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Lab resource: Stem Cell Line

Generation of a homozygous CRISPR/Cas9-mediated knockout human iPSC line for the *STUB1* locus

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A B S T R A C T

STUB1/CHIP is a central component of cellular protein homeostasis and interacts with key proteins involved in the pathogenesis of many neurodegenerative diseases. Missense and truncating mutations in *STUB1* lead to SCAR16. For ideal *in vitro* disease modelling with isogenic controls, we generated a CHIP knockout cell line from a healthy control with no CHIP functionality, but remaining genomic integrity and verified pluripotency.

Resource table

Unique stem cell line identifier	HIHCNi004-A-1
Alternative name(s) of stem cell line	iPSC-STUB1_KO
Institution	Hertie Institute for Clinical Brain Research, University of Tübingen, and German Center for Neurodegenerative Diseases (DZNE), Germany
Contact information of distributor	Stefanie Schuster Stefanie.schuster@klinikum.uni-tuebingen.de Ludger Schöls ludger.schoels@uni-tuebingen.de
Type of cell line	iPSC
Origin	Human
Additional origin info	Female, 37 years
Cell Source	Fibroblasts
Clonality	Clonal
Method of reprogramming	Non-integrating episomal plasmids
Genetic Modification	YES
Type of Modification	CRISPR/Cas9-mediated gene knockout
Associated disease	Spinocerebellar ataxia, autosomal recessive 16 (SCAR16), OMIM #615768
Gene/locus	*STUB1*; c.283-438del, p.Val94Alafs*5 (homozygous)
Method of modification	CRISPR/Cas9
Name of transgene or resistance	N/A
Inducible/constitutive system	N/A
Date archived/stock date	September 2018
Cell line repository/bank	N/A
Ethical approval	Institutional Review Board of the Medical Faculty, University of Tübingen Approval Number: 598/2011BO1

Resource utility

CHIP is a central component of cellular protein homeostasis and interacts with several key proteins associated with neurodegenerative diseases, with mutations in *STUB1* leading to SCAR16. For ideal *in vitro* disease modelling with isogenic controls, we generated a CHIP knockout cell line with no CHIP functionality.

Resource details

A skin biopsy was obtained from a healthy 37-year old woman (Control, CO) and cultured fibroblasts were reprogrammed by the delivery of episomal plasmids encoding human OCT4, SOX2, KLF4, L-MYC and LIN28. iPSCs exhibited a morphology similar to those of human embryonic stem cells (hESCs) and were assessed after manual picking and expansion for several passages. iPSC-CO (HIHCNi004-A) were then nucleofected with two crRNA-Atto550 tracrRNA RNP complexes targeting exon 2 and 3 of *STUB1*, followed by fluorescence-activated cell sorting (FACS) of Atto550^{+}-cells, single cell seeding and manual picking. Homozygous knock-out state was confirmed to be c.283-438del, p.Val94Alafs*5 (Fig. 1B), leading to nonsense-mediated decay and a loss of CHIP protein as shown by protein expression analysis (Suppl. Fig. 1) (Table 1).

iPSC-STUB1_KO (HIHCNi004-A-1) was verified to be pluripotent by the expression of pluripotency-associated surface markers such as

