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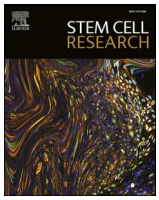

Lab Resource: Genetically-Modified Multiple Cell Lines

CRISPR/Cas9-mediated generation of hESC lines with homozygote and heterozygote p.R331W mutation in CTBP1 to model HADDTS syndrome

Enes Yağız Akdaş [a], Soeren Turan [b], Debarpan Guhathakurta [a], Arif Ekici [c], Seda Salar [a], D. Chichung Lie [b], Beate Winner [d], Anna Fejtova [a,*]

[a] Department of Psychiatry and Psychotherapy, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
[b] Institute of Biochemistry, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
[c] Institute of Human Genetics, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany
[d] Department of Stem Cell Biology, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen, Germany

A B S T R A C T

C-terminal Binding Protein 1 (CTBP1) is a ubiquitously expressed transcriptional co-repressor and membrane trafficking regulator. A recurrent *de novo* c.991C>T mutation in *CTBP1* leads to expression of p.R331W CTBP1 and causes hypotonia, ataxia, developmental delay, and tooth enamel defects syndrome (HADDTS), a rare early onset neurodevelopmental disorder. We generated hESCs lines with heterozygote and homozygote c.991C>T in *CTBP1* using CRISPR/Cas9 genome editing and validated them for genetic integrity, off-target mutations, and pluripotency. They will be useful for investigation of HADDTS pathophysiology and for screening for potential therapeutics.

Resource Table

Unique stem cell lines identifier	1. UKERe008-A-1 2. UKERe008-A-2 3. UKERe008-A-3 4. UKERe008-A-4 5. UKERe008-A-5 6. UKERe008-A-6 7. UKERe008-A-7 8. UKERe008-A-8 9. UKERe008-A-9
Alternative name(s) of stem cell lines	Not applicable
Institution	Department of Psychiatry and Psychotherapy, Universitätsklinikum Erlangen, Friedrich-Alexander-Universität Erlangen-Nürnberg (FAU), Erlangen, Germany
Contact information of the reported cell line distributor	Juliana MontiJuliana.monti@uk-erlangen.de
Type of cell lines	H9 human embryonic stem cell line (WA09)
Origin	Human
Additional origin info (applicable for human ESC or iPSC)	Sex: Female Ethnicity: unknown
Cell Source	Line H9 (WA09) (WiCell Research Institute, Inc.)
Method of reprogramming	Not applicable
Clonality	Clonal selection was achieved using FACS.

(*continued on next column*)

(*continued*)

Evidence of the reprogramming transgene loss (including genomic copy if applicable)	Not applicable
Cell culture system used	Feeder-free cell culture
Type of Genetic Modification	CRISPR-cas9 mediated substitutions g.1,213,033C>T (exonic, corresponds to c.991C>T) and g.1,213,28C>T (intronic)
Associated disease	Hypotonia, ataxia, developmental delay, and tooth enamel defect syndrome (HADDTS)
Gene/locus	rs869320802
Method of modification/site-specific nuclease used	CRISPR eSpCas9
Site-specific nuclease (SSN) delivery method	Plasmid nucleofection
All genetic material introduced into the cells	pCAG-eSpCas9-GFP-U6-gRNA plasmid, single-stranded oligonucleotide donor DNA (ssODN)
Analysis of the nuclease-targeted allele status	Sanger sequencing for target region.
Method of the off-target nuclease activity surveillance	Sanger sequencing for possible off-target regions.
Name of transgene	Not applicable
Eukaryotic selective agent resistance (including inducible/gene expressing cell-specific)	eGFP-based FACS selection
Inducible/constitutive system details	Not applicable

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* Corresponding author.
E-mail address: Anna.Fejtova@uk-erlangen.de (A. Fejtova).

