lys112Serfs*54	Fig. 1 panel B
	PCR-based analyses	N/A	N/A
	Southern Blot or WGS; western blotting (for knockouts, KOs)	Western blot shows truncation of REEP1 in knockout lines	Data not shown
Off-target nuclease analysis-	PCR and sequencing across top 8 predicted most likely exonic off-target sites	No off targets detected	Data not shown
Specific pathogen-free status	Mycoplasma	Mycoplasma testing by RT-PCR, negative	Data not shown
Multilineage differentiation potential	Embryoid body formation	ß-III-tubulin (TUJ), smooth muscle actin (SMA) and SOX17, FOXA2	Fig. 1 panel C
Donor screening (OPTIONAL)	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype - additional histocompatibility info (OPTIONAL)	Blood group genotyping HLA tissue typing	N/A N/A	N/A N/A

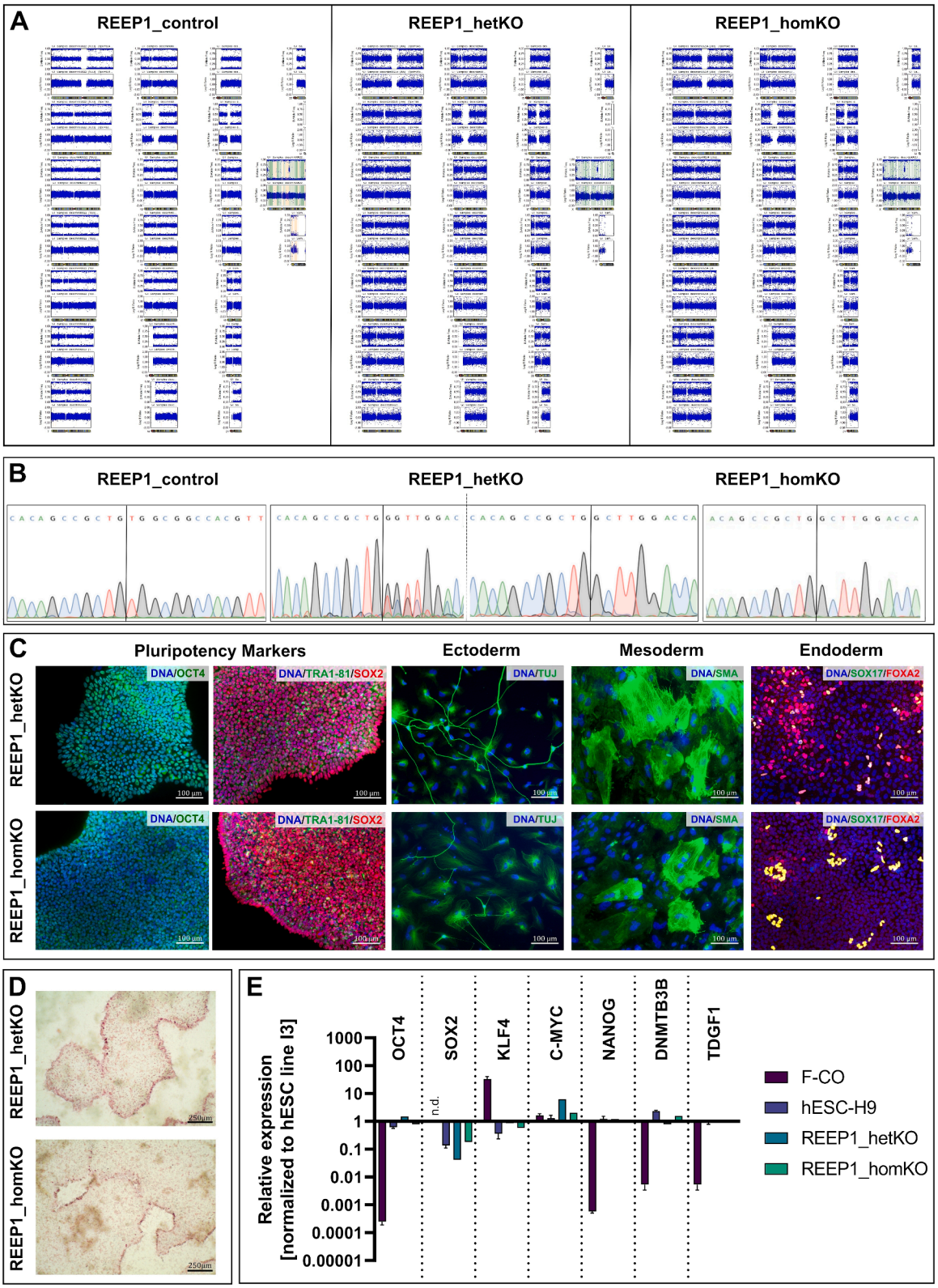


Fig. 1. Both knock-out lines were genetically and functionally tested. **A**SNP arrays of both KO lines compared to the control line.**B**Sanger sequencing reveals the cutting site in the homozygous clone and in one allele of the heterozygous clone. **C** Both clones stained positive for pluripotency markers OCT4 (panel 1, green), SOX2 (panel 2, red) and TRA1-81 (panel 2, green) were positive in both clones. Trilineage markers TUJ (panel 3, ectoderm, green), SMA (panel 4, mesoderm, green), SOX17 (panel 5, endoderm, green) and FOXA2 (panel 5, endoderm, red) were also stained positive in differentiations of both clones. Hoechst staining of the nuclei is shown blue in all panels. **D** Alkaline phosphatase activity was tested in the knock-out iPSCs. **E** Pluripotency marker mRNA levels were assessed in the iPSCs using RT-qPCR. Data represent mean ± SD, normalized to a hESC line (I3).

