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Generation of two *SPAST* knockout human induced pluripotent stem cell lines to create a model for Hereditary Spastic Paraplegia type 4

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A B S T R A C T

Spastin is a an ATPase that severs microtubules therby regulating amount and mobility of these structures. Mutations in the *SPAST* gene (SPG4) are the most common form of Hereditary Spastic Paraplegia (HSP). Here, we report the generation of a homozygous and a heterozygous *SPAST* knockout induced pluripotent stem cell (iPSC) line from a healthy control iPSC line using CRISPR/Cas9 technology.

1. Resource Table:

Unique stem cell lines identifier	HIHCNi006-B HIHCNi006-C
Alternative name(s) of stem cell lines	iPSC-SPAST_hetKO iPSC-SPAST_homKO
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: 47 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 4, autosomal dominant (SPG4), OMIM #182601
Gene/locus	*SPAST*, 2p22.3
Method of modification/site-specific nuclease used	Site-specific nuclease (SSN) CRISPR/Cas9 Nucleofection
Site-specific nuclease (SSN) delivery method	
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 five 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/HIHCNi006-B https://hpscreg.eu/cell-line/HIHCNi006-C
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

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2. Resource utility

Spastin holds an important role in microtubule severing and shaping and when mutated causes SPG4, the most common form of Hereditary Spastic Paraplegia. Here, we generated isogenic *SPAST* knockout iPSC

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Table 1
Characterization and validation.

Classification (optional *italicized*)	**Test**	**Result**	**Data**
Morphology	Photography	normal	Supplementary file 2
Pluripotency status evidence for the described cell line	Qualitative analysis (Immunocytochemistry)	Expression of pluripotency markers OCT4 and TRA1-81 Alkaline phosphatase expression expression	Figure 1 panel C Figure 1 panel D
	Quantitative analysis (RT-qPCR)	qRT-PCR for OCT4, NANOG, SOX2, KLF4, c-MYC and DNMT3B	Figure 1 panel E
Karyotype	Whole genome SNP genotyping with Infinium OmniExpressExome-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 Transgene-specific PCR	homo-/ heterozygous status verified N/A	Figure 1 panel B N/A
Verification of the absence of random plasmid integration events	PCR	Non-integration of reprogramming plasmids verified	Supplemental file 1
Parental and modified cell line genetic identity evidence	STR analysis N/A	6 sites, all matched D12S1648, D12S345, D6S1624, D6S265, D12S85, D12S1687, D12S1713	Submitted in archive with journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR product) PCR-based analyses Southern Blot or WGS; western blotting (for knock-outs, KOs)	c.955-1032del p.Asn319Lysfs*20 N/A N/A	Figure 1 panel B N/A N/A
Off-target nuclease analysis-	PCR and sequencing across top 5 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	Figure 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

lines to create a valuable *in vitro* tool to further investigate the disease mechanism behind SPG4.Table 1.

3. Resource details

Hereditary Spastic Paraplegia type 4 (SPG4) is the most common form of autosomal-dominant HSPs (Schüle et al., 2016). It is caused by mutations in SPAST, which encodes for Spastin, a microtubule severing enzyme present in two isoforms. In the cytosol, the shorter M87 isoform is found while the longer M1 form is ER membrane bound and, due to its weak Kozak sequence, less frequently translated (Parodi et al., 2018). The CRISPR/Cas9 technology makes it possible to create isogenic knockouts from control induced pluripotent stem cell lines (iPSCs) that share the genetic background of their cell line of origin. Especially in OMICS studies this proves to be a great advantage as observed effects are most likely attributed to the non-functioning protein since genetic background differences can be excluded.