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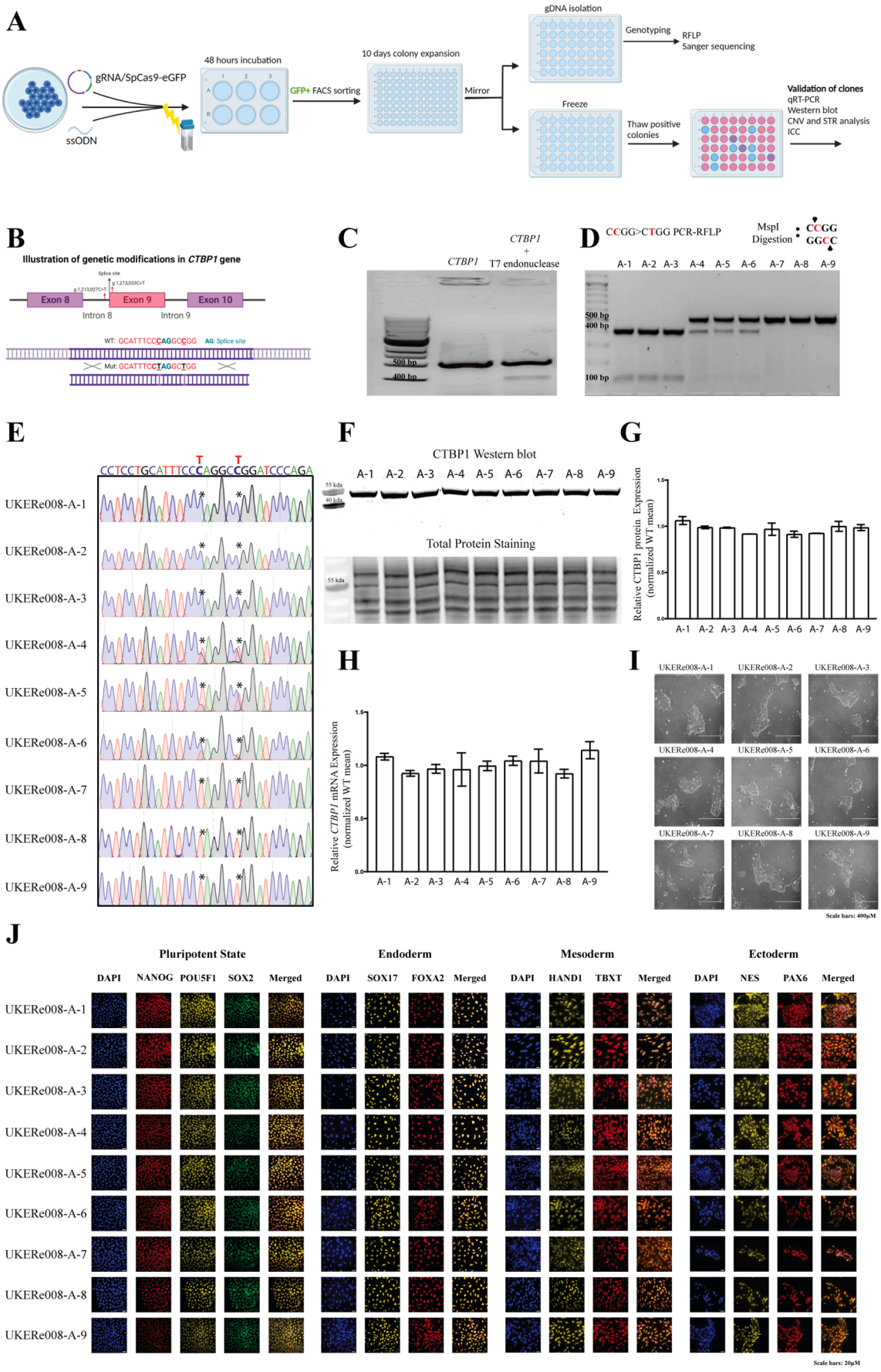

Fig. 1. Generation and validation of new cell lines

Table 1
Summary of cell lines

hESC line names	Gender	Genotype of CtBP1 locus	Disease
UKERe008-A-1	Female	WT / WT	Control
UKERe008-A-2	Female	WT / WT	Control
UKERe008-A-3	Female	WT / WT	Control
UKERe008-A-4	Female	g.1,213,033C>T and g.1,213,028C>T / WT	HADDTS syndrome
UKERe008-A-5	Female	g.1,213,033C>T and g.1,213,028C>T / WT	HADDTS syndrome
UKERe008-A-6	Female	g.1,213,033C>T and g.1,213,028C>T / WT	HADDTS syndrome
UKERe008-A-7	Female	g.1,213,033C>T and g.1,213,028C>T / g.1,213,033C>T and g.1,213,028C>T	Strongest phenotype for HADDTS syndrome
UKERe008-A-8	Female	g.1,213,033C>T and g.1,213,028C>T / g.1,213,033C>T and g.1,213,028C>T	Strongest phenotype for HADDTS syndrome
UKERe008-A-9	Female	g.1,213,033C>T and g.1,213,028C>T / g.1,213,033C>T and g.1,213,028C>T	Strongest phenotype for HADDTS syndrome

(*continued*)