3. Materials and methods

3.1. iPSC culture

iPSCs were cultivated in standard Essential 8 medium with daily media changes and were passaged in a ratio between 1:8 and 1:15 using 0.2 % EDTA/PBS when 70 % confluence was reached. All cells were cultured on 1:60 Matrigel at 37 °C, 5 % CO_2.

3.2. CRISPR/Cas9-mediated knockout

The fully characterized iPSC line HIHCNi006-A (healthy control) was

Table 2
Reagents details.

Antibodies and stains used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:100	Proteintech AB_2167545
	Rabbit anti-SOX2Mouse anti-TRA1-81	1:200 1:500	Proteintech AB_2118-1-AP Millipore AB_177638
Differentiation Markers Western Blot	Mouse anti-SMA	1:100	Dako, AB_2223500
	Goat anti-SOX17	1:250	R&D Systems, AB_355060
	Rabbit anti-FOXA2	1:300	Millipore, AB_390153
	Mouse anti-TUJRabbit anti-REEP1	1:10001:2000	Sigma Aldrich, AB_477590 Abcam, ab230026
Secondary antibodies	Alexa Fluor 488 Goat anti-Mouse IgG	1:1000	Invitrogen
	Alexa Fluor 488 Goat anti-Rabbit IgG	1:1000	Invitrogen
	Alexa Fluor 488 Donkey anti-Goat IgG	1:1000 1:10.000	Invitrogen Life Technologies
	Alexa Fluor 568 Donkey anti-Rabbit IgG		
	Goat anti-Rabbit HRP		
Nuclear stain	DAPI	1 µg/ml	ThermoFisher Scientific cat #D1306
Site-specific nuclease			
Nuclease information	Alt-R™ S.p. Cas9 Nuclease 3NLS		
Delivery method	Nucleofection		
Selection/enrichment strategy	FACS		