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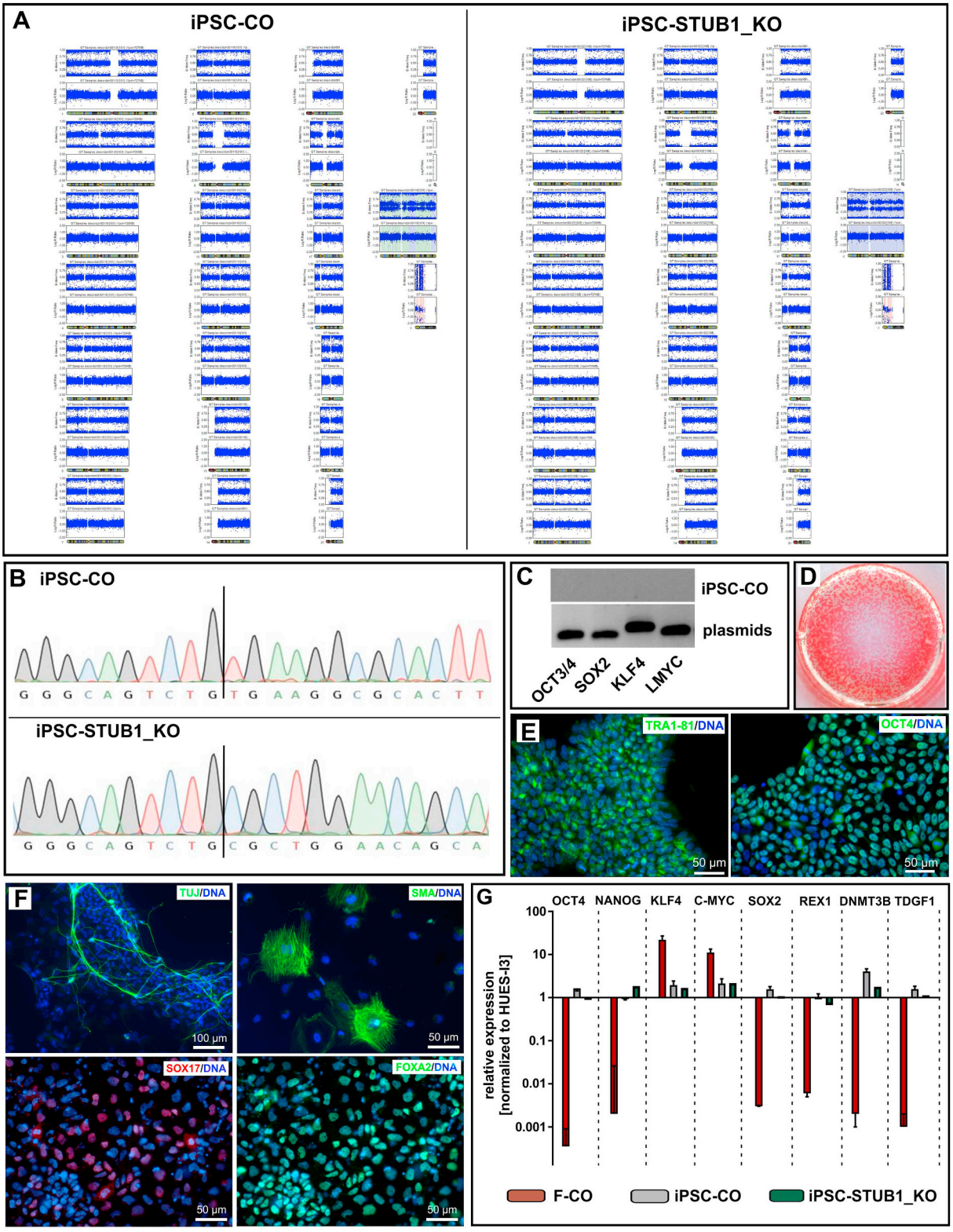

Fig. 1. Characterization and validation of HIHCNi004-A-1.

alkaline phosphatase (Fig. 1D), the absence of exogenous reprogramming factors after passage 5 (Fig. 1C) and the expression of endogenous pluripotency genes OCT4 and TRA1–81 on protein level as shown by immunocytochemistry (Fig. 1E). OCT4, NANOG, KLF4, c-MYC, SOX2, REX1, DNMT3B and TDGF1 were further transcriptionally expressed in a similar pattern compared to the hESC line HUES-I3 and the original iPSC line iPSC-CO (Fig. 1G). Pluripotency was furthermore demonstrated by embryoid body-based differentiation to endodermal, mesodermal and ectodermal cell lineage (Fig. 1F). Genomic integrity was confirmed by whole genome SNP genotyping (Fig. 1A). Top 5 off-target

Table 1
Characterization and validation.