Date archived/stock date	11.10.2021
Cell line repository/bank	https://hpscreg.eu/cell-line/UKERe008-A-1 https://hpscreg.eu/cell-line/UKERe008-A-2 https://hpscreg.eu/cell-line/UKERe008-A-3 https://hpscreg.eu/cell-line/UKERe008-A-4 https://hpscreg.eu/cell-line/UKERe008-A-5 https://hpscreg.eu/cell-line/UKERe008-A-6 https://hpscreg.eu/cell-line/UKERe008-A-7 https://hpscreg.eu/cell-line/UKERe008-A-8 https://hpscreg.eu/cell-line/UKERe008-A-9
Ethical/GMO work approvals	RKI AZ: 3.04.02/0164
Addgene/public access repository recombinant DNA sources' disclaimers (if applicable)	Not applicable

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1. Resource Utility

The mechanism by which the CTBP1 mutation p.R331W leads to HADDTS is unclear. Here described hESC lines are useful disease models to study the impact of mutation on development and cellular functions, to understand HADDTS pathophysiology, and for potential treatment development.

2. Resource Details

C-terminal Binding Protein 1 (CTBP1) is a ubiquitously expressed transcriptional co-repressor and membrane trafficking regulator. Till date, 17 patients with *CTBP1*-linked Hypotonia, Ataxia, Developmental Delay, and Tooth enamel defects Syndrome (HADDTS) were reported (Beck et al., 2016; Wong et al., 2022). *CTBP1* mutation c.991C>T mutation leads to p.R331W change in the substrate binding region of CTBP1 thereby affecting its interaction with its targets. To investigate HADDTS pathophysiology in various relevant tissues, we generated a human embryonic stem cell (hESC)-based disease model. The experimental flow is depicted in Fig. 1A. We employed single-stranded oligo DNA (ssODN)-mediated homologous DNA repair upon a Cas9-mediated double-strand DNA break strategy to introduce HADDTS-linked c.991C>T mutation into WA09 hESCs. Additionally, an intronic g.1,213,028C>T substitution was introduced for silencing of Cas9 restriction site as described earlier (Fig 1B) (Turan et al., 2019). Guide RNAs (gRNA) were designed *in silico* and validated using T7 endouclease assay (Fig 1C). The most effective gRNA was subcloned into pCAG-eSpCas9-GFP-U6-gRNA plasmids, which were nucleofected along with ssODN into hESCs.

Table 2
Characterization and validation

Classification	Test	Result	Data
Morphology	Phase contrast image	stem cell-like morphology	Fig. 1I
Pluripotency status evidence for the described cell line	Qualitative analysis: Immunocytochemistry	Pluripotency markers: POU5F1, NANOG and SOX2	Fig. 1J
	Quantitative analysis: Immunocytochemistry counting	Percentage of cells positive for POU5F1 and NANOG was assessed	Fig. S1B
Karyotype	Affymetrix CytoScan HD SNP based Karyotyping	46 XX Resolution filtered for >20kb	Fig. S1C
Genotyping for the desired genomic alteration/allelic status of the gene of interest	Sequencing	g.1,213,033C>T and g.1,213,028C>T	Fig. 1E
	Transgenic region PCR-RFLP	Loss of restriction digest	Fig. 1D
Verification of the absence of random plasmid integration events	PCR	Negative PCR	submitted in archive with journal
Parental and modified cell line genetic identity evidence	PowerPlex® 21 System	STR Analysis: 21 sites were tested and all the loci matched the parental line	submitted in the archive with journal
Mutagenesis / genetic modification outcome analysis	Sequencing (genomic DNA PCR)	Mutation confirmed	Fig. 1E
	qRT-PCR	*CtBP1*$^{c.991C>T}$ mRNA is stable	Fig. 1H
	Western blotting	CtBP1$^{p.R331W}$ protein is translated	Fig. 1F,G
Off-target nuclease analysis	PCR and Sanger sequencing of top predicted off-target sites	No mutation detected	Fig. S1A
Specific pathogen-free status	Mycoplasma	No mycoplasma detected	submitted in the archive with journal
Multilineage differentiation potential	Trilineage differentiation followed by ICC for markers for endoderm (SOX17 & FOXA2), ectoderm (NES & PAX6), and mesoderm: HAND1 & TBXT)	All clones have proven to differentiate into the 3 germ layers	Fig. F1J
Donor screening	HIV 1 + 2 Hepatitis B, Hepatitis C	Not applicable	
Genotype - additional histocompatibility info	Blood group genotyping	Not applicable	
	HLA tissue typing	Not applicable	