Primers and Oligonucleotides used in this study

	Target	Forward/Reverse primer (5′-3′)
Episomal plasmids	*OCT4*	CATTCAAACTGAGGTAAGGG/TAGCGTAAAAGGAGCAACATAG
	L-MYC	GGCTGAGAAGAGGATGGCTACT/TTTGTTTGACAGGAGCGACAA
	KLF4SOX2	CCACCTCGCCTTACACATGAAG/TAGCGTAAAAGGAGCAACATAGTTCACATGTCCCAGCACTACCAG/TTTGTTTGACAGGAGCGACAAT
Pluripotency Markers (qPCR)	*OCT3/4*	GGAAGGTATTCAGCCAAACG/CTCCAGGTTGCCTCTCACTC
	c-MYC	ATTCTCTGCTCTCCTCGACG/CTGTGAGGAGGTTTGCTGTG
	KLF4	CCATCTTTCTCCACGTTCGC/CGTTGAACTCCTCGGTCTCT
	SOX2	TGATGGAGACGGAGCTGAAG/GCTTGCTGATCTCCGAGTTG
	NANOG	CAAAGGCAAACAACCCACTT/TGCGTCACACCATTGCTATT
	TDGF1	GGTCTGTGCCCCATGACA/AGTTCTGGAGTCCTGGAAGC
	DNMT3B	ACGACACAGAGGACACACAT/AAGCCCTTGATCTTTCCCCA
House-Keeping Genes (qPCR)	*GAPDHTBP*	AGGTCGGAGTCAACGGATTT/ATCTCGCTCCTGGAAGATGGCTTCGGAGAGTTCTGGGATTG/CACGAAGTGCAATGGTCTTTAG
e.g. Genotyping (desired allele/ transgene presence detection)	N/A	N/A
Targeted sequencing	Exon 5 FExon 5 R	GCCCAGCAAGAACAAGGATTTAGCCTGTTCTGTGTGGTCG
Potential random integration-detecting PCRs	N/A	N/A
crRNA sequence	crRNA 1crRNA 2	CGUAACUUCGGUCUUUUGCUGUUUUAGAGCUAUGCUAGCCAUCACAGCCGCUGUGGGUUUUAGAGCUAUGCU
Genomic target sequences	Exon 5	GCATTGAAGCCAGAAAACGACAAAATCTCGATACGATCGGTAGTGTCGGCGACACCCAAAATCTCGATACGA
Top off-target mutagenesis predicted site sequencing (for CRISPR/Cas9) primers	*RPL17*	ACCACTGACTTGAAGGGAGG/CAGGCTGCACAGGAATTGAG
	ANK2	GCAAGCATCGCACCAGATAA/GCTCTTAACTGGCAATCGGG
	UBE2	TCTGACGGAACTACTGCCAG/AGGTGCTGTTCCGAGAAGAA
	SLC22	GTCTTTCTCCTTGCCGCAG/TGGCTGCGAATGGGAAGT
	PER2	TGGAGGAGGTCTGGCTCATA/GGTCTCTGCCATGTAGCCTT
	BCAR3	GCATCTTTCGGTCTTCAGTCC/CGAGAATTGAGCGAGTGGATC
	SDK2C1orf145	GCGGAACAGGTAGACAGACT/CAAGGTGGCCATGTACAGACGGGTGGGACGATGCACTTA/AGCACCGAGAGAACACACA
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.	N/A	N/A

edited at passage 9 using the CRISPR/Cas9 system. The two selected crRNAs were delivered by nucleofection with RNP complexes containing the crRNA, a different ATTO-tracrRNA for each crRNA, and the Cas9 (Table 2) (Integrated DNA technologies) using the Amaxa nucleofection system (Lonza). To increase editing efficiency, double-positive cells containing both crRNAs were sorted using FACS (SONY cell sorter SH800). Cells were seeded in single cell suspension on 10 cm petri dishes at low density (25,000 cells/dish) and colonies were picked after 7–10 days. Clones were transferred to 24-well plates and PCR screening was performed to pre-select edited clones. To verify the heterozygous and homozygous knockouts, Sanger sequencing was performed using knockout-specific primers (Table 2) (3130xl Genetic Analyzer, Applied Biosystems). The top 8 potential exonic off-targets for the crRNAs were sequenced (data not shown).

3.3. Genomic integrity and identity

DNA was isolated using the DNeasy Blood&Tissue Kit (Qiagen) according to the manufacturer's instructions. STR analysis of seven different loci was performed to link the knockout lines to their origin to ensure cell identity. Whole genome SNP genotyping (OminExpressExome-8-BeadChip, Illumina) was conducted to ensure the genomic integrity of the knockout lines. Data was analyzed with the GenomeStudio software from Illumina (V2.0.4) using the CNV-partition plugin (V3.2.0). Copy number events are reported by the algorithm if they are larger than 350.000 base pairs or 1 Mbp for loss-of-heterozygosity regions. If an event were to be reported, the number indicated would refer to the copy number variants that span the reported event completely. Mosaicism states were manually reviewed on the provided B-allele frequency plots. Additionally, RT-PCR was performed to verify non-integration of the reprogramming plasmids using plasmid-specific primers (Table 2).