Here, we created a heterozygous and a homozygous knockout of both Spastin isoforms from a healthy control iPSC line. Fibroblasts were obtained from a healthy 47 year old male donor and reprogrammed by transfecting episomal factors encoding human OCT4, LIN28, SOX2, L-MYC and KLF4 (HIHCNi006-A, iPSC-CO2). After several passages, cells exhibited pluripotent stem cell morphology and were assessed and expanded. iPSC-CO2 were then electroporated with two different crRNA-ATTO tracrRNA RNP complexes cutting in exon 6 and 7 of the *SPAST* gene. Only double positive cells containing both crRNAs were selected with fluorescence-activated cell sorting (FACS) and seeded as single cells. Colonies were picked, screened using RT-PCR and the knockout was verified with Sanger sequencing to be c.955-1032del, p.Asn319Lysfs*20. We obtained a heterozygous clone where one allele had the wildtype sequence and the other the knockout sequence missing 187 base pairs and a homozygous clone carrying the deletion on both alleles (Fig. 1B). These knockout iPSCs (HIHCNi006-B, HIHCNi006-C) showed the typical morphology of pluripotent stem cells and stained positive for the pluripotency markers alkaline phosphatase (ALP), OCT4 and TRA-1-81 (Fig. 1C,D). Integration of plasmids was excluded (Supplementary file 1) and the transcription levels of pluripotency related genes (OCT3/4, NANOG, SOX2, KLF4, C-MYC and DMNT3B) were comparable to levels seen in human embryonic stem cells (hESCs) (Fig. 1E). The capacity of differentiation into cells of all three germ layers was confirmed by expression of ecto- (TUJ) meso- (SMA) and endodermal (FOXA2, SOX17) markers after embryoid body-based differentiation (Fig. 1C). Genomic integrity was shown by whole genome short nucleotide polymorphism (SNP) array (Fig. 1A) and cell identity was confirmed by short tandem repeat (STR) analysis.

In summary, we generated a homozygous and a heterozygous human SPAST knockout iPSC line. Together with previous established SPG4 patient lines (Hauser et al., 2016), these lines will prove to be valuable tools to further investigate the pathophysiology of SPG4.

4. Materials and methods

4.1. iPSC culture

The iPSCs received Essential 8 medium changes every day and were passaged 1:10 using 0.2% EDTA/PBS when 70% confluency was reached. All cells were cultured on 1:60 Matrigel at 37 °C, 5% CO_2.

4.2. CRISPR/Cas9-mediated knockout

The fully characterized iPSC line HIHCNi006-A (healthy control) was edited at passage 18 using CRISPR/Cas9. The two chosen crRNAs were delivered via nucleofection with RNP-complexes containing the crRNA, a different ATTO-tracrRNA for each crRNA and the Cas9 (Table2) (Integrated DNA technologies) using the Amaxa nucleofection system (Lonza). To increase editing efficiency, double-positive cells containing both crRNAs were FACS-sorted directly after nucleofection (SONY cell sorter SH800). Cells were seeded in single-cell suspension onto 10 cm petri-dishes in low density (25.000 cells/dish) and colonies were picked after 7–10 days. The clones were transferred to 24well-plates and PCR-