Table 3
Reagents and primers used in study

Antibodies used for immunocytochemistry/flow-cytometry

	Antibody	Dilution	Company Cat #	RRID
Gene of interest	Mouse anti-CTBP1	*1:250*	DB Biosciences Cat# 612042	RRID:AB_399429
Pluripotency Marker	Rabbit anti-SOX2	*1:300*	Cell Signalling Cat# 3579	RRID:AB_2195767
Pluripotency Marker	Mouse anti- POU5F1 (C-10)	*1:300*	Santa Cruz Cat# Sc-5279	RRID: AB_628051
Pluripotency Marker	Goat anti-NANOG	*1:300*	R&D Systems Cat# AF1997	RRID:AB_355097
Mesoderm Marker	Goat anti-HAND1	*1:300*	R&D Systems Cat# 3168	RRID:AB_2115853
Mesoderm Marker	Mouse anti-TBXT	*1:300*	SinoBiological Cat# 100338-MM07	RRID:AB_2923107
Endoderm Marker	Mouse anti-SOX17	*1:300*	Biolegend Cat # 698502	RRID:AB_2687317
Endoderm Marker	Goat anti-FOXA2	*1:300*	R&D Systems Cat# AF2400	RRID:AB_2294104
Ectoderm Marker	Rabbit anti-PAX6	*1:300*	Biolegend Cat # 901301	RRID:AB_2565003
Ectoderm Marker	Mouse anti-NES	*1:300*	Millipore Cat# Mab5326	RRID:AB_2251134
Secondary Antibody	Alexa Fluor™ anti-goat 647	1:500	Thermo Scientific Cat# A32728	RRID:AB_2633277
Secondary Antibody	Alexa Fluor™ anti-rabbit 488	1:500	Thermo Scientific Cat# A32790	RRID:AB_2762833
Secondary Antibody	Alexa Fluor™ anti-mouse 546	1:500	Thermo Scientific Cat# A32773	RRID:AB_2762848
Secondary Antibody	IRDye® 800CW anti-mouse	1:10000	LI-COR Biosciences Cat# 926-32210	RRID:AB_2687825

Primers used in study

	Target	Size of band	Forward/Reverse primer (5′-3′)
gRNA cloning primer	-	-	5'CACCGTCTTCAGGCTGTCTGGGATC 3' //5'AAACCTAGGGTCTGTCGGACTTCTC 3'
SSO	-	-	*5'GCTCCTGGGGCCCCCTCAGAGCCACAGCTGTTCCTTCCTCCTGCATCTCCTAGGCTGGATCCCAGACAGCCTGAAGAACTGTGTCAACAAGGACCATCTGACAGCC 3'*
Off-target sequencing	*ATP1A4*	500 bp	5'ACAGTACACAGACGGCATGG 3' // 5'TTGACGCAGCCTCAATCCAT 3'
Off-target sequencing	*VEZT*	819 bp	5'CTTGCAAGGACAACTTCTAC 3' // 5'TGAATTACTTATGGGTTCCACT 3'
Off-target sequencing	*RBM22*	491 bp	5'GCAGAGCAATGTTCACCACA 3' //5'GTGTGCTGAGGAGGAGGTAGA 3'
Off-target sequencing	*ANPEP*	497 bp	5'CTCCTGGGAGTACACCACTG 3' //5'GTTCTGATGCTGTTCCCACCC 3'
Mutation sequencing	*CTBP1*	508 bp	5'GAGGAGCACACTGCCAAGAATC 3' //5'TGAACATGAGGTCTTCGCCTC 3'
qRT-PCR	*CTBP1*	533 bp	5'CAGGACCTGCTCTTCCACA 3' //5'ATACCTTCCACAGCAGCTGG 3'
Vector integration control	pCAG-eCas9-GFP-U6-gRNA	512 bp	5'GAAGAACCTGATCGGAGCCCT 3' //5'TGGCAGACAGGATGGCCTT 3'

Site-specific nuclease	
Nuclease information	eSpCas9
Delivery method	Nucleofection
Selection/enrichment strategy	eGFP based FACS sorting

Table 4
Differentiation mediums used in study

Lineage Specific Medium	Components	Concentration	Duration
Ectoderm	Neurobasal	48.75%	12 days
	DMEM/F12	48.75%	
	B27+VitA (50X)	1%	
	N2 (100X)	0.5%	
	NEAA (100X)	0.5%	
	ß-ME (55mM)	55μM	
	LDN193189	100nM	
	SB431542	10μM	
	Penicillin-Streptomycin (100X)	0.5%	
Mesoderm I	DMEM/F12	98.5%	Initial 2 days and then
	ITS-G	1%	
	CHIR99021	3μM	Mesoderm II
	Penicillin-Streptomycin (100X)	0.5%	
Mesoderm II	DMEM/F12	98.5%	3 days
	ITS-G	1%	
	CHIR99021	3μM	
	Penicillin-Streptomycin (100X)	0.5%	
	LDN193189	200nM	
	SB431542	10μM	
Endoderm I	RPMI-1640	98.5%	Initial 2 days and then
	Activin A	100nM	
	FGF2	20nM	Endoderm II
	BMP4	20nM	
	B27-Insulin (50X)	1%	
	Penicillin-Streptomycin (100X)	0.5%	
Endoderm II	RPMI-1640	98.5%	3 days
	Activin A	100nM	
	B27-Insulin (50X)	1%	
	Penicillin-Streptomycin (100X)	0.5%	