3.4. Pluripotency assessment

Immunocytochemistry was performed to detect the expression of pluripotent stem cell-specific markers at passage 9 + 9. Cells were fixed with 4 % paraformaldehyde (PFA) for 10 min at 37 °C, permeabilized and blocked in 5 % BSA/T-PBS for 1 h, followed by primary antibody staining overnight at 4 °C (Table 2). AlexaFluor secondary antibodies were incubated 1 h at RT, the samples were counterstained with 1 µg/ml DAPI for 20 min at RT and embedded in mounting medium (DAKO). Images were captured using the Axio Observer Z1 (Zeiss).

At the transcriptional level, RT-qPCR was performed to assess markers of pluripotency (Table 2) by comparing the expression levels to hESCs (I3, H9) and fibroblasts. RNA was isolated using the RNeasy-Kit (Qiagen) and transcribed into cDNA using the RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific) according to manufacturer's instructions. cDNA was used at a concentration of 1.25 ng/µl with SYBR Select Master Mix (Applied Biosystems) and measured in triplicate.

The ability to differentiate into cells of all three germ layers was determined using an embryonic body (EB)-based protocol in which $1.2x10^6$ iPSC were seeded in AggreWell800 plates (StemCell technologies) in EB medium (77 % DMEM/F-12, 20 % knockout serum replacement, 1 % MEM non-essential-amino-acid solution, 1 % Pen/Strep, 1 % GlutaMAX, 0.0035 % 50 µM β-Mercaptoethanol) and plated on coverslips on day 4 for further differentiation. Specific marker expression (TUJ or SMA (Table 2)) was assessed after 10 days as described above. For endodermal differentiation, $2x10^5$ cells were seeded on coverslips, supplied with endoderm induction medium (RPMI1640 advanced, 1xB27, 1xPen/Strep, 0.2 % FCS, ±2µM CHIR-99021, 50 ng/ml Activin A) for 4 days and then stained for FOXA2 and SOX17 (Table 2).

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Stefan Hauser reports financial support was provided by Federal Ministry of Education and Research Berlin Office. 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.

Acknowledgements

cDNA of the hESC lines I3 and H9 was kindly provided by the Institute of Reconstructive Neurobiology, Bonn, Germany. This work was supported by the German Federal Ministry of Education and Research (01GM1905A) to LS and SH. LS is member of the European Reference Network for Rare Neurological Diseases-Project ID No. 739510. We acknowledge support by the Open Access Publishing Fund of the University of Tuebingen.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scr.2024.103378.

CRediT authorship contribution statement

M. Korneck: Conceptualization, Data curation, Writing – original draft, Writing – review & editing. **A. Leonhardt:** Data curation. **L. Schöls:** Funding acquisition. **S. Hauser:** Conceptualization, Funding acquisition, Writing – review & editing.

References

Beetz, Christian; Schüle, Rebecca; Deconinck, Tine; Tran-Viet, Khanh-Nhat; Zhu, Hui; Kremer, Berry P. H. et al. (2008): REEP1 mutation spectrum and genotype/phenotype correlation in hereditary spastic paraplegia type 31. In: *Brain: a journal of neurology* 131 (Pt 4), S. 1078–1086. DOI: 10.1093/brain/awn026.

Hauser, Stefan; Schuster, Stefanie; Heuten, Elena; Höflinger, Philip; Admard, Jakob; Schelling, Yvonne et al. (2020): Comparative Transcriptional Profiling of Motor Neuron Disorder-Associated Genes in Various Human Cell Culture Models. In: *Frontiers in cell and developmental biology* 8, S. 544043. DOI: 10.3389/fcell.2020.544043.

Park, S.H., Zhu, P.-P., Parker, R.L., Blackstone, C., 2010. Hereditary spastic paraplegia proteins REEP1, spastin, and Atlastin-1 coordinate microtubule interactions with the tubular ER network. J Clin Invest 120 (4), 1097–1110. https://doi.org/10.1172/JCI40979.