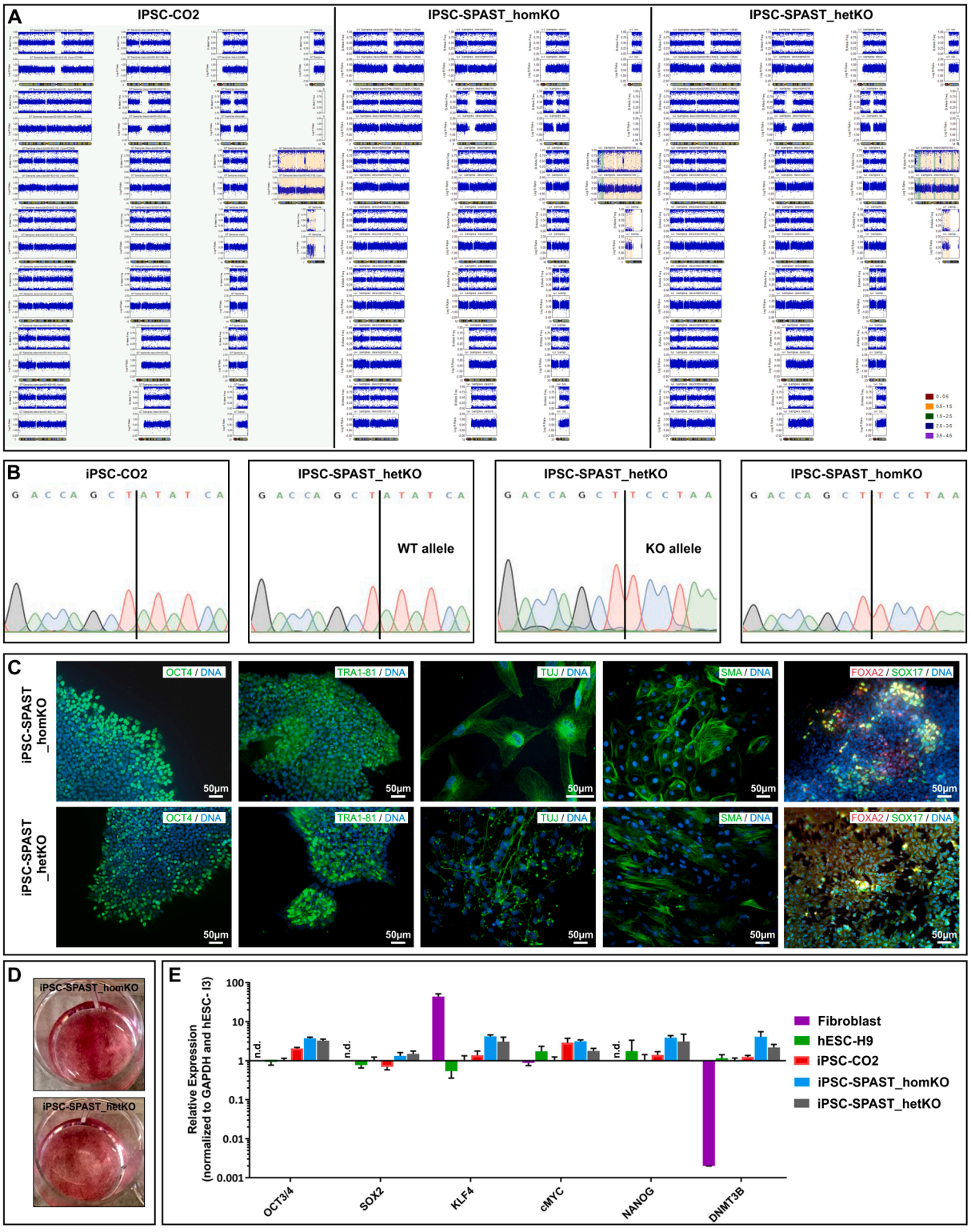

Fig. 1. Characterization and validation of HIHCNi006-B and HIHCNi006-C. (A) Whole genome SNP genotyping of the origin and knockout iPSCs HIHCNi006-B and -C. (B) Sanger sequencing of the cutting sites of both SPAST knockout iPSCs. (C) Immunofluorescence stainings of iPSCs (OCT4, TRA1-81) and differentiated cells expressing SMA (mesodermal), TUJ (ectodermal) and SOX17/FOXA2 (endodermal). Scale: 50 µm. (D) Alkaline phosphatase (ALP) staining. (E) qRT-PCR validation of pluripotency markers. GAPDH was used as housekeeping genes and relative quantification (RQ) values (RQmin/RQmax) were determined using hESC line I3 as reference.

screening was performed to preselect edited clones. To verify the heterozygous and homozygous knockout, Sanger-Sequencing using knockout-specific primer (Table2) (3130xl Genetic Analyzer, Applied Biosystems) was performed . The top 5 exonic off-targets for the crRNAs were sequenced (data not shown).