Fluorescence-activated cell sorting (FACS) was employed to isolate nucleofected cells in individual clones. The single-cell clones were passaged once to obtain one plate for clone screening, while a mirror plate was cryopreserved for later validation. PCR restriction fragment length polymorphism (PCR-RFLP) assay was applied to detect WT (wild-type) clones and clones with heterozygote or homozygote c991C>T mutation (Fig. 1D). For at least three clones per genotype the correct gene editing of the *CTBP1* locus was confirmed by Sanger sequencing (Fig. 1E). Expression of *CTBP1* mRNA was comparable in all clones confirming no deleterious effect of intronic nucleotide exchange (Fig. 1F, G). All clones had a typical pluripotent stem cell morphology 24h upon Rho-kinase inhibitor (Y-27632) depletion (Fig. 1H), expressed core pluripotency markers POU5F1, NANOG, and SOX2, and showed potential to differentiate into the three-germ layer derivatives, namely, endoderm, mesoderm, and ectoderm (Fig. 1I and Fig. S1C). No unintended variants were detected at the predicted off-target loci (Fig. S1A). A single nucleotide polymorphism (SNP) based genomic copy number variation (CNV) analysis validated the genetic integrity of at least two clones per line (Fig. S1B). All clones are regularly proven mycoplasma free. A summary of the independent cell lines and analysis methods are given in Table 1 and Table 2**,** respectively.

3. Materials and Methods

3.1. Cell culture

H9 hESC line was obtained from WiCell and handled according to the published protocol (Turan et al., 2019). Mycoplasma was regularly checked using Venor®GeM Classic (Minerva-Biolabs, Cat.# 11-1025). All analyses were performed at passage 5 after clonal selection.

3.2. Genome editing

For gene editing, a previously published protocol was utilized (Turan et al., 2019). Briefly, gRNAs were designed using (http://www.crispor.tefor.net) and cloned into pCAG-eCas9-GFP-U6-gRNA (Addgene, #79145) as previously described (Ran et al., 2013; Turan et al., 2019). $1.5x10^6$ cells were nucleofected with 5μg gRNA plasmid, 100pmol ssODN in 2μM Alt-R™ HDR Enhancer V2 (IDT, Cat.#1075916) using hESC nucleofector kit 2 (Lonza, Cat.#VPH-5022), protocol of manufacturer and program B-16. 48h later, $GFP^{\pm}$ cells were isolated using FACS and seeded into 96-well plates for 10 days of clonal expansion. A replica was prepared for all clones as described previously to be used for cryopreservation and DNA extraction, respectively (Turan et al., 2019). To preselect desired clones, region within *CTBP1* exon 9 was amplified using primers given in Table 3 as described before (Turan et al., 2019). Amplicons were digested using Msp1 restriction enzyme (NEB, Cat. #R0106S). For clones with correct digest pattern, undigested amplicons were sequenced. T7 endonuclease assay was done as described before (Turan et al., 2019).

3.3. Off-target analysis

CRISPOR was used to determine highly possible off-target regions (Turan et al., 2019). These regions were amplified using primers given in Table 3 and examined via Sanger sequencing.

3.4. Immunoblot, stainings, and qPCR

Immunohistochemistry, Western blot, and qPCR analysis were performed using antibodies and primers listed in Table 3 as previously described (Ivanova et al., 2020; Turan et al., 2019).

3.5. Chromosomal microarray and STR analysis

Affymetrix CytoScan HD Array kit (Thermo, Cat.#901835) was employed to assess *de novo* mosaic and non-mosaic copy number variants (CNVs). CNVs > 20 kb within exonic regions were considered for their pathogenicity. Short tandem repeat (STR) analysis was made using PowerPlex® 21 system (Promega, Cat.#DC8902).

3.6. Trilineage differentiation

Differentiation into mesoderm, endoderm, and ectoderm was induced in 12-well plate seeding $125*10^5$, $250*10^5$, and $800*10^5$ cells respectively. Detailed differentiation mediums and durations are given in Table 4. All reagents and proteins were obtained from Thermo Fisher Scientific.

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

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