Classification	Test	Result	Data
Morphology	Photography	Normal	Supplementary file 2
Phenotype	Qualitative analysis	Immunocytochemistry of pluripotency markers OCT4 and TRA1–81;	Fig. 1 panel E
		Expression of alkaline phosphatase	Fig. 1 panel D
	Quantitative analysis	qRT-PCR for OCT4, NANOG, KLF4, c-MYC, SOX2, REX1, DNMT3B and TDGF1	Fig. 1 panel G
Genotype	Whole genome SNP genotyping with Infinium OmniExpressExome-8 BeadChip (Illumina) Spacing (kb): Mean: 3,03; Median: 1,36	No larger chromosomal aberrations or copy number variations upon CRISPR/Cas9 mediated genome editing	Fig. 1 panel A
Identity	STR analysis	7 sites; F-CO, iPSC-CO and iPSC-STUB1_KO all matched	Submitted in archive with journal
Mutation analysis	Sequencing	c.283-438del p.Val94Alafs*5	Fig. 1 panel B
	Southern Blot OR WGS	N/A	
Microbiology and virology	Mycoplasma	Mycoplasma testing by RT-PCR, negative	Supplementary file 3
Differentiation potential	Embryoid body formation	Smooth muscle actin (SMA), β-tubulin (TUJ) and FOXA2, SOX17	Fig. 1 panel F
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	N/A	N/A
Genotype additional info	Blood group genotyping	N/A	N/A
	HLA tissue typing	N/A	N/A

Table 2
Reagents details.

Antibodies used for immunocytochemistry and Western Blotting

	Antibody	Dilution	Company Cat # and RRID
Pluripotency Markers	Rabbit anti-OCT4	1:100	Proteintech, AB_2167545 Millipore, AB_177638
	Mouse anti-TRA1–81	1:500	
In vitro Differentiation	Mouse anti-SMA	1:100	Dako, AB_2223500 R&D Systems, AB_355060
	goat anti-SOX17	1:250	
	rabbit anti-FOX-A2	1:300	Millipore, AB_390153 Sigma Aldrich, AB_477590
	mouse anti-TUJ	1:1000	
Western Blotting	Rabbit anti-CHIP	1:10.000	Abcam, AB_2751008 Meridian Life Science, AB_151542
	Mouse anti-GAPDH	1:10.000	
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-Rabbit IgG	1:1000	Life Technologies
	Alexa Fluor 568 Donkey anti-Goat IgG	1:1000	Life Technologies
	Peroxidase-conjugated AffiniPure goat anti mouse	1:10.000	Jackson ImmunoResearch
	Peroxidase-conjugated AffiniPure goat anti-rabbit	1:10.000	Jackson ImmunoResearch

Primers

	Target	Forward primer Reverse primer (5′-3′)
Episomal Plasmids	KLF4	CCACCTCGCCTTACACATGAAG TAGCGTAAAAGGAGCAACATAG
	L-MYC	GGCTGAGAAGAGGATGGCTAC TTTGTTTGACAGGAGCGACAAT
	OCT3/4	CATTCAAACTGAGGTAAGGG TAGCGTAAAAGGAGCAACATAG
	SOX2	TTCACATGTCCCAGCACTACCAG TTTGTTTGACAGGAGCGACAAT
Pluripotency Markers (qPCR)	c-MYC	GACTCTGAGGAGGAACAAGA TGATCCAGACTCTGACCTTT
	DNMT3B	GAGTATCAGGATGGGAAGGA ATAGCCTGTCGCTTGGA
	KLF4	CCATCTTTCTCCACGTTCGC CGTTGAACTCCTCGGTCTCT
	NANOG	CAAAGGCAAACAACCCACTT TGCGTCACACCATTGCTATT
	OCT4	GGAAGGTATTCAGCCAAACG CTCCAGGTTGCCTCTCACTC
	SOX2	TGATGGAGACGGAGCTGAAG GCTTGCTGATCTCCGAGTTG
	TDGF1	GGTCTGTGCCCCATGACA AGTTCTGGAGTCCTGGAAGC
Housekeeping Gene (qPCR)	GAPDH	AGGTCGGAGTCAACGGATTT ATCTCGCTCCTGGAAGATGG
Targeted sequencing of CHIP KO	CHIP	TGATTCTAGCCAGAGCGCAG TCGGGAGTCGGTGATTCAGA

CRISPR Guide RNAs

	Target	Sequence PAM Sequence
crRNAs	CHIP exon 2	GCTGGACGGGCAGTCTGTGA AGG
	CHIP exon 3	GAATCGCGAAGAAGAAGCGC TGG

effects of Cas9 for both crRNAs were excluded by Sanger sequencing (data not shown). The cell line was confirmed to be mycoplasma-free (Suppl. Fig.3). STR analysis of 7 loci confirmed cell identity of fibroblasts, iPSC-CO and iPSC-STUB1_KO.