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
	Mouse anti-TRA1-81	1:500	Millipore AB_177638
Differentiation Markers	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-TUJ	1:1000	Sigma Aldrich, AB_477590
Secondary antibodies	Alexa Fluor 488 Goat anti-Mouse IgG	1:1000	Life Technologies
	Alexa Fluor 488 Goat anti-Rabbit IgG	1:1000	Life Technologies
	Alexa Fluor 488 Donkey anti-Goat IgG	1:1000	Life Technologies
	Alexa Fluor 568 Mouse anti-Rabbit IgG	1:1000	Life Technologies
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/	
	L-MYC	TAGCGTAAAAGGAGCAACATAG	
	KLF4	GGCTGAGAAGAGGATGGCTACT/	
	SOX2	TTTGTTTGACAGGAGCGACAA	
		CCACCTCGCCTTACACATGAAG/	
		TAGCGTAAAAGGAGCAACATAG	
		TTCACATGTCCCAGCACTACCAG/	
		TTTGTTTGACAGGAGCGACAAT	
Pluripotency Markers (qPCR)	OCT3/4	GGAAGGTATTCAGCCAAACG/	
	c-MYC	CTCCAGGTTGCCTCTCACTC	
	KLF4	ATTCTCTGCTCTCCTCGACG/	
	SOX2	CTGTGAGGAGGTTTGCTGTG	
	NANOG	CCATCTTTCTCCACGTTCGC/	
	DNMT3B	CGTTGAACTCCTCGGTCTCT	
		TGATGGAGACGGAGCTGAAG/	
		GCTTGCTGATCTCCGAGTTG	
		CAAAGGCAAACAACCCACTT/	
		TGCGTCACACCATTGCTATT	
		ACGACACAGAGGACACACAT/	
		AAGCCCTTGATCTTTCCCCA	
House-Keeping Genes (qPCR)	GAPDH	AGGTCGGAGTCAACGGATTT/	
		ATCTCGCTCCTGGAAGATGG	
e.g. Genotyping (desired allele/transgene presence detection)	N/A	N/A	
Targeted sequencing	Exon 6F	GGACAAATAAACCTTCTACCCCT	
	Exon 7 R	AGTTCTTACCTCAGGCCTCA	
Potential random integration-detecting PCRs	N/A	N/A	
crRNA sequence	crRNA 1	CUUGAAGAAUUUUAGGAAUGGUUUUAGAGCUAUGCU	
	crRNA 2	GUUAAAUUUGAUGAUAUAGCGUUUUAGAGCUAUGCU	
Genomic target sequences	PAM: NGG	CTTGAAGAATTTTAGGAATG TGG (52.824 bp)	
	Spastin: 2:32,063,550–32,157,636	GTTAAATTTGATGATATAGC TGG (53.183 bp)	
Top off-target mutagenesis predicted site sequencing (for CRISPR/Cas9) primers	VAMP3 F/R	TGAAGAACCAGCGGAACTCA/	
	RP11-362F19.1F/R	GCCGGCCTTCTAATTTGGAG	
	PLXDC F/R	TCTTCCTTTCTGCAGCACCT/	
	RP5-1166F10.1F/R	GCAGGGGAGCTGTAGAAGAA	
	MAP3K19 F/R	TGTCCTTGGCTAACAGTCTGT/	
		AGAGCACTGACATAAAACGCA	
		CCATTCGGAAGCTGCTGATC/	
		CATGAGCAGCGTAGGAGAGA	
		ATCTTGGTCGAACTCCTGCA/	
		AACCATGACTGTCTGAGCCT	
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	

4.3. Genomic integrity and identity

DNA was isolated using the DNeasy Blood&Tissue Kit (Qiagen) according to manufacturer's guidelines. 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, Illumnia) was conducted to ensure the genomic integrity of the knockout lines. RT-PCR was performed to verify non-integration of the reprogramming plasmids using plasmid-specific primers (Table2).

4.4. Pluripotency assessment

Immunocytochemistry was performed to show expression of pluripotent stem cell specific markers at passage 18 + 6. Cells were fixed with 4% Paraformaldehyde for 10 min at 37 °C, permeablized and blocked for 1 h in 5% BSA/T-PBS, followed by overnight staining at 4 °C

with primary antibodies (Table 2). AlexaFluor secondary antibodies were incubated 1 h at RT, the samples were counterstained with 1 μg/ml DAPI for 20 m at RT and embedded in mounting medium (DAKO) (Table2). Images were taken at 20x with the Axio Observer Z1 (Zeiss).

On transcription level, RT-qPCR was performed to assess markers of pluripotency (Table2) and compare the expression levels to hESCs (I3, H9) and fibroblasts. RNA was isolated with the RNeasy-Kit (Qiagen) and transcribed into cDNA with the RevertAid First-Strand cDNA Synthesis Kit (Thermo Scientific). cDNA was used at a concentration of 1.25 ng/μl with SYBR Select Master Mix (Applied Biosystems) and measured in triplicates.

The differentiation capacity into cells of all three germ layers was shown with an embryonic body (EB)-based protocol where $1.2x10^6$ iPSC were seeded in AggreWell800 plates (Stem cell technologies) in EB medium (77% DMEM/F-12, 20% Knockout Serum Replacement, 1% MEM Non-essential-amino-acid solution 100x, 1% Pen/Strep 100x, 1% GlutaMAX 100x, 0.0035% 50 μM β-Mercaptoethanol) and plated onto coverslips at day 4 for further differentiation (Table2). Specific marker expression (TUJ or SMA) was assessed after 10 days as described above. For endodermal differentiation, $2x10^5$ cells were seeded onto 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.

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.2022.102741.

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