In summary, we have generated a human disease-specific homozygous CHIP knockout iPSC line. This will serve, together with patient-derived generated iPSCs (Schuster et al., 2018), as an ideal tool for *in vitro* disease modelling and pathological study of SCAR16.

Materials and methods

Fibroblast reprogramming

For reprogramming, 10^5 human dermal fibroblasts were nucleofected with 1 μg of each episomal plasmid pCXLE-hUL, pCXLE-hSK and pCXLE-hOCT4, as described by Okita et al., 2011 After electroporation, fibroblasts were cultivated in fibroblast medium [DMEM high glucose + 10% FBS (Life Technologies)] before adding FGF2 (2 ng/ml (Peprotech)) on day 2. The following day, medium was changed to Essential 8 (E8) medium with 100 μM NaB (Sigma-Aldrich). After 3–4 weeks with medium change every other day, colonies were picked and expanded on matrigel-coated plates in E8 medium. iPSCs were frozen in E8 medium with 40% KO-SR (Life Technologies), 10% DMSO (Sigma-Aldrich) and 1 μM Y-27632 (Abcam Biochemicals).

CRISPR/Cas9-mediated gene knockout

For *STUB1*/CHIP knockout, 6×10^5 iPSC-CO cells of passage 14 were nucleofected with two crRNA-Atto550 tracrRNA RNP complexes (Table 2) (Integrated DNA Technologies) in Amaxa Nucleofection Solution (Lonza) with supplement, followed by FACS of Atto550$^+$-iPSCs, single-cell seeding and picking after 5–8 days. Homozygous knockout state was confirmed by PCR analysis of DNA and Sanger sequencing using knockout-specific primers (Table 2) according to standard procedures, using 3130xl Genetic Analyzer (Applied Biosystems) and CRISP-ID (Dehairs et al., 2016) for visualisation. Top 5 off-target effects were excluded for both crRNAs by Sanger sequencing (data not shown).

Genomic integrity analysis

To verify genomic integrity, DNA of iPSC-CO and iPSC-STUB1_KO was isolated with DNeasy Blood & Tissue Kit (Qiagen) according to manufacturer's instructions. Whole-genome SNP genotyping was conducted using Infinium OmniExpressExome-8-BeadChip (Illumina) and GenomeStudio V2.0.3 for evaluation. Copy number analysis was performed using CNVPartition plugin (Illumina). Early mosaicism states were evaluated by manual review on B-allele frequency plots on chromosomal level. STR analysis of 7 loci confirmed cell identity. To verify non-integration of plasmids, RT-PCR was performed with plasmid-specific primers (Table 2).

Pluripotency assessment

iPSCs were fixed with 4% PFA and assessed for alkaline phosphatase expression. For immunocytochemical analysis, fixed iPSCs were permeabilized and blocked, followed by overnight staining with primary antibodies at 4 °C and staining with Alexa Fluor 488- or 568-conjugated secondary antibodies (Table 2) for 1 h at room temperature. Nuclei were counterstained with Hoechst 33342 (1:10.000, Invitrogen). Immunofluorescence was visualized with AxioImager Z1 (Zeiss).

On transcript level, qRT-PCR with pluripotency genes-specific primers (Table 2) was performed: RNA was extracted with High Pure RNA Isolation Kit (Roche) according to manufacturer's instructions and reverse-transcribed to cDNA, using Transcriptor High Fidelity cDNA Synthesis Kit (Roche). qRT-PCR was performed as triplicates with SYBR Select Master Mix (Applied Biosystems). C_T values were normalized to GAPDH and the reference hESC line I3 with the $2^{-\Delta\Delta Ct}$ method. Pluripotency was further confirmed by embryoid body-based differentiation by cultivating iPSCs in EB medium (80% DMEM/F12 (Life Technologies), 20% KO-SR, 1× NEAA (Sigma-Aldrich), 1× Penicillin-Streptomycin (Merck Millipore), 2 mM L-Glutamine (Gibco), 0.1 mM β-Mercaptoethanol (Merck)) on AggreWell800 Plates (StemCell Technologies) with medium change on day 2. Embryoid bodies were collected (day 4) and plated onto 0.1% gelatine- or matrigel-coated plates, for *endo*- and mesodermal or ectodermal differentiation, respectively. Cells were cultivated for 2–3 weeks and immunocytochemically stained for SMA, TUJ, FOXA2 and SOX17 (Table 2).

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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.2018.101378.

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